(75) The immunologically functional molecule composition according to (73) or (74), wherein the transgenic non-human animal is a transgenic non-human animal prepared by using the embryonic stem cell or the fertilized egg cell according to (21).

(76) The immunologically functional molecule composition according to any one of (73) to (75), wherein the transgenic non-human animal is selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

(77) The immunologically functional molecule composition according to any one of (60) to (76), wherein the immunologically functional molecule is a protein or a peptide.

(78) The immunologically functional molecule composition according to (77), wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(0033)

(79) The immunologically functional molecule composition according to (78), wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(80) The immunologically functional molecule composition according to (79), wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor

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receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(81) A medicament comprising, as an active ingredient, comprising the immunologically functional molecule according to (59) or the immunologically functional molecule composition according to any one of (60) to (80).

(82) The medicament according to (81), which is a diagnostic drug, a preventive drug or a therapeutic drug for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

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(83) A method for preparing a medicament having a desired effector function, which comprises changing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain.
(0034)

(84) The method according to (83), wherein the medicament having a desired effector function is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain and has a higher effector function than the original medicament.

(85) The method according to (84), wherein the higher effector function is 10 times or more than the effector function of the original medicament.

(86) The method according to (84) or (85), wherein the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain is controlled to be 20% or more of the total *N*-glycoside-linked sugar chains in the composition.

(87) The method according to (83), wherein the medicament having a desired effector function is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain and has a lower effector function than the original medicament.

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(88) The method according to (87), wherein the lower effector function is 1/10 times or less than the effector function of the original medicament.

(89) The method according to (87) or (88), wherein the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain is controlled to be 10% or less of the total *N*-glycoside-linked sugar chains in the composition.

(0035)

(90) The method according to any one of (83) to (89), wherein the effector function is a cytotoxic activity through an Fc region.

(91) The method according to (90), wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(92) The method according to any one of (83) to (91), wherein the *N*-glycoside-linked sugar chain is a sugar chain selected from the group consisting of the following (a), (b) and (c):

(a) a high mannose type sugar chain;

(b) a complex type sugar chain;

(c) a hybrid type sugar chain.

(93) The method according to any one of (83) to (92), wherein the sugar which is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain is a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(94) A medicament which is prepared by the method of any one of (83) to (93).

(95) The medicament according to (94), which is a diagnostic drug, a preventive drug or a therapeutic drug

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for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

(96) A method for preparing a cell in which activity of an enzyme relating to modification of a sugar chain of a glycoprotein is controlled, which comprises selecting or modifying a cell by the artificial technique selected from the group consisting of (a) to (e) according to (5; and selecting a desired cell as a measure of an activity of an enzyme relating to modification of a sugar chain of a glycoprotein or a sugar chain structure of a glycoprotein.

(0036)

(97) A method for screening a cell in which an activity of an enzyme relating to modification of a glycoprotein is controlled, which comprises selecting a desired cell as a measure of an activity of an enzyme relating to modification of a sugar chain of a glycoprotein or a sugar chain structure of a glycoprotein.

(98) The method according to (96) or (97), wherein said selecting of a desired cell as a measure of a sugar chain structure of a glycoprotein is carried out by selecting a desired cell as a measure of the presence or absence of a sugar bound to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(99) The method according to (98), wherein the sugar to be bound is fucose.

(100) The method according to any one of (96) to (99), wherein the enzyme relating to modification of a glycoprotein is glycosyltransferase.

(101) The method according to (100), wherein the glycosyltransferase is glycosyltransferase having no

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influence on growth of a cell comprising the glycosyltransferase by decrease of the activity thereof.

(102) The method according to (100), wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by increase of the activity thereof.

(103) The method according to any one of (100) to (102), wherein the glycosyltrasnferase is an enzyme having an activity of binding a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(104) The method according to (103), wherein the sugar to be bound is fucose.

(105) The method according to any one of (100) to (105), wherein the glycosyltransferase is fucosyltransferase.

(0037)

(106) The method according to any one of (100) to (105), wherein the glycosyltransferase is α-1,6-fucosyltransferase.

(107) The method according to any one of (96) to (106), wherein the glycoprotein is an immunologically functional molecule.

(108) The method according to (107), wherein the immunologically functional molecule is an immunologically functional molecule having a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(109) The method according to (107), wherein the immunologically functional molecule is an immunologically functional molecule having a sugar chain in which fucose is bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(110) The method according to (108) or (109), wherein the sugar to be bound is fucose.

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(111) The method according to any one of (107) to (110), wherein the immunologically functional molecule is a protein or a peptide.

(112) The method according to (111), wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(0038)

(113) The method according to (112), wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(114) The method according to (113), wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody,

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or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(115) A DNA comprising the nucleotide sequence represented by SEQ ID NO:1 in the Sequence Listings.

(116) A DNA comprising the nucleotide sequence represented by SEQ ID NO:2 in the Sequence Listings.

(117) A genome DNA selected from the group consisting of the following (a), (b) and (c):

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) an anisogenome DNA corresponding to a synteny of genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:3 under stringent conditions.

(118) A target vector comprising the DNA according to any one of (115 to 117.

(0039)

(Embodiment for carrying out the invention)

In the present invention, an enzyme relating to the modification of a sugar chain of a glycoprotein may be any enzyme so long as it can influence the addition of a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycoside-linked sugar chain. The expression "to influence the addition of a sugar" means that the addition of the

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sugar is directly or indirectly inhibited or accelerated. Herein, the expression "the addition of the sugar is indirectly inhibited or accelerated" means that the enzyme activity of an enzyme which directly inhibits or accelerates the addition of a sugar (e.g., fucosyltransferase) is influenced or a sugar chain structure as a substrate is influenced.

Examples include α-1,2-glucosidase I, α-1,3-glucosidase II, α-1,2-mannosidase, *N*-acetylglucosamine phosphotransferase, *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase, α-mannosidase I, *N*-acetylglucosamine transferase (GnTI), α-mannosidase II, *N*-acetylglucosamine transferase (GnTII), galactose transferase, sialic acid transferase, *N*-acetylglucosamine transferase III (GnTIII), *N*-acetylglucosamine transferase V (GnTV), FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT8, FUT9 (with regard to FUTs 1-9, see *Protein, Nucleic Acid, Enzyme*, extra number, 43(16), 2394 (Dec., 1994) and the like. Among these, fucosyltransferases such as FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT8, FUT9 and the like are preferred, and α-1,6-fucosyltransferase is more preferred.

(0040)

In the present invention, the method which controls the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein by an artificial technique may be any method, so long as it can artificially control the activity of an enzyme which influences the addition of a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycoside-linked sugar chain.

(0041)

In the present invention, the cell in which the activity of an enzyme relating to the modification of a sugar chain is controlled by an artificial technique includes a cell in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein,

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i.e., the activity of an enzyme which influences the addition of a sugar chain to *N*-acetylglucosamine in the reducing end of *N*-glycoside-linked sugar chain, is decreased or increased in comparison with the parent strain. In the glycoprotein expressed by the cell, a difference of the modification state of a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycoside-linked sugar chain is found in comparison with a glycoprotein expressed by the parent cell.

(0042)

In the present invention, the cell in which the activity of an enzyme relating to the modification of a sugar chain is decreased by an artificial technique includes a cell in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein, i.e., the activity of an enzyme which influences the addition of a sugar chain to *N*-acetylglucosamine in the reducing end of *N*-glycoside-linked sugar chain, is decreased in comparison with the parent strain or deleted.

(0043)

In the present invention, the cell in which the activity of an enzyme relating to the modification of a sugar chain is increased by an artificial technique includes a cell in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein, i.e., the activity of an enzyme which influences the addition of a sugar chain to *N*-acetylglucosamine in the reducing end of *N*-glycoside-linked sugar chain, is increased in comparison with the parent strain.

(0044)

The parent strain in the present invention may be any cell line, so long as the activity of an enzyme relating to the modification of is not changed by an artificial technique.

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The sugar which is bound to *N*-acetylglucosamine in the reducing end of an *N*-acetylglucosamine in the present invention may be any sugar, so long as the effect of the present invention can be obtained by the sugar. Examples of the sugar include troise, tetrose, pentose, hexsose, heptose and the like, and hexsoses are preferred. Also, examples of the hexsose include glucose, galactose, mannose, fucose and the like, and fucose is particularly preferred.
(0045)

In the present invention, the artificial technique includes

(a) a technique for adding an inhibitor or activator for the activity of the enzyme to a medium;

(b) a technique for selecting a mutant relating to the enzyme;

(c) a technique for introducing a gene encoding the enzyme;

(d) a technique of a gene disruption for targeting a gene encoding the enzyme; and

(e) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme.
(0046)

In the present invention, the glycoprotein may be any one, but a complex protein having a sugar chain structure other than proteoglycan is preferred, and an immunologically functional molecule is more preferred.

The immunologically functional molecule may be any molecule, so long as it related to various immunological reactions in the living body. Specifically, it includes a protein, a peptide and the like. The protein and the peptide include an antibody, an antibody fragment, a fusion protein comprising an Fc region, and the like.
(0047)

As the present invention, the sugar chain of a glycoprotein, an *N*-glycoside-linked sugar chain is

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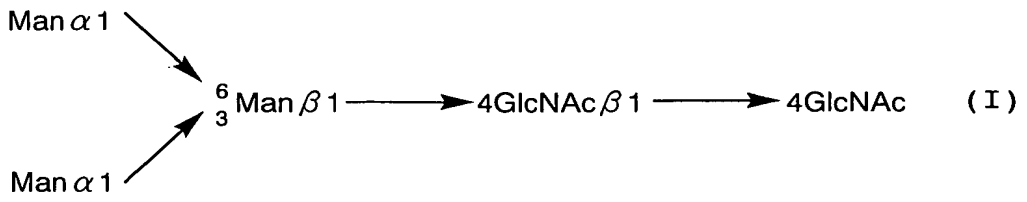
exemplified, the *N*-glycoside-linked sugar chain includes a high mannose type in which mannose alone binds to the non-reducing end of the core structure; a complex type in which the non-reducing end side of the core structure has at least one parallel branches of galactose-*N*-acetylglucosamine (hereinafter referred to as "Gal-GlcNAc") and the non-reducing end side of Gal-GlcNAc has a structure of sialic acid, bisecting *N*-acetylglucosamine or the like; a hybrid type in which the non-reducing end side of the core structure has branches of both of the high mannose type and complex type; and the like.

In one antibody, the Fc region has positions to which an *N*-glycoside-linked sugar chain is bound which will be described later. Accordingly, two sugar chains are bound per one antibody molecule. Since the *N*-glycoside-linked sugar chain which binds to an antibody includes any sugar chain having the core structure represented by the structural formula (I), a number of combinations of sugar chains may possible for the two *N*-glycoside-linked sugar chains which bind to the antibody. Accordingly, identity of substances can be judged from the viewpoint of the sugar structure bound to the Fc region.

(Chem. 2)

Formula (I)



(I)

(0049)

Accordingly, in the immunologically functional molecule of the present invention may comprise a structurally single compound or different molecules, so

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long as the effect of the present invention is obtained from the composition. The term "structurally" means that the sugar chain structures binding to the Fc region is different.

(0050)

In the present invention, when the activity of an enzyme relating to the modification in the reducing end of an *N*-glycoside-linked sugar chain of the immunologically functional molecule is decreased, it is preferred that the effector function of the immunologically functional molecule is higher than that prior to the treatment of the artificially technique.

On the other hand, in the present invention, when the activity of an enzyme relating to the modification in the reducing end of an *N*-glycoside-linked sugar chain of the immunologically functional molecule is increased, it is preferred that the effector function of the immunologically functional molecule is lower than that prior to the treatment of the artificially technique.

In the present invention, the immunologically functional molecule composition comprises a structurally single immunologically functional molecule or structurally plural immunologically functional molecules (so-called mixture).

(0051)

In the present invention, the ratio of the *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the composition comprising an immunologically functional molecule having an *N*-acetylglucosamine in the reducing end in the composition is a ratio of the number of the *N*-glycoside-linked sugar chains in which a sugar is not bound to *N*-acetylglucosamine in the composition against the total number of the *N*-glycoside-linked sugar chains in which a sugar is bound to *N*-acetylglucosamine and the *N*-glycoside-linked sugar chains

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in which a sugar is bound to *N*-acetylglucosamine in the composition.

The ratio of a sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in the immunologically functional molecule composition is preferably 20% or more, more preferably 30% or more, still more preferably 50% or more, far preferably 80% or more, particularly preferably 90% or more, and the most preferably 100%. The immunologically functional molecule composition has a higher effector function than an immunologically functional molecule composition having lower ratio of *N*-glycoside-linked sugar chain to which a sugar is not bound to *N*-acetylglucosamine in the reducing end.

(0052)

For example, when the immunologically functional molecule composition is an immunologically functional molecule prepared from a cell in which the activity of an enzyme is decreased by the artificial technique, the higher effector function means that the cytotoxic activity is at least two times or more, preferably 5 times or more, more preferably 10 times or more, and most preferably 100 times or more, higher than that in the immunologically functional molecule prepared from the parent strain at an antibody concentration of 0.00001 to 10 μg/ml.

(0053)

In the present invention, the immunologically functional molecule composition having a ratio of *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in the immunologically functional molecule composition has a lower effector function than the immunologically functional molecule composition having a high rate of *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end.

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(0054)

For example, when the immunologically functional molecule composition is an immunologically functional molecule prepared from a cell in which the activity of an enzyme is increased by the artrificial technique, the lower effector function means that the cytotoxic activity is at least 1/2 times or less, preferably 1/5 times or less, more preferably 1/10 times or less, and most preferably 1/100 times or less, lower than that in the immunologically functional molecule prepared from the parent strain at an antibody concentration of 0.00001 to 10 μg/ml.

(0055)

The ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in the composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain can be determined by releasing the sugar chain from the antibody molecule using a known method such as hydrazinolysis, enzyme digestion or the like [*Biochemical Experimentation Methods 23 - Method for Studying Glycoprotein Sugar Chain* (Japan Scientific Societies Press), edited by Reiko Takahashi (1989)], carrying out fluorescence labeling or radioisotope labeling of the released sugar chain and then separating the labeled sugar chain by chromatography. Also, the released sugar chain can also be determined by analyzing it with the HPAED-PAD method [*J. Liq. Chromatogr.*, 6, 1557 (1983)].

(0056)

In the present invention, the antibody is a protein which is produced in the living body by immune reaction as a result of exogenous antigen stimulation and has an activity to specifically bind to the antigen. Examples of the antibody include an antibody secreted by a hybridoma cell prepared from a spleen cell of an animal immunized with an antigen; an antibody prepared by a genetic

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recombination technique, namely an antibody obtained by introducing an antibody gene-inserted antibody expression vector into a host cell; and the like. Specific examples include an antibody produced by a hybridoma, a humanized antibody, a human antibody and the like.

(0057)

A hybridoma is a cell which is obtained by cell fusion between a B cell obtained by immunizing a mammal other than human with an antigen and a myeloma cell derived from mouse or the like and can produce a monoclonal antibody having the desired antigen specificity.

Examples of the humanized antibody include a human chimeric antibody, a human complementarity determining region (CDR)-grafted antibody and the like.

A human chimeric antibody is an antibody which comprises an antibody heavy chain variable region (hereinafter referred to as "HV" or "VH", the heavy chain being "H chain") and an antibody light chain variable region (hereinafter referred to as "LV" or "VL", the light chain being "L chain"), both of an animal other than human, a human antibody heavy chain constant region (hereinafter also referred to as "CH") and a human antibody light chain constant region (hereinafter also referred to as "CL"). As the animal other than human, any animal such as mouse, rat, hamster, rabbit or the like can be used, so long as a hybridoma can be prepared therefrom.

(0058)

The human chimeric antibody can be produced by obtaining cDNA's encoding VH and VL from a monoclonal antibody-producing hybridoma, inserting them into an expression vector for host cell having genes encoding human antibody CH and human antibody CL to thereby construct a human chimeric antibody expression vector, and then introducing the vector into a host cell to express the antibody.

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(0059)

As the CH of human chimeric antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg") can be used. But those belonging to the hIgG class are preferable and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Also, as the CL of human chimeric antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the κ class or λ class can also be used.

A human CDR-grafted antibody is an antibody in which amino acid sequences of CDR's of VH and VL of an antibody derived from an animal other than human are grafted into appropriate positions of VH and VL of a human antibody.

The human CDR-grafted antibody can be produced by constructing cDNA's encoding V regions in which CDR's of VH and VL of an antibody derived from an animal other than human are grafted into CDR's of VH and VL of a human antibody, inserting them into an expression vector for host cell having genes encoding human antibody CH and human antibody CL to thereby construct a human CDR-grafted antibody expression vector, and then introducing the expression vector into a host cell to express the human CDR-grafted antibody.

(0060)

As the CH of human CDR-grafted antibody, any CH can be used, so long as it belongs to the hIg, but those of the hIgG class are preferable and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Also, as the CL of human CDR-grafted antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the κ class or λ class can also be used.

(0061)

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A human antibody is originally an antibody naturally existing in the human body, but it also includes antibodies obtained from a human antibody phage library, a human antibody-producing transgenic animal and a human antibody-producing transgenic plant, which are prepared based on the recent advance in genetic engineering, cell engineering and developmental engineering techniques.

Regarding the antibody existing in the human body, a lymphocyte capable of producing the antibody can be cultured by isolating a human peripheral blood lymphocyte, immortalizing it by its infection with EB virus or the like and then cloning it, and the antibody can be purified from the culture.

(0062)

The human antibody phage library is a library in which antibody fragments such as Fab, single chain antibody and the like are expressed on the phage surface by inserting a gene encoding an antibody prepared from a human B cell into a phage gene. A phage expressing an antibody fragment having the desired antigen binding activity can be recovered from the library, using its activity to bind to an antigen-immobilized substrate as the marker. The antibody fragment can be converted further into a human antibody molecule comprising two full H chains and two full L chains by genetic engineering techniques.

(0063)

A human antibody-producing transgenic non-human animal is an animal in which a human antibody gene is introduced into cells. Specifically, a human antibody-producing transgenic animal can be prepared by introducing a human antibody gene into ES cell of a mouse, transplanting the ES cell into an early stage embryo of other mouse and then developing it. By introducing a human chimeric antibody gene into a fertilized egg and developing it, the transgenic animal can be also prepared. Regarding

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the preparation method of a human antibody from the human antibody-producing transgenic animal, the human antibody can be produced and accumulated in a culture by obtaining a human antibody-producing hybridoma by a hybridoma preparation method usually carried out in mammals other than human and then culturing it.

Examples of the transgenic non-human animal include cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit and the like.

(0064)

Also, in the present invention, it is preferable that the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen or an antibody which recognizes a viral or bacterial infection-related antigen, and a human antibody which belongs to the IgG class is preferable.

(0065)

Examples of the antibody which recognizes a tumor-related antigen include anti-GD2 antibody (Ohta *et al.*, *Anticancer Res.*, 13, 331-336, 1993), anti-GD3 antibody (Ohta *et al.*, *Cancer Immunol. Immunother.*, 36, 260-266, 1993), anti-GM2 antibody (Nakamura *et al.*, *Cancer Res.*, 54, 1511-1516, 1994), anti-HER2 antibody (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 4285-4289, 1992), anti-CD52 antibody (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 4285-4289, 1992), anti-MAGE antibody (Jungbluth *et al.*, *British J. Cancer*, 83, 493-497, 2000), anti-HM1.24 antibody (Ono *et al.*, *Molecular Immunol.*, 36, 387-395, 1999), anti-parathyroid hormone-related protein (PTHrP) antibody (Ogata *et al.*, *Cancer*, 88, 2909-2911, 2000), anti-basic fibroblast growth factor antibody and anti-FGF8 antibody (Matsuzaki *et al.*, *Proc. Natl. Acad. Sci. USA*, 86, 9911-9915, 1989),

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anti-basic fibroblast growth factor receptor antibody and anti-FGF8 receptor antibody (Kuo *et al.*, *J. Biol. Chem.*, 265, 16455-16463, 1990), anti-insulin-like growth factor antibody (Yao *et al.*, *J. Neurosci. Res.*, 40, 647-659, 1995), anti-insulin-like growth factor receptor antibody (Yao *et al.*, *J. Neurosci. Res.*, 40, 647-659, 1995), anti-PMSA antibody (Murphy *et al.*, *J. Urology*, 160, 2396-2401, 1998), anti-vascular endothelial cell growth factor antibody (Presta *et al.*, *Cancer Res.*, 57, 4593-4599, 1997), anti-vascular endothelial cell growth factor receptor antibody (Kanno *et al.*, *Oncogene*, 19, 2138-2146, 2000) and the like. Examples of the antibody which recognizes an allergy- or inflammation-related antigen include anti-interleukin 6 antibody (Abrams *et al.*, *Immunol. Rev.*, 127, 5-24, 1992), anti-interleukin 6 receptor antibody (Sato *et al.*, *Molecular Immunol.*, 31, 371-381, 1994), anti-interleukin 5 antibody (Abrams *et al.*, *Immunol. Rev.*, 127, 5-24, 1992), anti-interleukin 5 receptor antibody and anti-interleukin 4 antibody (Biord *et al.*, *Cytokine*, 3, 562-567, 1991), anti-tumor necrosis factor antibody (Tempest *et al.*, *Hybridoma*, 13, 183-190, 1994), anti-tumor necrosis factor receptor antibody (Amrani *et al.*, *Molecular Pharmacol.*, 58, 237-245, 2000), anti-CCR4 antibody (Campbell *et al.*, *Nature*, 400, 776-780, 1999), anti-chemokine antibody (Peri *et al.*, *J. Immuno. Meth.*, 174, 249-257, 1994), anti-chemokine receptor antibody (Wu *et al.*, *J. Exp. Med.*, 186, 1373-1381, 1997) and the like. Examples of the antibody which recognizes a circulatory organ disease-related antigen include anti-GpIIb/IIIa antibody (Co *et al.*, *J. Immunol.*, 152, 2968-2976, 1994), anti-platelet-derived growth factor antibody (Ferns *et al.*, *Science*, 253, 1129-1132, 1991), anti-platelet-derived growth factor receptor antibody (Shulman *et al.*, *J. Biol. Chem.*, 272, 17400-17404, 1997) and anti-blood coagulation factor antibody (Peter *et al.*, *Circulation*, 101, 1158-1164, 2000) and the like. Examples of the antibody

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which recognizes a viral or bacterial infection-related antigen include anti-gp120 antibody (Tugarinov *et al.*, *Structure*, 8, 385-395, 2000), anti-CD4 antibody (Schulze-Koops *et al.*, *J. Rheumatology*, 25, 2065-2076, 1998), anti-CCR4 antibody and anti-Vero toxin antibody (Karnali *et al.*, *J. Clin. Microbiol.*, 37, 396-399, 1999) and the like.

These antibodies can be obtained from public organizations such as ATCC (The American Type Culture Collection), RIKEN Gene Bank at The Institute of Physical and Chemical Research, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology and the like, or private reagent sales companies such as Dainippon Pharmaceutical, R & D SYSTEMS, PharMingen, Cosmo Bio, Funakoshi and the like.

(0066)

In the present invention, the effector function of the immunologically functional molecule means cytotoxic activity through Fc region (ADCC activity) and/or complement-dependent cytotoxic activity (CDC activity).

The cytotoxic activity through Fc region is an activity in which an antibody bound to a cell surface antigen on a tumor cell in the living body activate an effector cell through an Fc receptor existing on the antibody Fc region and the surface of an effector cell such as a killer cell, a natural killer cell, an activated macrophage and the like and thereby obstruct the tumor cell and the like [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1955)].

(0067)

As the protein or peptide as the above immunologically functional molecule, any one can be used, so long as it can activate various immunological reactions. Examples include interferon molecules, such as interleukin-2 (IL-2) (*Science*, 193, 1007 (1976)) and interleukin-12 (IL-12) (*J. Leuc. Biol.*, 55, 280 (1994)); colony-

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stimulating factors, such as granulocyte colony-stimulating factor (G-CSF) (*J. Biol. Chem.*, 258, 9017 (1983)), macrophage colony-stimulating factor (M-CSF) (*J. Exp. Med.*, 173, 269 (1992)) and granulocyte macrophage colony-stimulating factor (MG-CSF) (*J. Biol. Chem.*, 252, 1998 (1977)); growth factors, such as erythropoietin (EPO) (*J. Biol. Chem.*, 252, 5558 (1977)) and thrombopoietin (TPO) (*Nature*, 369, 533 (1994)); and the like.

(0068)

The activities of protein and peptide of the present invention are activities of various immunocompetent cells including lymphocytes (T cell, B cell and the like) and macrophage, or various immune response reactions, when the sugar chain-containing protein and peptide are administered into the living body.

The promotion of activities of protein and peptide of the present invention includes activation of NK cell and T cell by IL-2 and IL-12, promotion activities of erythrocyte production by EPO and the like which are further increased.

The host cell of the present invention may be any host, so long as it can express an antibody molecule. Examples include a yeast cell, an animal cell, an insect cell, a plant cell and the like. Examples of the cells include those which will be later in the item 3. Among animal cells, preferred examples include a CHO cell derived from a Chinese hamster ovary tissue, a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell, a mouse myeloma cell line NSO cell, a mouse myeloma SP2/0-Ag14 cell, a BHK cell derived from a syrian hamster kidney tissue, an antibody producing-hybridoma cell, a human leukemia cell line Namalwa cell, an embryonic stem cell, a fertilized egg cell and the like.

(0069)

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In the present invention, the synteny means a genome gene region preserved on chromosome between heterogeneous species. That is, it is known that during evolution in organisms, there is a region in which a gene sequence is preserved between different organisms in a mosaic form in the genome. The part is called synteny. The synteny can be exemplified by The Dysmorphic Human-Mouse Homology Database (GHMHD, http://www.hgmp.mrc.ac.uk/DHMHD/dysmorph. html) or National Center for Biotechnology Information (http://www3.ncbi.nlm.nih.gov/Homology).

(0070)

In the present invention, a DNA which hybridizes under stringent conditions is a DNA obtained, e.g., by a method such as colony hybridization, plaque hybridization or Southern blot hybridization using a DNA such as the DNA having the nucleotide sequence represented by SEQ ID NO:1, 2 or 3 or a partial fragment thereof as the probe, and specifically includes a DNA which can be identified by carrying out hybridization at 65°C in the presence of 0.7 to 1.0 M sodium chloride using a filter to which colony- or plaque-derived DNA fragments are immobilized, and then washing the filter at 65°C using 0.1 to 2 × SSC solution (composition of the 1 × SSC solution comprising 150 mM sodium chloride and 15 mM sodium citrate). The hybridization can be carried out in accordance with the methods described, e.g., in *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as "*Molecular Cloning*, Second Edition"), *Current Protocols in Molecular Biology*, John Wiley & Sons, 1987-1997 (hereinafter referred to as "*Current Protocols in Molecular Biology*"); *DNA Cloning 1: Core Techniques, A Practical Approach*, Second Edition, Oxford University (1995); and the like. Examples of the hybridizable DNA include a DNA having at least 60% or more,

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preferably 70% or more, more preferably 80% or more, still more preferably 90% or more, far more preferably 95% or more, and most preferably 98% or more, of homology with the nucleotide sequence represented by SEQ ID NO:1, 2 or 3.

Explanation of the target vector comprising the DNA is in accordance with the explanation described in the 1(4)(b) described below.

(0071)

The present invention will be described below in detail.

1. Preparation of cell of the present invention

The cell in which the activity of an enzyme relating to the modification of a sugar chain of a sugar protein is controlled by an artificial technique of the present invention can be prepared by (a) adding an inhibitor or activator for the activity of the enzyme to a medium; (b) selecting a mutant relating to the enzyme; (c) introducing a gene encoding the enzyme; (d) a gene disruption for targeting a gene encoding the enzyme; (e) inhibiting transcription and/or translation of a gene encoding the enzyme. They are specifically explained below.

(0072)

(1) Preparation of cell of the present invention by addition of enzyme inhibitor or activator to medium

The cell of the present invention can be produced by adding an inhibitor or activator of the activity of an enzyme relating to the modification of a sugar strain of a glycoprotein.

The host cell of the present invention can be produced by culturing the cell in a medium to which an inhibitor or activator of the activity of an enzyme relating to the modification of a sugar strain of a glycoprotein has been added.

The enzyme inhibitor or activator to be added for the preparation of the host cell of the present invention

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may be any compound, so long as it can change the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein. Examples of the compound which can change the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein include inhibitors of enzyme activity such as tunicamycin, caspatospermine, N-methyl-1-deoxynojirimycin, bromoconduritol, 1-deoxynojirimycin, 1,4-dioxy-1,4-imino-D-mannitol, swansonin, 1-deoxynojirimycin and the like. Also, it is known that deoxy derivatives of a substrate of an enzyme relating to the modification of a sugar chain influence the activity of an enzyme, and are exemplified as the compound which can change the activity of an enzyme relating to the modification of a sugar chain of a sugar protein. Moreover, the compound which can change the activity of an enzyme relating to the modification of a sugar chain of a sugar protein includes a transcription activator or inhibitor which control the transcription of an enzyme gene relating to the modification of a sugar chain of a glycoprotein. The inhibitors or activators of the enzyme relating to the modification of a sugar chain of a glycoprotein may be added to a medium alone or to a medium culturing the host cell as a combination of plural compounds.

(0073)

(2) Preparation of cell of the present invention by selection of mutant

The method for selecting a desired cell line from mutants obtained by treating the parent strain by a mutation-inducing treatment or mutants spontaneously obtained includes a method in which a desired cell line is selected from mutants obtained by treating the parent strain by a mutation-inducing treatment or mutants spontaneously obtained as a measure of the activity of an enzyme relating to the modification of a sugar protein of a

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glycoprotein, a method in which a desired cell line is selected from mutants obtained by treating the parent strain by a mutation-inducing treatment or mutants spontaneously obtained as a measure of the sugar chain structure of a glycoprotein produced from the mutants obtained by treating the parent strain by a mutation-inducing treatment or the mutants spontaneously obtained.

As the mutation-inducing treatment, any treatment can be used, so long as it can induce a point mutation or a deletion or frame shift mutation in the DNA of cells of the parent cell line. Examples include treatment with ethyl nitrosourea, nitrosoguanidine, benzopyrene or an acridine pigment and treatment with radiation. Also, various alkylating agents and carcinogens can be used as mutagens. Examples of the method for allowing a mutagen to act upon cells include the methods described in *Tissue Culture Techniques*, 3rd edition (Asakura Shoten), edited by Japanese Tissue Culture Association (1996), *Nature Genet.*, 24, 314 (2000) and the like.

Examples of the spontaneously generated mutant include mutants which are spontaneously formed by continuing subculture under general cell culture conditions without applying special mutation-inducing treatment.

(0074)

A method for selecting and measuring the activity of an enzyme relating to the modification of a glycoprotein include biochemical method, genetic method and the like described in the 7 below. Examples of the method for identifying the sugar chain structure of a glycoprotein include a method using lectin described in *Somatic Cell Mol. Genet.*, 12, 51 (1986) and the like. Examples of the lectin include WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (cocanavalin A derived from *C. ensiformis*), RIC (a toxin derived from *R. communis*), L-PHA (leucoagglutinin derived from *P. vulgaris*), LCA (lentil

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agglutinin derived from *L. culinaris*), PSA (pea lectin derived from *P. sativum*), AAL (*Aleuria aurantia* lectin), ACL (*Amaranthus caudatus* lectin), BPL (*Bauhinia purpurea* lectin), DSL (*Datura stramonium* lectin), DBA (*Dolichos biflorus* agglutinin), EBL (elderberry balk lectin), ECL (*Erythrina cristagalli* lectin), EEL (*Euonymus eoropaeus* lectin), GNL (*Galanthus nivalis* lectin), GSL (*Griffonia simplicifolia* lectin), HPA (*Helix pomatia* agglutinin), HHL (*Hippeastrum* hybrid lectin), Jacalin, LTL (*Lotus tetragonolobus* lectin), LEL (*Lycopersicon esculentum* lectin), MAL (*Maackia amurensis* lectin), MPL (*Maclura pomifera* lectin), NPL (*Narcissus pseudonarcissus* lectin), PNA (peanut agglutinin), E-PHA (*Phaseolus vulgaris* erythroagglutinin), PTL (*Psophocarpus tetragonolobus* lectin), RCA (*Ricinus communis* agglutinin), STL (*Solanum tuberosum* lectin), SJA (*Sophora japonica* agglutinin), SBA (soybean agglutinin), UEA (*Ulex europaeus* agglutinin), VVL (*Vicia villosa* lectin) and WFA (*Wisteria floribunda* agglutinin).

(0075)

(3) Preparation of cell of the present invention by introduction of gene encoding enzyme relating to modification of sugar chain of glycoprotein

The cell of the present invention can be prepare, for example, as follows using the gene introduction method described in *Molecular Cloning*, 2nd ed., *Current Protocols in Molecular Biology*, *Manipulating the Mouse Embryo*, 2nd ed. and the like.

(0076)

cDNA of an enzyme relating to the modification of a sugar chain of a sugar protein is prepared.

If necessary, using the prepared full cDNA of an enzyme relating to the modification of a sugar chain of a sugar protein, a DNA fragment having an appropriate length comprising a part encoding the protein.

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The DNA fragment or the full length cDNA is inserted into downstream of a promoter of an appropriate expression vector.

The recombinant vector is introduced to a host cell suitable for the expression vector to obtain a transformant.

The transformant is selected as a measure of the activity of an enzyme relating to the modification of the glycoprotein or the sugar chain structure of the glycoprotein.

The host cell may be any host, so long as it can express an antibody molecule. Examples include a yeast cell, an animal cell, an insect cell, a plant cell and the like, such as the host cell described in the item 1.

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the DNA encoding the desired enzyme relating to the modification of a sugar chain of a glycoprotein can be transferred is used. Examples include expression vectors which will be described later in the item 3.

(0077)

As the introduction of a gene into various host cells, the introduction method of a recombinant vector suitable for various host cells described later in the item 3 is used.

The method for selecting the transformant as a measure of the activity of the introduced enzyme relating to the modification of a glycoprotein or the sugar chain structure of a glycoprotein includes the methods described in the item 1(2).

cDNA of the enzyme relating to the modification of a sugar chain of a glycoprotein can be prepared, for example, as follows from human or non-human tissue or cell.

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A total RNA or mRNA is prepared from a human or non-human animal tissue or cell.

A cDNA library is prepared from the prepared total RNA or mRNA.

(0078)

Degenerative primers are produced based on the amino acid sequence of an enzyme relating to the modification of a sugar chain of a glycoprotein, and a gene fragment encoding the enzyme relating to modification of a sugar chain of a glycoprotein is obtained by PCR using the prepared cDNA library as the template.

A DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein can be obtained by screening the cDNA library using the obtained gene fragment as a probe.

(0078)

Regarding the mRNA of a human or non-human tissue or cell, a commercially available product (e.g., manufactured by Clontech) may be used or it may be prepared from a human or non-human animal tissue or cell in the following manner. Examples of the method for preparing a total RNA from a human or non-human animal tissue or cell include the guanidine thiocyanate-cesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)], the acidic guanidine thiocyanate phenol chloroform (AGPC) method [*Analytical Biochemistry*, 162, 156 (1987); *Experimental Medicine*, 9, 1937 (1991)] and the like.

(0080)

Also, examples of the method for preparing mRNA from a total RNA as poly(A)$^+$ RNA include an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like.

In addition, mRNA can be prepared using a kit such as Fast Track mRNA Isolation Kit (manufactured by

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Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) or the like.

A cDNA library is prepared from the prepared mRNA of a human or non-human animal tissue or cell. Examples of the method for preparing cDNA libraries include the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; *A Laboratory Manual*, Second Edition (1989); and the like, or methods using commercially available kits such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies), ZAP-cDNA Synthesis Kit (manufactured by STRATAGENE) and the like.

(0081)

As the cloning vector for use in the preparation of the cDNA library, any vector such as a phage vector, a plasmid vector or the like can be used, so long as it is autonomously replicable in *Escherichia coli* K12. Examples include ZAP Express [manufactured by STRATAGENE, *Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)], Lambda ZAP II (manufactured by STRATAGENE), λgt10 and λgt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)], λTriplEx (manufactured by Clontech), λExCell (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

(0082)

Any microorganism can be used as the host microorganism, but *Escherichia coli* is preferably used. Examples include *Escherichia coli* XL1-Blue MRF' [manufactured by STRATAGENE, *Strategies*, 5, 81 (1992)], *Escherichia coli* C600 [*Genetics*, 39, 440 (1954)], *Escherichia coli* Y1088 [*Science*, 222, 778 (1983)], *Escherichia coli* Y1090 [*Science*, 222, 778 (1983)], *Escherichia coli* NM522 [*J. Mol. Biol.*, 166, 1 (1983)],

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Escherichia coli K802 [*J. Mol. Biol.*, 16, 118 (1966)], *Escherichia coli* JM105 [*Gene*, 38, 275 (1985)] and the like
(0083)

The cDNA library may be used as such in the succeeding analysis, and in order to obtain a full length cDNA as efficient as possible by decreasing the ratio of an infull length cDNA, a cDNA library prepared using the oligo cap method developed by Sugano *et al.* [*Gene*, 138, 171 (1994); *Gene*, 200, 149 (1997); *Protein, Nucleic Acid and Protein*, 41, 603 (1996); *Experimental Medicine*, 11, 2491 (1993); *cDNA Cloning* (Yodo-sha) (1996); *Methods for Preparing Gene Libraries* (Yodo-sha) (1994)] may be used in the following analysis.
(0084)

Degenerative primers specific for the 5'-terminal and 3'-terminal nucleotide sequences of a nucleotide sequence presumed to encode the amino acid sequence are prepared based on the amino acid sequence of the enzyme relating to modification of a sugar chain of a glycoprotein, and DNA is amplified by PCR [*PCR Protocols*, Academic Press (1990)] using the prepared cDNA library as the template to obtain a gene fragment encoding the enzyme relating to modification of a sugar chain of a glycoprotein.
(0085)

It can be confirmed that the obtained gene fragment is a DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein, by a method usually used for analyzing a nucleotide, such as the dideoxy method of Sanger *et al.* [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)], a nucleotide sequence analyzer such as ABIPRISM 377 DNA Sequencer (manufactured by PE Biosystems) or the like.

A DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein can be obtained by carrying out colony hybridization or plaque hybridization (*Molecular Cloning*, Second Edition) for the cDNA or cDNA

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library synthesized from the mRNA contained in the human or non-human animal tissue or cell, using the gene fragment as a DNA probe.

(0086)

Also, a DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein can also be obtained by carrying out screening by PCR using the cDNA or cDNA library synthesized from the mRNA contained in a human or non-human animal tissue or cell as the template and using the primers used for obtaining the gene fragment encoding the enzyme relating to modification of a sugar chain of a glycoprotein.

The nucleotide sequence of the obtained DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein is analyzed from its terminus and determined by a method usually used for analyzing a nucleotide, such as the dideoxy method of Sanger *et al.* [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)], a nucleotide sequence analyzer such as ABIPRISM 377 DNA Sequencer (manufactured by PE Biosystems) or the like.

(0087)

A gene encoding the enzyme relating to modification of a sugar chain of a glycoprotein can also be determined from genes in data bases by searching nucleotide sequence data bases such as GenBank, EMBL, DDBJ and the like using a homology retrieving program such as BLAST based on the determined cDNA nucleotide sequence.

Examples of the nucleotide sequence of the gene obtained by the method encoding the enzyme relating to modification of a sugar chain of a glycoprotein include the nucleotide sequence represented by SEQ ID NO:1 or 2.

The cDNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein can also be obtained by chemically synthesizing it with a DNA synthesizer such as DNA Synthesizer model 392 manufactured

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by Perkin Elmer or the like using the phosphoamidite method, based on the determined DNA nucleotide sequence.

(0088)

(4) Preparation of cell of the present invention by disruption of gene encoding enzyme relating to modification of sugar chain of sugar protein

The host cell of the present invention can be prepared using a gene disruption technique by targeting an enzyme relating to the modification of a sugar chain of a glycoprotein. The gene as used herein includes DNA and RNA.

The gene disruption method may be any method, so long as it can disrupt the gene of the target enzyme relating to the modification of a sugar chain of a glycoprotein is included. Examples include an antisense method, a ribozyme method, a homologous recombination method, an RDO method, an RNAi method, a retrovirus-employed method, a transposon-employed method and the like. The methods are specifically described below.

(a) Preparation of the host cell of the present invention by the antisense method or the ribozyme method

The host cell of the present invention can be prepared by the ribozyme method described in *Cell Technology*, 12, 239 (1993); *BIO/TECHNOLOGY*, 17, 1097 (1999); *Hum. Mol. Genet.*, 5, 1083 (1995); *Cell Technology*, 13, 255 (1994); *Proc. Natl. Acad. Sci. USA*, 96, 1886 (1999); or the like, e.g., in the following manner by targeting at an enzyme relating to the modification of a sugar chain of a glycoprotein.

(0089)

A cDNA or a genome DNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

The nucleotide sequence of the prepared cDNA or genome DNA is determined.

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Based on the determined DNA sequence, an appropriate length of an antisense gene or ribozyme construct comprising a DNA moiety which encodes the enzyme relating to the modification of a sugar chain of a glycoprotein, a part of its non-translation region or an intron, is designed.

In order to express the antisense gene or ribozyme in a cell, a recombinant vector is prepared by inserting a fragment or total length of the prepared DNA into downstream of the promoter of an appropriate expression vector.

A transformant is obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

The host cell of the present invention can be obtained by selecting a transformant using, as a marker, the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein. The host cell of the present invention can also be obtained by selecting a transformant as a measure of the sugar chain structure of a glycoprotein on the cell membrane or the sugar chain structure of the produced antibody molecule.

As the host cell used for the production of the host cell of the present invention, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include host cells which will be described later in the item 3.

(0090)

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the designed antisense gene or ribozyme can be transferred is used. Examples include

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expression vectors which will be described later in the item 3.

Regarding the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

As the method for selecting a transformant as a measure of the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein, the method described in the item 1(2) can be exemplified.

(0091)

A method for preparing cDNA of an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, includes the method described in the item 1(3).

A method for preparing a genome DNA of an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, includes the method described in the item 1(4)(b).

Furthermore, the host cell of the present invention can be obtained by directly introducing an antisense oligonucleotide or ribozyme decided based on the nucleotide sequence of an enzyme relating to the modification of a sugar chain of a glycoprotein into a host cell.

(0092)

The antisense oligonucleotide or ribozyme can be prepared in the usual method or using a DNA synthesizer. Specifically, it can be prepared based on the sequence information of an oligonucleotide having a corresponding sequence of continued 5 to 150 bases, preferably 5 to 60 bases, and more preferably 10 to 40 bases, among nucleotide sequences of a cDNA and a genome DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein, by synthesizing an oligonucleotide which corresponds to a sequence complementary to the

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oligonucleotide (antisense oligonucleotide) or a ribozyme comprising the oligonucleotide sequence.

(0093)

Examples of the oligonucleotide include oligo RNA and derivatives of the oligonucleotide (hereinafter referred to as "oligonucleotide derivatives").

Examples of the oligonucleotide derivatives include oligonucleotide derivatives in which a phosphodiester bond in the oligonucleotide is converted into a phosphorothioate bond, an oligonucleotide derivative in which a phosphodiester bond in the oligonucleotide is converted into an N3'-P5' phosphoamidate bond, an oligonucleotide derivative in which ribose and a phosphodiester bond in the oligonucleotide are converted into a peptide-nucleic acid bond, an oligonucleotide derivative in which uracil in the oligonucleotide is substituted with C-5 propynyluracil, an oligonucleotide derivative in which uracil in the oligonucleotide is substituted with C-5 thiazoleuracil, an oligonucleotide derivative in which cytosine in the oligonucleotide is substituted with C-5 propynylcytosine, an oligonucleotide derivative in which cytosine in the oligonucleotide is substituted with phenoxazine-modified cytosine, an oligonucleotide derivative in which ribose in the oligonucleotide is substituted with 2'-*O*-propylribose and an oligonucleotide derivative in which ribose in the oligonucleotide is substituted with 2'-methoxyethoxyribose [*Cell Technology*, 16, 1463 (1997)].

(0094)

(b) Preparation of cell of the present invention by homologous recombination

The cell of the present invention can be produced by modifying a target gene on chromosome through a homologous recombination technique, using a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein as the target gene.

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The target gene on the chromosome can be modified by using a method described in *Manipulating the Mouse Embryo, A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1994) (hereinafter referred to as "*Manipulating the Mouse Embryo, A Laboratory Manual*"); *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995) (hereinafter referred to as "*Preparation of Mutant Mice using ES Cells*"); or the like, for example, as follows.
(0095)

A genome DNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

Based on the nucleotide sequence of the genome DNA, a target vector is prepared for homologous recombination of a target gene to be modified (e.g., structural gene of the enzyme relating to the modification of a sugar chain of a glycoprotein, or a promoter gene).

The host cell of the present invention can be produced by introducing the prepared target vector into a host cell and selecting a cell in which homologous recombination occurred between the target gene and target vector.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include the host cells which will be described later in the item 3.
(0096)

Examples of the method for preparing genome DNA include known methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; and the like. In addition, a genome DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein can also be isolated using a kit such as

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Genome DNA Library Screening System (manufactured by Genome Systems), Universal GenomeWalker™ Kits (manufactured by CLONTECH) or the like.

Examples of the nucleotide sequence of genome DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein include the nucleotide sequence represented by SEQ ID NO:3.

The target vector for use in the homologous recombination of the target gene can be prepared in accordance with a method described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995); or the like. The target vector can be used as either a replacement type or an insertion type.

(0097)

For introducing the target vector into various host cells, the methods for introducing recombinant vectors suited for various host cells, which will be described later in the item 3, can be used.

Examples of the method for efficiently selecting a homologous recombinant include a method such as the positive selection, promoter selection, negative selection or polyA selection described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995); or the like. Examples of the method for selecting the homologous recombinant of interest from the selected cell lines include the Southern hybridization method for genome DNA (*Molecular Cloning*, Second Edition), PCR [*PCR Protocols*, Academic Press (1990)], and the like.

(0098)

(c) Preparation of cell of the present invention by RDO method

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The cell of the present invention can be prepared by an RDO (RNA-DNA oligonucleotide) method by targeting at a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, as follows.

A cDNA or a genome DNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

The nucleotide sequence of the prepared cDNA or genome DNA is determined.

Based on the determined DNA sequence, an appropriate length of an RDO construct comprising a DNA moiety which encodes the enzyme relating to the modification of a sugar chain of a glycoprotein or a part of its non-translation region or an intron, is designed and synthesized.

The host cell of the present invention can be obtained by introducing the synthesized RDO into a host cell and then selecting a transformant in which a mutation occurred in the target enzyme relating to the modification of a sugar chain of a glycoprotein.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target . Examples include the host cells which will be described later in the item 3.

Examples of the method for introducing RDO into various host cells include the methods for introducing recombinant vectors suited for various host cells, which will be described later in the item 3.

Examples of the method for preparing cDNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein include the methods described in the preparation of DNA in the item 1(3) and the like.

(0099)

Examples of the method for preparing a genome DNA encoding the enzyme relating to the modification of a sugar

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chain of a glycoprotein include the methods in preparation of genome DNA described in the item 1(4)(a) and the like.

The method for determining the nucleotide sequence of DNA includes the method described in item 6.

The RDO can be prepared by a usual method or using a DNA synthesizer.

Examples of the method for selecting a cell in which a mutation occurred, by introducing the ROD into the host cell, in the gene encoding the enzyme relating to the modification of a sugar chain of a glycoprotein include the methods for directly detecting mutations in chromosomal genes described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology* and the like. Also, the method of the item 1(2) in which the transformant can be selected as a measure of the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein can be used.

(0100)

The construct of the ROD can be designed in accordance with the methods described in *Science*, 273, 1386 (1996); *Nature Medicine*, 4, 285 (1998); *Hepatology*, 25, 1462 (1997); *Gene Therapy*, 5, 1960 (1999); *J. Mol. Med.*, 75, 829 (1997); *Proc. Natl. Acad. Sci. USA*, 96, 8774 (1999); *Proc. Natl. Acad. Sci. USA*, 96, 8768 (1999); *Nuc. Acids. Res.*, 27, 1323 (1999); *Invest. Dematol.*, 111, 1172 (1998); *Nature Biotech.*, 16, 1343 (1998); *Nature Biotech.*, 18, 43 (2000); *Nature Biotech.*, 18, 555 (2000); and the like.

(0101)

(d) Preparation of cell of the present invention by RNAi method

The cell of the present invention can be prepared by the RNAi (RNA interference) method by targeting at a gene of an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, as follows.

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A cDNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

The nucleotide sequence of the prepared cDNA is determined.

Based on the determined DNA sequence, an appropriate length of an RNAi gene construct comprising the DNA coding moiety encoding the enzyme relating to the modification of a sugar chain of a glycoprotein or a part of its non-translation region, is designed.

In order to express the RNAi gene in a cell, a recombinant vector is prepared by inserting a fragment or full length of the prepared DNA into downstream of the promoter of an appropriate expression vector.

A transformant is obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

The host cell of the present invention can be obtained by selecting a transformant as a measure of the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein, or the sugar chain structure of a glycoprotein.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include the host cells which will be described later in the item 3.

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the designed RNAi gene can be transferred is used. Examples include the expression vectors which will be described later in the item 3.

(0102)

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As the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

Examples of the method for selecting a transformant as a measure of the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein include the methods described in the item 1(2).

Examples of the method for selecting a transformant as a measure of the sugar chain structure of a glycoprotein on the cell membrane include the methods which will be described later in the item 1(3).

In addition, the host cell of the present invention can also be obtained without using an expression vector, by directly introducing an RNAi gene designed based on the nucleotide sequence encoding the enzyme relating to the modification of a sugar chain of a glycoprotein.

The RNAi gene can be prepared in the usual method or using a DNA synthesizer.

(0103)

The RNAi gene construct can be designed in accordance with the methods described in *Nature*, 391, 806 (1998); *Proc. Natl. Acad. Sci. USA*, 95, 15502 (1998); *Nature*, 395, 854 (1998); *Proc. Natl. Acad. Sci. USA*, 96, 5049 (1999); *Cell*, 95, 1017 (1998); *Proc. Natl. Acad. Sci. USA*, 96, 1451 (1999); *Proc. Natl. Acad. Sci. USA*, 95, 13959 (1998); *Nature Cell Biol.*, 2, 70 (2000); and the like.

(0104)

(e) Preparation of cell of the present invention by method using transposon

The cell of the present invention can be prepared by inducing mutation using a transposon system described in *Nature Genet.*, 25, 35 (2000) or the like, and then by selecting a mutant as a measure of the activity of the

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enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein.

The transposon system is a system in which a mutation is induced by randomly inserting an exogenous gene into chromosome, wherein an exogenous gene interposed between transposons is generally used as a vector for inducing a mutation, and a transposase expression vector for randomly inserting the gene into chromosome is introduced into the cell at the same time.

(0105)

Any transposase can be used, so long as it is suitable for the sequence of the transposon to be used.

As the exogenous gene, any gene can be used, so long as it can induce a mutation in the DNA of a host cell.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include the host cells which will be described later in the item 3. For introducing the gene into various host cells, the method for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

Examples of the method for selecting a mutant as a measure of the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein include the methods described in the item 1(2).

(0106)

(5) Method for inhibiting transcription and/or translation of gene encoding enzyme relating to modification of sugar chain of glycoprotein

The cell of the present invention can be prepared by inhibiting transcription and/or translation of a target gene through a method such as the antisense RNA/DNA

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technique [*Bioscience and Industry*, 50, 322 (1992); *Chemistry*, 46, 681 (1991); *Biotechnology*, 9, 358 (1992); *Trends in Biotechnology*, 10, 87 (1992); *Trends in Biotechnology*, 10, 152 (1992); *Cell Engineering*, 16, 1463 (1997)], the triple helix technique [*Trends in Biotechnology*, 10, 132 (1992)] or the like, using a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein, as the target.

(0107)

2. Preparation of a transgenic non-human animal or plant or the progenies thereof of the present invention

The transgenic non-human animal or plant or the progenies thereof of the present invention is a transgenic non-human animal or plant or the progenies thereof in which a genome gene is modified in such a manner that the activity of an enzyme relating to the modification of a sugar chain of an antibody molecule can be controlled, and it can be prepared according to the method similar to that in the item 1, using a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein, as the target.

(0108)

In a transgenic non-human animal, the embryonic stem cell of the present invention in which the activity of the enzyme the modification of a sugar chain of a glycoprotein is controlled can be prepared applying the method similar to that in the item 1 to an embryonic stem cell of the intended non-human animal such as cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit or the like. Specifically, a mutant clone is prepared in which a gene encoding the enzyme relating to the modification of a sugar chain of a glycoprotein is inactivated or substituted with any sequence, by a known homologous recombination technique [e.g., *Nature*, 326, 6110, 295 (1987); *Cell*, 51, 3, 503 (1987); or the like]. Using the prepared mutant clone,

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a chimeric individual comprising an embryonic stem cell clone and a normal cell can be prepared by an injection chimera method into blastocyst of fertilized egg of an animal or by an aggregation chimera method. The chimeric individual is crossed with a normal individual, so that a transgenic non-human animal in which the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein is decreased or deleted in the whole body cells can be obtained.

(0109)

Also, a fertilized egg cell of the present invention in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased or deleted can be prepared by applying the method similar to that in the item 1 to fertilized egg of a non-human animal of interest such as cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit or the like. A transgenic non-human animal in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased can be prepared by transplanting the prepared fertilized egg cell into the oviduct or uterus of a pseudopregnant female using the embryo transplantation method described in *Manipulating Mouse Embryo*, Second Edition or the like, followed by childbirth by the animal.

(0110)

In a transgenic plant, the callus of the present invention in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased or deleted can be prepared by applying the method similar to that in the item 1 to a callus or cell of the plant of interest. A transgenic plant in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased can be prepared by culturing the prepared callus using a medium comprising auxin and cytokinin to redifferentite it in accordance with

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a known method [*Tissue Culture*, 20 (1994); *Tissue Culture*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)].
(0111)

3. Method for producing immunologically functional molecule expressed in various cells

The immunologically functional molecule can be obtained by expressing it in a host cell using the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988 (hereinafter referred also to as "*Antibodies*"); *Monoclonal Antibodies: Principles and Practice*, Third Edition, Acad. Press, 1993 (hereinafter referred also to as "*Monoclonal Antibodies*"); and *Antibody Engineering, A Practical Approach*, IRL Press at Oxford University Press (hereinafter referred also to as "*Antibody Engineering*"), for example, as follows.
(0102)

A full length cDNA encoding an immunologically functional molecule is prepared, and an appropriate length of a DNA fragment comprising a moiety encoding the antibody molecule is prepared.

A recombinant vector is prepared by inserting the DNA fragment or the full length cDNA into downstream of the promoter of an appropriate expression vector.

A transformant which produces the antibody molecule can be obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

As the host cell, any of yeast, animal cell, insect cell, plant cell or the like can be used, so long as it can express the gene of interest.

A cell such as yeast, animal cell, insect cell, plant cell or the like into which an enzyme relating to the modification of an *N*-glycoside-linked sugar chain which binds to the Fc region of the antibody molecule is

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introduced by a genetic engineering technique can also be used as the host cell.

(0103)

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the DNA encoding the immunologically functional molecule of interest can be transferred is used.

The cDNA can be prepared from a human or non-human tissue or cell using, e.g., a probe primer specific for the immunologically functional molecule of interest, in accordance with the methods described in the preparation of DNA in the item 1(1)(a).

(0114)

When a procaryote cell, such as a bacterium or the like, is used as the host cell, it is preferred that the recombinant vector containing the DNA encoding the polypeptide of the present invention can replicate autonomously in the bacterium and is a recombinant vector constituted by, at least a promoter, a ribosome binding sequence, the DNA of the present invention and a transcription termination sequence. A promoter controlling gene can also be contained therewith in operable combination.

(0115)

Examples of the expression vectors include a vector plasmid which is replicable in *Corynebacterium glutamicum*, such as pCG1 (Japanese Published Unexamined Patent Application No. 134500/82), pCG2 (Japanese Published Unexamined Patent Application No. 35197/83), pCG4 (Japanese Published Unexamined Patent Application No. 183799/82), pCG11 (Japanese Published Unexamined Patent Application No. 134500/82), pCG116, pCE54 and pCB101 (Japanese Published Unexamined Patent Application No. 105999/83), pCE51, pCE52 and pCE53 (*Mol. Gen. Genet.*, *196*: 175-178 (1984)), and the

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like; a vector plasmid which is replicable in *Escherichia coli*, such as pET3 and pET11 (manufactured by Stratagene), pBAD, pThioHis and pTrcHis (manufactured by Invitrogen), pKK223-3 and pGEX2T (manufactured by Amersham Pharmacia Biotech), and the like; and pBTrp2, pBTac1 and pBTac2 (manufactured by Boehringer Mannheim Co.), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 (*Agric. Biol. Chem.*, *48*: 669 (1984)), pLSA1 (*Agric. Biol. Chem.*, *53*: 277 (1989)), pGEL1 (*Proc. Natl. Acad. Sci. USA*, *82*: 4306 (1985)), pBluescript II SK(-) (manufactured by Stratagene), pTrs30 (prepared from *Escherichia coli* JM109/pTrS30 (FERM BP-5407)), pTrs32 (prepared from *Escherichia coli* JM109/pTrS32 (FERM BP-5408)), pGHA2 (prepared from *Escherichia coli* IGHA2 (FERM B-400), Japanese Published Unexamined Patent Application No. 221091/85), pGKA2 (prepared from *Escherichia coli* IGKA2 (FERM BP-6798), Japanese Published Unexamined Patent Application No. 221091/85), pTerm2 (U.S. Patents 4,686,191, 4,939,094 and 5,160,735), pSupex, pUB110, pTP5, pC194 and pEG400 (*J. Bacteriol.*, *172*: 2392 (1990)), pGEX (manufactured by Pharmacia), pET system (manufactured by Novagen), and the like.

Any promoter can be used so long as it can function in the host cell. Examples include promoters derived from *Escherichia coli*, phage and the like, such as *trp* promoter (P_{trp}), *lac* promoter, P_L promoter, P_R promoter, T7 promoter and the like. Also, artificially designed and modified promoters, such as a promoter in which two P*trp* are linked in series (P_{trp}×2), *tac* promoter, *lac*T7 promoter *let*I promoter and the like, can be used.

(0116)

It is preferred to use a plasmid in which the space between Shine-Dalgarno sequence which is the ribosome

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binding sequence and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 nucleotides).

The transcription termination sequence is not always necessary for the expression of the DNA of the present invention. However, it is preferred to arrange the transcription terminating sequence at just downstream of the structural gene.

(0117)

Examples of the host cell include microorganisms belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Bacillus*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Microbacterium*, the genus *Pseudomonas*, and the like. Specific examples include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* GI698, *Escherichia coli* TB1, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Corynebacterium ammoniagenes*, *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 13869, *Corynebacterium glutamicum* ATCC 14067 (prior genus and species: *Brevibacterium flavum*), *Corynebacterium glutamicum* ATCC 13869 (prior genus and species: *Brevibacterium lactofermentum*, or *Corynebacterium lactofermentum*), *Corynebacterium acetoacidophilum* ATCC 13870, *Corynebacterium thermoaminogenes* FERM 9244, *Microbacterium ammoniaphilum* ATCC 15354, *Pseudomonas putida*, *Pseudomonas* sp. D-0110, and the like.

(0118)

With regard to the method for the introduction of the recombinant vector, any method for introducing DNA into

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the above-described host cells, such as a method in which a calcium ion is used (*Proc. Natl. Acad. Sci. USA, 69*: 2110 (1972)), a protoplast method (Japanese Published Unexamined Patent Application No. 2483942/88), the methods described in *Gene, 17*: 107 (1982) and *Molecular & General Genetics, 168*: 111 (1979) and the like, can be used.

(0119)

When a yeast is used as the host cell, examples of the expression vector include YEP13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419) and the like.

Any promoter can be used, so long as it can function in yeast. Examples include a promoter of a gene of the glycolytic pathway such as a hexose kinase gene, *etc.*, PH05 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MF α1 promoter, CUP 1 promoter and the like.

Examples of the host cell include microorganisms belonging to the genus *Saccharomyces*, the genus *Schizosaccharomyces*, the genus *Kluyveromyces*, the genus *Trichosporon*, the genus *Schwanniomyces* and the like, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans* and *Schwanniomyces alluvius*, *etc.*

(0120)

As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into yeast. Examples include electroporation [*Methods in Enzymology*, 194, 182 (1990)], spheroplast method [*Proc. Natl. Acad. Sci. USA*, 84, 1929 (1978)], lithium acetate method [*J. Bacteriol.*, 153, 163 (1983)], a method described in *Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978) and the like.

(0121)

When an animal cell is used as the host, examples of the expression vector include pcDNAI, pcDM8 (available

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from Funakoshi), pAGE107 [Japanese Published Examined Patent Application No. 22979/91; *Cytotechnology*, 3, 133 (1990)], pAS3-3 (Japanese Published Examined Patent Application No. 227075/90), pCDM8 [*Nature*, 329, 840 (1987)], pcDNAI/Amp (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [*J. Biochemistry*, 101, 1307 (1987)], pAGE210 and the like.

Any promoter can be used, so long as it can function in an animal cell. Examples include a promoter of IE (immediate early) gene of cytomegalovirus (CMV), an early promoter of SV40, a promoter of retrovirus, a promoter of metallothionein, a heat shock promoter, an SRα promoter and the like. Also, an enhancer of the IE gene of human CMV may be used together with the promoter.

(0122)

Examples of the host cell include a human cell such as Namalwa cell, a monkey cell such as COS cell, a Chinese hamster cell such as CHO cell or HBT5637 (Japanese Published Examined Patent Application No. 299/88), a rat myeloma cell, a mouse myeloma cell, a cell derived from syrian hamster kidney, an embryonic stem cell, a fertilized egg cell and the like.

As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into an animal cell. Examples include electroporation [*Cytotechnology*, 3, 133 (1990)], the calcium phosphate method (Japanese Published Examined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)], the injection method [*Manipulating the Mouse Embryo, A Laboratory Manual*], a method using particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813), the DEAE-dextran method [*Biomanual Series 4-Gene Transfer and Expression Analysis* (Yodo-sha), edited by Takashi Yokota and Kenichi Arai

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(1994)], the virus vector method [*Manipulating Mouse Embryo*, Second Edition] and the like.

(0123)

When an insect cell is used as the host, the protein can be expressed by the method described in *Current Protocols in Molecular Biology*, *Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992), *Bio/Technology*, 6, 47 (1988) or the like.

That is, the protein can be expressed by simultaneously introducing a recombinant gene-introducing vector and a baculovirus into an insect cell to obtain a recombinant virus in an insect cell culture supernatant and then infecting the insect cell with the recombinant virus.

Examples of the gene introducing vector used in the method include pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen) and the like.

Examples of the baculovirus include *Autographa californica* nuclear polyhedrosis virus which is infected with an insect of the family *Barathra*.

(0124)

Examples of the insect cell include *Spodoptera frugiperda* oocytes Sf9 and Sf21 [*Current Protocols in Molecular Biology*, *Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992)], a *Trichoplusia ni* oocyte High 5 (manufactured by Invitrogen) and the like.

Examples of the method for the simultaneously introducing the recombinant gene-introducing vector and the baculovirus for preparing the recombinant virus include the calcium phosphate method (Japanese Published Examined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)] and the like.

(0125)

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When a plant cell is used as the host, examples of the expression vector include Ti plasmid, tobacco mosaic virus and the like.

As the promoter, any promoter can be used, so long as it can function in a plant cell. Examples include cauliflower mosaic virus (CaMV) 35S promoter, rice actin 1 promoter and the like.

Examples of the host cell include plant cells of tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, barley, *etc.*, and the like.

(0126)

As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into a plant cell. Examples include a method using *Agrobacterium* (Japanese Published Examined Patent Application No. 140885/84, Japanese Published Examined Patent Application No. 70080/85, WO 94/00977), electroporation (Japanese Published Examined Patent Application No. 251887/85), a method using a particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813) and the like.

As the method for expressing a gene, secretion production, expression of a fusion protein of the Fc region with other protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition or the like, in addition to the direct expression.

(0127)

When a gene is expressed by a bacterium, a yeast, an animal cell, an insect cell or a plant cell into which a gene relating to the synthesis of a sugar chain is introduced, an antibody molecule to which a sugar or a sugar chain is added by the introduced gene can be obtained.

(0128)

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An immunologically functional molecule can be obtained by culturing the obtained transformant in a medium to produce and accumulate the antibody molecule in the culture and then recovering it from the resulting culture. The method for culturing the transformant using a medium can be carried out in accordance with a general method which is used for the culturing of host cells.

As the medium for culturing a transformant obtained using a prokaryote such as *Escherichia coli etc.* or a eukaryote such as yeast *etc.* as the host cell, the medium may be either a natural medium or a synthetic medium, so long as it comprises materials such as a carbon source, a nitrogen source, an inorganic salt and the like which can be assimilated by the organism and culturing of the transformant can be efficiently carried out.

(0129)

As the carbon source, those which can be assimilated by the organism can be used. Examples include carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch, starch hydrolysate, *etc.*; organic acids such as acetic acid, propionic acid, *etc.*; alcohols such as ethanol, propanol, *etc.*; and the like.

Examples of the nitrogen source include ammonia; ammonium salts of inorganic acid or organic acid such as ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, *etc.*; other nitrogen-containing compounds; peptone; meat extract; yeast extract; corn steep liquor; casein hydrolysate; soybean meal; soybean meal hydrolysate; various fermented cells and hydrolysates thereof; and the like.

Examples of the inorganic material include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, and the like

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(0130)

The culturing is carried out generally under aerobic conditions such as a shaking culture, submerged-aeration stirring culture or the like. The culturing temperature is preferably 15 to 40°C, and the culturing time is generally 16 hours to 7 days. During the culturing, the pH is maintained at 3.0 to 9.0. The pH is adjusted using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia or the like.

If necessary, an antibiotic such as ampicillin, tetracycline or the like may be added to the medium during the culturing.

When a microorganism transformed with a recombinant vector obtained using an inducible promoter as the promoter is cultured, an inducer may be added to the medium, if necessary. For example, when a microorganism transformed with a recombinant vector obtained using *lac* promoter is cultured, isopropyl-β-D-thiogalactopyranoside may be added to the medium, and when a microorganism transformed with a recombinant vector obtained using *trp* promoter is cultured, indoleacrylic acid may be added to the medium.

(0131)

When a transformant obtained using an animal cell as the host cell is cultured, examples of the medium include generally used RPMI 1640 medium [*The Journal of the American Medical Association*, 199, 519 (1967)], Eagle's MEM medium [*Science*, 122, 501 (1952)], Dulbecco's modified MEM medium [*Virology*, 8, 396 (1959)], 199 medium [*Proceeding of the Society for the Biological Medicine*, 73, 1 (1950)] and Whitten's medium [*Developmental Engineering Experimentation Manual-Preparation of Transgenic Mice* (Kodan-sha), edited by M. Katshuki (1987)], the media to which fetal calf serum, *etc.* is added, and the like.

(0132)

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The culturing is carried out generally at a pH of 6 to 8 and 30 to 40°C for 1 to 7 days in the presence of 5% CO_2. If necessary, an antibiotic such as kanamycin, penicillin or the like may be added to the medium during the culturing.

Examples of the medium for use in the culturing of a transformant obtained using an insect cell as the host include usually used TNM-FH medium (manufactured by Pharmingen), Sf-900 II SFM medium (manufactured by Life Technologies), ExCell 400 and ExCell 405 (both manufactured by JRH Biosciences), Grace's Insect Medium [*Nature*, 195, 788 (1962)] and the like.

The culturing is carried out generally at a medium pH of 6 to 7 and 25 to 30°C for 1 to 5 days.

In addition, antibiotics such as gentamicin may be added to the medium during the culturing as occasion demands.

(0133)

A transformant obtained using a plant cell as the host can be cultured as a cell or by differentiating it into a plant cell or organ. Examples of the medium for culturing the transformant include generally used Murashige and Skoog (MS) medium and White medium, the media to which a plant hormone such as auxin, cytokinin, *etc.* is added, and the like.

The culturing is carried out generally at a pH of 5 to 9 and 20 to 40°C for 3 to 60 days.

If necessary, an antibiotic such as kanamycin, hygromycin or the like may be added to the medium during the culturing.

(134)

Accordingly, an immunologically functional molecule can be produced by culturing a transformant derived from a microorganism, an animal cell or a plant cell, which comprises a recombinant vector into which a DNA encoding an

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immunologically functional molecule is inserted, in accordance with a general culturing method, to thereby produce and accumulate the immunologically functional molecule, and then recovering the immunologically functional molecule from the culture.

As the method for expressing the gene, secretion production, expression of a fusion protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition, in addition to the direct expression.

Examples of the method for producing an immunologically functional molecule include a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, and a method of production on a host cell membrane outer envelope. The method can be selected by changing the host cell used or the structure of the immunologically functional molecule produced.

(0135)

When the immunologically functional molecule of the present invention is produced in a host cell or on a host cell membrane outer envelope, it can be positively secreted extracellularly in accordance with the method of Paulson *et al.* [*J. Biol. Chem.*, 264, 17619 (1989)], the method of Lowe *et al.* [*Proc. Natl. Acad. Sci. USA*, 86, 8227 (1989), *Genes Develop.*, 4, 1288 (1990)], the methods described in Japanese Published Examined Patent Application No. 336963/93 and Japanese Published Examined Patent Application No. 823021/94 and the like.

That is, an antibody molecule of interest can be positively secreted extracellularly from a host cell by inserting a DNA encoding the antibody molecule and a DNA encoding a signal peptide suitable for the expression of the antibody molecule into an expression vector using a gene recombination technique, introducing the expression

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vector into the host cell and then expressing the antibody molecule.

Also, its production amount can be increased in accordance with the method described in Japanese Published Examined Patent Application No. 227075/90 using a gene amplification system using a dihydrofolate reductase gene.

In addition, the immunologically functional molecule can also be produced using a gene-introduced animal individual (transgenic non-human animal) or a plant individual (transgenic plant) which is constructed by the redifferentiation of an animal or plant cell into which the gene is introduced.

(0136)

When the transformant is an animal individual or a plant individual, an immunologically functional molecule can be produced in accordance with a general method by rearing or cultivating it to thereby produce and accumulate the immunologically functional molecule and then recovering the immunologically functional molecule from the animal or plant individual.

Examples of the method for producing an immunologically functional molecule using an animal individual include a method in which the immunologically functional molecule of interest is produced in an animal constructed by introducing a gene in accordance with a known method [*American Journal of Clinical Nutrition*, 63, 627S (1996); *Bio/Technology*, 9, 830 (1991)].

(0137)

In the case of an animal individual, an immunologically functional molecule can be produced by rearing a transgenic non-human animal into which a DNA encoding an antibody molecule is introduced to thereby produce and accumulate the immunologically functional molecule in the animal, and then recovering the immunologically functional molecule from the animal.

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Examples of the place of the animal where the composition is produced and accumulated include milk (Japanese Published Examined Patent Application No. 309192/88) and eggs of the animal. As the promoter used in this case, any promoter can be used, so long as it can function in an animal. Preferred examples include mammary gland cell-specific promoters such as α casein promoter, β casein promoter, β lactoglobulin promoter, whey acidic protein promoter and the like.

(0138)

Example of the method for producing an immunologically functional molecule using a plant individual include a method in which an immunologically functional molecule is produced by cultivating a transgenic plant into which a DNA encoding an antibody molecule is introduced by a known method [*Tissue Culture*, 20 (1994); *Tissue Culture*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)] to produce and accumulate the immunologically functional molecule in the plant, and then recovering the immunologically functional molecule from the plant.

(0139)

Regarding purification of an immunologically functional molecule produced by a transformant into which a gene encoding an antibody molecule is introduced, for example, when the immunologically functional molecule is intracellularly expressed in a dissolved state, the cells after culturing are recovered by centrifugation, suspended in an aqueous buffer and then disrupted using ultrasonic oscillator, French press, Manton Gaulin homogenizer, dynomill or the like to obtain a cell-free extract. A purified product of the immunologically functional molecule can be obtained from a supernatant obtained by centrifuging the cell-free extract, by using a general enzyme isolation purification techniques such as solvent extraction; salting out; desalting with ammonium sulfate, *etc.*; precipitation

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with an organic solvent; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-Sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical), *etc.*; cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia), *etc.*; hydrophobic chromatography using a resin such as butyl-Sepharose, phenyl-Sepharose, *etc.*; gel filtration using a molecular sieve; affinity chromatography; chromatofocusing; electrophoresis such as isoelectric focusing, *etc.*; and the like which may be used alone or in combination.

(0140)

Also, when the immunologically functional molecule is expressed intracellularly by forming an insoluble body, the cells are recovered, disrupted and centrifuged in the same manner, and the insoluble body of the immunologically functional molecule is recovered as a precipitation fraction. The recovered insoluble body of the immunologically functional molecule is solubilized using a protein denaturing agent. The immunologically functional molecule is made into a normal three-dimensional structure by diluting or dialyzing the solubilized solution, and then a purified product of the immunologically functional molecule is obtained by the same isolation purification method.

When the immunologically functional molecule is secreted extracellularly, the immunologically functional molecule or derivatives thereof can be recovered from the culture supernatant. That is, the culture is treated by a technique such as centrifugation or the like to obtain a soluble fraction, and a purified preparation of the immunologically functional molecule can be obtained from the soluble fraction by the same isolation purification method.

Examples of the thus obtained immunologically functional molecule include an antibody, the fragment of

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the antibody, a fusion protein comprising the Fc region of the antibody, and the like.

(0141)

As an example for obtaining the immunologically functional molecule, a method for producing a composition of a humanized antibody is described below in detail, but other immunologically functional molecules can also be obtained in a manner similar to the method.

(1) Construction of vector for humanized antibody expression

A vector for humanized antibody expression is an expression vector for animal cell into which genes encoding the heavy chain (H chain) and light chain (L chain) C regions of a human antibody are inserted, which can be constructed by cloning each of genes encoding the H chain and L chain C regions of a human antibody into an expression vector for animal cell.

The C regions of a human antibody may be the H chain and L chain of any human antibody. Examples include the C region belonging to IgG1 subclass in the H chain of a human antibody (hereinafter referred to as "hCγ1"), the C region belonging to κ class in the L chain of a human antibody (hereinafter referred to as "hCκ"), and the like.

(0142)

As the genes encoding the H chain and L chain C regions of a human antibody, a chromosomal DNA comprising an exon and an intron can be used or a cDNA can also be used.

As the expression vector for animal cell, any vector can be used, so long as a gene encoding the C region of a human antibody can be inserted thereinto and expressed therein. Examples include pAGE107 [*Cytotechnology*, 3, 133 (1990)], pAGE103 [*J. Biochem.*, 101, 1307 (1987)], pHSG274 [*Gene*, 27, 223 (1984)], pKCR [*Proc. Natl. Acad. Sci. USA*, 78, 1527 (1981), pSG1 β d2-4 [*Cytotechnology*, 4, 173

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(1990)] and the like. Examples of the promoter and enhancer in the expression vector for animal cell include SV40 early promoter and enhancer [*J. Biochem.*, 101, 1307 (1987)], Moloney mouse leukemia virus LTR promoter [*Biochem. Biophys. Res. Commun.*, 149, 960 (1987)], immunoglobulin H chain promoter [*Cell*, 41, 479 (1985)] and enhancer [*Cell*, 33, 717 (1983)], and the like.

(0143)

The humanized antibody expression vector may be either of a type in which genes encoding the H chain and L chain of an antibody exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression amounts of the H and L chains of an antibody in animal cells, a tandem type of the humanized antibody expression vector is more preferred [*J. Immunol. Methods*, 167, 271 (1994)].

The constructed humanized antibody expression vector can be used for expression of a human chimeric antibody and a human CDR-grafted antibody in animal cells.

(0144)

(2) Preparation of cDNA encoding V region of antibody derived from animal other than human

cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human, such as a mouse antibody, can be obtained in the following manner.

A cDNA is synthesized by extracting mRNA from a hybridoma cell which produces the mouse antibody of interest. The synthesized cDNA is cloned into a vector such as a phage or a plasmid to obtain a cDNA library. Each of a recombinant phage or recombinant plasmid comprising a cDNA encoding the H chain V region and a recombinant phage or recombinant plasmid comprising a cDNA

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encoding the L chain V region is isolated from the library using a C region part or a V region part of an existing mouse antibody as the probe. Full nucleotide sequences of the H chain and L chain V regions of the mouse antibody of interest on the recombinant phage or recombinant plasmid are determined, and full amino acid sequences of the H chain and L chain V regions are deduced from the nucleotide sequences.

As the animal other than human, any animal such as mouse, rat, hamster, rabbit or the like can be used so long as a hybridoma cell can be produced therefrom.

(0145)

Examples of the method for preparing total RNA from a hybridoma cell include the guanidine thiocyanate-cesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)] and the like, and examples of the method for preparing mRNA from total RNA, an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like. In addition, examples of a kit for preparing mRNA from a hybridoma cell include Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) and the like.

(0146)

Examples of the method for synthesizing cDNA and preparing a cDNA library include the usual methods (*Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology*, Supplement 1-34), methods using a commercially available kit such as SuperScript™, Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by GIBCO BRL) or ZAP-cDNA Synthesis Kit (manufactured by Stratagene), and the like.

(0147)

In preparing the cDNA library, the vector into which a cDNA synthesized using mRNA extracted from a hybridoma cell as the template is inserted may be any

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vector so long as the cDNA can be inserted. Examples include ZAP Express [*Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)], λzapII (manufactured by Stratagene), λgt10 and λgt11 [*DNA Cloning, A Practical Approach*, I, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λExCell, pT7T3 18U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

(0148)

As *Escherichia coli* into which the cDNA library constructed from a phage or plasmid vector is introduced, any *Escherichia coli* can be used, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF' [*Strategies*, 5, 81 (1992)], C600 [*Genetics*, 39, 440 (1954)], Y1088 and Y1090 [*Science*, 222, 778 (1983)], NM522 [*J. Mol. Biol.*, 166, 1 (1983)], K802 [*J. Mol. Biol.*, 16, 118 (1966)], JM105 [*Gene*, 38, 275 (1985)] and the like.

(0149)

As the method for selecting a cDNA clone encoding the H chain and L chain V regions of an antibody derived from an animal other than human from the cDNA library, a colony hybridization or a plaque hybridization using an isotope- or fluorescence-labeled probe can be used (*Molecular Cloning*, Second Edition). The cDNA encoding the H chain and L chain V regions can also be prepared by preparing primers and carrying out polymerase chain reaction (hereinafter referred to as "PCR"; *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*, Supplement 1-34) using a cDNA synthesized from mRNA or a cDNA library as the template.

(0150)

The nucleotide sequences of the cDNAs can be determined by digesting the selected cDNAs with appropriate restriction enzymes, cloning the fragments into a plasmid

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such as pBluescript SK(-) (manufactured by Stratagene) or the like, carrying out the reaction of a generally used nucleotide sequence analyzing method such as the dideoxy method [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)] of Sanger *et al.* or the like and then analyzing the clones using an automatic nucleotide sequence analyzer such as A.L.F. DNA Sequencer (manufactured by Pharmacia) or the like.

(0151)

Whether or not the obtained cDNAs are encoding the full amino acid sequences of the H chain and L chain V regions of the antibody containing a secretory signal sequence can be confirmed by deducing the full amino acid sequences of the H chain and L chain V regions from the determined nucleotide sequence and comparing them with the full amino acid sequences of the H chain and L chain V regions of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)].

(0152)

(3) Analysis of amino acid sequence of V region of antibody derived from animal other than human

Regarding the full amino acid sequences of the H chain and L chain V regions of the antibody containing a secretory signal sequence, the length of the secretory signal sequence and the *N*-terminal amino acid sequences can be deduced and subgroups to which they belong can also be found, by comparing them with the full amino acid sequences of the H chain and L chain V regions of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services, (1991)]. In addition, the amino acid sequences of the H chain and L chain V regions of each CDR can also be found by comparing them with the amino acid sequences of the H chain and L chain V regions of known

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antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services, (1991)].

(0153)

(4) Construction of human chimeric antibody expression vector

A human chimeric antibody expression vector can be constructed by cloning cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human into upstream of genes encoding the H chain and L chain C regions of a human antibody in the vector for humanized antibody expression constructed in the item 3(1). For example, a human chimeric antibody expression vector can be constructed by linking each of cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human to a synthetic DNA comprising nucleotide sequences at the 3'-terminals of the H chain and L chain V regions of an antibody derived from an animal other than human and nucleotide sequences at the 5'-terminals of the H chain and L chain C regions of a human antibody and also having a recognition sequence of an appropriate restriction enzyme at both terminals, and by cloning them into upstream of genes encoding the H chain and L chain C regions of a human antibody contained in the vector for humanized antibody expression constructed described in the item 3(1).

(0154)

(5) Construction of cDNA encoding V region of human CDR-grafted antibody

cDNAs encoding the H chain and L chain V regions of a human CDR-grafted antibody can be obtained as follows. First, amino acid sequences of the frameworks (hereinafter referred to as "FR") of the H chain and L chain V regions of a human antibody for grafting CDR of the H chain and L chain V regions of an antibody derived from an animal other than human is selected. As the amino acid sequences of FRs

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of the H chain and L chain V regions of a human antibody, any amino acid sequences can be used so long as they are derived from a human antibody. Examples include amino acid sequences of FRs of the H chain and L chain V regions of human antibodies registered at databases such as Protein Data Bank, *etc.*, amino acid sequences common in each subgroup of FRs of the H chain and L chain V regions of human antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)] and the like. But in order to produce a human CDR-grafted antibody having potent activity, it is preferable to select an amino acid sequence having a homology as high as possible (at least 60% or more) with amino acid sequences of the H chain and L chain V regions of an antibody of interest derived from an animal other than human.

(0155)

Next, the amino acid sequences of CDRs of the H chain and L chain V regions of the antibody of interest derived from an animal other than human are grafted to the selected amino acid sequences of FRs of the H chain and L chain V regions of a human antibody to design amino acid sequences of the H chain and L chain V regions of the human CDR-grafted antibody. The designed amino acid sequences are converted into DNA sequences by considering the frequency of codon usage found in nucleotide sequences of antibody genes [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)], and the DNA sequences encoding the amino acid sequences of the H chain and L chain V regions of the human CDR-grafted antibody are designed. Based on the designed DNA sequences, several synthetic DNA fragments having a length of about 100 bases are synthesized, and PCR is carried out using them. In this case, it is preferable in each of the H chain and the L chain that 6 synthetic DNAs are designed in

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view of the reaction efficiency of PCR and the lengths of DNAs which can be synthesized.

(0156)

Also, they can be easily cloned into the vector for humanized antibody expression constructed in the item 3(1) by introducing recognition sequences of an appropriate restriction enzyme into the 5'-terminals of the synthetic DNA present on both terminals. After the PCR, the amplified product is cloned into a plasmid such as pBluescript SK(-) (manufactured by Stratagene) or the like and the nucleotide sequences are determined by the method in the item 3(2) to thereby obtain a plasmid having DNA sequences encoding the amino acid sequences of the H chain and L chain V regions of the desired human CDR-grafted antibody.

(0157)

(6) Construction of human CDR-grafted antibody expression vector

A human CDR-grafted antibody expression vector can be constructed by cloning the cDNAs encoding the H chain and L chain V regions of the human CDR-grafted antibody constructed in the item 3(5) into upstream of the gene encoding H chain and L chain C regions of a human antibody in the vector for humanized antibody expression described in the item 3(1). For example, the human CDR-grafted antibody expression vector can be constructed by introducing recognizing sequences of an appropriate restriction enzyme into the 5'-terminals of both terminals of a synthetic DNA fragment, among the synthetic DNA fragments which are used when PCR is carried out in the item 3(5) for constructing the H chain and L chain V regions of the human CDR-grafted antibody, so that they are cloned into upstream of the genes encoding the H chain and L chain C regions of a human antibody in the vector for

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humanized antibody expression described in the item 3(1) in such a manner that they can be expressed in a suitable form.
(0158)
(7) Stable production of humanized antibody

A transformant capable of stably producing a human chimeric antibody and a human CDR-grafted antibody (both hereinafter referred to as "humanized antibody") can be obtained by introducing the humanized antibody expression vectors described in the items 3(4) and (6) into an appropriate animal cell.

Examples of the method for introducing a humanized antibody expression vector into an animal cell include electroporation [Japanese Published Examined Patent Application No. 257891/90, *Cytotechnology*, 3, 133 (1990)] and the like.

As the animal cell into which a humanized antibody expression vector is introduced, any cell can be used so long as it is an animal cell which can produce the humanized antibody.

Examples include mouse myeloma cells such as NSO cell and SP2/0 cell, Chinese hamster ovary cells such as CHO/dhfr$^-$ cell and CHO/DG44 cell, rat myeloma such as YB2/0 cell and IR983F cell, BHK cell derived from a syrian hamster kidney, a human myeloma cell such as Namalwa cell, and the like, and a Chinese hamster ovary cell CHO/DG44 cell, a rat myeloma YB2/0 cell and the host cells of the present invention described in the item 5 are preferred.
(0159)

After introduction of the humanized antibody expression vector, a transformant capable of stably producing the humanized antibody can be selected using a medium for animal cell culture comprising an agent such as G418 sulfate (hereinafter referred to as "G418"; manufactured by SIGMA) and the like in accordance with the method disclosed in Japanese Published Examined Patent

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Application No. 257891/90. Examples of the medium for animal cell culture include RPMI 1640 medium (manufactured by Nissui Pharmaceutical), GIT medium (manufactured by Nihon Pharmaceutical), EX-CELL 302 medium (manufactured by JRH), IMDM medium (manufactured by GIBCO BRL), Hybridoma-SFM medium (manufactured by GIBCO BRL) media obtained by adding various additives such as fetal bovine serum (hereinafter referred to as "FBS") to these media, and the like. The humanized antibody can be produced and accumulated in the culture supernatant by culturing the obtained transformant in a medium. The expression level and antigen binding activity of the humanized antibody in the culture supernatant can be measured by a method such as enzyme-linked immunosorbent assay [hereinafter referred to as "ELISA"; *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1998), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)] or the like. Also, the expression level of the humanized antibody by the transformant can be increased using a DHFR gene amplification system in accordance with the method disclosed in Japanese Published Examined Patent Application No. 257891/90.

(0160)

The humanized antibody can be purified from a culture supernatant of the transformant using a protein A column [*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 8 (1988), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)]. In addition, purification methods generally used for the purification of proteins can also be used. For example, the purification can be carried out through the combination of a gel filtration, an ion exchange chromatography and an ultrafiltration. The molecular weight of the H chain, L chain or antibody molecule as a whole of the purified humanized antibody can be measured, e.g., by polyacrylamide

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gel electrophoresis [hereinafter referred to as "SDS-PAGE"; *Nature*, 227, 680 (1970)], Western blotting [*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 12, (1988), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)] or the like.

Thus, methods for producing an immunologically functional molecule using an animal cell as the host have been described, but, as described above, the immunologically functional molecule can also be produced by a yeast, an insect cell, a plant cell, an animal individual or a plant individual by the same methods on the animal cell.

(0161)

4. Production of immunologically functional molecule using cell of the present invention

The immunologically functional molecule of the present invention can be produced by preparing a cell expressing an antibody molecule using the method described in the item 1, culturing the cell using the method for preparing the immunologically functional molecule described in the item 3 and then purifying the immunologically functional molecule of interest from the resulting culture.

When a host cell has the ability to express an antibody molecule innately, it is not introduce the immunologically functional molecule of the present invention into the cell, and the immunologically functional molecule can be produced by preparing a cell expressing an antibody molecule using host cell as the parent cell line according to the method described in the item 1, culturing the cell using the method for preparing the immunologically functional molecule described in the item 3 and then purifying the immunologically functional molecule of interest from the resulting culture.

(0162)

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45. Activity evaluation of immunologically functional molecule

As the method for measuring the amount of the purified immunologically functional molecule, the activity to bind to an antibody and the effector function of the purified immunologically functional molecule, the known method described in *Monoclonal Antibodies, Antibody Engineering* and the like can be used. As the examples, when the immunologically functional molecule is a humanized antibody, the binding activity with an antigen and the binding activity with an antigen-positive cultured cell line can be measured by methods such as ELISA, an immunofluorescent method [*Cancer Immunol. Immunother.*, 36, 373 (1993)] and the like. The cytotoxic activity against an antigen-positive cultured cell line can be evaluated by measuring CDC activity, ADCC activity [*Cancer Immunol. Immunother.*, 36, 373 (1993)] and the like. Also, safety and therapeutic effect of the immunologically functional molecule in human can be evaluated using an appropriate model of animal species relatively close to human, such as *Macaca fascicularis* or the like.

(0163)

6. Analysis of sugar chains binding to antibody molecules expressed in various cells

The sugar chain structure binding to an antibody molecule expressed in various cells can be analyzed in accordance with the general analysis of the sugar chain structure of a glycoprotein. For example, the sugar chain which is bound to IgG molecule comprises a neutral sugar such as galactose, mannose, fucose or the like, an amino sugar such as *N*-acetylglucosamine or the like and an acidic sugar such as sialic acid or the like, and can be analyzed by a method such as a sugar chain structure analysis or the like using sugar composition analysis, two dimensional sugar chain mapping or the like.

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(0164)

(1) Analysis of neutral sugar and amino sugar compositions

The sugar chain composition binding to an antibody molecule can be analyzed by carrying out acid hydrolysis of sugar chains with an acid such as trifluoroacetic acid or the like to release a neutral sugar or an amino sugar and measuring the composition ratio.

Example include a method using a sugar composition analyzer (BioLC) manufactured by Dionex. The BioLC is an apparatus which analyzes a sugar composition by HPAEC-PAD (high performance anion-exchange chromatography-pulsed amperometric detection) [*J. Liq. Chromatogr.*, 6, 1577 (1983)].

The composition ratio can also be analyzed by a fluorescence labeling method using 2-aminopyridine. Specifically, the compositional ratio can be calculated in accordance with a known method [*Agric. Biol. Chem.*, 55(1), 283-284 (1991)], by labeling an acid-hydrolyzed sample with a fluorescence with 2-aminopyridylation and then analyzing the composition by HPLC.

(0165)

(2) Analysis of sugar chain structure

The sugar chain structure binding to an antibody molecule can be analyzed by the two dimensional sugar chain mapping method [*Anal. Biochem.*, 171, 73 (1988), *Biochemical Experimentation Methods 23 - Methods for Studying Glycoprotein Sugar Chains* (Japan Scientific Societies Press) edited by Reiko Takahashi (1989)]. The two dimensional sugar chain mapping method is a method for deducing a sugar chain structure by, e.g., plotting the retention time or elution position of a sugar chain by reverse phase chromatography as the X axis and the retention time or elution position of the sugar chain by normal phase chromatography as the Y axis, respectively, and comparing them with such results of known sugar chains.

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(0166)

Specifically, sugar chains are released from an antibody by subjecting the antibody to hydrazinolysis, and the released sugar chain is subjected to fluorescence labeling with 2-aminopyridine (hereinafter referred to as "PA") [*J. Biochem.*, 95, 197 (1984)], and then the sugar chains are separated from an excess PA-treating reagent by gel filtration, and subjected to reverse phase chromatography. Thereafter, each peak of the separated sugar chains are subjected to normal phase chromatography. The sugar chain structure can be deduced by plotting the results on a two dimensional sugar chain map and comparing them with the spots of a sugar chain standard (manufactured by Takara Shuzo) or a literature [*Anal. Biochem.*, 171, 73 (1988)].

The structure deduced by the two dimensional sugar chain mapping method can be confirmed by further carrying out mass spectrometry such as MALDI-TOF-MS of each sugar chain or the like.

(0167)

7. Comparison of activity of enzyme relating to modification of sugar chain in various host cells

The activity of an enzyme relating to the modification of a sugar chain, that is, a glycosyltransferase and a glycolytic enzyme, in various host cells can be carried out by biochemical methods or genetic engineering techniques described in *New Biochemical Experimentation Series 3-Saccharides I, Glycoprotein* (Tokyo Kagaku Dojin), edited by Japanese Biochemical society (1988); *Cell Engineering, Supplement, Experimental Protocol Series, Glycobiology Experimental Protocol, Glycoprotein, Glycolipid and Proteoglycan* (Shujun-sha), edited by Naoyuki Taniguchi, Akemi Suzuki, Kiyoshi Furukawa and Kazuyuki Sugawara (1996);, *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; and the like.

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Examples of the biochemical method include a method in which the enzyme activity is evaluated using an enzyme-specific substrate and the like. Examples of the genetic engineering technique include the Northern analysis, RT-PCR and the like which measures the amount of mRNA of a gene encoding the enzyme.

(0186)

8. Immunological determination method for discriminating sugar chain structure of antibody molecule

An immunologically functional molecule comprises an antibody molecule in which sugar chains binding to the Fc region of the antibody are different in structure. The immunologically functional molecule in which the ratio of a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end in the sugar chain is 20% or more among the total complex *N*-glycoside-linked sugar chains binding to the Fc region in the immunologically functional molecule reducing end has potent ADCC activity. The immunologically functional molecule can be identified by using the method for analyzing the sugar chain structure of an antibody molecule described in the item 6. Also, it can also be identified by an Immunological determination method using a lectin.

The sugar chain structure of an antibody molecule can be identified by the Immunological determination method using a lectin in accordance with the known Immunological determination method such as Western staining, IRA (radioimmunoassay), VIA (viroimmunoassay), EIA (enzymoimmunoassay), FIA (fluoroimmunoassay), MIA (metalloimmunoassay) and the like described in *Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc. (1995); Immunoassay, 3rd Ed., Igakushoin (1987); *Enzyme Antibody Method*, Revised Edition, Gakusai Kikaku (1985); and the like.

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A lectin which recognizes the sugar chain structure of an antibody molecule comprised in an immunologically functional molecule is labeled, and the labeled lectin is allowed to react with an immunologically functional molecule which is a sample. Then, the amount of the complex of the labeled lectin with the antibody molecule is measured.

Examples of the lectin used for identifying the sugar chain structure of an antibody molecule include WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (cocanavalin A derived from *C. ensiformis*), RIC (a toxin derived from *R. communis*), L-PHA (leucoagglutinin derived from *P. vulgaris*), LCA (lentil agglutinin derived from *L. culinaris*), PSA (pea lectin derived from *P. sativum*), AAL (*Aleuria aurantia* lectin), ACL (*Amaranthus caudatus* lectin), BPL (*Bauhinia purpurea* lectin), DSL (*Datura stramonium* lectin), DBA (*Dolichos biflorus* agglutinin), EBL (elderberry balk lectin), ECL (*Erythrina cristagalli* lectin), EEL (*Euonymus eoropaeus* lectin), GNL (*Galanthus nivalis* lectin), GSL (*Griffonia simplicifolia* lectin), HPA (*Helix pomatia* agglutinin), HHL (*Hippeastrum* hybrid lectin), Jacalin, LTL (*Lotus tetragonolobus* lectin), LEL (*Lycopersicon esculentum* lectin), MAL (*Maackia amurensis* lectin), MPL (*Maclura pomifera* lectin), NPL (*Narcissus pseudonarcissus* lectin), PNA (peanut agglutinin), E-PHA (*Phaseolus vulgaris* erythroagglutinin), PTL (*Psophocarpus tetragonolobus* lectin), RCA (*Ricinus communis* agglutinin), STL (*Solanum tuberosum* lectin), SJA (*Sophora japonica* agglutinin), SBA (soybean agglutinin), UEA (*Ulex europaeus* agglutinin), VVL (*Vicia villosa* lectin) and WFA (*Wisteria floribunda* agglutinin).

It is preferable to use a lectin which specifically recognizes a sugar chain structure wherein fucose binds to the *N*-acetylglucosamine in the reducing end in the complex *N*-glycoside-linked sugar chain. Examples include *Lens*

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culinaris lectin LCA (lentil agglutinin derived from *Lens culinaris*), pea lectin PSA (pea lectin derived from *Pisum sativum*), broad bean lectin VFA (agglutinin derived from *Vicia faba*) and *Aleuria aurantia* lectin AAL (lectin derived from *Aleuria aurantia*).

(0186)

8. Method for preparing medicament having desired effector function by changing ratio of *N*-glycoside-linked sugar chain in which sugar is not bound to *N*-acetylglucamine in reducing end in composition comprising immunologically functional molecule having *N*-glycoside-linked sugar chain

The immunologically functional molecule having an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the sugar chain reducing end can be prepared, for example, by the production method of the immunologically functional molecule of the present invention described in the item 4, and a medicament having a desired effector function can be prepared by changing the ratio of the *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the sugar chain reducing end using the obtained a composition comprising the immunologically functional molecule as a material. By changing the ratio of the *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetyglucosamine in the sugar chain reducing end, for example, an immunologically functional molecular composition having an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the sugar chain reducing end can be purified, and a medicament composition having any ratio by adding the purified immunologically functional molecule composition.

(0169)

9. Method for screening cell in which activity of enzyme relating to sugar chain in glycoprotein is controlled by artificial technique

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The screening method of the present invention comprises selecting a desired mutant as a measure of the activity of an enzyme relating to the modification of a glycoprotein or the sugar chain structure of a glycoprotein.

Specifically, the mutant can be screened as a measure of the activity of an enzyme relating to the modification of a glycoprotein or the sugar chain structure of a glycoprotein, and, for example, the desired mutant is selected by the method described in the item 1(2).

(0170)

10. Application of immunologically functional molecule of the present invention

The immunologically functional molecule of the present invention has potent antibody-dependent cell-mediated cytotoxic activity. An antibody having potent antibody-dependent cell-mediated cytotoxic activity is useful for preventing and treating various diseases including cancers, inflammatory diseases, immune diseases such as autoimmune diseases, allergies and the like, circulatory organ diseases and viral or bacterial infections.

In the case of cancers, namely malignant tumors, cancer cells grow. General anti-tumor agents inhibit the growth of cancer cells. In contrast, an antibody having potent antibody-dependent cell-mediated cytotoxic activity can treat cancers by injuring cancer cells through its cell killing effect, and therefore, it is more effective as a therapeutic agent than the general anti-tumor agents. At present, in the therapeutic agent for cancers, an anti-tumor effect of an antibody medicament alone is insufficient so that combination therapy with chemotherapy has been carried out [*Science*, 280, 1197 (1998)]. If more potent anti-tumor effect is found by the immunologically functional molecule of the present invention alone, the

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dependency on chemotherapy will be decreased and side effects will be reduced.

(0171)

In allergies, *in vivo* reactions of the diseases are induced by the release of a mediator molecule by immunocytes, so that the allergy reaction can be inhibited by eliminating immunocytes using an antibody having potent ADCC or CDC activity.

(0172)

Examples of the circulatory organ diseases include arteriosclerosis and the like. The arteriosclerosis is treated using balloon catheter at present, but circulatory organ diseases can be prevented and treated by inhibiting growth of arterial cells in restricture after treatment using an antibody having potent antibody-dependent cell-mediated cytotoxic activity.

(0173)

Various diseases including viral and bacterial infections can be prevented and treated by inhibiting proliferation of cells infected with a virus or bacterium using an antibody having potent ADCC or CDC activity.

Also, an antibody in which ADCC or CDC activity is inhibited is useful for preventing or treating autoimmune diseases in view of that the accelerated immune reaction in the autoimmune diseases can be inhibited. Also, they are useful for immunosuppressant agents, inhibition of unnecessary cell death, prevention and treatment of blood cancer. Furthermore, in pharmaceuticals in which activity other than the effector function of the immunologically functional molecule, for example, neutralizing activity against a recognized antigen molecule, side effects by potent effector function can be inhibited.

(0174)

The medicament comprising the immunologically functional molecule (antibody, etc.) of the present

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invention can be administered as a therapeutic agent alone, but generally, it is preferable to provide it as a pharmaceutical formulation produced by an appropriate method well known in the technical field of manufacturing pharmacy, by mixing it with at least one pharmaceutically acceptable carrier.

It is desirable to select a route of administration which is most effective in treatment. Examples include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular, intravenous or the like. In an antibody preparation, intravenous administration is preferable.

The dosage form includes sprays, capsules, tablets, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

Examples of the pharmaceutical preparation suitable for oral administration include emulsions, syrups, capsules, tablets, powders, granules and the like.

(0175)

Liquid preparations, such as emulsions and syrups, can be produced using, as additives, water; saccharides, such as sucrose, sorbitol, fructose, *etc.*; glycols, such as polyethylene glycol, propylene glycol, *etc.*; oils, such as sesame oil, olive oil, soybean oil, *etc.*; antiseptics, such as p-hydroxybenzoic acid esters, *etc.*; flavors, such as strawberry flavor, peppermint, *etc.*; and the like.

Capsules, tablets, powders, granules and the like can be produced using, as additive, fillers, such as lactose, glucose, sucrose, mannitol, *etc.*; disintegrating agents, such as starch, sodium alginate, *etc.*; lubricants, such as magnesium stearate, talc, *etc.*; binders, such as polyvinyl alcohol, hydroxypropylcellulose, gelatin, *etc.*; surfactants, such as fatty acid ester, *etc.*; plasticizers, such as glycerine, *etc.*; and the like.

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Examples of the pharmaceutical preparation suitable for parenteral administration include injections, suppositories, sprays and the like.

Injections may be prepared using a carrier, such as a salt solution, a glucose solution, a mixture of both thereof or the like. Also, powdered injections can be prepared by freeze-drying the immunologically functional molecule in the usual way and adding sodium chloride thereto.

(0176)

Suppositories may be prepared using a carrier such as cacao butter, hydrogenated fat, carboxylic acid or the like.

Also, sprays may be prepared using the immunologically functional molecule as such or using a carrier which does not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the immunologically functional molecule by dispersing it as fine particles.

Examples of the carrier include lactose, glycerol and the like. Depending on the properties of the immunologically functional molecule and the carrier, it is possible to produce pharmaceutical preparations such as aerosols, dry powders and the like. In addition, the components exemplified as additives for oral preparations can also be added to the parenteral preparations.

(0177)

Although the clinical dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually 10 μg/kg to 20 mg/kg per day and per adult.

Also, as the method for examining antitumor effect of the immunologically functional molecule against various tumor cells, *in vitro* tests include CDC activity measuring

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method, ADCC activity measuring method and the like, and *in vivo* tests include antitumor experiments using a tumor system in an experimental animal such as a mouse, *etc.* and the like.

CDC activity and ADCC activity measurements and antitumor experiments can be carried out in accordance with the methods described in *Cancer Immunology Immunotherapy*, 36, 373 (1993); *Cancer Research*, 54, 1511 (1994) and the like.

The present invention will be described below in detail based on Examples; however, Examples are only simple illustrations, and the scope of the present invention is not limited thereto.

(0178)

(Examples)

Example 1

Production of anti-ganglioside GD3 human chimeric antibody:

1. Construction of tandem expression vector pChiLHGM4 for anti-ganglioside GD3 human chimeric antibody

A plasmid pChi641LGM40 was constructed by ligating a fragment of about 4.03 kb containing an L chain cDNA, obtained by digesting an L chain expression vector, pChi641LGM4 [*J. Immunol. Methods*, 167, 271 (1994)] for anti-ganglioside GD3 human chimeric antibody (hereinafter referred to as "anti-GD3 chimeric antibody") with restriction enzymes *Mlu*I (manufactured by Takara Shuzo) and *Sal*I (manufactured by Takara Shuzo) with a fragment of about 3.40 kb containing a G418-resistant gene and a splicing signal, obtained by digesting an expression vector pAGE107 [*Cytotechnology*, 3, 133 (1990)] for animal cell with restriction enzymes *Mlu*I (manufactured by Takara Shuzo) and *Sal*I (manufactured by Takara Shuzo) using DNA Ligation Kit (manufactured by Takara Shuzo), and then transforming *E. coli* HB101 (*Molecular Cloning*, Second Edition) with the ligated product.

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(0179)

Next, a fragment of about 5.68 kb containing an L chain cDNA, obtained by digesting the constructed plasmid pChi641LGM40 with a restriction enzyme *Cla*I (manufactured by Takara Shuzo), blunt-terminating it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with *Mlu*I (manufactured by Takara Shuzo), was ligated with a fragment of about 8.40 kb containing an H chain cDNA, obtained by digesting an anti-GD3 chimeric antibody H chain expression vector, pChi641HGM4 [*J. Immunol. Methods*, 167, 271 (1994)] with a restriction enzyme, *Xho*I (manufactured by Takara Shuzo), blunt-terminating it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with *Mlu*I (manufactured by Takara Shuzo), using DNA Ligation Kit (manufactured by Takara Shuzo), and then *E. coli* HB101 (*Molecular Cloning*, Second Edition) was transformed with the ligated product to thereby construct a tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody.

(0180)

2. Preparation of cells stably producing anti-GD3 chimeric antibody

Cells capable of stably producing an anti-GD3 chimeric antibody were prepared using the tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody constructed in the item 1 of Example 1, as described below.

(1) Preparation of antibody-producing cell using rat myeloma YB2/0 cell

After introducing 5 μg of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into 4×10^6 cells of rat myeloma YB2/0 [ATCC CRL-1662, J.V. Kilmarin *et al.*, *J. Cell. Biol.*, 93, 576-582 (1982)] by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of RPMI1640-FBS(10) (RPMI1640 medium comprising 10% FBS (manufactured by GIBCO BRL)) and dispensed in 200

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μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24 hours in a 5% CO_2 incubator, G418 was added to a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

(0181)

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 50 nM DHFR inhibitor, methotrexate (hereinafter referred to as "MTX"; manufactured by SIGMA) to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 200 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount were finally obtained by the same method as described above. Among the obtained transformants,

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suitable cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0182)

The obtained anti-GD3 chimeric antibody-producing transformed cell clone 7-9-51 has been deposited on April 5, 1999, as FERM BP-6691 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan).

(0183)

(2) Preparation of antibody-producing cell using CHO/DG44 cell

After introducing 4 μg of the anti-GD3 chimeric antibody expression vector, pChi641LHGM4, into 1.6×10^6 cells of CHO/DG44 [G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA*, 77, 4216-4220 (1980)] by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-FBS(10) [IMDM medium comprising 10% FBS and 1× concentration of HT supplement (manufactured by GIBCO BRL)] and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing them at 37°C for 24 hours in a 5% CO_2 incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

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(0184)

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an IMDM-dFBS(10) medium [IMDM medium comprising 10% dialyzed fetal bovine serum (hereinafter referred to as "dFBS"; manufactured by GIBCO BRL)] comprising 0.5 mg/ml G418 and 10 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. Regarding the transformants in wells in which their growth was observed, the MTX concentration was increased to 100 nM, and transformants capable of growing in the IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 100 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount were finally obtained by the same method as described above. Among the obtained transformants, suitable cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0185)

(3) Preparation of antibody-producing cell using mouse myeloma NS0 cell

After introducing 5 μg of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into 4×10^6 cells of mouse myeloma NS0 by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of EX-CELL302-FBS(10) (EX-CELL302 medium comprising 10% FBS and 2

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mM L-glutamine [hereinafter referred to as "L-Gln"; manufactured by GIBCO BRL)] and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24 hours in a 5% CO_2 incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

(0186)

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an EX-CELL302-dFBS(10) medium (EX-CELL302 medium comprising 10% dFBS and 2 mM L-Gln) comprising 0.5 mg/ml G418 and 50 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the EX-CELL302-dFBS(10) medium comprising 0.5 mg/ml G418 and 200 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount was finally obtained by the same

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method as described above. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0187)

3. Measurement of binding activity of antibody to GD3 (ELISA)

The binding activity of the antibody to GD3 was measured as described below.

In 2 ml of ethanol solution containing 10 μg of dipalmitoylphosphatidylcholine (manufactured by SIGMA) and 5 μg of cholesterol (manufactured by SIGMA), 4 nmol of GD3 was dissolved. Into each well of a 96 well plate for ELISA (manufactured by Greiner), 20 μl of the solution (40 pmol/well in final concentration) was dispensed, followed by air-drying, 1% bovine serum albumin (hereinafter referred to as "BSA"; manufactured by SIGMA)-containing PBS (hereinafter referred to as "1% BSA-PBS") was dispensed in 100 μl/well, and then the reaction was carried out at room temperature for 1 hour for blocking remaining active groups. After discarding 1% BSA-PBS, a culture supernatant of a transformant or a diluted solution of a human chimeric antibody was dispensed in 50 μl/well to carry out the reaction at room temperature for 1 hour. After the reaction, each well was washed with 0.05% Tween 20 (manufactured by Wako Pure Chemical Industries)-containing PBS (hereinafter referred to as "Tween-PBS"), a peroxidase-labeled goat anti-human IgG (H & L) antibody solution (manufactured by American Qualex) diluted 3,000 times with 1% BSA-PBS was dispensed in 50 μl/well as a secondary antibody solution, and then the reaction was carried out at

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room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, ABTS substrate solution [solution prepared by dissolving 0.55 g of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt in 1 liter of 0.1 M citrate buffer (pH 4.2) and adding 1 μl/ml of hydrogen peroxide to the solution just before use (hereinafter the same solution was used)] was dispensed in 50 μl/well for color development, and then absorbance at 415 nm (hereinafter referred to as "OD415") was measured.

(0188)

4. Purification of anti-GD3 chimeric antibody

(1) Culturing of antibody-producing cell derived from YB2/0 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(1) of Example 1 was suspended in the Hybridoma-SFM medium comprising 0.2% BSA, 200 nM MTX and 100 nM triiodothyronine (hereinafter referred to as "T3"; manufactured by SIGMA) to give a density of 3×10^5 cells/ml and cultured using a 2.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 50 rpm. After culturing them at 37°C for 10 days in a temperature-controlling room, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named YB2/0-GD3 chimeric antibody.

(0189)

(2) Culturing of antibody-producing cell derived from CHO/DG44 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(2) of Example 1 was suspended in the EX-CELL302 medium comprising 3 mM L-Gln, 0.5% fatty acid concentrated solution (hereinafter

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referred to as "CDLC"; manufactured by GIBCO BRL) and 0.3% Pluronic F68 (hereinafter referred to as "PF68"; manufactured by GIBCO BRL) to give a density of 1×10^6 cells/ml, and the suspension was dispensed in 50 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 4 days in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named CHO/DG44-GD3 chimeric antibody. (0190)

(3) Culturing of antibody-producing cell derived from NS0 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(3) of Example 1 was suspended in the EX-CELL302 medium comprising 2 mM L-Gln, 0.5 mg/ml G418, 200 nM MTX and 1% FBS, to give a density of 1×10^6 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 4 days in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (302).

Also, the transformed cell clone was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 10 days in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by

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Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (GIT).

(0191)

(4) Culturing of antibody-producing cell derived from SP2/0 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone (KM-871 (FERM BP-3512)) described in Japanese Published Unexamined Patent Application No. 304989/93 (EP 533199) was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 8 days in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named SP2/0-GD3 chimeric antibody.

(0192)

5. Analysis of purified anti-GD3 chimeric antibody

In accordance with a known method [*Nature*, 227, 680 (1970)], 4 μg of each of the five kinds of the anti-GD3 chimeric antibodies produced by and purified from respective animal cells, obtained in the item 4 of Example 1, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. The results are shown in Fig. 1. As shown in Fig. 1, a single band of about 150 kilodaltons (hereinafter referred to as "Kd") in molecular weight was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd under reducing conditions, in each of the purified anti-GD3 chimeric antibodies. The molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide

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sequences of H chain and L chain of the antibody (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule: about 144 Kd), and also coincided with the reports stating that the IgG antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chains having a molecular weight of about 50 Kd and L chains having a molecular weight of about 25 Kd under reducing conditions due to cutting of the disulfide bond (hereinafter referred to as "S-S bond") in the molecule [*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1998); *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)], so that it was confirmed that each anti-GD3 chimeric antibody was expressed and purified as an antibody molecule having the true structure.

(0193)

Example 2

Activity evaluation of anti-GD3 chimeric antibody:

1. Binding activity of anti-GD3 chimeric antibody to GD3 (ELISA)

The activity of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1 to bind to GD3 (manufactured by Snow Brand Milk Products) was measured by the ELISA shown in the item 3 of Example 1. Fig. 2 shows a result of the examination of the binding activity measured by changing the concentration of the anti-GD3 chimeric antibody to be added. As shown in Fig. 2, the five kinds of the anti-GD3 chimeric antibodies showed almost the same binding activity to GD3. The result shows that antigen binding activities of these antibodies are constant independently of the antibody-producing animal cells and their culturing methods. Also, it was suggested from the comparison of the NS0-GD3 chimeric antibody (302) with the NS0-GD3 chimeric antibody (GIT) that the antigen

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binding activities are constant independently of the media used in the culturing.

(0194)

2. *In vitro* cytotoxic activity (ADCC activity) of anti-GD3 chimeric antibody

In order to evaluate *in vitro* cytotoxic activity of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1, the ADCC activity was measured in accordance with the following method.

(1) Preparation of target cell solution

A human melanoma cultured cell line G-361 (ATCC CRL 1424) was cultured using the RPMI1640-FBS(10) medium to prepare 1×10^6 cells, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance $Na_2{}^{51}CrO_4$ at 37°C for 1 hour. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, re-suspended in the medium and then incubated at 4°C for 30 minutes in ice for spontaneous dissolution of the radioactive substance. After centrifugation, the precipitate was adjusted to 2×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

(0195)

(2) Preparation of effector cell solution

From a healthy person, 50 ml of venous blood was collected, and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). The mixture was centrifuged to isolate a mononuclear cell layer using Lymphoprep (manufactured by Nycomed Pharma AS) in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting precipitate was re-suspended to give a density of 2×10^6 cells/ml using the medium and used as the effector cell solution.

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(0196)

(3) Measurement of ADCC activity

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50 μl of the target cell solution prepared in the above (1) (1×10^4 cells/well) was dispensed. Next, 100 μl of the effector cell solution prepared in the above (2) was added thereto (2×10^5 cells/well, the ratio of effector cells to target cells becomes 20:1). Subsequently, each of the anti-GD3 chimeric antibodies was added to give a final concentration from 0.0025 to 2.5 μg/ml, followed by reaction at 37°C for 4 hours. After the reaction, the plate was centrifuged, and the amount of ^{51}Cr in the supernatant was measured using a γ-counter. The amount of spontaneously released ^{51}Cr was calculated by the same operation using only the medium instead of the effector cell solution and the antibody solution, and measuring the amount of ^{51}Cr in the supernatant. The amount of total released ^{51}Cr was calculated by the same operation using only the medium instead of the antibody solution and adding 1 N hydrochloric acid instead of the effector cell solution, and measuring the amount of ^{51}Cr in the supernatant. The ADCC activity was calculated from the following equation (I):

(0197)

Equation (I)

$$\text{ADCC activity (\%)} = \frac{^{51}\text{Cr in sample supernatant - spontaneously released } ^{51}\text{Cr}}{\text{total released } ^{51}\text{Cr - spontaneously released } ^{51}\text{Cr}} \times 100 \quad \text{(II)}$$

(0198)

The results are shown in Fig. 3. As shown in Fig. 3, among the five kinds of the anti-GD3 chimeric antibodies, the YB2/0-GD3 chimeric antibody showed the most potent ADCC activity, followed by the SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody and CHO-GD3 chimeric

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antibody in that order. No difference in the ADCC activity was found between the NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT) prepared using different media in the culturing. The above results show that the ADCC activity of antibodies greatly varies depending on the kind of the animal cells to be used in their production. As its mechanism, since their antigen binding activities were identical, it was considered that it is caused by a difference in the structure binding to the antibody Fc region.

(0199)

Example 3

Preparation of anti-human interleukin 5 receptor α chain human CDR-grafted antibody:

1. Preparation of cell stably producing anti-human interleukin 5 receptor α chain human CDR-grafted antibody

(1) Preparation of antibody-producing cell using rat myeloma YB2/0 cell

Using the anti-human interleukin 5 receptor α chain human CDR-grafted antibody (hereinafter referred to as "anti-hIL-5Rα CDR-grafted antibody") expression vector, pKANTEX1259HV3LV0, described in WO 97/10354, cells capable of stably producing anti-hIL-5Rα CDR-grafted antibody were prepared as described below.

After introducing 5 μg of the anti-hIL-5Rα CDR-grafted antibody expression vector pKANTEX1259HV3LV0 into 4×10^6 cells of rat myeloma YB2/0 by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of RPMI1640-FBS(10) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24 hours in a 5% CO_2 incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were

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formed and growth of colonies was observed, and the antigen binding activity of the anti-hIL-5Rα CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

(0200)

Regarding the transformants in wells in which production of the anti-hIL-5Rα CDR-grafted antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of the them was suspended in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 50 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-hIL-5Rα CDR-grafted antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 2 of Example 3. Regarding the transformants in wells in which production of the anti-hIL-5Rα CDR-grafted antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 200 nM MTX and of producing the anti-hIL-5Rα CDR-grafted antibody in a large amount were finally obtained in the same manner as described above. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line. The obtained anti-hIL-5Rα CDR-grafted antibody-producing

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transformed cell clone No. 3 has been deposited on April 5, 1999, as FERM BP-6690 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan).

(0201)

(2) Preparation of antibody-producing cell using CHO/dhfr$^-$ cell

After introducing 4 μg of the anti-hIL-5Rα CDR-grafted antibody expression vector pKANTEX1259HV3LV0 described in WO 97/10354 into 1.6×10^6 cells of CHO/dhfr$^-$ by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-FBS(10) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing them at 37°C for 24 hours in a 5% CO_2 incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-hIL-5Rα CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

(0202)

Regarding the transformants in wells in which production of the anti-hIL-5Rα CDR-grafted antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of the transformants was suspended in an IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 10 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO_2 incubator. Regarding the transformants in wells in which their growth

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was observed, the MTX concentration was increased to 100 nM and then to 500 nM, and transformants capable of growing in the IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 500 nM MTX and of producing the anti-hIL-5Rα CDR-grafted antibody in a large amount were finally obtained in the same manner as described above. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0203)

(3) Production of antibody-producing cell using mouse myeloma NS0 cell

An anti-hIL-5Rα CDR-grafted antibody expression vector was prepared in accordance with the method of Yarranton *et al.* [*BIO/TECHNOLOGY*, 10, 169 (1992)] and using the antibody H chain cDNA and L chain cDNA on the anti-hIL-5Rα CDR-grafted antibody expression vector pKANTEX1259HV3LV0 described in WO 97/10354, and NS0 cell was transformed to obtain transformants capable of producing the anti-hIL-5Rα CDR-grafted antibody in a large amount. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription production of an α-1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0204)

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2. Measurement of binding activity of antibody to hIL-5Rα (ELISA)

The binding activity of the antibody to hIL-5Rα was measured as described below.

A solution was prepared by diluting the anti-hIL-5Rα mouse antibody KM1257 described in WO 97/10354 with PBS to give a concentration of 10 μg/ml, and 50 μl of the resulting solution was dispensed into each well of a 96 well plate for ELISA (manufactured by Greiner), followed by reaction at 4°C for 20 hours. After the reaction, 1% BSA-PBS was dispensed in 100 μl/well, and then the reaction was carried out at room temperature for 1 hour to block remaining active groups. After discarding 1% BSA-PBS, a solution prepared by diluting the soluble hIL-5Rα described in WO 97/10354 with 1% BSA-PBS to give a concentration of 0.5 μg/ml was dispensed in 50 μl/well, followed by reaction at 4°C for 20 hours. After the reaction, each well was washed with Tween-PBS, culture supernatants of transformants or diluted solutions of a purified human CDR-grafted antibodies were dispensed in 50 μg/well to carry out the reaction at room temperature for 2 hours. After the reaction, each well was washed with Tween-PBS, a peroxidase-labeled goat anti-human IgG (H & L) antibody solution (manufactured by American Qualex) diluted 3,000 times with 1% BSA-PBS was dispensed in 50 μl/well as a secondary antibody solution, followed by reaction at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was dispensed in 50 μl/well for color development, and then the absorbance at OD415 was measured.

(0205)

3. Purification of anti-hIL-5Rα CDR-grafted antibody

(1) Culturing of antibody-producing cell derived from YB2/0 cell and purification of antibody

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The anti-hIL-5Rα CDR-grafted antibody-producing transformed cell clone obtained in the item 1(1) of Example 3 was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of 3×10^5 cells/ml and dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). After culturing them at 37°C for 8 days in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-hIL-5Rα CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5Rα CDR-grafted antibody was named YB2/0-hIL-5R CDR antibody.

(2) Culturing of antibody-producing cell derived from CHO/dhfr$^-$ cell and purification of antibody

The anti-hIL-5Rα CDR-grafted antibody-producing transformed cell clone obtained in the item 1(2) of Example 3 was suspended in the EX-CELL302 medium comprising 3 mM L-Gln, 0.5% CDLC and 0.3% PF68 to give a density of 3×10^5 cells/ml and cultured using a 4.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 100 rpm. After culturing them at 37°C for 10 days in a temperature-controlling room, the culture supernatant was recovered. The anti-hIL-5Rα CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5Rα CDR-grafted antibody was named CHO/d-hIL-5R CDR antibody.

(0206)

(3) Culturing of antibody-producing cell derived from NS0 cell and purification of antibody

The anti-hIL-5Rα CDR-grafted antibody-producing transformed cell clone obtained in the item 1(3) of Example 3 was cultured in accordance with the method of Yarranton *et al.* [*BIO/TECHNOLOGY*, 10, 169 (1992)] and then a culture supernatant was recovered. The anti-hIL-5Rα CDR-grafted antibody was purified from the culture supernatant using

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ion exchange chromatography and the gel filtration method. The purified anti-hIL-5Rα CDR-grafted antibody was named NS0-hIL-5R CDR antibody.

(0207)

4. Analysis of purified anti-hIL-5Rα CDR-grafted antibodies

In accordance with a known method [*Nature*, 227, 680 (1970)], 4 μg of each of the three kinds of the anti-hIL-5Rα CDR-grafted antibodies produced by and purified from each animal cells, obtained in the item 3 of Example 3, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. The results are shown in Fig. 4. As shown in Fig. 4, a single band of about 150 Kd in molecular weight was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd under reducing conditions, in each of the purified anti-hIL-5Rα CDR-grafted antibodies. The molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide sequences of H chain and L chain of the antibody (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule: about 144 Kd), and also coincided with the reports stating that the IgG antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chains having a molecular weight of about 50 Kd and L chains having a molecular weight of about 25 Kd under reducing conditions due to cutting of the S-S bond in the molecule [*Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory*, Chapter 14 (1998); *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)], so that it was confirmed that each anti-hIL-5Rα CDR-grafted antibody was expressed and purified as an antibody molecule having the true structure.

(0208)

Example 4

Activity evaluation of anti-hIL-5Rα CDR-grafted antibody:

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1. Binding activity of anti-hIL-5Rα CDR-grafted antibody to hIL-5Rα (ELISA)

The activity of the three kinds of the purified anti-hIL-5Rα CDR-grafted antibodies obtained in the item 3 of Example 3 to bind to hIL-5Rα was measured by the ELISA shown in the item 2 of Example 3. Fig. 5 shows a result of the examination of the binding activity measured by changing concentration of the anti-hIL-5Rα CDR-grafted antibody to be added. As shown in Fig. 5, the three kinds of the anti-hIL-5Rα CDR-grafted antibodies showed almost the same binding activity to hIL-5Rα. The result shows that the antigen binding activities of these antibodies are constant independently of the antibody-producing animal cells and their culturing methods, similar to the result of the item 1 of Example 2.

(0209)

2. *In vitro* cytotoxic activity (ADCC activity) of anti-hIL-5Rα CDR-grafted antibody

In order to evaluate *in vitro* cytotoxic activity of the three kinds of the purified anti-hIL-5Rα CDR-grafted antibodies obtained in the item 3 of Example 3, the ADCC activity was measured in accordance with the following method.

(1) Preparation of target cell solution

A mouse T cell line CTLL-2(h5R) expressing the hIL-5Rα chain and β chain described in WO 97/10354 was cultured using the RPMI1640-FBS(10) medium to give a density of 1×10^6 cells/0.5 ml, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance $Na_2{}^{51}CrO_4$ at 37°C for 1.5 hours. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, resuspended in the medium and then incubated at 4°C for 30 minutes in ice for spontaneous dissolution of the radioactive substance. After the

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centrifugation, the precipitate was adjusted to give a density of 2×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

(0210)

(2) Preparation of effector cell solution

From a healthy person, 50 ml of venous blood was collected and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). The mixture was centrifuged to separate a mononuclear cell layer using Polymorphprep (manufactured by Nycomed Pharma AS) and in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting cells were resuspended to give a density of 9×10^6 cells/ml using the medium and used as the effector cell solution.

(0211)

(3) Measurement of ADCC activity

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50 μl of the target cell solution prepared in the above (1) (1×10^4 cells/well) was dispensed. Next, 100 μl of the effector cell solution prepared in the above (2) was dispensed (9×10^5 cells/well, the ratio of effector cells to target cells becomes 90:1). Subsequently, each of the anti-hIL-5Rα CDR-grafted antibodies was added to give a final concentration of 0.001 to 0.1 μg/ml, followed by reaction at 37°C for 4 hours. After the reaction, the plate was centrifuged, and the amount of ^{51}Cr in the supernatant was measured using a γ-counter. The amount of spontaneously released ^{51}Cr was calculated by the same operation using only the medium instead of the effector cell solution and the antibody solution, and measuring the amount of ^{51}Cr in the supernatant. The amount of total released ^{51}Cr was calculated by the same operation using only the medium instead of the antibody solution and adding 1 N

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hydrochloric acid instead of the effector cell solution, and measuring the amount of ^{51}Cr in the supernatant. The ADCC activity was calculated from the above equation (I).
(0212)

The results are shown in Fig. 6. As shown in Fig. 6, among the three kinds of the anti-hIL-5Rα CDR-grafted antibodies, the YB2/0-hIL-5R CDR antibody showed the most potent ADCC activity, followed by the CHO/d-hIL-5R CDR antibody and the NS0-hIL-5R CDR antibody in this order. Similar to the result of the item 2 of Example 2, the above results show that the ADCC activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since the antibodies produced by the YB2/0 cell showed the most potent ADCC activity in both cases of the two kinds of the humanized antibodies, it was revealed that an antibody having potent ADCC activity can be produced by using the YB2/0 cell.
(0213)

3. *In vivo* activity evaluation of anti-hIL-5Rα CDR-grafted antibody

In order to evaluate *in vivo* activity of the three kinds of the purified anti-hIL-5Rα CDR-grafted antibodies obtained in the item 3 of Example 3, the inhibition activity in an hIL-5-induced eosinophilia increasing model of *Macaca faseicularis* was examined in accordance with the following method.

The hIL-5 (preparation method is described in WO 97/10354) was administered to *Macaca faseicularis* under the dorsal skin at a dose of 1 μg/kg, starting on the first day and once a day for a total of 14 times. Each anti-hIL-5Rα CDR-grafted antibody was intravenously administered at a dose of 0.3 mg/kg one hour before the hIL-5 administration on the day zero. An antibody-non-added group was used as the control. In the antibody-administered groups, three animals of *Macaca faseicularis* were used in each group

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(No. 301, No. 302, No. 303, No. 401, No. 402, No. 403, No. 501, No. 502 and No. 503), and two animals (No. 101 and No. 102) were used in the antibody-non-added group. Starting 7 days before commencement of the administration and until 42 days after the administration, about 1 ml of blood was periodically collected from a saphena or a femoral vein, and the number of eosinophils in 1 μl of peripheral blood was measured. The results are shown in Fig. 7. As shown in Fig. 7, increase in the blood eosinophil was completely inhibited in the group to which the YB2/0-hIL-5R CDR antibody was administered. On the other hand, complete inhibition activity was found in one animal in the group to which the CHO/d-hIL-5R CDR antibody was administered, but the inhibition activity was not sufficient in two animals. In the group to which NS0-hIL-5R CDR antibody was administered, complete inhibition activity was not found and its effect was not sufficient.
(0214)

The above results show that the *in vivo* activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since a positive correlation was found between the degree of the *in vivo* activity of the anti-hIL-5Rα CDR-grafted antibody and the degree of its ADCC activity described in the item 2 of Example 4, it was indicated that the degree of ADCC activity is remarkably important for its activity expression.

Based on the above results, it is expected that an antibody having potent ADCC activity is useful also in the clinical field for various diseases in human.
(0215)

Example 5

Analysis of sugar chain which enhances ADCC activity:

1. Preparation of 2-aminopyridine-labeled sugar chain (PA-treated sugar chain)

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The humanized antibody of the present invention was acid-hydrolyzed with hydrochloric acid to remove sialic acid. After hydrochloric acid was completely removed, the sugar chain was cleaved from the protein by hydrazinolysis [*Method of Enzymology*, 83, 263 (1982)]. Hydrazine was removed, and *N*-acetylation was carried out by adding an aqueous ammonium acetate solution and acetic anhydride. After lyophilizing, fluorescence labeling with 2-aminopyridine was carried out [*J. Biochem.*, 95, 197 (1984)]. The fluorescence-labeled sugar chain (PA-treated sugar chain) was separated from impurity using Surperdex Peptide HR 10/30 Column (manufactured by Pharmacia). The sugar chain fraction was dried using a centrifugal concentrator and used as a purified PA-treated sugar chain.
(0216)

2. Reverse phase HPLC analysis of PA-treated sugar chain of purified anti-hIL-5Rα CDR-grafted antibody

According to the method in the item 1 of Example 5, various anti-hIL-5Rα CDR-grafted antibodies produced in Example 3 were subjected to PA-treated sugar chain treatment, and reverse phase HPLC analysis was carried out by CLC-ODS column (manufactured by Shimadzu). An excess amount of α-L-fucosidase (derived from bovine kidney, manufactured by SIGMA) was added to the PA-treated sugar chain for digestion (37°C, 15 hours), and then the products were analyzed by reverse phase HPLC (Fig. 8). It was confirmed that the asparagine-linked sugar chain is eluted for 30 minutes to 80 minute using PA-treated sugar chain standards manufactured by Takara Shuzo. The ratio of sugar chains whose reverse phase HPLC elution positions were shifted (sugar chains eluted for 48 minutes to 78 minutes) by the α-L-fucosidase digestion was calculated. The results are shown in Table 1.
(0217)
(Table 1)

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Table 1

Antibody-producing cell	α-1,6-Fucose-linked sugar chain (%)
YB2/0	47
NS0	73

(0218)

About 47% of the anti-hIL-5R CDR-grafted antibody produced by the YB2/0 cell and about 73% of the anti-hIL-5R CDR-grafted antibody produced by the NS0 cell were sugar chains in which 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the *N*-glycoside-linked sugar chain (hereinafter referred to as "sugar chain having α-1,6-fucose"). Thus, the ratio of sugar chains in which 1-position of fucose is not bound to 6-position of *N*-acetylglucosamine in the reducing end through α-bond in the *N*-glycoside-linked sugar chain (hereinafter referred to as "α-1,6-fucose-free sugar chain") is higher in the antibody produced by the YB2/0 cell than in the antibody produced by the NS0 cell.

(0219)

3. Analysis of monosaccharide composition of purified anti-hIL-5Rα CDR-grafted antibody

Sugar chains of anti-hIL-5Rα CDR-grafted antibodies produced by the YB2/0 cell, NS0 cell and CHO/d cell were hydrolyzed into monosaccharides by acid hydrolysis with trifluoroacetic acid, and monosaccharide composition analysis was carried out using BioLC (manufactured by Dionex).

Among *N*-glycoside-linked sugar chains, there are 3 mannose units in one sugar chain in the complex type *N*-glycoside-linked sugar chain. A relative ratio of each monosaccharide obtained by calculating the number of mannose as 3 is shown in Table 2.

(0220)

(Table 3)

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Table 2

Antibody-producing cell	Fuc	GlcNAc	Gal	Man	ADCC activity (%)*
YB2/0	0.60	4.98	0.30	3.00	42.27
NS0	1.06	3.94	0.66	3.00	16.22
CHO/dhFr$^-$	0.85	3.59	0.49	3.00	25.73
CHO/dhFr$^-$	0.91	3.80	0.27	3.00	25.73

*: Antibody concentration: 0.01 μg/ml

(0221)

Since the relative ratios of fucose were in an order of YB2/0 < CHO/d < NS0, the sugar chain produced in the antibody produced by YB2/0 cell showed the lowest fucose content as also shown in the present results.

(0222)

Example 6

Sugar chain analysis of antibody produced by CHO/dhfr$^-$ cell:

PA-treated sugar chains were prepared from purified anti-hIL-5Rα CDR-grafted antibody produced by CHO/dhfr$^-$ cell, and reverse phase HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (Fig. 9). In Fig. 9, an elution time from 35 to 45 minutes corresponded to sugar chains having no fucose and an elution time from 45 to 60 minutes corresponded to sugar chains having fucose. Similar to the case of the antibody produced by mouse myeloma NS0 cell, the anti-hIL-5Rα CDR-grafted antibody produced by CHO/dhfr$^-$ cell had less fucose-free sugar chain content than the antibody produced by rat myeloma YB2/0 cell.

(0223)

Example 7

Separation of potent ADCC activity antibody:

The anti-hIL-5Rα CDR-grafted antibody produced by rat myeloma YB2/0 cell was separated using a lectin column which binds to sugar chains having fucose. HPLC was

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carried out using LC-6A manufactured by Shimadzu at a flow rate of 1 ml/min and at room temperature as the column temperature. After equilibration with 50 mM Tris-sulfate buffer (pH 7.3), the purified anti-hIL-5Rα CDR-grafted antibody was injected and then eluted by a linear density gradient (60 minutes) of 0.2 M α-methylmannoside (manufactured by Nakalai Tesque). The anti-hIL-5Rα CDR-grafted antibody was separated into non-adsorbed fraction and adsorbed fraction. When the non-adsorbed fraction and a part of the adsorbed fraction were sampled and their binding activity to hIL-5Rα was measured, they showed similar binding activity (Fig. 10 upper). When the ADCC activity was measured, the non-adsorbed fraction showed potent ADCC activity (100 to 1000 folds) than that of the part of adsorbed fraction (Fig. 10 lower). In addition, PA-treated sugar chains were prepared from the non-adsorbed fraction and a part of the adsorbed fraction, and reverse HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (Fig. 11). In the non-adsorbed fraction, an antibody binding to fucose-free sugar chains was mainly present, and in the part of adsorbed fraction, an antibody binding to sugar chains having fucose was mainly present.

(0224)

Example 8

Determination of transcription product of α-1,6-fucosyltransferase (FUT8) gene in host cell line:

(1) Preparation of single-stranded cDNA from various cell lines

Single-stranded cDNA samples were prepared from dihydrofolate reductase gene (dhfr)-deleted CHO/DG44 cells derived from Chinese hamster ovary and rat myeloma YB2/0 cells by the following procedure.

The CHO/DG44 cells were suspended in IMDM medium (manufactured by Life Technologies) supplemented with 10%

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fetal bovine serum (manufactured by Life Technologies) and 1 × concentration HT supplement (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for adhesion cell culture use (manufactured by Greiner) at a density of 2×10^5 cells/ml. Also, the YB2/0 cells were suspended in RPMI 1640 medium (manufactured by Life Technologies) supplemented with 10% fetal bovine serum (manufactured by Life Technologies) and 4 mmol/l L-GLN (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture (manufactured by Greiner) at a density of 2×10^5 cells/ml. They were cultured at 37°C in a 5% CO_2 incubator, and 1×10^7 of respective host cells were recovered on the 1st, 2nd, 3rd, 4th and 5th days of the culturing to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

(0225)

The total RNA was dissolved in 45 μl of sterile water, 1 μl of RQ1 RNase-Free DNase (manufactured by Promega), 5 μl of the attached 10 × DNase buffer and 0.5 μl of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 μl of sterile water.

In a 20 μl of the reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μg of each of the obtained total RNA samples by reverse transcription reaction using SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) and in accordance with the manufacture's instructions. A 1 × concentration solution of the reaction solution was used for the cloning of α-

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1,6-fucosyltransferase (hereinafter referred sometimes to as "FUT8") and β-actin derived from respective host cells, and 50 folds-diluted aqueous solution of the reaction solution for the determination of each gene transcription amount by competitive PCR, and the solutions were stored at -80°C until use.

(0226)

(2) Preparation of cDNA partial fragments of Chinese hamster FUT8 and rat FUT8

Each cDNA partial fragment of Chinese hamster FUT8 and rat FUT8 was prepared by the following procedure (Fig. 12).

First, primers (shown in SEQ ID NOs:4 and 5) specific for nucleotide sequences common to human FUT8 cDNA [*J. Biochem.*, 121, 626 (1997)] and swine FUT8 cDNA [*J. Biol. Chem.*, 271, 27810 (1995)] were designed.

Next, 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5 μmol/l gene-specific primers (SEQ ID NOs:4 and 5)] containing 1 μl of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item (1) 2 days after culturing, and polymerase chain reaction (PCR) was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo). The PCR was carried out by heating at 94°C for 1 minute, subsequent 30 cycles of heating at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes as one cycle, and final heating at 72°C for 10 minutes.

(0227)

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 979 bp was purified using GENECLEAN Spin Kit (manufactured by BIO 101) and eluted with 10 μl of sterile water (hereinafter, the method was used for the purification of DNA fragments from agarose gel). Into a

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plasmid pCR2.1, 4 μl of the amplified fragment was employed to insert in accordance with the manufacture's instructions of TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* XL1-Blue was transformed with the reaction solution by the method of Cohen et al. [*Proc. Natl. Acad. Sci. USA*, 69, 2110 (1972)] (hereinafter, the method was used for the transformation of *E. coli*). Plasmid DNA samples were isolated in accordance with a known method [*Nucleic Acids Research*, 7, 1513 (1979)] (hereinafter, the method was used for the isolation of plasmid) from cDNA-inserted 6 clones among the obtained kanamycin-resistant colonies.

(0228)

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined by the method encode the open reading frame (ORF) partial sequences of Chinese hamster FUT8 or rat FUT8 (shown in SEQ ID NOs:6 and 7). Among these, plasmid DNA samples containing absolutely no reading error by the PCR in the sequences were selected. Herein, these plasmids are referred to as CHFUT8-pCR2.1 and YBFUT8-pCR2.1.

(0229)

(3) Preparation of Chinese hamster β-actin and rat β-actin cDNA

Chinese hamster β-actin and rat β-actin cDNA were prepared by the following procedure (Fig. 13).

First, a forward primer specific for a common sequence containing translation initiation codon (shown in SEQ ID NO:8) and reverse primers specific for respective sequences containing translation termination codon (shown in SEQ ID NOs:9 and 10) were designed from Chinese hamster

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β-actin genomic sequence (GenBank, U20114) and rat β-actin genomic sequence [*Nucleic Acids Research*, 11, 1759 (1983).
(0230)

Next, 25 μl of a reaction solution [KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l $MgCl_2$, 0.4 μmol/l gene-specific primers (SEQ ID NOs:8 and 9, or SEQ ID NOs:8 and 10) and 5% DMSO] containing 1 μl of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item (1) 2 days after culturing was prepared, and PCR was carried out using a DNA polymerase KOD (manufactured by Toyobo). The PCR was carried out by heating at 94°C for 1 minute and subsequent 25 cycles of heating at 98°C for 15 seconds, 65°C for 2 seconds and 74°C for 30 seconds as one cycle.
(0231)

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 1128 bp was purified. The DNA fragment was subjected to DNA 5'-terminal phosphorylation using MEGALABEL (manufactured by Takara Shuzo) in accordance with the manufacture's instructions. The DNA fragment was recovered from the reaction solution using ethanol precipitation method and dissolved in 10 μl of sterile water.

Separately, 3 μg of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 16 units of a restriction enzyme *Eco*RV (manufactured by Takara Shuzo) were added thereto for digestion reaction at 37°C for 3 hours. To the reaction solution, 35 μl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes to thereby dephophorylate the DNA terminus. The reaction solution was extracted with phenol/chloroform, followed by

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ethanol precipitation, and the recovered DNA fragment was dissolved in 100 μl of sterile water.

(0232)

Each 4 μl of the amplified fragment prepared from Chinese hamster cDNA or the amplified fragment (1192 bp) prepared from rat cDNA was mixed with 1 μl of the *Eco*RV-*Eco*RV fragment (about 3.0 Kb) prepared from plasmid pBluescript II KS(+) and 5 μl of Ligation High (manufactured by Toyobo) for ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* XL1-Blue was transformed, and plasmid DNA samples were isolated respectively in accordance with a known method from the obtained ampicillin-resistant clones.

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined by the method encode the ORF full sequences of Chinese hamster β-actin or rat β-actin. Among these, plasmid DNA samples containing absolutely no reading error of bases by the PCR in the sequences were selected. Herein, the plasmids are called CHAc-pBS and YBAc-pBS.

(0233)

(4) Preparation of FUT8 standard and internal control

In order to measure a transcription level of FUT8 gene mRNA in each cell, CHFT8-pCR2.1 or YBFT8-pCR2.1, as plasmids in which cDNA partial fragments prepared in the item (2) from Chinese hamster FUT8 or rat FUT8 were inserted into pCR2.1, respectively, were digested with a restriction enzyme *Eco*RI, and the obtained linear DNAs were used as the standards for the preparation of a calibration curve. CHFT8d-pCR2.1 and YBFT8d-pCR2.1, which were

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obtained from the CHFT8-pCR2.1 and YBFT8-pCR2.1, by deleting 203 bp between *Sca*I and *Hin*dIII, an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, were digested with a restriction enzyme *Eco*RI, and the obtained linear DNAs were used as the internal standards for FUT8 amount determination. Details thereof are described below.

(0234)

Chinese hamster FUT8 and rat FUT8 standards were prepared as follows. In 40 μl of NEBuffer 2 (manufactured by New England Biolabs), 2 μg of the plasmid CHFT8-pCR2.1 was dissolved, 24 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 μg of the plasmid YBFT8-pCR2.1 was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. By subjecting a portion of each of the reaction solutions to 0.8% agarose gel electrophoresis, it was confirmed that an *Eco*RI-*Eco*RI fragment (about 1 Kb) containing each of cDNA partial fragments of Chinese hamster FUT8 and rat FUT8 was separated from the plasmids CHFT8-pCR2.1 and YBFT8-pCR2.1 by the restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1 μg/ml of baker's yeast t-RNA (manufactured by SIGMA) to give a concentration of 0.02 fg/μl, 0.2 fg/μl, 1 fg/μl, 2 fg/μl, 10 fg/μl, 20 fg/μl and 100 fg/μl and used as the Chinese hamster FUT8 and rat FUT8 standards.

(0235)

Internal standards of Chinese hamster FUT8 and rat FUT8 were prepared as follows (Fig. 14). A reaction solution [KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l $MgCl_2$, 0.4 μmol/l gene-specific primers (SEQ ID NOs:11 and 12) and 5% DMSO] containing 5 ng

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of CHFT8-pCR2.1 or YBFT8-pCR2.1 was prepared, and PCR was carried out using a DNA polymerase KOD (manufactured by Toyobo). The PCR was carried out by heating at 94°C for 4 minutes and subsequent 25 cycles of heating at 98°C for 15 seconds, 65°C for 2 seconds and 74°C for 30 seconds as one cycle. After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of about 4.7 Kb was purified. The DNA 5'-terminal was phosphorylated using MEGALABEL (manufactured by Takara Shuzo) in accordance with the manufacture's instructions, and then the DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 50 μl of sterile water. The obtained DNA fragment (5 μl, about 4.7 kb) and 5 μl of Ligation High (manufactured by Toyobo) were mixed, followed by self-cyclization reaction at 16°C for 30 minutes.
(0236)

Using the reaction solution, *E. coli* DH5α was transformed, and plasmid DNA samples were isolated in accordance with a known method from the obtained ampicillin-resistant clones. The nucleotide sequence of each plasmid DNA was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer), and it was confirmed that a 203 bp inner nucleotide sequence between *Sca*I and *Hin*dIII of Chinese hamster FUT8 or rat FUT8 was deleted. The obtained plasmids are referred to as CHFT8d-pCR2.1 or YBFT8d-pCR2.1, respectively.
(0237)

Next, 2 μg of the plasmid CHFT8d-pCR2.1 was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 μg of the plasmid YBFT8d-pCR2.1 was dissolved

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in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an *Eco*RI-*Eco*RI fragment (about 800 bp) containing a fragment from which 203 bp of the inner nucleotide sequences of Chinese hamster FUT8 or rat FUT8 partial fragments was deleted was separated from the plasmids CHFT8d-pCR2.1 or YBFT8d-pCR2.1 by the restriction enzyme digestion reactions. Dilutions of 2 fg/μl were prepared from the reaction solutions using 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster FUT8 or rat FUT8 internal controls.

(0238)

(5) Preparation of β-actin standard and internal control

In order to measure the transcription amount of β-actin gene mRNA in various host cells, CHAc-pBS and YBAc-pBS, as plasmids in which the ORF full length of each cDNA of Chinese hamster β-actin and rat β-actin prepared in the item (3) was inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes *Hin*dIII and *Pst*I and restriction enzymes *Hin*dIII and *Kpn*I, respectively, and the digested linear DNAs were used as the standards for the preparation of a calibration curve. CHAcd-pBS and YBAcd-pBS which were obtained from the CHAc-pBS and YBAc-pBS by deleting 180 bp between *Dra*III and *Dra*III of an inner nucleotide sequence of Chinese hamster β-actin and rat β-actin were digested with restriction enzymes *Hin*dIII and *Pst*I and restriction enzymes *Hin*dIII and *Kpn*I, respectively, and the digested linear DNAs were used as the internal standards for β-actin amount determination. Details thereof are described below.

(0239)

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Chinese hamster β-actin and rat β-actin standards were prepared as follows. In 40 μl of NEBuffer 2 (manufactured by New England Biolabs), 2 μg of the plasmid CHAc-pBS was dissolved, and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 20 units of *Pst*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 μg of the plasmid YBAc-pBS was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 25 units of *Kpn*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that a *Hin*dIII-*Pst*I fragment and a *Hin*dIII-*Kpn*I fragment (about 1.2 Kb) containing the full length ORF of each cDNA of Chinese hamster β-actin and rat β-actin were separated from the plasmids CHAc-pBS and YBAc-pBS by the restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) to give a concentration 2 pg/μl, 1 pg/μl, 200 fg/μl, 100 fg/μl and 20 fg/μl and used as the Chinese hamster β-actin and or β-actin standards.

(0240)

Chinese hamster β-actin and rat β-actin internal standards were prepared as follows (Fig. 15). In 100 μl of NEBuffer 3 (manufactured by New England Biolabs) containing 100 ng/μl of BSA (manufactured by New England Biolabs), 2 μg of CHAc-pBS was dissolved, and 10 units of a restriction enzyme *Dra*III (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 3 hours. DNA fragments were recovered from the reaction solution by ethanol precipitation and the DNA termini were changed to blunt ends using DNA Blunting Kit (manufactured

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by Takara Shuzo) in accordance with the manufacture's instructions, and then the reaction solution was divided into two equal parts. First, to one part of the reaction solution, 35 μl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes for dephosphorylating the DNA termini. The DNA fragment was recovered by carrying out dephosphorylation treatment, phenol/chloroform extraction treatment and ethanol precipitation treatment and then dissolved in 10 μl of sterile water. The remaining part of the reaction solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.1 Kb containing the ORF partial fragment of Chinese hamster β-actin.

(0241)

The dephosphorylated *Dra*III-*Dra*III fragment (4.5 μl), 4.5 μl of the *Dra*III-*Dra*III fragment of about 1.1 Kb and 5 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and plasmid DNAs were isolated in accordance with a known method from the obtained ampicillin-resistant clones. The nucleotide sequence of each plasmid DNA was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer), and it was confirmed that a Chinese hamster β-actin *Dra*III-*Dra*III 180 bp inserted into the plasmid was deleted. The plasmid is referred to as CHAcd-pBS.

(0242)

Also, a plasmid in which rat β-actin *Dra*III-*Dra*III 180 bp was deleted was prepared via the same steps of CHAcd-pBS. The plasmid is referred to as YBAcd-pBS.

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Next, 2 μg of the plasmid CHAcd-pBS was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 20 units of *Pst*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 μg of the plasmid YBAcd-pBS was dissolved in 40 μl of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hin*dIII (manufactured by Takara Shuzo) and 24 units of *Kpn*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an *Hin*dIII-*Pst*I fragment and *Hin*dIII-*Kpn*I fragment (about 1.0 Kb) containing a fragment in which 180 bp of the inner nucleotide sequence of the ORF full length of each cDNA of Chinese hamster β-actin and rat β-actin was deleted were separated from the plasmids CHAcd-pBS and YBAcd-pBS by the restriction enzyme digestion reactions. Dilutions of 200 fg/μl were prepared from the reaction solutions using 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster β-actin and rat β-actin internal controls.

(0243)

(6) Determination of transcription amount by competitive PCR

Competitive PCR was carried out using the FUT8 internal control DNA prepared in the item (4) and the host cell-derived cDNA obtained in the item (1) as the templates, the determined value of the FUT8 transcription product in the host cell line was calculated from the relative value of the amount of the amplified product derived from each template. On the other hand, since it is considered that the β-actin gene is transcribed continuously in each cell and its transcription level is approximately the same

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between cells, transcription level of the β-actin gene was determined as a measure of the efficiency of synthesis reaction of cDNA in each host cell line. That is, the PCR was carried out using the β-actin internal control DNA prepared in the item (5) and the host cell-derived cDNA obtained in the item (1) as the templates, the determined value of the β-actin transcription product in the host cell line was calculated from the relative value of the amount of the amplified product derived from each template. Details thereof are described below.

(0244)

The FUT8 transcription product was determined by the following procedure. First, a set of sequence-specific primers (shown in SEQ ID NOs:13 and 14) common to the inner sequences of the ORF partial sequences of Chinese hamster FUT8 and rat FUT8 obtained in the item (2) were designed. Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of respective host cell line in the item (1) and 5 μl (10 fg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 32 cycles of heating at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute as one cycle.

(0245)

Also, PCR was carried out in a series of reaction in which 5 μl (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid obtained in the item (4) was added instead of the each host cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription level.

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The β-actin transcription product was determined by the following procedure. First, two sets of respective gene-specific primers common to the inner sequences of the ORF full lengths of Chinese hamster β-actin and rat β-actin obtained in the item (3) were designed (the former are shown in SEQ ID NOs:15 and 16, and the latter are shown in SEQ ID NOs:17 and 18).

Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l gene-specific primers (SEQ ID NOs:15 and 16, or SEQ ID NOs:17 and 18) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from respective host cell line in the item (1) and 5 μl (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 17 cycles of heating at 94°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes as one cycle.

(0246)

Also, PCR was carried out in a series of reaction in which 5 μl (10 pg, 5 pg, 1 pg, 500 fg or 100 fg) of the β-actin standard plasmid obtained in the item (5) was added instead of the each host cell line-derived cDNA, and used in the preparation of a calibration curve for the β-actin transcription level.

(0247)

(Table 3)

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Table 3

Target gene	Primer set *	Size (bp) of PCR amplification product	
		Target	Competitor
FUT8	F: 5'-GTCCATGGTGATCCTGCAGTGTGG-3' R: 5'-CACCAATGATATCTCCAGGTTCC-3'	638	431
β-Actin (Chinese hamster)	F: 5'-GATATCGCTGCGCTCGTTGTCGAC-3' R: 5'-CAGGAAGGAAGGCTGGAAAAGAGC-3'	789	609
β-Actin (Rat)	F: 5'-GATATCGCTGCGCTCGTCGTCGAC-3' R: 5'-CAGGAAGGAAGGCTGGAAGAGAGC-3'	789	609

* F: forward primer, R: reverse primer

(0248)

By carrying out PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 3 can be amplified from each internal control.

A 7 μl portion of each of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1 × concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). The amount of the amplified DNA fragment was measured by calculating luminescence intensity of each amplified DNA using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics).

(0249)

The amount of an amplified product formed by PCR using a standard plasmid as the template was measured by the method, and a calibration curve was prepared by plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each cell line was

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calculated from the amount of the amplified product when each expression cell line-derived cDNA was used as the template, and the amount was defined as the mRNA transcription amount in each cell line.

The amount of the FUT8 transcription product in each host cell line when a rat FUT8 sequence was used in the standard and internal control is shown in Fig. 16. Throughout the culturing period, the CHO cell line showed a transcription amount 10 folds or higher than that of the YB2/0 cell line. The tendency was also found when a Chinese hamster FUT8 sequence was used in the standard and internal control.

Also, the FUT8 transcription amounts are shown in Table 4 as relative values to the amount of the β-actin transcription product. Throughout the culturing period, the FUT8 transcription amount in the YB2/0 cell line was around 0.1% of β-actin while it was 0.5% to 2% in the CHO cell line.

The results shows that the amount of the FUT8 transcription product in YB2/0 cell line was significantly smaller than that in the CHO cell line.

(0250)

(Table 4)

Table 4

	Culture days				
Cell line	1st	2nd	3rd	4th	5th
CHO	1.95	0.90	0.57	0.52	0.54
YB2/0	0.12	0.11	0.14	0.08	0.07

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(0251)

Example 9

Determination of transcription product of α-1,6-fucosyltransferase (FUT8) gene in anti-ganglioside GD3 chimeric antibody-producing cell line:

(1) Preparation of single-stranded cDNA from various antibody-producing cell lines

Single-stranded cDNA was prepared from anti-ganglioside GD3 chimeric antibody-producing cell lines DCHI01-20 and 61-33 as follows. The DCHI01-20 is a transformant clone derived from the CHO/DG44 cell described in item 2(2) of Example 1. Also, the 61-33 is a clone obtained by carrying out serum-free adaptation of YB2/0-derived transformant cell 7-9-51 (FERM BP-6691, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan)) and then carrying out single cell isolation by two limiting dilution.

(0252)

Cells of the DCHI01-20 were suspended in EXCELL 302 medium (manufactured by JRH BIOSCIENCES) supplemented with 3 mmol/l L-GLN (manufactured by Life Technologies), 0.3% PLURONIC F-68 (manufactured by Life Technologies) and 0.5% fatty acid concentrate (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture use (manufactured by Greiner) at a density of 2×10^5 cells/ml. Also, cells of the 61-33 were suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.2% bovine serum albumin fraction V (manufactured by Life Technologies) (hereinafter referred to as "BSA"), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture (manufactured by Greiner) at a density of 2×10^5 cells/ml. They were cultured at 37°C in a 5% CO_2

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incubator, and 1, 2, 3, 4 and 5 days after culturing, 1×10^7 of respective host cells were recovered to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

(0253)

The total RNA was dissolved in 45 μl of sterile water, and 1 μl of RQ1 RNase-Free DNase (manufactured by Promega), 5 μl of the attached 10 × DNase buffer and 0.5 μl of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 μl of sterile water.

In a 20 μl reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μg of each of the obtained total RNA samples by reverse transcription reaction using SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions. The reaction solution was diluted 50 folds with water and stored at -80°C until use.

(0254)

(2) Determination of transcription amounts of each gene by competitive PCR

The transcription amount of each of the genes on the cDNA derived from the antibody-producing cell line obtained in the item (1) was determined by competitive PCR in accordance with Example 9(6).

The FUT8 gene-derived mRNA transcription amount in each of the antibody-producing cell lines was determined by the following procedure.

CHFT8-pCR2.1 and YBFT8-pCR2.1, as plasmids in which cDNA partial fragments prepared in Example 9(2) from Chinese hamster FUT8 and rat FUT8, respectively, were

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inserted into pCR2.1, were digested with a restriction enzyme *Eco*RI, and the obtained linear DNAs were used as the standards in the preparation of a calibration curve for determining the FUT8 transcription amount.

CHFT8d-pCR2.1 and YBFT8d-pCR2.1, which were obtained by deleting 203 bp between *Sca*I and *Hin*dIII of an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, in Example 8(4) were digested with a restriction enzyme *Eco*RI, and the obtained linear DNAs were used as the internal standards for FUT8 amount determination.

(0255)

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l FUT8 gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of the antibody-producing cell line in the item (1) and 5 μl (10 fg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 32 cycles of heating at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute as one cycle.

(0256)

Also, PCR was carried out in a series of reaction in which 5 μl (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid was added instead of the each antibody-producing cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount. In this case, 1 μg/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of the standard plasmid.

On the other hand, since it is considered that the β-actin gene is transcribed constantly in each cell and its transcription amount is approximately the same between

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cells, the transcription amount of the β-actin gene was determined as an index of the efficiency of synthesis reaction of cDNA in each antibody-producing cell line.

CHAc-pBS and YBAc-pBS as plasmids in which the ORF full length of each cDNA of Chinese hamster β-actin and rat β-actin prepared in Example 8(3) were inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNA samples were used as the standards in the preparation of a calibration curve for determining the β-actin transcription amount.

(0257)

CHAcd-pBS and YBAcd-pBS which were obtained by deleting 180 bp between *Dra*I and *Dra*I of an inner nucleotide sequence of Chinese hamster β-actin and rat β-actin, respectively in Example 8(5), were digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNAs were used as the internal standards for β-actin determination.

(0258)

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l β-actin-specific primers (SEQ ID NOs:17 and 18) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of the antibody-producing cell lines and 5 μl (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 17 cycles of heating at 94°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes as one cycle. Also, PCR was carried out in a series of reaction in which 10 pg, 5 pg, 1 pg, 500 fg or 100 fg of the β-actin standard plasmid was added instead of the each antibody-producing cell line-derived cDNA, and used in the preparation of a calibration curve for the β-

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actin transcription amount. In this case, 1 μg/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of standard plasmid.

(0259)

By PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 3 can be amplified from each internal control.

A 7 μl portion of each of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1 × concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). The amount of the amplified DNA fragment was measured by calculating luminescence intensity of each amplified DNA using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics).

(0260)

The amount of the amplified product formed by PCR which used a standard plasmid as the template was measured by the method, and a calibration curve was prepared by plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each cell line was calculated from the amount of the amplified product when each antibody-producing cell line-derived cDNA was used as the template, and the value was defined as the mRNA transcription amount in each cell line.

(0261)

The FUT8 transcription amounts are shown in Table 5 as relative values to the amount of the β-actin transcription product. Throughout the culturing period, the FUT8 transcription amount in the YB2/0 cell-derived

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antibody-producing 61-33 was 0.3% or less of β-actin while it was 0.7% to 1.5% in the CHO cell-derived antibody-producing cell. The results shows that the amount of the FUT8 transcription product in the YB2/0 cell-derived antibody-producing cell line was significantly less than that in the antibody-producing cell line derived from the CHO cell.

(0262)

(Table 5)

Table 5

	Culture days				
Cell line	1st	2nd	3rd	4th	5th
DCHI01-20	0.75	0.73	0.99	1.31	1.36
61-33	0.16	0.19	0.24	0.30	<0.10

(0263)

Example 10

Preparation of mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line:

(1) Construction of mouse α-1,6-fucosyltransferase (FUT8) expression plasmid

Total RNA was extracted from 1×10^7 cells of a mouse myeloma NSO cell (RCB0213, Cell Bank at The Institute of Physical and Chemical Research) subcultured using IMDM medium (manufactured by Life Technologies) containing 10% fetal bovine serum (manufactured by Life Technologies), using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions. The total RNA was dissolved in 45 μl of sterile water, and 1 μl of RQ1 RNase-Free DNase (manufactured by Promega), 5 μl of the attached 10 × DNase buffer and 0.5 μl of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction,

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the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 μl of sterile water. In a 20 μl reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 μg of the obtained total RNA by reverse transcription reaction using SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions.

(0264)

Mouse FUT8 cDNA was prepared by the following procedure (Fig. 17).

First, a forward primer specific for a sequence containing a translation initiation codon (shown in SEQ ID NO:19) and a reverse primer specific for a sequence containing translation termination codon (shown in SEQ ID NO:20) were designed from a mouse FUT8 cDNA sequence (GenBank, AB025198).

Next, 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 4% DMSO and 0.5 μmol/l specific primers (SEQ ID NO:19 and SEQ ID NO:20)] containing 1 μl of the NSO cell-derived cDNA was prepared, and PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo). The PCR was carried out by heating at 94°C for 1 minute, subsequent 30 cycles of heating at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes as one cycle, and final heating at 72°C for 10 minutes.

(0265)

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 1728 bp was purified. Into a plasmid pCR2.1, 4 μl of the DNA fragment was employed to insert in accordance with the manufacturer's instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5α was transformed with the reaction solution.

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Plasmid DNAs were isolated in accordance with a known method from cDNA-inserted 6 clones among the obtained kanamycin-resistant colonies.

(0266)

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined encode the ORF full sequence of mouse FUT8. Among these, a plasmid DNA containing absolutely no reading error of bases by the PCR in the sequences were selected (its DNA sequence and amino acid sequence are shown in SEQ ID NOs:2 and 24, respectively). Also, inconsistency of 3 bases due to amino acid substitution was found in the sequence when compared with the mouse FUT8 sequence registered on GenBank. Herein, the plasmid is referred to mfFUT8-pCR2.1.

(0267)

Next, a plasmid pBSmfFUT8 containing the ORF full sequence of mouse FUT8 was constructed as follows (Fig. 18). First, 1 μg of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 2 hours. To the reaction solution, 35 μl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added, followed by reaction at 65°C for 30 minutes for dephosphorylate the DNA termini. The reaction solution was extracted with phenol/chloroform, followed by ethanol precipitated, and the recovered DNA fragment was dissolved in 10 μl of sterile water.

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(0268)

Separately, 1 μg of the plasmid mfFUT8-pCR2.1 was dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Eco*RI (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 2 hours. The reaction solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.7 Kb containing the ORF full sequence of mouse FUT8 cDNA.

The obtained plasmid pBluescript II KS(+)-derived *Eco*RI-*Eco*RI fragment (1 μl, 2.9 Kb), 4 μl of the *Eco*RI-*Eco*RI fragment (1.7 Kb) prepared from the plasmid mfFUT8-pCR2.1 and 5 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and plasmid DNAs were isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pBSmfFUT8.

(0269)

Using the pBSmfFUT8 and pAGE249, a mouse FUT8 expression vector pAGEmfFUT8 was constructed by the following procedure (Fig. 19). The pAGE249 is a derivative of pAGE248 [*J. Biol. Chem.*, 269, 14730 (1994)], as a vector in which an *Sph*I-*Sph*I fragment (2.7 Kb) containing a dihydrofolate reductase gene (dhfr) expression unit was removed from the pAGE248.

In 50 μl of Universal Buffer H (manufactured by Takara Shuzo), 1 μg of the pAGE249 was dissolved, and 20 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Bam*HI (manufactured by New England Biolabs) were added

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thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, to the reaction solution, 35 μl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 μl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes to dephosphorylate the DNA termini. The reaction solution was extracted with phenol/chloroform extraction, followed by ethanol precipitation, and the recovered DNA fragment was dissolved in 10 μl of sterile water.

(0270)

Separately, 1 μg of pBSmfFUT8 was dissolved in 50 μl of Universal Buffer H (manufactured by Takara Shuzo), and 20 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 μl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Bam*HI (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.7 Kb containing the ORF full sequence of mouse FUT8 cDNA.

(0271)

The obtained plasmid pAGE249-derived *Bam*HI-*Sal*I fragment (1 μl, 6.5 Kb), 4 μl of the *Bam*HI-*Sal*I fragment (1.7 Kb) prepared from the plasmid pBSmfFUT8 and 5 μl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pAGEmfFUT8.

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(0272)

(2) Preparation of mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

A stable FUT8 gene-expressing cell line was obtained by introducing the mouse FUT8 expression vector pAGEmfFUT8 constructed in the item (1) into 61-33. The 61-33 is a clone obtained by carrying out serum-free adaptation of a transformant cell 7-9-51 (FERM BP-6691, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) derived from a YB2/0 cell highly producing an anti-ganglioside GD3 chimeric antibody, and then carrying out single cell isolation by two limiting dilution.

(0273)

The plasmid pAGEmfFUT8 was transferred into 61-33 by the following procedure in accordance with the electroporation [*Cytotechnology*, 3, 133 (1990)]. First, 30 μg of the plasmid pAGEmfFUT8 was dissolved in 600 μl of NEBuffer 4 (manufactured by New England Biolabs), and 100 units of a restriction enzyme *Fsp*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours to obtain a linear fragment. The reaction solution was subjected to ethanol precipitation, and the recovered linear plasmid was made into a 1 μg/μl aqueous solution. Next, the 61-33 was suspended in a K-PBS buffer (137 mol/l KCl, 2.7 mol/l NaCl, 8.1 mol/l Na_2HPO_4, 1.5 mol/l KH_2PO_4, 4.0 mol/l $MgCl_2$) to give a density of 2×10^7 cells/ml, and 200 μl of the cell suspension (4×10^6 cells) was mixed with 10 μl (10 μg) of the linear plasmid. The cell-DNA mixture was transferred into Gene Pulser Cuvette (inter-electrode distance, 2 mm) (manufactured by BIO-RAD) and then electroporation was carried out using a cell fusion apparatus Gene Pulser (manufactured by BIO-RAD) at 0.2 KV pulse voltage and 250 μF electric capacity. The cell suspension was mixed with

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10 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) and dispensed in 100 μl portions into a 96 well plate for suspension cell use (manufactured by Greiner). After culturing them at 37°C for 24 hours in 5% CO_2, 50 μl of the culture supernatant was removed, and Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) was dispensed at 100 μl. They were cultured for 3 weeks while repeating the medium exchange step at intervals of 3 to 4 days, and 14 cell lines showing hygromycin resistance were obtained.

(0274)

On the other hand, a negative control cell line was prepared by introducing the plasmid pAGE249 as a parent vector of the pAGEmfFUT8 into the 61-33. According to the above procedure, 10 μg of the plasmid pAGE249 converted into linear form with a restriction enzyme *Fsp*I was introduced into 4×10^6 cells of the 61-33 using the electroporation. The cells were mixed with 15 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies), transferred into a T75 flask for suspension cell (manufactured by Greiner) and then cultured at 37°C for 24 hours in 5% CO_2. After culturing them, a half of the culture supernatant (7.5 ml) was removed by centrifugation at 800 rpm for 4 minutes, and the cells were suspended in 7.5 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical

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Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) and transferred into the T75 flask for suspension cell (manufactured by Greiner). They were cultured for 3 weeks while repeating the medium exchange at intervals of 3 to 4 days, a hygromycin-resistant cell line was obtained.

(0275)

(3) Analysis of expression level of α-1,6-fucosyltransferase (FUT8) gene in cell lines over-expressing the gene

Using 6 cell lines optionally selected from the 14 mouse FUT8-over expressing cell lines prepared from 61-33 in the item (2) and the negative control cell line, the FUT8 expression levels were compared using competitive RT-PCR.

Each of these over-expression cell lines was suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) to give a density of 3×10^5 cells/ml and then transferred into a T75 flask for suspension cell culture use (manufactured by Greiner). After culturing them at 37°C for 24 hours in 5% CO_2, 1×10^7 of intact cells were recovered to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions. The total RNA was dissolved in 45 μl of sterile water, and 0.5 U/μl of RQ1 RNase-Free DNase (manufactured by Promega), 5 μl of the attached 10 × DNase buffer and 0.5 μl of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy

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(manufactured by QIAGEN) and dissolved in 50 μl of sterile water.

(0276)

In a 20 μl reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 2.5 μg of the obtained total RNA by reverse transcription reaction using SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions. The reaction solution was diluted 50 folds with water and the transcription amount of each gene was determined by the competitive PCR in accordance with Example 8(6).

The FUT8 gene-derived mRNA transcription amount in each expression cell line was determined by the following procedure.

YBFT8-pCR2.1, as a plasmid in which a cDNA partial fragment prepared in Example 8(2) from rat FUT8 was inserted into pCR2.1, was digested with a restriction enzyme *Eco*RI, and the obtained linear DNA was used as the standard in the preparation of a calibration curve for determining the FUT8 transcription amount.

(0277)

Among the YBFT8-pCR2.1 prepared in Example 8(4), YBFT8d-pCR2.1 obtained by deleting 203 bp between *Sca*I and *Hin*dIII of an inner nucleotide sequence of rat FUT8 was digested with a restriction enzyme *Eco*RI, and the obtained linear DNA was used as the internal control for FUT8 determination.

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l FUT8 gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from respective expression cell line in the above and 5 μl (10 fg) of the

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plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 32 cycles of heating at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute as one cycle.

(0278)

Also, PCR was carried out in a series of reaction in which 5 μl (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid was added instead of the each expression cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount. In this case, 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of standard plasmid.

On the other hand, since it is considered that the β-actin gene is transcribed constantly in each cell and its transcription level is approximately the same between cells, the transcription amount of the β-actin gene was determined as an index of the efficiency of synthesis reaction of cDNA in each expression cell line.

YBAc-pBS, as a plasmid in which the ORF full sequence of cDNA of rat β-actin was inserted into pBluescript II KS(+) prepared in Example 8(3), was digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNA was used as the standard in the preparation of a calibration curve for determining the β-actin gene transcription amount.

YBAcd-pBS obtained from the YBAc-pBS prepared in Example 8(5) by deleting 180 bp between *Dra*I and *Dra*I of an inner nucleotide sequence of rat β-actin was digested with restriction enzymes *Hin*dIII and *Kpn*I, and the obtained linear DNA was used as the internal standards for β-actin amount determination.

(0279)

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of

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a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5 μmol/l β-actin-specific primers (SEQ ID NOs:17 and 18) and 5% DMSO] containing 5 μl of 50 folds-diluted cDNA solution prepared from each of the expression cell lines and 5 μl (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 17 cycles of heating at 94°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes as one cycle.

Also, PCR was carried out in a series of reaction in which 10 pg, 5 pg, 1 pg, 500 fg or 100 fg of the β-actin standard plasmid was added instead of the each expression cell line-derived cDNA, and used in the preparation of a calibration curve for the β-actin transcription amount. In this case, 1 μg/ml baker's yeast t-RNA (manufactured by SIGMA) was used for diluting the standard plasmid.

(0280)

By carrying out PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 3 can be amplified from each internal control.

Each (7 μl) of the solutions after PCR was subjected to a 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1 × concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). By calculating luminescence intensity of each amplified DNA fragment using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics), the amount of the amplified DNA fragment was measured.

The amount of an amplified product formed by PCR using the standard plasmid as the template was measured by the method, and a calibration curve was prepared by

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plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each cell line was calculated from the amount of an amplified product when each expression cell line-derived cDNA was used as the template, and the amount was defined as the mRNA transcription amount in each cell line.

(0281)

Fig. 20 shows the FUT8 transcription amounts as relative values to the amount of β-actin transcription product. Three cell lines mfFUT8-1, mfFUT8-2 and mfFUT8-4 and the pAGE249-introduced cell line were cell lines having a relatively small FUT8 transcription amount, which was equivalent to 0.3 to 10% of a β-actin transcription amount. On the other hand, other three cell lines mfFUT8-3, mfFUT8-6 and mfFUT8-7 were cell lines having a relatively large FUT8 transcription amount, which was equivalent to 20 to 40% of a β-actin transcription amount.

(0282)

(4) Purification of antibody produced by α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

Each of the six FUT8 gene over-expressing cell lines and one negative control cell line obtained in the item (2) was suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 200 nmol/l MTX, 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries) and 0.2% BSA (manufactured by Life Technologies) to give a density of 2×10^5 cells/ml, and then 100 ml in total of the suspension was inoculated into three T225 flasks for suspension cell culture use (manufactured by IWAKI). After culturing them at 37°C for 7 to 9 days in a 5% CO_2 incubator, the number of intact cells was counted to confirm that their viability was almost the same (each 30% or less), and then each cell

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suspension was recovered. Each of the cell suspensions was centrifuged at 3,000 rpm at 4°C for 10 minutes, and the recovered supernatant was centrifuged at 10,000 rpm at 4°C for 1 hour and then filtered using PES Filter Unit (manufactured by NALGENE) having a pore diameter of 0.22 μm with 150 ml capacity.

(0283)

Prosep-A HighCapacity (manufactured by bioPROCESSING) was packed in a 0.8 cm diameter column to a thickness of 2 cm and washed with 10 ml of 0.1 mol/l citrate buffer (pH 3.0) and 10 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6) in that order to effect equilibration the carrier. Next, 100 ml of each of the culture supernatant was passed through the column and washed with 50 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6). After washing them, the antibody absorbed to Prosep-A was eluted using 2.5 ml of a 0.1 mol/l citrate buffer (pH 3.0), the eluate was fractionated at 500 μl and each fraction was neutralized by mixing with 100 μl of 2 mol/l Tris-HCl (pH 8.5). Two fractions containing the antibody at a high concentration (1.2 ml in total) were selected by the BCA method [*Anal. Biochem.*, 150, 76 (1985)], combined and then dialyzed against 10 mol/l citrate buffer (pH 6.0) at 4°C for a whole day and night. After the dialysis, the antibody solution was recovered and subjected to sterile filtration using a 0.22 μm pore size Millex GV (manufactured by MILLIPORE).

(0284)

(5) *In vitro* cytotoxic activity (ADCC activity) of antibody produced by mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

In order to evaluate *in vitro* cytotoxic activity of the anti-GD3 antibodies purified in the item (4), ADCC activity was measured using a GD3-positive cell, human

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melanoma cultured cell line G-361 (RCB0991, Cell Bank at The Institute of Physical and Chemical Research).

The G-361 cells subcultured in RPMI1640 medium (manufactured by Life Technologies) containing 10% fetal bovine serum (manufactured by Life Technologies) (hereinafter referred to as "RPMI1640-FBS(10)") were suspended in 500 μl of RPMI1640-FBS(10) at a density of 1×10^6 cells, and 3.7 MBq of $Na_2{}^{51}CrO_4$ was added thereto, followed by culturing at 37°C for 30 minutes for labeling the cells with a radioisotope. After centrifugation at 1,200 rpm for 5 minutes, the supernatant was discarded and the target cells were suspended in 5 ml of RPMI1640-FBS(10). The washing step was repeated three times and then the cell suspension was incubated for 30 minutes on ice for spontaneous dissociation of the radioactive substance. The washing step was again repeated twice and then the cells were suspended in 5 ml of RPMI1640-FBS(10) to thereby prepare 2×10^5 cells/ml of a target cell suspension.
(0285)

On the other hand, 30 ml of peripheral blood was collected from a healthy person and gently mixed with 0.5 ml of heparin sodium (manufactured by Shimizu Pharmaceutical) and then mixed with 30 ml of physiological saline (manufactured by Otsuka Pharmaceutical). After the mixing, 10 ml of the mixture was gently overlaid on 4 ml of Lymphoprep (manufactured by NYCOMED PHARMA AS) and centrifuged at room temperature at 2,000 rpm for 30 minutes. The separated mononuclear cell fractions were collected from the centrifugation tubes, combined and then suspended in 30 ml of RPMI1640-FBS(10). After centrifugation at room temperature at 1,200 rpm for 5 minutes, the supernatant was discarded and the cells were suspended in 20 ml of RPMI1640-FBS(10). The washing step was repeated twice and then 2×10^6 cells/ml of an effector cell suspension was prepared using RPMI1640-FBS(10).

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(0286)

The target cell suspension was dispensed at 50 μl (1×10^4 cells/well) into each well of a 96 well U-bottom plate (manufactured by Falcon). Subsequently, the effector cell suspension was dispensed at 100 μl (2×10^5 cells/well) into each well to thereby adjust the ratio of the effector cells to the target cells to 20 : 1. Next, using a 10 M citrate buffer (pH 6.0), a series of dilution solution of 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml and 10 μg/ml of each anti-GD3 antibody obtained in the item (4) was prepared, and the diluted solutions were dispensed at 50 μl into the wells to give final concentrations of 0.0025 μg/ml, 0.025 μg/ml, 0.25 μg/ml and 2.5 μg/ml, respectively. After carrying out the reaction at 37°C for 4 hours in 5% CO_2, the plate was centrifuged at 1,200 rpm for 5 minutes. Into a 12 mm diameter RIA tube (manufactured by IWAKI), 50 μl of the supernatant in each well was transferred and, and the amount of the dissociated ^{51}Cr was measured using MINAX-γ auto-gamma counter 5550 (manufactured by PACKARD).

(0287)

Also, the amount of the spontaneously dissociated ^{51}Cr was calculated by carrying out the same reaction in a reaction mixture in which 150 μl of RPMI1640-FBS(10) was added instead of the effector cell suspension and antibody solution. The amount of the total dissociated ^{51}Cr was calculated by carrying out the same reaction in a reaction mixture in which 100 μl of 1 N hydrochloric acid and 50 μl of RPMI1640-FBS(10) were added instead of the effector cell suspension and antibody solution. Using these values, the ADCC activity was calculated based on the formula (II) described in the item 2(3) of Example 2.

(0288)

Fig. 21 shows ADCC activity of each of the anti-GD3 antibodies for G-361 cell. Three cell lines mfFUT8-1, mfFUT8-2 and mfFUT8-4 having a low FUT8 expression level as

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shown in Fig. 20 showed potent ADCC activity equivalent to that of the negative control pAGE249-introduced cell line. On the other hand, other three cell lines mfFUT8-3, mfFUT8-6 and mfFUT8-7 having a high FUT8 expression level as shown in Fig. 20 showed low ADCC activity equivalent to that of the anti-GD3 antibody produced from CHO cell. Based on these results, it was shown that the ADCC activity of produced antibodies can be controlled by regulating the expression level of FUT8 in host cells.

(0289)

(6) Sugar chain analysis of antibody produced by mouse α-1,6-fucosyltransferase (FUT8) gene over-expressing cell line

Sugar chains of the anti-GD3 antibodies purified in the item (4) were analyzed. The sugar chains binding to the antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines were cleaved from proteins by subjecting the antibodies to hydrazinolysis [*Method of Enzymology*, 83, 263 (1982)]. After removing hydrazine by evaporation under a reduced pressure, *N*-acetylation was carried out by adding an aqueous ammonium acetate solution and acetic anhydride. After freeze-drying, fluorescence labeling by 2-aminopyridine [*J. Biochem.*, 95, 197 (1984)] was carrying out. A fluorescence-labeled sugar chain group (PA-treated sugar chain group) was separated from excess reagents using Superdex Peptide HR 10/30 column (manufactured by Pharmacia). The sugar chain fractions were dried using a centrifugation concentrator and used as a purified PA-treated sugar chain group. Next, the purified PA-treated sugar chain group was subjected to reverse phase HPLC analysis using a CLC-ODS column (manufactured by Shimadzu) (Fig. 23). When calculated from the peak area, the content of α-1,6-fucose-free sugar chains in mfFUT8-6 was 10%, and the content of α-1,6-fucose-bound sugar chains was 90%. The content of α-1,6-fucose-free sugar chains in pAGE249

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was 20%, and the content of α-1,6-fucose-bound sugar chains was 80%. Based on these results, it was found that the content of α-1,6-fucose-bound sugar chains of a produced antibody is increased by over-expressing the FUT8 gene.

Fig. 23 shows elution patterns obtained by carrying out reverse phase HPLC analysis of each of PA-treated sugar chains prepared from antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines. Figs. 23 (upper) and 23 (lower) show elution patterns of mfFUT8-6 and pAGE249, respectively. The relative fluorescence intensity and the elution time are plotted as the ordinate and the abscissa, respectively. Using a sodium phosphate buffer (pH 3.8) as buffer A and a sodium phosphate buffer (pH 3.8) + 0.5% 1-butanol as buffer B, the analysis was carried out by the following gradient.

Time (minute)	0	80	90	90.1	120
Buffer B (%)	0	60	60	0	0

Peaks (i) to (ix) shown in Fig. 23 and Fig. 24 show the following structures.

(0290)

(Chem. 3)

(0291)

(Chem. 4)

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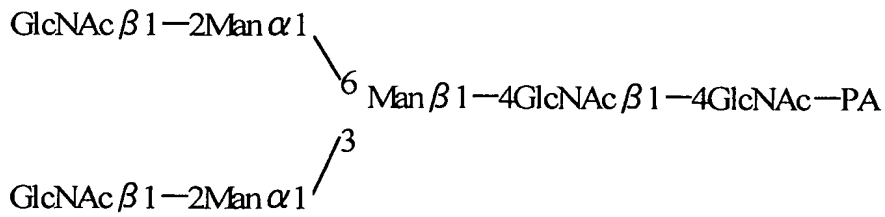
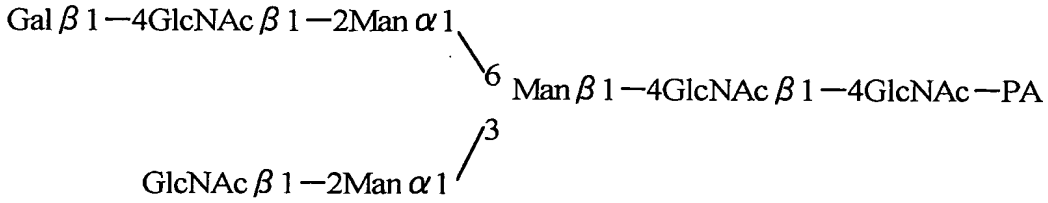
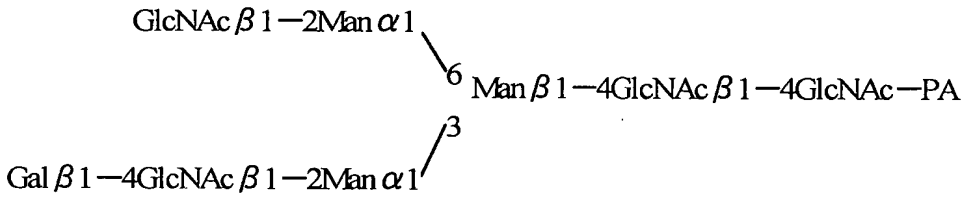
(i)



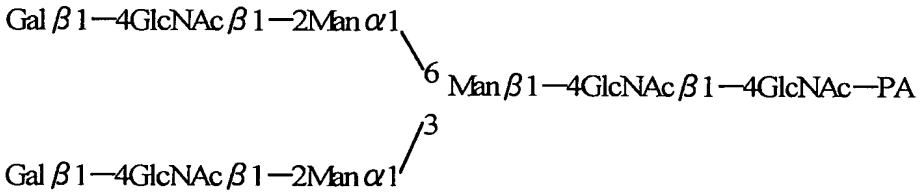
(ii)



(iii)



(iv)



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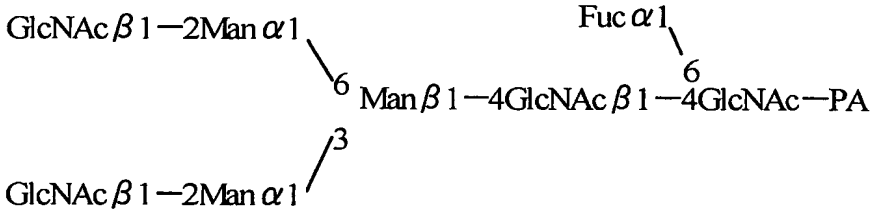

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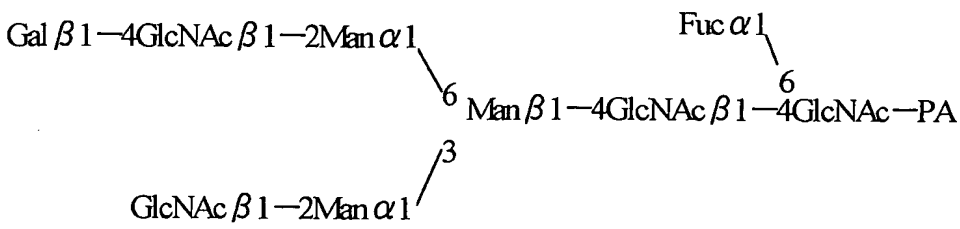

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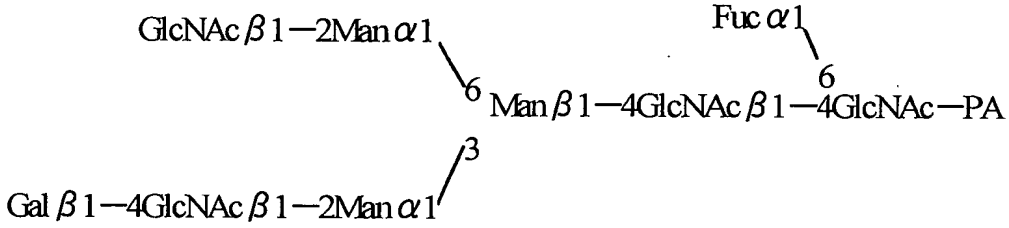

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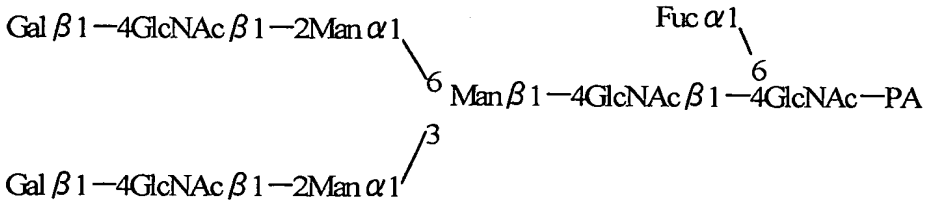

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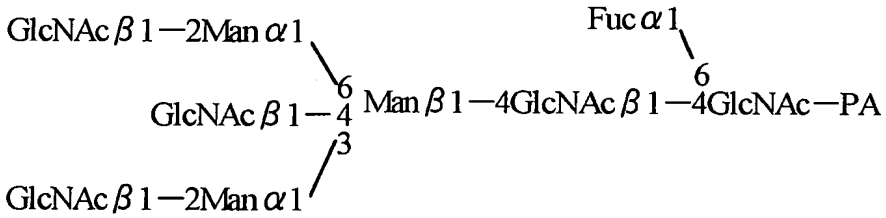

(0292)

GlcNAc, Gal, Man, Fuc and PA indicate *N*-acetylglucosamine, galactose, mannose, fucose and a pyridylamino group, respectively. In Figs. 23 and 24, the ratio of the α-1,6-fucose-free sugar chain group was calculated from the area occupied by the peaks (i) to (iv) among (i) to (ix), and the ratio of the α-1,6-fucose-bound sugar chain group from the area occupied by the peaks (v) to (ix) among (i) to (ix).

(0293)

Example 11

Preparation of CHO cell α-1,6-fucosyltransferase (FUT8) gene:

(1) Preparation of CHO cell α-1,6-fucosyltransferase (FUT8) cDNA sequence

From a single-stranded cDNA prepared from CHO/DG44 cells on the 2nd day of culturing in Example 8(1), Chinese hamster FUT8 cDNA was obtained by the following procedure (Fig. 22).

First, a forward primer specific for a 5'-terminal non-translation region (shown in SEQ ID NO:21) and a reverse primer specific for a 3'-terminal non-translation region (shown in SEQ ID NO:22) were designed from a mouse FUT8 cDNA sequence (GenBank, AB025198).

Next, 25 μl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 4% DMSO and 0.5 μmol/l specific primers (SEQ ID NOs:21 and 22)] containing 1 μl of the CHO/DG44 cell-derived cDNA was prepared and PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo). The PCR was carried out by heating at 94°C for 1 minute, subsequent 30 cycles of heating at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes as one cycle, and final heating at 72°C for 10 minutes.

(0294)

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After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of about 2 Kb was purified. Into a plasmid pCR2.1, 4 μl of the DNA fragment was employed to insert in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5α was transformed with the reaction solution. Plasmid DNAs were isolated in accordance with a known method from cDNA-inserted 8 clones among the obtained kanamycin-resistant colonies.

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed by the method that all of the inserted cDNAs encode a sequence containing the full ORF of CHO cell FUT8. Among these, a plasmid DNA containing absolutely no reading error of bases by the PCR in the sequences was selected. Herein, the plasmid is referred to as CHfFUT8-pCR2.1. The determined nucleotide sequence and the amino acid sequence of the cDNA of CHO FUT8 are shown in SEQ ID NOs:1 and 23, respectively.

(0295)

(2) Preparation of CHO cell α-1,6-fucosyltransferase (FUT8) genomic sequence

Using the ORF full length cDNA fragment of CHO cell FUT8 obtained in the item (1) as a probe, a CHO cell FUT8 genomic clone was obtained in accordance with a known genome screening method described, e.g., in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology*, *A Laboratory Manual*, Second Edition (1989). Next, after digesting the obtained genomic clone using various restriction enzymes, the Southern hybridization was carried out using an *Afa*I-*Sau*3AI fragment (about 280 bp) containing

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initiation codon of the CHO cell FUT8 cDNA as a probe, and then a *Xba*I-*Xba*I fragment (about 2.5 Kb) and a *Sac*I-*Sac*I fragment (about 6.5 Kb) were selected from restriction enzyme fragments showing positive reaction, inserted into pBluescript II KS(+) (manufactured by Stratagene), respectively.

(0296)

The nucleotide sequence of each of the obtained genomic fragments was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. Thereby, it was confirmed that the *Xba*I-*Xba*I fragment encodes a sequence of an upstream intron of about 2.5 Kb containing exon 2 of the CHO cell FUT8, and the *Sac*I-*Sac*I fragment encodes a sequence of a downstream intron of about 6.5 Kb containing exon 2 of the CHO cell FUT8. Herein, the plasmid containing *Xba*I-*Xba*I fragment is referred to as pFUT8fgE2-2, and the plasmid containing *Sac*I-*Sac*I fragment is referred to as pFUT8fgE2-4. The determined nucleotide sequence (about 9.0 Kb) of the genome region containing exon 2 of the CHO cell FUT8 is shown in SEQ ID NO:3.

(0297)

Example 12

Sugar chain analysis of conventionally available antibodies:

Sugar chains binding to a conventionally available anti-HER2/neu antibody Herceptin (manufactured by GENENTECH and Roche) produced by CHO cell as the host cell was analyzed in accordance with the method of Example 10(6) (Fig. 24). When calculated from each peak area of elution diagram, the content of α-1,6-fucose-free sugar chains of Herceptin was 16%, and the content of α-1,6-fucose-bound sugar chains was 84%. The same analysis was carried out on

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other commercially available antibodies, Rituxan (manufactured by GENENTECH, Roche and IDEC) and Zenapax (manufactured by Roche and PDL), and the α-1,6-fucose-free sugar chain content of was less than that in Herceptin.

Fig. 24 is a graph showing elution pattern of PA-treated sugar chains prepared from Herceptin, obtained by analyzing them by reverse phase HPLC. The relative fluorescence intensity and the elution time are plotted as the ordinate and the abscissa, respectively. The reverse phase HPLC analysis conditions, sugar chain structure analysis and calculation of the ratio of sugar chain group containing no α-1,6-fucose sugar chain were carried out.

(0298)

(Effect of the invention)

The present invention provides a cell for the production of a glycoprotein such as an immunologically functional molecule or the like, a method for producing an immunologically functional molecule using the cell, an immunologically functional molecule, and use thereof.

(0299)

(Sequence listing free text)

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SEQ ID NO:5- Explanation of artificial sequence: primer sequence of artificialy synthesized primer sequences

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(0300)

(Sequence listing)

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aatgtaaata caatctagaa catttttata aataagcata ttaactcagt aaaaataaat 5340
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Aragen/Transposagen Ex. 1036 - Part B

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Aragen/Transposagen Ex. 1036 - Part B

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aggatcccaa gagctc 9196

<210> 4
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 4
actcatcttg gaatctcaga attgg 25

<210> 5
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 5
cttgaccgtt tctatcttct ctcg 24

<210> 6
<211> 979
<212> DNA
<213> Cricetulus griseus

<400> 6
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gaagatagaa acggtcaag 979

<210> 7
<211> 979
<212> DNA
<213> Rattus norvegicus

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agtgaatgac aaaaatattc aagtggtgga gctccccatt gtagacagcc ttcatcctcg 180

gcctccttac ttaccactgg ctgttccaga agaccttgca gatcgactcg taagagtcca 240

tggtgatcct gcagtgtggt gggtgtccca gttcgtcaaa tatttgattc gtccacaacc 300

ttggctagaa aaggaaatag aagaagccac caagaagctt ggcttcaaac atccagtcat 360

tggagtccat gtcagacgca cagacaaagt gggaacagag gcagccttcc atcccatcga 420

agagtacatg gtacatgttg aagaacattt tcagcttctc gcacgcagaa tgcaagtgga 480

taaaaaaaga gtatatctgg ctaccgatga ccctgctttg ttaaaggagg caaagacaaa 540

gtactccaat tatgaattta ttagtgataa ctctatttct tggtcagctg gactacacaa 600

tcggtacaca gaaaattcac ttcggggcgt gatcctggat atacactttc tctctcaggc 660

tgacttccta gtgtgtactt tttcatccca ggtctgtcgg gttgcttatg aaatcatgca 720

aaccctgcat cctgatgcct ctgcaaactt ccactcttta gatgacatct actattttgg 780

aggccaaaat gcccacaacc agattgccgt ttatcctcac aaacctcgaa ctgatgagga 840

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taaaggtgtc aacagaaaac ttggaaaaac aggcttatat ccctcctaca aagtccgaga 960

gaagatagaa acggtcaag 979

<210> 8
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 8
aagtataagc ttacatggat gacgatatcg ctgcgctcgt 40

<210> 9
<211> 40
<212> DNA
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 9
atttaactgc aggaagcatt tgcggtggac gatggagggg 40

<210> 10
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 10
atttaaggta ccgaagcatt tgcggtgcac gatggagggg 40

<210> 11
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 11
ctccaattat gaatttatta gtg 23

<210> 12
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 12
ggatgtttga agccaagctt cttgg 25

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 13
gtccatggtg atcctgcagt gtgg 24

<210> 14
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

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<400> 14
caccaatgat atctccaggt tcc 23

<210> 15
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 15
gatatcgctg cgctcgttgt cgac 24

<210> 16
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 16
caggaaggaa ggctggaaaa gagc 24

<210> 17
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 17
gatatcgctg cgctcgtcgt cgac 24

<210> 18
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 18
caggaaggaa ggctggaaga gagc 24

<210> 19
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 19
atgcgggcat ggactggttc ctgg 24

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<210> 20
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 20
ctatttttca gcttcaggat atgtggg 27

<210> 21
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 21
gtctgaagca ttatgtgttg aagc 24

<210> 22
<211> 23
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequense: Synthetic DNA

<400> 22
gtgagtacat tcattgtact gtg 23

<210> 23
<211> 575
<212> PRT
<213> Cricetulus griseus

<400> 23
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1 5 10 15

Ala Trp Gly Thr Leu Leu Phe Tyr Ile Gly Gly His Leu Val Arg Asp
20 25 30

Asn Asp His Pro Asp His Ser Ser Arg Glu Leu Ser Lys Ile Leu Ala
35 40 45

Lys Leu Glu Arg Leu Lys Gln Gln Asn Glu Asp Leu Arg Arg Met Ala
50 55 60

Glu Ser Leu Arg Ile Pro Glu Gly Pro Ile Asp Gln Gly Thr Ala Thr
65 70 75 80

Gly Arg Val Arg Val Leu Glu Glu Gln Leu Val Lys Ala Lys Glu Gln
85 90 95

Ile Glu Asn Tyr Lys Lys Gln Ala Arg Asn Asp Leu Gly Lys Asp His
100 105 110

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Glu Ile Leu Arg Arg Arg Ile Glu Asn Gly Ala Lys Glu Leu Trp Phe
115 120 125

Phe Leu Gln Ser Glu Leu Lys Lys Leu Lys Lys Leu Glu Gly Asn Glu
130 135 140

Leu Gln Arg His Ala Asp Glu Ile Leu Leu Asp Leu Gly His His Glu
145 150 155 160

Arg Ser Ile Met Thr Asp Leu Tyr Tyr Leu Ser Gln Thr Asp Gly Ala
165 170 175

Gly Glu Trp Arg Glu Lys Glu Ala Lys Asp Leu Thr Glu Leu Val Gln
180 185 190

Arg Arg Ile Thr Tyr Leu Gln Asn Pro Lys Asp Cys Ser Lys Ala Arg
195 200 205

Lys Leu Val Cys Asn Ile Asn Lys Gly Cys Gly Tyr Gly Cys Gln Leu
210 215 220

His His Val Val Tyr Cys Phe Met Ile Ala Tyr Gly Thr Gln Arg Thr
225 230 235 240

Leu Ile Leu Glu Ser Gln Asn Trp Arg Tyr Ala Thr Gly Gly Trp Glu
245 250 255

Thr Val Phe Arg Pro Val Ser Glu Thr Cys Thr Asp Arg Ser Gly Leu
260 265 270

Ser Thr Gly His Trp Ser Gly Glu Val Lys Asp Lys Asn Val Gln Val
275 280 285

Val Glu Leu Pro Ile Val Asp Ser Leu His Pro Arg Pro Pro Tyr Leu
290 295 300

Pro Leu Ala Val Pro Glu Asp Leu Ala Asp Arg Leu Leu Arg Val His
305 310 315 320

Gly Asp Pro Ala Val Trp Trp Val Ser Gln Phe Val Lys Tyr Leu Ile
325 330 335

Arg Pro Gln Pro Trp Leu Glu Arg Glu Ile Glu Glu Thr Thr Lys Lys
340 345 350

Leu Gly Phe Lys His Pro Val Ile Gly Val His Val Arg Arg Thr Asp
355 360 365

Lys Val Gly Thr Glu Ala Ala Phe His Pro Ile Glu Glu Tyr Met Val
370 375 380

His Val Glu Glu His Phe Gln Leu Leu Glu Arg Arg Met Lys Val Asp
385 390 395 400

Lys Lys Arg Val Tyr Leu Ala Thr Asp Asp Pro Ser Leu Leu Lys Glu
405 410 415

Ala Lys Thr Lys Tyr Ser Asn Tyr Glu Phe Ile Ser Asp Asn Ser Ile
420 425 430

Ser Trp Ser Ala Gly Leu His Asn Arg Tyr Thr Glu Asn Ser Leu Arg
435 440 445

Aragen/Transposagen Ex. 1036 - Part B

Gly Val Ile Leu Asp Ile His Phe Leu Ser Gln Ala Asp Phe Leu Val
450 455 460

Cys Thr Phe Ser Ser Gln Val Cys Arg Val Ala Tyr Glu Ile Met Gln
465 470 475 480

Thr Leu His Pro Asp Ala Ser Ala Asn Phe His Ser Leu Asp Asp Ile
485 490 495

Tyr Tyr Phe Gly Gly Gln Asn Ala His Asn Gln Ile Ala Val Tyr Pro
500 505 510

His Gln Pro Arg Thr Lys Glu Glu Ile Pro Met Glu Pro Gly Asp Ile
515 520 525

Ile Gly Val Ala Gly Asn His Trp Asn Gly Tyr Ser Lys Gly Val Asn
530 535 540

Arg Lys Leu Gly Lys Thr Gly Leu Tyr Pro Ser Tyr Lys Val Arg Glu
545 550 555 560

Lys Ile Glu Thr Val Lys Tyr Pro Thr Tyr Pro Glu Ala Glu Lys
565 570 575

<210> 24
<211> 575
<212> PRT
<213> Mus musculus

<400> 24
Met Arg Ala Trp Thr Gly Ser Trp Arg Trp Ile Met Leu Ile Leu Phe
1 5 10 15

Ala Trp Gly Thr Leu Leu Phe Tyr Ile Gly Gly His Leu Val Arg Asp
20 25 30

Asn Asp His Pro Asp His Ser Ser Arg Glu Leu Ser Lys Ile Leu Ala
35 40 45

Lys Leu Glu Arg Leu Lys Gln Gln Asn Glu Asp Leu Arg Arg Met Ala
50 55 60

Glu Ser Leu Arg Ile Pro Glu Gly Pro Ile Asp Gln Gly Thr Ala Thr
65 70 75 80

Gly Arg Val Arg Val Leu Glu Glu Gln Leu Val Lys Ala Lys Glu Gln
85 90 95

Ile Glu Asn Tyr Lys Lys Gln Ala Arg Asn Gly Leu Gly Lys Asp His
100 105 110

Glu Ile Leu Arg Arg Arg Ile Glu Asn Gly Ala Lys Glu Leu Trp Phe
115 120 125

Phe Leu Gln Ser Glu Leu Lys Lys Leu Lys His Leu Glu Gly Asn Glu
130 135 140

Leu Gln Arg His Ala Asp Glu Ile Leu Leu Asp Leu Gly His His Glu
145 150 155 160

Arg Ser Ile Met Thr Asp Leu Tyr Tyr Leu Ser Gln Thr Asp Gly Ala

Aragen/Transposagen Ex. 1036 - Part B

```
                 165                 170                 175
Gly Asp Trp Arg Glu Lys Glu Ala Lys Asp Leu Thr Glu Leu Val Gln
            180                 185                 190

Arg Arg Ile Thr Tyr Leu Gln Asn Pro Lys Asp Cys Ser Lys Ala Arg
        195                 200                 205

Lys Leu Val Cys Asn Ile Asn Lys Gly Cys Gly Tyr Gly Cys Gln Leu
    210                 215                 220

His His Val Val Tyr Cys Phe Met Ile Ala Tyr Gly Thr Gln Arg Thr
225                 230                 235                 240

Leu Ile Leu Glu Ser Gln Asn Trp Arg Tyr Ala Thr Gly Gly Trp Glu
                245                 250                 255

Thr Val Phe Arg Pro Val Ser Glu Thr Cys Thr Asp Arg Ser Gly Leu
            260                 265                 270

Ser Thr Gly His Trp Ser Gly Glu Val Asn Asp Lys Asn Ile Gln Val
        275                 280                 285

Val Glu Leu Pro Ile Val Asp Ser Leu His Pro Arg Pro Pro Tyr Leu
    290                 295                 300

Pro Leu Ala Val Pro Glu Asp Leu Ala Asp Arg Leu Leu Arg Val His
305                 310                 315                 320

Gly Asp Pro Ala Val Trp Trp Val Ser Gln Phe Val Lys Tyr Leu Ile
                325                 330                 335

Arg Pro Gln Pro Trp Leu Glu Lys Glu Ile Glu Glu Ala Thr Lys Lys
            340                 345                 350

Leu Gly Phe Lys His Pro Val Ile Gly Val His Val Arg Arg Thr Asp
        355                 360                 365

Lys Val Gly Thr Glu Ala Ala Phe His Pro Ile Glu Glu Tyr Met Val
    370                 375                 380

His Val Glu Glu His Phe Gln Leu Leu Ala Arg Arg Met Gln Val Asp
385                 390                 395                 400

Lys Lys Arg Val Tyr Leu Ala Thr Asp Asp Pro Thr Leu Leu Lys Glu
                405                 410                 415

Ala Lys Thr Lys Tyr Ser Asn Tyr Glu Phe Ile Ser Asp Asn Ser Ile
            420                 425                 430

Ser Trp Ser Ala Gly Leu His Asn Arg Tyr Thr Glu Asn Ser Leu Arg
        435                 440                 445

Gly Val Ile Leu Asp Ile His Phe Leu Ser Gln Ala Asp Phe Leu Val
    450                 455                 460

Cys Thr Phe Ser Ser Gln Val Cys Arg Val Ala Tyr Glu Ile Met Gln
465                 470                 475                 480

Thr Leu His Pro Asp Ala Ser Ala Asn Phe His Ser Leu Asp Asp Ile
                485                 490                 495

Tyr Tyr Phe Gly Gly Gln Asn Ala His Asn Gln Ile Ala Val Tyr Pro
```


Aragen/Transposagen Ex. 1036 - Part B

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          500                 505                 510
His Lys Pro Arg Thr Glu Glu Glu Ile Pro Met Glu Pro Gly Asp Ile
    515                 520                 525

Ile Gly Val Ala Gly Asn His Trp Asp Gly Tyr Ser Lys Gly Ile Asn
530                 535                 540

Arg Lys Leu Gly Lys Thr Gly Leu Tyr Pro Ser Tyr Lys Val Arg Glu
545                 550                 555                 560

Lys Ile Glu Thr Val Lys Tyr Pro Thr Tyr Pro Glu Ala Glu Lys
            565                 570                 575
```

(Brief explanation of the drawings)

(Fig. 1)

Fig. 1 shows electrophoresis patterns of SDS-PAGE of five purified anti-GD3 chimeric antibodies (using gradient gel from 4 to 15%). Fig. 1 (upper) and Fig. 1 (lower) show a result of the electrophoresis under non-reducing conditions and that under reducing conditions, respectively. Lanes 1 to 7 show electrophoresis patterns of high molecular weight markers, YB2/0-GD3 chimeric antibody, CHO/DG44-GD3 chimeric antibody, SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody (302), NS0-GD3 chimeric antibody (GIT) and low molecular weight markers, respectively.

(Fig. 2)

Fig. 2 shows activities of five purified anti-GD3 chimeric antibodies to bind to GD3, measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with GD3 and the antibody concentration, respectively. "○", "●", "□", "■" and "△" show the activities of YB2/0-GD3 chimeric antibody, CHO/DG44-GD3 chimeric antibody, SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT), respectively.

(Fig. 3)

Fig. 3 shows ADCC activities of five purified anti-GD3 chimeric antibodies for a human melanoma cell line G-361. The ordinate and abscissa show the cytotoxic activity

Aragen/Transposagen Ex. 1036 - Part B

and the antibody concentration, respectively. "○", "●", "□", "■" and "△" show the activities of YB2/0-GD3 chimeric antibody, CHO/DG44-GD3 chimeric antibody, SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT), respectively.

(Fig. 4)

Fig. 4 shows electrophoresis patterns of SDS-PAGE of three purified anti-hIL-5Rα CDR-grafted antibodies (using gradient gel from 4 to 15%). Fig. 4 (upper) and Fig. 4 (lower) show results of the electrophoresis carried out under non-reducing conditions and those under reducing conditions, respectively. Lanes 1 to 5 show electrophoresis patterns of high molecular weight markers, YB2/0-hIL-5R CDR antibody, CHO/d-hIL-5R CDR antibody, NS0-hIL-5R CDR antibody and low molecular weight markers, respectively.

(Fig. 5)

Fig. 5 shows activities of three purified anti-hIL-5Rα CDR-grafted antibodies to bind to hIL-5Rα, measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with hIL-5Rα and the antibody concentration, respectively. "○", "●" and "□" show the activities of YB2/0-hIL-5R CDR antibody, CHO/d-hIL-5R CDR antibody and NS0-hIL-5R CDR antibody, respectively.

(Fig. 6)

Fig. 6 show ADCC activities of three purified anti-hIL-5Rα CDR-grafted antibodies for an hIL-5R expressing mouse T cell line CTLL-2(h5R). The ordinate and the abscissas show the cytotoxic activity and the antibody concentration, respectively. "○", "●" and "□" show the activities of YB2/0-hIL-5RCDR antibody, CHO/d-hIL-5R CDR antibody and NS0-hIL-5R CDR antibody, respectively.

(Fig. 7)

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Fig. 7 shows inhibition activities of three purified anti-hIL-5Rα CDR-grafted antibodies in an hIL-5-induced eosinophil increasing model of *Macaca faseicularis*. The ordinate and the abscissa show the number of eosinophils in peripheral blood and the number of days (the day of the commencement of antibody and hIL-5 administration was defined as 0 day). "101 and 102", "301, 302 and 303", "401, 402 and 403" and "501, 502 and 503" show results in the antibody non-administration group, the YB2/0-hIL-5R CDR antibody administered group, the CHO/d-hIL-5R CDR antibody administered group and the NS0-hIL-5R CDR antibody administered group, respectively.

(Fig. 8)

Fig. 8 shows elution patterns of reverse phase HPLC elution of a PA-treated sugar chain (left side), and an elution pattern obtained by treating the PA-treated sugar chain with α-L-fucosidase and then analyzed by reverse phase HPLC (right side), of the purified anti-hIL-5Rα CDR-grafted antibody produced by YB2/0 (Fig. 8, upper) and the purified anti-hIL-5Rα CDR-grafted antibody produced by NS0 (Fig. 8, lower). The ordinates and the abscissas show the relative fluorescence intensity and the elution time, respectively.

(Fig. 9)

Fig. 9 shows an elution pattern obtained by preparing a PA-treated sugar chain from the purified anti-hIL-5Rα CDR-grafted antibody produced by CHO/d cell and analyzing it by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

(Fig. 10)

In Fig. 10, Fig. 10 (upper) shows the GD3-binding activities of a non-adsorbed fraction and a part of an adsorbed fraction, measured by changing the antibody concentration. The ordinate and the abscissa show the

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binding activity with GD3 and the antibody concentration, respectively. "●" and "○" show the non-adsorbed fraction and a part of the adsorbed fraction, respectively. Fig. 10 (lower) shows the ADCC activities of the non-adsorbed fraction and a part of the adsorbed fraction for a human melanoma line G-361. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. "●" and "○" show the non-adsorbed fraction and a part of the adsorbed fraction, respectively.

(Fig. 11)

Fig. 11 shows elution patterns obtained by analyzing PA-treated sugar chains prepared from a non-adsorbed fraction and a part of an adsorbed fraction by a reverse HPLC. Fig. 11 (left) and Fig. 11 (light) show an elution pattern of the non-adsorbed fraction and an elution pattern of a part of the adsorbed fraction, respectively. The ordinates and the abscissas show the relative fluorescence strength and the elution time, respectively.

(Fig. 12)

Fig. 12 shows construction of plasmids CHFT8-pCR2.1 and YBFT8-pCR2.1.

(Fig. 13)

Fig. 13 shows construction of plasmids CHAc-pBS and YBAc-pBS.

(Fig. 14)

Fig. 14 shows construction of plasmids CHFT8d-pCR2.1 and YBFT8d-pCR2.1.

(Fig. 15)

Fig. 15 shows construction of plasmids CHAcd-pBS and YBAcd-pBS.

(Fig. 16)

Fig. 16 shows results of determination of an FUT8 transcription product in each host cell line using competitive RT-PCR. Amounts of the FUT8 transcription product in each host cell line when rat FUT8 sequence was

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used as the standard and internal control are shown. "■" and "□" show results when CHO cell line and YB2/0 cell line, respectively, were used as the host cell.

(Fig. 17)

Fig. 17 shows construction of a plasmid mfFUT8-pCR2.1.

(Fig. 18)

Fig. 18 shows construction of a plasmid pBSmfFUT8.

(Fig. 19)

Fig. 19 shows construction of a plasmid pAGEmfFUT8.

(Fig. 20)

Fig. 20 shows results of analysis of expression levels of FUT8 gene by a cell line excessively expressing the gene using a competitive RT-PCR. The ordinate shows relative values of amounts of FUT8 transcription to amounts of β-actin transcription.

(Fig. 21)

Fig. 21 shows ADCC activities of an anti-GD3 chimeric antibody purified from a cell line excessively expressing FUT8 gene against a human melanoma cell line G-361. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively.

(Fig. 22)

Fig. 22 shows construction of a plasmid CHfFUT8-pCR2.1.

(Fig. 23)

Fig. 23 shows elution patterns of PA-treated sugar chains prepared from antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines, obtained by analyzing them by reverse phase HPLC. Fig. 30 (upper) and Fig. 30 (lower) show elution patterns of PA-treated sugar chains prepared from an antibody produced by mfFUT8-6-introduced cell line and PA-treated sugar chains prepared from an antibody produced by pAGE249-introduced cell line, respectively.

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The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively. (Fig. 24)

Fig. 24 shows an elution pattern of PA-treated sugar chains prepared from Herceptin, obtained by analyzing them by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

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(Document name)

Abstract

(Abstract)

(Problem)

It is to provide a cell useful as a host cell for the production of a glycoprotein which can control the sugar chain structure of the glycoprotein, especially, a cell capable of producing an immunologically functional molecule of which effector function is controlled, a method for producing an immunologically functional molecule using the cell, an immunologically functional molecule produced by the production method, and their use.

(Means for solution)

A cell for the production of a glycoprotein such as an immunologically functional molecule or the like, a method for producing an immunologically functional molecule using the cell, an immunologically functional molecule, and use thereof.

(Selected drawing)

None

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(Document name)

Drawings

OIPE JC99 DEC 28 2004 PATENT & TRADEMARK OFFICE

FIG. 1

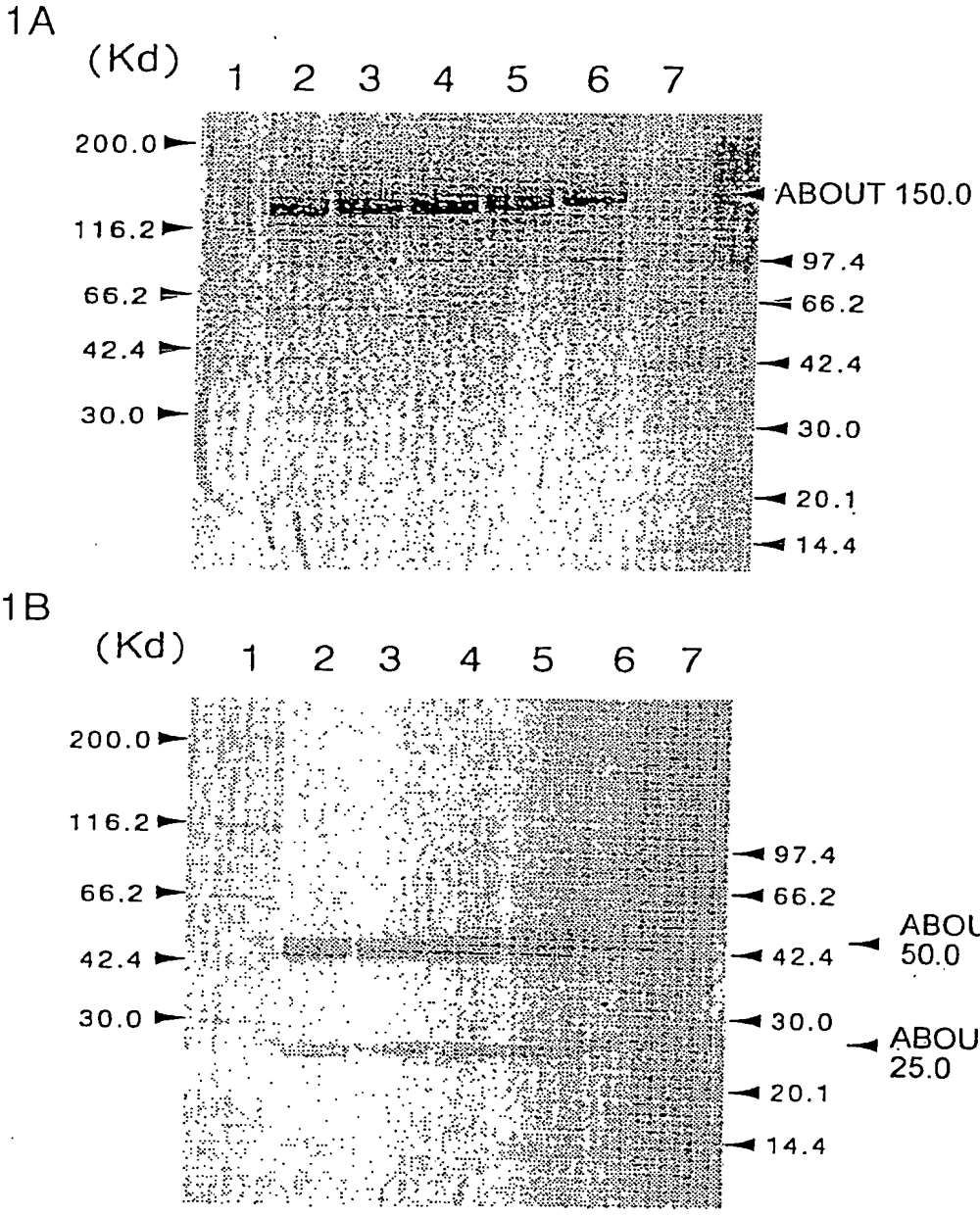


FIG. 2

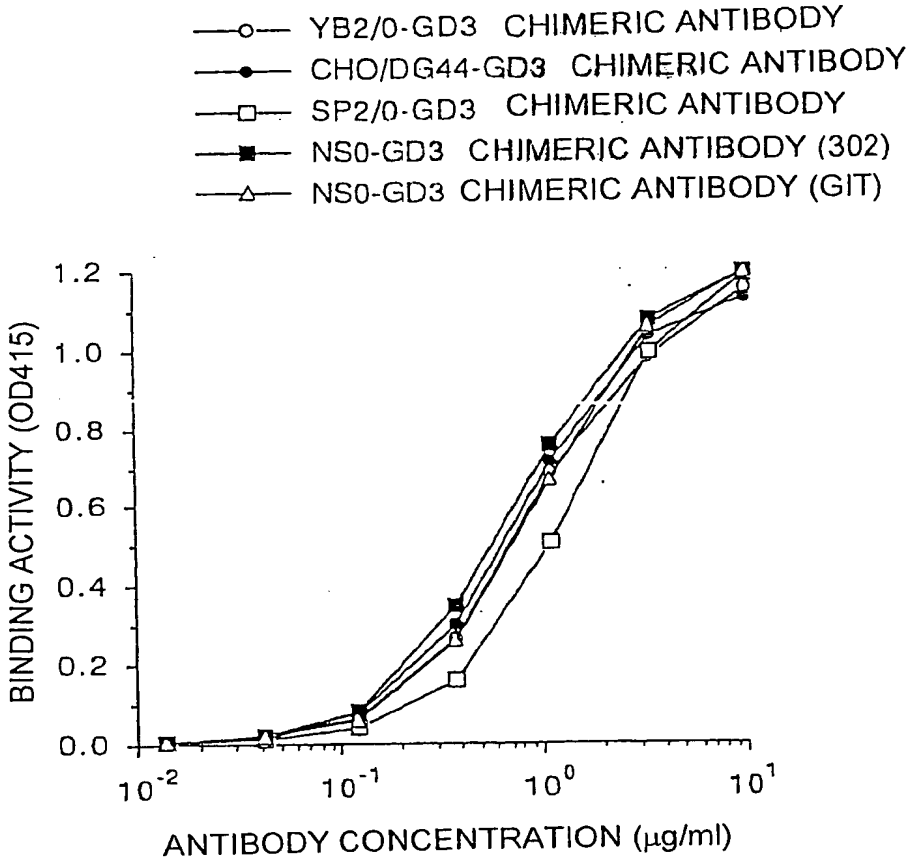

YB2/0-GD3 CHIMERIC ANTIBODY
CHO/DG44-GD3 CHIMERIC ANTIBODY
SP2/0-GD3 CHIMERIC ANTIBODY
NS0-GD3 CHIMERIC ANTIBODY (302)
NS0-GD3 CHIMERIC ANTIBODY (GIT)
BINDING ACTIVITY (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^{-2}
10^{-1}
10^{0}
10^{1}
ANTIBODY CONCENTRATION (μg/ml)

FIG. 3

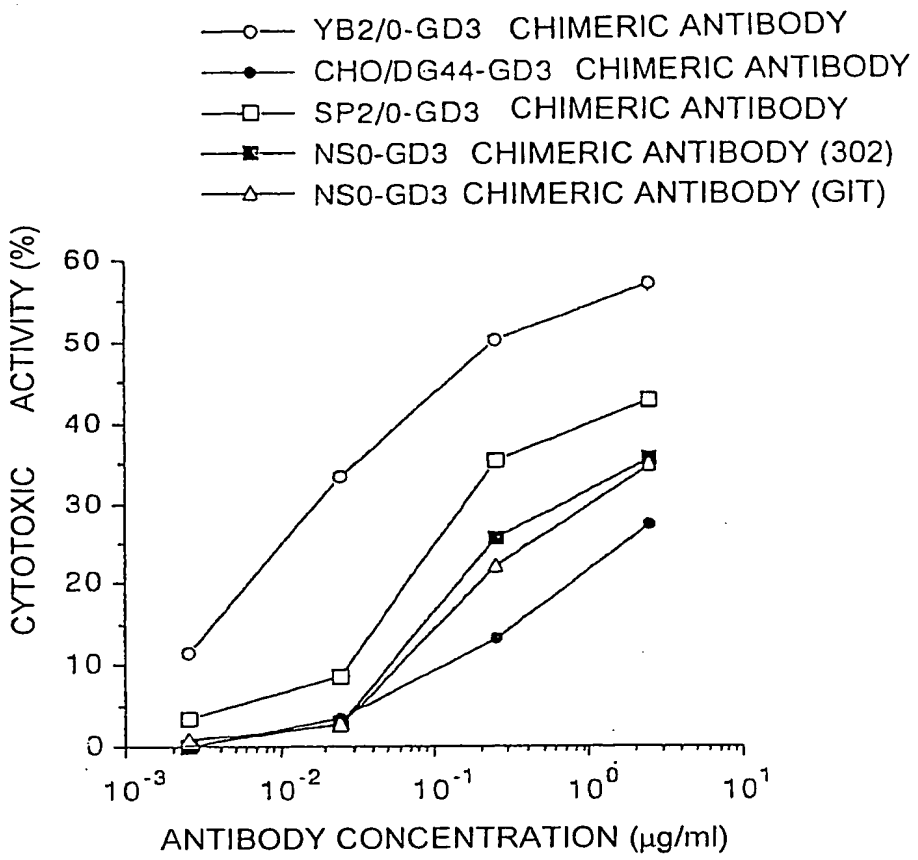

YB2/0-GD3 CHIMERIC ANTIBODY
CHO/DG44-GD3 CHIMERIC ANTIBODY
SP2/0-GD3 CHIMERIC ANTIBODY
NS0-GD3 CHIMERIC ANTIBODY (302)
NS0-GD3 CHIMERIC ANTIBODY (GIT)
CYTOTOXIC ACTIVITY (%)
60
50
40
30
20
10
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
ANTIBODY CONCENTRATION (μg/ml)

FIG. 4

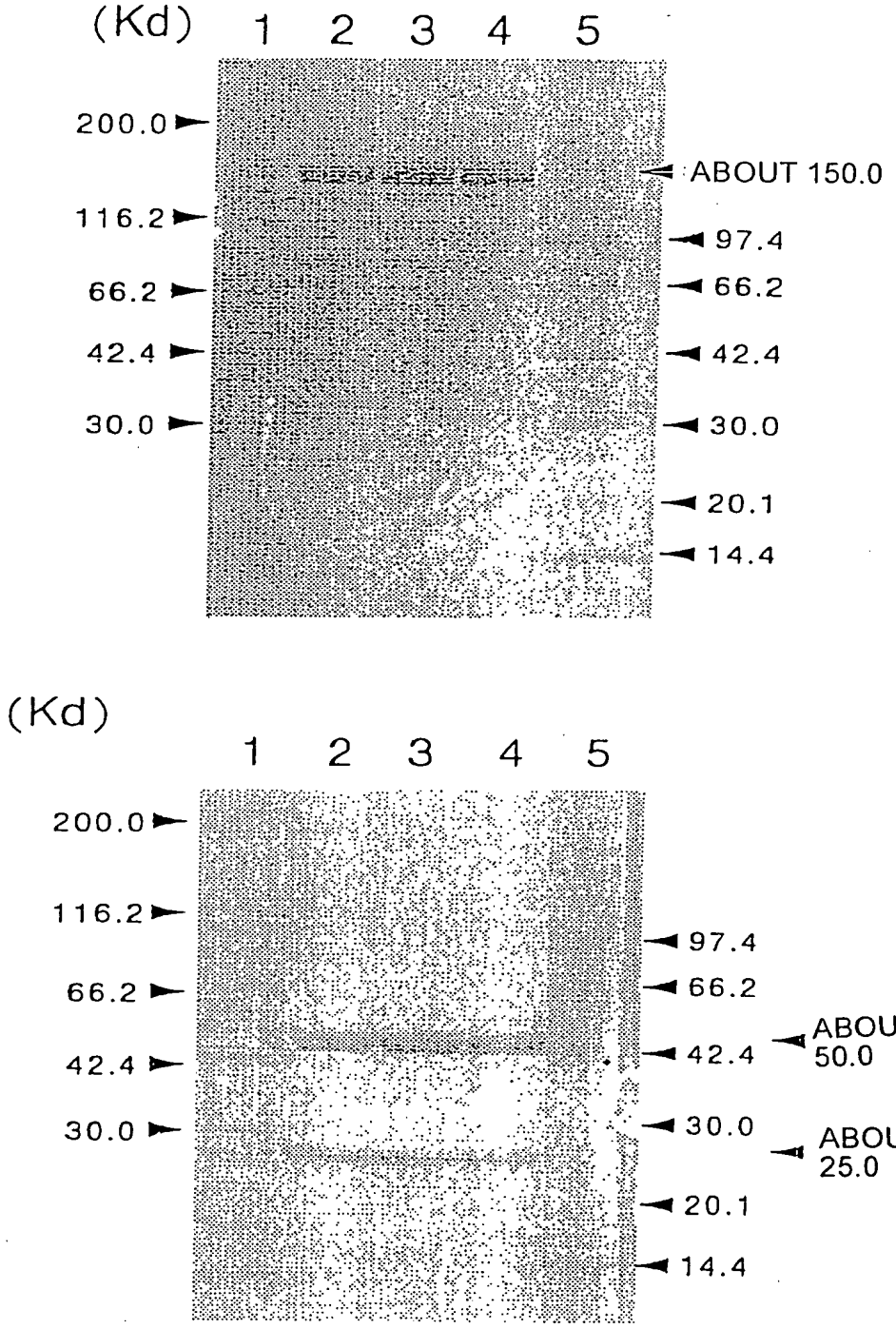

(Kd)
1 2 3 4 5
200.0
116.2
66.2
42.4
30.0
ABOUT 150.0
97.4
66.2
42.4
30.0
20.1
14.4
(Kd)
1 2 3 4 5
200.0
116.2
66.2
42.4
30.0
97.4
66.2
ABOUT 50.0
42.4
30.0
ABOUT 25.0
20.1
14.4

FIG. 5

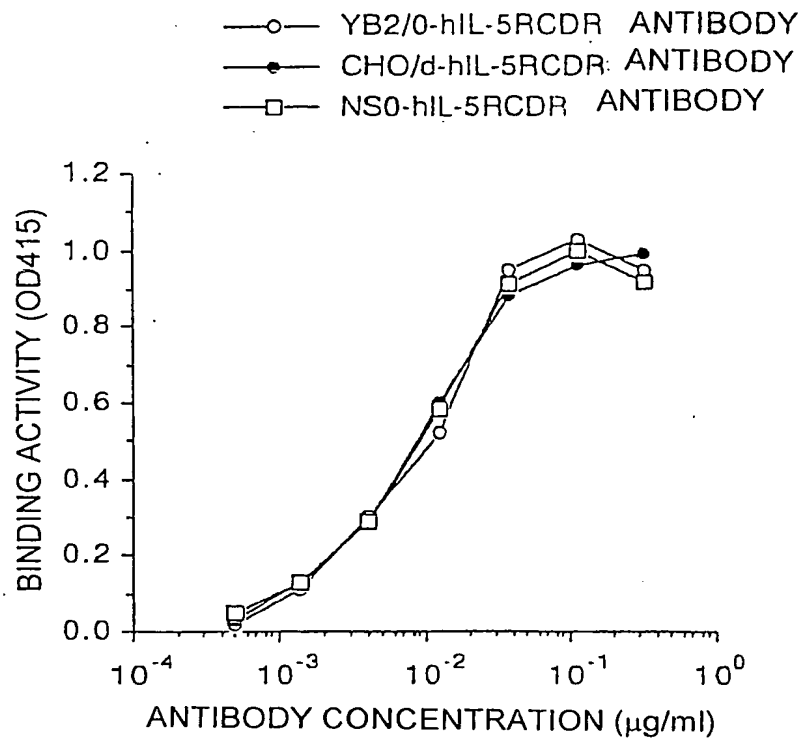

YB2/0-hIL-5RCDR ANTIBODY
CHO/d-hIL-5RCDR ANTIBODY
NS0-hIL-5RCDR ANTIBODY
BINDING ACTIVITY (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
ANTIBODY CONCENTRATION (μg/ml)

FIG. 6

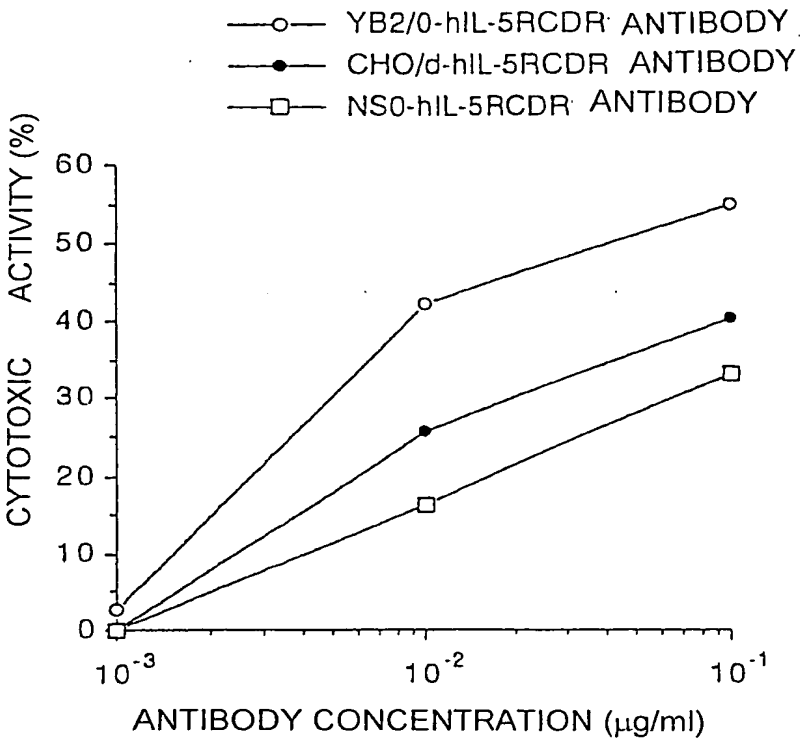

YB2/0-hIL-5RCDR ANTIBODY
CHO/d-hIL-5RCDR ANTIBODY
NS0-hIL-5RCDR ANTIBODY
CYTOTOXIC ACTIVITY (%)
60
50
40
30
20
10
0
10^{-3}
10^{-2}
10^{-1}
ANTIBODY CONCENTRATION (μg/ml)

FIG. 7

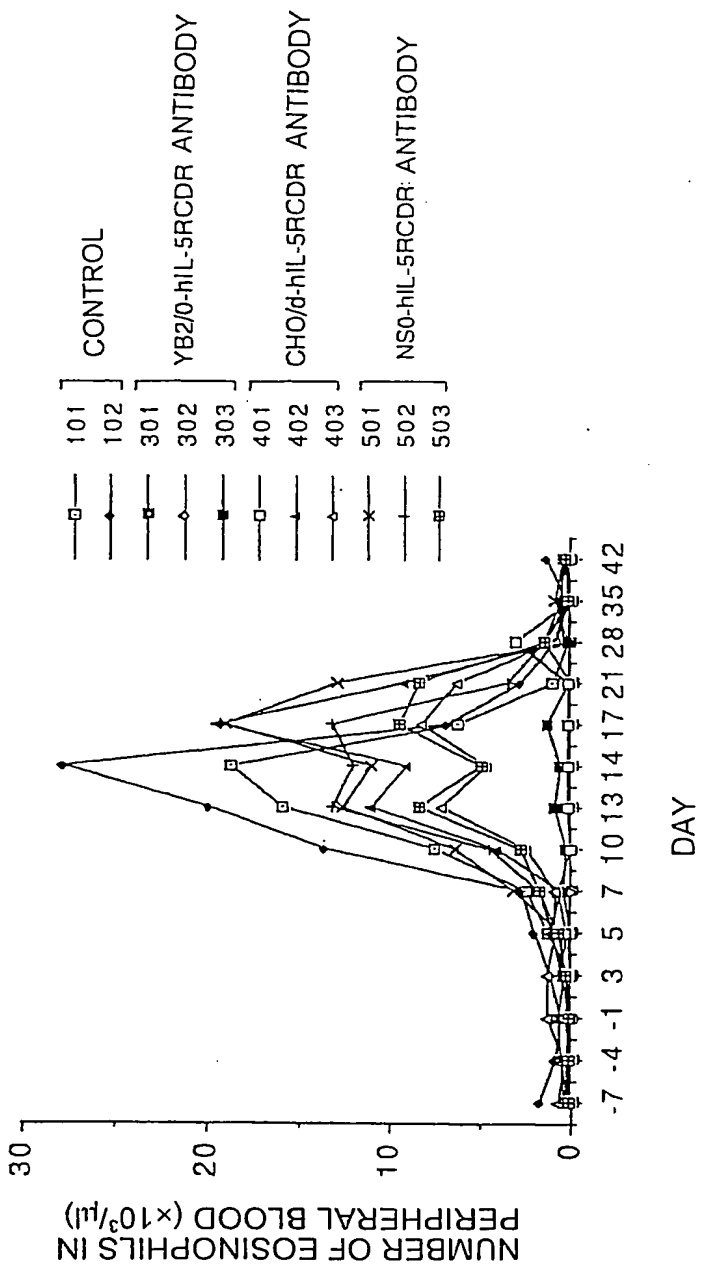
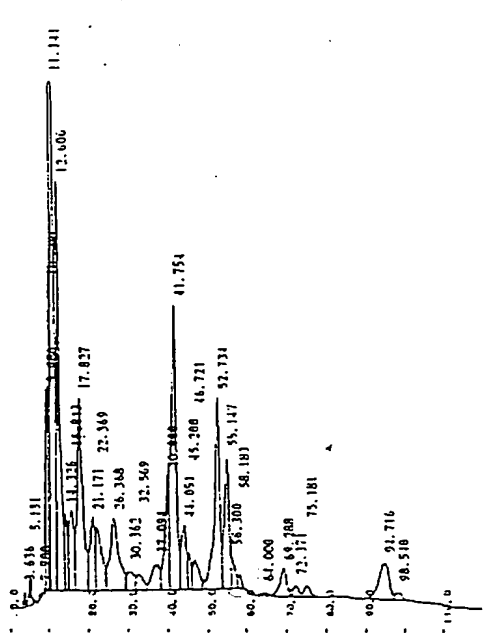
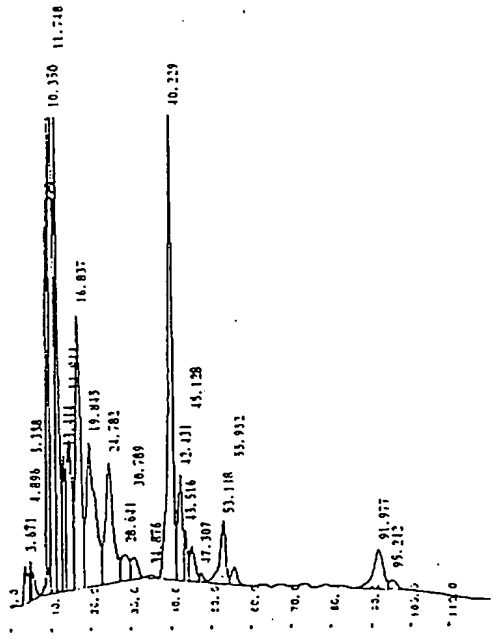

CONTROL
101
102
YB2/0-hIL-5RCDR ANTIBODY
301
302
303
CHO/d-hIL-5RCDR ANTIBODY
401
402
403
NS0-hIL-5RCDR ANTIBODY
501
502
503
NUMBER OF EOSINOPHILS IN PERIPHERAL BLOOD (×10³/μl)
30
20
10
0
-7 -4 -1 3 5 7 10 13 14 17 21 28 35 42
DAY

FIG. 8

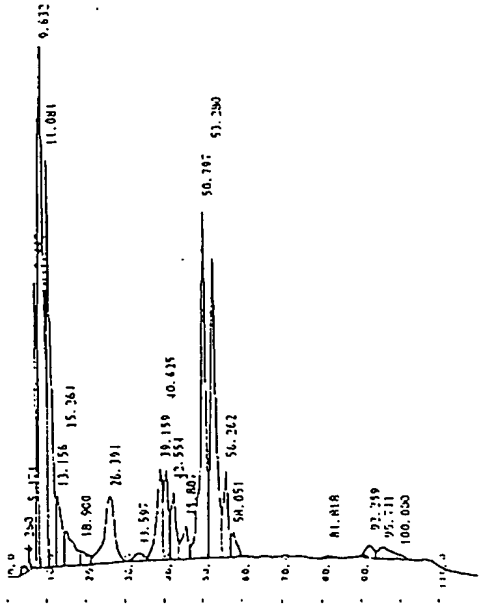
YB2/0-hIL-5RCDR ANTIBODY

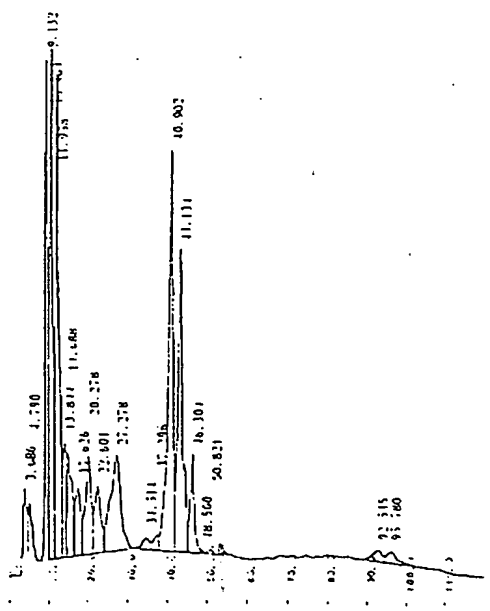




AFTER DIGESTION WITH α-L-FUCOSIDASE

NS0-hIL-5RCDR ANTIBODY





AFTER DIGESTION WITH α-L-FUCOSIDASE

FIG. 9

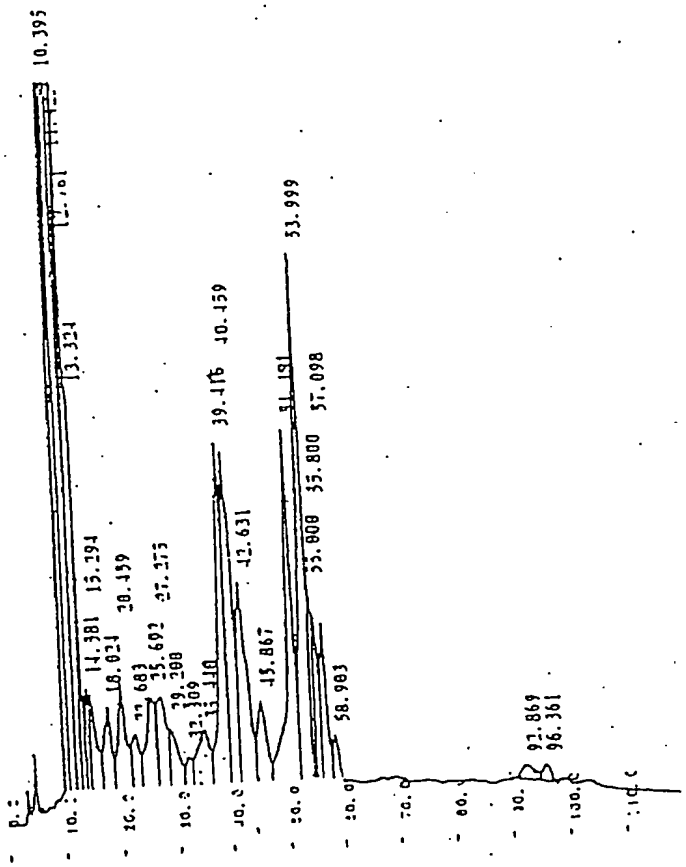

10.395
15.294
20.459
39.476
40.459
42.631
53.999
55.800
57.098
58.983
92.869
96.361

FIG. 10

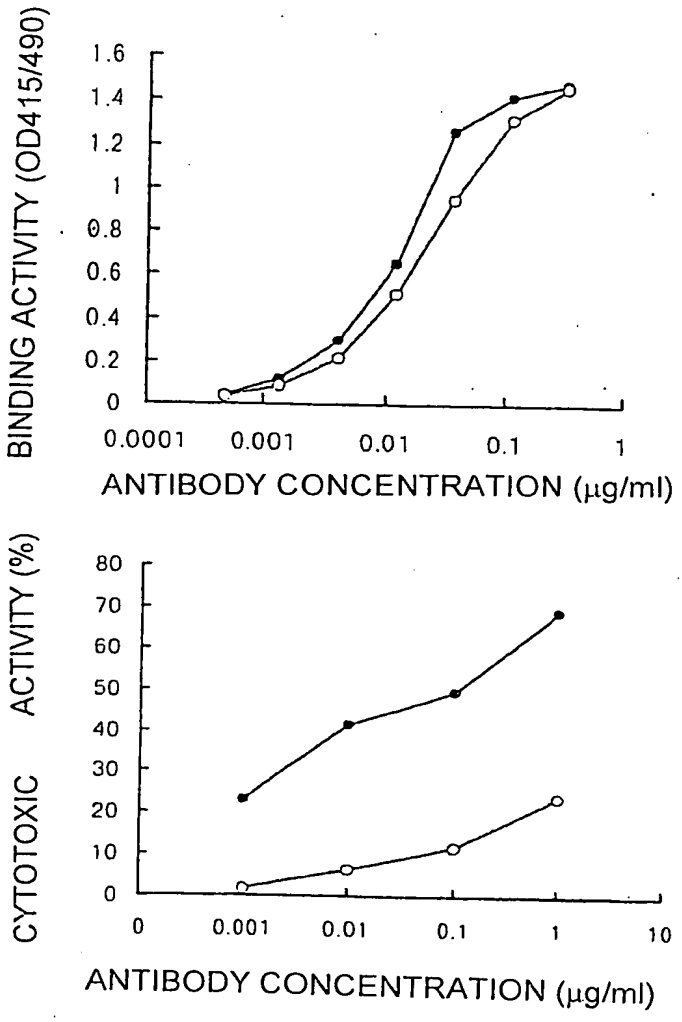

BINDING ACTIVITY (OD415/490)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.0001
0.001
0.01
0.1
1
ANTIBODY CONCENTRATION (μg/ml)
CYTOTOXIC ACTIVITY (%)
80
70
60
50
40
30
20
10
0
0
0.001
0.01
0.1
1
10
ANTIBODY CONCENTRATION (μg/ml)

FIG. 11

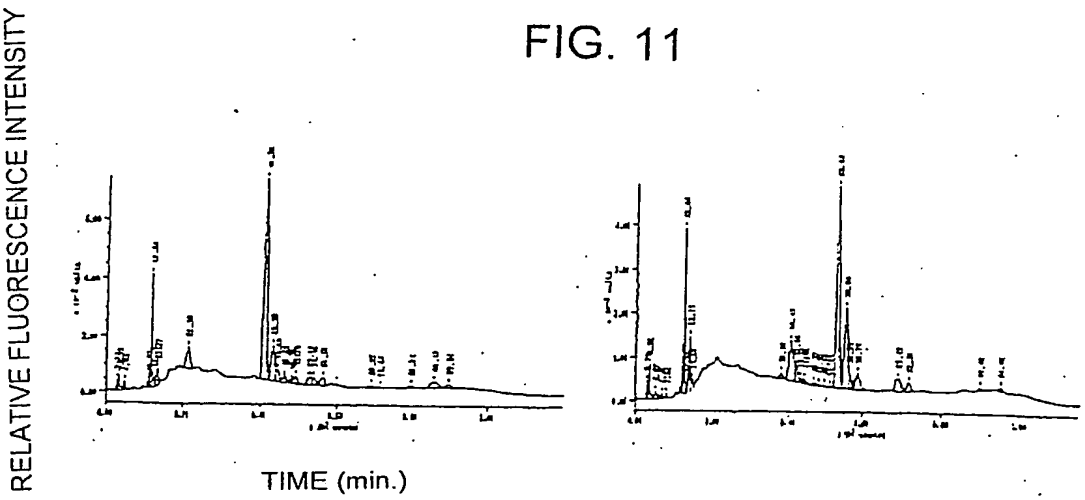


FIG. 12

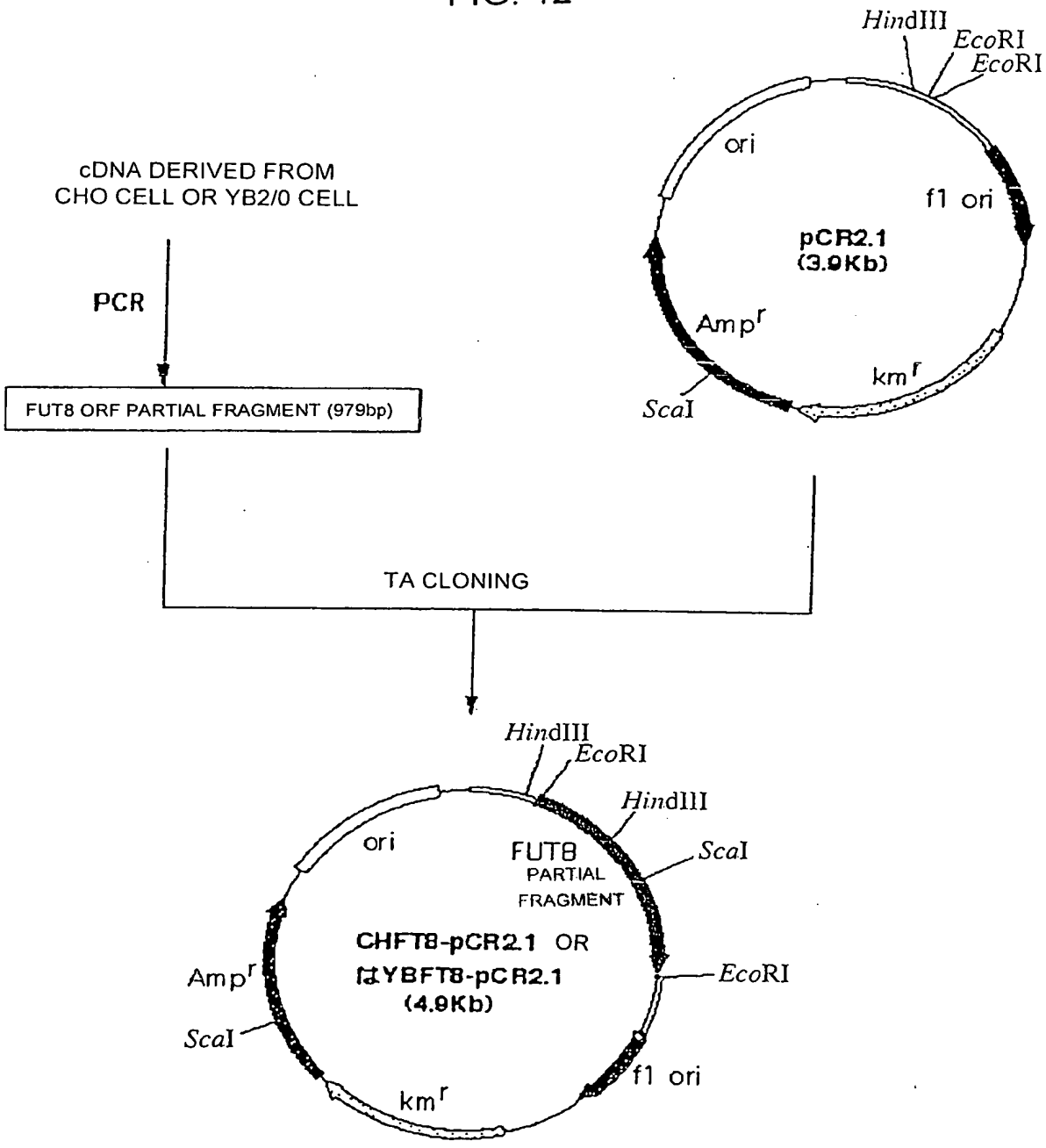

cDNA DERIVED FROM
CHO CELL OR YB2/0 CELL
PCR
FUT8 ORF PARTIAL FRAGMENT (979bp)
HindIII
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Ampr
kmr
ScaI
TA CLONING
HindIII
EcoRI
HindIII
ScaI
ori
FUT8
PARTIAL
FRAGMENT
CHFT8-pCR2.1 OR
YBFT8-pCR2.1
(4.9Kb)
Ampr
EcoRI
ScaI
f1 ori
kmr

FIG. 13

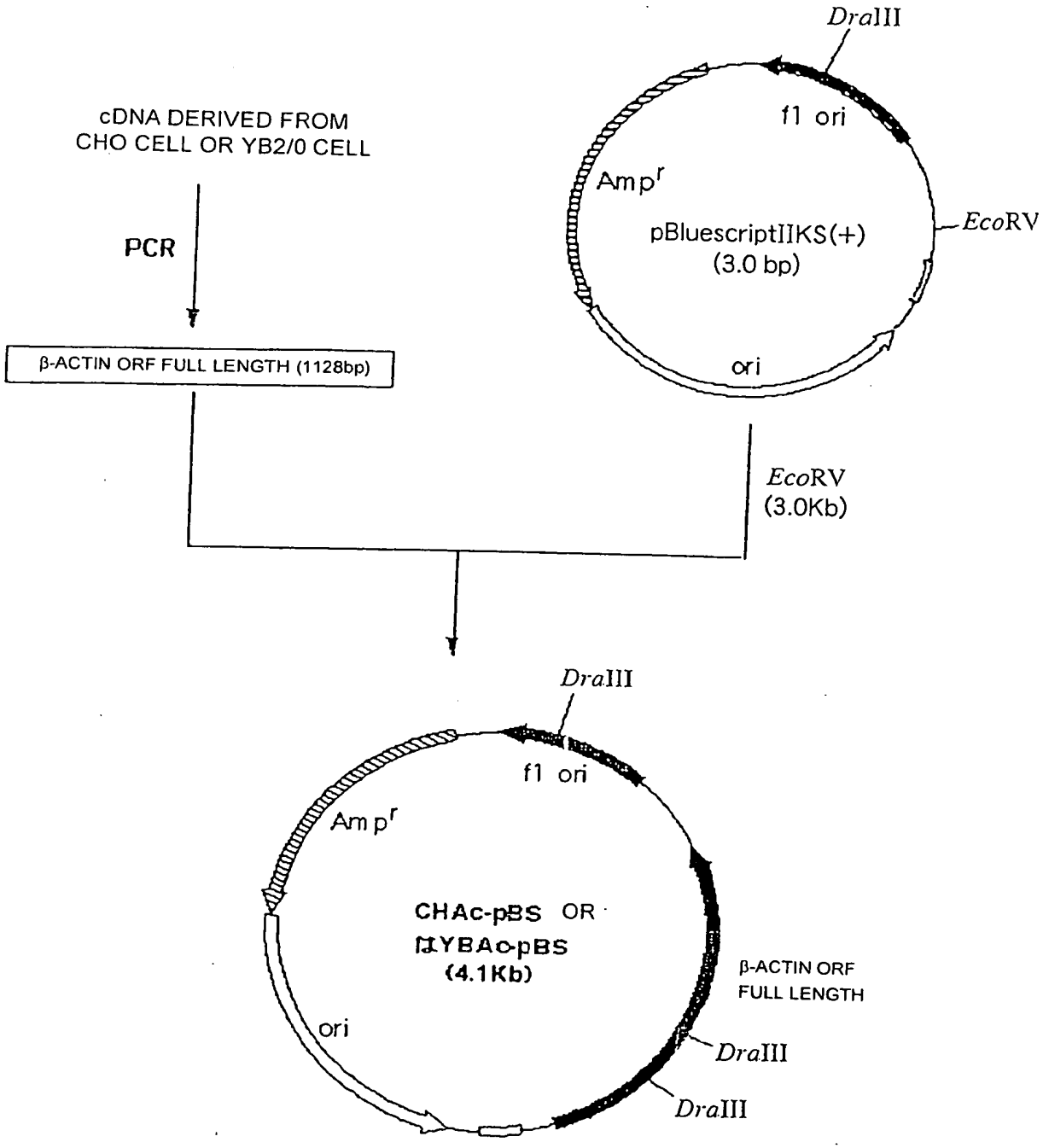

cDNA DERIVED FROM
CHO CELL OR YB2/0 CELL
PCR
β-ACTIN ORF FULL LENGTH (1128bp)
DraIII
f1 ori
Ampr
pBluescriptIIKS(+)
(3.0 bp)
EcoRV
ori
EcoRV
(3.0Kb)
DraIII
f1 ori
Ampr
CHAc-pBS OR
MYBAc-pBS
(4.1Kb)
β-ACTIN ORF
FULL LENGTH
ori
DraIII
DraIII

FIG. 14

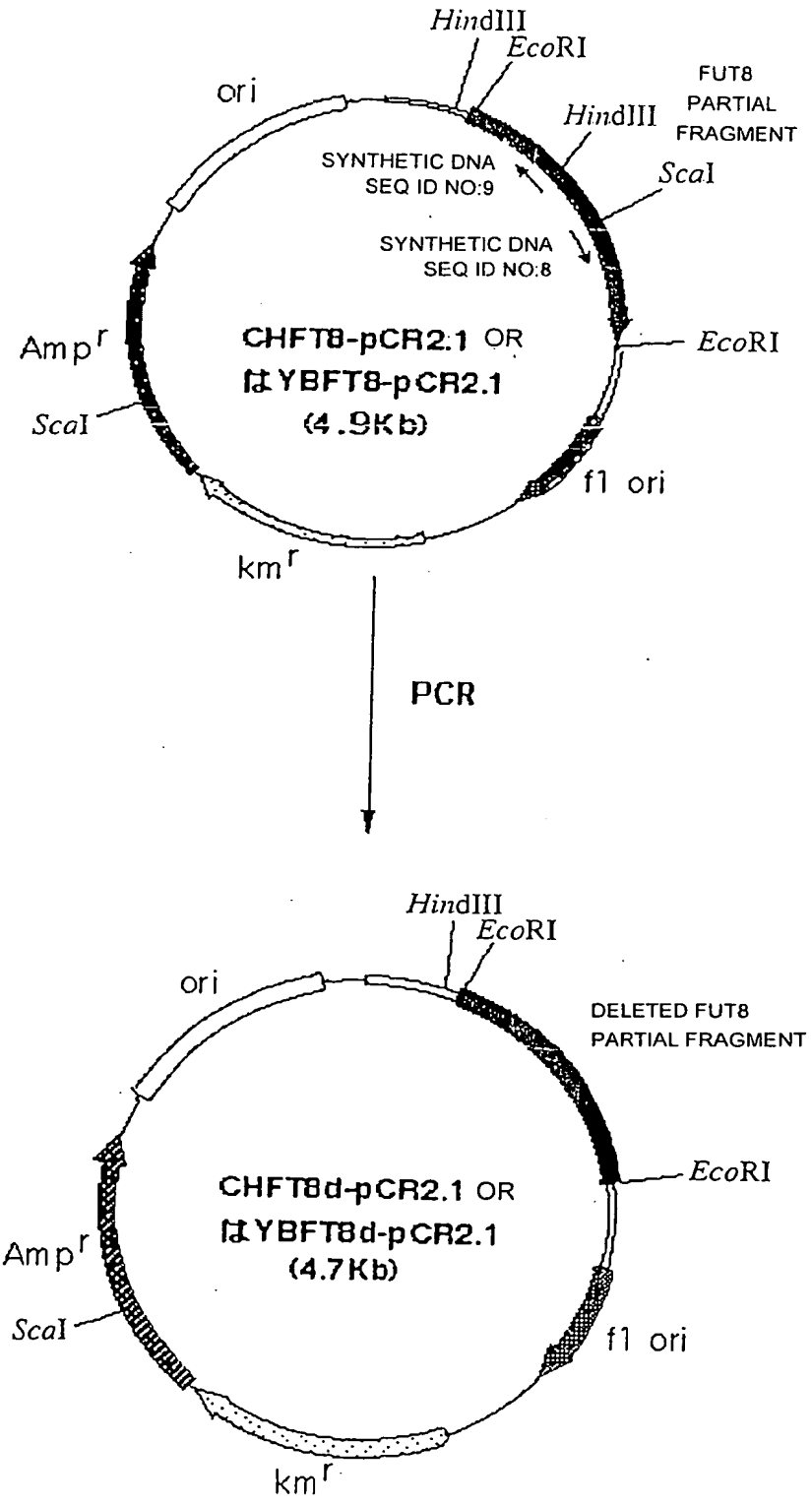

HindIII
EcoRI
ori
FUT8
PARTIAL
FRAGMENT
HindIII
SYNTHETIC DNA
SEQ ID NO:9
ScaI
SYNTHETIC DNA
SEQ ID NO:8
Ampr
CHFT8-pCR2.1 OR
はYBFT8-pCR2.1
(4.9Kb)
EcoRI
ScaI
f1 ori
kmr
PCR
HindIII
EcoRI
ori
DELETED FUT8
PARTIAL FRAGMENT
EcoRI
CHFT8d-pCR2.1 OR
はYBFT8d-pCR2.1
(4.7Kb)
Ampr
ScaI
f1 ori
kmr

FIG. 15

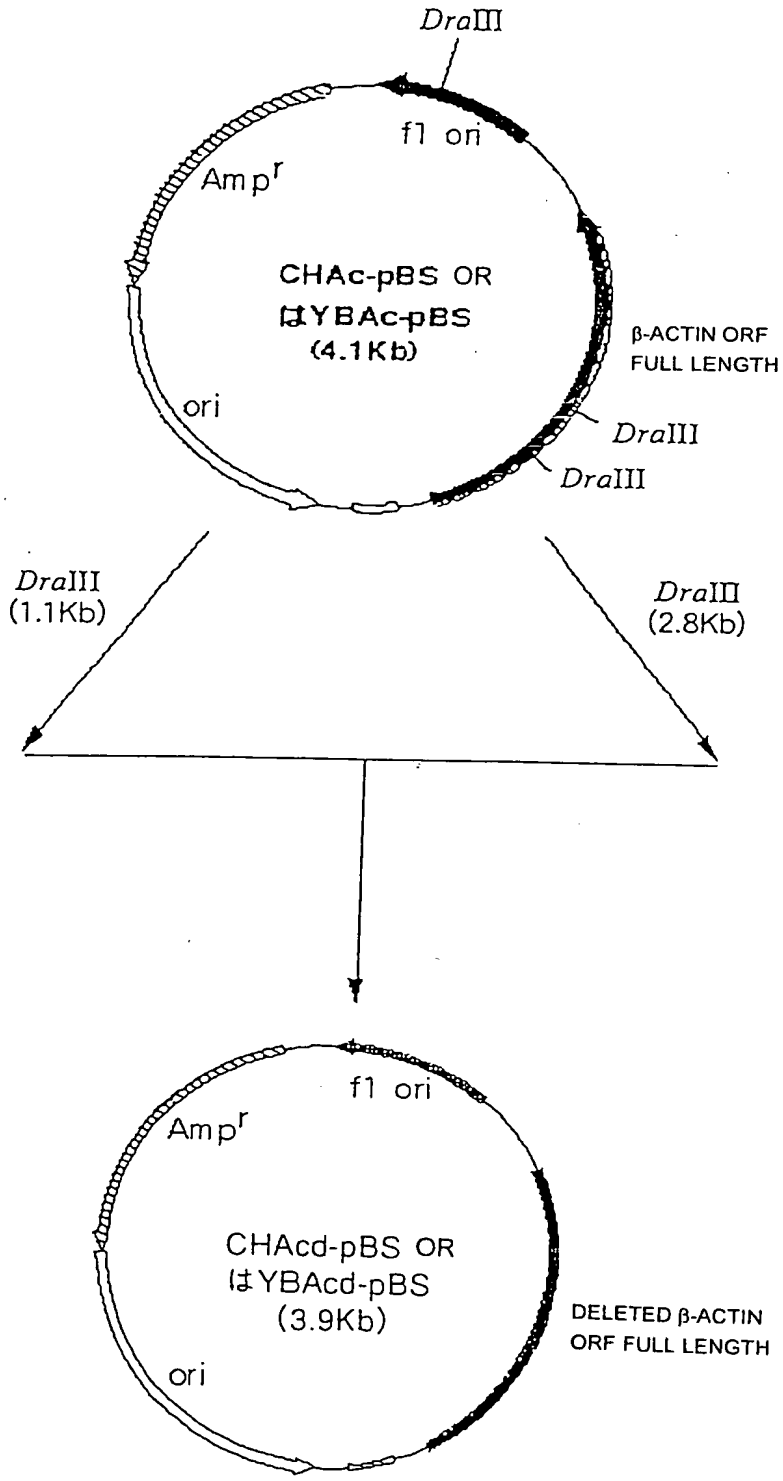

DraIII
f1 ori
Amp^r
CHAc-pBS OR
βYBAc-pBS
(4.1Kb)
ori
β-ACTIN ORF
FULL LENGTH
DraIII
DraIII
DraIII
(1.1Kb)
DraIII
(2.8Kb)
f1 ori
Amp^r
CHAcd-pBS OR
βYBAcd-pBS
(3.9Kb)
ori
DELETED β-ACTIN
ORF FULL LENGTH

FIG. 16

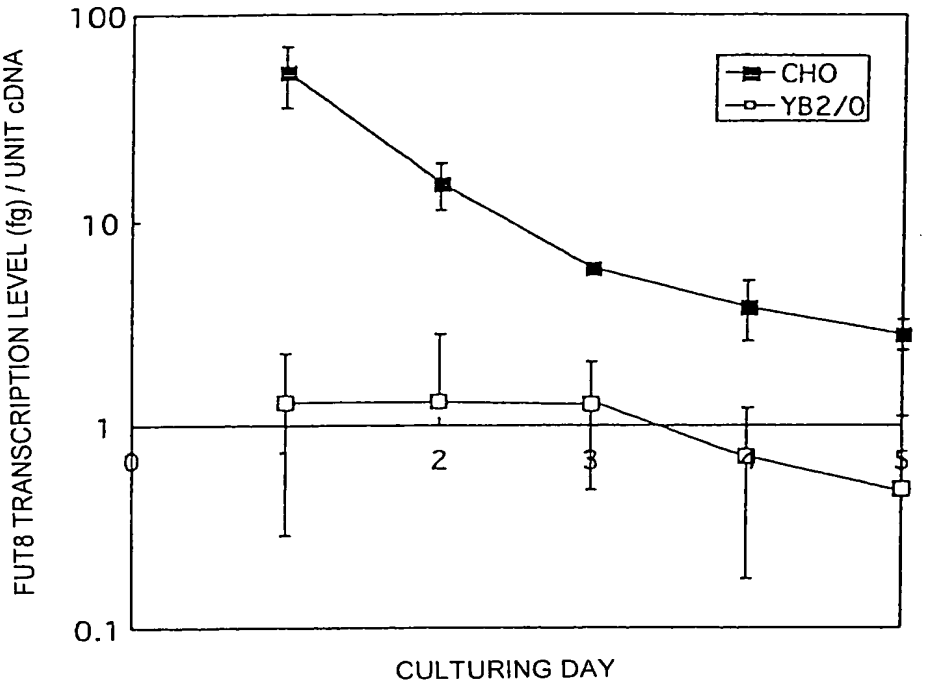

100
10
1
0.1
0
1
2
3
4
5
CHO
YB2/0
FUT8 TRANSCRIPTION LEVEL (fg) / UNIT cDNA
CULTURING DAY

FIG. 17

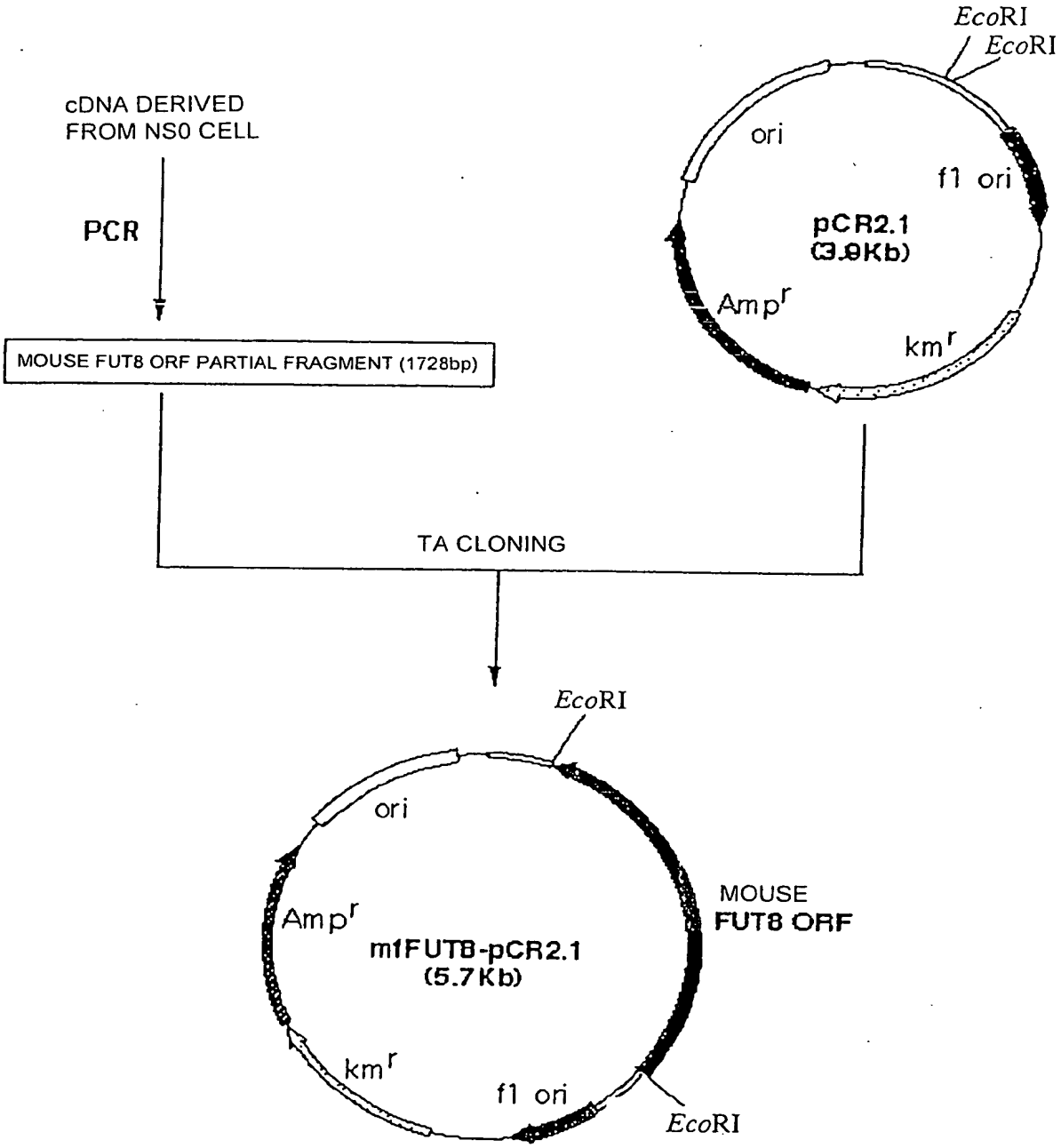

cDNA DERIVED
FROM NS0 CELL
PCR
MOUSE FUT8 ORF PARTIAL FRAGMENT (1728bp)
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Amp^r
km^r
TA CLONING
EcoRI
ori
Amp^r
m1FUT8-pCR2.1
(5.7Kb)
km^r
f1 ori
EcoRI
MOUSE
FUT8 ORF

FIG. 18

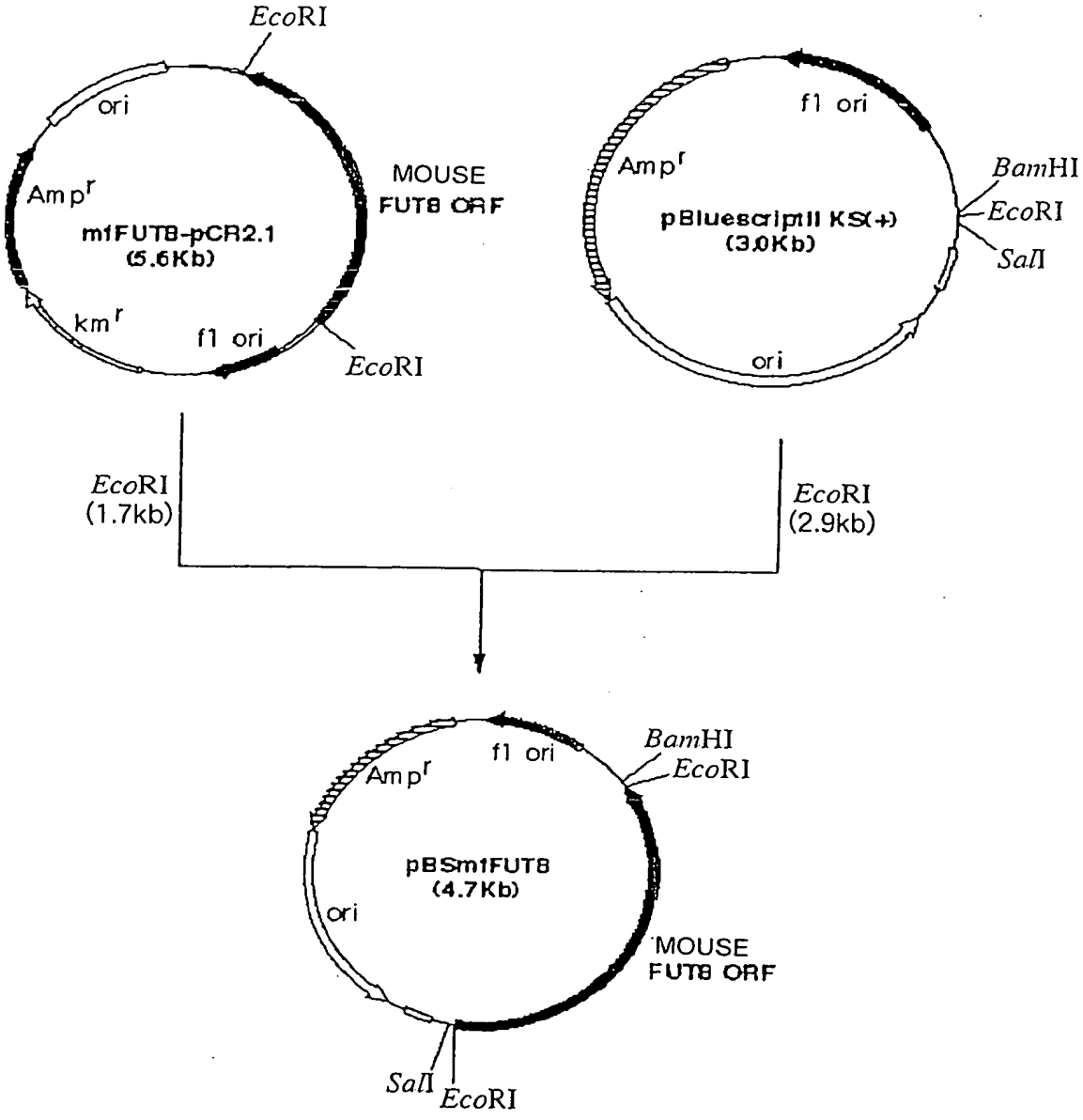

EcoRI
ori
Amp r
m1FUT8-pCR2.1
(5.6Kb)
km r
f1 ori
EcoRI
MOUSE
FUT8 ORF
f1 ori
Amp r
pBluescriptII KS(+)
(3.0Kb)
ori
BamHI
EcoRI
SalI
EcoRI
(1.7kb)
EcoRI
(2.9kb)
f1 ori
Amp r
pBSm1FUT8
(4.7Kb)
ori
BamHI
EcoRI
MOUSE
FUT8 ORF
SalI
EcoRI

FIG. 19

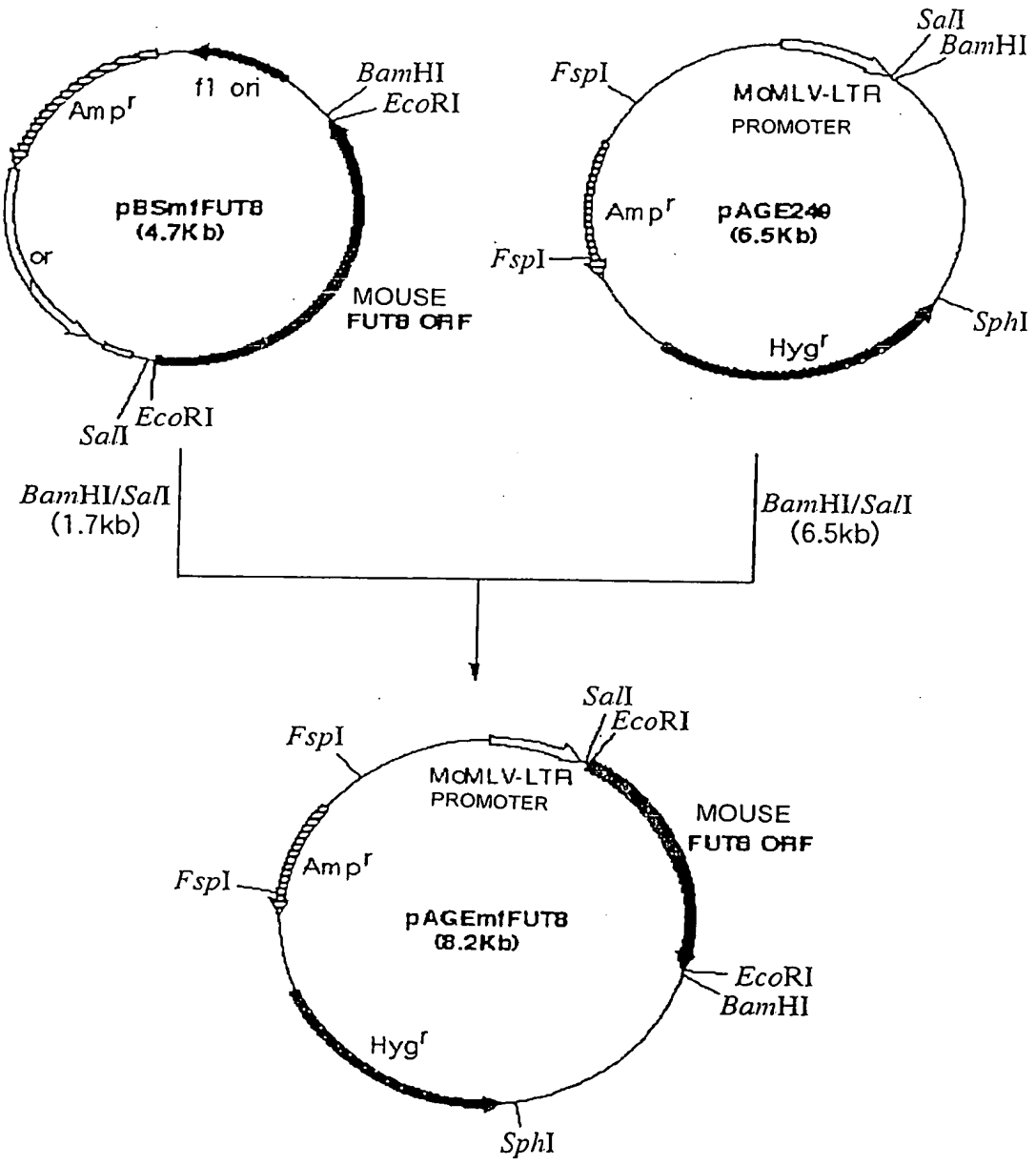

f1 ori
BamHI
EcoRI
Ampr
pBSmfFUT8
(4.7Kb)
or
MOUSE
FUT8 ORF
SalI
EcoRI
SalI
BamHI
FspI
MoMLV-LTR
PROMOTER
Ampr
pAGE249
(6.5Kb)
FspI
Hygr
SphI
BamHI/SalI
(1.7kb)
BamHI/SalI
(6.5kb)
SalI
EcoRI
FspI
MoMLV-LTR
PROMOTER
MOUSE
FUT8 ORF
FspI
Ampr
pAGEmfFUT8
(8.2Kb)
EcoRI
BamHI
Hygr
SphI

FIG. 20

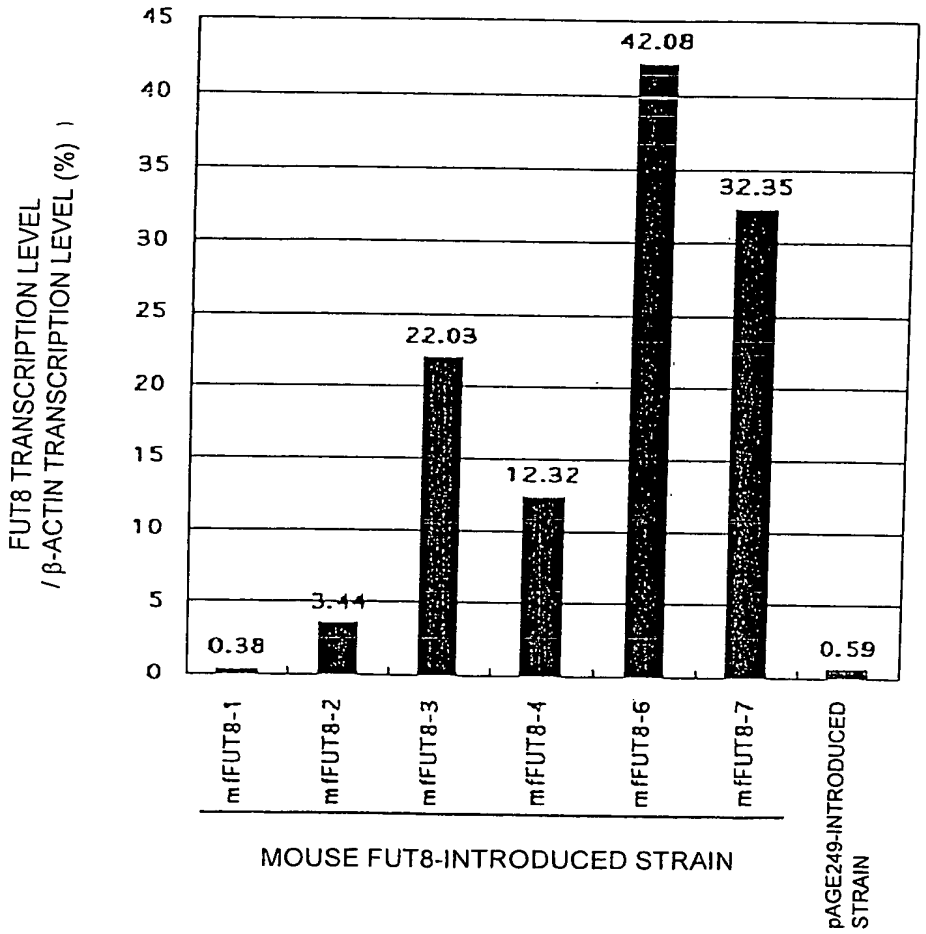

FUT8 TRANSCRIPTION LEVEL
/ β-ACTIN TRANSCRIPTION LEVEL (%))
45
40
35
30
25
20
15
10
5
0
0.38
3.44
22.03
12.32
42.08
32.35
0.59
mfFUT8-1
mfFUT8-2
mfFUT8-3
mfFUT8-4
mfFUT8-6
mfFUT8-7
pAGE249-INTRODUCED STRAIN
MOUSE FUT8-INTRODUCED STRAIN

FIG. 21

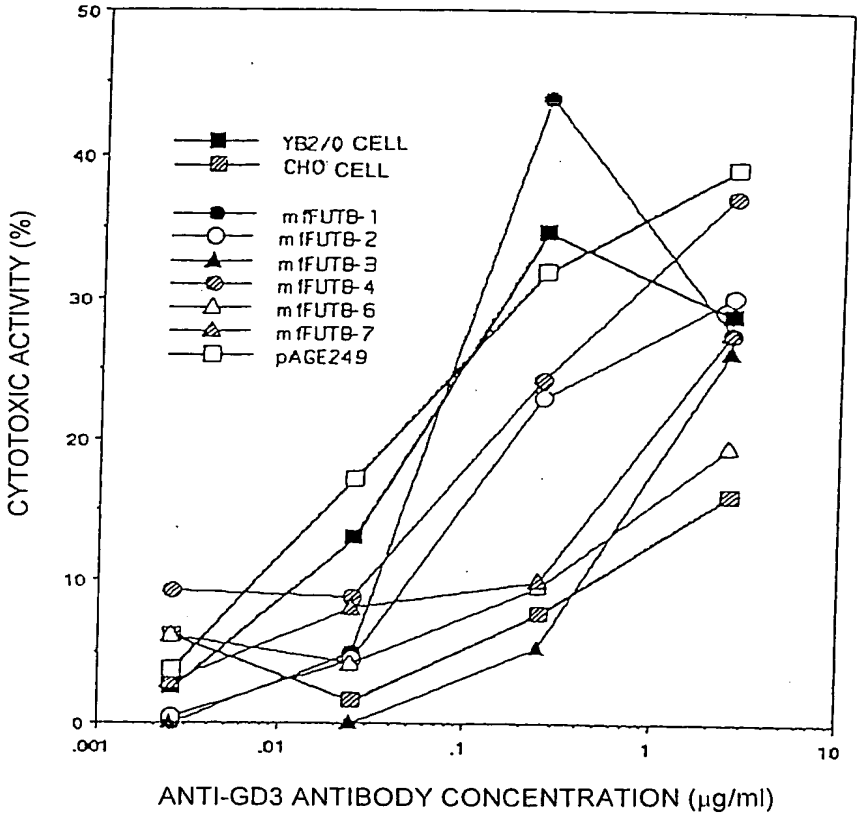

50
40
30
20
10
0
.001
.01
.1
1
10
CYTOTOXIC ACTIVITY (%)
ANTI-GD3 ANTIBODY CONCENTRATION (µg/ml)
YB2/0 CELL
CHO CELL
mFUT8-1
mFUT8-2
mFUT8-3
mFUT8-4
mFUT8-6
mFUT8-7
pAGE249

FIG. 22

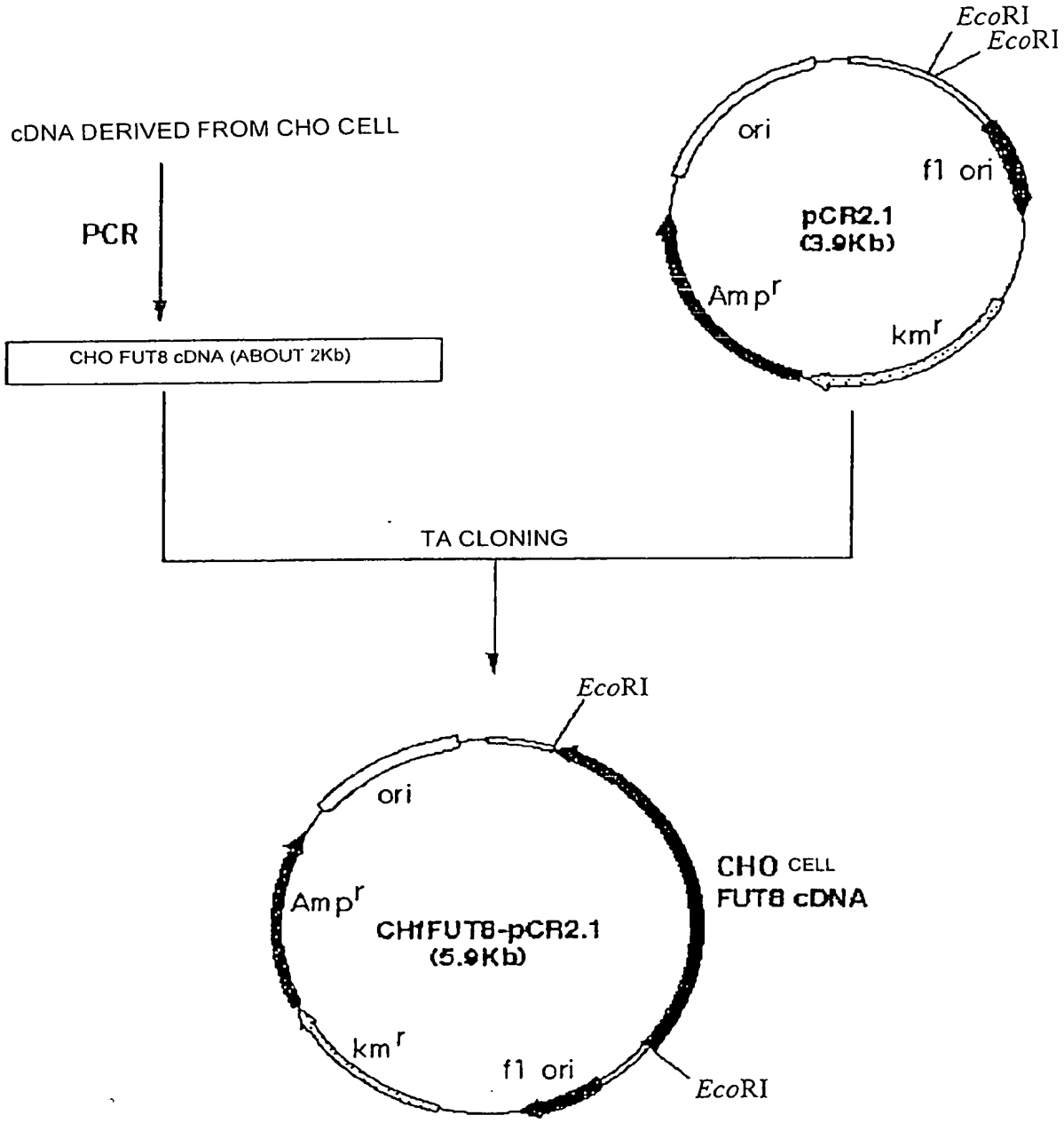

cDNA DERIVED FROM CHO CELL
PCR
CHO FUT8 cDNA (ABOUT 2Kb)
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Amp r
km r
TA CLONING
EcoRI
ori
Amp r
CHFUT8-pCR2.1
(5.9Kb)
km r
f1 ori
EcoRI
CHO CELL
FUT8 cDNA

FIG. 23

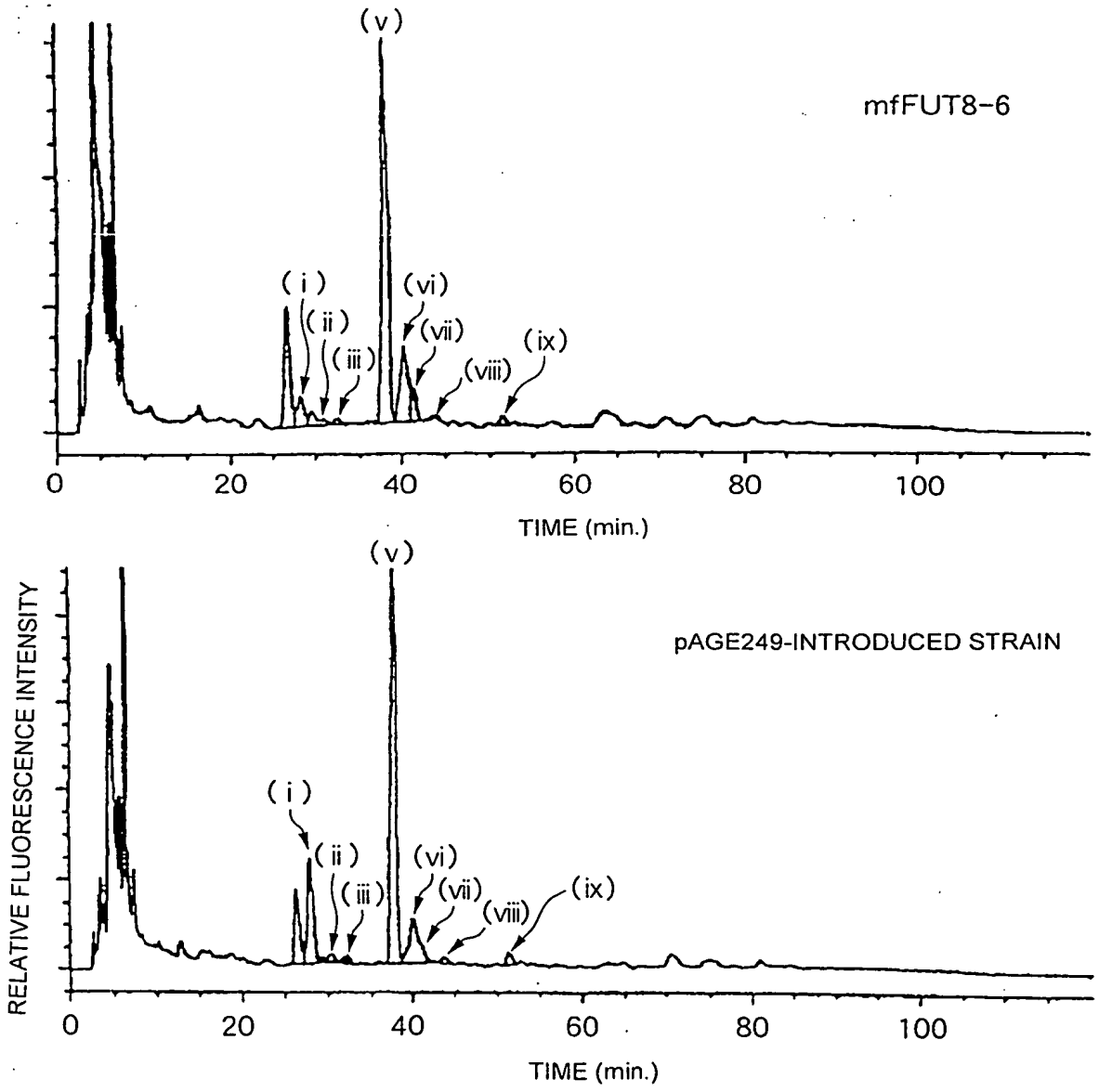


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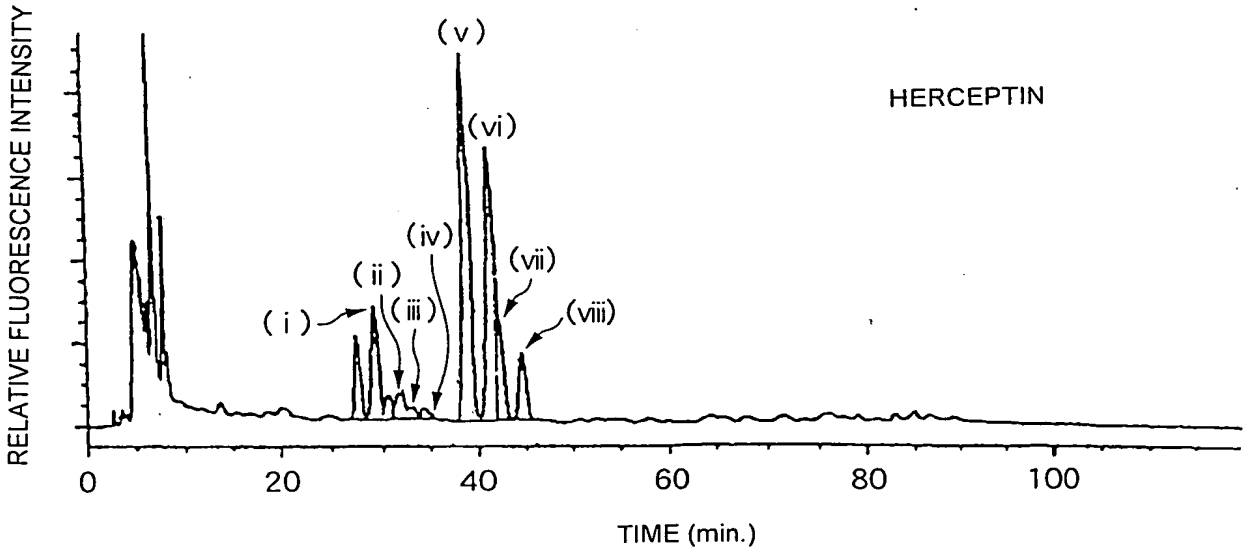
FIG. 24



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Nixon & Vanderhye P.C.
ATTORNEYS AT LAW

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WRITER'S DIRECT DIAL NUMBER:
(703) 816-4091

FACSIMILE COVER SHEET
PLEASE DELIVER IMMEDIATELY!!!!

Our Ref.: 249-202
Your Ref.: Appln No. 09/971,773 Date: December 17, 2004

To: Examiner Kelly, Robert
Firm: USPTO - Group 1632
Facsimile No.: **Facsimile No.: 7038729306 (Examiner's Tel. No.: 571-272-0729)**
From: B. J. Sadoff

Number of Pages (including cover sheet): 57

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I hereby certify that this paper and any noted attachments are being facsimile transmitted to the Patent and Trademark Office on December 17, 2004.

B. J. Sadoff
Reg. No. 36663

RESPONSE UNDER RULE 116
EXPEDITED HANDLING PROCEDURES

ATTACHMENT/S: Amendment, 2 cvr sheets with charge authorization, copy of substance of slide presentation, copy of post card from August 12, 2004, copy of noted ISR.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE			
In re Patent Application of			
KANDA, et al.		Atty. Ref.:	249-202
Serial No.	09/971,773	Group:	1632
Filed:	October 9, 2001	Examiner:	Kelly, Robert
For:	ANTIBODY COMPOSITION-PRODUCING CELL		

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RESPONSE UNDER RULE 116
EXPEDITED HANDLING PROCEDURES

In re Patent Application of

KANDA, et al.
Serial No. 09/971,773
Filed: October 9, 2001
Title: ANTIBODY COMPOSITION-PRODUCING CELL

Atty Dkt. 249-202
C# M#
TC/A.U. 1632
Examiner: Kelly, Robert
Date: December 17, 2004

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

AMENDMENT & SUBSTANCE OF SLIDE PRESENTATION

This is a response/amendment/letter in the above-identified application and includes an attachment which is hereby incorporated by reference and the signature below serves as the signature to the attachment in the absence of any other signature thereon.

☐ **Correspondence Address Indication Form Attached.**

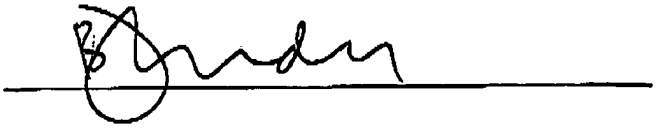
Fees are attached as calculated below:

Total effective claims after amendment 0 minus highest number previously paid for 20 (at least 20) = 0 x $ 50.00	$	0.00
Independent claims after amendment 0 minus highest number previously paid for 3 (at least 3) = 0 x $ 200.00	$	0.00
If proper multiple dependent claims now added for first time, add $360.00 (ignore improper)	$	0.00
Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s) ($120.00/1 month; $450.00/2 months; $1020.00/3 months)	$	0.00
Terminal disclaimer enclosed, add $ 130.00	$	0.00
☐ First/second submission after Final Rejection pursuant to 37 CFR 1.129(a) ($790.00) ☐ Please enter the previously unentered , filed ☐ Submission attached	$	0.00
Subtotal	$	0.00
If "small entity," then enter half (1/2) of subtotal and subtract ☐ Applicant claims "small entity" status. ☐ Statement filed herewith	-$	0.00
Rule 56 Information Disclosure Statement Filing Fee ($180.00)	$	0.00
Assignment Recording Fee ($40.00)	$	0.00
Other:		0.00
TOTAL FEE ENCLOSED	$	0.00

The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Account No. 14-1140. A duplicate copy of this sheet is attached.

1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100
BJS:

NIXON & VANDERHYE P.C.
By Atty: B. J. Sadoff, Reg. No. 36,663

Signature: 

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

RESPONSE UNDER RULE 116
EXPEDITED HANDLING PROCEDURES

In re Patent Application of Atty Dkt. 249-202
C# M#

KANDA, et al. TC/A.U. 1632

Serial No. 09/971,773 Examiner: Kelly, Robert

Filed: October 9, 2001 Date: December 17, 2004

Title: ANTIBODY COMPOSITION-PRODUCING CELL

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Examiner's Tel. no. (571-272-0729)

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P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

AMENDMENT & SUBSTANCE OF SLIDE PRESENTATION

This is a response/amendment/letter in the above-identified application and includes an attachment which is hereby incorporated by reference and the signature below serves as the signature to the attachment in the absence of any other signature thereon.

☐ **Correspondence Address Indication Form Attached.**

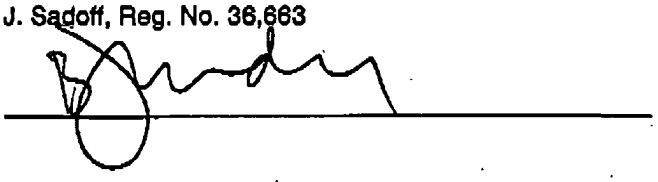
Fees are attached as calculated below:

Total effective claims after amendment 0 minus highest number previously paid for 20 (at least 20) = 0 x $ 50.00	$	0.00
Independent claims after amendment 0 minus highest number previously paid for 3 (at least 3) = 0 x $ 200.00	$	0.00
If proper multiple dependent claims now added for first time, add $360.00 (ignore improper)	$	0.00
Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s) ($120.00/1 month; $450.00/2 months; $1020.00/3 months)	$	0.00
Terminal disclaimer enclosed, add $ 130.00	$	0.00
☐ First/second submission after Final Rejection pursuant to 37 CFR 1.129(a) ($790.00) ☐ Please enter the previously unentered , filed ☐ Submission attached	$	0.00
Subtotal	$	0.00
If "small entity," then enter half (1/2) of subtotal and subtract ☐ Applicant claims "small entity" status. ☐ Statement filed herewith	-$	0.00
Rule 56 Information Disclosure Statement Filing Fee ($180.00)	$	0.00
Assignment Recording Fee ($40.00)	$	0.00
Other:		0.00
TOTAL FEE ENCLOSED	$	0.00

The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Account No. 14-1140. A duplicate copy of this sheet is attached.

1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100
BJS:

NIXON & VANDERHYE P.C.
By Atty: B. J. Sadoff, Reg. No. 36,663

Signature: 

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

KANDA, et al. Atty. Ref.: 249-202; Confirmation No. 2525

Appl. No. 09/971,773 TC/A.U. 1632

Filed: October 9, 2001 Examiner: Kelly, Robert

For: ANTIBODY COMPOSITION-PRODUCING CELL

* * * * * * * * * * *

December 17, 2004

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

VIA FACSIMILE (7038729306)

Examiner's Tel. no. (571-272-0729)

Sir:

AMENDMENT UNDER RULE 116

Responsive to the Official Action dated November 3, 2004, and in furtherance to the Examiner interview of December 10, 2004, entry and consideration of the following amendments and remarks are requested.

AMENDMENTS TO THE CLAIMS:

Amend the claims as follows:

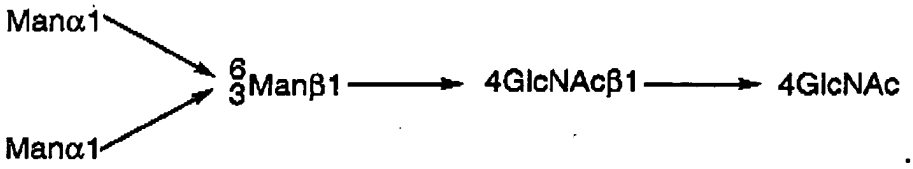
Claims 1-68. (Canceled)

69. (New) An isolated fucosyltransferase knock-out host cell wherein when a gene encoding an antibody molecule is introduced in to said host cell, said host cell produces an antibody composition comprising the antibody molecule,

said antibody molecule comprising a Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region,

said sugar chains comprising a reducing end which contains an N-acetylglucosamine, wherein the sugar chains do not contain fucose bound to N-acetylglucosamine in the reducing end of the sugar chains.

70. (new) The isolated host cell of claim 69 wherein said sugar chains comprise:



71. (new) The isolated host cell of claim 69 wherein said host cell is a CHO cell.

72. (new) The isolated host cell of claim 69 wherein said host cell is a NS0 cell.

73. (new) The isolated host cell of claim 69 wherein said host cell is a SP2/0 cell.

74. (new) The isolated host cell of claim 69 wherein said host cell is a YB2/0 cell.

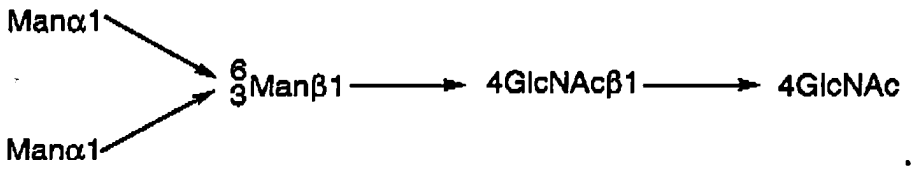
75. (new) The isolated host cell of claim 69 wherein said host cell is a plant cell.

76. (New) A fucosyltransferase knock-out plant wherein when a gene encoding an antibody molecule is introduced in to said plant, said plant produces an antibody composition comprising the antibody molecule,

said antibody molecule comprising a Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region,

said sugar chains comprising a reducing end which contains an N-acetylglucosamine, wherein the sugar chains do not contain fucose bound to N-acetylglucosamine in the reducing end of the sugar chains.

77. (new) The plant of claim 76 wherein said sugar chains comprise:



PAGE 6/57 * RCVD AT 12/17/2004 3:31:32 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-1/5 * DNIS:8729306 * CSID:703 816 4100 * DURATION (mm-ss):20-38

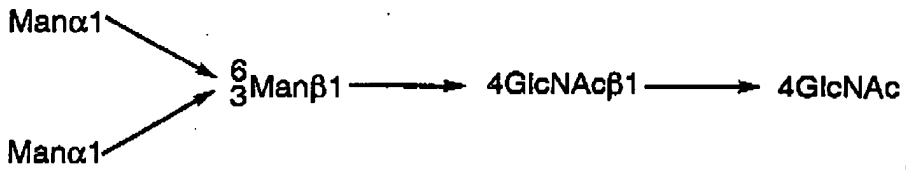
Aragen/Transposagen Ex. 1036 - Part B

78. (New) A non-human fucosyltransferase knock-out animal wherein when a gene encoding an antibody molecule is introduced in to said animal, said non-human animal produces an antibody composition comprising the antibody molecule,

said antibody molecule comprising a Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region,

said sugar chains comprising a reducing end which contains an N-acetylglucosamine, wherein the sugar chains do not contain fucose bound to N-acetylglucosamine in the reducing end of the sugar chains.

79. (new) The non-human animal of claim 78 wherein said sugar chains comprise:

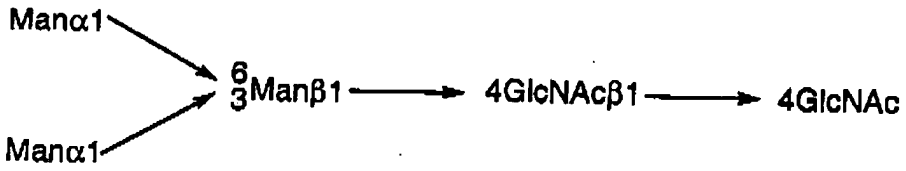


80. (New) An isolated fucosyltransferase knock-out host cell comprising a gene encoding an antibody molecule, wherein said host cell produces an antibody composition comprising the antibody molecule,

said antibody molecule comprising a Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region,

said sugar chains comprising a reducing end which contains an N-acetylglucosamine, wherein the sugar chains do not contain fucose bound to N-acetylglucosamine in the reducing end of the sugar chains.

81. (new) The isolated host cell of claim 80 wherein said sugar chains comprise:



82. (new) The isolated host cell of claim 80 wherein said host cell is a CHO cell.

83. (new) The isolated host cell of claim 80 wherein said host cell is a NS0 cell.

84. (new) The isolated host cell of claim 80 wherein said host cell is a SP2/0 cell.

85. (new) The isolated host cell of claim 80 wherein said host cell is a YB2/0 cell.

86. (new) The isolated host cell of claim 80 wherein said host cell is a plant cell.

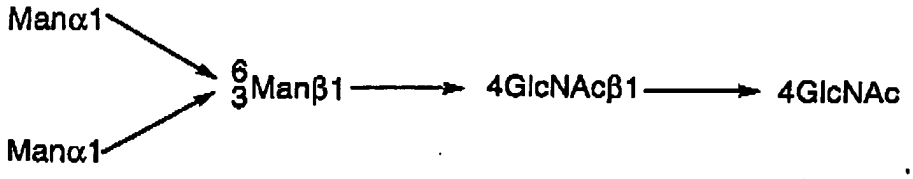
87. (New) A fucosyltransferase knock-out plant comprising a gene encoding an antibody molecule, wherein said plant produces an antibody composition comprising the antibody molecule,

said antibody molecule comprising a Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region,

PAGE 8/57 * RCVD AT 12/17/2004 3:31:32 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-1/5 * DNIS:8729306 * CSID:703 816 4100 * DURATION (mm-ss):20-38

said sugar chains comprising a reducing end which contains an N-acetylglucosamine, wherein the sugar chains do not contain fucose bound to N-acetylglucosamine in the reducing end of the sugar chains.

88. (new) The plant of claim 87 wherein said sugar chains comprise:

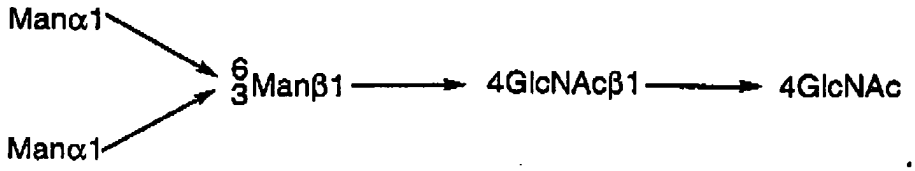


89. (New) A non-human fucosyltransferase knock-out animal comprising a gene encoding an antibody molecule, wherein said non-human animal produces an antibody composition comprising the antibody molecule,

said antibody molecule comprising a Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region,

said sugar chains comprising a reducing end which contains an N-acetylglucosamine, wherein the sugar chains do not contain fucose bound to N-acetylglucosamine in the reducing end of the sugar chains.

90. (new) The non-human animal of claim 89 wherein said sugar chains comprise:



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91. (new) The isolated host cell of claim 69 wherein said antibody molecule is an IgG antibody.

92. (new) The plant of claim 76 wherein said antibody molecule is an IgG antibody.

93. (new) The non-human animal of claim 78 wherein said antibody molecule is an IgG antibody.

94. (new) The isolated host cell of claim 80 wherein said antibody molecule is an IgG antibody.

95. (new) The plant of claim 87 wherein said antibody molecule is an IgG antibody.

96. (new) The non-human animal of claim 89 wherein said antibody molecule is an IgG antibody.

PAGE 10/57 * RCVD AT 12/17/2004 3:31:32 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-1/5 * DNIS:8729306 * CSID:703 816 4100 * DURATION (mm-ss):20-38

REMARKS

Reconsideration is requested.

The Examiner Interview of December 10, 2004, is acknowledged, with appreciation. The Interview Summary is accurate in its brief review of the issues discussed during the interview. Attached is a paper copy of the substance of the slide presentation discussed during the interview.

Claims 1-68 have been canceled, without prejudice. Claims 69-96 have been added.

At a minimum, the new claims 69-75, 80-86, 91 and 94 are believed to read on the elected invention. Specifically, the applicants elected the subject matter of the Examiner's Group II of the Office action dated June 17, 2003 ("drawn to a cell in which the activity of an enzyme relating to the synthesis of an intracellular nucleotide GDP-fucose and/or relating to the modification of a sugar chain is decreased or deleted, and a method of using such a cell for antibody production.").

Moreover, the following species elections were made for the purpose of initial search (see, page 2 of the Office Action dated February 13, 2004):

(1) a CHO cell;

(2) a gene disruption technique targeting a gene encoding the enzyme as the method to produce the cell; and

(3) the enzyme α-1,6-fucosyltransferase as the enzyme that is disrupted.

The applicants understand from the Examiner's comments during the interview that perhaps a more rapid allowance may be obtained if the claims were rewritten to define the elected subject matter, and specifically with regard to the enzyme to be

affected. Moreover, in view of the data presented in the slides discussed during the interview, the applicants understood that perhaps the claims to a plant and/or non-human animal such as defined above, although arguably defined by the Examiner's restriction requirement as being separately patentable, may also be examined and potentially allowed with the claims of Group II. The Examiner is requested to contact the undersigned after reviewing the above claims if the Examiner believes that further amendments are required to place the claims in condition for allowance.

The Examiner will appreciate that claims 76-77, 87-88, 92 and 95 define plants and claims 78-79, 89-90, 93 and 96 define non-human animals.

The claims are believed to be supported by the specification. No new matter has been added. The claims are not believed to raise new issues requiring further search and/or consideration.

Acknowledgement of the certified copies of the priority document filed August 12, 2004 is requested. Attached is a copy of the undersigned's post card receipt from the filing of August 12, 2004 as evidence of the Patent Office receipt of the noted documents. A verified English language translation of the priority document is being prepared and will be filed under separate cover, via facsimile, as required by the Examiner on page 3 of the Office Action dated November 3, 2004.

The specification was amended August 12, 2004 to include a cross-reference to the parent applications.

Acknowledgement of the acceptance of the drawings in the Office Action of November 3, 2004 is noted with appreciation.

PAGE 12/57 * RCVD AT 12/17/2004 3:31:32 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-1/5 * DNIS:8729306 * CSID:703 816 4100 * DURATION (mm-ss):20-38

Aragen/Transposagen Ex. 1036 - Part B

The Examiner's comments relating to the previously-cited Furukawa et al (Protein, Nucleic Acid and Enzyme, 43, 2309-2317 (1998)) reference are noted. The applicants believe however that the relevance of the non-English language document was adequately described in the submission of the International Search Report from the corresponding application no. PCT/JP01/08804, wherein the reference was cited. A further copy of the indicated Search Report is attached.

The applicants further submit, as a supplemental concise explanation of the alleged relevance of the document, that the cited document is understood to disclose that (i) the survival ratio of beta-1,4-galactosyltransferase knockout mice is low, (ii) beta-1,4-galactosyltransferase activity was found even in the above knockout mice, although the activity is low, and (iii) there are plural beta-1,4-galactosyltransferases in the living body.

Consideration of the reference and return of an initialed copy of the previously-submitted PTO 1449 Form, pursuant to MPEP § 609, are requested.

The Rule 75 objection of claims 2, 19 and 63 will be moot upon entry of the above amendments. Entry of the amendments and withdrawal of the objection are requested.

The Section 112, second paragraph, rejection of claims 1, 12, 19 and 63 will be moot upon entry of the above amendments. The applicants have amended the claims, without prejudice, to advance prosecution. Entry of the above amendments is requested.

The Section 112, first paragraph "written description", rejection of Claims 4 and 23 will be moot upon entry of the above amendments. The claims have been amended

PAGE 13/57 * RCVD AT 12/17/2004 3:31:32 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-1/5 * DNIS:8729306 * CSID:703 816 4100 * DURATION (mm-ss):20-38

Aragen/Transposagen Ex. 1036 - Part B

to clearly indicate that the enzymes involved in the invention are not those which modify sugars containing fucose, for example, after they are bound to the recited antibody, as the Examiner had apparently interpreted claims 4 and 23. Entry of the above amendments is requested to advance prosecution.

The Section 112, first paragraph "enablement", rejection of Claims 1-4, 12-20, 23, 31-34, 36-40, 62-64 and 65-68, will be moot upon entry of the above amendments. The Examiner's indication that the specification teaches one of ordinary skill in the art the subject matter described on page 9 of the Office Action dated November 3, 2004, is acknowledged, with appreciation. As discussed during the Examiner interview however, the applicants believe that the specification provides a broader teaching, such as defined in the above claims. The attached evidence from the Examiner Interview is believed to adequately demonstrate that the specification provides an enabling teaching of the above claims. Entry of the present Amendment is requested.

The claims, as amended above, are submitted to be in condition for allowance and entry of the above amendments and a Notice of Allowance are requested.

The Examiner is requested to contact the undersigned in the event anything further is required.

KANDA, et al.
Appl. No. 09/971,773
December 17, 2004

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____
B. J. Sadoff
Reg. No. 36,663

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C#/M#: 249-202
Atty: B. J. Sadoff
Date: August 12, 2004

Serial No.: 09/971,773
Inventor/s: KANDA, et al.
Title: ANTIBODY COMPOSITION-PRODUCING CELL

XX Amendment & Cover Sheet (2 copies), four (4) references & marked-up copy of Proposed Amended Figure (Figure 22)
XX Submission of Formal Drawings & fifty-two (52) sheets formal drawings (Figs. 1A-55)
XX Submission of Priority Documents, certified copies of Japanese Patent Application No. 2000-308526 and PCT/JP01/08804
XX IDS, PTO-1449 Form & cited references

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$1220.00 Total Fee Enclosed

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国際調査報告

(法8条、法施行規則第40、41条)
〔PCT18条、PCT規則43、44〕

出願人又は代理人の書類記号 P-38524	今後の手続きについては、国際調査報告の送付通知様式(PCT/ISA/220)及び下記5を参照すること。	
国際出願番号 PCT/JP01/08804	国際出願日 (日.月.年) 05.10.01	優先日 (日.月.年) 06.10.00
出願人(氏名又は名称) 協和醗酵工業株式会社		

国際調査機関が作成したこの国際調査報告を法施行規則第41条(PCT18条)の規定に従い出願人に送付する。
この写しは国際事務局にも送付される。

この国際調査報告は、全部で 6 ページである。

☐ この調査報告に引用された先行技術文献の写しも添付されている。

1. 国際調査報告の基礎
   a. 言語は、下記に示す場合を除くほか、この国際出願がされたものに基づき国際調査を行った。
      ☐ この国際調査機関に提出された国際出願の翻訳文に基づき国際調査を行った。
   b. この国際出願は、ヌクレオチド又はアミノ酸配列を含んでおり、次の配列表に基づき国際調査を行った。
      ☐ この国際出願に含まれる書面による配列表
      ☒ この国際出願と共に提出されたフレキシブルディスクによる配列表
      ☐ 出願後に、この国際調査機関に提出された書面による配列表
      ☐ 出願後に、この国際調査機関に提出されたフレキシブルディスクによる配列表
      ☐ 出願後に提出した書面による配列表が出願時における国際出願の開示の範囲を超える事項を含まない旨の陳述書の提出があった。
      ☒ 書面による配列表に記載した配列とフレキシブルディスクによる配列表に記録した配列が同一である旨の陳述書の提出があった。

2. ☐ 請求の範囲の一部の調査ができない(第I欄参照)。

3. ☒ 発明の単一性が欠如している(第II欄参照)。

4. 発明の名称は
   ☒ 出願人が提出したものを承認する。
   ☐ 次に示すように国際調査機関が作成した。

5. 要約は
   ☐ 出願人が提出したものを承認する。
   ☒ 第III欄に示されているように、法施行規則第47条(PCT規則38.2(b))の規定により国際調査機関が作成した。出願人は、この国際調査報告の発送の日から1カ月以内にこの国際調査機関に意見を提出することができる。

6. 要約書とともに公表される図は、
   第 ＿＿＿ 図とする。 ☐ 出願人が示したとおりである。 ☒ なし
   ☐ 出願人は図を示さなかった。
   ☐ 本図は発明の特徴を一層よく表している。

様式PCT/ISA/210(第1ページ)(1998年7月)

CONFIRMATION COPY

国際調査報告

国際出願番号 PCT/JP01/08804

第Ⅰ欄 請求の範囲の一部の調査ができないときの意見（第１ページの２の続き）

法第８条第３項（ＰＣＴ１７条(2)(a)）の規定により、この国際調査報告は次の理由により請求の範囲の一部について作成しなかった。

1. ☐ 請求の範囲 _____ は、この国際調査機関が調査をすることを要しない対象に係るものである。
つまり、

2. ☐ 請求の範囲 _____ は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、

3. ☐ 請求の範囲 _____ は、従属請求の範囲であってＰＣＴ規則6.4(a)の第２文及び第３文の規定に従って記載されていない。

第Ⅱ欄 発明の単一性が欠如しているときの意見（第１ページの３の続き）

次に述べるようにこの国際出願に二以上の発明があるとこの国際調査機関は認めた。

請求項１－５６に係る発明は、還元末端のＮ－アセチルグルコサミンにフコースが結合していないＮ－結合複合型糖鎖の割合を増加させることに関するものである。請求項５７－６１に係る発明は、糖鎖合成に関与する酵素に関するものであり、これらの酵素は請求項１－５６に係る発明において直接用いられるものではない。
したがって、請求項１－５６、５７－６１に係るそれぞれの発明の間にＰＣＴ規則１３．２の意味における技術的な関係はなく、発明の単一性の要件は満たされていない。
また請求項５７－６１に係る発明についてみると、これらはGMD、Fx、GFPP、２種類のα-1,6-フコシルトランスフェラーゼという酵素に関する５つの発明を含んでいる。

（特別ページに続く）

1. ☒ 出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求の範囲について作成した。

2. ☐ 追加調査手数料を要求するまでもなく、すべての調査可能な請求の範囲について調査することができたので、追加調査手数料の納付を求めなかった。

3. ☐ 出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求の範囲のみについて作成した。

4. ☐ 出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載されている発明に係る次の請求の範囲について作成した。

追加調査手数料の異議の申立てに関する注意
☐ 追加調査手数料の納付と共に出願人から異議申立てがあった。
☒ 追加調査手数料の納付と共に出願人から異議申立てがなかった。

PAGE 53/57 * RCVD AT 12/17/2004 3:31:32 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-1/5 * DNIS:8729306 * CSID:703 816 4100 * DURATION (mm-ss):20-38

Aragen/Transposagen Ex. 1036 - Part B

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第III欄 要約（第1ページの5の続き）

本発明は、種々の疾患に有用な抗体依存性細胞障害活性の高い、抗体、抗体の断片、抗体のFc領域を有する融合タンパク質等の抗体組成物の製造に用いる細胞、該細胞を用いた抗体組成物の製造方法、抗体組成物、およびそれらの用途に関する。該抗体組成物は、Fc領域に結合する全N-グリコシド結合複合型糖鎖のうち、糖鎖還元末端のN-アセチルグルコサミンにフコースが結合していない糖鎖の割合が20%以上であるものである。また新規なGDP-マンノース 4,6-デヒドロゲナーゼ、GDP-ケト-6-デオキシマンノース 3,5-エピメラーゼ 4-レダクターゼ、GDP-ベータ-L-フコース ピロフォスソリラーゼ、アルファ-1,6-フコシルトランスフェラーゼ及びこれをコードするDNAをも提供する。

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Aragen/Transposagen Ex. 1036 - Part B

COPY NATION

国際調査報告 | 国際出願番号 PCT/JP01/08804

A. 発明の属する分野の分類（国際特許分類（IPC））

Int.Cl' C12N5/10, C12P21/08, C07K16/00, A01K67/00, A61K39/395, C12N9/00, C12N15/52, G01N33/53

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int.Cl' C12N5/10, C12P21/08, C07K16/00, A01K67/00, A61K39/395, C12N9/00, C12N15/52, G01N33/53

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）
WPIDS/BIOSIS/BIOTECHABS/MEDLINE/CA(STN)
GenBank/EMBL/DDBJ/GeneSeq
PIR/Swissprot/GeneSeq

C. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
X A	Shitara K. et al., A new veator for the high level expression of chimeric antibodies in myeloma cells, Journal of Immunological Methods, 1994, Vol.167, p.271-278	41,54-56 1-40,42-53,57-61
Y A	古川清，生体における糖蛋白質糖鎖の機能解析，蛋白質核酸酵素，1998，Vol.43，No.16，p.2309-2317	23-36,42-50 1-22,37-41,51-61

☒ C欄の続きにも文献が列挙されている。 ☐ パテントファミリーに関する別紙を参照。

＊ 引用文献のカテゴリー
「A」特に関連のある文献ではなく、一般的技術水準を示すもの
「E」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「O」口頭による開示、使用、展示等に言及する文献
「P」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「T」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「&」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
11.01.02	12.01.02

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）	
日本国特許庁（ISA/JP） 郵便番号100-8915 東京都千代田区霞が関三丁目4番3号	傑草 亜子	4B 9548
	電話番号 03-3581-1101 内線 3448	

様式PCT/ISA/210（第2ページ）（1998年7月）

国際調査報告 国際出願番号 PCT/JP01/08804

C（続き）. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
Y A	US 5728568 A (Genetic Institute Inc.) 1998.05.17 (ファミリーなし)	23-36,42-50,57-61 1-22,37-41,51-56
Y A	WO 99/64618 A1 (DCV Inc.) 1999.12.16 & EP 1084267 A1 & AU 9942051 A	23-36,42-50,57-61 1-22,37-41,51-56
Y A	WO 97/37683 A1 (CYTEL CORPORATION) 1997.10.16 & EP 904101 A1	23-36,42-50,57-61 1-22,37-41,51-56
Y A	WO 97/27303 A1 (東洋紡績株式会社) 1997.07.31 & JP 9-201191 A & EP 816503 A1 & JP 10-4959 A & JP 10-84975 A & US 6054304 A & US 6291219 A	23-36,42-50,57-61 1-22,37-41,51-56
PX PA	WO 00/61739 A1 (協和醗酵工業株式会社) 2000.10.19 & AU 200036728 A	1-56 57-61
A	WO 99/54342 A1 (UMANA Pablo) 1999.10.28 & EP 1071700 A1 & AU 9936578 A	1-61

様式PCT/ISA/210（第2ページの続き）（1998年7月）

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Aragen/Transposagen Ex. 1036 - Part B

第Ⅱ欄の続き

これらの活性を有する酵素は既に知られていることから、この５つの酵素に係るそれぞれの発明の間には、ＰＣＴ規則１３．２の意味における技術的な関係はなく、発明の単一性の要件は満たされていない。

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Aragen/Transposagen Ex. 1036 - Part B

PATENT APPLICATION FEE DETERMINATION RECORD
Effective January 1, 2003

Application or Docket Number: 09/971,723

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS		
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	minus 20=	*
INDEPENDENT CLAIMS	minus 3 =	*
MULTIPLE DEPENDENT CLAIM PRESENT		☐

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐		OR	OTHER THAN SMALL ENTITY	
RATE	FEE		RATE	FEE
BASIC FEE	$375	OR	BASIC FEE	$750
X$ 9=		OR	X$18=	
X42=		OR	X84=	
+140=		OR	+280=	
TOTAL		OR	TOTAL	

8/12/04

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	* 69	Minus	** 74	= 5
Independent	* 7	Minus	*** 7	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	90
X42=		OR	X84=	84
+140=		OR	+280=	5
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

12/17/04

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	* 28	Minus	** 74	= 0
Independent	* 6	Minus	*** 7	= 0
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X42=		OR	X84=	
+140=		OR	+280=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X42=		OR	X84=	
+140=		OR	+280=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
***If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/971,773	10/09/2001	Yutaka Kanda	249-202	2525

23117 7590 12/14/2004

NIXON & VANDERHYE, PC
1100 N GLEBE ROAD
8TH FLOOR
ARLINGTON, VA 22201-4714

EXAMINER	
KELLY, ROBERT M	

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 12/14/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARK
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
09/971,773	9 October 2001	Kanda, Y.	249-202

EXAMINER	
Robert M. Kelly	
ART UNIT	**PAPER NUMBER**
1632	12132004

DATE MAILED:

INTERVIEW SUMMARY

All participants (applicant, applicant's representative, PTO personnel):

(1) Robert M Kelly; Anne Marie Wehbe (3) Nobou Hanai; B.J. Sadoff

(2) Kazuyuki Iwahashi; Kenya Shitara (4) Martina Molsbergen

Date of Interview 10 December 2004

Type: ☐ Telephonic ☐ Televideo Conference ☒ Personal (copy is given to ☐ applicant ☒ applicant's representative).

Exhibit shown or demonstration conducted: ☒ Yes ☐ No If yes, brief description: A general description of the technology and its use in treating patients was presented.

Agreement ☐ was reached. ☐ was not reached.

Claim(s) discussed: All pending claims were discussed

Identification of prior art discussed:

Description of the general nature of what was agreed to if an agreement was reached, or any other comments:

Claims were discussed w/r/t overcoming the rejections, and it was discussed that cells other than CHO cells may be rejoined, but less likely that other enzymes may be rejoined. Also, it was discussed that Applicant may amend to elimate all FUT 8 activity.

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

☒ It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph above has been checked to indicate to the contrary. A FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has are ready been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW.

Examiner Note: You must sign this form unless it is an attachment to another form.

ANNE M. WEHBE' PH.D
PRIMARY EXAMINER

FORM PTOL-413 (REV. 2-98)

Manual of Patent Examining Procedure, Section 713.04 Substance of Interview Must Be Made of Record

A complete written statement as to the substance of *any* face-to-face or telephone *interview* with regard to an application *must be made of record in the application*, whether or not an agreement with the examiner was reached at the interview.

§ 1.133 Interviews

• • • • •

(b) In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be *filed* by the applicant. An interview does not remove the necessity for response to Office actions as specified in § § 1.111, 1.135. (35 U.S.C. 132)

§ 1.2 Business to be transacted in writing. All business with the Patent and Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete a two-sheet carbon interleaf interview Summary Form for each interview held after January 1, 1978 where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks in neat handwritten form using a ball point pen. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below.

The interview Summary Form shall be given an appropriate paper number, placed in the right hand portion of the file, and listed on the "Contents" list on the file wrapper. The docket and serial register cards need not be updated to reflect interviews. In a personal interview, the duplicate copy of the Form is removed and given to the applicant (or attorney or agent) at the conclusion of the interview. In case of a telephonic interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the telephonic interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Serial Number of the application
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (personal or telephonic)
- Name of participant(s) (applicant, attorney or agent, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the claims discussed
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). (Agreements as to allowability are tentative and do not restrict further action by the examiner to the contrary.)
- The signature of the examiner who conducted the interview
- Names of other Patent and Trademark Office personnel present.

The Form also contains a statement reminding the applicant of his responsibility to the record the substance of the interview.

It is desirable that the examiner orally remind the applicant of his obligation to record the substance of the interview in each case unless both applicant and examiner agree that the examiner will record same. Where the examiner agrees to record the substance of the interview, or when it is adequately recorded on the Form in an attachment to the form, the examiner should check a box at the Form informing the applicant that he need not supplement the Form by submitting a separate record of the interview.

It should be noted, however, that the interview Summary Form will not be considered and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview:

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted.
2) an identification of the claims discussed.
3) an identification of specific prior art discussed.
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the examiner.
5) a brief identification of the general thrust of the principal arguments presented to the examiner. The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application office. Of course, the applicant may desire to emphasize and fully describe those arguments which he feels were or might be persuasive to the examiner.
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete or accurate, the examiner will give the applicant one month from the date of the notifying letter or the remainder of any period for response, whichever is longer, to complete the response and thereby avoid abandonment of the application (37 CFR 1.135(c)).

Examiner to Check for Accuracy

Applicant's summary of what took place at the interview should be carefully checked to determine the accuracy of any argument or statement attributed to the examiner during the interview. If there is an inaccuracy and it bears directly on the question of patentability, it should be pointed out in the next Office letter. If the claims are allowable for other reasons of record, the examiner should send a letter setting forth his or her version of the statement attributed to him. If the record is complete and accurate, the examiner should place the indication "interview record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/971,773	10/09/2001	Yutaka Kanda	249-202	2525

23117 7590 11/03/2004

NIXON & VANDERHYE, PC
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ARLINGTON, VA 22201-4714

EXAMINER	
KELLY, ROBERT M	

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 11/03/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/971,773	KANDA ET AL.	
	Examiner	Art Unit	
	Robert M Kelly	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 August 2004*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-34,36-61 and 63-68* is/are pending in the application.

4a) Of the above claim(s) *5-11,21,22,24-30 and 41-61* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-4,12-20,23,31-40 and 63-68* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *12 August 2004* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☒ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *8/12/04*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Applicant's amendments and arguments of 12 August 2004 have been entered.

Claims 35 and 62 have been cancelled.

Claims 1-2, 4, 12, 16-19, 23, and 63 are amended.

Claims 65-68 are newly added.

Election/Restrictions

Applicant now traverses the restriction requirement of 10 June 2003, arguing that Group I should be rejoined with Group II because the subject matter necessarily requires a review of the same art, and the fact that the Examiner considered the common claim to both groups: Claim 1 (Applicant's Response of 12 August 2004, pp. 29-30).

It is noted that Applicant did not traverse the restriction requirement in the response to restriction, received by this office on 8 December 2003, pp. 24-25. As such, the Response to Restriction Requirement of 8 December 2003 was treated as an election without traverse (Official Action of 13 February 2004, p. 2). However, the Examiner recognizes that Applicant has made a persuasive argument, and that, indeed, the Examiner did examine some aspects of Group I. Claim 2 was substantively considered in Claim 19, Claim 3 was substantively considered in Claim 64, and Claim 20 was substantively considered in Claim 39. (Further evidence that Applicant was aware of the substantive consideration of these claims is evidenced by Applicant's amendments, which amends Claim 2 in a manner consistent with Claim 19. (Applicant's Response of 12 August 2004, pp. 3 and 9).) Moreover, it is recognized that Applicant has amended Claims 2-3 and 20 to read within the scope of the presently considered invention. Therefore, Claims 2-3 and 20 will be rejoined with the consideration of Group II;

however, since the Applicant did not traverse this requirement until after a First Action on the Merits, and because these aspects were already rejected in the First Action on the Merits, new rejections to these claims are not considered to preclude finality, unless such rejections were not applied to the previously examined claims in the prior Office Action.

Therefore, Claims 1-20 and 23-40 are now all within the scope of the invention of Group II, and Claims 5-11 and 24-30 remain withdrawn from prosecution until such time as a generic claim is found otherwise allowable with respect to the elected species.

The restriction requirement is made FINAL.

Hence, Claims 1-4, 12-20, 23, 31-40, and 63-68 are presently considered with respect to the elected species.

This application contains claims 21-22 and 41-61 drawn to an invention nonelected with traverse in Applicant's responses of 17 September 2003 and 12 August 2004. A complete reply to the final rejection must include cancellation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

Priority

If applicant desires priority under 35 U.S.C. 119(e) based upon a previously filed application, specific reference to the earlier filed application must be made in the instant application. For benefit claims under 35 U.S.C. 120, 121 or 365(c), the reference must include the relationship (i.e., continuation, divisional, or continuation-in-part) of the applications. This should appear as the first sentence of the specification following the title, preferably as a separate paragraph unless it appears in an application data sheet. The status of

nonprovisional parent application(s) (whether patented or abandoned) should also be included. If a parent application has become a patent, the expression "now Patent No. _____" should follow the filing date of the parent application. If a parent application has become abandoned, the expression "now abandoned" should follow the filing date of the parent application.

If the application is a utility or plant application filed under 35 U.S.C. 111(a) on or after November 29, 2000, the specific reference must be submitted during the pendency of the application and within the later of four months from the actual filing date of the application or sixteen months from the filing date of the prior application. If the application is a utility or plant application which entered the national stage from an international application filed on or after November 29, 2000, after compliance with 35 U.S.C. 371, the specific reference must be submitted during the pendency of the application and within the later of four months from the date on which the national stage commenced under 35 U.S.C. 371(b) or (f) or sixteen months from the filing date of the prior application. See 37 CFR 1.78(a)(2)(ii) and (a)(5)(ii). This time period is not extendable and a failure to submit the reference required by 35 U.S.C. 119(e) and/or 120, where applicable, within this time period is considered a waiver of any benefit of such prior application(s) under 35 U.S.C. 119(e), 120, 121 and 365(c). A priority claim filed after the required time period may be accepted if it is accompanied by a grantable petition to accept an unintentionally delayed claim for priority under 35 U.S.C. 119(e), 120, 121 and 365(c). The petition must be accompanied by (1) the reference required by 35 U.S.C. 120 or 119(e) and 37 CFR 1.78(a)(2) or (a)(5) to the prior application (unless previously submitted), (2) a surcharge under 37 CFR 1.17(t), and (3) a statement that the entire delay between the date the claim was

due under 37 CFR 1.78(a)(2) or (a)(5) and the date the claim was filed was unintentional. The Director may require additional information where there is a question whether the delay was unintentional. The petition should be addressed to: Mail Stop Petition, Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

While the PCT document may be obtained, a certified copy of the Japanese language Applications, translated into English, is required.

Priority – Response to Arguments

Applicant has amended the first paragraph of the specification to properly recite priority to Applicant's priority documents. Moreover, Applicant has stated that "Certified copies and English translations of the priority documents will be forwarded under separate cover." (Applicant's response of 12 August 2004, p. 28, first paragraph). However, no such certified copies have been received at this time.

Therefore, the objection to Applicant's priority for lacking English translations, while currently held, will also be held in abeyance until such time as the English translations are made available to the Examiner or Applicant's claims are found otherwise allowable.

Information Disclosure Statement

The information disclosure statement filed 12 August 2004 fails to comply with 37 CFR 1.98(a)(2), which requires a legible copy of each U.S. and foreign patent; each publication or that portion which caused it to be listed; and all other information or that portion which caused it to be listed. It has been placed in the application file, but the information referred to therein has not been considered. It is noted that WIPO documents 97/27303A1 and 00/61739A1 are written in a

foreign language, but provide an English language abstract, and therefore, these abstracts have been considered, as noted on the IDS. It is further noted that the reference to Furukawa, et al. (1998) Nucleic Acid and Enzyme, 43: 2309-17 was not provided with such English-language translation; hence a line has been drawn through such reference on the IDS. The examiner has not considered the reference of Furukawa, et al.

Specification

In light of Applicant's amendments and arguments of 12 August 2004, the objection to the specification is withdrawn.

Claim Objections – Old

In light of Applicant's amendments and arguments of 12 August 2004, the objection to Claim 1 is withdrawn.

Claim Objections – New – Necessitated by Amendment

Claims 2, 19, and 63 are objected to under 37 CFR 1.75(c), as being of improper dependent form for failing to further limit the subject matter of a previous claim. Applicant is required to cancel the claim(s), or amend the claim(s) to place the claim(s) in proper dependent form, or rewrite the claim(s) in independent form. Claims 2, 19, and 63 require that fucose is not linked to a reducing-end N-Acetylglucosamine through alpha-1,6 linkages in complex sugar chains; however, they depend from Claims 1, 18, and 4, respectively, referring to complex sugar chains in which fucose is not linked to a penultimate N-Acetylglucosamine at all. Therefore, Claims 2, 19, and 63 do not further limit Claim 1, 18, and 4, respectively. Such rejection is made now because it was unclear what the Applicant was claiming during the first action on the merits, as reflected in the rejection under 35 USC 112, second paragraph rejection in the Official Action

of 13 February 2004, p. 6. Moreover, as noted in rejoinder of Claim 2 (Above, pp. 2-3) Claim 2 has been rejoined as of this action, but rejections and objections to this Claim do not remove the necessity of making this action final.

Claim Rejections - 35 USC § 112, second paragraph – Old rejections

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

In light of Applicant's amendments and arguments of 12 August 2004, the rejection of Claims 4, 12-19, 23, 31-40, and 63 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention, is withdrawn.

In light of Applicant's cancellation of Claim 62, the rejection of Claim 62 under 35 USC 112, second paragraph, as being indefinite, is moot, and therefore, withdrawn.

Claim Rejections - 35 USC § 112, second paragraph, Necessiated by Amendment

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 1, 12, 19, and 63 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 12 limits the enzyme relating the modification of complex N-glycoside-linked sugar chains in which fucose is bound to the 6-position of the N-acetylglucosamine in the

reducing end to the enzyme alpha-1,6-fucosyltransferase. It is not clear how this enzyme, which places such fucose onto N-acetylglucosamine is related to the modification of chains already containing such fucose linked to N-acetylaglucosamine.

Claims 2, 19, and 63 limit Claims 1, 18, and 62, respectively, to reducing-end N-Acetylglucosamines substituted with fucose at the 6 position of N-Acetylglucoseamine. It is unclear how fucose could be bound through the 6 position in Claims 2, 19, and 63, if it is already not bound in parent claims 1, 18, and 62.

Claim Rejections - 35 USC § 112 – New Matter, Necessiated by Amendment

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 4 and 23 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Claims 4 and 23 encompass enzymes relating to the modification of a complex N-glycoside-linked sugar chain in which fucose is bound to the 6-position of N-Acetylglucosamine. While the specification does discuss alpha-1,6-N-Fucosyltransferase, no enzymes are disclosed for the modification of such bound sugars once they are bound. While Applicant directs the attention of the Examiner to Example 14 for support (Applicant's response of 12 August 2004, p. 31, first paragraph), such Example discusses the same alpha-1,6-N-Fucosyltransferase and not

any enzyme relating to the modification of existing fucose bound to N-Acetylglucosamine in such complex sugars.

This is a new matter rejection.

Claim Rejections - 35 USC § 112 – Enablement, old rejection

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1, 4, 12-19, 23, 31-40 and 62-64 remain rejected, and Claims 2-3, 20, and 65-68 are newly rejected, under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a CHO cell comprising a deletion of at least exon 2 of one FUT8 gene, which deletion produces a non-functional enzyme, into which a gene encoding an antibody is introduced, such antibody gene being expressed and producing antibodies having complex N-glycoside-linked sugar chains bound to the Fc region, wherein among the total complex N-glycoside-linked sugar chains bound to the Fc region in the composition, the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine at the 6 position is 20% or more, does not reasonably provide enablement for any CHO cell or any CHO cell comprising any deletion of a gene encoding FUT8 that produces any decrease in such enzyme, into which a gene encoding an antibody is introduced, such antibody gene being expressed and producing antibodies having complex N-glycoside-linked sugar chains bound to the Fc region, wherein among the total complex N-glycoside-linked sugar chains bound to the Fc region in the composition, the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine is

20% or more. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make the invention commensurate in scope with these claims.

It is noted that newly rejected Claims 2-3, 20, and 65-68, while encompassing limitations in glyclosylation characteristics, antibody characteristics, methods of making antibodies, methods of making the CHO cells, do not encompass limitations which are considered lacking in this rejection.

Response - 35 USC § 112 – Enablement, old rejection

Applicant argues that the Specification enables teaches much more than the exemplified embodiments; that the Artisan would be able to make the mutants with the decreased or deleted activity; and reviews examples of mutants that could be made to meet the requirements of the claims. Therefore, Applicant argues, the rejections of the claims under 35 USC 112, first paragraph, as lacking an enabling disclosure, should be withdrawn (Applicant's response of 12 August 2004, pp. 32-35).

Applicants arguments have been fully considered but are not persuasive. Applicant appears to be arguing that these mutants could be made, by way of examples of mutants that delete the activity of the enzyme being mutated (Id., all examples). Applicant's papers discuss the divergent evolution of fucosyltransferases (Oriol, et al. (1999) Glycobiology, 9: 323-334); conserved structural features of such fucosyltransferases (Breton, et al. (1998) Glycobiology, 8(1) 87-94); knockout mice of a specific fucosyltransferase (Asano, et al. (1997) EMBO J., 16(8): 1850-57); and fucosyltransferase roles in selectin ligand biosynthesis (Maly, et al. (1996) Cell, 86: 643-53). Through these, Applicant argues that specific areas of the protein are

considered important, and therefore the Artisan would know how to mutate the fucosyltransferase in a reasonably predictable manner in order to knock-out the activity of the enzyme and thus prepare the cell (Applicant's arguments of 12 August 2004, p. 35, paragraph 1). However, Applicant's claims specifically embrace mutants that have decreased activity (e.g., Claims 4 and 23). It is maintained that Applicant still has not provided an enabling disclosure based on even one single enzyme mutation that decreases the activity of such enzyme to the proper amount, in CHO cells and thereby allows such cells to produce the claimed characteristic glycosylations (e.g., Official Action of 13 February 2004, p. 7, first paragraph, "... that produces any decrease in such enzyme [activity] ..."). Applicant has only demonstrated the ability to completely remove activity in a reasonably predictable manner (Id.).

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

In light of Applicant's amendments and arguments of 12 August 2004, the rejection of Claim 1 under 35 U.S.C. 102(b) as being anticipated by Lifely, et al. (1995) Glycobiology, 5(8): 813-22, is withdrawn.

CONCLUSION

The claims are free of the art of record.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Robert M Kelly whose telephone number is (571) 272-0729. The examiner can normally be reached on M-F, 9:00am-5:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amy Nelson can be reached on (571) 272-0804. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Robert M. Kelly, Ph.D.
Examiner, USPTO, AU 1632
2C55 Remsen Building
(571) 272-0729

ANNE M. WEHBE' PH.D
PRIMARY EXAMINER

Sheet 1 of 1

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AUG 12 2004
PATENT & TRADEMARK OFFICE

INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

ATTY. DOCKET NO.	SERIAL NO.
249-202	09/971,773
APPLICANT	
KANDA, et al.	
FILING DATE	**GROUP**
October 9, 2001	1632

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
RMK		5728568	5/1998	Sullivan et al	435	232	
RMK		6054304	4/2000	Taniguchi	435	232	

FOREIGN PATENT DOCUMENTS

		DOCUMENT	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
RMK		99/64618A1	12/1999	WO				
↓		97/37683A1	10/1997	WO				
↓		97/27303A1	7/1997	WO Abstract				
↓		00/61739A1	10/2000	WO Abstract				
RMK		99/54342A1	10/1999	WO				

OTHER DOCUMENTS (including Author, Title, Date, Pertinent pages, etc.)

RMK		Shitara et al. Journal of Immunological Methods, 167, 271-278 (1994)
—		~~Furukawa et al Protein, Nucleic Acid and Enzyme, 43, 2309-2317 (1998)~~
RMK		Oriol et al, Glycobiology, 9, no. 4, 323-334 (1999)
↓		Breton et al, Glycobiology, 8, no. 1, 87-94 (1998)
↓		Asano et al, The EMBO Journal, vol. 16, No. 8, pp 1850-1857 (1997)
RMK		Maly et al, Cell , Vol 86, 643-653, August 23, 1996

*Examiner R. A. Kelley — Date Considered 10/25/04

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to application.

Form PTO-FB-A820 (Also PTO-1449)

Index of Claims

Application No.	Applicant(s)
09/971,773	KANDA ET AL.
Examiner	**Art Unit**
Robert M Kelly	1632

√	Rejected	–	(Through numeral) Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

Claim		Date
Final	Original	11/1/04
	1	√
	2	√
	3	√
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	5	N
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	7	N
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	10	N
	11	N
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	50	N

Claim		Date
Final	Original	1/11/04
	51	N
	52	N
	53	N
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	58	N
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	60	N
	61	N
	62	N
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Claim		Date
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Search Notes

Application No.	Applicant(s)	
09/971,773	KANDA ET AL.	
Examiner	**Art Unit**	
Robert M Kelly	1632	

SEARCHED			
Class	Subclass	Date	Examiner
800	8	11/1/2004	RMK
435	326	11/1/2004	RMK
435	70.1	11/1/2004	RMK
435	183	11/1/2004	RMK
435	232	11/1/2004	RMK

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
CHO/Chinese hamster ovary, fucosyltransferase, glycosylation, antibody, IgG, n-acetyl glucosamine, fucose	11/1/2004	RMK
antibody dependent (cellular) cytoxicity, ADCC	11/1/2004	RMK
MEDLINE, BIOSIS, EMBASE, CAPLUS, WIPDS, SCISEARCH	11/1/2004	RMK

Freeform Search

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US OCR Full-Text Database
EPO Abstracts Database
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Derwent World Patents Index
IBM Technical Disclosure Bulletins

Term: L4 and (adcc or antibody adj dependent)

Display: 10 **Documents in Display Format:** CIT **Starting with Number** 1

Generate: ○ **Hit List** ◉ **Hit Count** ○ **Side by Side** ○ **Image**

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Search History

DATE: Monday, October 25, 2004 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=PGPB,USPT; PLUR=YES; OP=OR			
L5	L4 and (adcc or antibody adj dependent)	12	L5
L4	L3 and antibody	110	L4
L3	L2 and glycosylation	113	L3
L2	L1 and fucosyltransferase	157	L2
L1	@ad<20001006 and (cho or chinese adj hamster adj ovary)	41644	L1

END OF SEARCH HISTORY

Aragen/Transposagen Ex. 1036 - Part 45

DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199701
ENTRY DATE: Entered STN: 19970219
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L1 28338 S CHO OR CHINESE(W)HAMSTER(W)OVARY
L2 98 S L1 AND FUCOSYLTRANSFERASE
L3 27 S L2 AND GLYCOSYLATION
L4 6 S L3 AND ANTIBODY
L5 0 S L3 AND ANTIBODY(W)DEPENDENT

FILE 'MEDLINE, BIOSIS, EMBASE, CAPLUS, SCISEARCH, WPIDS' ENTERED AT 07:45:45 ON 25 OCT 2004
L6 31 S L4
L7 14 DUP REM L6 (17 DUPLICATES REMOVED)

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files searched

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

KANDA, et al. Atty. Ref.: 249-202; Confirmation No. 2525

Appl. No. 09/971,773 TC/A.U. 1632

Filed: October 9, 2001 Examiner: Kelly, Robert

For: ANTIBODY COMPOSITION-PRODUCING CELL

* * * * * * * * * * * *

August 12, 2004

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

AMENDMENT

Responsive to the Official Action dated February 13, 2004, entry and consideration of the following amendments and remarks are requested; the period for response having been extended up to and including August 13, 2004, by submission of the requisite three (3) month extension petition and fee, attached.

08/13/2004 EABUBAK1 00000008 09971773
01 FC:1202 90.00 OP
02 FC:1253 950.00 OP

AMENDMENTS TO THE SPECIFICATION:

Amend the specification as follows:

Page 1, after the Title, insert the following new paragraph:

The present application claims benefit of U.S. Provisional Application Serial No. 60/268,916, filed February 16, 2001, and Japanese applications P.2000-308526, filed October 6, 2000 and PCT/JP01/08804, filed October 5, 2001.

Page 4, delete the paragraph spanning lines 15-24, after the structure of formula (I) (i.e., the last paragraph on page 4), and insert the following therefor:

In the Fc region of an antibody of an IgG type, two N-glycoside-linked sugar chain binding sites are present. In serum IgG, to the sugar chain binding site, generally, binds a complex type sugar chain having plural branches and in which addition of sialic acid or bisecting *N*-acetylglucosamine is low. It is known that there is variety in the manner and form in which ~~regarding the~~ addition of galactose is made to the non-reducing end of the complex type sugar chain and the addition of fucose to the *N*-acetylglucosamine in the reducing end [Leppanen, A, et al., *Biochemistry (1997) 36, 7026-7036,* "in vitro Biosynthesis of a Decasacharide Prototype of Multiply Branched Polylactoaminoglycan Backbones" ~~*Biochemistry*, 36, 130 (1997)~~].

AMENDMENTS TO THE CLAIMS:

Amend the claims as follows:

1. (Currently Amended) An isolated Chinese hamster ovary (CHO)~~tissue-derived CHO~~ cell into which a gene encoding an antibody molecule is introduced, said cell producing ~~which produces~~ an antibody composition comprising an antibody molecule having complex N-glycoside-linked sugar chains bound to the Fc region, wherein ~~among the total complex N-glycoside-linked sugar chains bound to the Fc region in the composition,~~ the ratio of ~~a sugar chain~~sugar chains in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain, among the total complex N-glycoside-linked sugar chains bound to the Fc region in the composition, is 20% or more.

2. (Withdrawn) The CHO cell according to claim 1, wherein the complex N-glycoside-linked sugar chains to which fucose is not bound ~~is a~~ are complex N-glycoside-linked sugar chains in which fucose is not bound to the 6-position of N-acetylglucosamine of a complex N-glycoside linked sugar chain in the reducing end ~~through α(1→6)glycosyl bond~~.

3. (Withdrawn) The CHO cell according to claim 1, wherein the antibody molecule belongs to an IgG class.

4. (Currently Amended) The CHO cell according to claim 1, wherein the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose

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and/or the activity of an enzyme relating to the modification of a complex N-glycoside-linked sugar chain in whichwhich of fucose is bound to the 6-position of N-acetylglucosamine in the reducing end through α(1→6)glycosyl bond in of the complex N-glycoside-linked sugar chain is decreased or deleted or decreased such that said CHO cell are resistant to a lectin which binds a complex N-glycoside-linked sugar chain in which fucose is bound to the 6-position of *N*-acetylglucosamine in the reducing end of the complex N-glycoside-linked sugar chain.

5. (Withdrawn) The CHO cell according to claim 4, wherein the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose is an enzyme selected from the group consisting of the following (a), (b) and (c):

(a) GMD (GDP-mannose 4,6-dehydratase);

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase);

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase).

6. (Withdrawn) The CHO cell according to claim 5, wherein the GMD is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:65;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:65 under stringent conditions and encodes a protein having GMD activity.

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7. (Withdrawn) The CHO cell according to claim 5, wherein the GMD is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:71;

(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:71 and has GMD activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:71 and has GMD activity.

8. (Withdrawn) The CHO cell according to claim 5, wherein the Fx is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:48;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under stringent conditions and encodes a protein having Fx activity.

9. (Withdrawn) The CHO cell according to claim 5, wherein the Fx is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:72;

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(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:72 and has Fx activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:72 and has Fx activity.

10. (Withdrawn) The CHO cell according to claim 5, wherein the GFPP is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:51;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:51 under stringent conditions and encodes a protein having GFPP activity.

11. (Withdrawn) The CHO cell according to claim 5, wherein the GFPP is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:73;

(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity;

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(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity.

12. (Currently Amended) The CHO cell according to claim 4, wherein the enzyme relating to the modification of a complex N-glycoside-linked sugar chain in which fucose ~~is~~ bound to the 6-position of the N-acetylglucosamine in the reducing end ~~through α(1→6)glycosyl bond in~~ of the complex N-glycoside-linked sugar chain is α-1,6-fucosyltransferase.

13. (Original) The CHO cell according to claim 12, wherein the α-1,6-fucosyltransferase is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions and encodes a protein having α-1,6-fucosyltransferase activity.

14. (Original) The CHO cell according to claim 12, wherein the α-1,6-fucosyltransferase is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:23;

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(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity.

15. (Previously Presented) The CHO cell according to claim 4, wherein the enzyme activity is decreased or deleted by a technique selected from the group consisting of the following (a), (b), (c), (d) and (e):

(a) a gene disruption technique targeting a gene encoding the enzyme;

(b) a technique for introducing a dominant negative mutant of a gene encoding the enzyme;

(c) a technique for introducing mutation into the enzyme;

(d) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme;

(e) a technique for selecting a cell line resistant to a lectin which recognizes a sugar chain in which fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α(1→6)glycosyl bond in the complex N-glycoside-linked sugar chain.

16. (Currently Amended) The CHO cell according to claim 1~~claim 4~~, which is resistant to at least ~~a~~ one lectin which recognizes a complex N-glycoside-linked sugar

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chain in which fucose is bound to the 6-position of N-acetylglucosamine in the reducing end ~~through α(1→6)glycosyl bond in~~ of the complex N-glycoside-linked sugar chain.

17. (Currently Amended) The CHO cell according to claim 4, which produces an antibody composition having greater ~~higher~~ antibody-dependent cell-mediated cytotoxic activity than an antibody composition produced by its parent CHO cell.

18. (Currently Amended) The CHO cell according to claim 17, which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition in which ~~among the total complex N-glycoside-linked sugar chains bound to the Fc region contained in the antibody composition,~~ the ratio of ~~a~~ sugar ~~chain~~chains in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain, among the total complex N-glycoside-linked sugar chains bound to the Fc region in the composition, is less than 20%.

19. (Currently Amended) The CHO cell according to claim 18, wherein the complex N-glycoside-linked sugar chains to which fucose is not bound ~~is a~~ are complex N-glycoside-linked sugar chains in which fucose is not bound to the 6-position of N-acetylglucosamine of the complex N-glycoside linked sugar chain in the reducing end ~~through α(1→6)glycosyl bond~~.

20. (Withdrawn) A method for producing an antibody composition, which comprises culturing the CHO cell according to claim 1 in a medium to produce and

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accumulate an antibody composition in the culture; and recovering the antibody composition from the culture.

21. (Withdrawn) An antibody composition which is produced using the method according to claim 20.

22. (Withdrawn) An antibody composition which comprises an antibody molecule having complex N-glycoside-linked sugar chains bound to the Fc region which is produced by a CHO cell, wherein among the total complex N-glycoside-linked sugar chains bound to the Fc region in the composition, the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain is 20% or more.

23. (Currently Amended) A cell in which the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose, and/or the activity of an enzyme relating to the modification of a complex N-glycoside-linked sugar chain in which ~~wherein~~ fucose is bound to the 6-position of N-acetylglucosamine in the reducing end ~~through α(1→6)glycosyl bond in~~of the complex N-glycoside-linked sugar chain is ~~decreased or~~ deleted or decreased such that said cell is resistant to a lectin which recognizes a complex N-glycoside-linked sugar chain in which fucose is bound to the 6-position of *N*-acetylglucosamine in the reducing end of the complex N-glycoside-linked sugar chain by a genetic engineering technique.

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24. (Withdrawn) The cell according to claim 23, wherein the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose is an enzyme selected from the group consisting of the following (a), (b) and (c):

(a) GMD (GDP-mannose 4,6-dehydratase);

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase);

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase).

25. (Withdrawn) The cell according to claim 24, wherein the GMD is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:65;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:65 under stringent conditions and encodes a protein having GMD activity.

26. (Withdrawn) The cell according to claim 24, wherein the GMD is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:71;

(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:71 and has GMD activity;

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(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:71 and has GMD activity.

27. (Withdrawn) The cell according to claim 24, wherein the Fx is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:48;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under stringent conditions and encodes a protein having Fx activity.

28. (Withdrawn) The cell according to claim 24, wherein the Fx is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:72;

(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:72 and has Fx activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:72 and has Fx activity.

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29. (Withdrawn) The cell according to claim 24, wherein the GFPP is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:51;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:51 under stringent conditions and encodes a protein having GFPP activity.

30. (Withdrawn) The cell according to claim 24, wherein the GFPP is a protein selected from the group consisting of the following (a), (b) and (c):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:73;

(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity;

(c) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity.

31. (Previously Presented) The cell according to claim 23, wherein the enzyme relating to the modification of a sugar chain in which fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α(1→6)glycosyl bond in the N-glycoside-linked sugar chain is α-1,6-fucosyltransferase.

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32. (Original) The cell according to claim 31, wherein the α-1,6-fucosyltransferase is a protein encoded by a DNA selected from the group consisting of the following (a), (b), (c) and (d):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

(b) a DNA comprising the nucleotide sequence represented by SEQ ID NO:2;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions and encodes a protein having α-1,6-fucosyltransferase activity,;

(d) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions and encodes a protein having α-1,6-fucosyltransferase activity.

33. (Original) The cell according to claim 31, wherein the α-1,6-fucosyltransferase is a protein selected from the group consisting of the following (a), (b), (c), (d), (e) and (f):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:23;

(b) a protein comprising the amino acid sequence represented by SEQ ID NO:24;

(c) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity;

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(d) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:24 and has α-1,6-fucosyltransferase activity;

(e) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity;

(f) a protein which comprises an amino acid sequence having a homology of at least 80% with the amino acid sequence represented by SEQ ID NO:24 and has α-1,6-fucosyltransferase activity

34. (Previously Presented) The cell according to claim 23, wherein the genetic engineering technique is a technique selected from the group consisting of the following (a), (b), (c) and (d):

(a) a gene disruption technique targeting a gene encoding the enzyme;

(b) a technique for introducing a dominant negative mutant of a gene encoding the enzyme;

(c) a technique for introducing mutation into the enzyme;

(d) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme.

Claim 35. (Canceled)

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36. (Previously Presented) The cell according to claim 23, which is a cell selected from the group consisting of the following (a) to (i):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line, YB2/3HL.P2.G11.16Ag.20 cell;

(c) a mouse myeloma cell line, NSO cell;

(d) a mouse myeloma cell line, SP2/0-Ag14 cell;

(e) a BHK cell derived from a syrian hamster kidney tissue;

(f) an antibody-producing hybridoma cell;

(g) a human leukemia cell line Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

37. (Previously Presented) The cell according to claim 23 into which a gene encoding an antibody molecule is introduced.

38. (Original) The cell according to claim 37, wherein the antibody molecule belongs to an IgG class.

39. (Previously Presented) A method for producing an antibody composition, which comprises culturing the cell according to claim 37 in a medium to produce and accumulate the antibody composition in the culture; and recovering the antibody composition from the culture.

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40. (Original) The method according to claim 39, which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition obtained from its parent cell line.

41. (Withdrawn) An antibody composition which is produced using the method according to claim 39.

42. (Withdrawn) A transgenic non-human animal or plant or the progenies thereof, comprising a genome which is modified such that the activity of an enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose and/or the activity of an enzyme relating to the modification of a sugar chain in which fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α(1→6)glycosyl bond in the N-glycoside-linked sugar chain is decreased.

43. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 42, wherein a gene encoding the enzyme relating to the synthesis of an intracellular sugar nucleotide, GDP-fucose or a gene encoding the enzyme relating to the modification of a sugar chain in which fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α(1→6)glycosyl bond in the N-glycoside-linked sugar chain is knocked out.

44. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 42, wherein the enzyme relating to the synthesis of an

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intracellular sugar nucleotide, GDP-fucose is an enzyme selected from the group consisting of the following (a), (b) and (c):

(a) GMD (GDP-mannose 4,6-dehydratase);

(b) Fx (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase);

(c) GFPP (GDP-beta-L-fucose pyrophosphorylase).

45. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 44, wherein the GMD is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:65;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:65 under stringent conditions and encodes a protein having GMD activity.

46. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 44, wherein the Fx is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:48;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:48 under stringent conditions and encodes a protein having Fx activity.

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47. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 44, wherein the GFPP is a protein encoded by a DNA of the following (a) or (b):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:51;

(b) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:51 under stringent conditions and encodes a protein having GFPP activity.

48. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 42, wherein the enzyme relating to the modification of a sugar chain in which fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α(1→6)glycosyl bond in the N-glycoside-linked sugar chain is α-1,6-fucosyltransferase.

49. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 48, wherein the α-1,6-fucosyltransferase is a protein encoded by a DNA selected from the group consisting of the following (a), (b), (c) and (d):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

(b) a DNA comprising the nucleotide sequence represented by SEQ ID NO:2;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions and encodes a protein having α-1,6-fucosyltransferase activity;

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(d) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions and encodes a protein having α-1,6-fucosyltransferase activity.

50. (Withdrawn) The transgenic non-human animal or plant or the progenies thereof according to claim 42, wherein the transgenic non-human animal is an animal selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

51. (Withdrawn) A method for producing an antibody composition, which comprises introducing a gene encoding an antibody molecule into the transgenic non-human animal or plant or the progenies thereof according to claim 42; rearing the animal or plant; isolating tissue or body fluid comprising the introduced antibody composition from the reared animal or plant; and recovering the antibody composition from the isolated tissue or body fluid.

52. (Withdrawn) The method according to claim 51, wherein the antibody molecule belongs to an IgG class.

53. (Withdrawn) The method according to claim 51, which produces an antibody composition having higher antibody-dependent cell-mediated cytotoxic activity than an antibody composition obtained from a non-human animal or plant or the progenies thereof whose genome is not modified.

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54. (Withdrawn) An antibody composition which is produced using the method according to claim 51.

55. (Withdrawn) A medicament comprising the antibody composition according to any one of claims 21, 22, 41 and 54 as an active ingredient.

56. (Withdrawn) The medicament according to claim 55, wherein the medicament is a diagnostic drug, a preventive drug or a therapeutic drug for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

57. (Withdrawn) A protein selected from the group consisting of the following (a), (b), (c), (d), (e), (f), (g), (h), (i) and (j):

(a) a protein comprising the amino acid sequence represented by SEQ ID NO:71;

(b) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:71 and has GMD activity;

(c) a protein comprising the amino acid sequence represented by SEQ ID NO:72;

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(d) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:72 and has Fx activity;

(e) a protein comprising the amino acid sequence represented by SEQ ID NO:73;

(f) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:73 and has GFPP activity;

(g) a protein comprising the amino acid sequence represented by SEQ ID NO:23;

(h) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:23 and has α-1,6-fucosyltransferase activity;

(i) a protein comprising the amino acid sequence represented by SEQ ID NO:24;

(j) a protein which comprises an amino acid sequence in which at least one amino acid is deleted, substituted, inserted and/or added in the amino acid sequence represented by SEQ ID NO:24 and the α-1,6-fucosyltransferase activity.

58. (Withdrawn) A DNA which encodes the protein according to claim 57.

59. (Withdrawn) A DNA selected from the group consisting of the following (a), (b), (c), (d) and (e):

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

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(b) a DNA comprising the nucleotide sequence represented by SEQ ID NO:2;

(c) a DNA comprising the nucleotide sequence represented by SEQ ID NO:65;

(d) a DNA comprising the nucleotide sequence represented by SEQ ID NO:48;

(e) a DNA comprising the nucleotide sequence represented by SEQ ID NO:51.

60. (Withdrawn) A genome DNA selected from the group consisting of the following (a), (b) and (c):

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:67;

(c) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:70.

61. (Withdrawn) A target vector for homologous recombination, comprising a full length of the DNA according to any one of claims 58 to 60, or a part thereof.

Claim 62. (Canceled)

63. (Currently Amended) The CHO cell according to claim 4, wherein the complex N-glycoside-linked sugar chains to which fucose is not bound ~~is a~~ are complex *N*-glycoside-linked sugar chains in which fucose is not bound to the 6-position of *N*-

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acetylglucosamine of a complex N-glycoside linked sugar chain in the reducing end ~~through α(1→6)glycosyl bond~~.

64. (Previously Presented) The CHO cell according to claim 4, wherein the antibody molecule belongs to an IgG class.

65. (New) The CHO cell according to claim 4, wherein the CHO cell is obtained by culturing a parent CHO cell line in a medium comprising lectin, and selecting cells which can be cultured with 80% survival rate when cultured in a lectin concentration of at least 2 times or more than the lectin concentration which is toxic to the parent cell line.

66. (New) The CHO cell according to claim 4, wherein the resistance of the CHO cell is obtained by culturing CHO cells for 1 day to 2 weeks using a medium comprising the lectin at a concentration of 1 μg/ml to 1 mg/ml, and selecting a surviving cell.

67.(New) The cell according to claim 23, wherein the cell is obtained by culturing a parent cell line in a medium comprising lectin, and selecting cells which can be cultured with 80% survival rate when cultured in a lectin concentration of at least 2 times or more than the lectin concentration which is toxic to the parent cell line.

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68.(New) The cell according to claim 23, wherein the resistance of the cell is obtained by culturing cells for 1 day to 2 weeks using a medium comprising the lectin at a concentration of 1 μg/ml to 1 mg/ml, and selecting a surviving cell.

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AMENDMENTS TO THE DRAWINGS

Please insert the attached 52 sheets of formal drawings in place of the drawings filed June 17, 2002.

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REMARKS

Reconsideration is requested.

The specification has been amended to include a cross reference to the noted U.S. provisional application and foreign applications. Attached is a new inventors' Declaration which includes a claim to benefit of PCT/JP01/08804 under 35 U.S.C. § 119, as the PCT application did not designate the U.S.

A Petition under 37 CFR § 1.78(a) and surcharge under 37 CFR § 1.17(t), as described by the Examiner on pages 2-4 of the Office Action dated February 13, 2004 (Paper No. 02022004), or anything further, is not believed to be required to comply with Rule 78. The Examiner is requested to see the attached Notice ("Claiming the Benefit of a Prior-Filed Application under 35 U.S.C. §§ 119(e), 120, 121, and 365(c)") dated February 24, 2003 from Stephen G. Kuhnin, Deputy Commissioner for Patent Examination Policy, as well as the applicants' Transmittal Letter of June 17, 2002 and the Patent Office Filing Receipt dated September 27, 2002, which includes a correct statement of the priority documents and their relationship to the above-identified U.S. application Serial No. 09/971,773.

Specifically, the attached Notice states the following in Part IV ("Office Practice to Not Require Petition and Surcharge if Benefit Claim is Not Present in the Proper Place But is Recognized By Office Continues But Applicants Are Advised That Proper Reference Must be Presented"):

> "Previously the Office indicated that if an applicant includes a benefit claim in the application but not in the manner specified by 37 CFR 1.78(a) (e.g., if the claim is included in an oath or declaration or the application transmittal letter) within the time period set forth in 37 CFR 1.78(a), the Office

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> will not require a petition under 37 CFR 1.78(a) and the surcharge under 37 CFR 1.17(t) to correct the claim if the information concerning the claim was recognized by the Office as shown by its inclusion on the filing receipt. ... The Office will continue to follow this practice."

Certified copies and English language translations of the priority documents will be forwarded under separate cover.

The specification has been amended at page 4 in response to the Examiner's objection of the same at page 4 of Paper No. 02022004. The citation of the reference was readily determined from an internet search by the undersigned with Google and the information of the original disclosure. No new matter has been added.

Claims 35 and 62 have been canceled, without prejudice.

The withdrawn claims have been amended with an indication of their current status, as required by the PTO "REVISED AMENDMENT PRACTICE: 37 CFR 1.121 CHANGED COMPLIANCE IS MANDATORY - Effective Date: July 30, 2003" Flyer (Rev. 3 (07/24/03). See, specifically Example 8 of the Flyer.

Claims 64-68 have been added. Support for new claims 65 and 67 may be found, for example, in the paragraph bridging pages 48 and 49 of the specification. Support for new claims 66 and 68 may be found, for example, in the first full paragraph on page 81 of the specification. No new matter has been added.

Figure 22 has been amended in the attached formal drawings to include SEQ ID NOs: 11 and 12, in place of the originally-filed Figure 22 which recited SEQ ID NOs: 8 and 9, based on the description of page 162 of the specification. A hand-written marked-up copy of originally-filed Figure 22 is attached wherein the change has been

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indicated. No new matter has been added. The Examiner is requested to advise the undersigned if anything further is required in this regard.

Claims 2, 3, 20-22, 24-30 and 41-61 have been withdrawn from consideration. The Examiner has indicated that claims 1, 4, 12-19, 23, 31-40 and 62-64 are under active consideration. Claims 5-11 and 24-30 however "will be rejoined for consideration, along with the non-elected species upon allowance of all the elected subject matter." See, page 2 of Paper No. 02022004. The applicants also however request rejoinder of claims 2, 3 and 20, which are not believed to be patentably distinct from the subject matter of the Examiner's Group II of the Office Action dated June 17, 2003 (Paper No. 13). Specifically, the Examiner has asserted in Paper No. 13 that "the CHO cells of group I [as defined by claims 1-3 and 20] have a normal activity of an enzyme relating to the synthesis of an intracellular nucleotide GDP-fucose and/or relating to the modification of a sugar chain, whereas the activity of such enzyme is decreased or deleted in the CHO cells of group II [as defined by claims 1, 4-19 and 23-40]." See, pages 4-5 of Paper No. 13.

The applicants note however that the subject matter of the Examiner's Groups I and II have been defined by the Examiner in the same Class and Subclass (i.e., Class 435, Subclasses 69.1 and 455) such that examination of the subject matter of the Examiner's Group II (i.e., the elected subject matter) and the subject matter of the Examiner's Group I (i.e., as defined by claims 1-3 and 20) will necessarily require a review of the same art.

Moreover, the indication by the Examiner that the subject matter of the Examiner's Groups I and II are defined by the same Class and Subclasses is strong

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evidence that the subject matter of the examiner's Groups I and II have not been established as distinct in the art, which would otherwise require separate classification by the Patent Office. Restriction is not believed to be appropriate.

Finally, the applicants note in this regard that claim 1 has been indicated by the examiner as being defined by both of the Examiner's Groups I and II and has been examined. Claim 4 (of Group II), like claims 2 and 3 and 20 (of Group I), is dependent from claim 1. None of claims 2, 3 or 20 specify that the claimed CHO cells have a normal activity of an enzyme relating to the synthesis of an intracellular nucleotide GDP-fucose and/or modification of a sugar chain, to the exclusion of the decreased or deleted activity of an enzyme relating to the synthesis of an intracellular nucleotide GDP-fucose, which is the Examiner's stated basis for requiring restriction between the Examiner's Groups I and II. In fact, claim 2 further defines the CHO cell of claim 1 as producing an antibody composition wherein the sugar chain to which fucose is not bound is a complex N-glycoside-linked sugar chain in which fucose is not bound in the 6-position of N-acetylglucosamine in the reducing end. The Examiner's basis for requiring restriction between the subject matter of the Examiner's Groups I and II is not believed to reflect the claimed subject matter.

Clarification, or examination of the subject matter of the Examiner's Groups I and II, is requested.

Examination of all of the pending claims, or an indication that the pending claims will be considered upon allowance of the elected subject matter, is requested.

The Section 112, second paragraph, rejection of claims 4 and 12-19 stated on page 5 of the Office Action dated February 13, 2004, is obviated by the above

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amendments. Claim 4 has been amended to recite an amount of "activity of an enzyme relating to the modification of a sugar chain in which fucose is bound to the 6-position of N-acetylglucosamine in the reducing end through α(1→6) glycosyl bond in the complex N-glycoside-linked sugar chain must be decreased." See, page 5 of the Office Action dated February 13, 2004. Specifically, claim 4 recites that the activity of an enzyme relating to the modification of a sugar chain in which fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α(1→6)glycosyl bond in the complex N-glycoside-linked sugar chain is decreased to the extent that the CHO cell will be resistant to a lectin which recognizes a sugar chain in which fucose is bound to the 6-position of N-acetylglucosamine in the reducing end through an α(1→6)glycosyl bond in the complex N-glycoside-linked sugar chain. The specification teaches that resistance to such a lectin is related to decreased activity of the recited enzyme. The Examiner is requested to see, for example, Example 14 of the specification in this regard.

Withdrawal of the Section 112, second paragraph, rejection of claims 4 and 12-19 stated on page 5 of the Office Action dated February 13, 2004, is requested.

The Section 112, second paragraph, rejection of claims 23 and 31-40 stated on pages 5-6 of the Office Action dated February 13, 2004, is similarly believed to be obviated by the above amendments. Claims 23 has been amended above in manner similar to claim 4 discussed above. As the Examiner's comments in rejecting claims 23 and 31-40 are similar to the Examiner's comments rejecting claims 4 and 12-19, the Examiner is requested to see the above comments relating to claims 4 and 12-19. Claims 23 and 31-40 are submitted to be definite. Withdrawal of the Section 112, second paragraph, rejection of claims 23 and 31-40 is requested.

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The Section 112, second paragraph, rejection of claim 19 is obviated by the above amendments. Withdrawal of the rejection is requested.

The Section 112, second paragraph, rejection of claim 62 is moot in view of the above.

The Section 112, second paragraph, rejection of claim 63 is obviated by the above amendments. Claim 63 is submitted to be definite and withdrawal of the Section 112, second paragraph, rejection of claim 63 is requested.

The Section 112, first paragraph, rejection of claims 1, 4, 12-19, 23, 31-40 and 62-64 is traversed. Reconsideration and withdrawal of the rejection are requested in view of the following as well as the attached.

The Examiner's indication that the specification is

> "enabling for a CHO cell comprising a deletion of at least exon 2 of one FUT8 gene, which deletion produces a non-functional enzyme, into which a gene encoding an antibody is introduced, such antibody gene being expressed and producing antibodies having complex N-glycoside-linked sugar chains bound o the Fc region, wherein among the total complex N-glycoside-linked sugar chains bound to the Fc region in the composition, the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine at the 6 position is 20% or more"

is acknowledged, with appreciation. See, page 7 of the Office Action dated February 13, 2004. The applicants respectfully submit however that the specification teaches more than the exemplified embodiments which appear to be the extent of the subject matter which the Examiner believes is enabled by the description. In fact, the state of the art in the field of, for example, genetic manipulation techniques, at the time of the present invention, were quite advanced. Moreover, the knowledge in the art relating to antibody production from CHO cells, manipulation of CHO cells and enzymes relating to

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the synthesis of an intracellular sugar nucleotide, GDP-fucose and/or modification of a sugar chain in which fucose is bound to the 6-position of N-acetylglucosamine in the reducing end through an α(1→6)glycosyl bond in a complex N-glycoside-linked sugar chain, were advanced at the time of the present invention.

The applicants exemplified the presently claimed Invention by producing a cell in which a gene encoding an antibody molecule was introduced into a CHO cell having a decreased or deleted enzyme activity of a1,6-fucosyltransferase as the host cell. As noted above, the Examiner appears to have accepted that the specification teaches one of ordinary skill in the art how to make and use a CHO cell in which the exon 2 of a gene encoding a1,6-fucosyltransferase is deleted in order to delete the enzyme activity of α1,6-fucosyltransferase of the cell. The Examiner appears to believe the applicants have not taught how to make and use cells in which a gene in another region is destroyed, for example.

The applicants submit however, that one of ordinary skill in the art would have been able to prepare a cell in which the enzyme activity of α1,6-fucosyltransferase, for example, is deleted or decreased without limitation to the exon 2, based on the present specification. The Examiner is requested to consider the attached in this regard, with the following discussion.

One of ordinary skill in the art will appreciate that in order to obtain a knock-out cell, the intron and exon structures of the target gene should be, advantageously, recognized. One of ordinary skill in the art would appreciate the intron and exon structures of, for example, α1,6-fucosyltransferase, by using a method similar to the method described in Example 12 of the present specification, if the cDNA of the target

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gene is known. The following references (copies of which are attached) describe that the relevant structures can be determined based on the cDNA:

(i) Glycobiology, vol.9, 323-334 (1999) and

(ii) Glycobiology, vol.8, 87-94 (1998).

In reference (i), the structure motif which is important to the activity of the fucosyltransferase was expected from fucosyltransferases derived from various species (see Figs. 2, 3, 4 and 6). In the reference (ii), the structure which is important to the activity of the fucosyltransferase was similarly expected (Fig. 3).

As for the region of the gene to be deleted, one of ordinary skill will appreciate that after the determination of the structure, any region can be deleted, so long as the activity of the gene is deleted. Generally, one of the ordinary skill in the art would delete the following regions.

(1) ATG site,

(2) promoter region, and/or

(3) active site of protein.

The deletion of these region is apparent for one of ordinary skill in the art based on the following references (copies of which are attached), for example:

(iii) The EMBO Journal, vol.16, 1850-1857, (1997), and

(iv) Cell, vol.86, 643-653, (1996)

In reference (iii), the exon I containing ATG of β1,4-garalactosyltransferase is deleted to prepare a knock-out mouse (p. 1851, right column, Fig. 1A). Also, in the reference (iv), the region containing a catalyst domain of fucosyltransferase VII is deleted (p. 644, right column, Fig. 2A).

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The inventors of the presently claimed invention found cDNA encoding α1,6-fucosyltransferase in CHO cells and the exon 2 genomic region, as described in Example 12 of the present specification. Since the exon 2 contains ATG site, this selection was carried out according to ordinary, well-known methods in the production of knock-out cells. It will be apparent for one of ordinary skill in the art that a knock-out cell could be prepared, without an undue amount of experimentation, by deleting, for example, regions containing an ATG site, a promoter region, and/or an active site of a protein of interest in addition to or in place of the exon 2 region exemplified in the present application.

The claims are submitted to be supported by an enabling disclosure and withdrawal of the Section 112, first paragraph, rejection of claims 1, 4, 12-19, 23, 31-40 and 62-64 is requested.

The Section 102 rejection of claim 1 over Lifely et al ((1995) Glycobiology 5(8): 813-22) is traversed. Reconsideration and withdrawal of the Section 102 rejection of claim 1 are requested as the cited reference discloses sugar chain analysis data of an antibody produced by CHO cells in Figure 3A. There are five (5) kinds of sugar chains detected in the cited art, as described by peaks 004300, 004301, 014-00, 004300 and 014300. The areas of peaks 004300 and 014300 based on the total are calculated to be less than 20%. As the cited art fails to teach each and every aspect of claim 1, claim 1 is submitted to be patentable over the cited art. Withdrawal of the Section 102 rejection of claim 1 is requested.

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The claims are submitted to be in condition for allowance and a Notice to that effect, along with further rejoinder and allowance of the above-noted claims, are requested.

The Examiner is requested to contact the undersigned in the event anything further is required.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____
B. J. Sadoff
Reg. No. 36,663

BJS:
1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

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844139

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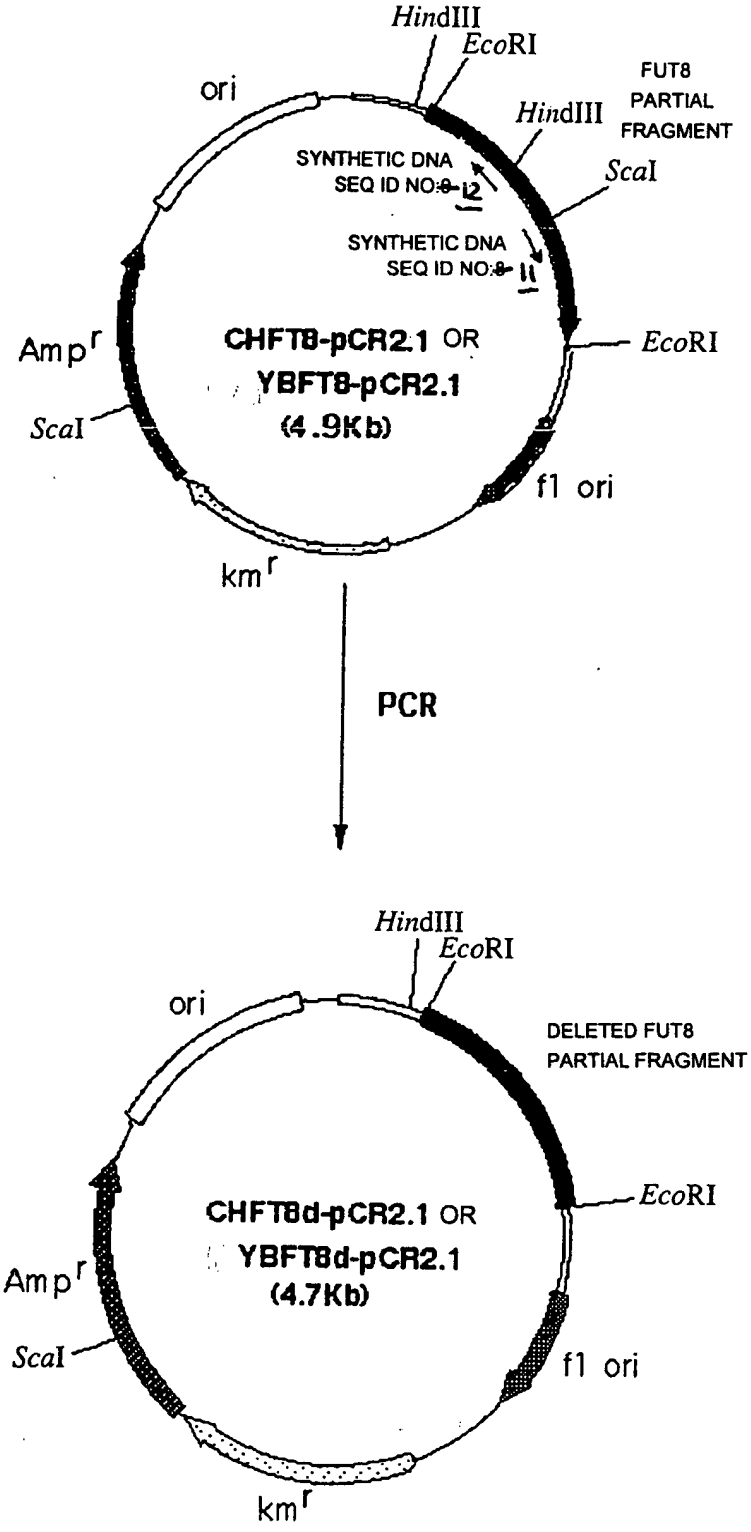
Marked-up copy of Proposed Amended figure

FIG. 22



OIPE JC174
AUG 1 2 2004
PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of Confirmation No. 2525

KANDA, et al.

Serial No. 09/971,773 Atty Ref.: 249-202

Filed: October 9, 2001 Group: 1632

For: ANTIBODY COMPOSITION-PRODUCING CELL Examiner: Kelly, Robert

* * * * * * * * * * * *

August 12, 2004

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBMISSION OF FORMAL DRAWINGS

Sir:

Enclosed herewith are fifty-two (52) sheets of formal, inked drawings for the above-identified application.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. 36,663

BJS:pp
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Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

OIPE JC174 AUG 12 2004 PATENT & TRADEMARK OFFICE

1632

In re Patent Application of Atty Dkt. 249-202
C# M#

KANDA, et al. TC/A.U. 1632

Serial No. 09/971,773 Examiner: Kelly, Robert

Filed: October 9, 2001 Date: August 12, 2004

Title: ANTIBODY COMPOSITION-PRODUCING CELL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

RESPONSE/AMENDMENT/LETTER

This is a response/amendment/letter in the above-identified application and includes an attachment which is hereby incorporated by reference and the signature below serves as the signature to the attachment in the absence of any other signature thereon.

☐ **Correspondence Address Indication Form Attached.**

Fees are attached as calculated below:

Total effective claims after amendment **74** minus highest number previously paid for **69** (at least 20) = 5 x $ 18.00	$	90.00
Independent claims after amendment **7** minus highest number previously paid for **7** (at least 3) = 0 x $ 86.00	$	0.00
If proper multiple dependent claims now added for first time, add $290.00 (ignore improper)	$	0.00
Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s) ($110.00/1 month; $420.00/2 months; $950.00/3 months)	$	950.00
Terminal disclaimer enclosed, add $ 110.00	$	0.00
☐ First/second submission after Final Rejection pursuant to 37 CFR 1.129(a) ($770.00) ☐ Please enter the previously unentered , filed ☐ Submission attached	$	0.00
Subtotal	$	**1040.00**
If "small entity," then enter half (1/2) of subtotal and subtract ☐ Applicant claims "small entity" status. ☐ Statement filed herewith	-$	0.00
Rule 56 Information Disclosure Statement Filing Fee ($180.00)	$	180.00
Assignment Recording Fee ($40.00)	$	0.00
Other:		**0.00**
TOTAL FEE ENCLOSED	$	**1220.00**

The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Account No. 14-1140. A duplicate copy of this sheet is attached.

1100 North Glebe Road, 8th Floor
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Facsimile: (703) 816-4100
BJS:pp

NIXON & VANDERHYE P.C.
By Atty: B. J. Sadoff, Reg. No. 36,663

Signature: _____

Inventor: KANDA et al.
SN 09/971,773/Sheet 1 of 52
Atty. Dkt.: 249-202

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AUG 12 2004
PATENT & TRADEMARK OFFICE

FIG. 1A

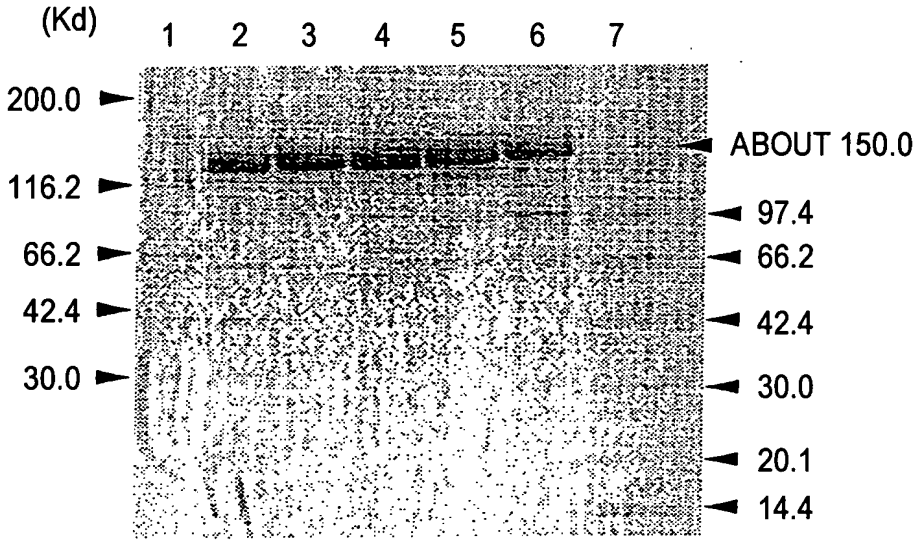

FIG. 1B

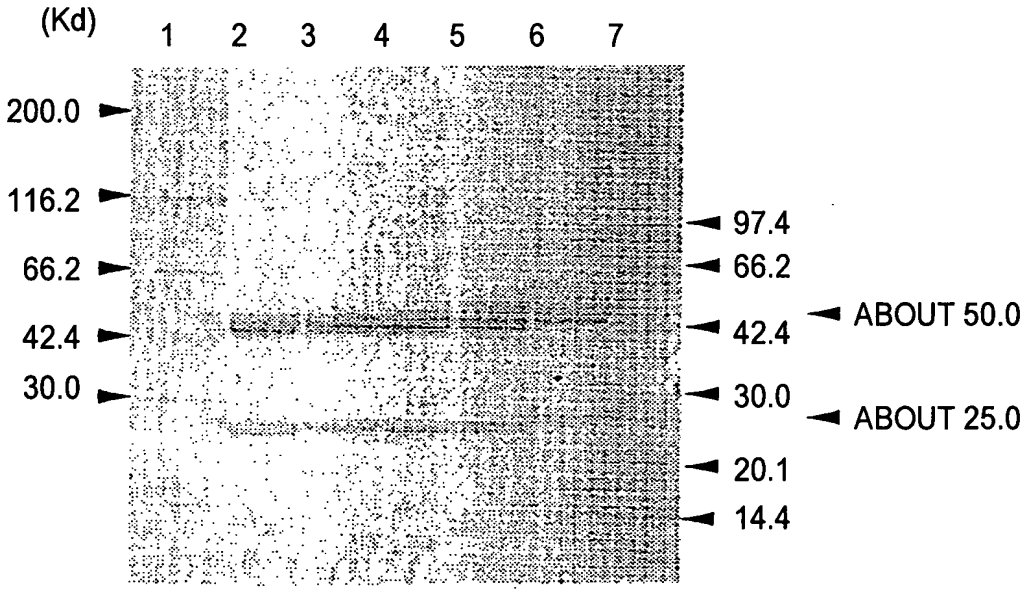

FIG. 2

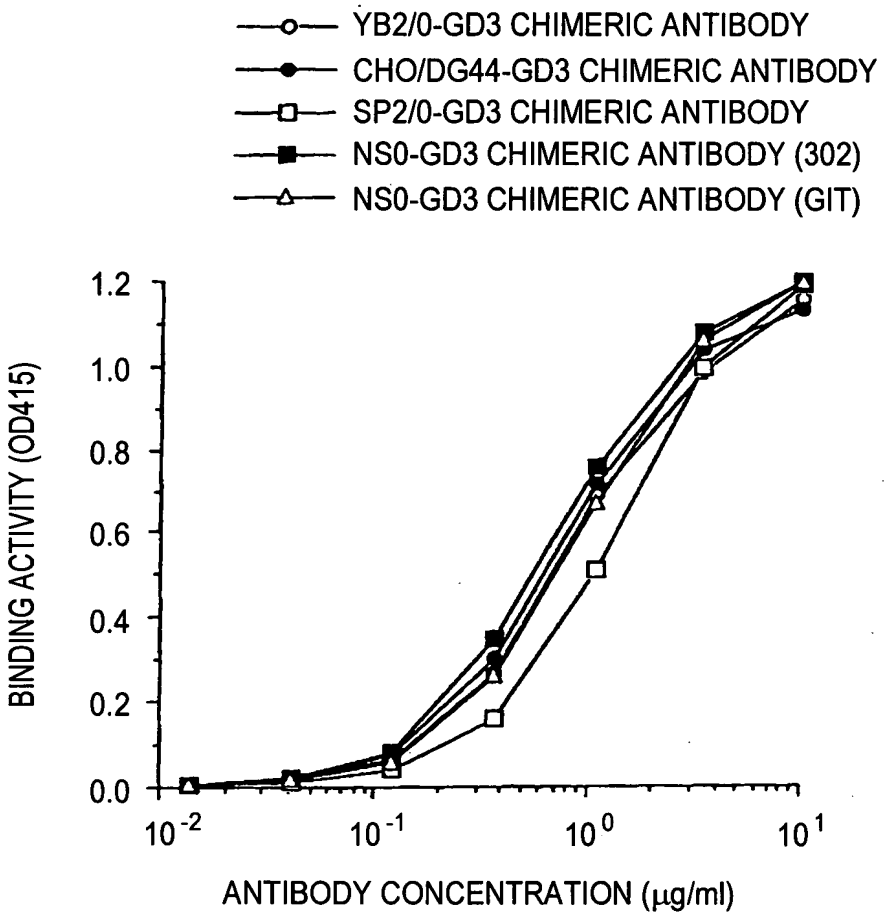
YB2/0-GD3 CHIMERIC ANTIBODY
CHO/DG44-GD3 CHIMERIC ANTIBODY
SP2/0-GD3 CHIMERIC ANTIBODY
NS0-GD3 CHIMERIC ANTIBODY (302)
NS0-GD3 CHIMERIC ANTIBODY (GIT)
BINDING ACTIVITY (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10 -2
10 -1
10 0
10 1
ANTIBODY CONCENTRATION (μg/ml)

FIG. 3

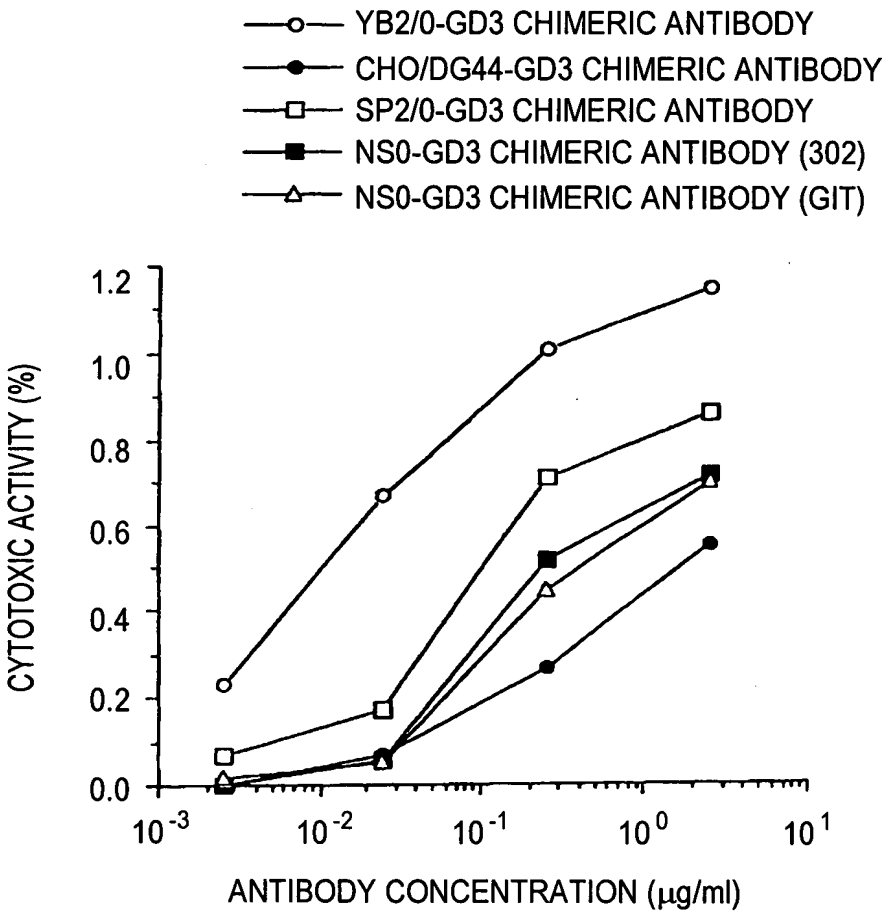
YB2/0-GD3 CHIMERIC ANTIBODY
CHO/DG44-GD3 CHIMERIC ANTIBODY
SP2/0-GD3 CHIMERIC ANTIBODY
NS0-GD3 CHIMERIC ANTIBODY (302)
NS0-GD3 CHIMERIC ANTIBODY (GIT)
CYTOTOXIC ACTIVITY (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-3
10-2
10-1
100
101
ANTIBODY CONCENTRATION (μg/ml)

FIG. 4A

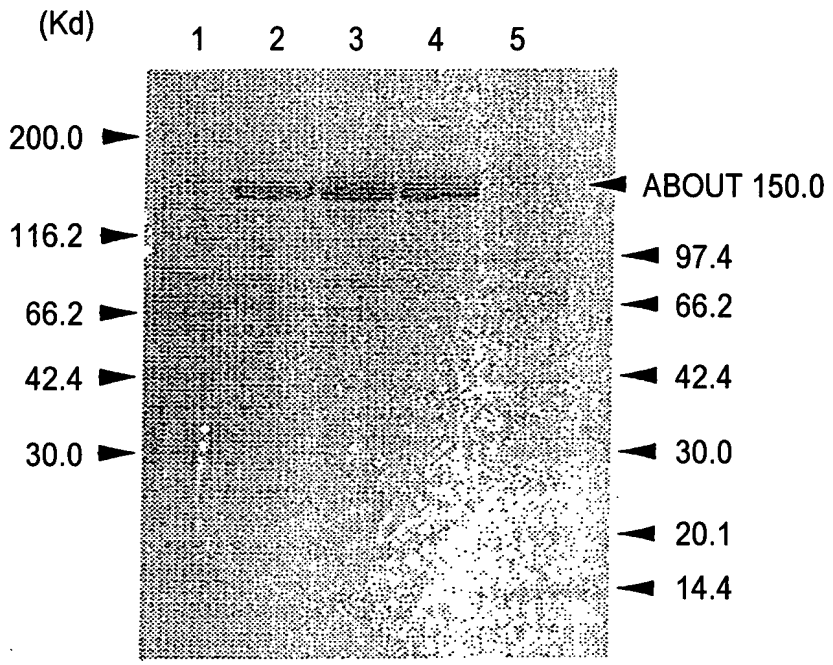
(Kd)
1
2
3
4
5
200.0
116.2
66.2
42.4
30.0
ABOUT 150.0
97.4
66.2
42.4
30.0
20.1
14.4

FIG. 4B

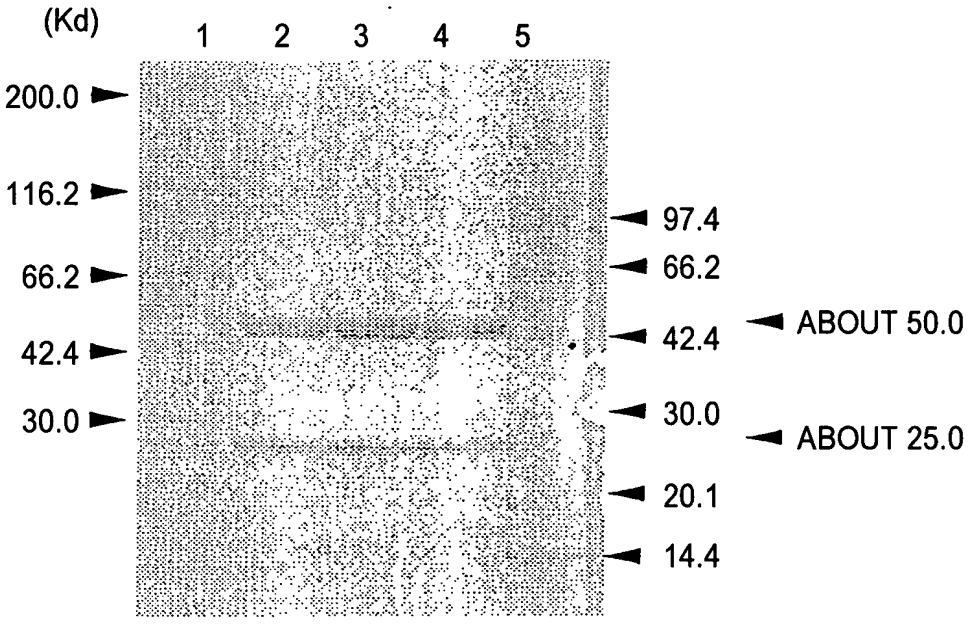
(Kd)
1
2
3
4
5
200.0
116.2
66.2
42.4
30.0
97.4
66.2
42.4
30.0
20.1
14.4
ABOUT 50.0
ABOUT 25.0

FIG. 5

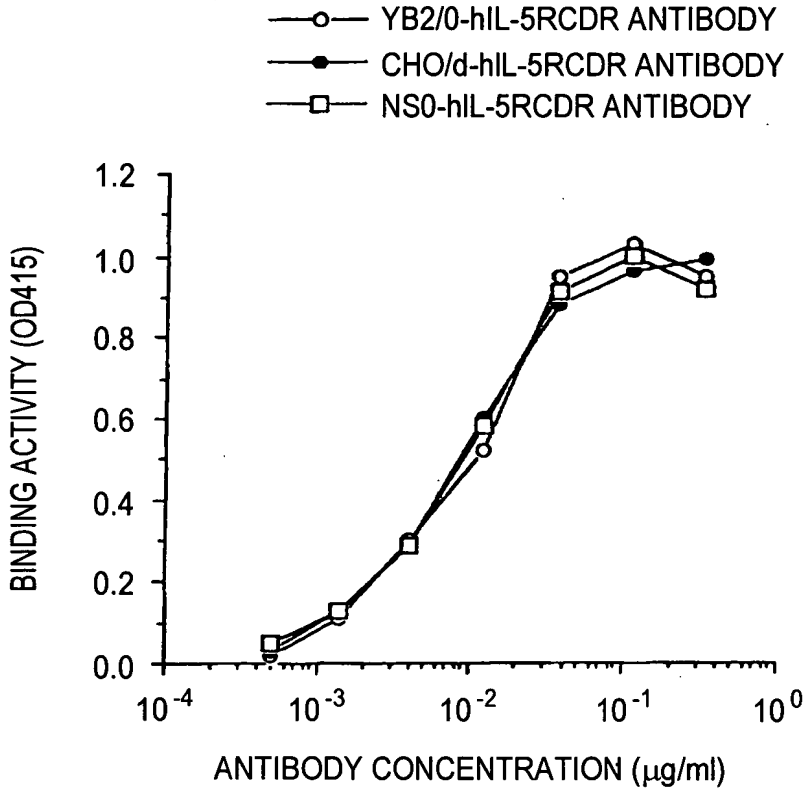
YB2/0-hIL-5RCDR ANTIBODY
CHO/d-hIL-5RCDR ANTIBODY
NS0-hIL-5RCDR ANTIBODY
BINDING ACTIVITY (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-4
10-3
10-2
10-1
100
ANTIBODY CONCENTRATION (μg/ml)

FIG. 6

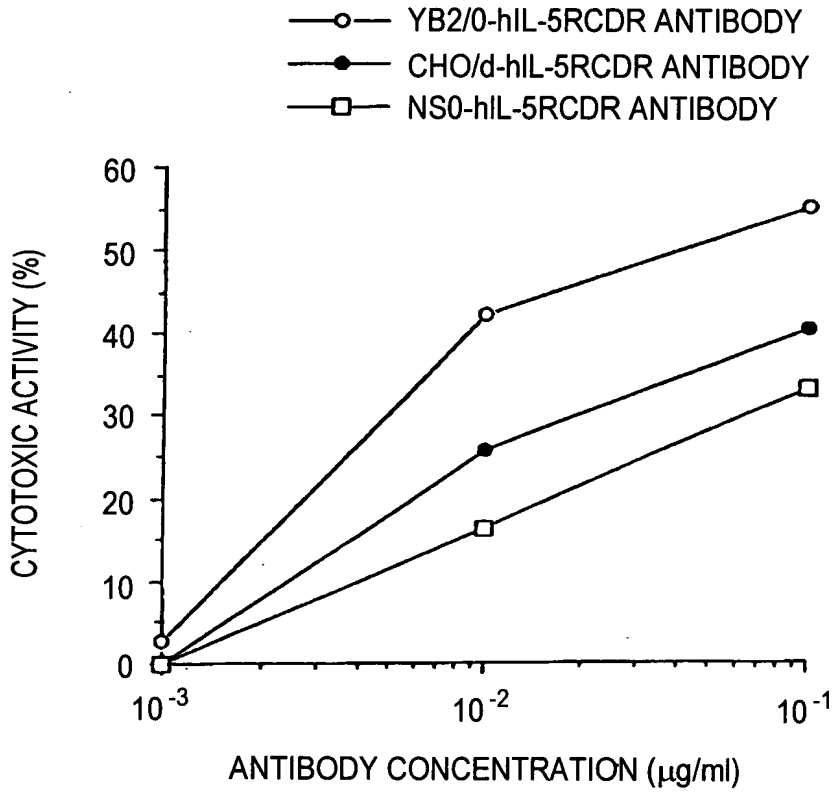
YB2/0-hIL-5RCDR ANTIBODY
CHO/d-hIL-5RCDR ANTIBODY
NS0-hIL-5RCDR ANTIBODY
CYTOTOXIC ACTIVITY (%)
60
50
40
30
20
10
0
10 -3
10 -2
10 -1
ANTIBODY CONCENTRATION (μg/ml)

FIG. 7

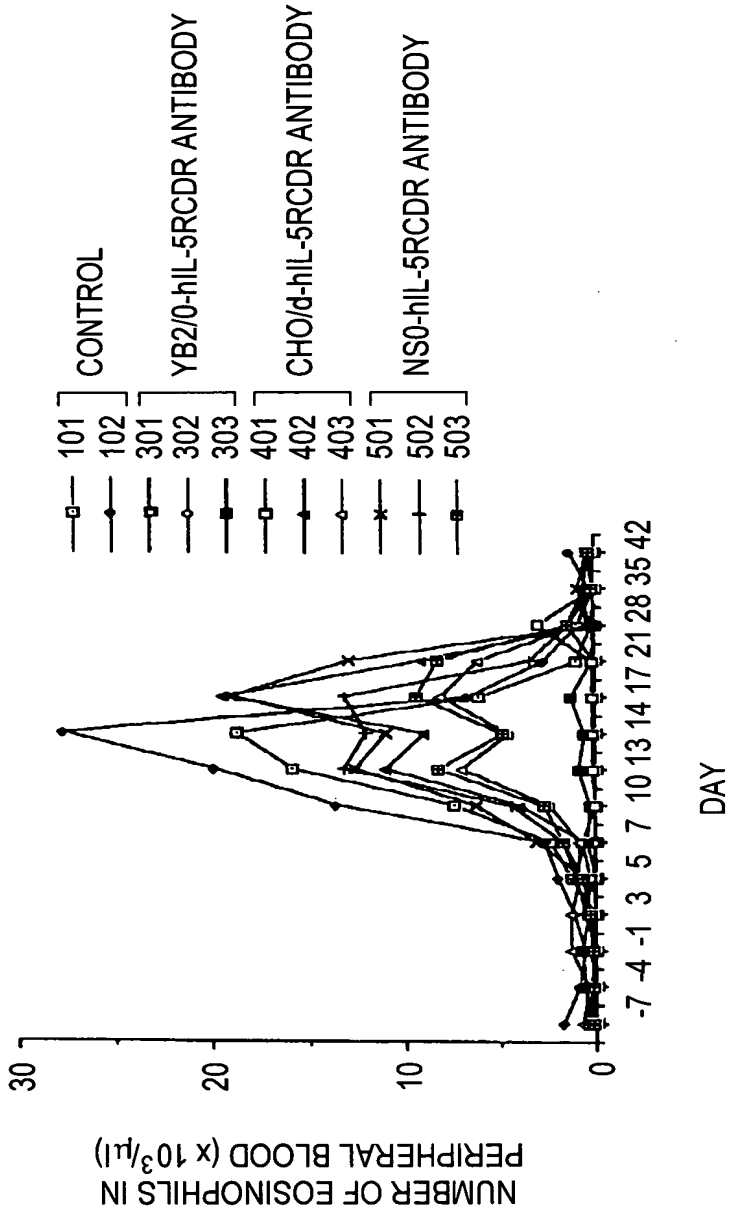
101 CONTROL
102
301 YB2/0-hIL-5RCDR ANTIBODY
302
303
401 CHO/d-hIL-5RCDR ANTIBODY
402
403
501 NS0-hIL-5RCDR ANTIBODY
502
503
30
20
10
0
-7 -4 -1 3 5 7 10 13 14 17 21 28 35 42
DAY
NUMBER OF EOSINOPHILS IN PERIPHERAL BLOOD (x 10³/μl)

FIG. 8A

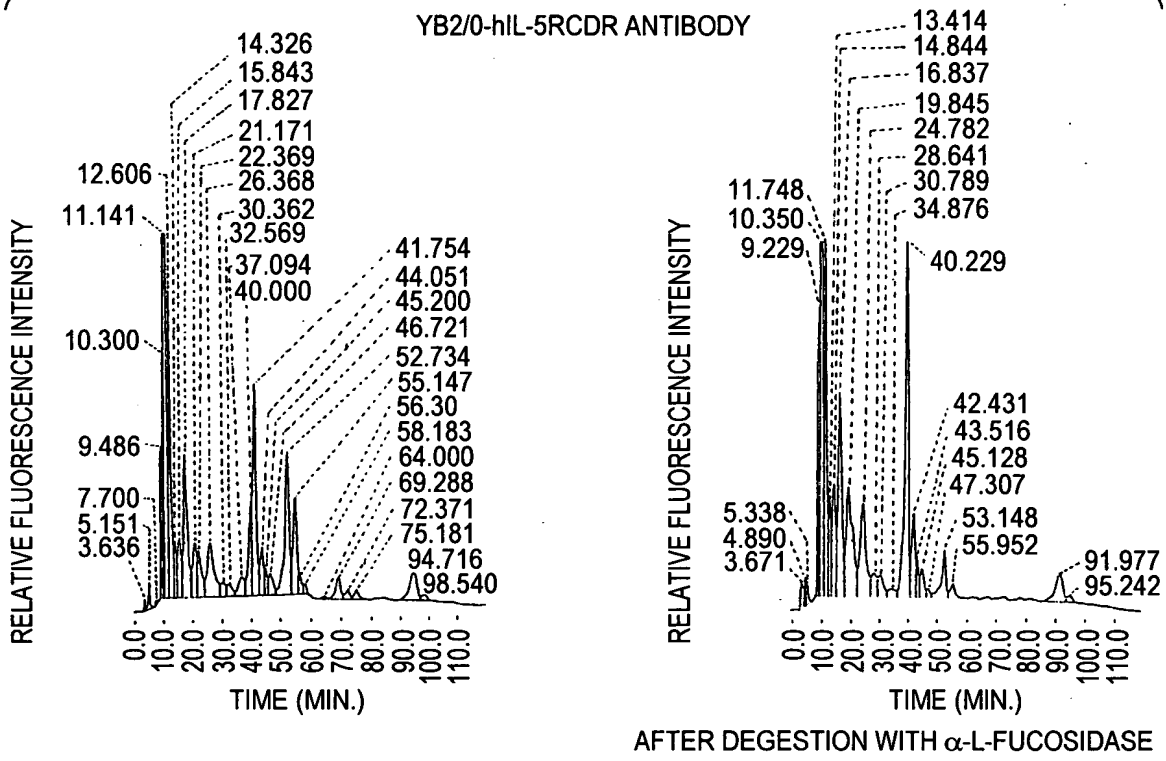
YB2/0-hIL-5RCDR ANTIBODY
RELATIVE FLUORESCENCE INTENSITY
14.326
15.843
17.827
21.171
22.369
26.368
30.362
32.569
37.094
40.000
12.606
11.141
10.300
9.486
7.700
5.151
3.636
41.754
44.051
45.200
46.721
52.734
55.147
56.30
58.183
64.000
69.288
72.371
75.181
94.716
98.540
0.0 10.0 20.0 30.0 40.0 50.0 60.0 70.0 80.0 90.0 100.0 110.0
TIME (MIN.)
13.414
14.844
16.837
19.845
24.782
28.641
30.789
34.876
40.229
11.748
10.350
9.229
42.431
43.516
45.128
47.307
53.148
55.952
91.977
95.242
5.338
4.890
3.671
AFTER DEGESTION WITH α-L-FUCOSIDASE

FIG. 8B

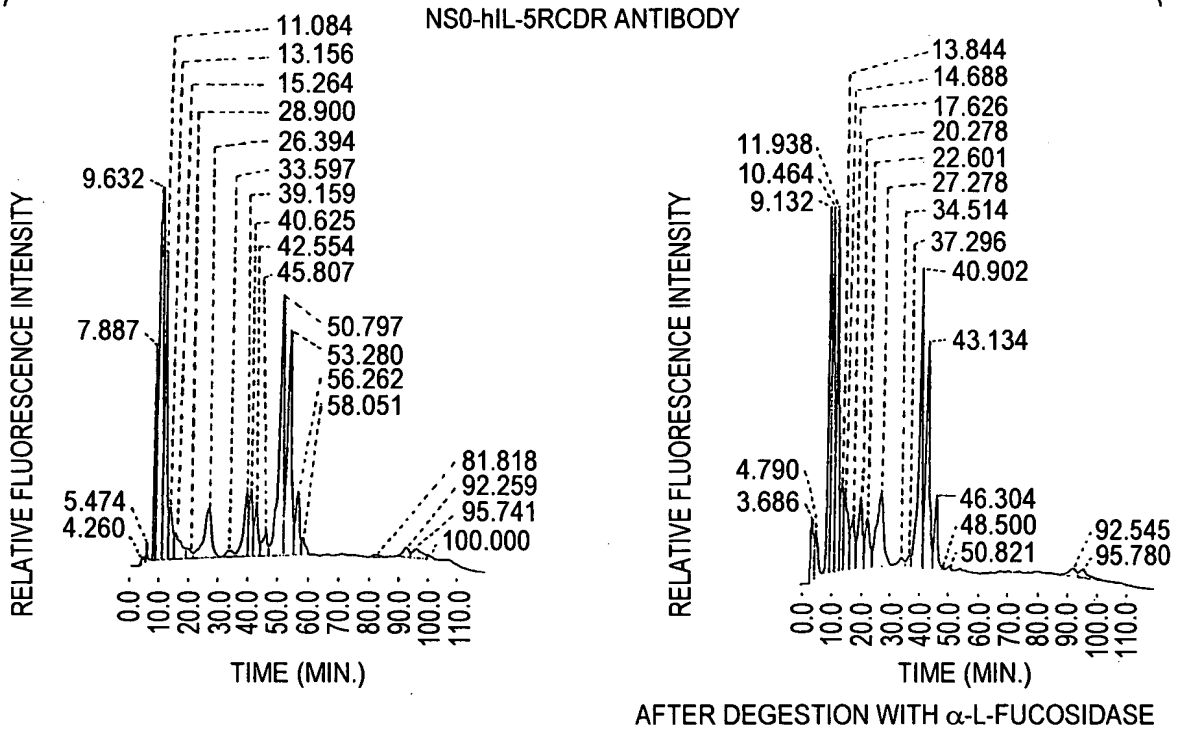
NS0-hIL-5RCDR ANTIBODY
RELATIVE FLUORESCENCE INTENSITY
11.084
13.156
15.264
28.900
26.394
33.597
39.159
40.625
42.554
45.807
9.632
7.887
5.474
4.260
50.797
53.280
56.262
58.051
81.818
92.259
95.741
100.000
0.0 10.0 20.0 30.0 40.0 50.0 60.0 70.0 80.0 90.0 100.0 110.0
TIME (MIN.)
13.844
14.688
17.626
20.278
22.601
27.278
34.514
37.296
40.902
43.134
11.938
10.464
9.132
4.790
3.686
46.304
48.500
50.821
92.545
95.780
AFTER DEGESTION WITH α-L-FUCOSIDASE

FIG. 9

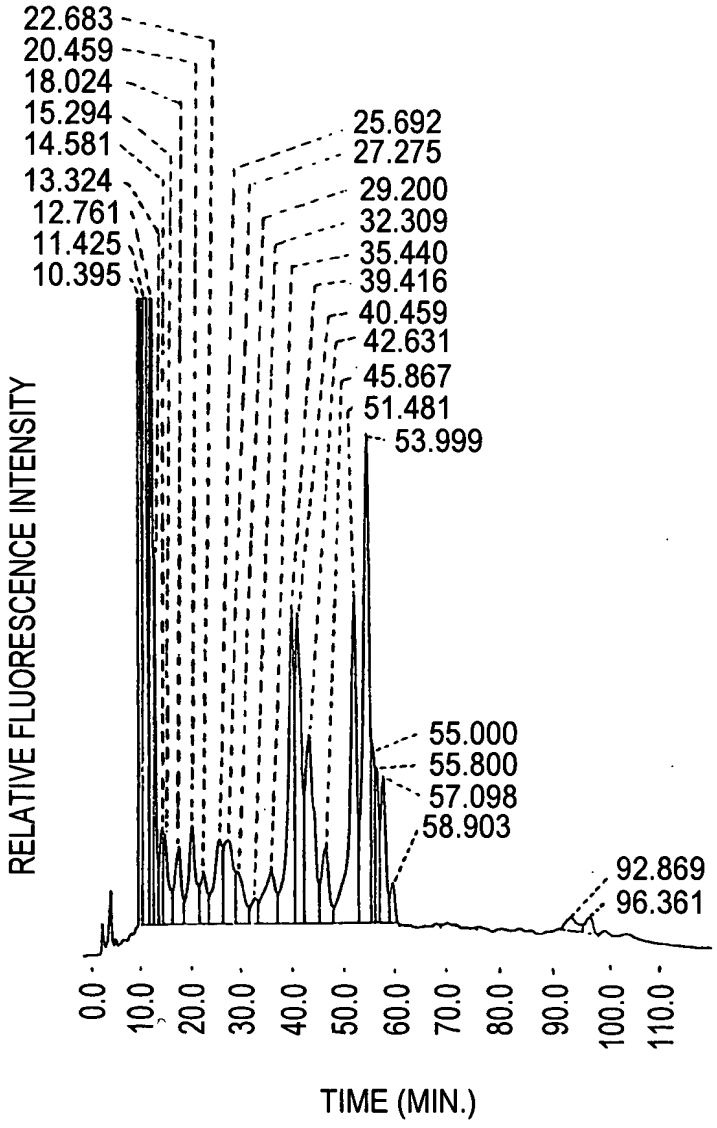
22.683
20.459
18.024
15.294
14.581
13.324
12.761
11.425
10.395
25.692
27.275
29.200
32.309
35.440
39.416
40.459
42.631
45.867
51.481
53.999
55.000
55.800
57.098
58.903
92.869
96.361
RELATIVE FLUORESCENCE INTENSITY
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0
110.0
TIME (MIN.)

FIG. 10

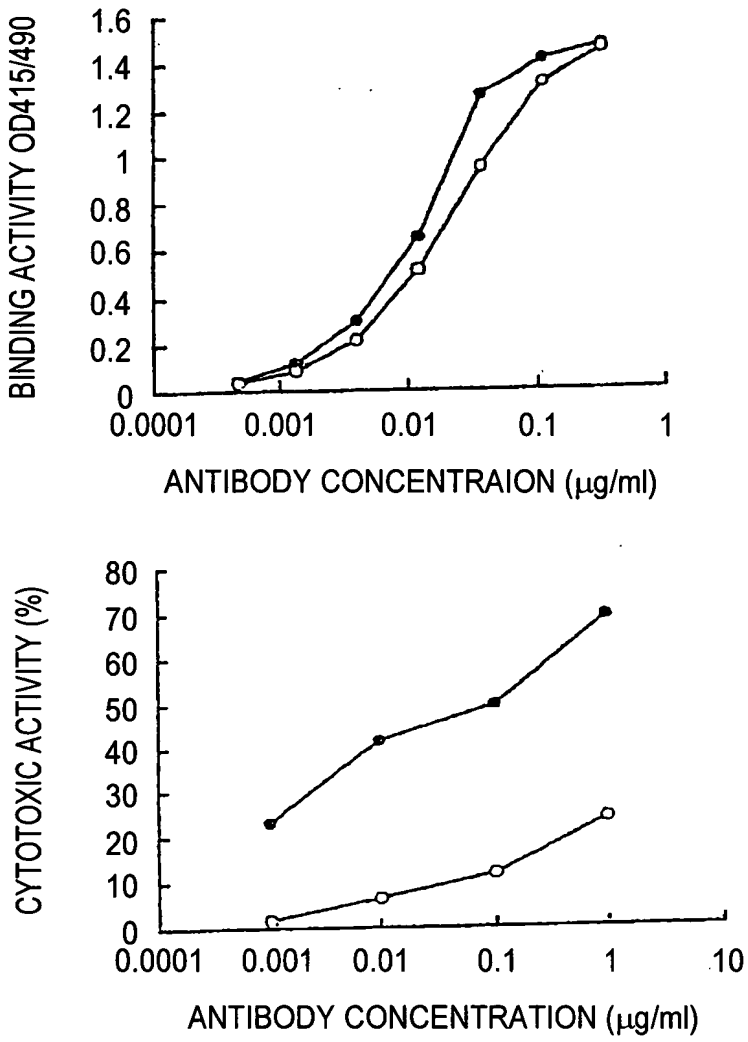
BINDING ACTIVITY OD415/490
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.0001
0.001
0.01
0.1
1
ANTIBODY CONCENTRAION (μg/ml)
CYTOTOXIC ACTIVITY (%)
80
70
60
50
40
30
20
10
0
0.0001
0.001
0.01
0.1
1
10
ANTIBODY CONCENTRATION (μg/ml)

FIG. 11B

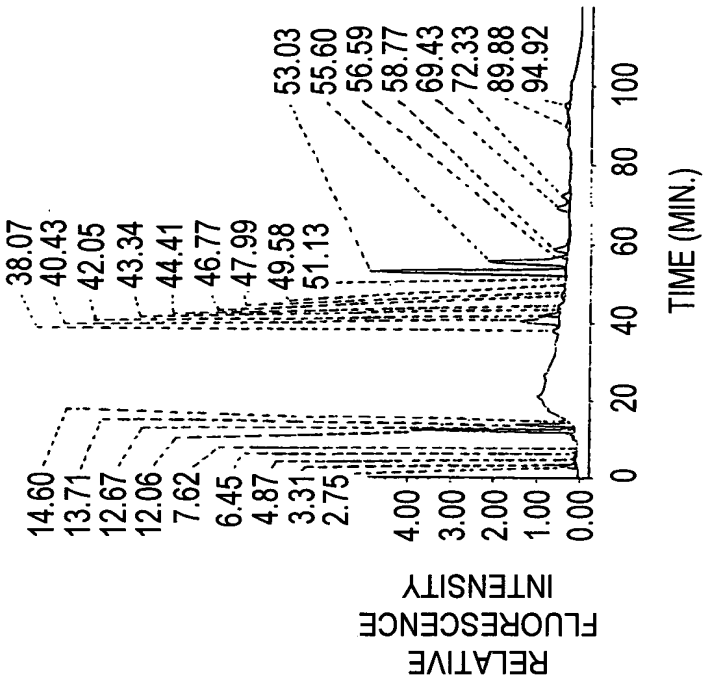

FIG. 11A

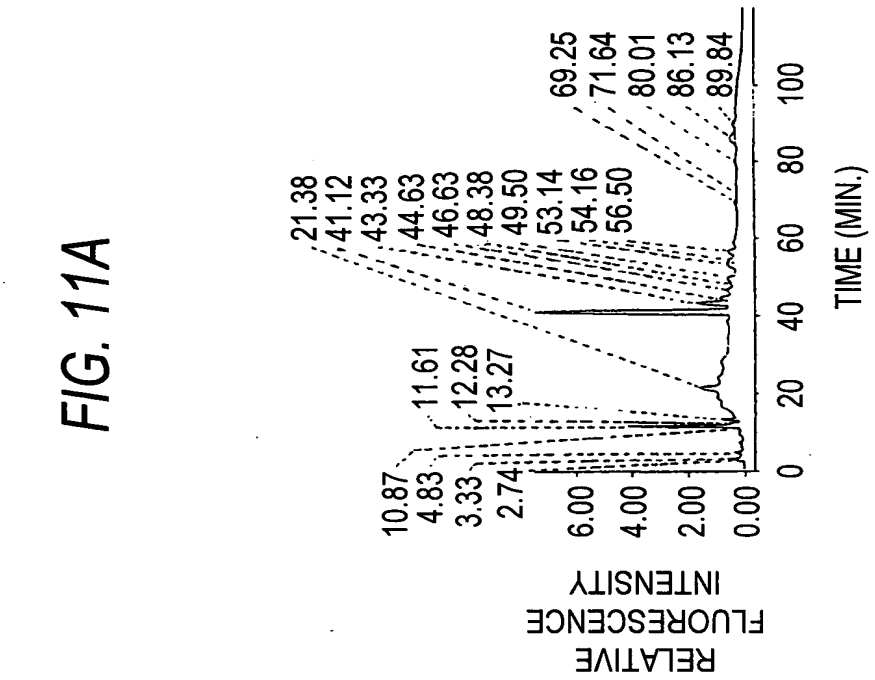

FIG. 12A

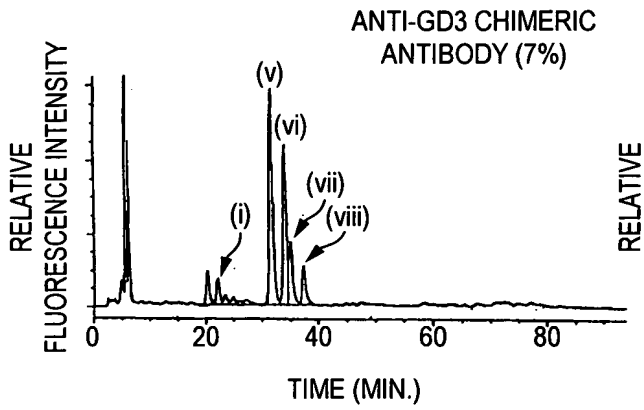

FIG. 12B

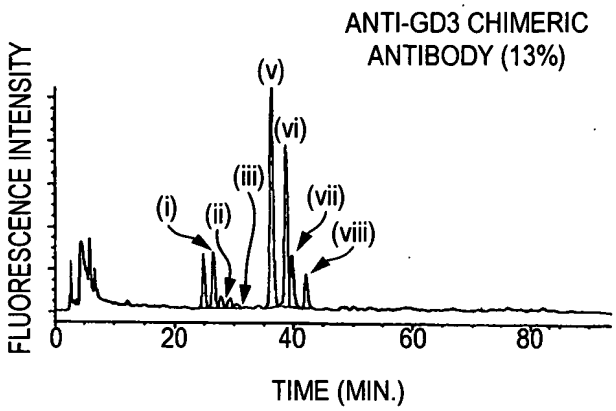

FIG. 12C

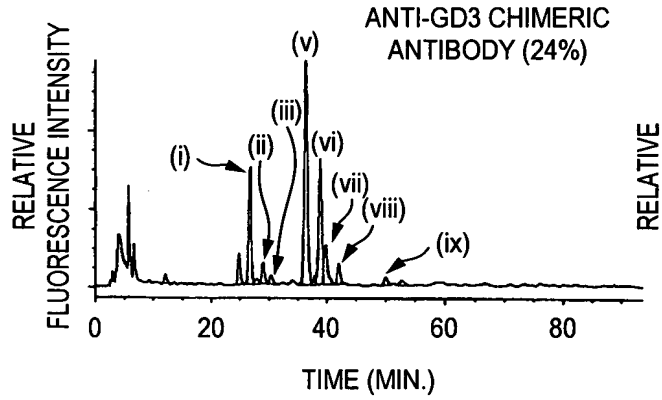

FIG. 12D

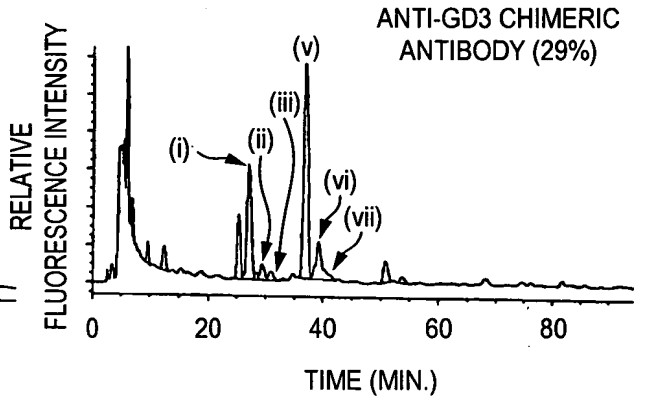

FIG. 12E

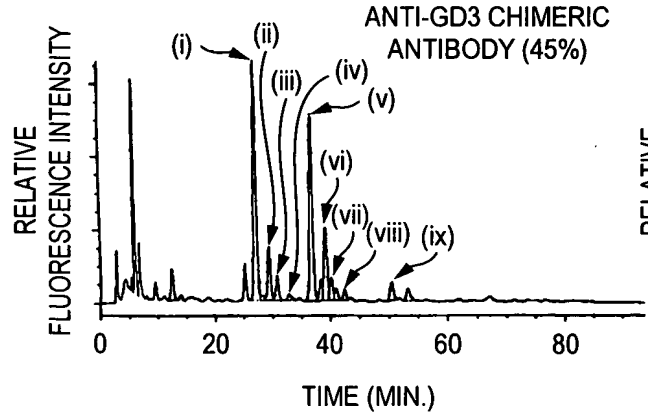

FIG. 12F

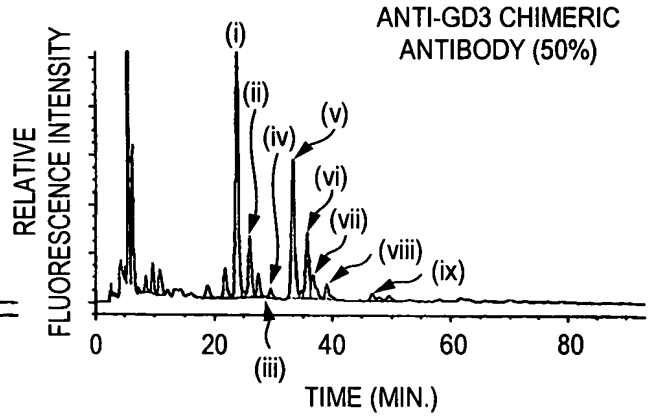

FIG. 13

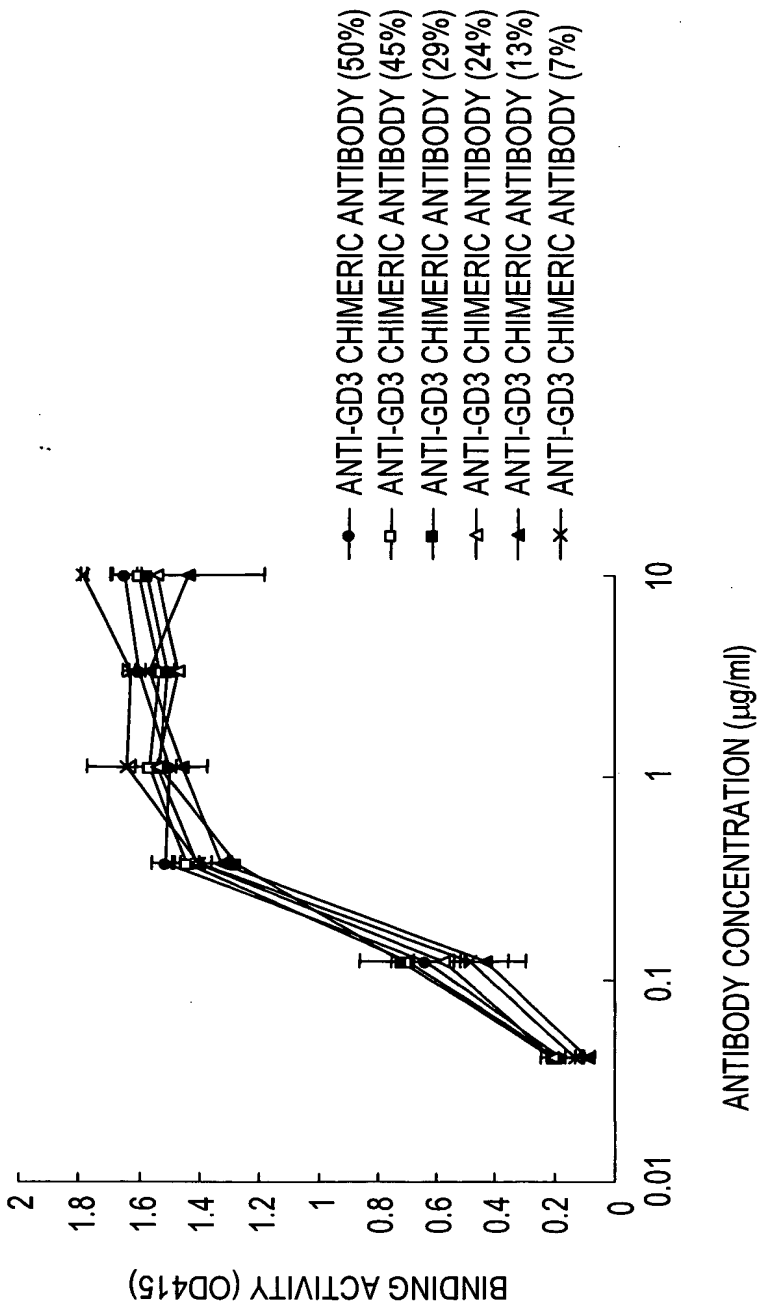
ANTI-GD3 CHIMERIC ANTIBODY (50%)
ANTI-GD3 CHIMERIC ANTIBODY (45%)
ANTI-GD3 CHIMERIC ANTIBODY (29%)
ANTI-GD3 CHIMERIC ANTIBODY (24%)
ANTI-GD3 CHIMERIC ANTIBODY (13%)
ANTI-GD3 CHIMERIC ANTIBODY (7%)
BINDING ACTIVITY (OD415)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
ANTIBODY CONCENTRATION (μg/ml)

FIG. 14

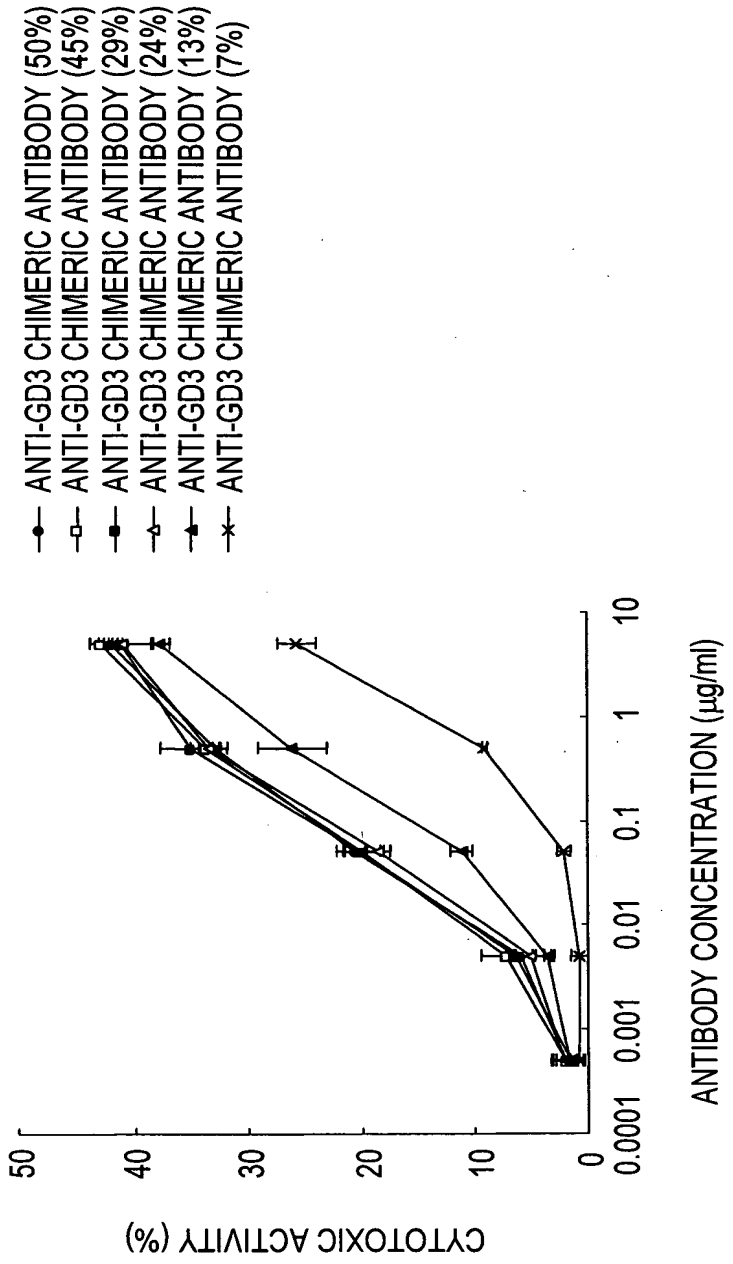
ANTI-GD3 CHIMERIC ANTIBODY (50%)
ANTI-GD3 CHIMERIC ANTIBODY (45%)
ANTI-GD3 CHIMERIC ANTIBODY (29%)
ANTI-GD3 CHIMERIC ANTIBODY (24%)
ANTI-GD3 CHIMERIC ANTIBODY (13%)
ANTI-GD3 CHIMERIC ANTIBODY (7%)
CYTOTOXIC ACTIVITY (%)
50
40
30
20
10
0
0.0001
0.001
0.01
0.1
1
10
ANTIBODY CONCENTRATION (μg/ml)

FIG. 15

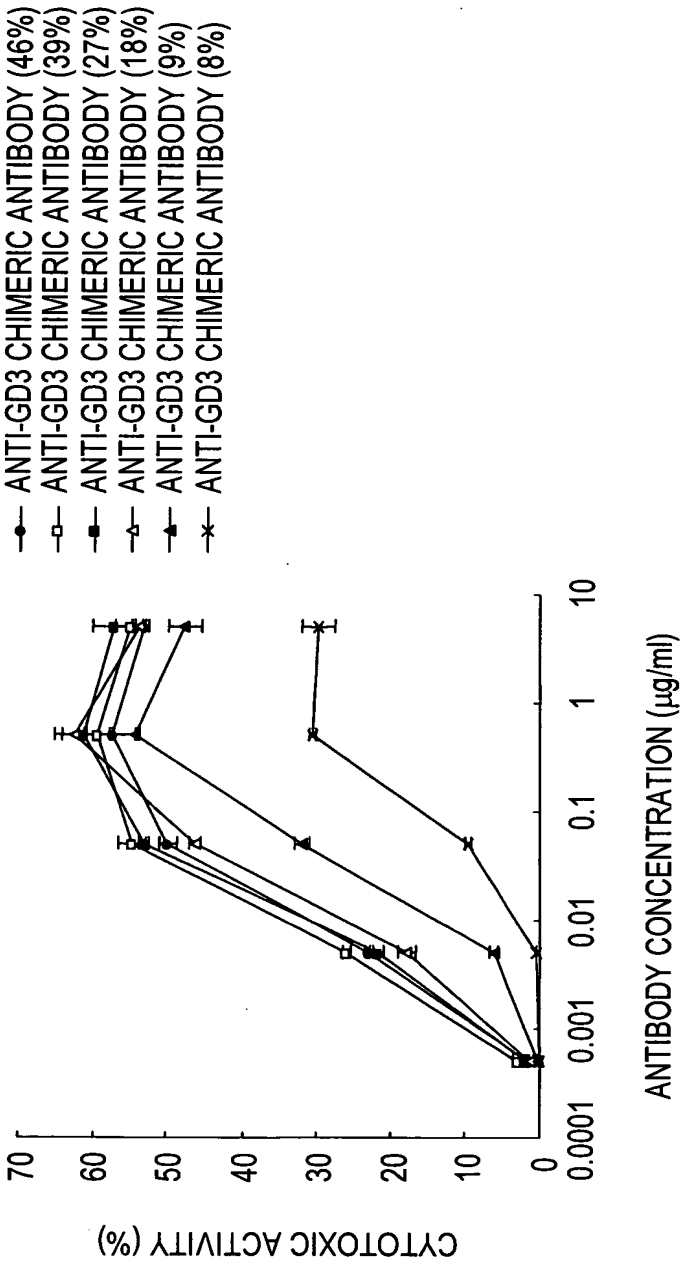
ANTI-GD3 CHIMERIC ANTIBODY (46%)
ANTI-GD3 CHIMERIC ANTIBODY (39%)
ANTI-GD3 CHIMERIC ANTIBODY (27%)
ANTI-GD3 CHIMERIC ANTIBODY (18%)
ANTI-GD3 CHIMERIC ANTIBODY (9%)
ANTI-GD3 CHIMERIC ANTIBODY (8%)
CYTOTOXIC ACTIVITY (%)
70
60
50
40
30
20
10
0
0.0001
0.001
0.01
0.1
1
10
ANTIBODY CONCENTRATION (μg/ml)

FIG. 16A

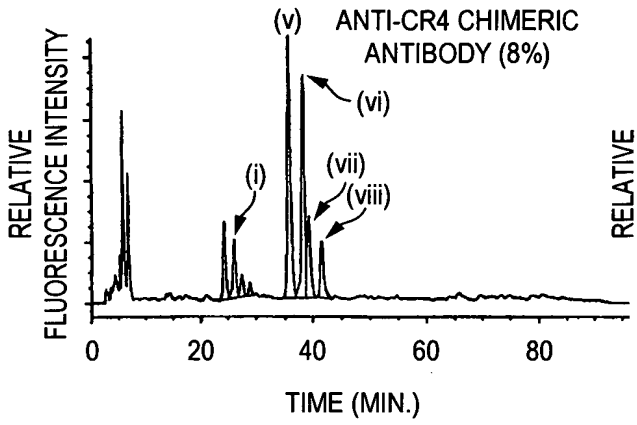

FIG. 16B

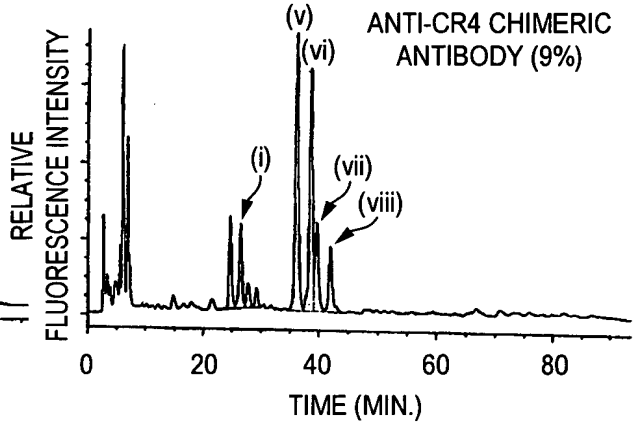

FIG. 16C

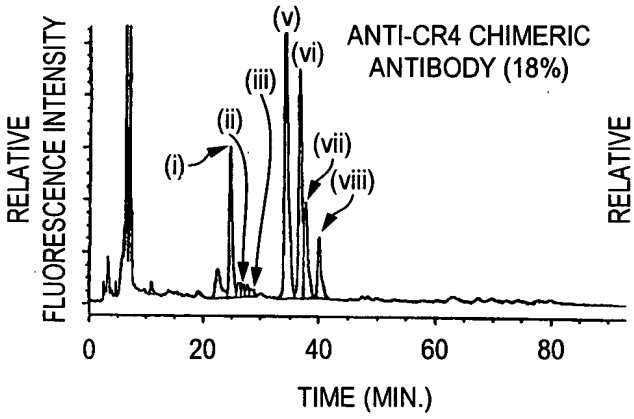

FIG. 16D

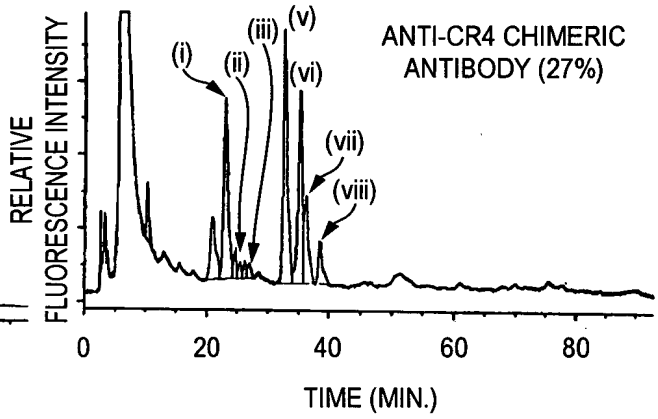

FIG. 16E

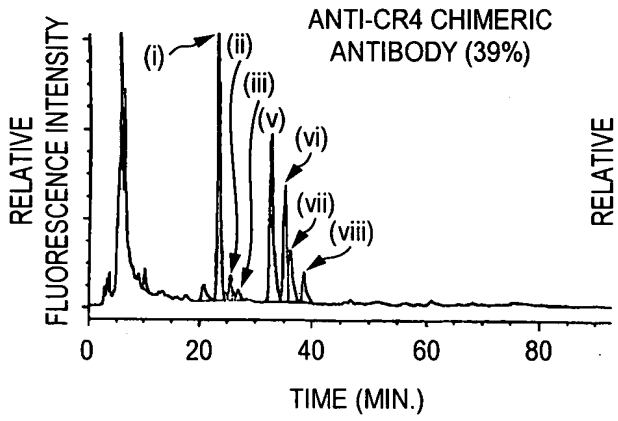

FIG. 16F

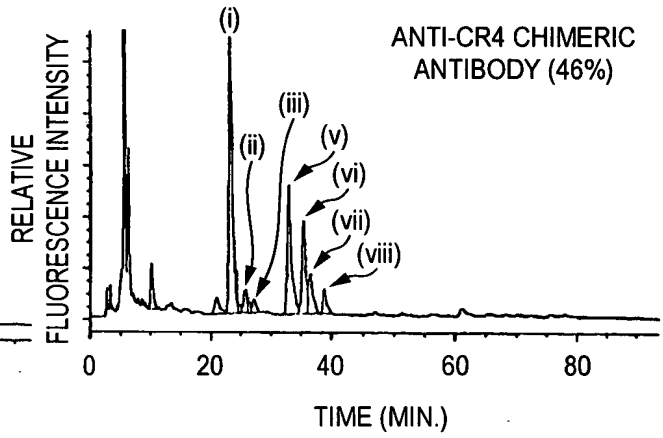

FIG. 17

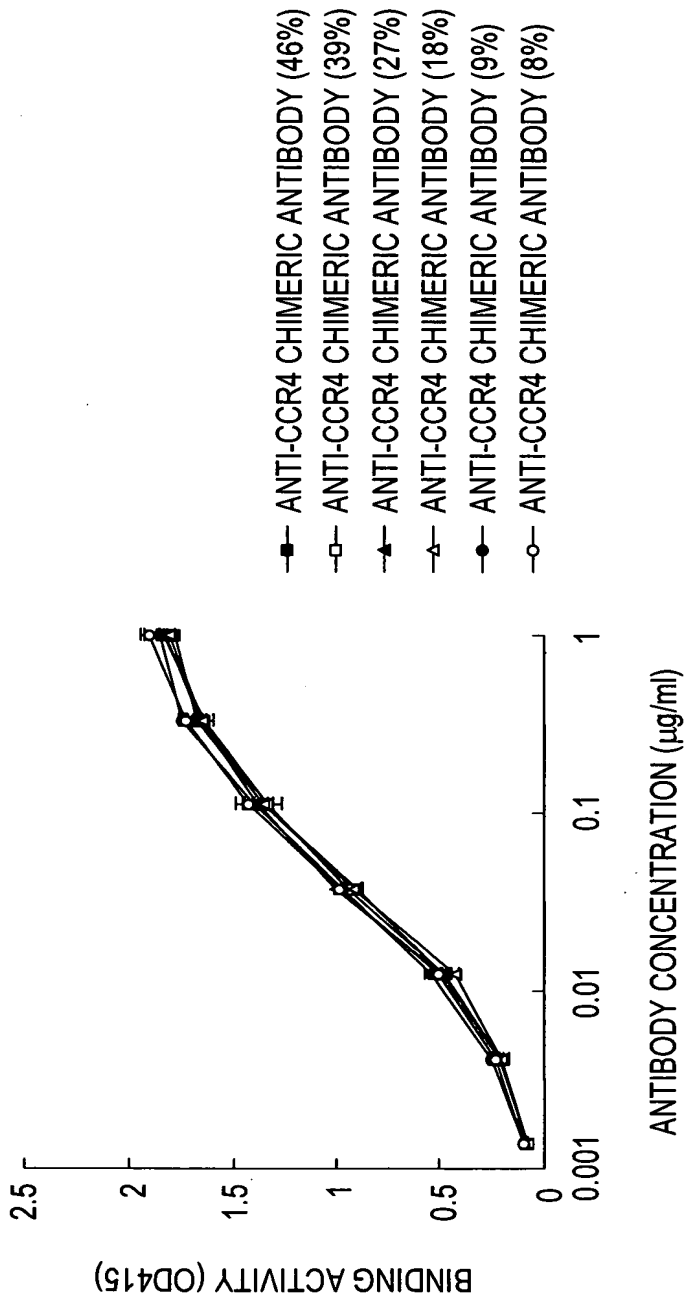
ANTI-CCR4 CHIMERIC ANTIBODY (46%)
ANTI-CCR4 CHIMERIC ANTIBODY (39%)
ANTI-CCR4 CHIMERIC ANTIBODY (27%)
ANTI-CCR4 CHIMERIC ANTIBODY (18%)
ANTI-CCR4 CHIMERIC ANTIBODY (9%)
ANTI-CCR4 CHIMERIC ANTIBODY (8%)
BINDING ACTIVITY (OD415)
2.5
2
1.5
1
0.5
0
0.001
0.01
0.1
1
ANTIBODY CONCENTRATION (μg/ml)

FIG. 18

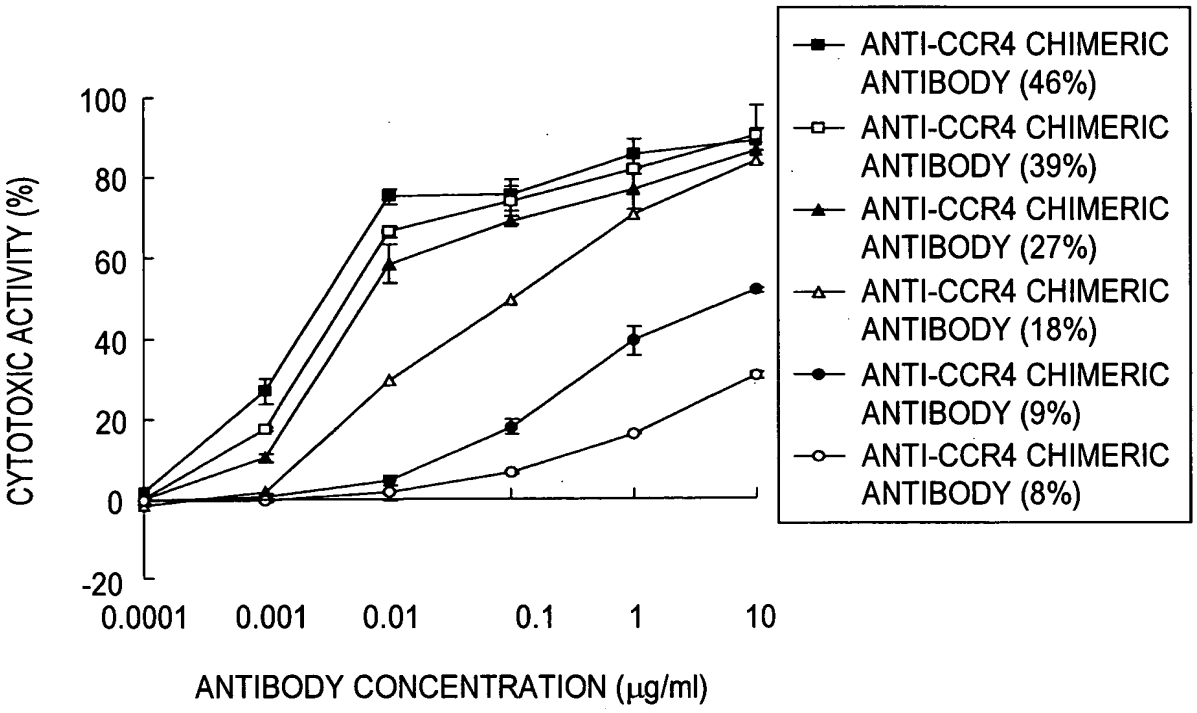

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FIG. 19

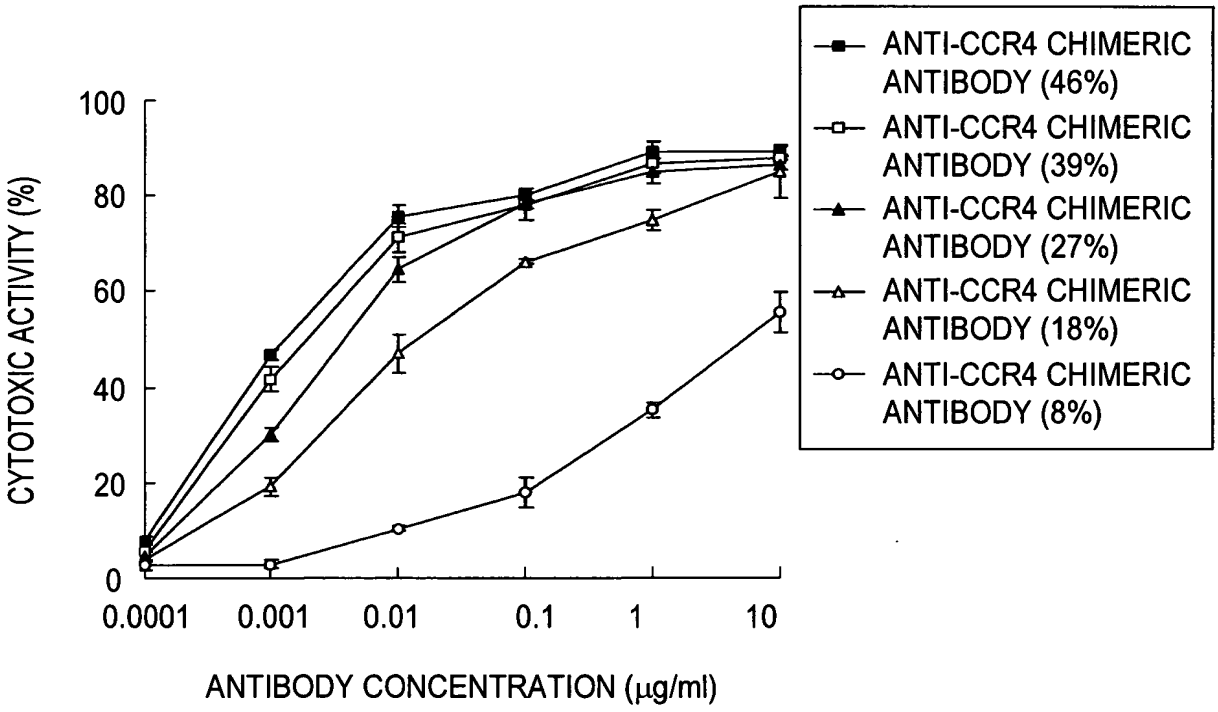
ANTI-CCR4 CHIMERIC ANTIBODY (46%)
ANTI-CCR4 CHIMERIC ANTIBODY (39%)
ANTI-CCR4 CHIMERIC ANTIBODY (27%)
ANTI-CCR4 CHIMERIC ANTIBODY (18%)
ANTI-CCR4 CHIMERIC ANTIBODY (8%)
CYTOTOXIC ACTIVITY (%)
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
10
ANTIBODY CONCENTRATION (μg/ml)

FIG. 20

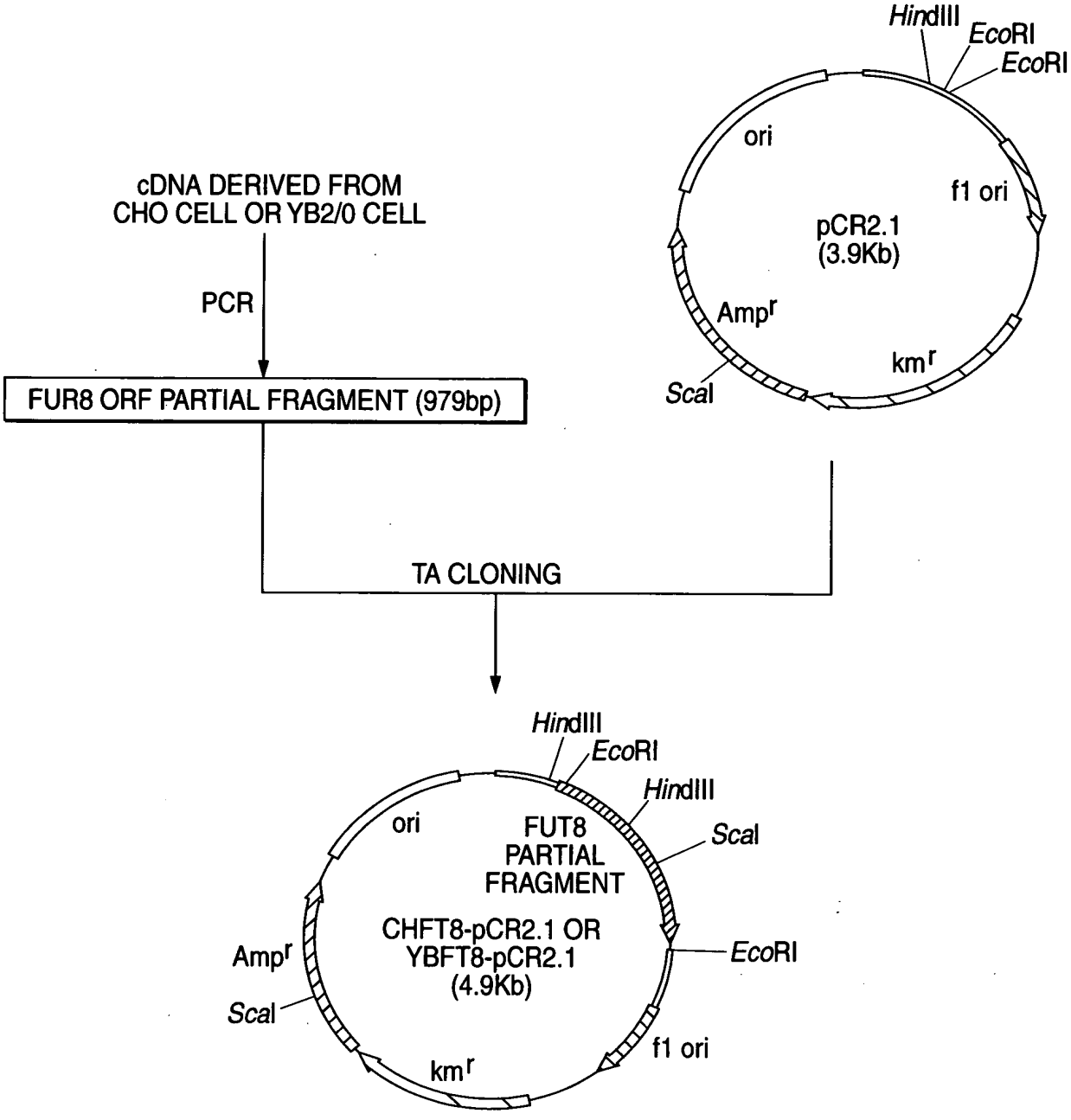
cDNA DERIVED FROM
CHO CELL OR YB2/0 CELL
PCR
FUR8 ORF PARTIAL FRAGMENT (979bp)
HindIII
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Ampr
kmr
ScaI
TA CLONING
HindIII
EcoRI
HindIII
ScaI
ori
FUT8
PARTIAL
FRAGMENT
CHFT8-pCR2.1 OR
YBFT8-pCR2.1
(4.9Kb)
EcoRI
Ampr
ScaI
kmr
f1 ori

FIG. 21

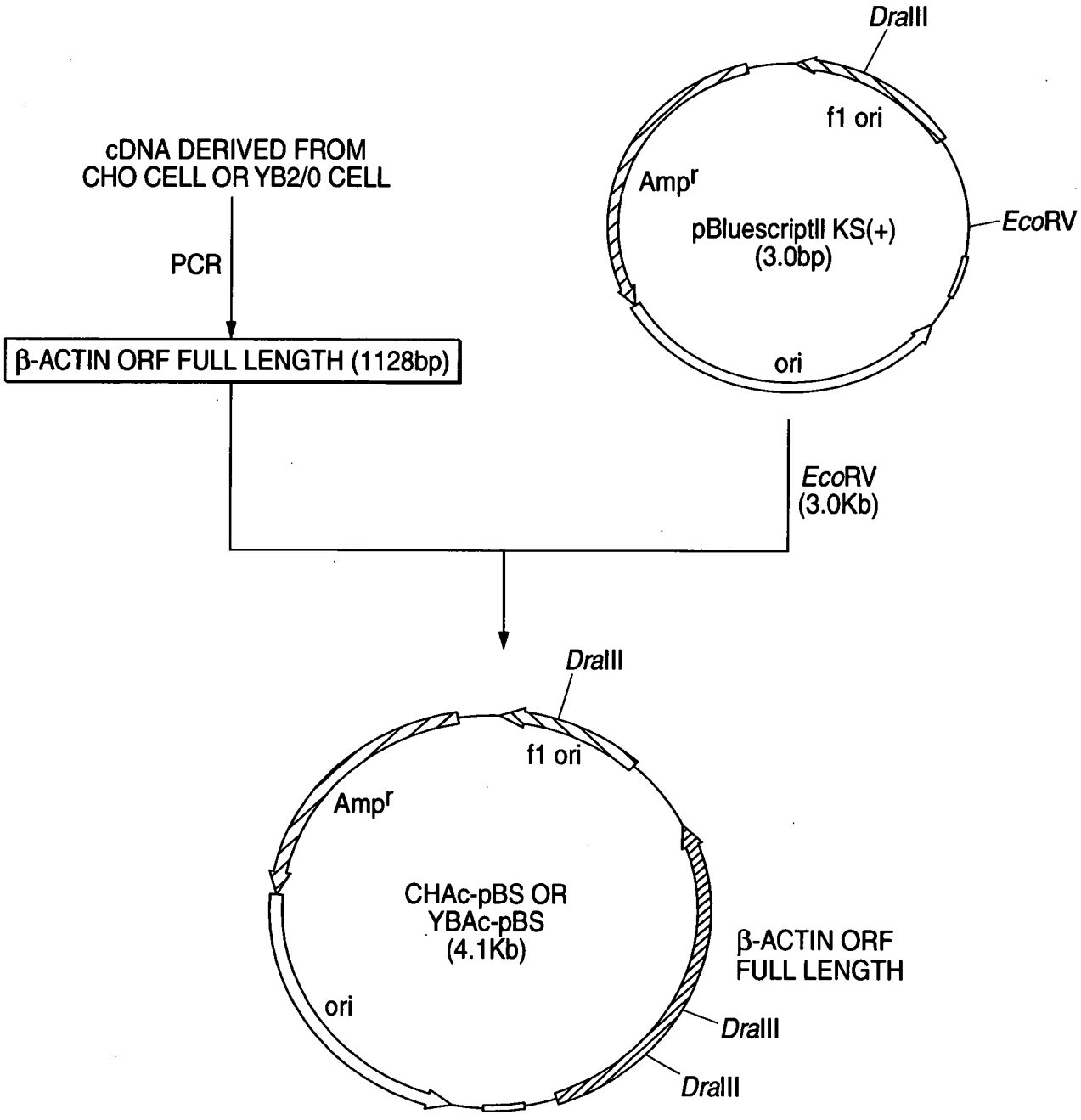
DraIII
f1 ori
Ampr
pBluescriptII KS(+)
(3.0bp)
EcoRV
ori
cDNA DERIVED FROM
CHO CELL OR YB2/0 CELL
PCR
β-ACTIN ORF FULL LENGTH (1128bp)
EcoRV
(3.0Kb)
DraIII
f1 ori
Ampr
CHAc-pBS OR
YBAc-pBS
(4.1Kb)
β-ACTIN ORF
FULL LENGTH
ori
DraIII
DraIII

FIG. 22

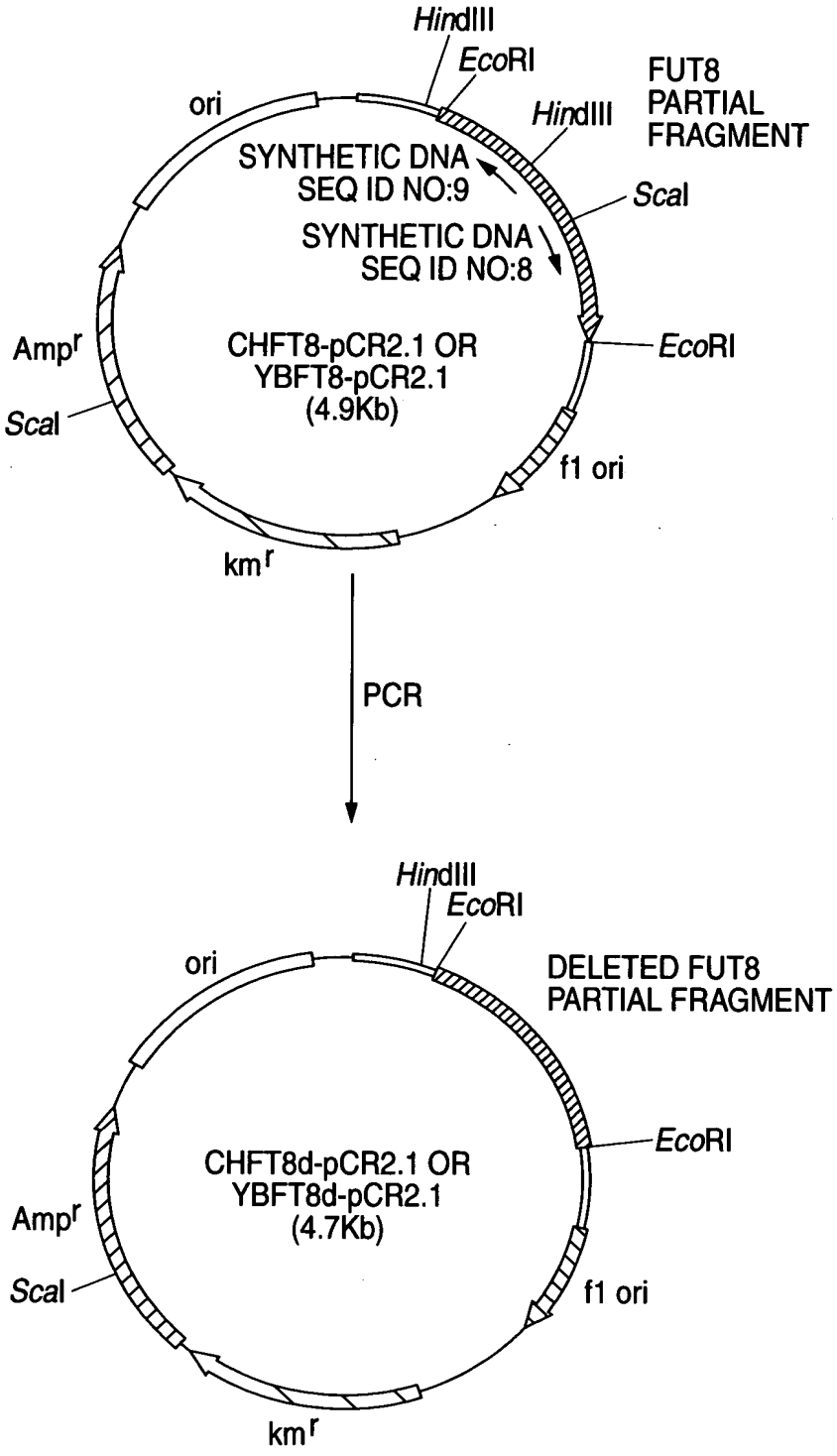
HindIII
EcoRI
FUT8
PARTIAL
FRAGMENT
ori
HindIII
SYNTHETIC DNA
SEQ ID NO:9
ScaI
SYNTHETIC DNA
SEQ ID NO:8
Ampr
CHFT8-pCR2.1 OR
YBFT8-pCR2.1
(4.9Kb)
EcoRI
ScaI
f1 ori
kmr
PCR
HindIII
EcoRI
ori
DELETED FUT8
PARTIAL FRAGMENT
EcoRI
CHFT8d-pCR2.1 OR
YBFT8d-pCR2.1
(4.7Kb)
Ampr
ScaI
f1 ori
kmr

FIG. 23

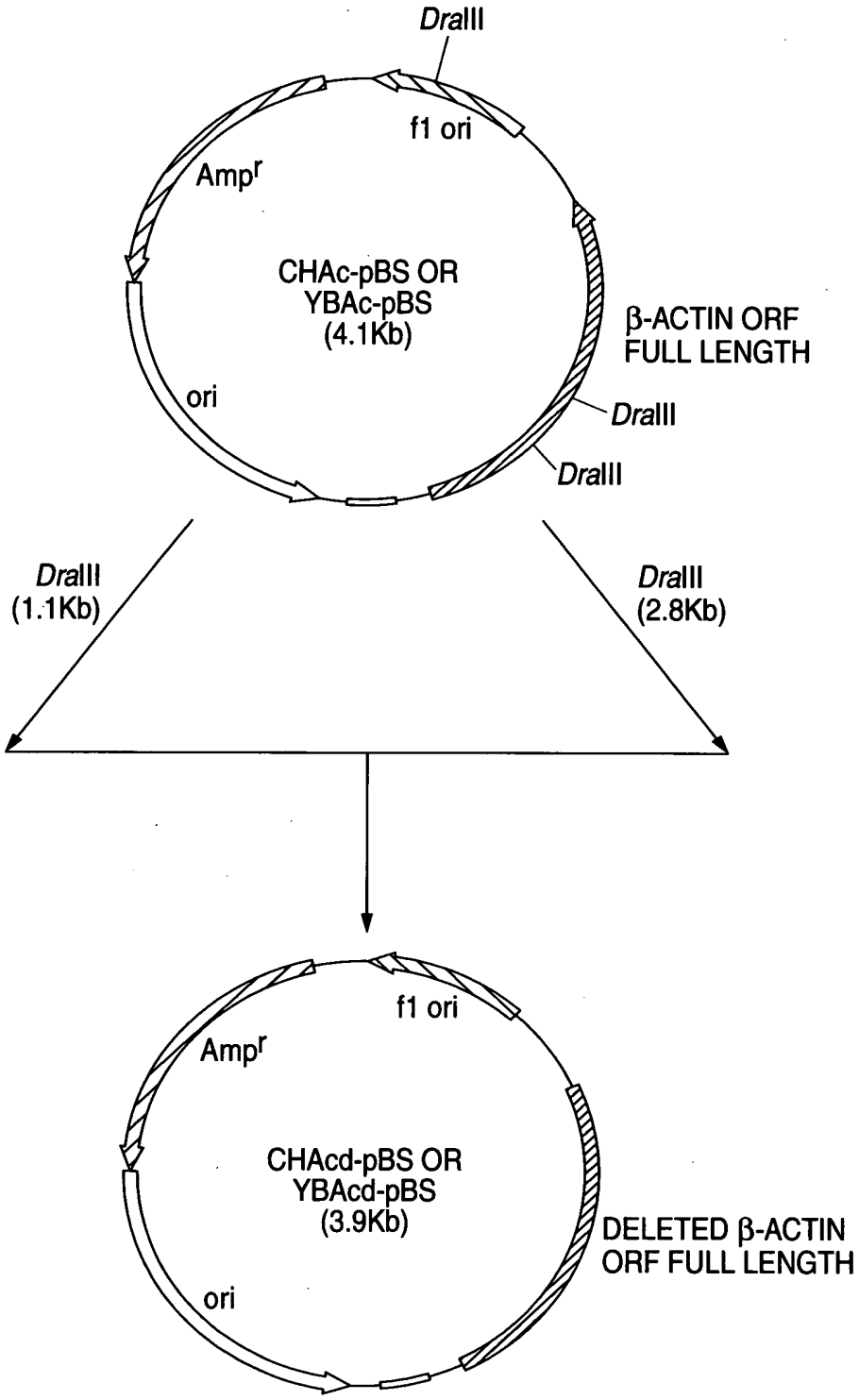
DraIII
f1 ori
Ampr
CHAc-pBS OR
YBAc-pBS
(4.1Kb)
β-ACTIN ORF
FULL LENGTH
ori
DraIII
DraIII
DraIII
(1.1Kb)
DraIII
(2.8Kb)
f1 ori
Ampr
CHAcd-pBS OR
YBAcd-pBS
(3.9Kb)
DELETED β-ACTIN
ORF FULL LENGTH
ori

FIG. 24

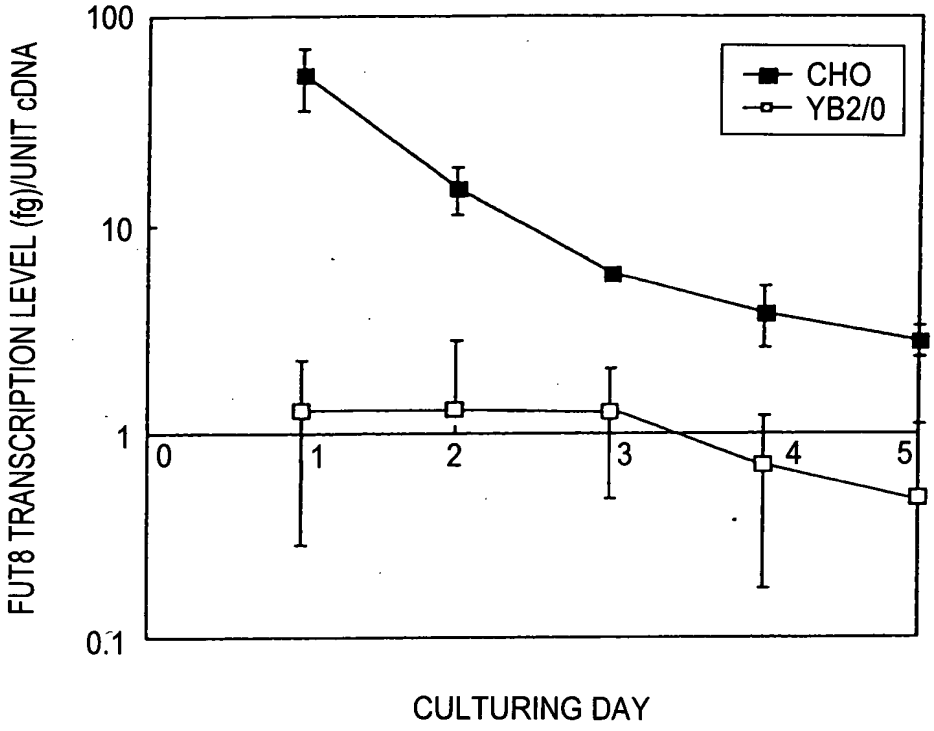
100
10
1
0.1
0
1
2
3
4
5
CHO
YB2/0
FUT8 TRANSCRIPTION LEVEL (fg)/UNIT cDNA
CULTURING DAY

FIG. 25

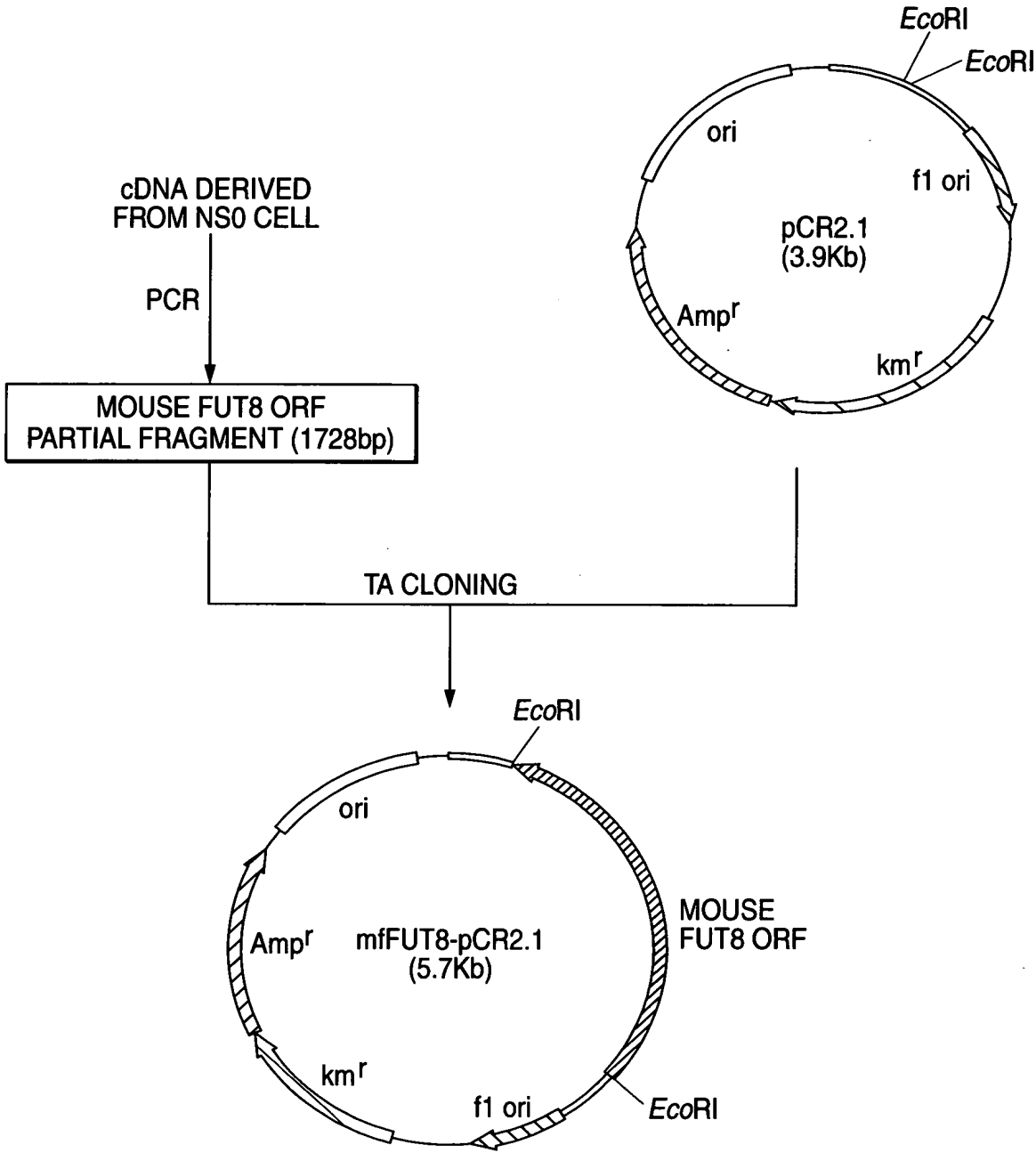
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Amp^r
km^r
cDNA DERIVED
FROM NS0 CELL
PCR
MOUSE FUT8 ORF
PARTIAL FRAGMENT (1728bp)
TA CLONING
EcoRI
ori
MOUSE
FUT8 ORF
Amp^r
mfFUT8-pCR2.1
(5.7Kb)
km^r
f1 ori
EcoRI

FIG. 26

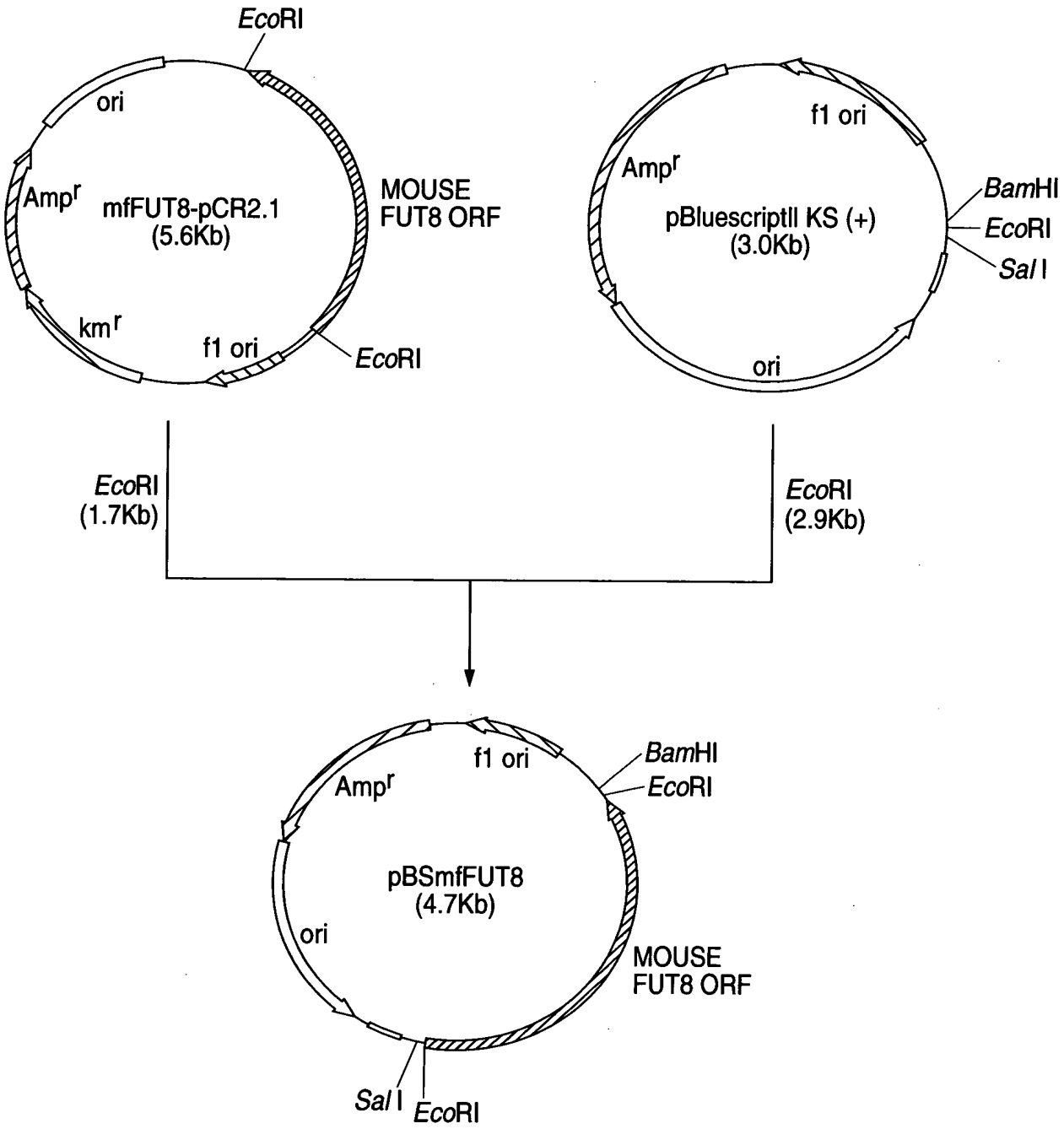
EcoRI
ori
Ampr
mfFUT8-pCR2.1
(5.6Kb)
MOUSE
FUT8 ORF
kmr
f1 ori
EcoRI
f1 ori
Ampr
pBluescriptII KS (+)
(3.0Kb)
BamHI
EcoRI
SalI
ori
EcoRI
(1.7Kb)
EcoRI
(2.9Kb)
f1 ori
BamHI
Ampr
EcoRI
pBSmfFUT8
(4.7Kb)
ori
MOUSE
FUT8 ORF
SalI
EcoRI

FIG. 27

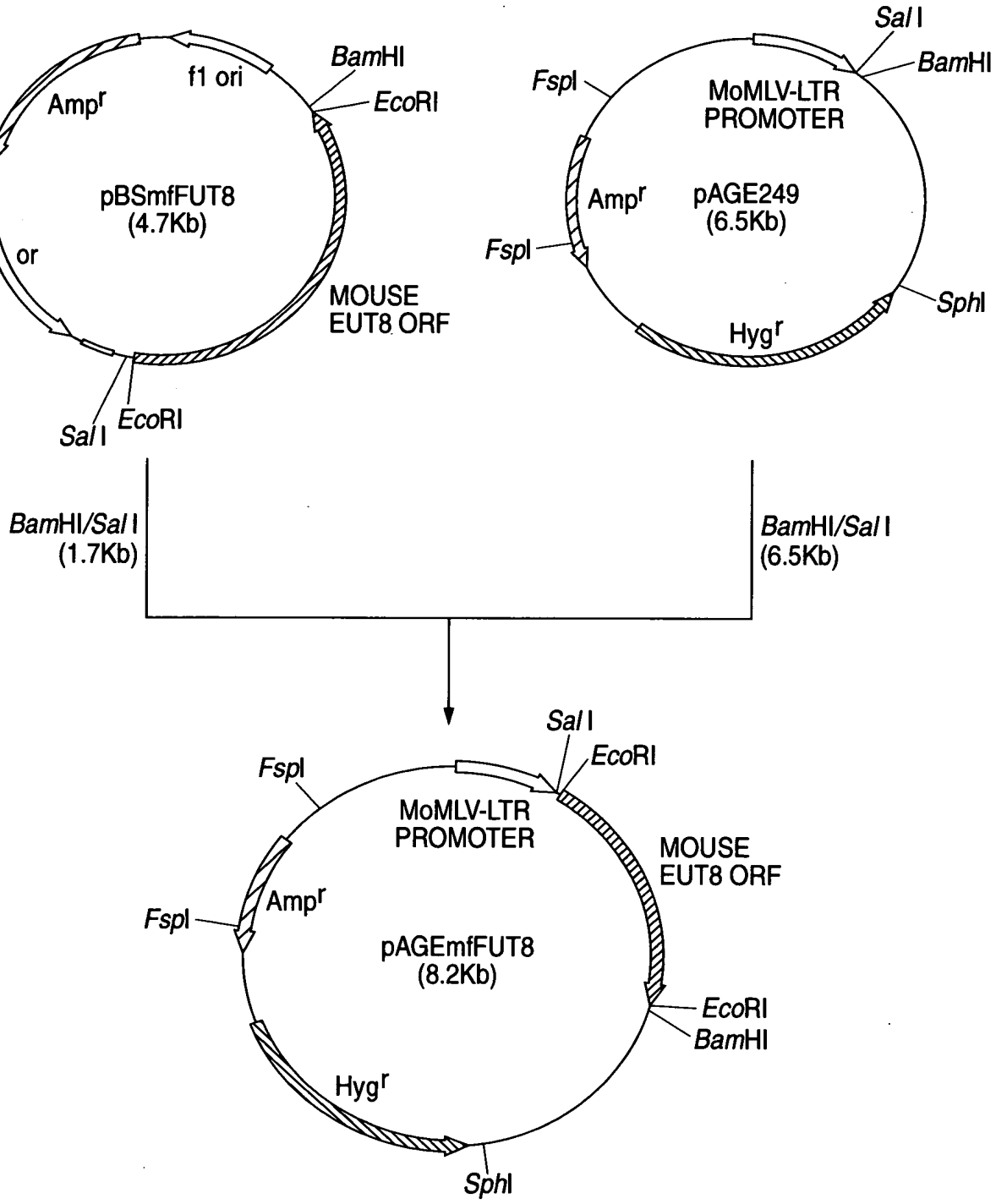
BamHI
f1 ori
Ampr
EcoRI
pBSmfFUT8
(4.7Kb)
or
MOUSE
EUT8 ORF
SalI
EcoRI
SalI
BamHI
FspI
MoMLV-LTR
PROMOTER
Ampr
pAGE249
(6.5Kb)
FspI
SphI
Hygr
BamHI/SalI
(1.7Kb)
BamHI/SalI
(6.5Kb)
SalI
EcoRI
FspI
MoMLV-LTR
PROMOTER
MOUSE
EUT8 ORF
Ampr
FspI
pAGEmfFUT8
(8.2Kb)
EcoRI
BamHI
Hygr
SphI

FIG. 28

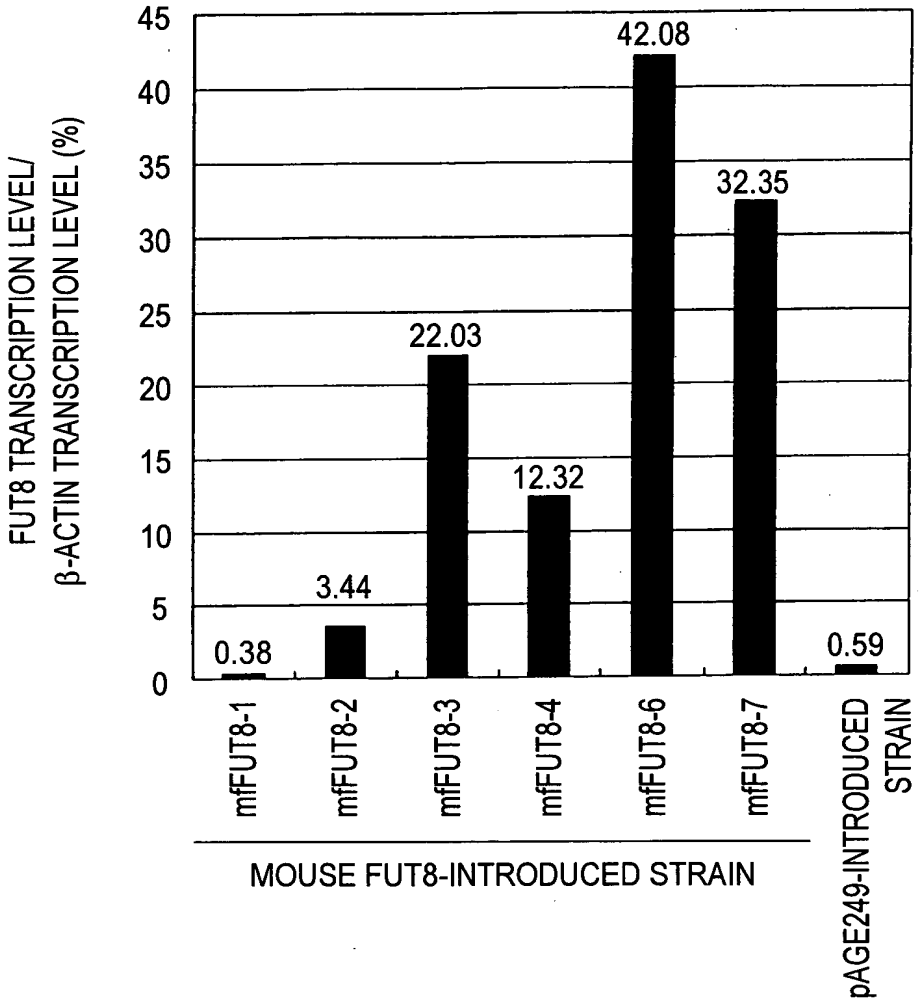

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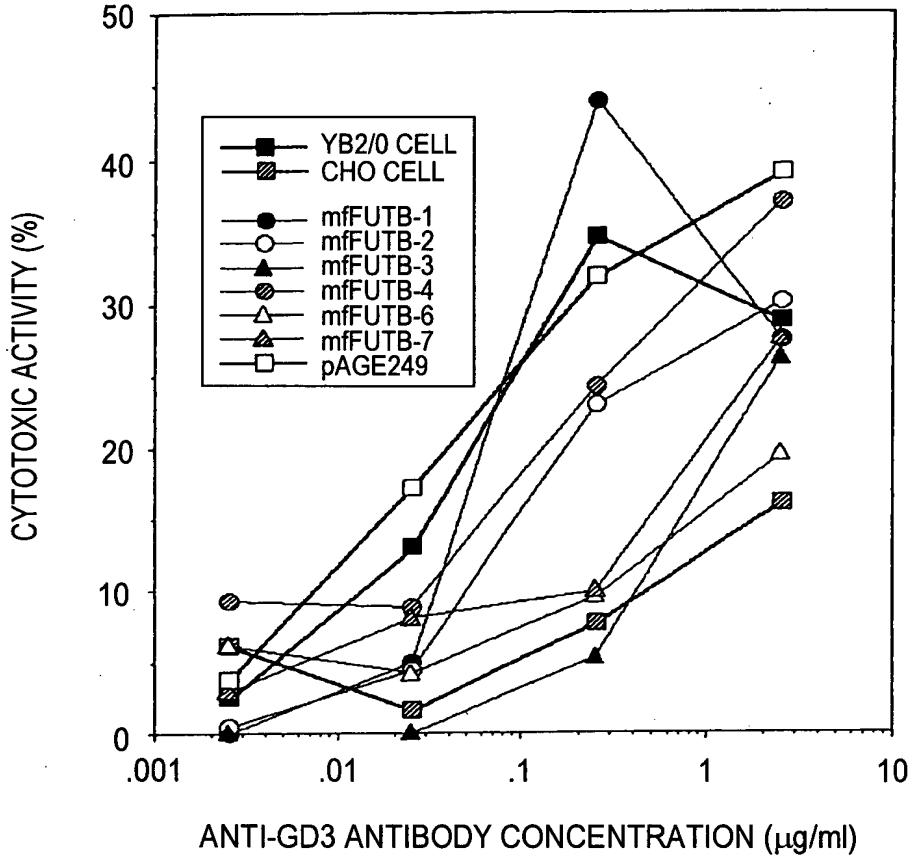
FIG. 29


50
40
30
20
10
0
.001
.01
.1
1
10
CYTOTOXIC ACTIVITY (%)
ANTI-GD3 ANTIBODY CONCENTRATION (μg/ml)
YB2/0 CELL
CHO CELL
mfFUTB-1
mfFUTB-2
mfFUTB-3
mfFUTB-4
mfFUTB-6
mfFUTB-7
pAGE249

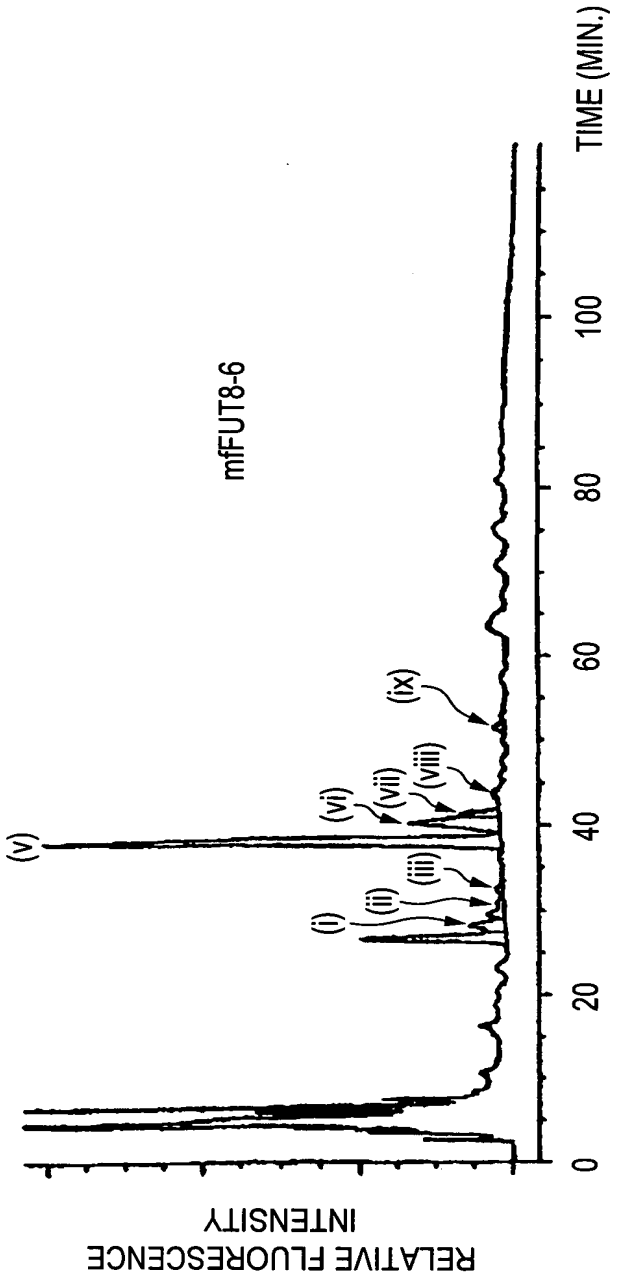


FIG. 30A

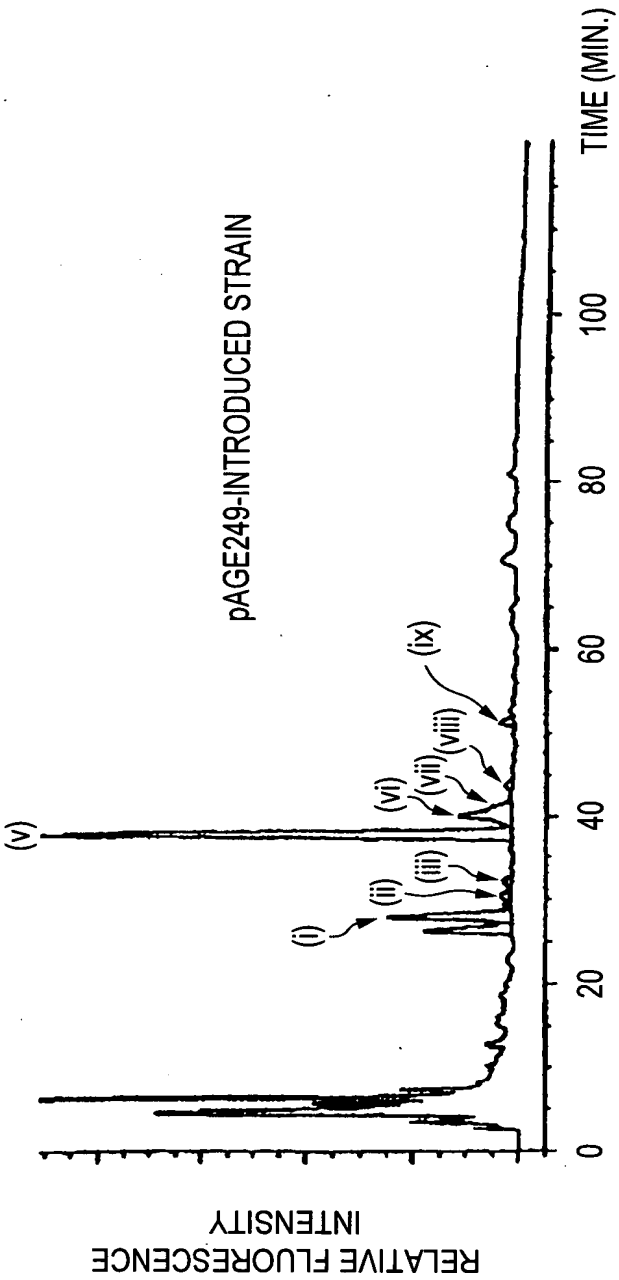


FIG. 30B

FIG. 31

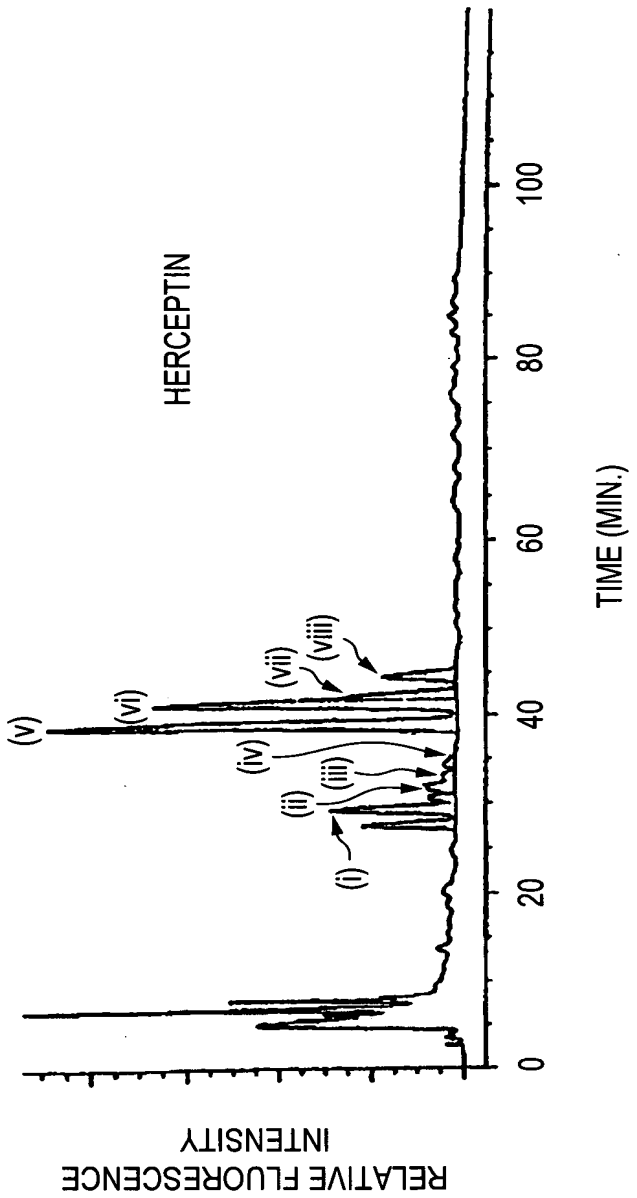


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FIG. 32

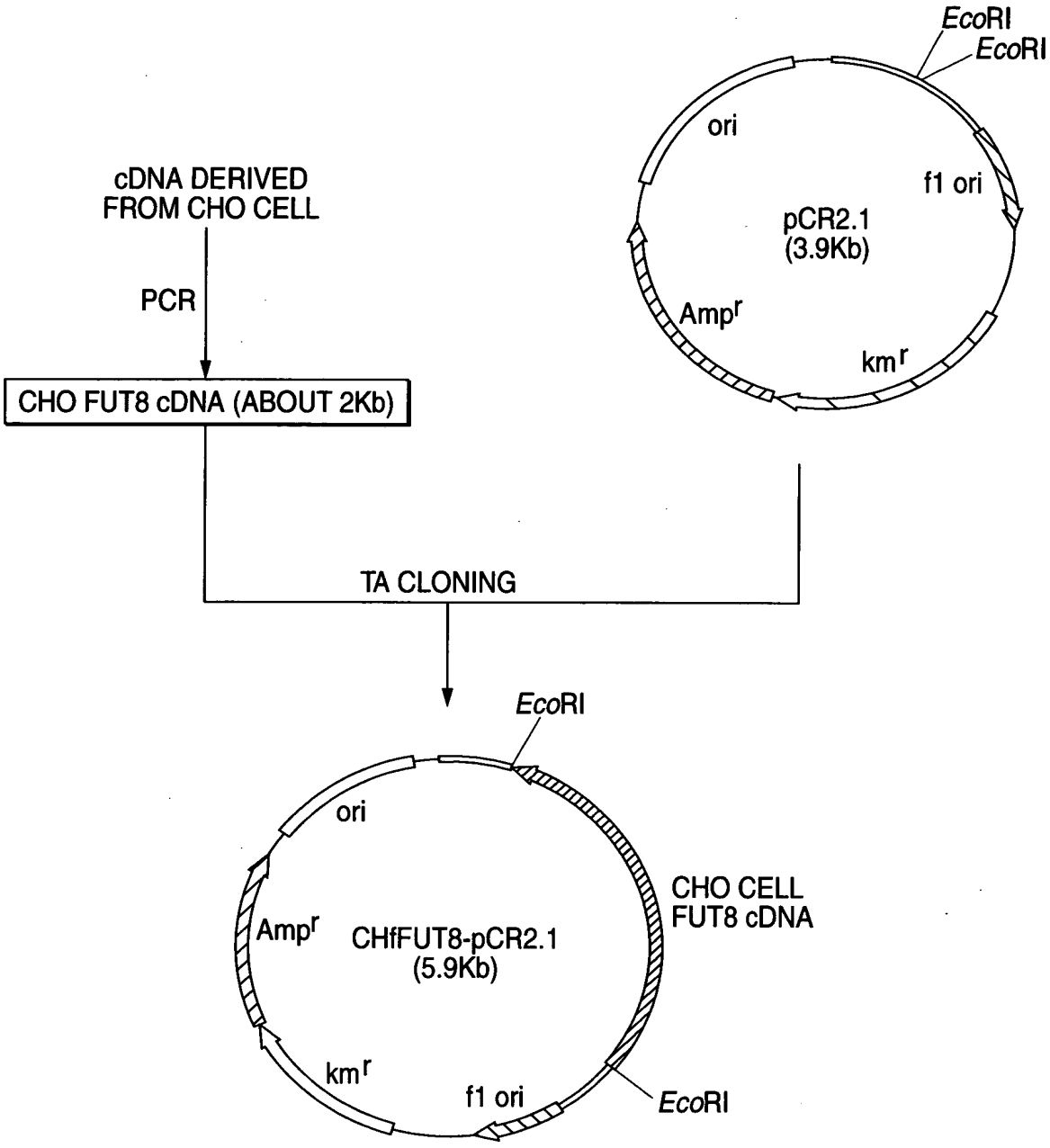
EcoRI
EcoRI
ori
f1 ori
pCR2.1
(3.9Kb)
Ampr
kmr
cDNA DERIVED
FROM CHO CELL
PCR
CHO FUT8 cDNA (ABOUT 2Kb)
TA CLONING
EcoRI
ori
CHO CELL
FUT8 cDNA
Ampr
CHfFUT8-pCR2.1
(5.9Kb)
kmr
f1 ori
EcoRI

FIG. 33

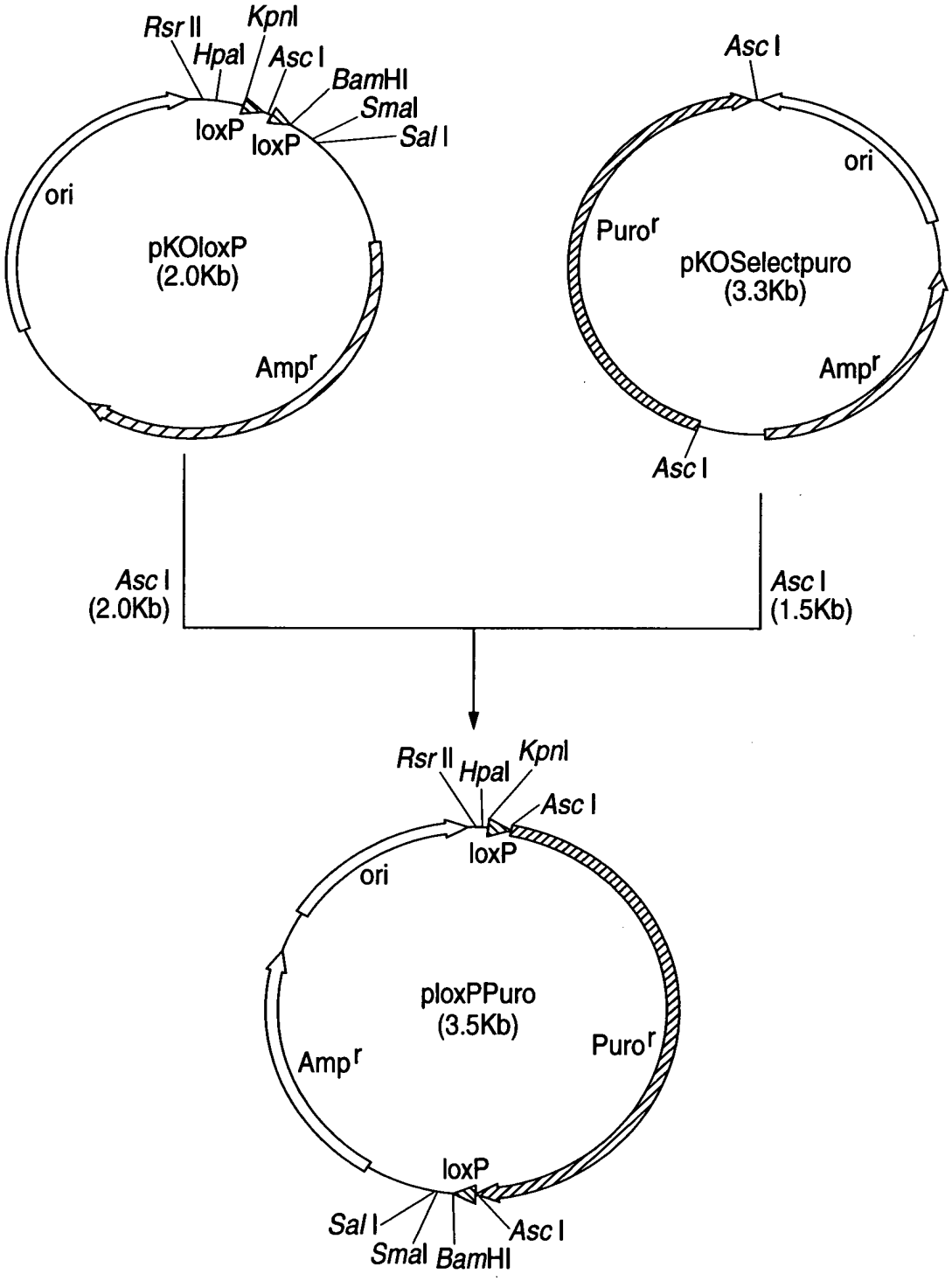
Rsr II
KpnI
HpaI
Asc I
BamHI
SmaI
SalI
loxP
loxP
ori
pKOloxP
(2.0Kb)
Ampr
Asc I
ori
Puror
pKOSelectpuro
(3.3Kb)
Ampr
Asc I
Asc I
(2.0Kb)
Asc I
(1.5Kb)
Rsr II
HpaI
KpnI
Asc I
loxP
ori
ploxPPuro
(3.5Kb)
Puror
Ampr
loxP
SalI
SmaI
BamHI
Asc I

FIG. 34

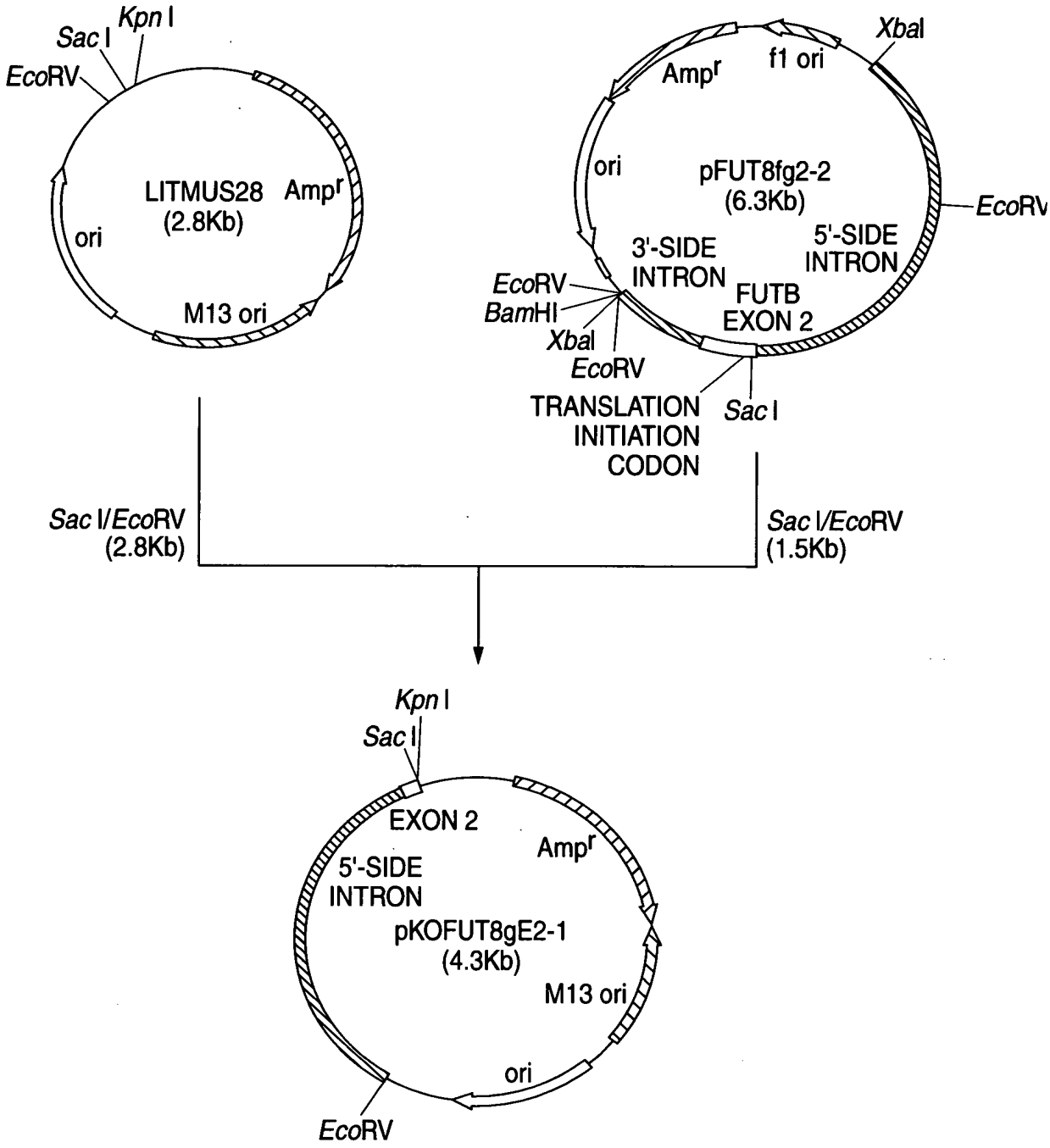
Kpn I
Sac I
EcoRV
LITMUS28
(2.8Kb)
Ampr
ori
M13 ori
XbaI
Ampr
f1 ori
ori
pFUT8fg2-2
(6.3Kb)
EcoRV
3'-SIDE
INTRON
5'-SIDE
INTRON
EcoRV
BamHI
XbaI
EcoRV
FUTB
EXON 2
TRANSLATION
INITIATION
CODON
Sac I
Sac I/EcoRV
(2.8Kb)
Sac I/EcoRV
(1.5Kb)
Kpn I
Sac I
EXON 2
Ampr
5'-SIDE
INTRON
pKOFUT8gE2-1
(4.3Kb)
M13 ori
ori
EcoRV

FIG. 35

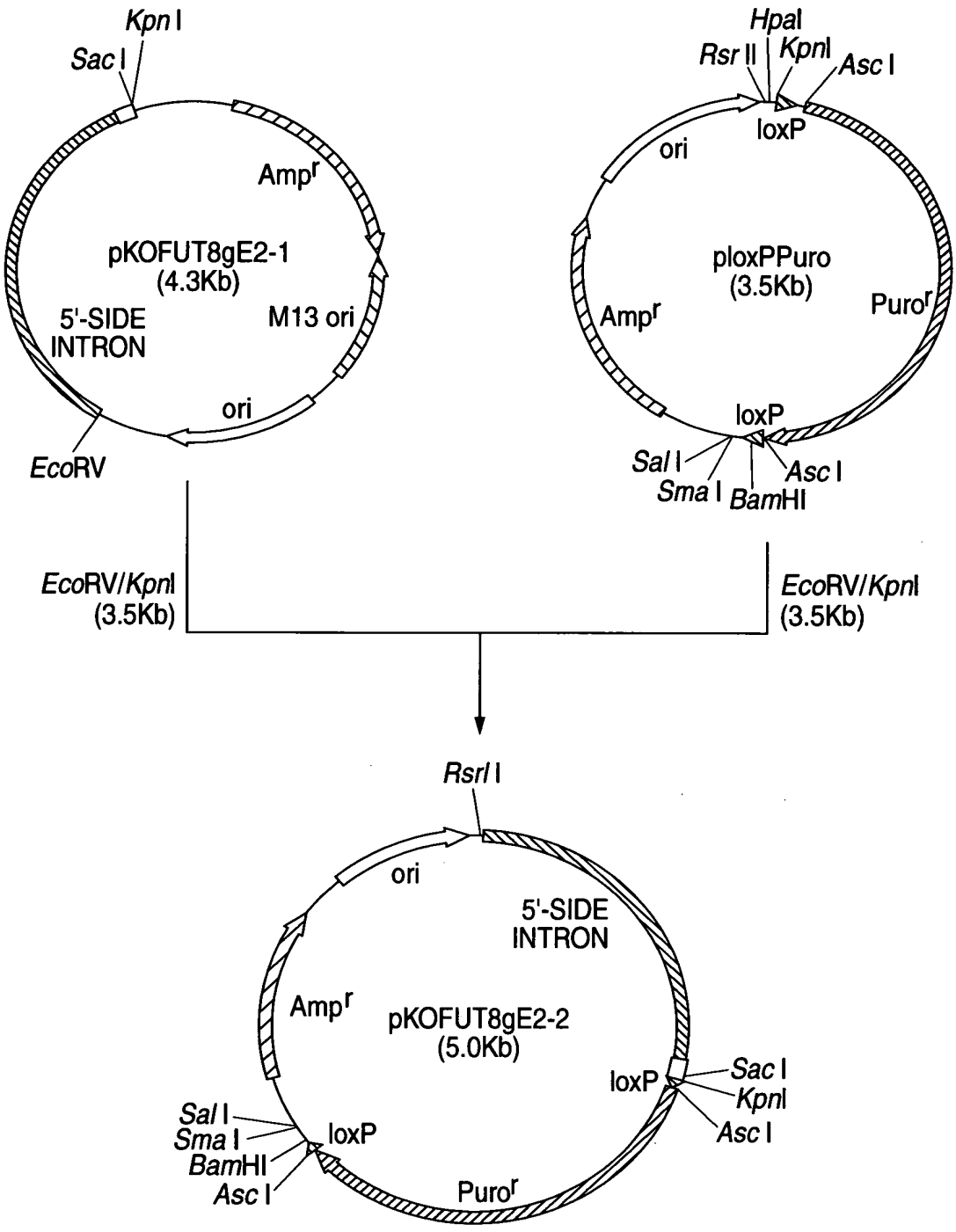
Kpn I
Sac I
Amp^r
pKOFUT8gE2-1
(4.3Kb)
5'-SIDE
INTRON
M13 ori
ori
EcoRV
HpaI
Rsr II
KpnI
Asc I
loxP
ori
ploxPPuro
(3.5Kb)
Amp^r
Puro^r
loxP
Sal I
Sma I
BamHI
Asc I
EcoRV/KpnI
(3.5Kb)
EcoRV/KpnI
(3.5Kb)
RsrI I
ori
5'-SIDE
INTRON
Amp^r
pKOFUT8gE2-2
(5.0Kb)
loxP
Sac I
KpnI
Asc I
Sal I
Sma I
BamHI
Asc I
loxP
Puro^r

FIG. 36

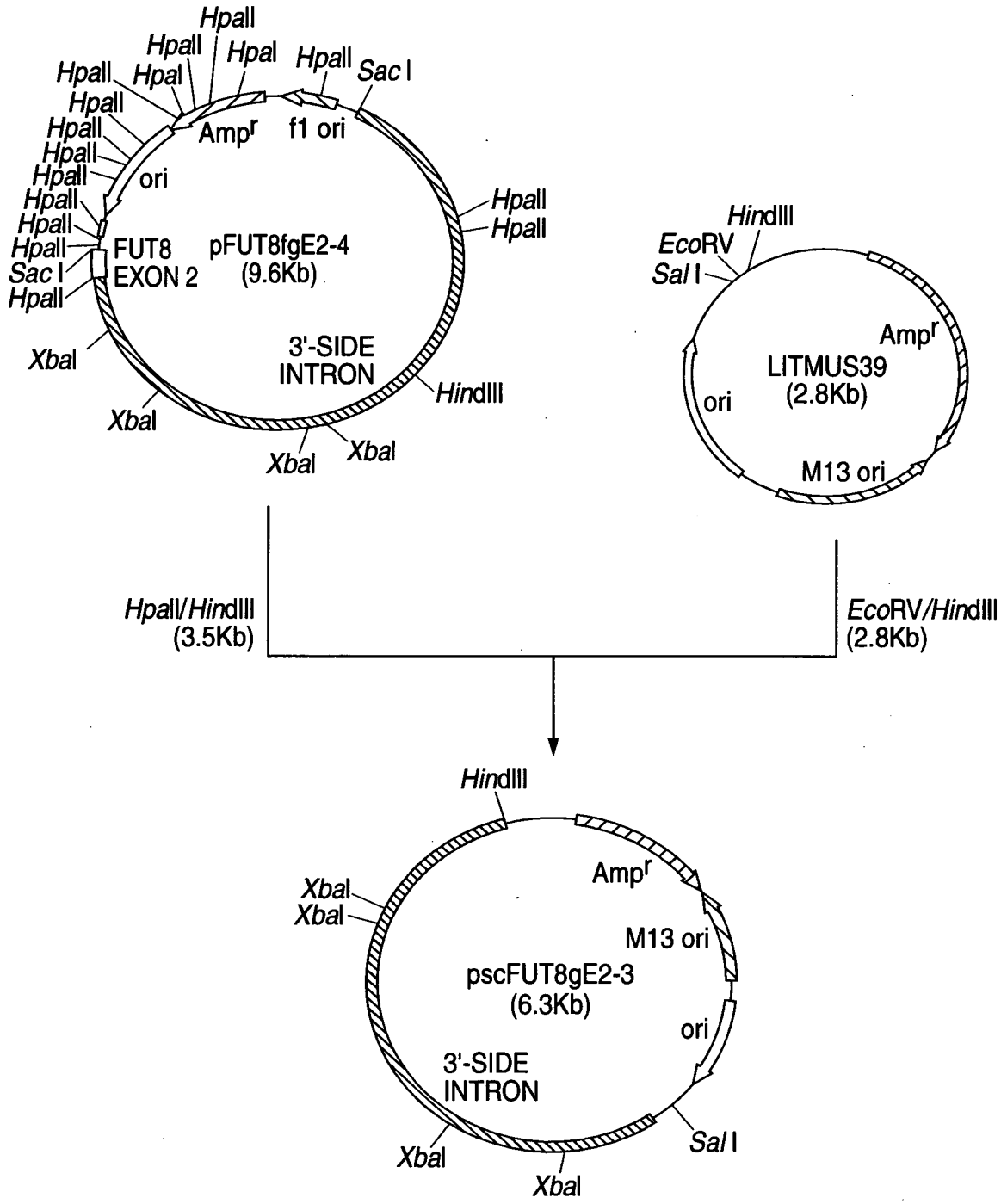
HpaII
HpaII
HpaI
HpaII
SacI
HpaII
HpaI
HpaII
Ampr
f1 ori
HpaII
HpaII
ori
HpaII
HpaII
HpaII
FUT8
EXON 2
pFUT8fgE2-4
(9.6Kb)
SacI
HpaII
HpaII
HpaII
XbaI
3'-SIDE
INTRON
HindIII
XbaI
XbaI
XbaI
HindIII
EcoRV
SalI
Ampr
LITMUS39
(2.8Kb)
ori
M13 ori
HpaII/HindIII
(3.5Kb)
EcoRV/HindIII
(2.8Kb)
HindIII
Ampr
XbaI
XbaI
M13 ori
pscFUT8gE2-3
(6.3Kb)
ori
3'-SIDE
INTRON
SalI
XbaI
XbaI

FIG. 37

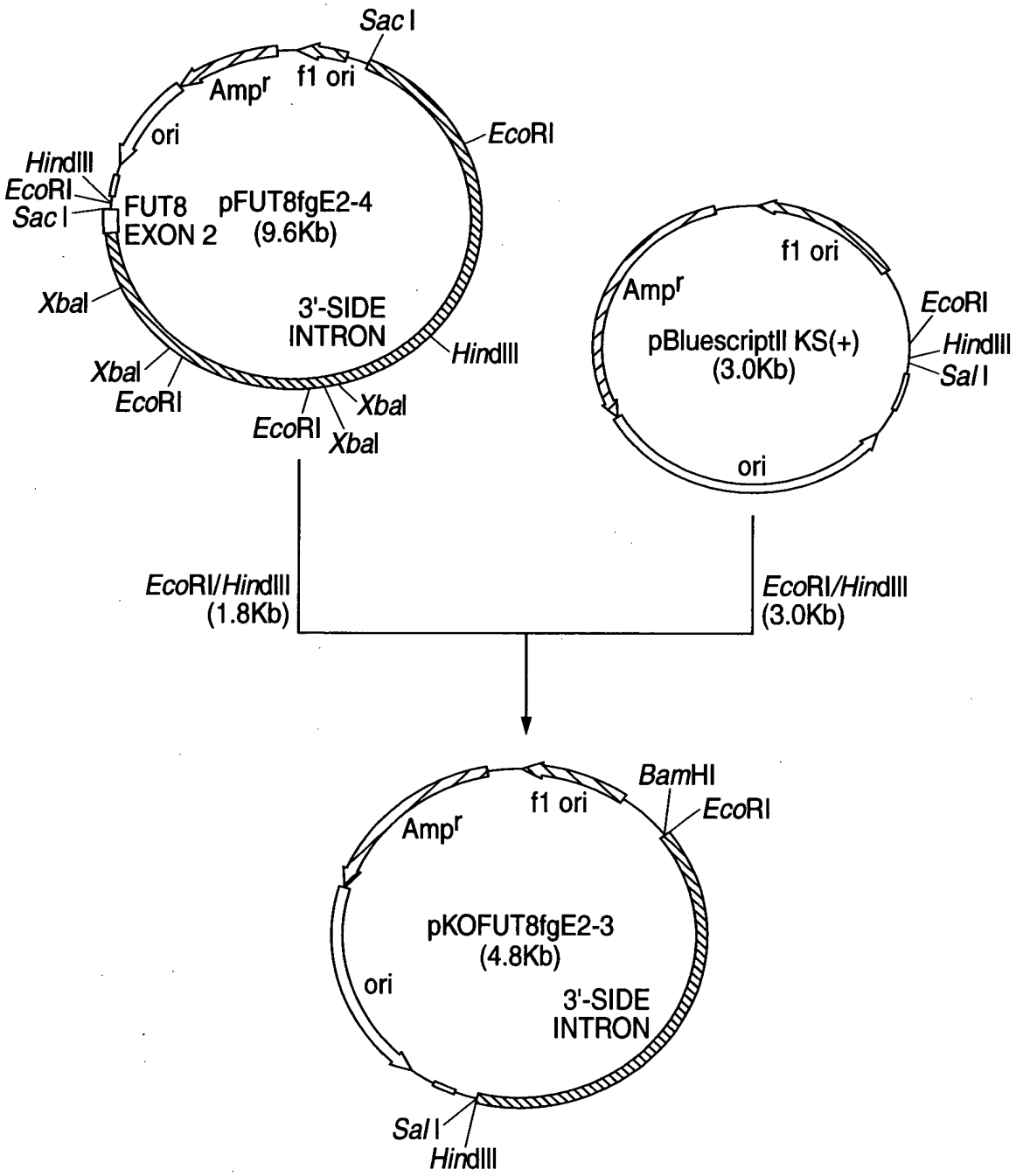
Sac I
Ampr
f1 ori
EcoRI
ori
HindIII
EcoRI
Sac I
FUT8 EXON 2
pFUT8fgE2-4
(9.6Kb)
XbaI
3'-SIDE
INTRON
HindIII
XbaI
EcoRI
EcoRI
XbaI
f1 ori
Ampr
EcoRI
HindIII
SalI
pBluescriptII KS(+)
(3.0Kb)
ori
EcoRI/HindIII
(1.8Kb)
EcoRI/HindIII
(3.0Kb)
BamHI
f1 ori
EcoRI
Ampr
pKOFUT8fgE2-3
(4.8Kb)
ori
3'-SIDE
INTRON
SalI
HindIII

FIG. 38

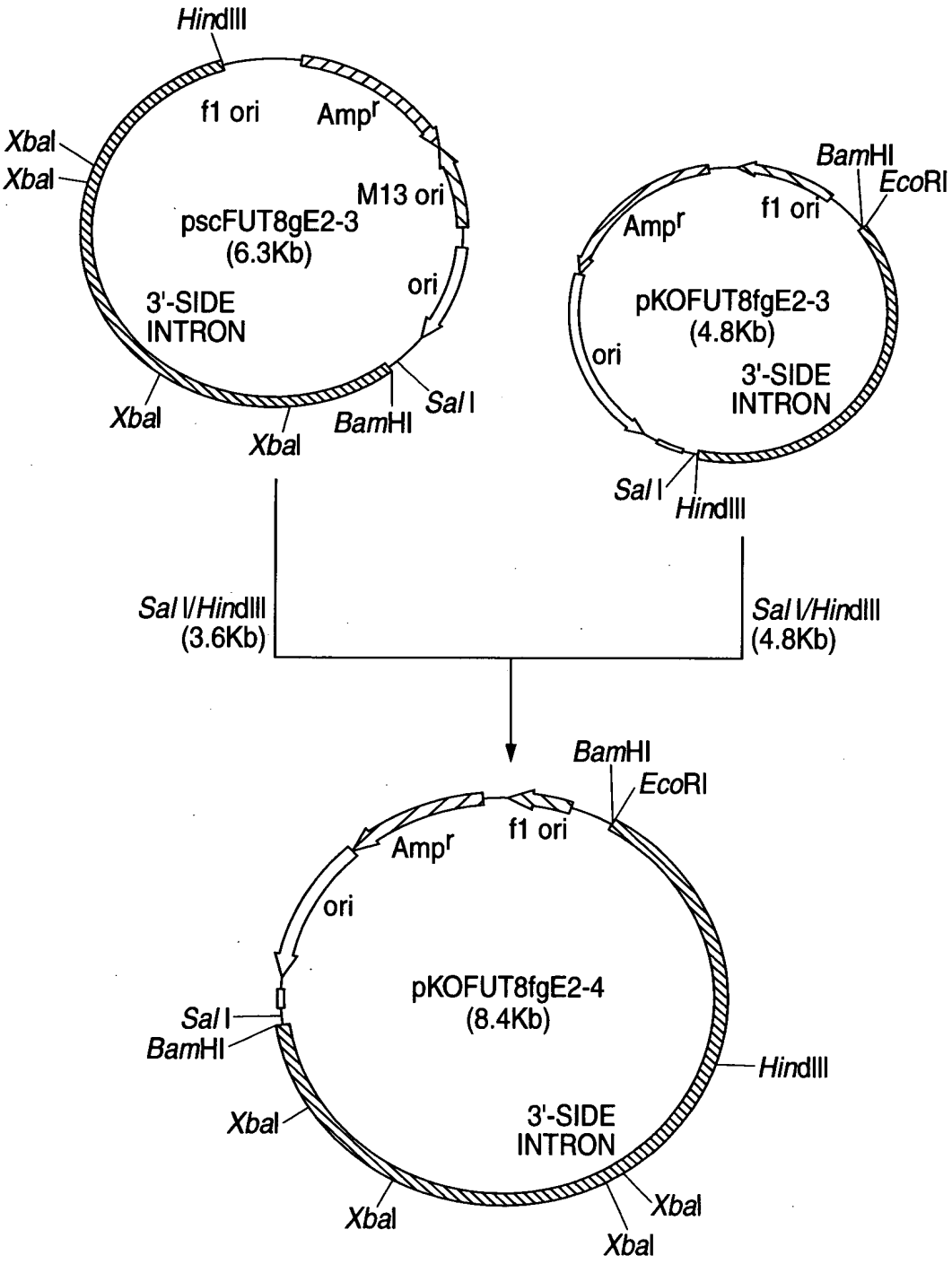
HindIII
f1 ori
Ampr
XbaI
XbaI
M13 ori
pscFUT8gE2-3
(6.3Kb)
ori
3'-SIDE
INTRON
XbaI
XbaI
BamHI
SalI
BamHI
EcoRI
f1 ori
Ampr
pKOFUT8fgE2-3
(4.8Kb)
ori
3'-SIDE
INTRON
SalI
HindIII
SalI/HindIII
(3.6Kb)
SalI/HindIII
(4.8Kb)
BamHI
EcoRI
f1 ori
Ampr
ori
pKOFUT8fgE2-4
(8.4Kb)
SalI
BamHI
HindIII
XbaI
3'-SIDE
INTRON
XbaI
XbaI
XbaI

FIG. 39

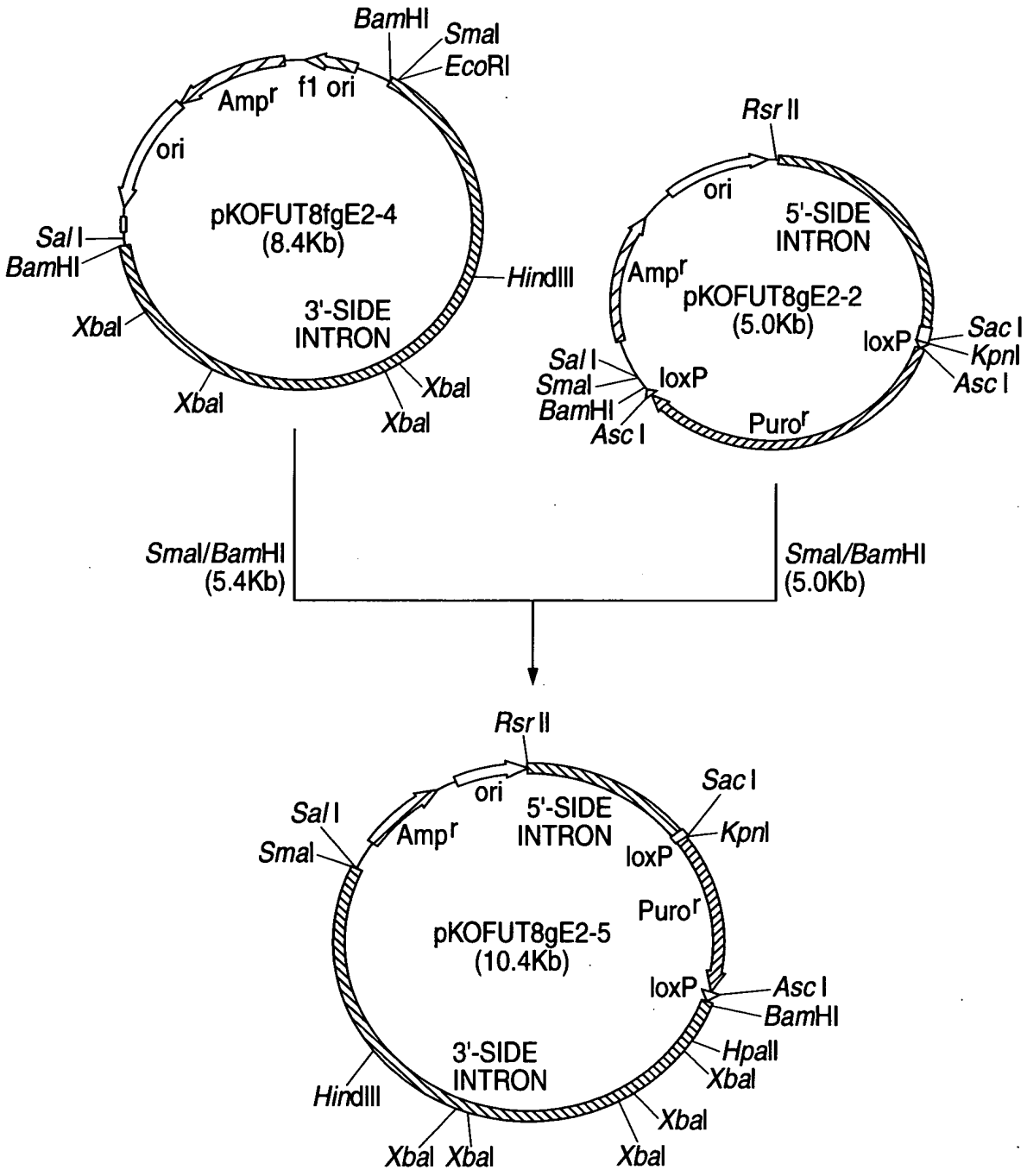
BamHI
SmaI
EcoRI
Ampr
f1 ori
ori
pKOFUT8fgE2-4
(8.4Kb)
SalI
BamHI
HindIII
XbaI
3'-SIDE
INTRON
XbaI
XbaI
XbaI
RsrII
ori
5'-SIDE
INTRON
Ampr
pKOFUT8gE2-2
(5.0Kb)
loxP
SacI
KpnI
AscI
SalI
SmaI
BamHI
AscI
loxP
Puror
SmaI/BamHI
(5.4Kb)
SmaI/BamHI
(5.0Kb)
RsrII
ori
5'-SIDE
INTRON
SacI
SalI
SmaI
Ampr
loxP
KpnI
Puror
pKOFUT8gE2-5
(10.4Kb)
loxP
AscI
BamHI
HpaII
XbaI
3'-SIDE
INTRON
HindIII
XbaI
XbaI
XbaI
XbaI

FIG. 40

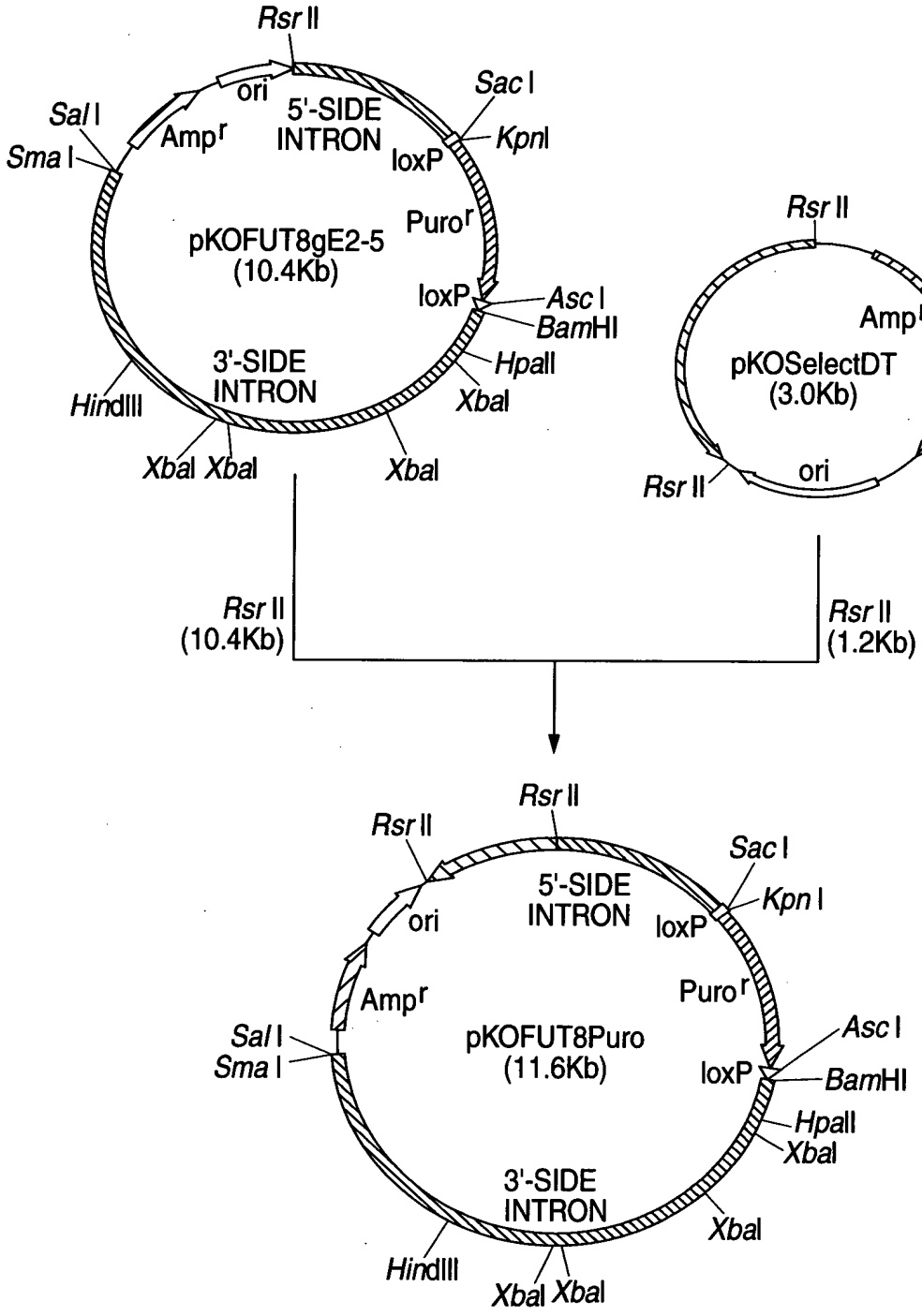
Rsr II
ori
5'-SIDE INTRON
Sac I
Sal I
Amp r
Kpn I
Sma I
loxP
Puro r
pKOFUT8gE2-5
(10.4Kb)
loxP
Asc I
BamHI
HpaII
3'-SIDE INTRON
HindIII
XbaI
XbaI XbaI
XbaI
Rsr II
Amp r
pKOSelectDT
(3.0Kb)
ori
Rsr II
Rsr II
(10.4Kb)
Rsr II
(1.2Kb)
Rsr II
Rsr II
5'-SIDE INTRON
Sac I
ori
Kpn I
loxP
Amp r
Puro r
Sal I
Sma I
pKOFUT8Puro
(11.6Kb)
Asc I
loxP
BamHI
HpaII
XbaI
3'-SIDE INTRON
XbaI
HindIII
XbaI XbaI

FIG. 41

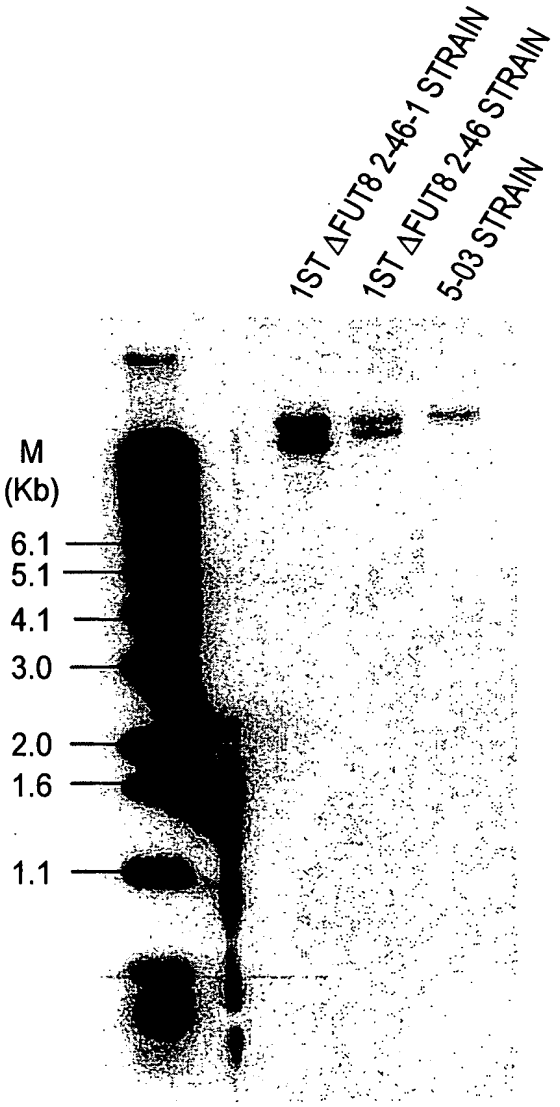
1ST ΔFUT8 2-46-1 STRAIN
1ST ΔFUT8 2-46 STRAIN
5-03 STRAIN
M
(Kb)
6.1
5.1
4.1
3.0
2.0
1.6
1.1

FIG. 42

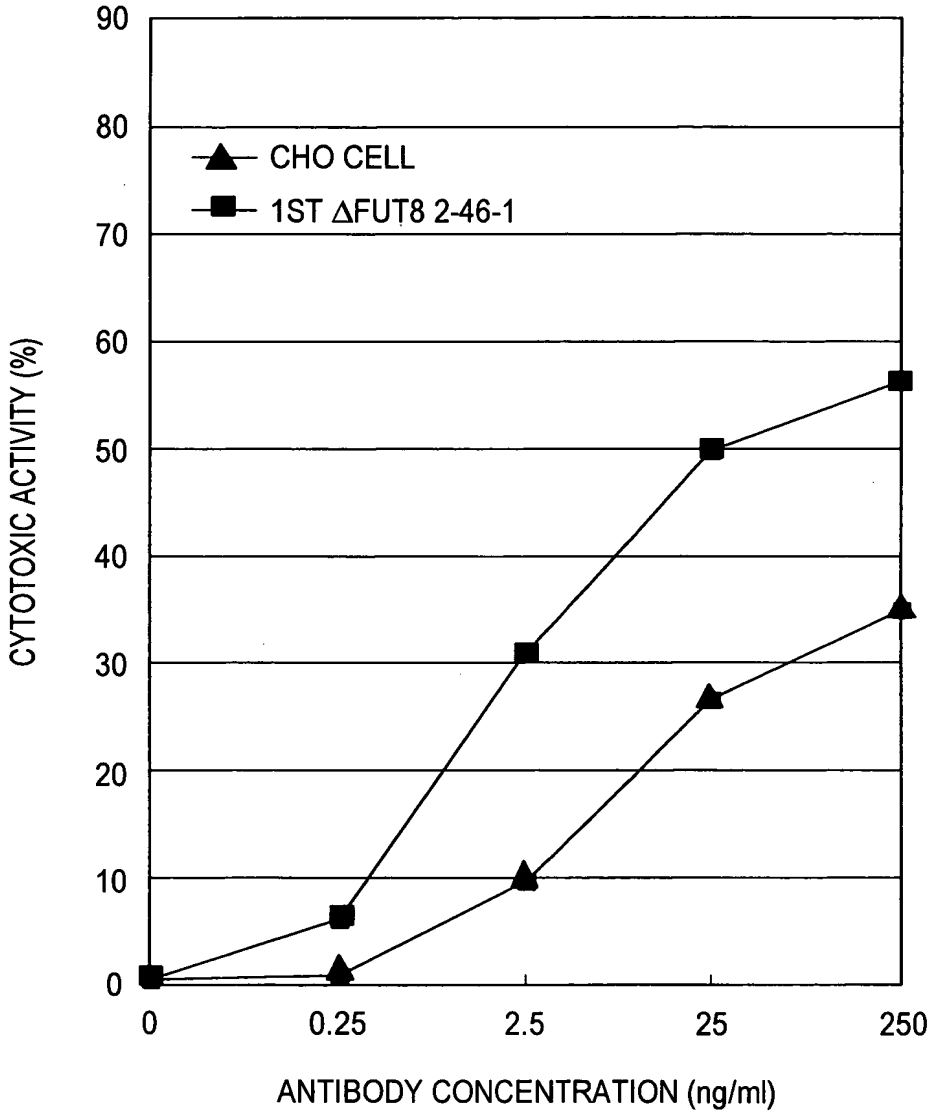
90
80
70
60
50
40
30
20
10
0
CHO CELL
1ST ΔFUT8 2-46-1
CYTOTOXIC ACTIVITY (%)
0
0.25
2.5
25
250
ANTIBODY CONCENTRATION (ng/ml)

FIG. 43

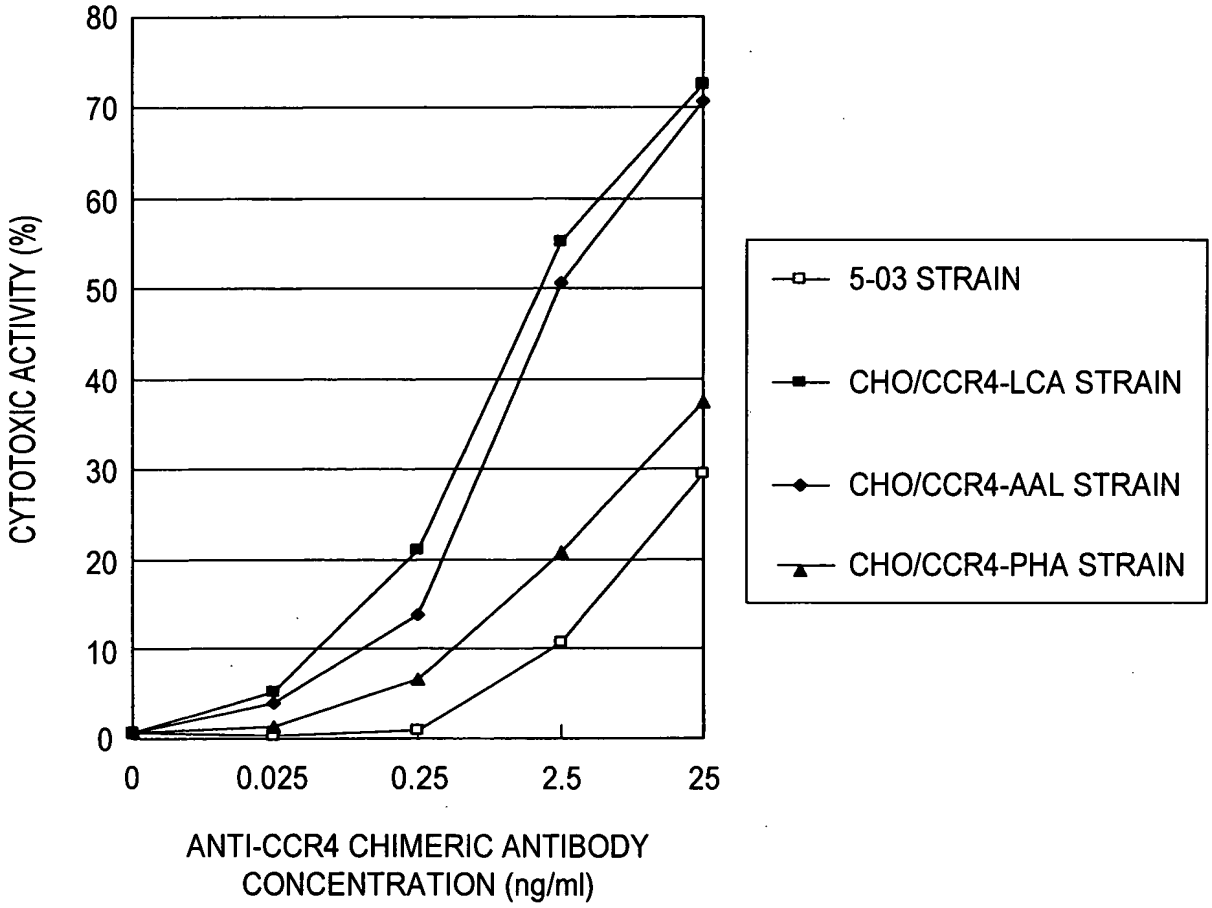
80
70
60
50
40
30
20
10
0
CYTOTOXIC ACTIVITY (%)
0
0.025
0.25
2.5
25
ANTI-CCR4 CHIMERIC ANTIBODY CONCENTRATION (ng/ml)
5-03 STRAIN
CHO/CCR4-LCA STRAIN
CHO/CCR4-AAL STRAIN
CHO/CCR4-PHA STRAIN

FIG. 44

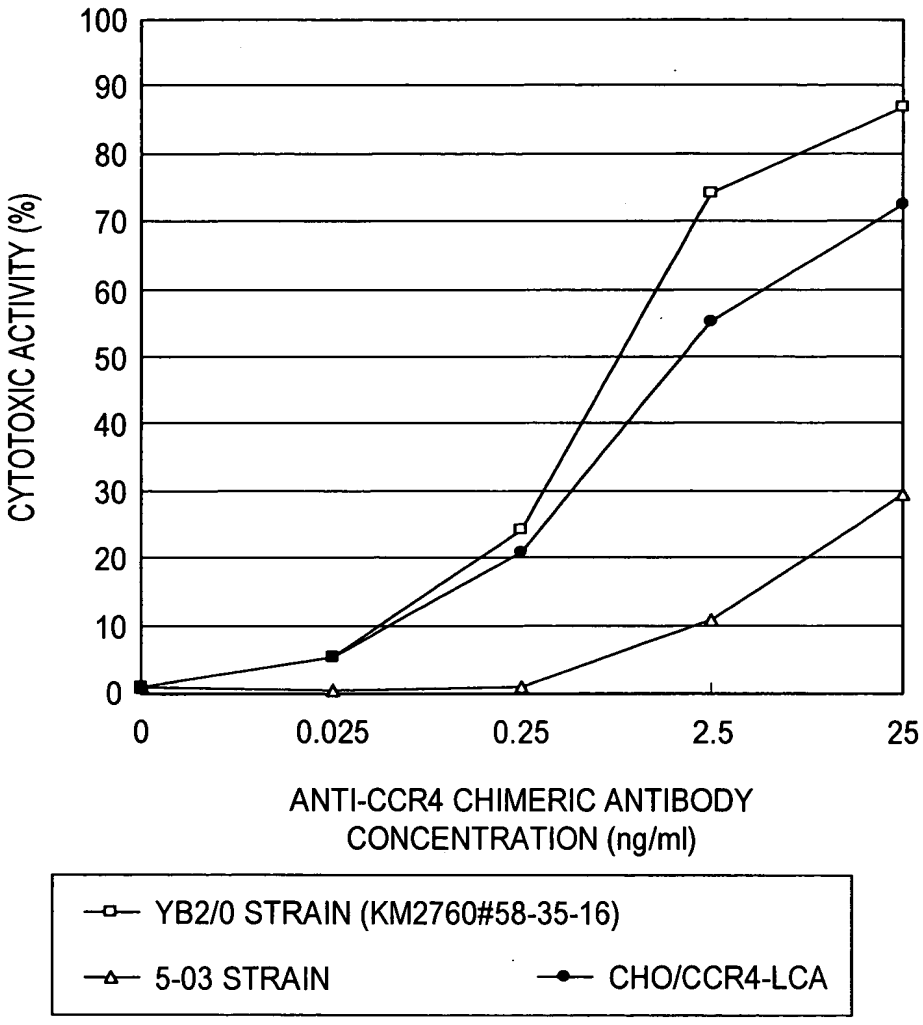
100
90
80
70
60
50
40
30
20
10
0
CYTOTOXIC ACTIVITY (%)
0
0.025
0.25
2.5
25
ANTI-CCR4 CHIMERIC ANTIBODY CONCENTRATION (ng/ml)
YB2/0 STRAIN (KM2760#58-35-16)
5-03 STRAIN
CHO/CCR4-LCA

FIG. 45A

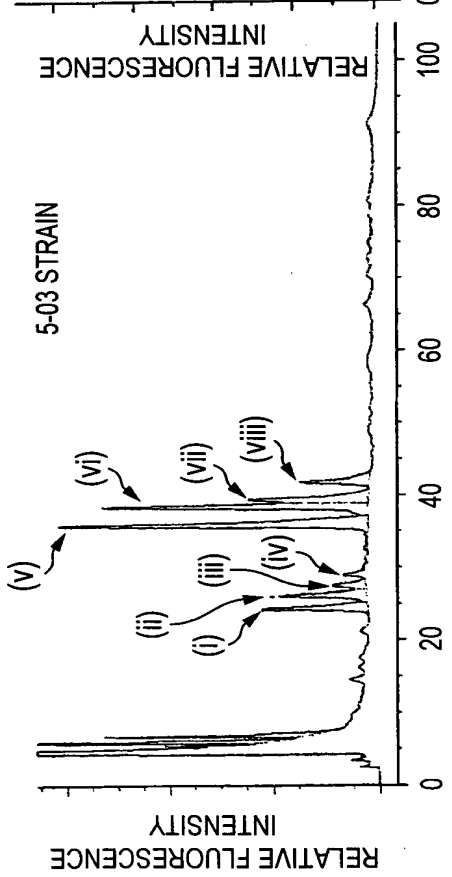

FIG. 45B

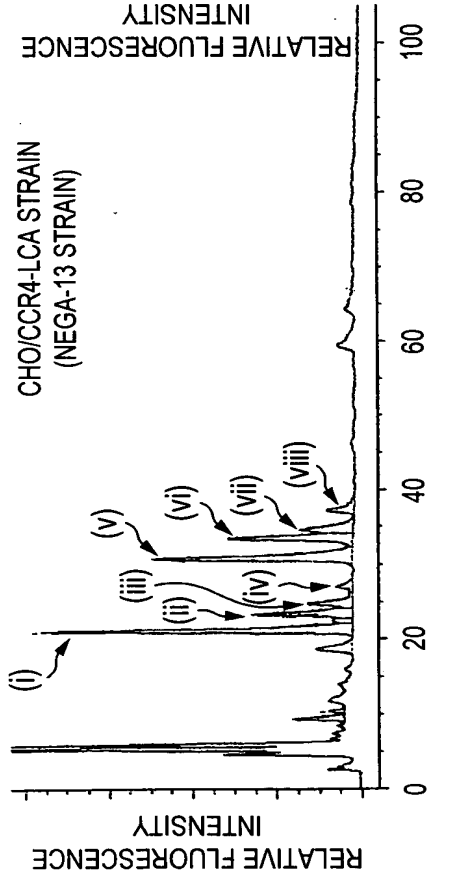

FIG. 45C

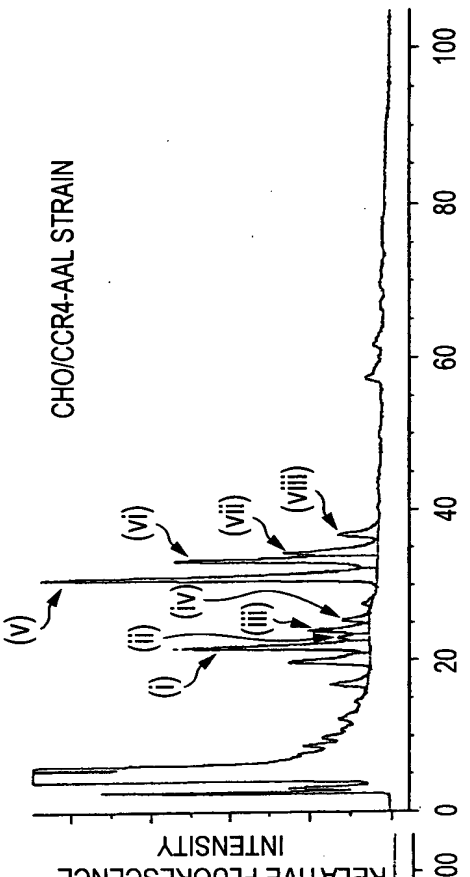

FIG. 45D

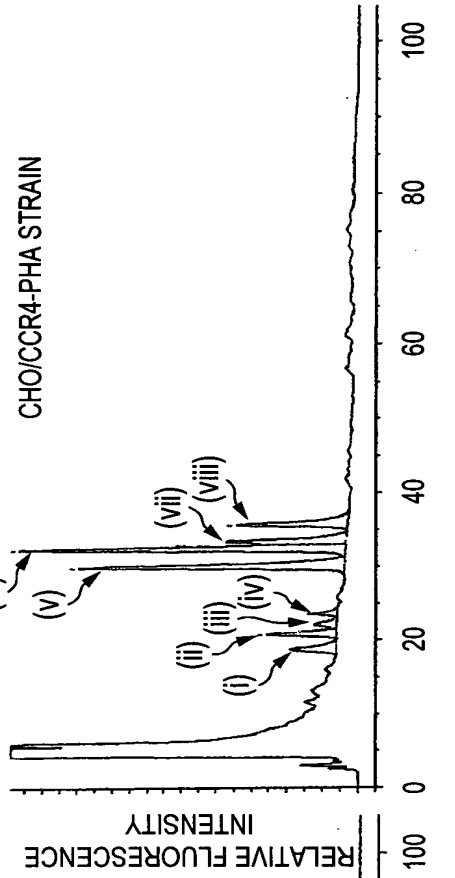

FIG. 46

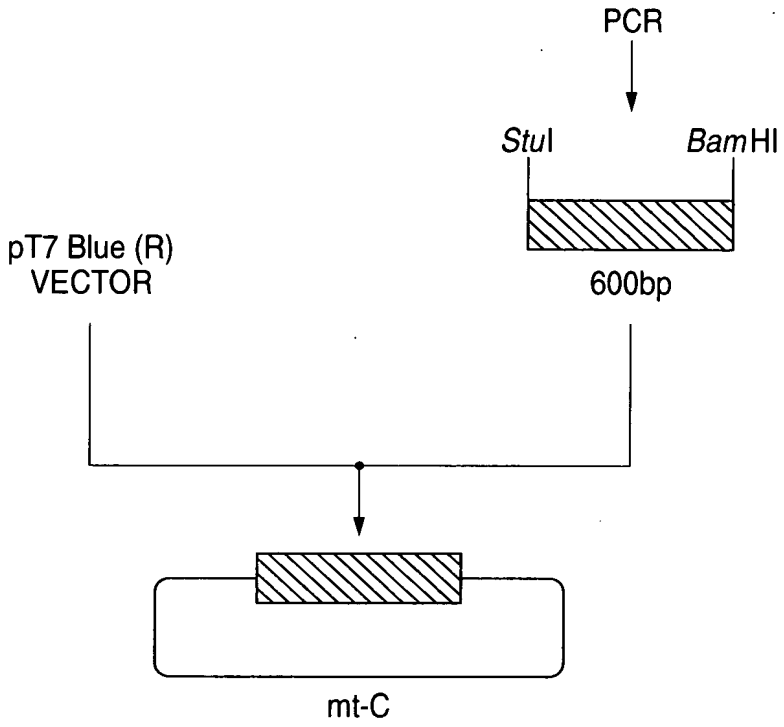
PCR
StuI
BamHI
pT7 Blue (R)
VECTOR
600bp
mt-C

FIG. 47

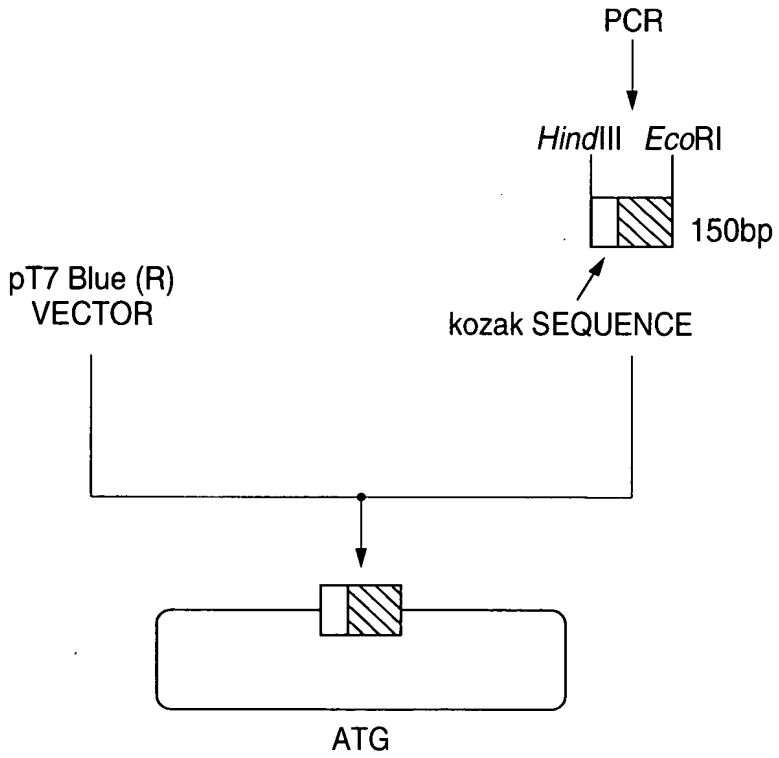
PCR
HindIII
EcoRI
150bp
pT7 Blue (R)
VECTOR
kozak SEQUENCE
ATG

FIG. 48

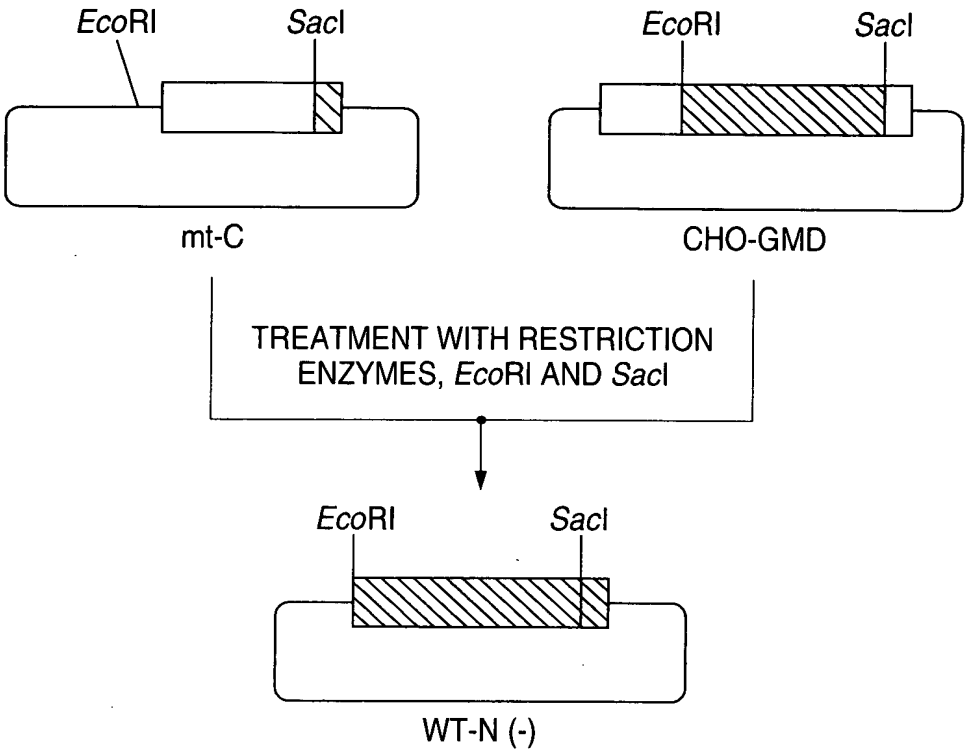
EcoRI
SacI
EcoRI
SacI
mt-C
CHO-GMD
TREATMENT WITH RESTRICTION ENZYMES, EcoRI AND SacI
EcoRI
SacI
WT-N (-)

FIG. 49

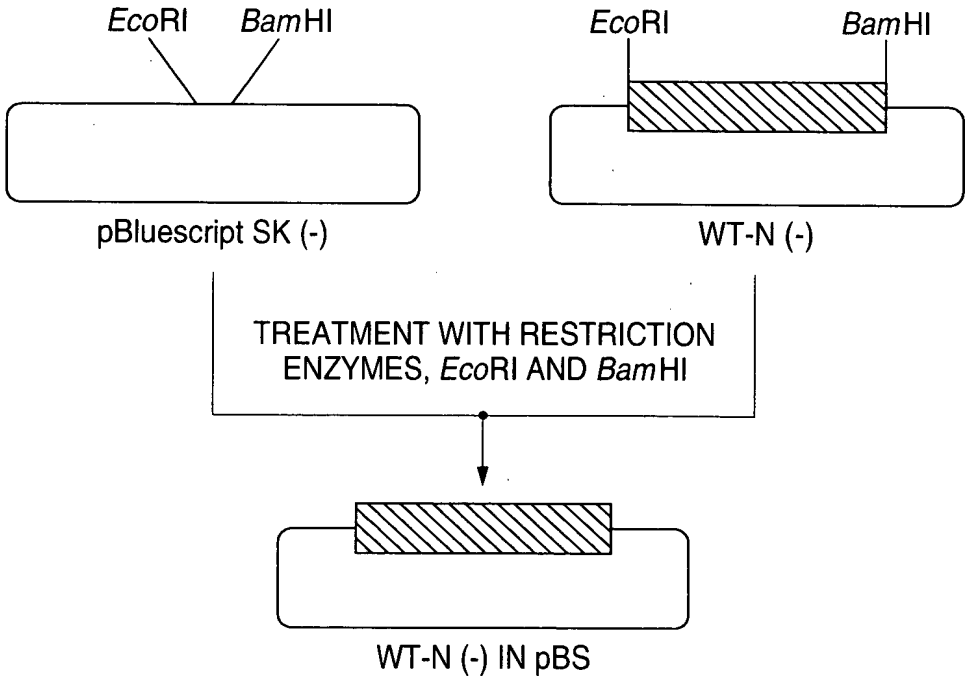
EcoRI
BamHI
EcoRI
BamHI
pBluescript SK (-)
WT-N (-)
TREATMENT WITH RESTRICTION ENZYMES, EcoRI AND BamHI
WT-N (-) IN pBS

FIG. 50

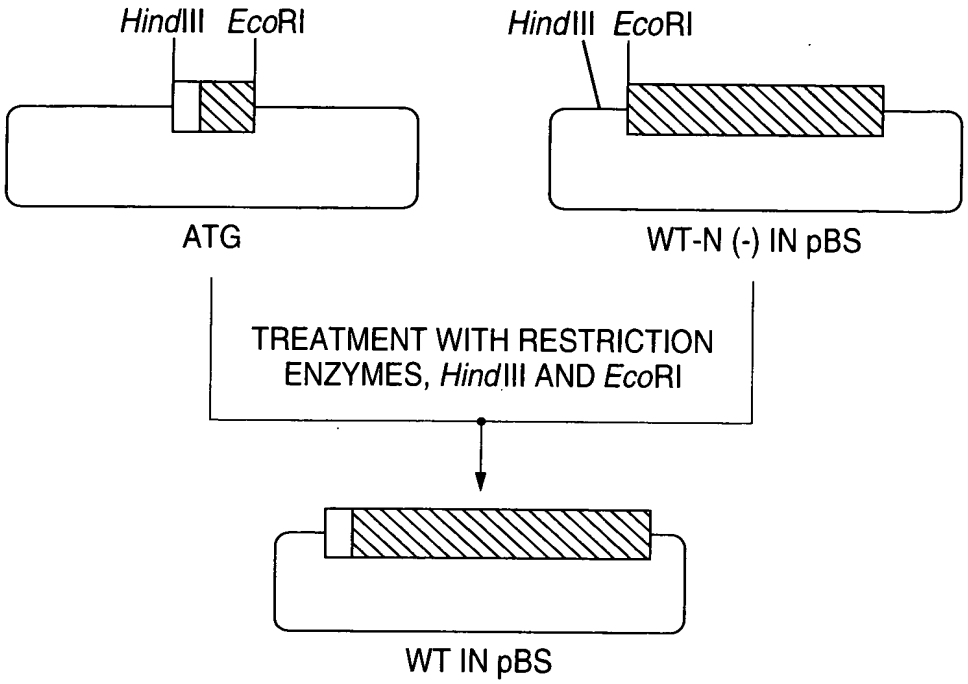
HindIII EcoRI
HindIII EcoRI
ATG
WT-N (-) IN pBS
TREATMENT WITH RESTRICTION ENZYMES, HindIII AND EcoRI
WT IN pBS

FIG. 51

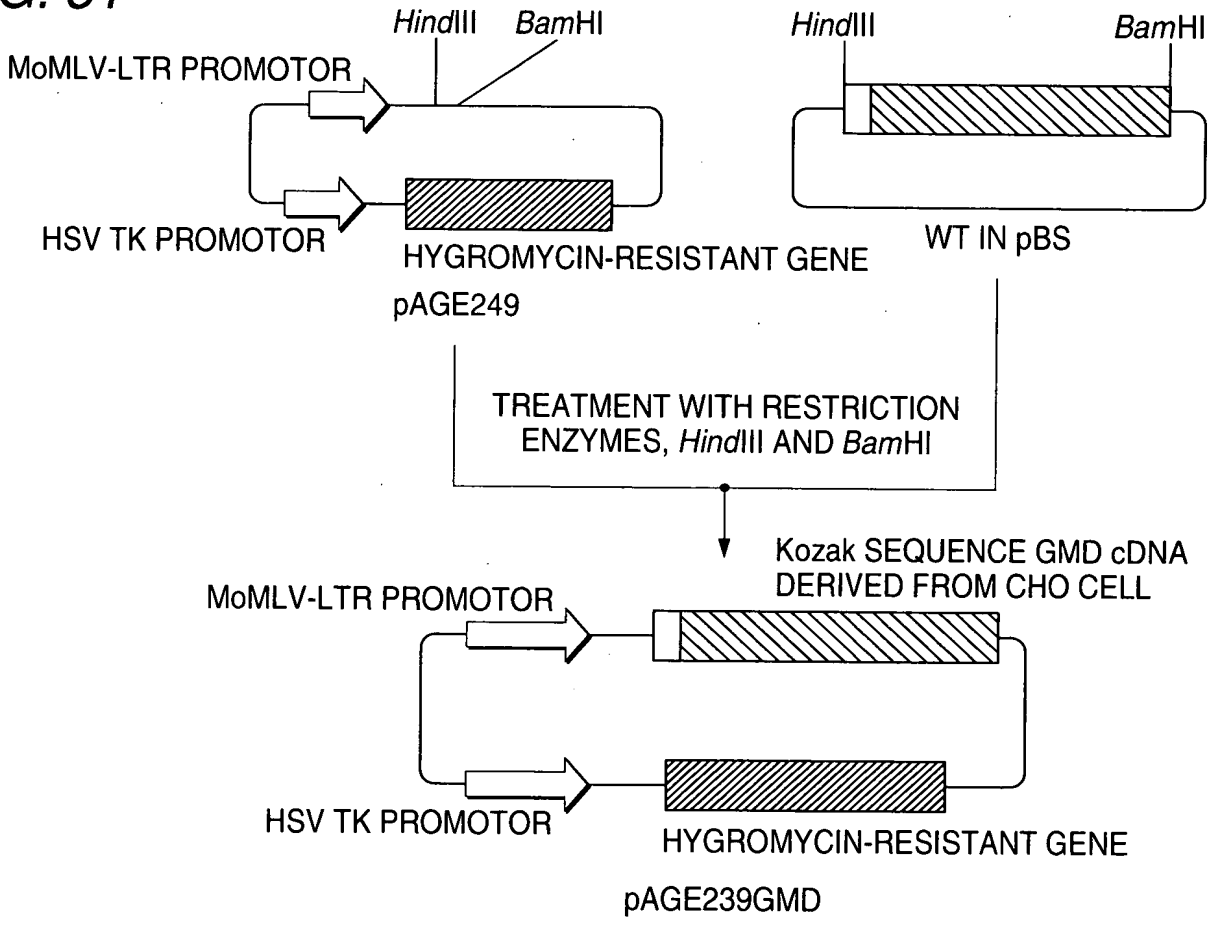
HindIII BamHI
HindIII BamHI
MoMLV-LTR PROMOTOR
HSV TK PROMOTOR
HYGROMYCIN-RESISTANT GENE
WT IN pBS
pAGE249
TREATMENT WITH RESTRICTION ENZYMES, HindIII AND BamHI
Kozak SEQUENCE GMD cDNA DERIVED FROM CHO CELL
MoMLV-LTR PROMOTOR
HSV TK PROMOTOR
HYGROMYCIN-RESISTANT GENE
pAGE239GMD

FIG. 52

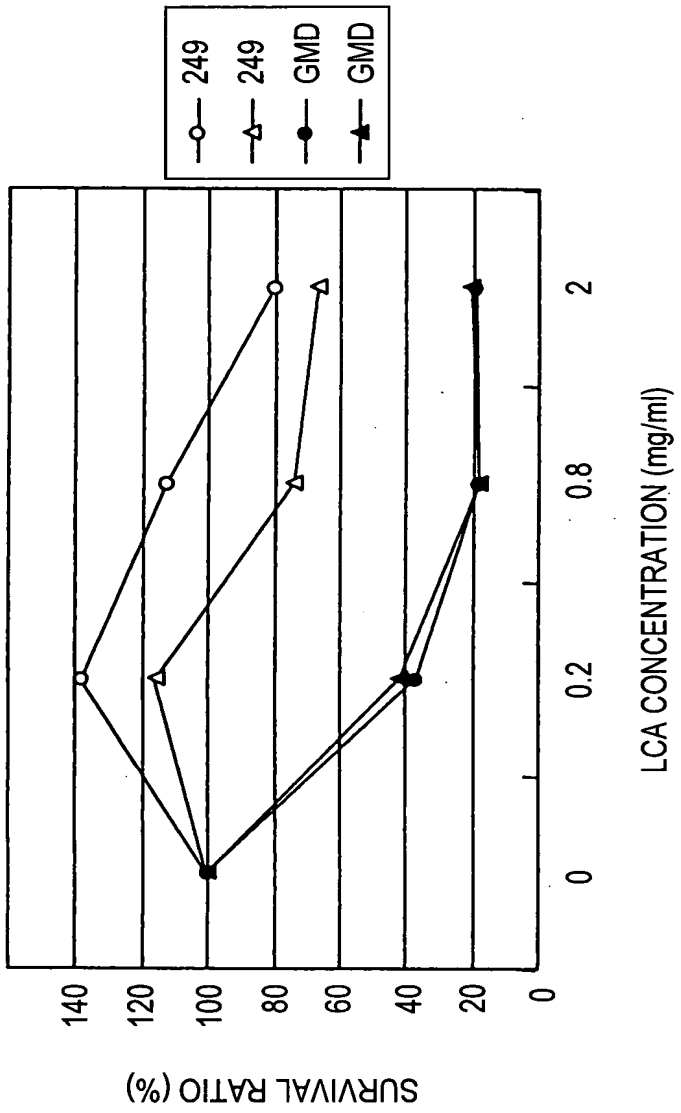
249
249
GMD
GMD
SURVIVAL RATIO (%)
140
120
100
80
60
40
20
0
0
0.2
0.8
2
LCA CONCENTRATION (mg/ml)

FIG. 53

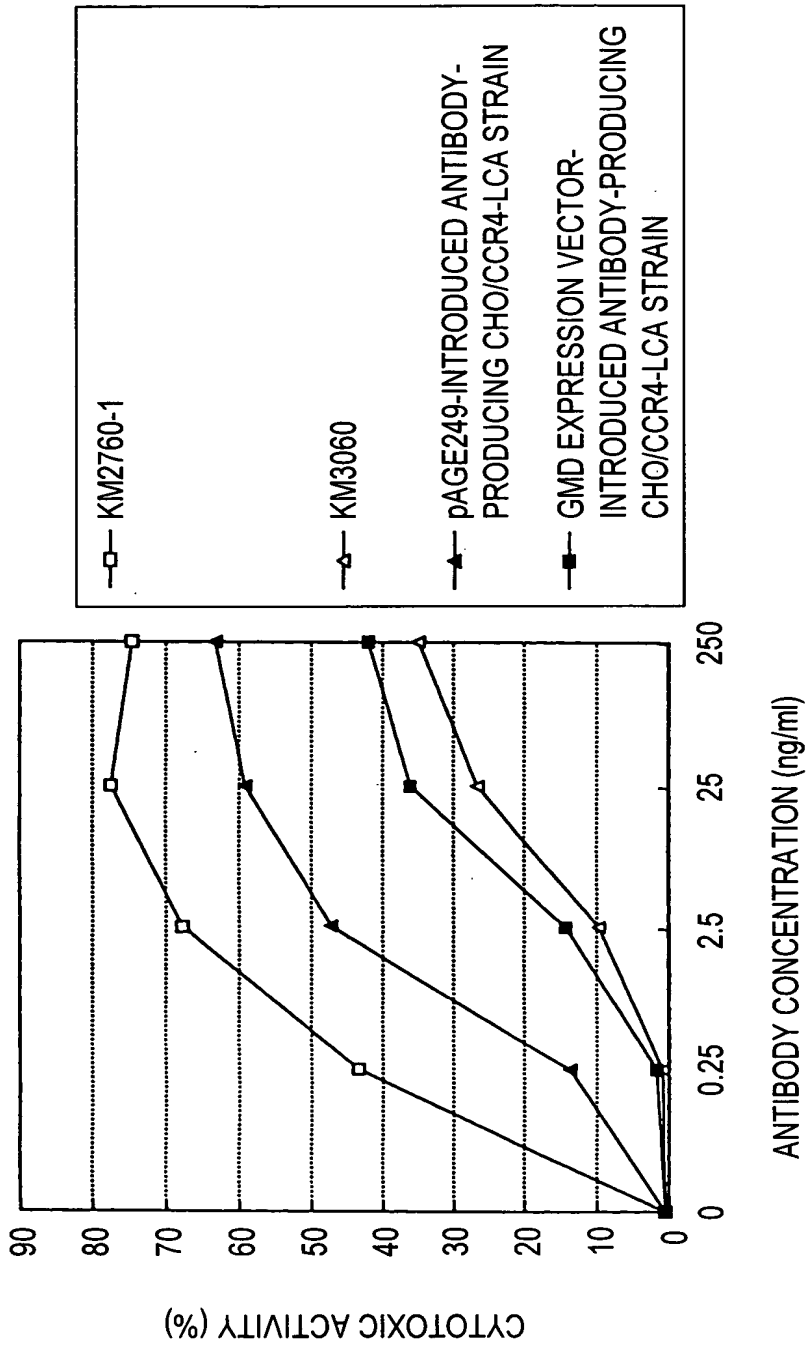
KM2760-1
KM3060
pAGE249-INTRODUCED ANTIBODY-PRODUCING CHO/CCR4-LCA STRAIN
GMD EXPRESSION VECTOR-INTRODUCED ANTIBODY-PRODUCING CHO/CCR4-LCA STRAIN
CYTOTOXIC ACTIVITY (%)
0
10
20
30
40
50
60
70
80
90
ANTIBODY CONCENTRATION (ng/ml)
0
0.25
2.5
25
250

FIG. 54

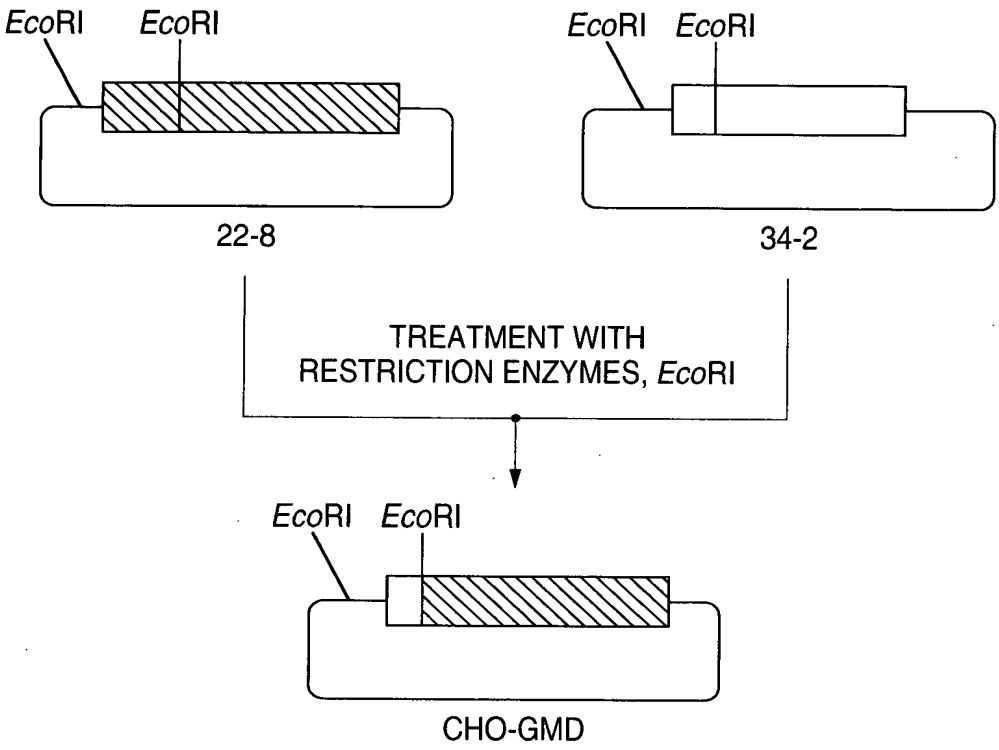

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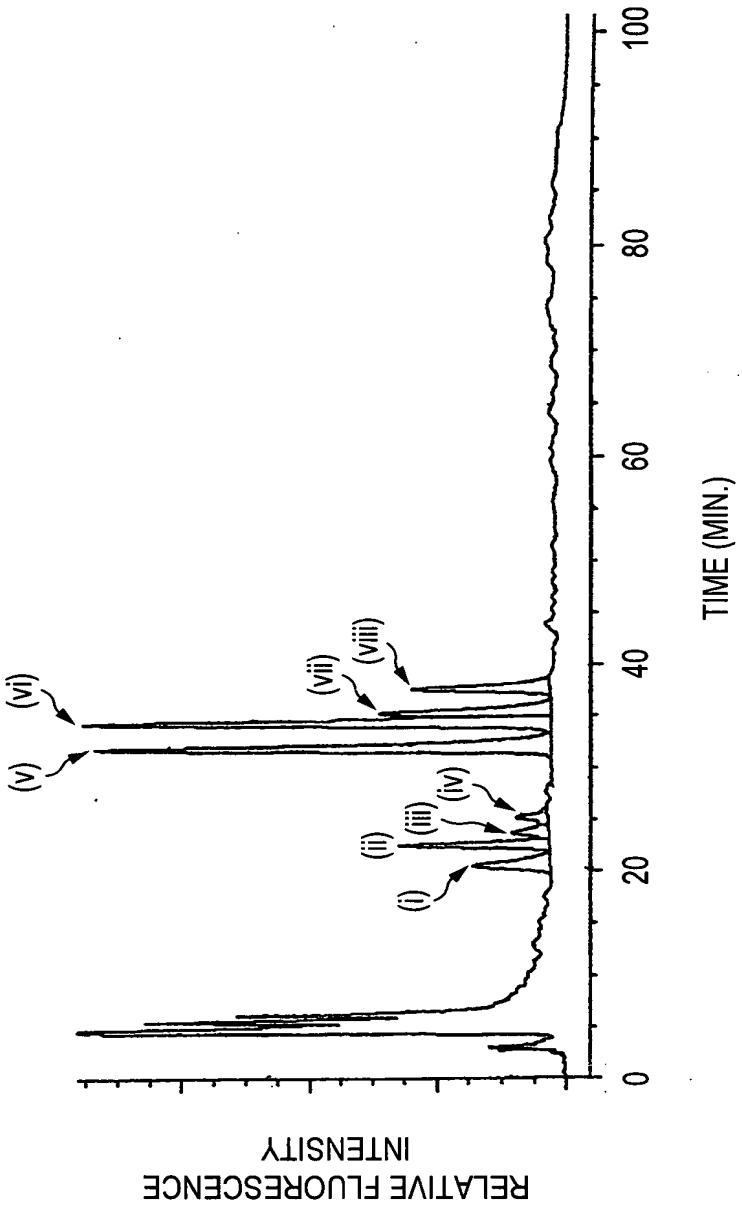
FIG. 55


RELATIVE FLUORESCENCE INTENSITY
TIME (MIN.)
0
20
40
60
80
100
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)

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In re Patent Application of

KANDA, et al. Atty. Ref.: 249-202

Serial No. 09/971,773 Group: 1632

Filed: October 9, 2001 Examiner: Kelly, Robert

For: ANTIBODY COMPOSITION-PRODUCING CELL

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

August 12, 2004

Sir:

INFORMATION DISCLOSURE STATEMENT

☐ 1. **PTO-1449 Pursuant to 37 CFR 1.97(b)**
[within 3 months of filing or prior to 1st Office Action on the merits]
N/C

☐ 2.(a) **Statement Pursuant to 37 CFR 1.97(c)**
[before Final Office Action or Allowance (requires Rule 97(e) Statement or Rule 17(p) fee)] N/C

☒ 2.(b) **Fee Payment Pursuant to 37 CFR 1.97(c)**
[before Final Office Action or Allowance (requires Rule 97(e) Statement or Rule 17(p) fee)] *$180.00*

☐ 3. **Pursuant to 37 CFR 1.97(d)**
[after Final Office Action or Allowance (requires Rule 97(e) Statement and Rule 17(p) fee), but before final fee payment]
$180.00

The following are submitted in the above-identified application in compliance with 37 C.F.R. §§ 1.97 and 1.98:

☒ 4. A list of documents on Form PTO-1449 together with copies of each identified document and a translation or a concise explanation of each non-English language document (such as the attached Search

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Report issued in PCT/JP01/08804 dated January 22, 2002) is enclosed herewith.

This paper is submitted in accordance with:

☐ 5. 37 CFR 1.97(b): [within 3 months of filing or prior to 1st Office Action]

☒ 6. 37 CFR 1.97(c): [before Final Office Action or Allowance, whichever is earlier]; and

☐ a) The required Statement made in item 8 below; **or**

☒ b) The $180.00 fee specified in 37 CFR §1.17(p) for submission of this Information Disclosure Statement is authorized in item 9 below.

☐ 7. 37 CFR §1.97(d): [after Final Office Action or Allowance (requires Rule 97(e) Statement and Rule 17(p) fee), but before final fee payment]; and

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☐ 8. Statement under 37 CFR 1.97(e)

☐ a) The undersigned attorney of record hereby certifies under 37 C.F.R. §1.97(e) that each item of information contained in this Information Disclosure Statement was first cited in a communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement (each item contained in this IDS was the first citation of that item by a foreign patent office in a counterpart foreign application which occurred no more than three months prior to the filing of this IDS); or

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☒ 9. Please charge all deficiency fees associated with the submission of this Information Disclosure Statement and any other fees applicable to this application to Deposit Account No. 14-1140. An original and one (1) copy of this document are enclosed.

871762
Aragen/Transposagen Ex. 1036 - Part B

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Sheet 1 of 1

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U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
		5728568	5/1998	Sullivan et al			
		6054304	4/2000	Taniguchi			

FOREIGN PATENT DOCUMENTS

		DOCUMENT	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
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		97/37683A1	10/1997	WO				
		97/27303A1	7/1997	WO				
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*Examiner		Date Considered	

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(57) Abstract

A biosynthetic method for producing vitamin C (ascorbic acid, L–ascorbic acid, or AA) is disclosed. Such a method includes fermentation of a genetically modified microorganism or plant to produce L–ascorbic acid. In particular, the present invention relates to the use of microorganisms and plants having at least one genetic modification to increase the action of an enzyme involved in the ascorbic acid biosynthetic pathway. Included is the use of nucleotide sequences encoding epimerases, including the endogenous GDP–D–mannose:GDP–L–galactose epimerase from the L–ascorbic acid pathway and homologues thereof for the purposes of improving the biosynthetic production of ascorbic acid. The present invention also relates to genetically modified microorganisms, such as strains of microalgae, bacteria and yeast useful for producing L–ascorbic acid, and to genetically modified plants, useful for producing consumable plant food products.

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VITAMIN C PRODUCTION IN MICROORGANISMS AND PLANTS

FIELD OF THE INVENTION

The present invention relates to vitamin C (L-ascorbic acid) production using genetically modified microorganisms and plants. In particular, the present invention relates to the use of nucleotide sugar epimerase enzymes for the biological production of ascorbic acid in plants and microorganisms.

BACKGROUND OF THE INVENTION

Nearly all forms of life, both plant and animal, either synthesize ascorbic acid (vitamin C) or require it as a nutrient. Ascorbic acid was first identified to be useful as a dietary supplement for humans and animals for the prevention of scurvy. Ascorbic acid, however, also affects human physiological functions such as the adsorption of iron, cold tolerance, the maintenance of the adrenal cortex, wound healing, the synthesis of polysaccharides and collagen, the formation of cartilage, dentine, bone and teeth, the maintenance of capillaries, and is useful as an antioxidant.

For use as a dietary supplement, ascorbic acid can be isolated from natural sources, such as rosehips, synthesized chemically through the oxidation of L-sorbose, or produced by the oxidative fermentation of calcium D-gluconate by *Acetobacter suboxidans*. Considine, "Ascorbic Acid," *Van Nostrand's Scientific Encyclopedia*, Vol. 1, pp. 237-238, (1989). Ascorbic acid (predominantly intracellular) has also been obtained through the fermentation of strains of the microalga, *Chlorella pyrenoidosa*. See U.S. Patent No. 5,001,059 by Skatrud, which is assigned to the assignee of the present application. It is believed that ascorbic acid is produced inside the chloroplasts of photosynthetic microorganisms and functions to neutralize energetic electrons produced during photosynthesis. Accordingly, ascorbic acid production is known in photosynthetic organisms as a protective mechanism.

Therefore, products and processes which improve the ability to biosynthetically produce ascorbic acid are desirable and beneficial for the improvement of human health.

SUMMARY OF THE INVENTION

One embodiment of the present invention relates to a method for producing ascorbic acid or esters thereof in a microorganism. The method includes the steps of: (a)

culturing a microorganism having a genetic modification to increase the action of an enzyme selected from the group of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-D-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase; and (b) recovering the ascorbic acid or esters produced by the microorganism. Preferably, the genetic modification is a genetic modification to increase the action of an enzyme selected from the group of GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase. In one embodiment of the method of the present invention, the microorganism further includes a genetic modification to decrease the action of an enzyme having GDP-D-mannose as a substrate, other than GDP-D-mannose:GDP-L-galactose epimerase. Such a genetic modification can include, for example, a genetic modification to decrease the action of GDP-D-mannose-dehydrogenase.

In one embodiment, the genetic modification is a genetic modification to increase the action of an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, which can include GDP-D-mannose:GDP-L-galactose epimerase. In one embodiment, the epimerase binds NADPH. In one embodiment of this method, the genetic modification includes transformation of the microorganism with a recombinant nucleic acid molecule that expresses the epimerase. Such an epimerase can have a tertiary structure that substantially conforms to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. Preferably, the epimerase has a structure having an average root mean square deviation of less than about 2.5 Å, and more preferably less than about 1 Å, over at least about 25% of Cα positions of the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

In one embodiment, the epimerase comprises a substrate binding site having a tertiary structure that substantially conforms to the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by

atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. Such a substrate binding site preferably has a tertiary structure with an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

In another embodiment, the epimerase comprises a catalytic site having a tertiary structure that substantially conforms to the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. Such a catalytic site preferably has a tertiary structure with an average root mean square deviation of less than about 1 Å over at least about 25% of Cα positions of the tertiary structure of a catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. The catalytic site preferably includes the amino acid residues serine, tyrosine and lysine and in one embodiment, the tertiary structure positions of the amino acid residues serine, tyrosine and lysine substantially conform to tertiary structure positions of residues Ser107, Tyr136 and Lys140, respectively, as represented by atomic coordinates in Brookhaven Protein Data Bank Accession Code 1bws.

In yet another embodiment of this method, the epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a CLUSTAL alignment program, wherein amino acid residues in the amino acid sequence align with 100% identity with at least about 50%, and in another embodiment with at least about 75%, and in yet another embodiment with at least about 90% of non-Xaa residues in SEQ ID NO:11. In another embodiment, the epimerase comprises an amino acid sequence having at least 4 contiguous amino acid residues that are 100% identical to at least 4 contiguous amino acid residues of an amino acid sequence selected from the group of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10. In yet another embodiment, the recombinant nucleic acid molecule comprises a nucleic acid sequence comprising at least about 12 contiguous nucleotides having 100% identity with at least about 12

contiguous nucleotides of a nucleic acid sequence selected from the group of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9.

In yet another embodiment of this method of the present invention, the epimerase comprises an amino acid sequence having a motif: Gly-Xaa-Xaa-Gly-Xaa-Xaa-Gly. In yet another embodiment, the recombinant nucleic acid molecule comprises a nucleic acid sequence that is at least about 15% identical, and in another embodiment, at least about 20% identical, and in another embodiment, at least about 25% identical, to a nucleic acid sequence selected from the group of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, as determined using a Lipman-Pearson method with Lipman-Pearson standard default parameters.

In yet another embodiment of this method of the present invention, the recombinant nucleic acid molecule comprises a nucleic acid sequence that hybridizes under stringent hybridization conditions to a nucleic acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase. The nucleic acid sequence encoding the GDP-4-keto-6-deoxy-D-mannose epimerase/reductase includes nucleic acid sequences selected from the group of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5, and the GDP-4-keto-6-deoxy-D-mannose epimerase/reductase can include an amino acid sequence selected from the group of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.

In one embodiment of the method of the present invention, the microorganism is selected from the group of bacteria, fungi and microalgae. In one embodiment, the microorganism is acid-tolerant. Preferred bacteria include, but are not limited to *Azotobacter* and *Pseudomonas*. Preferred fungi include, but are not limited to, yeast, including, but not limited to *Saccharomyces* yeast. Preferred microalgae include, but are not limited to, microalgae of the genera *Prototheca* and *Chlorella*, with microalgae of the genus *Prototheca* being particularly preferred.

In yet another embodiment of the method of the present invention, the microorganism is acid-tolerant and the step of culturing is conducted at a pH of less than about 6.0, and more preferably, at a pH of less than about 5.5, and even more preferably, at a pH of less than about 5.0. The step of culturing can be conducted in a fermentation medium that comprises a carbon source other than D-mannose in one embodiment, and

in another embodiment, the step of culturing is conducted in a fermentation medium that comprises glucose as a carbon source.

In yet another embodiment of the present method, the step of culturing is conducted in a fermentation medium that is magnesium (Mg) limited. Preferably, the step of culturing is conducted in a fermentation medium that is Mg limited during a cell growth phase. In one embodiment, the fermentation medium includes less than about 0.5 g/L of Mg during a cell growth phase, and more preferably, less than about 0.2 g/L of Mg during a cell growth phase, and even more preferably, less than about 0.1 g/L of Mg during a cell growth phase.

Another embodiment of the present invention relates to a microorganism for producing ascorbic acid or esters thereof. The microorganism has a genetic modification to increase the action of an enzyme selected from the group of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-D-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase. Preferably, the genetic modification is a genetic modification to increase the action of an enzyme selected from the group of GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase, and even more preferably, to increase the action of GDP-D-mannose:GDP-L-galactose epimerase.

In one embodiment, the microorganism has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein the epimerase has a tertiary structure having an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. In another embodiment, the microorganism has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein the epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a

CLUSTAL alignment program, wherein amino acid residues in the amino acid sequence align with 100% identity with at least about 50% of non-Xaa residues in SEQ ID NO:11. Preferred microorganisms are disclosed as for the method discussed above.

Yet another embodiment of the present invention relates to a plant for producing ascorbic acid or esters thereof. Such a plant has a genetic modification to increase the action of an enzyme selected from the group of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-D-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase. In a preferred embodiment, the genetic modification is a genetic modification to increase the action of an enzyme selected from the group of GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase, and in a more preferred embodiment, the genetic modification is a genetic modification to increase the action of GDP-D-mannose:GDP-L-galactose epimerase.

In one embodiment, the plant further comprises a genetic modification to decrease the action of an enzyme having GDP-D-mannose as a substrate other than GDP-D-mannose:GDP-L-galactose epimerase. Such a genetic modification includes a genetic modification to decrease the action of GDP-D-mannose-dehydrogenase. Such a plant also includes a plant that has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein the epimerase has a tertiary structure having an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. In another embodiment, such a plant has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein the epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a CLUSTAL alignment program, wherein amino acid residues in the amino acid sequence align with 100% identity with at least about 50% of non-Xaa residues in SEQ ID NO:11.

In one embodiment, a plant for producing ascorbic acid or esters thereof according to the present invention is a microalga. Preferred microalgae include, but are not limited to microalgae of the genera *Prototheca* and *Chlorella*, with microalga of the genus *Prototheca* being particularly preferred. In another embodiment, the plant is a higher plant, with consumable higher plants being more preferred.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1A is a schematic drawing of the pathway from glucose to GDP-D-mannose in plants.

Fig. 1B is a schematic drawing of the pathway from GDP-D-mannose to L-galactose-1-phosphate in plants.

Fig. 1C is a schematic drawing of the pathway from L-galactose to L-ascorbic acid in plants.

Fig. 2A is a schematic drawing of selected carbon flow from glucose in *Prototheca*.

Fig. 2B is a schematic drawing of selected carbon flow from glucose in *Prototheca*.

Fig. 3 is a schematic drawing that shows the lineage of mutants derived from *Prototheca moriformis* ATCC 75669, and their ability to produce L-ascorbic acid.

Fig. 4 is a bar graph illustrating the conversion of substrates by resting cells of strain NA45-3 following growth in media containing various magnesium concentrations and resuspension in media containing various magnesium concentrations.

Fig. 5 is a line graph showing the relationship between specific ascorbic acid formation in cultures of *Prototheca* strains and the specific activity of GDP-D-mannose:GDP-L-galactose epimerase in extracts prepared from cells harvested from the same cultures.

Fig. 6 is a line graph showing the relationship between specific epimerase activity and the degree of magnesium limitation in two strains, ATCC 75669 and EMS13-4.

Fig. 7 depicts the overall catalytic mechanism of GDP-D-mannose:GDP-L-galactose epimerase proposed by Barber (1979, *J. Biol. Chem.* 254:7600-7603).

Fig. 8A depicts the catalytic mechanism of GDP-D-mannose-4,6-dehydratase (converts GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose).

Fig. 8B depicts the catalytic mechanism of GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (converts GDP-4-keto-6-deoxy-D-mannose to GDP-L-fucose) (Chang, et al., 1988, *J. Biol. Chem.* 263:1693-1697; Barber, 1980, *Plant Physiol.* 66:326-329).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a biosynthetic method and production microorganisms and plants for producing vitamin C (ascorbic acid, L-ascorbic acid, or AA). Such a method includes fermentation of a genetically modified microorganism to produce L-ascorbic acid. In particular, the present invention relates to the use of nucleotide sequences encoding epimerases, including the endogenous GDP-D-mannose:GDP-L-galactose epimerase from the L-ascorbic acid pathway, as well as epimerases having structural homology (e.g., by nucleotide/amino acid sequence and/or tertiary structure of the encoded protein) to GDP-4-keto-6-deoxy-D-mannose epimerase/reductases, or UDP-galactose 4-epimerases, for the purposes of improving the biosynthetic production of ascorbic acid. The present invention also relates to genetically modified microorganisms, such as strains of microalgae, bacteria and yeast useful for producing L-ascorbic acid, and to genetically modified plants, useful for producing consumable plant food products.

One embodiment of the present invention relates to a method to produce L-ascorbic acid by fermentation of a genetically modified microorganism. This method includes the steps of (a) culturing in a fermentation medium a microorganism having a genetic modification to increase the action of an enzyme selected from the group of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and L-galactono-γ-lactone dehydrogenase; and (b) recovering L-ascorbic acid or esters thereof. The various enzymes in this list represent the enzymes involved in the vitamin C biosynthetic pathway in plants. It is uncertain at this time

whether the enzyme represented by GDP-L-galactose phosphorylase is actually a phosphorylase or a pyrophosphorylase (i.e., GDP-L-galactose pyrophosphorylase). Therefore, use of the term "GDP-L-galactose phosphorylase" herein refers to either GDP-L-galactose phosphorylase or GDP-L-galactose pyrophosphorylase. In one aspect of the invention, this method includes the step of culturing in a fermentation medium a microorganism having a genetic modification to increase the action of an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose. This aspect of the present invention is discussed in detail below.

Another embodiment of the present invention relates to a genetically modified microorganism for producing L-ascorbic acid or esters thereof. Another embodiment of the present invention relates to a genetically modified plant for producing L-ascorbic acid or esters thereof. Both genetically modified microorganisms (e.g., bacteria, yeast, microalgae) and plants (e.g., higher plants, microalgae) have a genetic modification to increase the action of an enzyme selected from the group of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase. In a preferred embodiment, both genetically modified microorganisms (e.g., bacteria, yeast, microalgae) and plants (e.g., higher plants, microalgae) have a genetic modification to increase the action of an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose. In one embodiment, the genetic modification includes the transformation of the microorganism or plant with the epimerase as described above.

To produce significantly high yields of L-ascorbic acid by the method of the present invention, a plant and/or microorganism is genetically modified to enhance production of L-ascorbic acid. As used herein, a genetically modified plant (such as a higher plant or microalgae) or microorganism, such as a microalga (*Prototheca, Chlorella*), *Escherichia coli*, or a yeast, is modified (i.e., mutated or changed) within its genome and/or by recombinant technology (i.e., genetic engineering) from its normal (i.e., wild-type or naturally occurring) form. In a preferred embodiment, a genetically modified plant or microorganism according to the present invention has been modified by

recombinant technology. Genetic modification of a plant or microorganism can be accomplished using classical strain development and/or molecular genetic techniques, include genetic engineering techniques. Such techniques are generally disclosed herein and are additionally disclosed, for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press; Roessler, 1995, *Plant Lipid Metabolism*, pp. 46-48; and Roessler et al., 1994, in Bioconversion for Fuels, Himmel et al. eds., American Chemical Society, Washington D.C., pp 255-70). These references are incorporated by reference herein in their entirety.

In some embodiments, a genetically modified plant or microorganism can include a natural genetic variant as well as a plant or microorganism in which nucleic acid molecules have been inserted, deleted or modified, including by mutation of endogenous genes (e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), in such a manner that the modifications provide the desired effect within the plant or microorganism. As discussed above, a genetically modified plant or microorganism includes a plant or microorganism that has been modified using recombinant technology.

As used herein, genetic modifications which result in a decrease in gene expression, an increase in inhibition of gene expression or inhibition of a gene product (i.e., the protein encoded by the gene), a decrease in the function of the gene, or a decrease in the function of the gene product can be referred to as inactivation (complete or partial), deletion, interruption, blockage, down-regulation, or decreased action of a gene. For example, a genetic modification in a gene which results in a decrease in the function of the protein encoded by such gene can be the result of a complete deletion of the gene encoding the protein (i.e., the gene does not exist, and therefore the protein does not exist), a mutation in the gene encoding the protein which results in incomplete or no translation of the protein (e.g., the protein is not expressed), or a mutation in the gene which decreases or abolishes the natural function of the protein (e.g., a protein is expressed which has decreased or no enzymatic activity).

Genetic modifications which result in an increase in gene expression or function can be referred to as amplification, overproduction, overexpression, activation, enhancement, addition, up-regulation or increased action of a gene. Additionally, a genetic modification to a gene which modifies the expression, function, or activity of the gene can

have an impact on the action of other genes and their expression products within a given metabolic pathway (e.g., by inhibition or competition). In this embodiment, the action (e.g., activity) of a particular gene and/or its product can be affected (i.e., upregulated or downregulated) by a genetic modification to another gene within the same metabolic pathway, or to a gene within a different metabolic pathway which impacts the pathway of interest by competition, inhibition, substrate formation, etc.

In general, a plant or microorganism having a genetic modification that affects L-ascorbic acid production has at least one genetic modification, as discussed above, which results in a change in the L-ascorbic acid production pathway as compared to a wild-type plant or microorganism grown or cultured under the same conditions. Such a modification in an L-ascorbic acid production pathway changes the ability of the plant or microorganism to produce L-ascorbic acid. According to the present invention, a genetically modified plant or microorganism preferably has an enhanced ability to produce L-ascorbic acid compared to a wild-type plant or microorganism cultured under the same conditions.

The present invention is based on the present inventors' discovery of the biosynthetic pathway for L-ascorbic acid (vitamin C) in plants and microorganisms. Prior to the present invention, the metabolic pathway by which plants produce L-ascorbic acid, was not completely elucidated. The present inventors have demonstrated that L-ascorbic acid production in plants, including L-ascorbic acid-producing microorganisms (e.g., microalgae), is a pathway which uses GDP-D-mannose and involves sugar phosphates and NDP-sugars. In addition, the present inventors have made the surprising discovery that both L-galactose and L-galactono-γ-lactone can be rapidly converted into L-ascorbic acid in L-ascorbic acid-producing microalgae, including *Prototheca* and *Chlorella pyrenoidosa*. The entire pathway for L-ascorbic acid production in plants is set forth in Figs. 1A-1C. More particularly, Fig. 1A shows that the production of L-ascorbic acid in plants proceeds through the production of mannose intermediates to GDP-D-mannose, followed by the conversion of GDP-D-mannose to GDP-L-galactose by GDP-D-mannose:GDP-L-galactose epimerase (also known as GDP-D-mannose-3,5-epimerase) (Fig. 1B), and then by the subsequent progression to L-galactose-1-P, L-galactose, L-galactonic acid (optional), L-galactono-γ-lactone, and L-ascorbic acid (Fig. 1C). Fig. 1B

also illustrates alternate pathways for the use of various intermediates, such as GDP-D-mannose. Certain aspects of this pathway have been independently described in a publication (Wheeler, et al., 1998, *Nature* 393:365-369), incorporated herein by reference in its entirety.

Points within the L-ascorbic acid production pathway which can be targeted by genetic modification to affect the production of L-ascorbic acid can generally be catagorized into at least one of the following pathways: (a) pathways affecting the production of GDP-D-mannose (e.g., pathways for converting a carbon source into GDP-D-mannose); (b) pathways for converting GDP-D-mannose into other compounds, (c) pathways associated with or downstream of the action of GDP-D-mannose:GDP-L-galactose epimerase, (d) pathways which compete for substrates involved in the production of any of the intermediates within the L-ascorbic acid production pathway, and in particular, with GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, L-galactono-γ-lactone, and/or L-ascorbic acid; and (e) pathways which inhibit production of any of the intermediates within the L-ascorbic acid production pathway, and in particular, with GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, L-galactono-γ-lactone, and/or L-ascorbic acid.

A genetically modified plant or microorganism useful in a method of the present invention typically has at least one genetic modification in the L-ascorbic acid production pathway which results in an enhanced production of L-ascorbic acid. In one embodiment, a genetically modified plant or microorganism has at least one genetic modification that results in: (a) an enhanced production of GDP-D-mannose; (b) an inhibition of pathways which convert GDP-D-mannose into compounds other than GDP-L-galactose; (c) an enhancement of action of the GDP-D-mannose:GDP-L-galactose epimerase; (d) an enhancement of the action of enzymes downstream of the GDP-D-mannose:GDP-L-galactose epimerase; (e) an inhibition of pathways which compete for substrates involved in the production of any of the intermediates within the L-ascorbic acid production pathway, and in particular, with GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, L-galactono-γ-lactone, and/or L-ascorbic acid; and (e) an inhibition of pathways which inhibit production of any of the intermediates within the L-ascorbic acid production pathway, and in particular, with GDP-D-mannose, GDP-L-

galactose, L-galactose-1-phosphate, L-galactose, L-galactono-γ-lactone, and/or L-ascorbic acid.

An enhanced production of GDP-D-mannose by genetic modification of the plant or microorganism can be achieved by, for example, overexpression of enzymes such as hexokinase, glucose phosphate isomerase, phosphomannose isomerase (PMI), phosphomannomutase (PMM) and/or GDP-D-mannose pyrophosphorylase (GMP). Inhibition of pathways which convert GDP-D-mannose to compounds other than GDP-L-galactose can be achieved, for example, by modifications which inhibit polysaccharide synthesis, GDP-D-rhamnose synthesis, GDP-L-fucose synthesis and/or GDP-D-mannuronic acid synthesis. An increase in the action of the GDP-D-mannose:GDP-L-galactose epimerase and of enzymes downstream of the epimerase in the L-ascorbic acid production pathway can be achieved by genetic modifications which include, but are not limited to: overexpression of the epimerase gene (i.e, by overexpression of a recombinant nucleic acid molecule encoding the epimerase gene or a homologue thereof (discussed in detail below), and/or by mutation of the endogenous or recombinant gene to enhance expression of the gene) and/or overexpression of genes downstream of the epimerase which encode subsequent enzymes in the L-ascorbic acid pathway. Finally, metabolic pathways which compete with or inhibit the L-ascorbic acid production pathway can be inhibited by deleting or mutating enzymes, substrates or products which either inhibit or compete for an enzyme, substrate or product in the L-ascorbic acid pathway.

As discussed above, a genetically modified plant or microorganism useful in the method of the present invention can have at least one genetic modification (e.g., mutation in the endogenous gene or addition of a recombinant gene) in a gene encoding an enzyme involved in the L-ascorbic acid production pathway. Such genetic modifications preferably increase (i.e., enhance) the action of such enzymes such that L-ascorbic acid is preferentially produced as compared to other possible end products in related metabolic pathways. Such genetic modifications include, but are not limited to, overexpression of the gene encoding such enzyme, and deletion, mutation, or downregulation of genes encoding competitors or inhibitors of such enzyme. Preferred enzymes for which the action of the gene encoding such enzyme can be genetically modified include: hexokinase, glucose phosphate isomerase, phosphomannose isomerase (PMI), phosphomannomutase

(PMM), GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase. More preferably, a genetically modified plant or microorganism useful in the present invention has a genetic modification which increases the action of an enzyme selected from the group of GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and/or L-galactono-γ-lactone dehydrogenase. Even more preferably, a genetically modified plant or microorganism useful in the present invention has a genetic modification which increases the action of GDP-D-mannose:GDP-L-galactose epimerase. These enzymes and the reactions catalyzed by such enzymes are illustrated in Figs. 1A-1C.

Prior to the present invention, without knowing the L-ascorbic acid biosynthetic (i.e., production) pathway, previous mutagenesis and screening efforts were limited in that only non-lethal mutations could be detected. One embodiment of the present invention relates to elimination of a key competing enzyme that diverts carbon flow from L-ascorbic acid synthesis. If such enzyme is absolutely required for growth on glucose, then mutants lacking the enzyme (and, therefore, having increased carbon flow to L-ascorbic acid) would have been nonviable and not have been detected during prior screening efforts. One such enzyme is phosphofructokinase (PFK) (See Fig. 2A). PFK is required for growth on glucose, and is the major step drawing carbon away from L-ascorbic acid biosynthesis (Fig. 2A). Elimination of PFK would render the cells nonviable on glucose-based media. Selection of a conditional mutant where PFK was inactivated by temperature shift, for example, may allow development of a L-ascorbic acid process where cell growth is achieved under permissive fermentation conditions, and L-ascorbic acid production (from glucose) is initiated by a shift to non-permissive condition. In this example, the temperature shift would eliminate carbon flow from glucose to glycolysis via PFK, thereby shunting carbon into the L-ascorbic acid branch of metabolism. This approach has application not only in natural L-ascorbic acid producing organisms, but also in L-ascorbic acid recombinant systems (genetically engineered plant or microorganisms) as discussed herein.

Knowing the identity and mechanism of the rate-limiting pathway enzymes in the L-ascorbic acid production pathway allows for design of specific inhibitors of the enzymes that are also growth inhibitory. Selection of mutants resistant to the inhibitors allows for the isolation of strains that contain L-ascorbic acid-pathway enzymes with more favorable kinetic properties. Therefore, one embodiment of the present invention is to identify inhibitors of the enzymes that are also growth inhibitory. These inhibitors are then used to select genetic mutants that overcome this inhibition and produce L-ascorbic acid at high levels. In this embodiment, the resultant plant or microorganism is a non-recombinant strain which can then be further modified by recombinant technology, if desired. In recombinant L-ascorbic acid producing strains, random mutagenesis and screening can be used as a final step to increase L-ascorbic acid production.

In yet another embodiment genetic modifications are made to an L-ascorbic acid producing organism directly. This allows one to build upon a base of data acquired during prior classical strain improvement efforts, and perhaps more importantly, allows one to take advantage of undefined beneficial mutations that occurred during classical strain improvement. Furthermore, fewer problems are encountered when expressing native, rather than heterologous, genes. The most advanced system for development of genetic systems for microalgae has been developed for *Chlamydomonas reinhardtii*. Preferably, development of such a genetically modified production organism would include: isolation of mutant(s) with a specific nutritional requirement for use with a cloned selectable marker gene (similar to the *ura3* mutants used in yeast and fungal systems); a cloned selectable marker such as *URA3* or alternatively, identification and cloning of a gene that specifies resistance to a toxic compound (this would be analogous to the use of antibiotic resistance genes in bacterial systems, and, as is the case in yeast and other fungi, a means of inserting/removing the marker gene repeatedly would be required, unless several different selectable markers were developed); a transformation system for introducing DNA into the production organism and achieving stable transformation and expression; and, a promoter system (preferably several) for high-level expression of cloned genes in the organism.

Another embodiment of the present invention, discussed in detail below, is to place key genes or allelic variants and homologues thereof from L-ascorbic acid producing

organisms (i.e., higher plants and microalgae) into a plant or microorganism that is more amenable to molecular genetic manipulation, including endogenous L-ascorbic acid producing microorganisms and suitable plants. For example, it is possible to identify a suitable non-pathogenic organism based on the requirement of growth (on glucose) at low pH (i.e., acid-tolerant organisms, discussed in detail below).

One suitable candidate for recombinant production in any suitable host organism is the gene (nucleic acid molecule) encoding GDP-D-mannose:GDP-L-galactose epimerase and homologues of the GDP-D-mannose:GDP-L-galactose epimerase, as well as any other epimerase that has structural homology at the primary (i.e., sequence) or tertiary (i.e., three dimensional) level, to a GDP-4-keto-6-deoxy-D-mannose epimerase/ reductase, or to a UDP-galactose 4-epimerase. Many microorganisms produce GDP-D-mannose as a precursor to exopolysaccharide and glycoprotein production, even though such organisms may not make L-ascorbic acid. This aspect of the present invention is discussed in detail below.

Referring to Figs. 1A-1C, at least some of the enzymes from glucose-6-phosphate to GDP-D-mannose are present in many organisms. In fact, the entire sequence is present in bacteria such as *Azotobacter vinelandii* and *Pseudomonas aeruginosa*, and make up the early steps in the biosynthesis of the exopolysaccharide alginate. In this regard, it is possible that the only thing preventing these organisms from producing L-ascorbic acid could be the lack of GDP-D-mannose:GDP-L-galactose epimerase. The presence of PMI, PMM and GMP (see Fig. 1A) in so many organisms is important for two reasons. First, these organisms themselves could serve as alternate hosts for L-ascorbic acid production, by building on the existing early pathway enzymes and adding the required cloned genes (the epimerase and possibly others). Second, the genes encoding PMI, PMM and GMP can be cloned into a new organism where, together with the cloned epimerase, they would encode the overall pathway from glucose-6-phosphate to GDP-L- galactose.

In order to screen genomic DNA or cDNA libraries from different organisms and to isolate nucleic acid molecules encoding these enzymes such as the GDP-D-mannose:GDP-L-galactose epimerase, one can use any of a variety of standard molecular and biochemical techniques. For example, the GDP-D-mannose:GDP-L-galactose epimerase can be purified from an organism such as *Prototheca*, the N-terminal amino

acid sequence can be determined (including, if necessary, the sequence of internal peptide fragments), and this information can be used to design degenerate primers for amplifying a gene fragment from the organism's DNA. This fragment would then be used to probe the library, and subsequently fragments that hybridize to the probe would be cloned in that organism or another suitable production organism. There is ample precedent for plant enzymes being expressed in an active form in bacteria, such as *E. coli*. Alternatively, yeast are also a suitable candidate for developing a heterologous system for L-ascorbic acid production.

It is to be understood that the present invention discloses a method comprising the use of a microorganism with an ability to produce commercially useful amounts of L-ascorbic acid in a fermentation process (i.e., preferably an enhanced ability to produce L-ascorbic acid compared to a wild-type microorganism cultured under the same conditions). This method is achieved by the genetic modification of one or more genes encoding a protein involved in an L-ascorbic acid pathway which results in the production (expression) of a protein having an altered (e.g., increased or decreased) function as compared to the corresponding wild-type protein. Preferably, such genetic modification is achieved by recombinant technology. It will be appreciated by those of skill in the art that production of genetically modified plants or microorganisms having a particular altered function as described elsewhere herein (e.g., an enhanced ability to produce GDP-D-mannose:GDP-L-galactose epimerase), such as by transformation of the plant or microorganism with a nucleic acid molecule which encodes a particular enzyme, can produce many organisms meeting the given functional requirement, albeit by virtue of a variety of different genetic modifications. For example, different random nucleotide deletions and/or substitutions in a given nucleic acid sequence may all give rise to the same phenotypic result (e.g., decreased enzymatic activity of the protein encoded by the sequence). The present invention contemplates any such genetic modification which results in the production of a plant or microorganism having the characteristics set forth herein.

A microorganism to be used in the fermentation method of the present invention is preferably a bacterium, a fungus, or a microalga which has been genetically modified according to the disclosure above. More preferably, a microorganism useful in the present

invention is a microalga which is capable of producing L-ascorbic acid, although the present invention includes microorganisms which are genetically engineered to produce L-ascorbic acid using the knowledge of the key components of the pathway and the guidance provided herein. Even more preferably, a microorganism useful in the present invention is an acid-tolerant microorganism, such as microalgae of the genera *Prototheca* and *Chlorella*. Acid-tolerant yeast and bacteria are also known in the art. Acid-tolerant microorganisms are discussed in detail below. Particularly preferred microalgae include microalgae of the genera, *Prototheca* and *Chlorella*, with *Prototheca* being most preferred. All known species of *Prototheca* produce L-ascorbic acid. Production of ascorbic acid by microalgae of the genera *Prototheca* and *Chlorella* is described in detail in U.S. Patent No. 5,792,631, issued August 11, 1998, and in U.S. Patent No. 5,900,370, issued May 4, 1999, both of which are incorporated herein by reference in their entirety. Preferred bacteria for use in the present invention include, but are not limited to, *Azotobacter*, *Pseudomonas*, and *Escherichia*, although acid-tolerant bacteria are more preferred. Preferred fungi for use in the present invention include yeast, and more preferably, yeast of the genus, *Saccharomyces*. A microorganism for use in the fermentation method of the present invention can also be referred to as a production organism. According to the present invention, microalgae can be referred to herein either as microorganisms or as plants.

A preferred plant to genetically modify according to the present invention is preferably a plant suitable for consumption by animals, including humans. More preferably, such a plant is a plant that naturally produces L-ascorbic acid, although other plants can be genetically modified to produce L-ascorbic acid using the guidance provided herein.

The L-ascorbic acid production pathways of the microalgae *Prototheca* and *Chlorella pyrenoidosa* will be addressed as specific embodiments of the present invention are described below. It will be appreciated that other plants and, in particular, other microorganisms, have similar L-ascorbic acid pathways and genes and proteins having similar structure and function within such pathways. It will also be appreciated that plants and microorganisms which do not naturally produce L-ascorbic acid can be modified according to the present invention to produce L-ascorbic acid. As such, the principles

discussed below with regard to *Prototheca* and *Chlorella pyrenoidosa* are applicable to other plants and microorganisms, including genetically modified plants and microorganisms.

In one embodiment of the present invention, the action of an enzyme in the L-ascorbic acid production pathway is increased by amplification of the expression (i.e., overexpression) of an enzyme in the pathway, and particularly, the GDP-D-mannose:GDP-L-galactose epimerase, homologues of the epimerase, and/or enzymes downstream of the epimerase. Overexpression of an enzyme can be accomplished, for example, by introduction of a recombinant nucleic acid molecule encoding the enzyme. It is preferred that the gene encoding an enzyme in the L-ascorbic acid production pathway be cloned under control of an artificial promoter. The promoter can be any suitable promoter that will provide a level of enzyme expression required to maintain a sufficient level of L-ascorbic acid in the production organism. Preferred promoters are constitutive (rather than inducible) promoters, since the need for addition of expensive inducers is therefore obviated. The gene dosage (copy number) of a recombinant nucleic acid molecule according to the present invention can be varied according to the requirements for maximum product formation. In one embodiment, the recombinant nucleic acid molecule encoding a gene in the L-ascorbic acid production pathway is integrated into the chromosomes of the microorganism.

It is another embodiment of the present invention to provide a microorganism having one or more enzymes in the L-ascorbic acid production pathway with improved affinity for its substrates. An enzyme with improved affinity for its substrates can be produced by any suitable method of genetic modification or protein engineering. For example, computer-based protein engineering can be used to design an epimerase protein with greater stability and better affinity for its substrate. See for example, Maulik et al., 1997, *Molecular Biotechnology: Therapeutic Applications and Strategies*, Wiley-Liss, Inc., which is incorporated herein by reference in its entirety.

Recombinant nucleic acid molecules encoding proteins in the L-ascorbic acid production pathway can be modified to enhance or reduce the function (i.e., activity) of the protein, as desired to increase L-ascorbic acid production, by any suitable method of genetic modification. For example, a recombinant nucleic acid molecule encoding an

enzyme can be modified by any method for inserting, deleting, and/or substituting nucleotides, such as by error-prone PCR. In this method, the gene is amplified under conditions that lead to a high frequency of misincorporation errors by the DNA polymerase used for the amplification. As a result, a high frequency of mutations are obtained in the PCR products. The resulting gene mutants can then be screened for enhanced substrate affinity, enhanced enzymatic activity, or reduced/increased inhibitory ability by testing the mutant genes for the ability to confer increased L-ascorbic acid production onto a test microorganism, as compared to a microorganism carrying the non-mutated recombinant nucleic acid molecule.

Another embodiment of the present invention includes a microorganism in which competitive side reactions are blocked, including all reactions for which GDP-D-mannose is a substrate other than the production of L-ascorbic acid. In a preferred embodiment, a microorganism having complete or partial inactivation (decrease in the action of) of genes encoding enzymes which compete with the GDP-D-mannose:GDP-L-galactose epimerase for the GDP-D-mannose substrate is provided. Such enzymes include GDP-D-mannase and/or GDP-D-mannose-dehydrogenase. As used herein, inactivation of a gene can refer to any modification of a gene which results in a decrease in the activity (i.e., expression or function) of such a gene, including attenuation of activity or complete deletion of activity.

As discussed above, a particularly preferred aspect of the method to produce L-ascorbic acid by fermentation of a genetically modified microorganism of the present invention includes the step of culturing in a fermentation medium a microorganism having a genetic modification to increase the action of an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose. According to the present invention, such an epimerase can include the endogenous GDP-D-mannose:GDP-L-galactose epimerase from the L-ascorbic acid pathway, described above, as well as any other epimerase that has structural homology at the primary (i.e., sequence) or tertiary (i.e., three dimensional) level, to a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase, or to a UDP-galactose 4-epimerase. Such structural homology is discussed in detail below. Preferably, such an epimerase is capable of catalyzing the conversion of GDP-D-mannose to GDP-L-galactose. In one embodiment, the genetic modification includes transformation of the

microorganism with a recombinant nucleic acid molecule that expresses such an epimerase.

Therefore, the epimerase encompassed in the method and organisms of the present invention includes the endogenous epimerase which operates in the naturally occurring ascorbic acid biosynthetic pathway (referred to herein as GDP-D-mannose:GDP-L-galactose epimerase), GDP-4-keto-6-deoxy-D-mannose epimerase/reductases, and any other epimerase which is capable of catalyzing the conversion of GDP-D mannose to GDP-L-galactose and which is structurally homologous to a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or a UDP-galactose 4-epimerase. An epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose according the present invention can be identified by biochemical and functional characteristics as well as structural characteristics. For example, an epimerase according to the present invention is capable of acting on GDP-D-mannose as a substrate, and more particularly, such an epimerase is capable of catalyzing the conversion of GDP-D-mannose to GDP-L-galactose. It is to be understood that such capabilities need not necessarily be the normal or natural function of the epimerase as it acts in its endogenous (i.e., natural) environment. For example, GDP-4-keto-6-deoxy-D-mannose epimerase/reductase in its natural environment under normal conditions, catalyzes the conversion of GDP-D-mannose to GDP-L-fucose and does not act directly on GDP-D-mannose (See Fig.8A, B), however, such an epimerase is encompassed by the present invention for use in catalyzing the conversion of GDP-D-mannose to GDP-L-galactose for production of ascorbic acid, to the extent that it is capable of, or can be modified to be capable of, catalyzing the conversion of GDP-D-mannose to GDP-L-galactose. Therefore, the present invention includes epimerases which have the desired enzyme activity for use in production of ascorbic acid, are capable of having such desired enzyme activity, and/or are capable of being modified or induced to have such desired enzyme activity.

In one embodiment, an epimerase according to the present invention includes an epimerase that catalyzes the reaction depicted in Fig. 7. In another embodiment, an epimerase according to the present invention includes an epimerase that catalyzes the first of the reactions depicted in Fig. 8B. In one embodiment, an epimerase according to the

present invention binds to NADPH. In another embodiment, an epimerase according to the present invention is NADPH-dependent for enzyme activity.

As discussed above, the present inventors have discovered that a key enzyme in L-ascorbic acid biosynthesis in plants and microorganisms is GDP-D-mannose:GDP-L-galactose epimerase (refer to Figs. 1A-1C). One embodiment of the invention described herein is directed to the manipulation of this enzyme and structural homologues of this enzyme to increase L-ascorbic acid production in genetically engineered plants and/or microorganisms. More particularly, the GDP-D-mannose:GDP-L-galactose epimerase of the L-ascorbic acid pathway and GDP-4-keto-6-deoxy-D-mannose epimerase/reductases are believed to be structurally homologous at both the sequence and tertiary structure level; a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase is believed to be capable of functioning in the L-ascorbic acid biosynthetic pathway; and a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or homologue thereof may be superior to a GDP-D-mannose-GDP-L-galactose epimerase for increasing L-ascorbic acid production in genetically engineered plants and/or microorganisms. Furthermore, the present inventors disclose the use of a nucleotide sequence encoding all or part of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase as a probe to identify the gene encoding GDP-D-mannose:GDP-L-galactose epimerase. Similarly, the present inventors disclose the use of a nucleotide sequence of the gene encoding GDP-4-keto-6-deoxy-D-mannose epimerase/reductase to design oligonucleotide primers for use in a PCR-based strategy for identifying and cloning a gene encoding GDP-D-mannose:GDP-L-galactose epimerase.

Without being bound by theory, the present inventors believe that the following evidence supports the novel concept that the GDP-D-mannose:GDP-L-galactose epimerase and GDP-4-keto-6-deoxy-D-mannose epimerase/reductases have significant structural homology at the level of sequence and/or tertiary structure, and that the GDP-4-keto-6-deoxy-D-mannose epimerase/reductases and/or homologues thereof would be useful for production of ascorbic acid and/or for isolating the endogenous GDP-D-mannose:GDP-L-galactose epimerase.

Although prior to the present invention, it was not known that the GDP-D-mannose:GDP-L-galactose epimerase enzyme (also known as GDP-D-mannose-3,5-epimerase) plays a critical role in L-ascorbic acid biosynthesis, this enzyme was previously

described to catalyze the overall reversible reaction between GDP-D-mannose and GDP-L-galactose (Barber, 1971, *Arch. Biochem. Biophys.* 147:619-623; Barber, 1975, *Arch. Biochem. Biophys.* 167:718-722; Barber, 1979, *J. Biol. Chem.* 254:7600-7603; Hebda, et al., 1979, *Arch. Biochem. Biophys.* 194:496-502; Barber and Hebda, 1982, *Meth. Enzymol.*, 83:522-525). Despite these studies, GDP-D-mannose:GDP-L-galactose epimerase has never been well characterized nor has the gene encoding this enzyme been cloned and sequenced. Since the original work by Barber, GDP-D-mannose:GDP-L-galactose epimerase activity has been detected in the colorless microalga *Prototheca moriformis* by the assignee of the present application, and in *Arabidopsis thaliana* and pea embryonic axes (Wheeler, et al., 1998, *ibid.*).

Barber (1979, *J. Biol. Chem.* 254:7600-7603) proposed a mechanism for GDP-D-mannose:GDP-L-galactose epimerase partially purified from the green microalga *Chlorella pyrenoidosa*. The overall conversion of GDP-D-mannose to GDP-L-galactose was proposed to proceed by oxidation of the hexosyl moiety at C-4 to a keto intermediate, ene-diol formation, and inversion of the configurations at C-3 and C-5 upon rehydration of the double bonds and stereospecific reduction of the keto group. The proposed mechanism is depicted in Fig. 7.

Based on Barber's work, Feingold and Avigad (1980, In *The Biochemistry of Plants*, Vol. 3: Carbohydrates; Structure and Function, P.K. Stompf and E.E. Conn, eds., Academic Press, NY) elaborated further on the proposed mechanism for GDP-D-mannose:GDP-L-galactose epimerase. This mechanism is based on the assumption that the epimerase contains tightly bound NAD^+, and transfer of a hydride ion from C-4 of the substrate (GDP-D-mannose) to enzyme-associated NAD^+ converts the enzyme to the reduced (NADH)form, generating enzyme-bound GDP-4-keto-D-mannose. The latter would then undergo epimerization by an ene-diol mechanism. The final product (GDP-L-galactose) would be released from the enzyme after stereospecific transfer of the hydride ion originally removed from C-4, simultaneously regenerating the oxidized form of the enzyme.

L-fucose (6-deoxy-L-galactose) is a component of bacterial lipopolysaccharides, mammalian and plant glycoproteins and polysaccharides of plant cell walls. L-fucose is synthesized *de novo* from GDP-D-mannose by the sequential action of GDP-D-mannose-

4,6-dehydratase (an NAD(P)-dependent enzyme), and a bifunctional GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (NADPH-dependent), also referred to in scientific literature as GDP-fucose synthetase (Rizzi, et al., 1998, *Structure* 6:1453-1465; Somers, et al., 1998, *Structure* 6:1601-1612). This pathway for L-fucose biosynthesis appears to be ubiquitous (Rizzi, et al., 1998, *Structure* 6:1453-1465). The mechanisms for GDP-D-mannose-4,6-dehydratase and GDP-4-keto-6-deoxy-D-mannose epimerase/reductase are shown in Fig. 8A, B (Chang, et al., 1988, *J. Biol. Chem.* 263:1693-1697; Barber, 1980, *Plant Physiol.* 66:326-329).

Comparison of Figs. 7 and 8A, B reveals that Barber's proposed mechanism for GDP-D-mannose:GDP-L-galactose epimerase is analogous to the reaction mechanism for GDP-4-keto-6-deoxy-D-mannose epimerase/reductase. The same mechanism has also been demonstrated for the epimerization reaction that occurs in the biosynthesis of two TDP-6-deoxy hexoses, TDP-L-rhamnose and TDP-6-deoxy-L-talose, from TDP-D-glucose (Liu and Thorson, 1994, *Ann. Rev. Microbiol.* 48:223-256). In the latter cases, however, the final reduction at C-4 is catalyzed by NADPH-dependent reductases that are separate from the epimerase enzyme. These reductases have opposite stereospecificity, providing either TDP-L-rhamnose or TDP-6-deoxy-L-talose (Liu and Thorson, 1994, *Ann. Rev. Microbiol.* 48:223-256).

In all of the mechanisms described above, NAD(P)H is required for the final reduction at C-4 (refer to Fig. 8B). In the work of Hebda, et al. (1979, *Arch. Biochem. Biophys.* 194:496-502), it was reported that GDP-D-mannose:GDP-L-galactose epimerase from *C. pyrenoidosa* did not require NAD, NADP or NADH for activity. Strangely, NADPH was not tested. Based on the analogous mechanisms shown in Figs. 7 and 8A, B, the present inventors believe that it is likely that GDP-D-mannose:GDP-L-galactose epimerase from *C. pyrenoidosa* requires NADPH for the final reduction step. Why activity was detected *in vitro* without NADPH addition is not known, but tight *binding of NADPH to the enzyme could explain this observation. On the other hand, if the proposed mechanism of Feingold and Avigad (1980, in *The Biochemistry of Plants*, Vol. 3, p. 101-170: Carbohydrates; Structure and Function, P.K. Stompf and E.E. Conn, ed., Academic Press, NY) is correct, the reduced enzyme-bound cofactor generated in the first oxidation step of the epimerase reaction would serve as the source of electrons for

the final reduction of the keto group at C-4 back to the alcohol. Thus no addition of exogenous reduced cofactor would be required for activity *in vitro*.

Recently, a human gene encoding the bifunctional GDP-4-keto-6-deoxy-D-mannose epimerase/reductase was cloned and sequenced (Tonetti, et al., 1996, *J. Biol. Chem.* 271-27274-27279). This amino acid sequence of the human GDP-4-keto-6-deoxy-D-mannose epimerase/reductase shows significant homology (29% identity) to the *E. coli* GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (Tonetti, et al., 1998, *Acta Cryst.* D54:684-686; Somers, et al., 1998, *Structure* 6:1601-1612, both of which are incorporated herein by reference in their entireties). Tonetti et al. and Somers et al. additionally disclosed the tertiary (three dimensional) structure of the *E. coli* GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (also known as GDP-fucose synthetase), and noted significant structural homology with another epimerase, UDP-galactose 4-epimerase (GalE). These epimerases also share significant homology at the sequence level. Since no gene encoding a GDP-D-mannose:GDP-L-galactose epimerase has been cloned and sequenced, homology with genes encoding GDP-4-keto-6-deoxy-D-mannose epimerase/reductases or with genes encoding a UDP-galactose 4-epimerase has not been demonstrated. However, based on the similarity of the reaction products for GDP-D-mannose:GDP-L-galactose epimerase and GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (i.e., GDP-L-galactose and GDP-6-deoxy-L-galactose [i.e., GDP-L-fucose], respectively) and the common catalytic mechanisms (Figs. 7 and 8A, B) the present inventors believe that the genes encoding the enzymes will have a high degree of sequence homology, as well as tertiary structural homology.

Significant structural homology between GDP-D-mannose:GDP-L-galactose epimerase and GDP-4-keto-6-deoxy-D-mannose epimerase/reductases may allow a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase, or a homologue thereof, to function in the L-ascorbic acid biosynthetic pathway, and a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase could potentially be even better than a GDP-D-mannose-GDP-L-galactose epimerase for increasing L-ascorbic acid production in genetically engineered plants and/or microorganisms. Furthermore, a nucleotide sequence encoding all or part of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase can be used as a probe to identify the gene encoding GDP-D-mannose:GDP-L-galactose epimerase. Likewise, the

nucleotide sequence of the gene encoding GDP-4-keto-6-deoxy-D-mannose epimerase/ reductase can be used to design oligonucleotide primers for use in a PCR-based strategy for identifying and cloning a gene encoding GDP-D-mannose:GDP-L-galactose epimerase.

The ability to substitute GDP-4-keto-6-D-mannose epimerase/reductase for GDP-D-mannose:GDP-L-galactose epimerase to enhance L-ascorbic acid biosynthesis in plants or microorganisms depends on the ability of GDP-4-keto-6-deoxy-D-mannose epimerase/ reductase to act directly on GDP-D-mannose to form GDP-L-galactose. Evidence supporting this possibility already exists. *Arabidopsis thaliana murl* mutants are defective in GDP-D-mannose-4,6-dehydratase activity (Bonin, et al., 1997, *Proc. Natl. Acad. Sci.* 94:2085-2090). These mutants are thus blocked in GDP-L-fucose biosynthesis, and consequently have less than 2% of the normal amounts of L-fucose in the primary cell walls of aerial portions of the plant (Zablackis, et al., 1996, *Science* 272:1808-1810). The *murl* mutants are more brittle than wild-type plants, are slightly dwarfed and have an apparently normal life cycle (Zablackis, et al., 272:1808-1810). When *murl* mutants are grown in the presence of exogenous L-fucose, the L-fucose content in the plant is restored to the wild-type state (Bonin, et al., 1997, *Proc. Natl. Acad. Sci.* 94:2085-2090). It was discovered (Zablackis, et al., 1996, *Science* 272:1808-1810) that *murl* mutants contain, in the hemicellulose xyloglucan component of the primary cell wall, L-galactose in place of the normal L-fucose. L-galactose is not normally found in the xyloglucan component, but in *murl* mutants L-galactose partly replaces the terminal L-fucosyl residue. Bonin, et al. (1997, *Proc. Natl. Acad. Sci.* 94:2085-2090) hypothesized that in the absence of a functional GDP-D-mannose-4,6-dehydratase in the *murl* mutants, the GDP-4-keto-6-deoxy-D-mannose epimerase/reductase normally involved in L-fucose synthesis may be able to use GDP-D-mannose directly, forming GDP-L-galactose. Another possibility, however, is that the enzymes involved in L-ascorbic acid biosynthesis in *A. thaliana* are responsible for forming GDP-L-galactose in the *murl* mutant. If this were true, it would suggest that in the wild-type plant, some mechanism exists that prevents GDP-L-galactose formed in the L-ascorbic acid pathway from entering cell wall biosynthesis and substituting for (competing with) GDP-L-fucose for incorporation into the xyloglucan

component (since L-galactose is not present in the primary cell wall of the wild-type plant).

Because of the similar reaction mechanisms of GDP-D-mannose:GDP-L-galactose epimerase and GDP-4-keto-6-deoxy-D-mannose epimerase/reductase, and because of the evidence that GDP-4-keto-6-deoxy-D-mannose epimerase/reductase can act directly on GDP-D-mannose to form GDP-L-galactose, the present inventors believe that genes encoding all epimerases and epimerase/reductases that act on GDP-D-mannose have high homology. As such, one aspect of the present invention relates to the use of any epimerase (and nucleic acid sequences encoding such epimerase) having significant homology (at the primary, secondary and/or tertiary structure level) to a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or to a UDP-galactose 4-epimerase for the purpose of improving the biosynthetic production of L-ascorbic acid.

Therefore, as described above, one embodiment of the present invention relates to a method for producing ascorbic acid or esters thereof in a microorganism, which includes culturing a microorganism having a genetic modification to increase the action of an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose. Also included in the present invention are genetically modified microorganisms and plants in which the genetic modification increases the action of an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose.

According to the present invention, an increase in the action of the GDP-D-mannose:GDP-L-galactose epimerase in the L-ascorbic acid production pathway can be achieved by genetic modifications which include, but are not limited to overexpression of the GDP-D-mannose:GDP-L-galactose epimerase gene, a homologue of such gene, or of any recombinant nucleic acid sequence encoding an epimerase that is homologous in primary (nucleic acid or amino acid sequence) or tertiary (three dimensional protein) structure to a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or a UDP-galactose 4-epimerase, such as by overexpression of a recombinant nucleic acid molecule encoding the epimerase gene or a homologue thereof, and/or by mutation of the endogenous or recombinant gene to enhance expression of the gene.

According to the present invention, an epimerase that has a tertiary structure that is homologous to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/

reductase is an epimerase that has a tertiary structure that substantially conforms to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws (Table 12). In another embodiment, an epimerase that has a tertiary structure that is homologous to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase is an epimerase that has a tertiary structure that substantially conforms to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1GFS. As used herein, a "tertiary structure" or "three dimensional structure" of a protein, such terms being interchangeable, refers to the components and the manner of arrangement of the components in three dimensional space to constitute the protein. The use of the term "substantially conforms" refers to at least a portion of a tertiary structure of an epimerase which is sufficiently spatially similar to at least a portion of a specified three dimensional configuration of a particular set of atomic coordinates (e.g., those represented by Brookhaven Protein Data Bank Accession Code 1bws) to allow the tertiary structure of at least said portion of the epimerase to be modeled or calculated (i.e., by molecular replacement) using the particular set of atomic coordinates as a basis for estimating the atomic coordinates defining the three dimensional configuration of the epimerase.

More particularly, a tertiary structure that substantially conforms to a given set of atomic coordinates is a structure having an average root-mean-square deviation (RMSD) of less than about 2.5 Å, and more preferably, less than about 2 Å, and, in increasing preference, less than about 1.5 Å, less than about 1 Å, less than about 0.5 Å, and most preferably, less than about 0.3 Å, over at least about 25% of the Cα positions as compared to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. In other embodiments, a structure that substantially conforms to a given set of atomic coordinates is a structure wherein such structure has the recited average root-mean-square deviation (RMSD) value over at least about 50% of the Cα positions as compared to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws, and in another embodiment, such structure has the

recited average root-mean-square deviation (RMSD) value over at least about 75% of the Cα positions as compared to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws, and in another embodiment, such structure has the recited average root-mean-square deviation (RMSD) value over about 100% of the Cα positions as compared to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. Methods to calculate RMSD values are well known in the art. Various software programs for determining the tertiary structural homology between one or more proteins are known in the art and are publicly available, such as QUANTA (Molecular Simulations Inc.).

A preferred epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose according to the method and genetically modified organisms of the present invention includes an epimerase that comprises a substrate binding site having a tertiary structure that substantially conforms to the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. Preferably, the tertiary structure of the substrate binding site of the epimerase has an average root-mean-square deviation (RMSD) of less than about 2.5 Å, and more preferably, less than about 2 Å, and, in increasing preference, less than about 1.5 Å, less than about 1 Å, less than about 0.5 Å, and most preferably, less than about 0.3 Å, over at least about 25% of the Cα positions as compared to the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. In other embodiments, the tertiary structure of the substrate binding site of the epimerase has the recited average root-mean-square deviation (RMSD) value over at least about 50% of the Cα positions as compared to the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws, and in another embodiment, the tertiary structure of the substrate binding site of the epimerase has the recited average root-mean-square deviation (RMSD) value over at least about 75% of the Cα positions

as compared to the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws, and in another embodiment, the tertiary structure of the substrate binding site of the epimerase has the recited average root-mean-square deviation (RMSD) value over about 100% of the Cα positions as compared to the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. The tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws is discussed in detail in Rizzi et al., 1998, *ibid.* Additionally, the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1GFS is discussed in detail in Somers et al., 1998, *ibid.*

Another preferred epimerase according to the present invention includes an epimerase that comprises a catalytic site having a tertiary structure that substantially conforms to the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. Preferably, the tertiary structure of the catalytic site of the epimerase has an average root-mean-square deviation (RMSD) of less than about 2.5 Å, and more preferably, less than about 2 Å, and, in increasing preference, less than about 1.5 Å, less than about 1 Å, less than about 0.5 Å, and most preferably, less than about 0.3 Å, over at least about 25% of the Cα positions as compared to the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws. In other embodiments, the tertiary structure of the catalytic site of the epimerase has the recited average root-mean-square deviation (RMSD) value over at least about 50% of the Cα positions as compared to the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws, and in another embodiment, the tertiary structure of the catalytic site of the epimerase has the recited

average root-mean-square deviation (RMSD) value over at least about 75% of the Cα positions as compared to the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws, and in another embodiment, the tertiary structure of the catalytic site of the epimerase has the recited average root-mean-square deviation (RMSD) value over 100% of the Cα positions as compared to the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

In one embodiment, an epimerase encompassed by the present invention includes an epimerase that has a catalytic site which includes amino acid residues: serine, tyrosine and lysine. In a preferred embodiment, the tertiary structure positions of the amino acid residues serine, tyrosine and lysine substantially conform to the tertiary structure position of residues Ser107, Tyr136 and Lys140, respectively, as represented by atomic coordinates in Brookhaven Protein Data Bank Accession Code 1bws. The tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws is discussed in detail in Rizzi et al., 1998, *ibid.* Additionally, the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by the atomic coordinates having Brookhaven Protein Data Bank Accession Code 1GFS is discussed in detail in Somers et al., 1998, *ibid.*

In an even more preferred embodiment, the above definition of "substantially conforms" can be further defined to include atoms of amino acid side chains. As used herein, the phrase "common amino acid side chains" refers to amino acid side chains that are common to both the structures which substantially conforms to a given set of atomic coordinates and the structure that is actually represented by such atomic coordinates. Preferably, a tertiary structure that substantially conforms to a given set of atomic coordinates is a structure having an average root-mean-square deviation (RMSD) of less than about 2.5 Å, and more preferably, less than about 2 Å, and, in increasing preference, less than about 1.5 Å, less than about 1 Å, less than about 0.5 Å, and most preferably, less than about 0.3 Å over at least about 25% of the common amino acid side chains as

compared to the tertiary structure represented by the given set of atomic coordinates. In another embodiment, a structure that substantially conforms to a given set of atomic coordinates is a structure having the recited average root-mean-square deviation (RMSD) value over at least about 50% of the common amino acid side chains as compared to the tertiary structure represented by the given set of atomic coordinates, and in another embodiment, such structure has the recited average root-mean-square deviation (RMSD) value over at least about 75% of the common amino acid side chains as compared to the tertiary structure represented by the given set of atomic coordinates, and in another embodiment, such a structure has the recited average root-mean-square deviation (RMSD) value over 100% of the common amino acid side chains as compared to the tertiary structure represented by the given set of atomic coordinates.

A tertiary structure of an epimerase which substantially conforms to a specified set of atomic coordinates can be modeled by a suitable modeling computer program such as MODELER (A. Sali and T.L. Blundell, *J. Mol. Biol.*, vol. 234:779-815, 1993 as implemented in the Insight II Homology software package (Insight II (97.0), MSI, San Diego)), using information, for example, derived from the following data: (1) the amino acid sequence of the epimerase; (2) the amino acid sequence of the related portion(s) of the protein represented by the specified set of atomic coordinates having a three dimensional configuration; and, (3) the atomic coordinates of the specified three dimensional configuration. Alternatively, a tertiary structure of an epimerase which substantially conforms to a specified set of atomic coordinates can be modeled using data generated from analysis of a crystallized structure of the epimerase. A tertiary structure of an epimerase which substantially conforms to a specified set of atomic coordinates can also be calculated by a method such as molecular replacement. Methods of molecular replacement are generally known by those of skill in the art (generally described in Brunger, *Meth. Enzym.*, vol. 276, pp. 558-580, 1997; Navaza and Saludjian, *Meth. Enzym.*, vol. 276, pp. 581-594, 1997; Tong and Rossmann, *Meth. Enzym.*, vol. 276, pp. 594-611, 1997; and Bentley, *Meth. Enzym.*, vol. 276, pp. 611-619, 1997, each of which are incorporated by this reference herein in their entirety) and are performed in a software program including, for example, XPLOR (Brunger, et al., *Science*, vol. 235, p. 458, 1987). In addition, a structure can be modeled using techniques generally described by,

for example, Sali, *Current Opinions in Biotechnology*, vol. 6, pp. 437-451, 1995, and algorithms can be implemented in program packages such as Homology 95.0 (in the program Insight II, available from Biosym/MSI, San Diego, CA). Use of Homology 95.0 requires an alignment of an amino acid sequence of a known structure having a known three dimensional structure with an amino acid sequence of a target structure to be modeled. The alignment can be a pairwise alignment or a multiple sequence alignment including other related sequences (for example, using the method generally described by Rost, *Meth. Enzymol.*, vol. 266, pp. 525-539, 1996) to improve accuracy. Structurally conserved regions can be identified by comparing related structural features, or by examining the degree of sequence homology between the known structure and the target structure. Certain coordinates for the target structure are assigned using known structures from the known structure. Coordinates for other regions of the target structure can be generated from fragments obtained from known structures such as those found in the Protein Data Bank maintained by Brookhaven National Laboratory, Upton, NY. Conformation of side chains of the target structure can be assigned with reference to what is sterically allowable and using a library of rotamers and their frequency of occurrence (as generally described in Ponder and Richards, *J. Mol. Biol.*, vol. 193, pp. 775-791, 1987). The resulting model of the target structure, can be refined by molecular mechanics (such as embodied in the program Discover, available from Biosym/MSI) to ensure that the model is chemically and conformationally reasonable.

According to the present invention, an epimerase that has a nucleic acid sequence that is homologous at the primary structure level (i.e., is a homologue of) to a nucleic acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or a UDP-galactose 4-epimerase includes any epimerase encoded by a nucleic acid sequence that is at least about 15%, and preferably at least about 20%, and more preferably at least about 25%, and even more preferably, at least about 30% identical to a nucleic acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or a UDP-galactose 4-epimerase, and preferably to a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9. Similarly, an epimerase that has an amino acid sequence that is homologous to an amino acid sequence of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or a UDP-

galactose 4-epimerase includes any epimerase having an amino acid sequence that is at least about 15%, and preferably at least about 20%, and more preferably at least about 25%, and even more preferably, at least about 30% identical to an amino acid sequence of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or a UDP-galactose 4-epimerase, and preferably to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:10.

According to one embodiment of the present invention, homology or percent identity between two or more nucleic acid or amino acid sequences is performed using methods known in the art for aligning and/or calculating percentage identity. To compare the homology/percent identity between two or more sequences as set forth above, for example, a module contained within DNASTAR (DNASTAR, Inc., Madison, Wisconsin) can be used. In particular, to calculate the percent identity between two nucleic acid or amino acid sequences, the Lipman-Pearson method, provided by the MegAlign module within the DNASTAR program, is preferably used, with the following parameters, also referred to herein as the Lipman-Pearson standard default parameters:

(1) Ktuple = 2;

(2) Gap penalty = 4;

(3) Gap length penalty = 12.

Using the Lipman-Pearson method with these parameters, for example, the percent identity between the amino acid sequence for *E. coli* GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (SEQ ID NO:4) and human GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (FX) (SEQ ID NO:6) is 27.7%, which is comparable to the 27% identity described for these enzymes in Tonetti et al., 1998, *Acta Cryst.* D54:684-686.

According to another embodiment of the present invention, to align two or more nucleic acid or amino acid sequences, for example to generate a consensus sequence or evaluate the similarity at various positions between such sequences, a CLUSTAL alignment program (e.g., CLUSTAL, CLUSTAL V, CLUSTAL W), also available as a module within the DNASTAR program, can be used using the following parameters, also referred to herein as the CLUSTAL standard default parameters:

Multiple Alignment Parameters (i.e., for more than 2 sequences):

(1) Gap penalty = 10;

(2) Gap length penalty = 10;

Pairwise Alignment Parameters (i.e., for two sequences):

(1) Ktuple = 1;

(2) Gap penalty = 3;

(3) Window = 5;

(4) Diagonals saved = 5.

According to the present invention, a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase can be a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from any organism, including *Arabidopsis thaliana*, *Escherichia coli*, and human. A nucleic acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from *Arabidopsis thaliana* is represented herein by SEQ ID NO:1. SEQ ID NO:1 encodes a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase having an amino acid sequence represented herein as SEQ ID NO:2. A nucleic acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from *Escherichia coli* is represented herein by SEQ ID NO:3. SEQ ID NO:3 encodes a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase having an amino acid sequence represented herein as SEQ ID NO:4. A nucleic acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from *homo sapiens* is represented herein by SEQ ID NO:5. SEQ ID NO:5 encodes a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase having an amino acid sequence represented herein as SEQ ID NO:6.

According to the present invention, a UDP-galactose 4-epimerase can be a UDP-galactose 4-epimerase from any organism, including *Escherichia coli* and human. A nucleic acid sequence encoding a UDP-galactose 4-epimerase from *Escherichia coli* is represented herein by SEQ ID NO:7. SEQ ID NO:7 encodes a UDP-galactose 4-epimerase having an amino acid sequence represented herein as SEQ ID NO:8. A nucleic acid sequence encoding a UDP-galactose 4-epimerase from *homo sapiens* is represented herein by SEQ ID NO:9. SEQ ID NO:9 encodes a UDP-galactose 4-epimerase having an amino acid sequence represented herein as SEQ ID NO:10.

In a preferred embodiment, an epimerase encompassed by the present invention has an amino acid sequence that aligns with the amino acid sequence of SEQ ID NO:11, for example using a CLUSTAL alignment program, wherein amino acid residues in the

amino acid sequence of the epimerase align with 100% identity with at least about 50% of non-Xaa residues in SEQ ID NO:11, and preferably at least about 75% of non-Xaa residues in SEQ ID NO:11, and more preferably, at least about 90% of non-Xaa residues in SEQ ID NO:11, and even more preferably 100% of non-Xaa residues in SEQ ID NO:11. The percent identity of residues aligning with 100% identity with non-Xaa residues can be simply calculated by dividing the number of 100% identical matches at non-Xaa residues in SEQ ID NO:11 by the total number of non-Xaa residues in SEQ ID NO:11. A preferred nucleic acid sequence encoding an epimerase encompassed by the present invention include a nucleic acid sequence encoding an epimerase having an amino acid sequence with the above described identity to SEQ ID NO:11. Such an alignment using a CLUSTAL alignment program is based on the same parameters as previously disclosed herein. SEQ ID NO:11 represents a consensus amino acid sequence of an epimerase which was derived by aligning at least portions of amino acid sequences SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8, as described in Somers et al., 1998, *Structure* 6:1601-1612, and can be approximately duplicated using CLUSTAL.

In another embodiment, an epimerase encompassed by the present invention includes an epimerase that has a catalytic site which includes amino acid residues: serine, tyrosine and lysine. Preferably, such serine, tyrosine and lysine residues are located at positions in the epimerase amino acid sequence which align using a CLUSTAL alignment program with positions Ser105, Tyr134 and Lys138 of consensus sequence SEQ ID NO:11, with positions Ser109, Tyr138 and Lys142 of sequence SEQ ID NO:2, with positions Ser107, Tyr136 and Lys140 of SEQ ID NO:4, with positions Ser114, Tyr143 and Lys147 of sequence SEQ ID NO:6, with positions Ser124, Tyr149 and Lys153 of sequence SEQ ID NO:8 or with positions Ser132, Tyr157 and Lys161 of sequence SEQ ID NO:10.

In another embodiment, an epimerase that has an amino acid sequence that is homologous to an amino acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase includes any epimerase that has an amino acid motif: Gly-Xaa-Xaa-Gly-Xaa-Xaa-Gly, which is found, for example in positions 8 through 14 of the consensus sequence SEQ ID NO:11, in positions 12 through 18 of SEQ ID NO:2, in positions 10 through 16 of SEQ ID NO:4, in positions 14 through 20 of SEQ ID NO:6, in positions

7 through 13 of SEQ ID NO:8, and in positions 9 through 15 of SEQ ID NO:10. Such a motif can be identified by its alignment with the same motif in the above-identified amino acid sequences using a CLUSTAL alignment program. Preferably, such motif is located within the first 25 N-terminal amino acids of the amino acid sequence of the epimerase.

In yet another embodiment, an epimerase encompassed by the present invention includes an epimerase that has a substrate binding site which includes amino acid residues that align using a CLUSTAL alignment program with at least 50% of amino acid positions Asn177, Ser178, Arg187, Arg209, Lys283, Asn165, Ser107, Ser108, Cys109, Asn133, Tyr136 and His179 of SEQ ID NO:4. Alignment with positions Ser107, Tyr136, Asn165, Arg209, is preferably with 100% identity (i.e., exact match of residue, under parameters for alignment).

In another embodiment of the present invention, an epimerase encompassed by the present invention comprises at least 4 contiguous amino acid residues having 100% identity with at least 4 contiguous amino acid residues of an amino acid sequence selected from the group of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:10, as determined using a Lipman-Pearson method with Lipman-Pearson standard default parameters or by comparing an alignment using a CLUSTAL program with CLUSTAL standard default parameters. According to the present invention, the term "contiguous" means to be connected in an unbroken sequence. For a first sequence to have "100% identity" with a second sequence means that the first sequence exactly matches the second sequence with no gaps between nucleotides or amino acids.

In another embodiment of the present invention, an epimerase encompassed by the present invention is encoded by a nucleic acid sequence that comprises at least 12 contiguous nucleic acid residues having 100% identity with at least 12 contiguous nucleic acid residues of a nucleic acid sequence selected from the group of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:10, as determined using a Lipman-Pearson method with Lipman-Pearson standard default parameters or by comparing an alignment using a CLUSTAL program with CLUSTAL standard default parameters.

In another embodiment of the present invention, an epimerase encompassed by the present invention is encoded by a nucleic acid sequence that hybridizes under stringent

hybridization conditions to a nucleic acid sequence selected from the group of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9. As used herein, stringent hybridization conditions refer to standard hybridization conditions under which nucleic acid molecules are used to identify similar nucleic acid molecules. Such standard conditions are disclosed, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989. Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety (see specifically, pages 9.31-9.62). In addition, formulae to calculate the appropriate hybridization and wash conditions to achieve hybridization permitting varying degrees of mismatch of nucleotides are disclosed, for example, in Meinkoth et al., 1984, *Anal. Biochem.* 138, 267-284; Meinkoth et al., *ibid.*, is incorporated by reference herein in its entirety.

More particularly, stringent hybridization and washing conditions, as referred to herein, refer to conditions which permit isolation of nucleic acid molecules having at least about 70% nucleic acid sequence identity with the nucleic acid molecule being used to probe in the hybridization reaction, more particularly at least about 75%, and most particularly at least about 80%. Such conditions will vary, depending on whether DNA:RNA or DNA:DNA hybrids are being formed. Calculated melting temperatures for DNA:DNA hybrids are 10°C less than for DNA:RNA hybrids. In particular embodiments, stringent hybridization conditions for DNA:DNA hybrids include hybridization at an ionic strength of 6X SSC (0.9 M Na^+) at a temperature of between about 20°C and about 35°C, more preferably, between about 28°C and about 40°C, and even more preferably, between about 35°C and about 45°C. In particular embodiments, stringent hybridization conditions for DNA:RNA hybrids include hybridization at an ionic strength of 6X SSC (0.9 M Na^+) at a temperature of between about 30°C and about 45°C, more preferably, between about 38°C and about 50°C, and even more preferably, between about 45°C and about 55°C. These values are based on calculations of a melting temperature for molecules larger than about 100 nucleotides, 0% formamide and a G + C content of about 40%. Alternatively, T_m can be calculated empirically as set forth in Sambrook et al., *supra*, pages 9.31 to 9.62.

In another embodiment of the present invention, an epimerase encompassed by the present invention is encoded by a nucleic acid sequence that comprises a nucleic acid

sequence selected from the group of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9 or a fragment thereof, wherein the fragment encodes a protein that is capable of catalyzing the conversion of GDP-D-mannose to GDP-L-galactose, such as under physiological conditions. In another embodiment, an epimerase encompassed by the present invention comprises an amino acid sequence selected from the group of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10 or a fragment thereof, wherein the fragment is capable of catalyzing the conversion of GDP-D-mannose to GDP-L-galactose. It is to be understood that the nucleic acid sequence encoding the amino acid sequences identified herein can vary due to degeneracies. As used herein, nucleotide degeneracies refers to the phenomenon that one amino acid can be encoded by different nucleotide codons.

One embodiment of the present invention relates to a method to identify an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose. Preferably, such a method is useful for identifying the GDP-D-mannose:GDP-L-galactose epimerase which catalyzes the conversion of GDP-D-mannose to GDP-L-galactose in the endogenous (i.e., naturally occurring L-ascorbic acid biosynthetic pathway of microorganisms and/or plants). Such a method can include the steps of: (a) contacting a source of nucleic acid molecules with an oligonucleotide at least about 12 nucleotides in length under stringent hybridization conditions, wherein the oligonucleotide is identified by its ability to hybridize under stringent hybridization conditions to a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5; and, (b) identifying nucleic acid molecules from the source of nucleic acid molecules which hybridize under stringent hybridization conditions to the oligonucleotide. Nucleic acid molecules identified by this method can then be isolated from the source using standard molecular biology techniques. Preferably, the source of nucleic acid molecules is obtained from a microorganism or plant that has an ascorbic acid production pathway. Such a source of nucleic acid molecules can be any source of nucleic acid molecules which can be isolated from an organism and/or which can be screened by hybridization with an oligonucleotide such as a probe or a PCR primer. Such sources include genomic and cDNA libraries and isolated RNA.

In order to screen cDNA libraries from different organisms and to isolate nucleic acid molecules encoding enzymes such as the GDP-D-mannose:GDP-L-galactose epimerase and related epimerases, one can use any of a variety of standard molecular and biochemical techniques. For example, oligonucleotide primers, preferably degenerate primers, can be designed using the most conserved regions of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase nucleic acid sequence, and such primers can be used in a polymerase chain reaction (PCR) protocol to amplify the same or related epimerases, including the GDP-D-mannose:GDP-L-galactose epimerase from the ascorbic acid pathway, from nucleic acids (e.g., genomic or cDNA libraries) isolated from a desired organism (e.g., a microorganism or plant having an L-ascorbic acid pathway). Similarly, oligonucleotide probes can be designed using the most conserved regions of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase nucleic acid sequence and such probe can be used to identify and isolate nucleic acid molecules, such as from a genomic or cDNA library, that hybridize under conditions of low, moderate, or high stringency with the probe.

Alternatively, the GDP-D-mannose:GDP-L-galactose epimerase can be purified from an organism such as *Prototheca*, the N-terminal amino acid sequence can be determined (including the sequence of internal peptide fragments), and this information can be used to design degenerate primers for amplifying a gene fragment from the organism cDNA. This fragment would then be used to probe the cDNA library, and subsequently fragments that hybridize to the probe would be cloned in that organism or another suitable production organism. There is ample precedent for plant enzymes being expressed in an active form in bacteria, such as *E. coli*. Alternatively, yeast are also a suitable candidate for developing a heterologous system for L-ascorbic acid production.

As discussed above in general for increasing the action of an enzyme in the L-ascorbic acid pathway according to the present invention, in one embodiment of the present invention, the action of an epimerase that catalyzes the conversion of GDP-D-mannose to GDP-L-galactose is increased by amplification of the expression (i.e., overexpression) of such an epimerase. Overexpression of an epimerase can be accomplished, for example, by introduction of a recombinant nucleic acid molecule encoding the epimerase. It is preferred that the gene encoding an epimerase according to

the present invention be cloned under control of an artificial promoter. The promoter can be any suitable promoter that will provide a level of epimerase expression required to maintain a sufficient level of L-ascorbic acid in the production organism. Preferred promoters are constitutive (rather than inducible) promoters, since the need for addition of expensive inducers is therefore obviated. The gene dosage (copy number) of a recombinant nucleic acid molecule according to the present invention can be varied according to the requirements for maximum product formation. In one embodiment, the recombinant nucleic acid molecule encoding an epimerase according to the present invention is integrated into the chromosome of the microorganism.

It is another embodiment of the present invention to provide a microorganism having one or more epimerases according to the present invention with improved affinity for its substrate. An epimerase with improved affinity for its substrate can be produced by any suitable method of genetic modification or protein engineering. For example, computer-based protein engineering can be used to design an epimerase protein with greater stability and better affinity for its substrate. See for example, Maulik et al., 1997, *Molecular Biotechnology: Therapeutic Applications and Strategies*, Wiley-Liss, Inc., which is incorporated herein by reference in its entirety.

As noted above, in the method for production of L-ascorbic acid of the present invention, a microorganism having a genetically modified L-ascorbic acid production pathway is cultured in a fermentation medium for production of L-ascorbic acid. An appropriate, or effective, fermentation medium refers to any medium in which a genetically modified microorganism of the present invention, when cultured, is capable of producing L-ascorbic acid. Such a medium is typically an aqueous medium comprising assimilable carbon, nitrogen and phosphate sources. Such a medium can also include appropriate salts, minerals, metals and other nutrients. One advantage of genetically modifying a microorganism as described herein is that although such genetic modifications can significantly alter the production of L-ascorbic acid, they can be designed such that they do not create any nutritional requirements for the production organism. Thus, a minimal-salts medium containing glucose as the sole carbon source can be used as the fermentation medium. The use of a minimal-salts-glucose medium for the L-ascorbic acid fermentation will also facilitate recovery and purification of the L-ascorbic acid product.

In one mode of operation of the present invention, the carbon source concentration, such as the glucose concentration, of the fermentation medium is monitored during fermentation. Glucose concentration of the fermentation medium can be monitored using known techniques, such as, for example, use of the glucose oxidase enzyme test or high pressure liquid chromatography, which can be used to monitor glucose concentration in the supernatant, e.g., a cell-free component of the fermentation medium. As stated previously, the carbon source concentration should be kept below the level at which cell growth inhibition occurs. Although such concentration may vary from organism to organism, for glucose as a carbon source, cell growth inhibition occurs at glucose concentrations greater than at about 60 g/L, and can be determined readily by trial. Accordingly, when glucose is used as a carbon source the glucose concentration in the fermentation medium is maintained in the range of from about 1 g/L to about 100 g/L, more preferably in the range of from about 2 g/L to about 50 g/L, and yet more preferably in the range of from about 5 g/L to about 20 g/L. Although the carbon source concentration can be maintained within desired levels by addition of, for example, a substantially pure glucose solution, it is preferred to maintain the carbon source concentration of the fermentation medium by addition of aliquots of the original fermentation medium. The use of aliquots of the original fermentation medium are desirable because the concentrations of other nutrients in the medium (e.g. the nitrogen and phosphate sources) can be maintained simultaneously. Likewise, the trace metals concentrations can be maintained in the fermentation medium by addition of aliquots of the trace metals solution.

In an embodiment of the fermentation process of the present invention, a fermentation medium is prepared as described above. This fermentation medium is inoculated with

an actively growing culture of genetically modified microorganisms of the present invention in an amount sufficient to produce, after a reasonable growth period, a high cell density. Typical inoculation cell densities are within the range of from about 0.1 g/L to about 15 g/L, preferably from about 0.5 g/L to about 10 g/L and more preferably from about 1 g/L to about 5 g/L, based on the dry weight of the cells. The cells are then grown to a cell density in the range of from about 10 g/L to about 100 g/L preferably from about

20 g/L to about 80 g/L, and more preferably from about 50 g/L to about 70 g/L. The residence times for the microorganisms to reach the desired cell densities during fermentation are typically less than about 200 hours, preferably less than about 120 hours, and more preferably less than about 96 hours.

The microorganisms useful in the method of the present invention can be cultured in conventional fermentation modes, which include, but are not limited to, batch, fed-batch, and continuous. It is preferred, however, that the fermentation be carried out in fed-batch mode. In such a case, during fermentation some of the components of the medium are depleted. It is possible to initiate fermentation with relatively high concentrations of such components so that growth is supported for a period of time before additions are required. The preferred ranges of these components are maintained throughout the fermentation by making additions as levels are depleted by fermentation. Levels of components in the fermentation medium can be monitored by, for example, sampling the fermentation medium periodically and assaying for concentrations. Alternatively, once a standard fermentation procedure is developed, additions can be made at timed intervals corresponding to known levels at particular times throughout the fermentation. As will be recognized by those in the art, the rate of consumption of nutrient increases during fermentation as the cell density of the medium increases. Moreover, to avoid introduction of foreign microorganisms into the fermentation medium, addition is performed using aseptic addition methods, as are known in the art. In addition, a small amount of anti-foaming agent may be added during the fermentation.

The present inventors have determined that high levels of magnesium in the fermentation medium inhibits the production of L-ascorbic acid due to repression of enzymes early in the production pathway, although enzymes late in the pathway (i.e., from L-galactose to L-ascorbic acid) are not negatively affected (See Examples). Therefore, in a preferred embodiment of the method of the present invention, the step of culturing is carried out in a fermentation medium that is magnesium (Mg^{2+}) limited. Even more preferably, the fermentation is magnesium limited during the cell growth phase. Preferably, the fermentation medium comprises less than about 0.5 g/L of Mg^{2+} during the cell growth phase of fermentation, and even more preferably, less than about 0.2 g/L of Mg^{2+}, and even more preferably, less than about 0.1 g/L of Mg^{2+}.

The temperature of the fermentation medium can be any temperature suitable for growth and ascorbic acid production, and may be modified according to the growth requirements of the production microorganism used. For example, prior to inoculation of the fermentation medium with an inoculum, the fermentation medium can be brought to and maintained at a temperature in the range of from about 20°C to about 45°C, preferably to a temperature in the range of from about 25°C to about 40°C, and more preferably in the range of from about 30°C to about 38°C.

It is a further embodiment of the present invention to supplement and/or control other components and parameters of the fermentation medium, as necessary to maintain and/or enhance the production of L-ascorbic acid by a production organism. For example, in one embodiment, the pH of the fermentation medium is monitored for fluctuations in pH. In the fermentation method of the present invention, the pH is preferably maintained at a pH of from about pH 6.0 to about pH 8.0, and more preferably, at about pH 7.0. In the method of the present invention, if the starting pH of the fermentation medium is pH 7.0, the pH of the fermentation medium is monitored for significant variations from pH 7.0, and is adjusted accordingly, for example, by the addition of sodium hydroxide. In a preferred embodiment of the present invention, genetically modified microorganisms useful for production of L-ascorbic acid include acid-tolerant microorganisms. Such microorganisms include, for example, microalgae of the genera *Prototheca* and *Chlorella* (See U.S. Patent No. 5,792,631, *ibid.* and U.S. Patent No. 5,900,370, *ibid.*).

The production of ascorbic acid by culturing acid-tolerant microorganisms provides significant advantages over known ascorbic acid production methods. One such advantage is that such organisms are acidophilic, allowing fermentation to be carried out under low pH conditions, with the fermentation medium pH typically less than about 6. Below this pH, extracellular ascorbic acid produced by the microorganism during fermentation is relatively stable because the rate of oxidation of ascorbic acid in the fermentation medium by oxygen is reduced. Accordingly, high productivity levels can be obtained for producing L-ascorbic acid with acid-tolerant microorganisms according to the methods of the present invention. In addition, control of the dissolved oxygen content to very low levels to avoid oxidation of ascorbic acid is unnecessary. Moreover, this

advantage allows for the use of continuous recovery methods because extracellular medium can be treated to recover the ascorbic acid product.

Thus, the present method can be conducted at low pH when acid-tolerant microorganisms are used as production organisms. The benefit of this process is that at low pH, extracellular ascorbic acid produced by the organism is degraded at a reduced rate than if the fermentation medium was at higher pH. For example, prior to inoculation of the fermentation medium with an inoculum, the pH of the fermentation medium can be adjusted, and further monitored during fermentation. Typically, the pH of the fermentation medium is brought to and maintained below about 6, preferably below 5.5, and more preferably below about 5. The pH of the fermentation medium can be controlled by the addition of ammonia to the fermentation medium. In such cases when ammonia is used to control pH, it also conveniently serves as a nitrogen source in the fermentation medium.

The fermentation medium can also be maintained to have a dissolved oxygen content during the course of fermentation to maintain cell growth and to maintain cell metabolism for L-ascorbic acid formation. The oxygen concentration of the fermentation medium can be monitored using known methods, such as through the use of an oxygen probe electrode. Oxygen can be added to the fermentation medium using methods known in the art, for example, through agitation and aeration of the medium by stirring or shaking. Preferably, the oxygen concentration in the fermentation medium is in the range of from about 20% to about 100% of the saturation value of oxygen in the medium based upon the solubility of oxygen in the fermentation medium at atmospheric pressure and at a temperature in the range of from about 30°C to about 40°C. Periodic drops in the oxygen concentration below this range may occur during fermentation, however, without adversely affecting the fermentation.

The genetically modified microorganisms of the present invention are engineered to produce significant quantities of extracellular L-ascorbic acid. Extracellular L-ascorbic acid can be recovered from the fermentation medium using conventional separation and purification techniques. For example, the fermentation medium can be filtered or centrifuged to remove microorganisms, cell debris and other particulate matter, and L-ascorbic acid can be recovered from the cell-free supernate by conventional methods, such

as, for example, ion exchange, chromatography, extraction, solvent extraction, membrane separation, electrodialysis, reverse osmosis, distillation, chemical derivatization and crystallization.

One such example of L-ascorbic acid recovery is provided in U.S. Patent No. 4,595,659 by Cayle, incorporated herein in its entirety be reference, which discloses the isolation of L-ascorbic acid from an aqueous fermentation medium by ion exchange resin adsorption and elution, which is followed by decoloration, evaporation and crystallization. Further, isolation of the structurally similar isoascorbic acid from fermentation medium by a continuous multi-bed extraction system of anion-exchange resins is described by K. Shimizu, *Agr. Biol. Chem.* 31:346-353 (1967), which is incorporated herein in its entirety by reference.

Intracellular L-ascorbic acid produced in accordance with the present invention can also be recovered and used in a variety of applications. For example, cells from the microorganisms can be lysed and the ascorbic acid which is released can be recovered by a variety of known techniques. Alternatively, intracellular ascorbic acid can be recovered by washing the cells to extract the ascorbic acid, such as through diafiltration.

Development of a microorganism with enhanced ability to produce L-ascorbic acid by genetic modification can be accomplished using both classical strain development and molecular genetic techniques, and particularly, recombinant technology (genetic engineering). In general, the strategy for creating a microorganism with enhanced L-ascorbic acid production is to (1) inactivate or delete at least one, and preferably more than one of the competing or inhibitory pathways in which production of L-ascorbic acid is negatively affected (e.g., inhibited), and more significantly to (2) amplify the L-ascorbic acid production pathway by increasing the action of a gene(s) encoding an enzyme(s) involved in the pathway.

In one embodiment, the strategy for creating a microorganism with enhanced L-ascorbic acid production is to amplify the L-ascorbic acid production pathway by increasing the action of GDP-D-mannose:GDP-L-galactose epimerase, as discussed above. Such strategy includes genetically modifying the endogenous GDP-D-mannose:GDP-L-galactose epimerase such that L-ascorbic acid production is increased, and/or expressing/overexpressing a recombinant epimerase that catalyzes the conversion

of GDP-D-mannose to GDP-L-galactose, which includes expression of recombinant GDP-D-mannose:GDP-L-galactose epimerase and/or homologues thereof, and of other recombinant epimerases such as GDP-4-keto-6-deoxy-D-mannose epimerase reductase and epimerases that share structural homology with such epimerase as discussed in detail above.

It is to be understood that a production organism can be genetically modified by recombinant technology in which a nucleic acid molecule encoding a protein involved in the L-ascorbic acid production pathway disclosed herein is transformed into a suitable host which is a different member of the plant kingdom from which the nucleic acid molecule was derived. For example, it is an embodiment of the present invention that a recombinant nucleic acid molecule encoding a GDP-D-mannose:GDP-L-galactose epimerase from a higher plant can be transformed into a microalgal host in order to overexpress the epimerase and enhance production of L-ascorbic acid in the microalgal production organism.

As previously discussed herein, in one embodiment, a genetically modified microorganism can be a microorganism in which nucleic acid molecules have been deleted, inserted or modified, such as by insertion, deletion, substitution, and/or inversion of nucleotides, in such a manner that such modifications provide the desired effect within the microorganism. A genetically modified microorganism is preferably modified by recombinant technology, such as by introduction of an isolated nucleic acid molecule into a microorganism. For example, a genetically modified microorganism can be transfected with a recombinant nucleic acid molecule encoding a protein of interest, such as a protein for which increased expression is desired. The transfected nucleic acid molecule can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transfected (i.e., recombinant) host cell in such a manner that its ability to be expressed is retained. Preferably, once a host cell of the present invention is transfected with a nucleic acid molecule, the nucleic acid molecule is integrated into the host cell genome. A significant advantage of integration is that the nucleic acid molecule is stably maintained in the cell. In a preferred embodiment, the integrated nucleic acid molecule is operatively linked to a transcription control sequence (described below) which can be induced to control expression of the nucleic acid molecule.

A nucleic acid molecule can be integrated into the genome of the host cell either by random or targeted integration. Such methods of integration are known in the art. For example, an *E. coli* strain ATCC 47002 contains mutations that confer upon it an inability to maintain plasmids which contain a ColE1 origin of replication. When such plasmids are transferred to this strain, selection for genetic markers contained on the plasmid results in integration of the plasmid into the chromosome. This strain can be transformed, for example, with plasmids containing the gene of interest and a selectable marker flanked by the 5'- and 3'-termini of the *E. coli lacZ* gene. The *lacZ* sequences target the incoming DNA to the *lacZ* gene contained in the chromosome. Integration at the *lacZ* locus replaces the intact *lacZ* gene, which encodes the enzyme β-galactosidase, with a partial *lacZ* gene interrupted by the gene of interest. Successful integrants can be selected for β-galactosidase negativity.

A genetically modified microorganism can also be produced by introducing nucleic acid molecules into a recipient cell genome by a method such as by using a transducing bacteriophage. The use of recombinant technology and transducing bacteriophage technology to produce several different genetically modified microorganism of the present invention is known in the art.

According to the present invention, a gene, for example the GDP-D-mannose:GDP-L-galactose epimerase gene, includes all nucleic acid sequences related to a natural epimerase gene such as regulatory regions that control production of the epimerase protein encoded by that gene (such as, but not limited to, transcription, translation or post-translation control regions) as well as the coding region itself. In another embodiment, a gene, for example the GDP-D-mannose:GDP-L-galactose epimerase gene, can be an allelic variant that includes a similar but not identical sequence to the nucleic acid sequence encoding a given GDP-D-mannose:GDP-L-galactose epimerase gene. An allelic variant of a GDP-D-mannose:GDP-L-galactose epimerase gene which has a given nucleic acid sequence is a gene that occurs at essentially the same locus (or loci) in the genome as the gene having the given nucleic acid sequence, but which, due to natural variations caused by, for example, mutation or recombination, has a similar but not identical sequence. Allelic variants typically encode proteins having similar activity to that of the protein encoded by the gene to which they are being

compared. Allelic variants can also comprise alterations in the 5' or 3' untranslated regions of the gene (e.g., in regulatory control regions). Allelic variants are well known to those skilled in the art and would be expected to be found within a given microorganism or plant and/or among a group of two or more microorganisms or plants.

In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation). As such, "isolated" does not reflect the extent to which the nucleic acid molecule has been purified. An isolated nucleic acid molecule can include DNA, RNA, or derivatives of either DNA or RNA. There is no limit, other than a practical limit, on the maximal size of a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof.

An isolated nucleic acid molecule of the present invention can be obtained from its natural source either as an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with that gene. An isolated nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated nucleic acid molecules include natural nucleic acid molecules and homologues thereof, including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications provide the desired effect within the microorganism. A structural homologue of a nucleic acid sequence has been described in detail above. Preferably, a homologue of a nucleic acid sequence encodes a protein which has an amino acid sequence that is sufficiently similar to the natural protein amino acid sequence that a nucleic acid sequence encoding the homologue is capable of hybridizing under stringent conditions to (i.e., with) a nucleic acid molecule encoding the natural protein (i.e., to the complement of the nucleic acid strand encoding the natural protein amino acid sequence). A nucleic acid molecule homologue encodes a protein homologue. As used herein, a homologue protein includes proteins in which amino acids have been deleted (e.g., a truncated version of the protein, such as a peptide), inserted, inverted, substituted and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation,

amidation and/or addition of glycosylphosphatidyl inositol) in such a manner that such modifications provide the desired effect on the protein and/or within the microorganism (e.g., increased or decreased action of the protein).

A nucleic acid molecule homologue can be produced using a number of methods known to those skilled in the art (see, for example, Sambrook et al., *ibid.*). For example, nucleic acid molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, PCR amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof. Nucleic acid molecule homologues can be selected from a mixture of modified nucleic acids by screening for the function of the protein encoded by the nucleic acid and/or by hybridization with a wild-type gene.

Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a gene involved in an L-ascorbic acid production pathway.

Knowing the nucleic acid sequences of certain nucleic acid molecules of the present invention allows one skilled in the art to, for example, (a) make copies of those nucleic acid molecules and/or (b) obtain nucleic acid molecules including at least a portion of such nucleic acid molecules (e.g., nucleic acid molecules including full-length genes, full-length coding regions, regulatory control sequences, truncated coding regions). Such nucleic acid molecules can be obtained in a variety of ways including traditional cloning techniques using oligonucleotide probes to screen appropriate libraries or DNA and PCR amplification of appropriate libraries or DNA using oligonucleotide primers. Preferred libraries to screen or from which to amplify nucleic acid molecule include bacterial and yeast genomic DNA libraries, and in particular, microalgal genomic DNA libraries. Techniques to clone and amplify genes are disclosed, for example, in Sambrook et al., *ibid.*

The present invention includes a recombinant vector, which includes at least one isolated nucleic acid molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule into a host microorganism of the present invention. Such a vector can contain nucleic acid sequences that are not naturally found adjacent to the isolated nucleic acid molecules to be inserted into the vector. The vector can be either RNA or DNA and typically is a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulating of nucleic acid molecules. One type of recombinant vector, referred to herein as a recombinant molecule and described in more detail below, can be used in the expression of nucleic acid molecules. Preferred recombinant vectors are capable of replicating in a transformed bacterial cells, yeast cells, and in particular, in microalgal cells.

Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection and biolistics.

A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules operatively linked to an expression vector containing one or more transcription control sequences. The phrase, operatively linked, refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. In the present invention, expression vectors are typically plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in a yeast host cell, a bacterial host cell, and preferably a microalgal host cell.

Nucleic acid molecules of the present invention can be operatively linked to expression vectors containing regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression

of nucleic acid molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in yeast or bacterial cells or preferably, in microalgal cells. A variety of such transcription control sequences are known to those skilled in the art.

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into the host cell chromosome, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals, modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing nucleic acid molecules encoding such a protein.

The following experimental results are provided for the purposes of illustration and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

The present example describes the elucidation of the pathway from glucose to L-ascorbic acid through GDP-D-mannose in plants.

Since the present inventors have previously shown that *Prototheca* makes L-ascorbic acid (AA) from glucose, it was worthwhile to examine cultures for some of the early conversion products of glucose. In the past, the present inventors had concentrated on pathways from glucose to organic acids, based on the published pathway of L-ascorbic acid synthesis in animals and proposed pathways in plants. The present inventors demonstrate herein that the pathway from glucose to L-ascorbic acid involves not organic acids, but rather sugar phosphates and nucleotide diphosphate sugars (NDP-sugars).

Prior to the present invention, it was known that all cells synthesize polysaccharides by first forming NDP-sugars. The sugar moiety is then incorporated into polymer, while the cleaved NDP is recycled. A variety of polysaccharides are known, and are usually named based on the relative proportions of the sugar residues in the polymers. For example, a "galactomannan" contains mostly galactose, and to a lesser degree, mannose residues. The "biopolymer" from *Prototheca* strains isolated by the present inventors was analyzed and found to be 80% D-galactose, 18% rhamnose (D- or L-configuration not determined), and 2% L-arabinose. The present inventors provide evidence herein of how the respective NDP-sugars that make up the *Prototheca* biopolymer are formed, and what correlations exist between L-ascorbic acid synthesis and the formation of the NDP-sugar forms of the sugar residues found in the biopolymer.

The common NDP-sugar UDP-glucose is shown in Fig. 2B. This is formed in plants from glucose-l-P by the action of UDP-D-glucose pyrophosphorylase. UDP-glucose can be epimerized in plants to form UDP-D-galactose, using UDP-D-glucose-4-epimerase. UDP-D-galactose can also be formed by phosphorylation of D-galactose by galactokinase to form D-galactose-l-P, which can be converted to UDP-D-galactose by UDP-D-galactose pyrophosphorylase. These known routes were believed to account for the D-galactose in the *Prototheca* biopolymer. The UDP-L-arabinose can be formed by known reactions beginning with the oxidation of UDP-D-glucose to UDP-D-glucuronic acid (by UDP-D-glucose dehydrogenase), decarboxylation to UDP-D-xylose, and epimerization to UDP-L-arabinose. This accounts for the arabinose residues in the

biopolymer. UDP-L-rhamnose is known to be formed from UDP-D-glucose, thus all three of the sugar moieties in the *Prototheca* biopolymer can be accounted for by a pathway through glucose-1-P and UDP-glucose. Alternatively, if the rhamnose in the biopolymer is D-rhamnose, it is not formed via UDP-D-glucose, but by oxidation of GDP-D-mannose (See Fig. 1).

GDP-D-rhamnose is formed by converting glucose, in turn, to D-glucose-6-P, D-fructose-6-P, D-mannose-6-P, D-mannose-1-P, GDP-D-mannose, and GDP-D-rhamnose. It was of interest to the present inventors that this route passes through GDP-D-mannose. Exogenous mannose is known to be converted to D-mannose-6-P in plants, and can enter the path above. D-mannose is converted to L-ascorbic acid by *Prototheca* cells cultured by the present inventors as well or better than glucose (see Example 4). The mechanism of conversion, in *Chlorella pyrenoidosa* , of GDP-D-mannose to GDP-L-galactose by GDP-D-mannose:GDP-L-galactose epimerase, has been known for years (See, Barber, 1971, *Arch. Biochem. Biophys.* 147:619-623, incorporated herein by reference in its entirety). The present inventors have discovered herein that L-galactose and L-galactono-γ-lactone are rapidly converted to L-ascorbic acid by strains *of Prototheca* and *Chlorella pyrenoidosa.* Prior to the present invention, it was known that L-galactono-γ-lactone is converted to L-ascorbic acid in several plant systems, but the synthesis steps prior to this step were unknown. Based on the published literature and the present experimental evidence, the present inventors have determined that the L-ascorbic acid biosynthetic pathway in plants passes through GDP-D-mannose and involves sugar phosphates and NDP-sugars. The proposed pathway is shown in Fig. 1. Salient points relevant to the design and production of genetically modified microorganisms useful in the present method include:

1. The enzymes leading from D-glucose to D-fructose-6-P are well known enzymes in the first, uncommitted steps of glycolysis.

2. The enzymes involved in the conversion of D-fructose-6-P to GDP-D-mannose have been well characterized in plants, yeast, and bacteria, particularly *Azotobacter vinelandii and Pseudomonas aeruginosa,* which convert GDP-D-mannose to GDP-D-mannuronic acid, which is the precursor for alginate (See for example, Sa-Correia et al., 1987, *J. Bacteriol.* 169:3224-3231; Koplin et al., 1992, *J. Bacteriol.* 174:191-199; Oesterhelt et al., 1996, *Plant Science* 121:19-27; Feingold et al., 1980, The

Biochemistry of Plants: Vol 3: Carbohydrates, structure and function, P.K. Stampf & E.E. Conn, eds., Academic Press, New York, pp. 101-170; Smith et al., 1992, *Mol. Cell Biol.* 12:2924-2930; Boles et al., 1994, *Eur. J. Med.* 220:83-96; Hashimoto et al., 1997, *J. Biol. Chem.* 272:16308-16314, all of which are incorporated herein by reference in their entirety).

3. Barber (1971, *supra*, and 1975) identified in *Chlorella pyrenoidosa* the enzyme activities for the conversion of GDP-D-mannose to GDP-L-galactose and L-galactose-l-P.

4. The present inventors have shown herein the rapid conversion of L-galactose and L-galactono-γ-lactone to L-ascorbic acid by *Prototheca* cells.

5. L-galactono-γ-lactone and L-galactonic acid can be interconverted in solution by changing the pH of the solution; addition of base shifts the equilibrium to L-galactonic acid, while addition of acid shifts the equilibrium to the lactone. Cells may have an enzymatic means for this conversion in addition to this non-enzymatic route.

6. In plants, GDP-L-fucose is also formed from GDP-D-mannose, presumably for incorporation into polysaccharide. Roberts (1971) fed labeled D-mannose to corn root tips and found the label in polysaccharide, specifically in the residues of D-mannose, L-galactose, and L-fucose. No label was detected in D-glucose, D-galactose, L-arabinose, or D-xylose. *Prototheca and C. pyrenoidosa* cells have the ability to convert L-fucose (6-deoxy-L-galactose) to a dipyridyl-positive product that was shown by HPLC not to be L-ascorbic acid. The present inventors believe that it is was the 6-deoxy analog of L-ascorbic acid.

Example 2

This example shows that in *Prototheca*, like other plants (Loewus, F.A. 1988. In: J. Priess (ed.), The Biochemistry of Plants, 14:85-107. New York, Academic Press) and the green microalga *Chlorella pyrenoidosa* (Renstrom, *et al.*, 1983. Plant Sci. Lett. 28:299-305), ascorbic acid (AA) production from glucose proceeds by a biosynthetic pathway that allows retention of the configuration of the carbon skeleton of glucose.

Cultures of the strain UV77-247 were grown to moderate cell density in shake flasks with 1-^{13}C-labeled glucose as 10% of the total glucose (40 g/L). Incubation was

as per the standard Mg-limited screen (see Example 3). The culture supernates were clarified, deionized to remove salts, lyophilized, and subjected to nuclear magnetic resonance (nmr) analysis to determine where in the AA molecule the ^{13}C was located. In each case, approximately 85% of the label was found at the C-1 position of AA, with most of the remaining label at the C-6 position. This strongly indicated that AA is synthesized from glucose by a pathway that retains the carbon chain configuration, *i. e.*, C-1 of glucose becomes C-1 of AA. This has typically been observed in plants (Loewus, F.A. 1988. Ascorbic acid and its metabolic products. In: The Biochemistry of Plants, ed. J. Priess, 14:85-107. New York, Academic Press). Animals (Mapson, L.W. and F.A. Isherwood 1956. Biochem. J. 64:151-157; Loewus, F.A. 1960. J. Biol. Chem. 235(4):937-939) and protists such as *Euglena* (Shigeoka, S., *et al.*, 1979. J. Nutr. Sci. Vitaminol. 25:299-307), on the other hand, synthesize AA by a pathway that involves the inversion of configuration, *i. e.*, C-1 of glucose becomes C-6 of AA. Demonstration of the inversion/non-inversion nature of the pathway was an important step in determining the pathway of AA biosynthesis since the two types of pathways require different types of enzymatic reactions. The label found at C-6 of AA is thought to be due to metabolism of glucose and subsequent gluconeogenesis. The metabolism of glucose in glycolysis proceeds through triose-phosphate intermediates. After this, the C-1 and C-6 carbons of glucose become biochemically equivalent. Hexose phosphates can be regenerated from the triose phosphates by gluconeogenesis, which is essentially a reversal of the degradative pathway. Consequently, metabolism of C-1-labeled glucose to triose phosphates with subsequent gluconeogenesis would result in the formation of hexose phosphate molecules labeled at either or both C-1 and C-6. If those hexose phosphates were precursors to AA, one would expect the AA to be similarly labeled. Consistent with this type of "isotopic mixing" is the observation that sucrose obtained from 1-^{13}C-labeled glucose was labeled at positions 1, 6, 1' and 6'.

Glucose can also be metabolized by the pentose phosphate pathway, the overall balanced equation for which is:

3 Glucose-6-phosphate → 2 Fructose-6-phosphate + Glyceraldehyde-3-phosphate + 3 CO_2

Based on the known biochemistry, it would then be expected that the label at each of the carbons in glucose (Table 1 left column) would appear at the positions for the other molecules shown, and that these patterns would be reflected in the AA formed from C-2- and C-3-labeled glucose.

TABLE 1

Predicted Carbon Labeling of Metabolites of Glucose in the Pentose Phosphate Pathway

Labeled Glucose Carbon	Position of Labeled Carbon in:			
	CO_2	F6P(1)	F6P(2)	G3P
1	+	-	-	-
2	-	1,3	1	-
3	-	2	2,3	-
4	-	4	4	1
5	-	5	5	2
6	-	6	6	3

AA recovered from cultures fed glucose labeled at C-2 or C-3 was also analyzed for its labeling patterns (Table 2).

TABLE 2

Labeling Pattern in AA after Cells were Fed 2-^{13}C and 3-^{13}C-glucose

Carbon Position in AA	Isotopic enhancement after growth on:	
	C-2 labeled glucose	C-3 labeled glucose
1	1.0	0.4
2	10.0	0.9
3	0.5	9.9
4	0	2.8
5	2.2	0.2
6	0	0

The data above again suggest a pathway from glucose to AA that proceeds by retention of configuration. As in the experiments with C-1 labeled glucose, approximately one-fifth of the label is present in "mirror image" position to the glucose label (C-5 for C-2 labeled glucose and C-4 for C-3 labeled glucose), indicating levels of gluconeogenesis consistent with those previously observed.

The small, but significant amount of enhancement observed in other positions is consistent with flux through the pentose phosphate pathway. As predicted above, carbon

flux through this pathway would result in isotopic enhancement at positions 1 and 3 when cells were grown on 2-^{13}C glucose and enhancement at position 2 when cells were grown on 3-^{13}C glucose. This is indeed observed. That there is twice as much enhancement at C-1 as there is at C-3 after growth on 2-^{13}C glucose is also predicted. These data indicate a small but measurable amount of carbon flux through the pentose phosphate pathway.

Example 3

This example shows the methods for generating, screening and isolating mutants of *Prototheca* with altered AA productivities compared to the starting strain ATCC 75669.

ATCC No. 75669, identified as *Prototheca moriformis* RSP1385 (unicellular green microalga), was deposited on February 8, 1994, with the American Type Culture Collection (ATCC), Rockville, Maryland, 20852, USA, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. Initial screening of *Prototheca* species and strains was reported in U.S. Patent No. 5,900,370, *ibid.* Table 3 lists the formulations of the media for growth and maintenance of the strains. Glucose for fermentors was supplied as glucose monohydrate and calculated on an anhydrous basis. The recipe for the trace metals solution is given in Table 4. The standard growth temperature was 35°C. All organisms were cultured axenically.

TABLE 3
Media for Growth and Maintenance of *Prototheca* Strains
All quantities are in g/L unless otherwise specified

Ingredient	Liquid		Agar		
	Standard	Mg-limiting	Slants	Ferrozine Plates	Standard Plates
Potassium phosphate monobasic	1.3	1.3	2.0	0.27	2.0
Potassium phosphate dibasic	3.8	3.8	2.0	1.4	2.0
Trisodium citrate dihydrate	7.7	7.7	2.6	1.3	2.6
Magnesium sulfate heptahydrate	0.40	0.02	0.4	0.01	0.4
Ammonium sulfate	3.7	3.7	1.0	1.0	1.0
Trace Metals Solution	2 mL	2 mL	2 mL	2 mL	2 mL
Ferrous sulfate heptahydrate	1.5 mg	4.5 mg	1.5 mg	-	1.5 mg
Calcium chloride dihydrate	-	0.25	-	-	-

Ingredient	Liquid		Agar		
	Standard	Mg-limiting	Slants	Ferrozine Plates	Standard Plates
Manganous sulfate monohydrate	-	0.08	-	-	-
Yeast extract	-	-	2.5	-	-
Agar	-	-	15	15 (Noble)	15
pH before autoclaving	7.2	7.2	7.2	7.2	7.2
Autoclave, then add					
Copper sulfate, pentahydrate, 100 g/L	-	-	-	2 mL	-
40 g/L Ferrozine in 5 mM phosphate (pH 7.5 final)	-	-	-	8.8 mL	-
Ferric ammonium sulfate dodecahydrate, 40 g/L	-	-	-	3.8 mL	-
50% glucose with 25 mg/L thiamine HCl	40 mL	60 mL	10 mL	10 mL	10 mL

TABLE 4

Trace Metals Solution

Compound	Molecular Weight	Conc. of Individ. Solutions, g/L	mL Indiv. Stock per liter of Working Stock
Distilled Water	—	—	823
Hydrochloric Acid	—	Conc.	20
Cobalt Chloride hexahydrate	237.9	24.0	6.5
Boric acid	61.8	38.1	24
Zinc sulfate heptahydrate	287.5	35.3	50
Manganous sulfate monohydrate	169.0	24.6	50
Sodium molybdate dihydrate	242.0	23.8	2.0
Calcium chloride dihydrate	147.0	—	11.4 g
Vanadyl sulfate dihydrate	199.0	10.0	8.0
Nickel nitrate hexahydrate	290.8	5.0	8.0
Sodium selenite	173.0	5.0	8.0

Mutant isolates were generated by treatment with one or more of the following agents: nitrous acid (NA); ethyl methane sulfonate (EMS); or ultraviolet light (UV). Typically, glucose-depleted cells grown in standard liquid medium were washed and resuspended in 25 mM phosphate buffer, pH 7.2, diluted to approximately 10^7 colony-forming units per mL (cfu/mL), exposed to the mutagen to achieve about 99% kill, incubated 4-8 hours in the dark, and spread onto standard agar medium, or agar media containing differential agents.

Some mutant colonies on standard agar medium were picked randomly and subcultured to master plates. Other isolation plates were inverted over chloroform to lyse cells on the surface of the colonies and allow them to release AA. Released AA was detected by spraying the treated plates with a solution of 2,6-dichrorophenol-indophenol (1.25 g/L in 70% EtOH). The ability of AA to reduce this blue redox dye to its colorless form is the basis for a standard assay of AA (Omaye, *et al.*, 1979. Meth. Enzymol. 62:3-11.). Colonies derived from mutagenized cells were saved to master plates for further evaluation if their clear halos were significantly larger than the halos typical of the other mutants in that group. Other mutagenized cells were spread onto plates containing an AA detection system incorporated directly into the agar. This system is based on the ability of AA to reduce ferric iron to ferrous iron. The compound ferrozine (3-(2-pyridyl)-5,6- bis(4-phenylsulfonic acid)-1,2,4-triazine) was present in the agar to complex with the ferrous iron and give a violet color reaction. The ferrozine agar formulation is shown in Table 3. Colonies giving the darkest color reactions were master-plated. When screening for non-AA-producing strains (blocked mutants), white colonies were chosen against a background of relatively dark colonies.

For primary screening of tube cultures, cells were inoculated from master plates into 4 mL of Mg-limiting medium in 16 x 125 mm test tubes, and tubes were shaken in a slanted position on a rotary shaker at 300 rpm for four days. After both three and four days of incubation aliquots were removed for AA assay and cell density determination. Those for AA assay were centrifuged at 1500 x g for 5 min and the resulting supernates were removed for either colorimetric assay or high pressure liquid chromatography (HPLC). Promising isolates were retested in tube culture. Those passing the tube screen were tested in shake flasks.

For secondary screening of flask cultures, cells were inoculated into 50 mL of standard flask medium in 250 mL baffled shake flasks, and incubated on a rotary shaker at 180 rpm until glucose depletion (24-48 hours). A second series of flasks of Mg-sufficient standard medium was inoculated from the first set to a cell density of 0.15 A_{620}, and incubated for 24 hours. A third series of Mg-limiting flask medium was inoculated from the second set by a 1/50 dilution and incubated for 96 hours. Flasks were sampled for AA analysis and cell density measurements during this time as required.

Aliquots for supernatant AA analysis were centrifuged at 5000 x g for 5 min. Aliquots for total whole broth AA analysis were first extracted for 15 min with an equal volume of 5% trichloroacetic acid (TCA) before centrifugation. Aliquots of the resulting supernates were removed for either colorimetric assay or HPLC analysis.

For colorimetric assay of AA, a modification of the method of Omaye, *et al.* (1979. Meth. Enzymol. 62:3-11) was used. Twenty-five μL aliquots of culture supernates were added to wells of 96-well microplates, and 125 μL of color reagent was added. The color reagent consisted of four parts 0.5% aqueous 2,2'-dipyridyl and one part 8.3 mM ferric ammonium sulfate in 27 % (v/v) *o*-phosphoric acid, the two components being mixed immediately before use. After one hour, the absorbance at 520 nm was read. AA concentration was calculated by comparison of the absorbances of AA standards.

HPLC analysis was based on that of Running, *et al.*, (1994). Supernates were chromatographed on a Bio-Rad HPX-87H organic acid column (Bio-Rad Laboratories, Richmond, CA) with 13 mM nitric acid as solvent, at a flow rate of 0.7 mL/min at room temperature. Detection was at either 254 nm using a Waters 441 detector (Millipore Corp., Milford, MA), or at 245 nm using a Waters 481 detector. This system can distinguish between the L- and D- isomers of AA.

For dry weight determinations of cell density, 5 mL whole broth samples were centrifuged at 5000 x g for 5 min, washed once with distilled water, and the pellet was washed into a tared aluminum weighing pan. Cells were dried for 8-24 h at 105°C. Cell weight was calculated by difference.

Table 5 shows the abilities of various mutants of *Prototheca* to synthesize AA.

TABLE 5

AA Synthesizing Ability of Various *Prototheca* Mutants in Flask Screen

Strain	Specific AA Formation, mg AA per L/Culture A_{620}, during Mg-limited Incubation	
	2 Days Incubation	4 Days Incubation
ATCC 75669	22	35
EMS13-4	79	166
UV213-1	0	0
UV218-1	0	0
UV244-1	0	0

Strain	Specific AA Formation, mg AA per L/Culture A_{620} during Mg-limited Incubation	
	2 Days Incubation	4 Days Incubation
UV244-15	58	68
UV77-247	56	83
UV140-1	67	100
UV164-6	91	131
NA21-14	27	78
UV82-21	0	0
UV127-10	50	95
SP2-3	3	4

The genealogy of these isolates is presented graphically in the "family tree" of Fig. 3. ATCC No. _____, identified as *Prototheca moriformis* EMS13-4 (unicellular green microalga), was deposited on May 25, 1999, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110, USA, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. ATCC No. _____, identified as *Prototheca moriformis* UV127-10 (unicellular green microalga), was deposited on May 25, 1999, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110, USA, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. ATCC No. _____, identified as *Prototheca moriformis* SP2-3 (unicellular green microalga), was deposited on May 25, 1999, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110, USA, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure.

Example 4

The following example shows that both growing and resting cells of *Prototheca* can rapidly convert L-galactose and L-galactono-γ-lactone to AA, and that conversion of D-mannose to AA by *Prototheca* is more rapid than conversion of D-glucose.

Shake flask cultures of the mutant strain UV77-247 were grown to glucose depletion in standard liquid medium (Table 3). Cells were washed twice and resuspended in complete medium with the glucose substituted by one of the compounds listed below.

Cell suspensions were incubated for 24 hours at 35° C with shaking, and the entire suspension was extracted with TCA as above and assayed for AA.

Tables 6-8 show that both growing and resting cells of strain UV77-247 can rapidly convert L-galactose and L-galactono-γ-lactone to AA. In these experiments, D-fructose and D-galactose were converted to AA at the same rate as D-glucose, suggesting that they are metabolized to AA through the same route as D-glucose. None of the organic acids suggested in the literature to be intermediates in the biosynthesis of AA were converted to AA, including sorbosone, which has been proposed as an intermediate by Saito *et al.*(1990 Plant Physiol. 94:1496-1500).

TABLE 6

Conversion of Compounds by Resting Cells of Strain UV77-247

Substrate (50 mM)	Total AA, mg/L	AA Relative to No Substrate Control
L-galactose	965	623
L-galactono-γ-lactone	818	476
D-fructose	590	248
D-glucosone	589	247
D-glucose	584	242
D-galactose	542	200
D-glucose (10 mM)	388	46
D-gluconolactone	382	40
D-gulono-γ-lactone	366	24
D-glucuronate	364	22
L-sorbosone	342	0
None	342	0
2-keto-D-gluconic acid	341	-1
D-isoascorbic acid (10 mM)	330	-12
D-glucuronolactone	329	-13
D-gluconic acid	309	-33
D-galacturonic acid	297	-45
L-idonate	296	-46

Since strain UV77-247 converted L-galactose and L-galactono-γ-lactone to AA much more rapidly than it did glucose, it suggests that these compounds are intermediates in the AA biosynthetic pathway and that they are "downstream" from glucose.

The data in Tables 7 and 8 also show that growing and resting cells of UV77-247 consistently convert D-mannose to AA at a rate greater than that of glucose.

TABLE 7

Conversion of Compounds to AA by Resting Cells of Strain UV77-247

Compound	Ascorbic Acid, mg/L		
	25.5 h	30 h	47 h
L-galactose	667	718	620
L-galactono-γ-lactone	644	681	749
D-glucosone	465	462	354
D-mannose	448	462	399
D-fructose	402	408	367
d-glucose	395	404	351
D-galactose	352	361	337
none	287	288	258

TABLE 8

Conversion of Compounds to AA by Growing Cells of Strain UV77-247

Compound	Ascorbic Acid, mg/L		A_{620}	AA/A_{620}
	25.5 h	44 h		
L-galactose	249	506	4.5	112
D-mannose	228	488	5.6	87
L-galactono-γ-lactone	214	342	5.0	68
D-glucose	178	398	5.9	67
D-fructose	181	383	5.9	65
D-glucosone	176	362	5.7	64
D-galactose	185	380	5.9	64
none	182	249	4.4	57
D-gluconic acid (K)	178	262	5.0	52
L-idonate (Na)	182	232	4.7	49
2-keto-D-gluconic acid	182	255	5.3	48
2-deoxy-D-glucose	181	227	4.8	47
D-glucuronic acid lactone	165	218	5.0	44
D-glucuronic acid (Na)	173	241	5.6	43
L-gulono-γ-lactone	152	195	5.0	39
L-sorbosone	178	160	4.7	34
D-glucono-δ-lactone	130	190	5.7	33
D-galacturonic acid	130	180	6.0	30

These cells converted L-galactose, L-galactono-γ-lactone and D-mannose to AA more rapidly than they did glucose, suggesting that mannose exerts its effect in the biosynthetic pathway "downstream" from glucose.

Example 5

Using the methods described above, a collection of mutants was assembled. The specific AA formation for representative mutants are shown in Table 5. The genealogy of these isolates is presented graphically in the "family tree" of Fig. 3.

These isolates were tested for their ability to convert compounds which could be converted to AA by strain UV77-247. Testing was done as in Example 4. Results are shown in Table 9.

TABLE 9

Conversion of Compounds to AA by Resting Cells of Mutant Strains of *Prototheca* of Varying Abilities to Synthesize AA

Strain	Absolute AA, mg/L					
	Buffer	Glucose	L-galactose	L-gal-γ-lact.	Mannose	Fructose
EMS13-4	53	97	191	173	139	ND
UV127-10	45	140	213	140	128	143
SP2-3	19	19	204	146	24	27
NA21-14	61	80	147	158	118	115
UV82-21	15	16	183	175	18	17
UV213-1	16	15	170	135	17	16
UV218-1	16	18	136	176	19	21
UV244-1	16	16	164	162	16	16
UV244-15	26	77	30	21	94	89
UV244-16	28	64	53	53	53	66

ND = Not Determined

These data suggest that the mutational blocks in those strains which convert fructose and mannose to AA poorly are before ("upstream" from) L-galactose and L-galactono-γ-lactone in the pathway.

Example 6

The following example shows that magnesium inhibits early steps in the production of AA.

To address the question of whether magnesium actually inhibits AA synthesis, strain NA45-3 (ATCC 209681) was grown in magnesium (Mg)-limited and Mg-sufficient medium. ATCC No. 209681, identified as *Prototheca moriformis* NA45-3 (Source:

repeated mutagenesis of ATCC No. 75669; Eucaryotic alga. Division Chlorophyta, Class Chlorophyceae, Order Chlorococcales), was deposited on March 13, 1998, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110, USA, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. Cells from both cultures were harvested and resuspended in the cell-free supernate from the Mg-limited culture, and to half of each cell suspension additional magnesium was added in order to bring the level in the suspension to the Mg-sufficient level. The four conditions under which assays were run were as follows.

TABLE 10

Conditions Used to Test the Effect of Magnesium on AA Production

Condition	Magnesium concentration, g/L, during:	
	Growth	Assay
1Mg>1Mg	0.02	0.02
1Mg>10Mg	0.02	0.2
10Mg>1Mg	0.2	0.02
10Mg>10Mg	0.2	0.2

Substrates previously shown to lead to the formation of AA, namely D-glucose, D-glucosone, D-fructose. D-galactose, D-mannose, and L-galactono-γ-lactone, were added at 20 g/L to the four cell suspensions. Accumulation of AA after 24 hours was measured and compared to a control in which no substrate was added. The results of this study are shown graphically in Fig. 4.

When cells growing under magnesium-limited conditions were incubated with substrates in low-magnesium broth (1Mg>1Mg condition), all showed significant and similar accumulation of AA over the control condition. When the same cells were incubated in high magnesium broth (1Mg>10Mg condition), the accumulation of AA was reduced about 40% for all substrates except D-mannose and L-galactono-γ-lactone, suggesting that 1) the rate-limiting step in the conversion of D-glucose, D-glucosone, D-fructose, and D-galactose to AA is inhibited by magnesium or 2) magnesium stimulates an enzyme which results in the conversion of these compounds to some other compound(s), reducing the amount of substrate available for AA synthesis. On the other

hand, conversion of D-mannose and L-galactono-γ-lactone appeared to be unaffected by the presence of magnesium in the resuspension buffer, indicating that either 1) magnesium-inhibited enzymes are not involved in the conversion of these substrates to AA or 2) D-mannose and L-galactono-γ-lactone enter the pathway far enough downstream from the point where they can be siphoned off by side reactions involving Mg-requiring enzymes.

When cells were grown under magnesium-sufficient conditions, very little AA accumulation from any of the D-sugars was observed, regardless of the level of magnesium in the resuspension broth. Accumulation of AA from L-galactono-γ-lactone, however, was enhanced over that observed when cells are grown in Mg-limited conditions. This suggests that enzymes early in the pathway are repressed under Mg-sufficient conditions. Thus, the D-substrates all behaved similarly, with the exception of the apparent lack of magnesium inhibition of D-mannose conversion to AA. This would suggest that D-mannose enters the AA biosynthetic pathway at a point other than the other D-sugars.

Figs. 2A and 2B represent some of the fates of glucose in plants. The first enzymatic step in this scheme which commits carbon to glycolysis is the conversion of fructose-6-P to fructose-1,6-diP by phosphofructokinase (PFK). This reaction is essentially irreversible, and leads to the well known TCA cycle and oxidative phosphorylation, with concomitant ATP and NADH/NADPH generation. PFK has an absolute requirement for magnesium. If magnesium is limiting, this reaction could slow and eventually stop, blocking the flow of carbon through glycolysis and beyond, and would result in cessation of cell division even in the presence of excess glucose. One would expect fructose-6-P to accumulate under these conditions, fueling AA synthesis by the pathway shown in Figs. 1 and 2.

Example 7

The following example shows the correlation in *Prototheca* between AA production and the activity levels of the enzymes in the AA pathway.

Phosphomannose isomerase (PMI) Assay

PMI activity was first assayed (See Fig. 1). Ten strains representing a range of AA productivities were grown according to the standard protocol to measure AA-synthesizing ability. Cells were harvested 96 hours into magnesium-limited incubation, washed and resuspended in buffer containing 50 mM Tris/10 mM $MgCl_2$, pH 7.5. The suspended cells were broken in a French press, spun at 30,000 x g for 30 minutes, and desalted through Sephadex G-25 (Pharmacia PD-10 columns). Reactions were carried out in the reverse direction by adding various volumes of extracts to solutions of Tris/Mg buffer containing 0.15 U phosphoglucose isomerase (EC 5.3.1.9), 0.5 U glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and 1.0 mM NADP. Reactions were initiated by addition of 3 mM (final) mannose-6-phosphate. Final reaction volume was 1.0 mL. All components were dissolved in Tris/Mg buffer. Activities were taken as the change in A_{340}/min. From these activities was subtracted the activities measured in identical reaction mixtures lacking the M-6-P substrate. Specific activities were calculated by normalizing the activities for protein concentration in the reactions. Protein in the original extracts was determined by the method of Bradford, using a kit from Bio-Rad Laboratories (Hercules, CA). All enzymes and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO).

Phosphomannomutase (PMM) Assay

Phosphomannomutase was measured in a similar manner in the same strains, but these assay reaction mixtures also contained 0.25 mM glucose-1,6-diphosphate, 0.5 U commercially available PMI, and the reactions were started with the addition of 3.0 mM (final) mannose-1-phosphate rather than mannose-6-phosphate.

Phosphofructokinase (PFK) Assay

To shed light on the possibility that the enhancement of AA concentration in cultures which were limited for magnesium was due to a diversion of carbon from normal metabolism by a reduced activity of the first committed step in glycolysis (PFK) the strains were also assayed to confirm the presence of this enzyme activity. Cells were cultured, washed and broken as above. Extracts were centrifuged at 100,000 x g for 90 min before

desalting. Reactions were carried out in the forward direction by adding various volumes of extracts to solutions of Tris/Mg buffer containing 1.5 mM dithiothreitol, 0.86 U aldolase (EC 4.1.2.13), 1.4 U α-glycerophosphate dehydrogenase (EC 1.1.1.8), 14 U triosephosphate isomerase (EC 5.3.1.1), 0.11 mM NADH, and 1.0 mM ATP. Reactions were initiated by addition of 5 mM (final) fructose-6-phosphate. Final reaction volume was 1.0 mL. All components were dissolved in Tris/Mg buffer. Activities were taken as the change in A_{340}/min. From these activities were subtracted the activities measured in identical reaction mixtures lacking the F-6-P substrate. Specific activities were calculated by normalizing the activities for protein concentration in the reaction. Protein in the original extracts was determined as above.

GDP-D-mannose pyrophosphorylase (GMP) Assay

These same mutant strains were assayed for the next enzyme in the proposed pathway, GMP. Strains were grown both according to the standard Mg-limiting protocol (harvested 43-48 hours into magnesium-limited incubation) and in standard Mg-sufficient medium (harvesting all cells before glucose depletion). Washed cell pellets were resuspended in 50 mM phosphate buffer, pH 7.0, containing 20% (v/v) glycerol and 0.1 M sodium chloride (3 mL buffer/g wet cells), and broken in a French press. Crude extracts were spun at 15,000 x g for 15 minutes. Reactions were carried out in the forward direction by adding various volumes of extracts to solutions of 50 mM phosphate/4 mM $MgCl_2$ buffer, pH 7.0, containing 1 mM GTP. Reactions were initiated by addition of 1 mM (final) mannose-1-phosphate. Final reaction volume was 0.1 mL. Reaction mixtures were incubated at 30 C for 10 min, filtered through a 0.45 µm PVDF syringe filter, and analyzed for GDP-mannose by HPLC. A Supelcosil SAX1 column (4.6 x 250 mm) was used with a solvent gradient (1 mL/min) of: A - 6 mM potassium phosphate, pH 3.6; B - 500 mM potassium phosphate, pH 4.5. The gradient was: 0-3 min, 100% A; 3-10 min, 79% A; 10-15 min, 29% A. Column temperature was 30 C. Two assays that showed enzyme activity proportional to the amount of protein were averaged. Control no-substrate and no-extract reactions were also run. Specific activity was calculated by normalizing the activity for protein concentration in the reaction. Protein in the original extracts was determined as above.

GDP-D-mannose:GDP-L-galactose Epimerase Assay

Further tests measured the activities of the next enzyme in the proposed pathway, GDP-D-mannose:GDP-L-galactose epimerase. Strains were grown according to the standard protocol, harvested 43-48 hours into magnesium-limited incubation, washed, and resuspended in buffer containing 50 mM MOPS/5 mM EDTA, pH 7.2. Washed pellets were broken in a French press, and spun at 20,000 x g for 20 min. Protein determinations were made as above and a dilution series of each was made, ranging from 0.4 to 2.2 mg protein/mL. 50 µL aliquots of these dilutions were added to 10 µL aliquots of 6.3 mM GDP-D-mannose in which a portion of this substrate was universally labeled with ^{14}C in the mannose moiety. This substrate had an activity of 16 µCi/mL before dilution into the reaction mixture. Reactions were stopped after 10 min by transferring 20 µL of the mixture into microfuge tubes containing 20 µL of 250 mM trifluoroacetic acid (TFA) containing 1.0 g/L each D-mannose and L-galactose. These tubes were sealed and boiled for 10 min, cooled, spun for 60 sec in a Beckman Microfuge E, and 5 µL of each hydrolysate was spotted on 20 x 20 cm plastic-backed EM Science Silica gel 60 thin-layer chromatography plates (#5748/7), with 1 cm lanes created by scoring with a blunt stylus. After drying, plates were twice chromatographed for 2.5 hours in ethyl acetate:isopropanol:water, 65:22.3:12.7 (plates were dried between runs). Spots of free sugars were visualized by spraying dried plates with 0.5% p-anisaldehyde in a 62% ethanolic solution of 0.89 M sulfuric acid and 0.17 mM glacial acetic acid, and heating at 105 C for about 15 min. Spots of L-galactose and D-mannose were cut from the plates and counted in a scintillation counter (Beckman model 2800). For time-zero control counts, 16.7 µL of each extract dilution was added to 23.3 µL of the labeled substrate above, which had been diluted 1:7 with the TFA/mannose/galactose solution.

Table 11 summarizes the results of the five enzyme assays for the strains tested, along with their specific AA formations.

TABLE 11

Specific Enzyme Activities (mU)* of Selected Mutant *Prototheca* Strains

Strain	AA Specific Form, mg/g	PMI	PMM	PFK	GMP		Epimerase
					Mg-limited	Mg-sufficient	
UV164-6	78.4						0.79
EMS13-4	73.7	10.8	69.6	13.5	2.6	6.8	0.78
UV140-1	69.9						0.78
NA45-3	61.4						0.58
UV77-247	44.4						0.52
UV127-10	40.1	11.1	45.8	24.4	4.3	5.9	0.39
UV244-15	24.5	14.3	41.5		3.1	5.3	0.42
NA21-14	23.6	12.1	60.3	47.4	2.4	7.6	0.27
ATCC 75669	21.9						0.28
UV244-16	5.0	16.5	85.6		4.3	5.2	
SP2-3	2.0	17.7	47.0	64.5	2.0	7.5	0.03
UV218-1	0.4	15.9	72.1		2.7	7.0	0.83
UV213-1	0.1	19.7	47.7	32.6	3.2	6.7	0.60
UV82-21	0.0	14.6	70.6	30.4	4.1	7.5	0.15
UV244-1	0.0	18.2	51.1		5.5	12	0.15

Units: PMI and PMM, nmoles NADP reduced per min/mg protein; PFK, nmoles NADH oxdized per min/mg protein; GMP, nmoles GDP-D-mannose formed per min/mg protein; epimerase, nmoles GDP-L-galactose formed per min/mg protein.

The only enzyme which showed a strong correlation between activity and the ability to synthesize AA was the GDP-D-mannose:GDP-L-galactose epimerase. This correlation is depicted in Fig. 5. All of the strains which produced measurable amounts of AA had measurable amounts of epimerase activity. The converse was not true: four of the strains which synthesize little or no AA had significant epimerase activities. These strains are candidates for having mutations which affect enzymatic steps downstream from the epimerase. Since all of the strains tested can synthesize AA from L-galactose and L-galactono-γ-lactone (see Examples 4 and 5), the genetic lesion(s) in these four mutants must lie between GDP-L-galactose and free L-galactose.

Example 8

The next example shows the relationship between GDP-D-mannose:GDP-L-galactose epimerase activity and the degree of magnesium limitation in two strains, the original unmutagenized parent strain ATCC 75669, and one of the best AA producers, EMS13-4 (ATCC _____).

Four flasks of each strain were grown according to the standard protocol. One culture of each was harvested 24 hours into magnesium-limited incubation, and every 24 hours thereafter for a total of four days. One flask of each strain was also harvested 24 hours into magnesium sufficient incubation. All cultures had glucose remaining when harvested. Fig. 6 shows graphically the AA productivity and epimerase activity in EMS13-4 and ATCC 75669 as the cultures became Mg-limited. Epimerase activity in EMS13-4 was significantly greater than that in ATCC 75669 at all time points. There was also a concurrent rapid rise in both AA productivity and epimerase activity in EMS13-4 as the cultures became increasingly Mg-limited. While there was a moderate increase in AA productivity in ATCC 75669 as Mg became more limiting, there was no effect on epimerase activity.

Example 9

The following example shows the results of epimerase assays performed with extracts of two *E. coli* strains into which were cloned the *E. coli* gene for GDP-4-keto-6-deoxy-D-mannose epimerase/reductase.

The *E. coli* K12 *wca* gene cluster is responsible for cholanic acid production; *wcaG* encodes a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase.

The *E. coli wcaG* sequence (nucleotides 4 through 966 of SEQ ID NO:3) was amplified by PCR from *E. coli* W3110 genomic DNA using primers WG EcoRI 5 (5' TAGAATTCAGTAAACAACGAGTTTTTATTGCTGG 3'; SEQ ID NO:12) and WG XhoI 3 (5' AACTCGAGTTACCCCCAAAGCGGTCTTGATTC 3'; SEQ ID NO:13). The 973-bp PCR product was ligated into the vector pPCR-Script SK(+) (Stratagene, LaJolla, CA). The 973-bp ExoRII/XhoI fragment was moved from this plasmid into the ExoRII/XhoI sites of pGEX-5X-1 (Amersham Pharmacia Biotech, Piscataway, NJ), creating plasmid pSW67-1. Plasmid pGEX-5X-1 is a GST gene fusion vector which adds a 26-kDa GST moiety onto the N-terminal end of the protein of interest. *E. coli* BL21(DE3) was transformed with pSW67-1 and pGEX-5X-1, resulting in strains BL21(DE3)/pSW67-1 and BL21(DE3)/pGEX-5X-1.

The *E. coli wcaG* sequence (nucleotides 1 through 966 of SEQ ID NO:3) was also amplified by PCR from *E. coli* W3110 genomic DNA using primers WG EcoRI 5-2 (5' CTGGAGTCGAATTCATGAGTAAACAACGAG 3'; SEQ ID NO:14) and WG PstI 3

(5' AACTGCAGTTACCCCCGAAAGCGGTCTTGATTC 3'; SEQ ID NO:15). The 976-bp PCR product was ligated into a pPCR-Script (Stratagene). The 976-bp ExoRII/PstI fragment was moved from this plasmid into the ExoRII/PstI sites of expression vector pKK223-3 (Amersham Pharmacia Biotech), creating plasmid pSW75-2. *E. coli* JM105 was transformed with pKK223-3 and pSW75-2, resulting in strains JM105/pKK223-3 and JM105/pSW75-2.

All six strains were grown in duplicate at 37°C with shaking in 2X YTA medium until an optical density of 0.8-1.0 at 600 nm was reached (about three hours). 2X YTA contains 16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride and 100 mg/L ampicillin. One of each culture was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to 1 mM final concentration. All 12 cultures were incubated for an additional four hours, washed in 0.9% NaCl, and the cells were frozen at -80°C. Prior to pelleting the cells for preparation of extracts, a portion of each culture was used for a plasmid DNA miniprep to confirm the presence of the appropriate plasmids in these strains. A protein preparation of each culture was also run on SDS gels to confirm expression of a protein of the appropriate size where expected. Frozen pellets were thawed, resuspended in 2.5 mL MOPS/EDTA buffer, pH 7.2, broken in a French Press (10,000 psi), spun for 20 min at 20,000 x g, assayed for protein as above and diluted to 0.01, 0.1, 1.0 and 3 mg/mL protein.

Induction of the strain BL21(DE3)/pGEX-5X-1 resulted in high-level expression of a 26-kDa protein indicating the synthesis of the native GST protein. Induction of strain BL21(DE3)/pSW67-1 resulted in high-level expression of a 62-kDa protein, indicating the synthesis of the native GST protein (26K) fused to the *wcaG* gene product (36K). An aliquot of the fusion protein was treated with the protease Factor Xa (New England Biolabs, Beverly, MA), which cleaves near the GST/*wcaG* junction. Induction of the strain JM105/pSW75-2 resulted in high level expression of a 36-kDa protein, indicating the synthesis of the *wcaG* gene product. No such protein was detected in JM105/pKK223-3 (vector only).

Next, it was of interest to test extracts in the standard epimerase assay described in Example 7 to determine if any of the extracts containing the *wcaG* product could bring

about the conversion of GDP-D-mannose to GDP-L-galactose. The extracts to be assayed are:

BL21(DE3) Group

1. BL21(DE3) uninduced
2. BL21(DE3) induced with 1mM IPTG
3. BL21(DE3)/pGEX-5X-1 uninduced
4. BL21(DE3)/pGEX-5X-1 induced with 1mM IPTG
5. BL21(DE3)/pSW67-1 uninduced
6. BL21(DE3)/pSW67-1 induced with 1 mM IPTG; fusion protein intact
7. BL21(DE3)/pSW67-1 induced with 1 mM IPTG; GST moiety cleaved

JM105 Group

1. JM105 uninduced
2. JM105 induced with 1mM IPTG
3. JM105/pKK223-3 uninduced
4. JM105/pKK223-3 induced with 1 mM IPTG
5. JM105/pSW75-2 uninduced
6. JM105/pSW75-2 induced with 1 mM IPTG

Extracts 1 and 7 from the BL21(DE3) group and extracts 1 and 6 from the JM105 group were tested for GDP-D-mannose:GDP-L-galactose epimerase-like activity in a pilot experiment. In this initial experiment, no epimerase activity was detected in any of the extracts. At this time, such a result can be attributed to a number of possibilities. First, it is possible that the *wcaG* gene product is incapable of catalyzing the conversion of GDP-D-mannose to GDP-L-galactose, although this conclusion can not be reached until several other parameters are tested. Second, it is possible that under the assay conditions which are satisfactory to measure activity for the endogenous GDP-D-mannose:GDP-L-galactose epimerase, the *wcaG* gene product does not have GDP-D-mannose:GDP-L-galactose epimerase-like activity. Therefore, alternate conditions should be tested. Additionally, confirmation experiments should be performed to confirm the accuracy of the pilot conditions. Third, although the BL21(DE3) and the JM105 clones produce proteins of the expected size, the constructs have not been sequenced to confirm the proper coding sequence for the *wcaG* gene product and thereby rule out PCR or cloning errors which may render the *wcaG* gene product inactive. Fourth, the protein formed from the cloned sequence is full-length, but inactive, for example, due to incorrect tertiary structure (folding). Fifth, the gene is overexpressed, resulting in accumulation of insoluble and inactive protein products (inclusion bodies). Future experiments will attempt to

determine whether the constructs have or can be induced to have the ability to catalyze the conversion of GDP-D-mannose to GDP-L-galactose, and to use the sequences to isolate the endogenous GDP-D-mannose:GDP-L-galactose epimerase.

Table 12 provides the atomic coordinates for Brookhaven Protein Data Bank Accession Code 1bws:

TABLE 12

```
HEADER    EPIMERASE/REDUCTASE                     27-SEP-98   1BWS
TITLE     CRYSTAL STRUCTURE OF GDP-4-KETO-6-DEOXY-D-MANNOSE
TITLE    2 EPIMERASE/REDUCTASE FROM ESCHERICHIA COLI A KEY ENZYME IN
TITLE    3 THE BIOSYNTHESIS OF GDP-L-FUCOSE
COMPND    MOL_ID: 1;
COMPND   2 MOLECULE: GDP-4-KETO-6-DEOXY-D-MANNOSE EPIMERASE/REDUCTASE;
COMPND   3 CHAIN: A;
COMPND   4 ENGINEERED: YES;
COMPND   5 BIOLOGICAL_UNIT: HOMODIMER
SOURCE    MOL_ID: 1;
SOURCE   2 ORGANISM_SCIENTIFIC: ESCHERICHIA COLI;
SOURCE   3 EXPRESSION_SYSTEM: ESCHERICHIA COLI
KEYWDS    EPIMERASE/REDUCTASE, GDP-L-FUCOSE BIOSYNTHESIS
EXPDTA    X-RAY DIFFRACTION
AUTHOR    DE M.RIZZITONETTIFLORA
REVDAT   1   13-JAN-99 1BWS     0
JRNL        AUTH   DE D.RIZZITONETTIVIGEVANISTURLABISSOFLORA
JRNL        TITL   GDP-4-KETO-6-DEOXYD-MANNOSE EPIMERASE/REDUCTASE
JRNL        TITL 2 FROM ESCHERICHIA COLI, A KEY ENZYME IN THE
JRNL        TITL 3 BIOSYNTHESIS OF GDP-L-FUCOSE, DISPLAYS THE
JRNL        TITL 4 STRUCTURAL CHARACTERISTICS OF THE RED PROTEIN
JRNL        TITL 5 HOMOLOGY SUPERFAMILY
JRNL        REF    STRUCTURE (LONDON)                1998
JRNL        REFN                                        9999
REMARK   1
REMARK   2
REMARK   2 RESOLUTION. 2.2  ANGSTROMS.
REMARK   3
REMARK   3 REFINEMENT.
REMARK   3   PROGRAM     : TNT
REMARK   3   AUTHORS     : TRONRUD,TEN EYCK,MATTHEWS
REMARK   3
REMARK   3 DATA USED IN REFINEMENT.
REMARK   3   RESOLUTION RANGE HIGH (ANGSTROMS) : 2.2
REMARK   3   RESOLUTION RANGE LOW  (ANGSTROMS) : 15.0
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REMARK   3   CROSS-VALIDATION METHOD           : NONE
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REMARK   3   R VALUE              (WORKING SET) : NONE
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REMARK   3
REMARK   3  USING ALL DATA, NO SIGMA CUTOFF.
REMARK   3   R VALUE    (WORKING + TEST SET, NO CUTOFF) : NULL
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REMARK   3   PROTEIN ATOMS            : 2527
REMARK   3   NUCLEIC ACID ATOMS       : NULL
REMARK   3   OTHER ATOMS              : 109
REMARK   3
REMARK   3  WILSON B VALUE (FROM FCALC, A**2) : NULL
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REMARK   3  RMS DEVIATIONS FROM IDEAL VALUES.      RMS     WEIGHT  COUNT
REMARK   3   BOND LENGTHS                  (A) : 0.016 ; NULL  ; NULL
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REMARK   3   TORSION ANGLES          (DEGREES) : NULL  ; NULL  ; NULL
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REMARK   3   METHOD USED : NULL
REMARK   3   KSOL        : NULL
REMARK   3   BSOL        : NULL
REMARK   3
```

```
REMARK   3  RESTRAINT LIBRARIES.
REMARK   3    STEREOCHEMISTRY : NULL
REMARK   3    ISOTROPIC THERMAL FACTOR RESTRAINTS : NULL
REMARK   3
REMARK   3  OTHER REFINEMENT REMARKS: NULL
REMARK   4
REMARK   4 1BWS COMPLIES WITH FORMAT V. 2.2, 16-DEC-1996
REMARK   5
REMARK   5 WARNING
REMARK   5 1BWS: THIS IS LAYER 1 RELEASE.
REMARK   5
REMARK   5 PLEASE NOTE THAT THIS ENTRY WAS RELEASED AFTER DEPOSITOR
REMARK   5 CHECKING AND APPROVAL BUT WITHOUT PDB STAFF INTERVENTION.
REMARK   5 AN AUXILIARY FILE, AUX1BWS.RPT, IS AVAILABLE FROM THE
REMARK   5 PDB FTP SERVER AND IS ACCESSIBLE THROUGH THE 3DB BROWSER.
REMARK   5 THE FILE CONTAINS THE OUTPUT OF THE PROGRAM WHAT_CHECK AND
REMARK   5 OTHER DIAGNOSTICS.
REMARK   5
REMARK   5 NOMENCLATURE IN THIS ENTRY, INCLUDING HET RESIDUE NAMES
REMARK   5 AND HET ATOM NAMES, HAS NOT BEEN STANDARDIZED BY THE PDB
REMARK   5 PROCESSING STAFF.  A LAYER 2 ENTRY WILL BE RELEASED SHORTLY
REMARK   5 AFTER THIS STANDARDIZATION IS COMPLETED AND APPROVED BY THE
REMARK   5 DEPOSITOR.  THE LAYER 2 ENTRY WILL BE TREATED AS A
REMARK   5 CORRECTION TO THIS ONE, WITH THE APPROPRIATE REVDAT RECORD.
REMARK   5
REMARK   5 FURTHER INFORMATION INCLUDING VALIDATION CRITERIA USED IN
REMARK   5 CHECKING THIS ENTRY AND A LIST OF MANDATORY DATA FIELDS
REMARK   5 ARE AVAILABLE FROM THE PDB WEB SITE AT
REMARK   5 HTTP://WWW.PDB.BNL.GOV/.
REMARK 200
REMARK 200 EXPERIMENTAL DETAILS
REMARK 200  EXPERIMENT TYPE                : X-RAY DIFFRACTION
REMARK 200  DATE OF DATA COLLECTION        : AUG-1997
REMARK 200  TEMPERATURE           (KELVIN) : 120
REMARK 200  PH                             : 6.5
REMARK 200  NUMBER OF CRYSTALS USED        : 1
REMARK 200
REMARK 200  SYNCHROTRON              (Y/N) : N
REMARK 200  RADIATION SOURCE               : NONE
REMARK 200  BEAMLINE                       : NULL
REMARK 200  X-RAY GENERATOR MODEL          : RIGAKU RU200
REMARK 200  MONOCHROMATIC OR LAUE    (M/L) : M
REMARK 200  WAVELENGTH OR RANGE        (A) : 1.5418
REMARK 200  MONOCHROMATOR                  : NULL
REMARK 200  OPTICS                         : NULL
```

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REMARK 200
REMARK 200  DETECTOR TYPE                    : IMAGE PLATE
REMARK 200  DETECTOR MANUFACTURER            : RAXIS
REMARK 200  INTENSITY-INTEGRATION SOFTWARE : MOSFLM
REMARK 200  DATA SCALING SOFTWARE            : SCALA
REMARK 200
REMARK 200  NUMBER OF UNIQUE REFLECTIONS   : 24481
REMARK 200  RESOLUTION RANGE HIGH      (A) : 2.2
REMARK 200  RESOLUTION RANGE LOW       (A) : 15.0
REMARK 200  REJECTION CRITERIA  (SIGMA(I)) : NONE
REMARK 200
REMARK 200 OVERALL.
REMARK 200  COMPLETENESS FOR RANGE     (%) : 99.7
REMARK 200  DATA REDUNDANCY                : 4.3
REMARK 200  R MERGE                    (I) : 0.057
REMARK 200  R SYM                      (I) : NONE
REMARK 200  <I/SIGMA(I)> FOR THE DATA SET  : 13.6
REMARK 200
REMARK 200 IN THE HIGHEST RESOLUTION SHELL.
REMARK 200  HIGHEST RESOLUTION SHELL, RANGE HIGH (A) : NULL
REMARK 200  HIGHEST RESOLUTION SHELL, RANGE LOW  (A) : NULL
REMARK 200  COMPLETENESS FOR SHELL     (%) : NULL
REMARK 200  DATA REDUNDANCY IN SHELL       : NULL
REMARK 200  R MERGE FOR SHELL          (I) : NULL
REMARK 200  R SYM FOR SHELL            (I) : NULL
REMARK 200  <I/SIGMA(I)> FOR SHELL         : NULL
REMARK 200
REMARK 200 DIFFRACTION PROTOCOL: NULL
REMARK 200 METHOD USED TO DETERMINE THE STRUCTURE: MIR
REMARK 200 SOFTWARE USED: NULL
REMARK 200 STARTING MODEL: NULL
REMARK 200
REMARK 200 REMARK: NULL
REMARK 280
REMARK 280 CRYSTAL
REMARK 280 SOLVENT CONTENT, VS   (%): NULL
REMARK 280 MATTHEWS COEFFICIENT, VM (ANGSTROMS**3/DA): NULL
REMARK 280
REMARK 280 CRYSTALLIZATION CONDITIONS: NULL
REMARK 290
REMARK 290 CRYSTALLOGRAPHIC SYMMETRY
REMARK 290 SYMMETRY OPERATORS FOR SPACE GROUP: P 32 2 1
REMARK 290
REMARK 290      SYMOP   SYMMETRY
REMARK 290     NNNMMM   OPERATOR
```

```
REMARK 290       1555   X,Y,Z
REMARK 290       2555   -Y,X-Y,Z+2/3
REMARK 290       3555   Y-X,-X,Z+1/3
REMARK 290       4555   Y,X,-Z
REMARK 290       5555   X-Y,-Y,1/3-Z
REMARK 290       6555   -X,Y-X,2/3-Z
REMARK 290
REMARK 290      WHERE NNN -> OPERATOR NUMBER
REMARK 290            MMM -> TRANSLATION VECTOR
REMARK 290
REMARK 290 CRYSTALLOGRAPHIC SYMMETRY TRANSFORMATIONS
REMARK 290 THE FOLLOWING TRANSFORMATIONS OPERATE ON THE ATOM/HETATM
REMARK 290 RECORDS IN THIS ENTRY TO PRODUCE CRYSTALLOGRAPHICALLY
REMARK 290 RELATED MOLECULES.
REMARK 290   SMTRY1   1  1.000000  0.000000  0.000000        0.00000
REMARK 290   SMTRY2   1  0.000000  1.000000  0.000000        0.00000
REMARK 290   SMTRY3   1  0.000000  0.000000  1.000000        0.00000
REMARK 290   SMTRY1   2 -0.500045 -0.865974  0.000000        0.00000
REMARK 290   SMTRY2   2  0.866077 -0.499955  0.000000        0.00000
REMARK 290   SMTRY3   2  0.000000  0.000000  1.000000       50.58553
REMARK 290   SMTRY1   3 -0.499955  0.865974  0.000000        0.00000
REMARK 290   SMTRY2   3 -0.866077 -0.500045  0.000000        0.00000
REMARK 290   SMTRY3   3  0.000000  0.000000  1.000000       25.29276
REMARK 290   SMTRY1   4 -0.500045  0.865922  0.000000        0.00000
REMARK 290   SMTRY2   4  0.866077  0.500045  0.000000        0.00000
REMARK 290   SMTRY3   4  0.000000  0.000000 -1.000000        0.00000
REMARK 290   SMTRY1   5  1.000000  0.000104  0.000000        0.00000
REMARK 290   SMTRY2   5  0.000000 -1.000000  0.000000        0.00000
REMARK 290   SMTRY3   5  0.000000  0.000000 -1.000000       25.29276
REMARK 290   SMTRY1   6 -0.499955 -0.866026  0.000000        0.00000
REMARK 290   SMTRY2   6 -0.866077  0.499955  0.000000        0.00000
REMARK 290   SMTRY3   6  0.000000  0.000000 -1.000000       50.58553
REMARK 290
REMARK 290 REMARK: NULL
REMARK 465
REMARK 465 MISSING RESIDUES
REMARK 465 THE FOLLOWING RESIDUES WERE NOT LOCATED IN THE
REMARK 465 EXPERIMENT. (M=MODEL NUMBER; RES=RESIDUE NAME; C=CHAIN
REMARK 465 IDENTIFIER; SSSEQ=SEQUENCE NUMBER; I=INSERTION CODE):
REMARK 465
REMARK 465   M RES C SSSEQI
REMARK 465     MET A     1
REMARK 465     SER A     2
REMARK 465     ASP A   317
REMARK 465     ARG A   318
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REMARK 465     PHE A   319
REMARK 465     ARG A   320
REMARK 465     GLY A   321
REMARK 800
REMARK 800 SITE
REMARK 800 SITE_IDENTIFIER: CAT
REMARK 800 SITE_DESCRIPTION:
REMARK 800 CATALYTIC RESIDUE
REMARK 800
REMARK 800 SITE_IDENTIFIER: CAT
REMARK 800 SITE_DESCRIPTION:
REMARK 800 CATALYTIC RESIDUE
REMARK 800
REMARK 800 SITE_IDENTIFIER: CAT
REMARK 800 SITE_DESCRIPTION:
REMARK 800 CATALYTIC RESIDUE
REMARK 800
DBREF  1BWS A    3   316  SWS    P32055   FCL_ECOLI
SEQRES   1 A  321  MET SER LYS GLN ARG VAL PHE ILE ALA GLY HIS ARG GLY
SEQRES   2 A  321  MET VAL GLY SER ALA ILE ARG ARG GLN LEU GLU GLN ARG
SEQRES   3 A  321  GLY ASP VAL GLU LEU VAL LEU ARG THR ARG ASP GLU LEU
SEQRES   4 A  321  ASN LEU LEU ASP SER ARG ALA VAL HIS ASP PHE PHE ALA
SEQRES   5 A  321  SER GLU ARG ILE ASP GLN VAL TYR LEU ALA ALA ALA LYS
SEQRES   6 A  321  VAL GLY GLY ILE VAL ALA ASN ASN THR TYR PRO ALA ASP
SEQRES   7 A  321  PHE ILE TYR GLN ASN MET MET ILE GLU SER ASN ILE ILE
SEQRES   8 A  321  HIS ALA ALA HIS GLN ASN ASP VAL ASN LYS LEU LEU PHE
SEQRES   9 A  321  LEU GLY SER SER CYS ILE TYR PRO LYS LEU ALA LYS GLN
SEQRES  10 A  321  PRO MET ALA GLU SER GLU LEU LEU GLN GLY THR LEU GLU
SEQRES  11 A  321  PRO THR ASN GLU PRO TYR ALA ILE ALA LYS ILE ALA GLY
SEQRES  12 A  321  ILE LYS LEU CYS GLU SER TYR ASN ARG GLN TYR GLY ARG
SEQRES  13 A  321  ASP TYR ARG SER VAL MET PRO THR ASN LEU TYR GLY PRO
SEQRES  14 A  321  HIS ASP ASN PHE HIS PRO SER ASN SER HIS VAL ILE PRO
SEQRES  15 A  321  ALA LEU LEU ARG ARG PHE HIS GLU ALA THR ALA GLN ASN
SEQRES  16 A  321  ALA PRO ASP VAL VAL VAL TRP GLY SER GLY THR PRO MET
SEQRES  17 A  321  ARG GLU PHE LEU HIS VAL ASP ASP MET ALA ALA ALA SER
SEQRES  18 A  321  ILE HIS VAL MET GLU LEU ALA HIS GLU VAL TRP LEU GLU
SEQRES  19 A  321  ASN THR GLN PRO MET LEU SER HIS ILE ASN VAL GLY THR
SEQRES  20 A  321  GLY VAL ASP CYS THR ILE ARG ASP VAL ALA GLN THR ILE
SEQRES  21 A  321  ALA LYS VAL VAL GLY TYR LYS GLY ARG VAL VAL PHE ASP
SEQRES  22 A  321  ALA SER LYS PRO ASP GLY THR PRO ARG LYS LEU LEU ASP
SEQRES  23 A  321  VAL THR ARG LEU HIS GLN LEU GLY TRP TYR HIS GLU ILE
SEQRES  24 A  321  SER LEU GLU ALA GLY LEU ALA SER THR TYR GLN TRP PHE
SEQRES  25 A  321  LEU GLU ASN GLN ASP ARG PHE ARG GLY
HET    NDP     1       0
HETNAM     NDP NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE
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HETSYN     NDP NADP
FORMUL   2  NDP    C21 H23 N7 O17 P3 3-
FORMUL   3  HOH   *109(H2 O1)
HELIX    1   1 MET A   14  GLN A   25  1                                  12
HELIX    2   2 SER A   44  GLU A   54  1                                  11
HELIX    3   3 ILE A   69  THR A   74  1                                   6
HELIX    4   4 PRO A   76  ASN A   97  1                                  22
HELIX    5   5 SER A  108  ILE A  110  5                                   3
HELIX    6   6 GLU A  121  GLU A  123  5                                   3
HELIX    7   7 GLU A  134  TYR A  154  1                                  21
HELIX    8   8 VAL A  180  ALA A  193  1                                  14
HELIX    9   9 VAL A  214  GLU A  226  1                                  13
HELIX   10  10 HIS A  229  GLU A  234  1                                   6
HELIX   11  11 ILE A  253  VAL A  264  1                                  12
HELIX   12  12 THR A  288  GLN A  292  1                                   5
HELIX   13  13 LEU A  301  GLU A  314  1                                  14
SHEET    1   A 6 VAL A  29  VAL A  32  0
SHEET    2   A 6 GLN A   4  ALA A   9  1  N  GLN A   4   O  GLU A  30
SHEET    3   A 6 GLN A  58  LEU A  61  1  N  GLN A  58   O  PHE A   7
SHEET    4   A 6 LYS A 101  LEU A 105  1  N  LYS A 101   O  VAL A  59
SHEET    5   A 6 ASP A 157  PRO A 163  1  N  ASP A 157   O  LEU A 102
SHEET    6   A 6 ILE A 243  VAL A 245  1  N  ILE A 243   O  MET A 162
SHEET    1   B 2 ASN A 165  TYR A 167  0
SHEET    2   B 2 PHE A 211  HIS A 213  1  N  LEU A 212   O  ASN A 165
SHEET    1   C 2 ASP A 198  TRP A 202  0
SHEET    2   C 2 ARG A 269  ASP A 273  1  N  ARG A 269   O  VAL A 199
SITE     1 CAT  1 TYR  136
SITE     2 CAT  1 LYS  140
SITE     3 CAT  1 SER  107
CRYST1  104.200  104.200   75.880  90.00  90.00 120.00 P 32 2 1      6
ORIGX1      1.000000  0.000000  0.000000        0.00000
ORIGX2      0.000000  1.000000  0.000000        0.00000
ORIGX3      0.000000  0.000000  1.000000        0.00000
SCALE1      0.009597  0.005541  0.000000        0.00000
SCALE2      0.000000  0.011081  0.000000        0.00000
SCALE3      0.000000  0.000000  0.013179        0.00000
HETATM   1  O   HOH     1      55.652 -16.806  22.535  1.00  8.73           O
HETATM   2  O   HOH     3      58.494 -10.639  18.740  1.00 13.17           O
HETATM   3  O   HOH     4      58.230 -11.715  27.770  1.00 19.07           O
HETATM   4  O   HOH     5      57.252  -3.759  30.107  1.00 11.21           O
HETATM   5  O   HOH     6      58.298 -10.011  25.527  1.00 15.74           O
HETATM   6  O   HOH     7      49.321   6.583  38.815  1.00 19.33           O
HETATM   7  O   HOH     8      53.785  -4.262  22.464  1.00 10.94           O
HETATM   8  O   HOH    10      74.652   2.888   9.141  1.00 17.80           O
HETATM   9  O   HOH    11      49.761   0.826  32.896  1.00 22.02           O
```

HETATM	10	O	HOH	12	55.530	-11.162	28.526	1.00	11.39	O
HETATM	11	O	HOH	13	75.027	7.034	27.353	1.00	16.30	O
HETATM	12	O	HOH	14	49.994	-2.314	11.032	1.00	21.33	O
HETATM	13	O	HOH	15	61.323	-8.959	29.657	1.00	22.84	O
HETATM	14	O	HOH	16	61.029	-11.560	29.131	1.00	21.24	O
HETATM	15	O	HOH	17	50.684	5.881	10.130	1.00	15.88	O
HETATM	16	O	HOH	18	64.506	-6.302	32.989	1.00	21.05	O
HETATM	17	O	HOH	19	57.856	-16.398	25.085	1.00	22.86	O
HETATM	18	O	HOH	20	38.979	26.536	19.070	1.00	21.08	O
HETATM	19	O	HOH	21	38.042	33.487	21.909	1.00	19.01	O
HETATM	20	O	HOH	24	38.172	35.775	20.827	1.00	33.46	O
HETATM	21	O	HOH	25	70.916	-11.128	15.244	1.00	31.37	O
HETATM	22	O	HOH	26	54.205	19.360	28.396	1.00	35.76	O
HETATM	23	O	HOH	27	50.436	2.654	16.783	1.00	12.25	O
HETATM	24	O	HOH	28	69.692	19.108	38.979	1.00	49.77	O
HETATM	25	O	HOH	29	56.432	-8.877	19.303	1.00	22.52	O
HETATM	26	O	HOH	30	60.832	3.415	42.349	1.00	17.39	O
HETATM	27	O	HOH	31	53.889	-12.706	29.764	1.00	22.40	O
HETATM	28	O	HOH	32	37.887	26.373	28.058	1.00	18.09	O
HETATM	29	O	HOH	33	49.201	11.173	26.867	1.00	33.95	O
HETATM	30	O	HOH	34	46.762	-0.278	31.394	1.00	20.63	O
HETATM	31	O	HOH	35	41.731	27.568	43.302	1.00	27.39	O
HETATM	32	O	HOH	36	66.827	11.202	28.929	1.00	13.23	O
HETATM	33	O	HOH	37	46.834	14.396	40.819	1.00	46.02	O
HETATM	34	O	HOH	38	61.342	1.064	43.868	1.00	26.68	O
HETATM	35	O	HOH	42	70.597	16.422	37.837	1.00	19.26	O
HETATM	36	O	HOH	44	72.275	-9.089	33.407	1.00	22.11	O
HETATM	37	O	HOH	45	42.685	34.461	33.955	1.00	17.32	O
HETATM	38	O	HOH	46	53.480	13.394	38.364	1.00	20.19	O
HETATM	39	O	HOH	47	56.085	21.757	44.744	1.00	33.50	O
HETATM	40	O	HOH	48	35.741	32.691	23.517	1.00	19.49	O
HETATM	41	O	HOH	49	40.458	36.700	34.312	1.00	34.53	O
HETATM	42	O	HOH	50	75.440	7.267	29.948	1.00	18.07	O
HETATM	43	O	HOH	51	47.476	18.347	20.851	1.00	34.16	O
HETATM	44	O	HOH	53	52.837	-16.344	19.587	1.00	25.92	O
HETATM	45	O	HOH	55	46.415	9.073	20.108	1.00	31.91	O
HETATM	46	O	HOH	57	45.912	35.170	36.133	1.00	35.55	O
HETATM	47	O	HOH	58	60.247	-2.880	41.919	1.00	16.85	O
HETATM	48	O	HOH	60	64.974	6.086	24.501	1.00	32.16	O
HETATM	49	O	HOH	61	52.103	4.683	4.978	1.00	35.72	O
HETATM	50	O	HOH	62	50.888	40.154	36.463	1.00	38.35	O
HETATM	51	O	HOH	63	44.373	31.233	37.336	1.00	20.07	O
HETATM	52	O	HOH	64	57.280	27.757	42.451	1.00	21.74	O
HETATM	53	O	HOH	65	58.409	23.769	45.517	1.00	58.42	O
HETATM	54	O	HOH	66	68.690	-11.764	35.335	1.00	57.07	O

HETATM	55	O	HOH	67	42.746	25.153	23.465	1.00	27.05	O
HETATM	56	O	HOH	68	53.638	-16.457	32.292	1.00	31.71	O
HETATM	57	O	HOH	69	33.390	41.716	31.408	1.00	29.92	O
HETATM	58	O	HOH	70	57.768	17.897	42.434	1.00	25.75	O
HETATM	59	O	HOH	71	75.647	9.164	11.766	1.00	35.13	O
HETATM	60	O	HOH	72	62.032	33.292	44.749	1.00	46.18	O
HETATM	61	O	HOH	73	47.310	14.312	34.285	1.00	31.18	O
HETATM	62	O	HOH	74	79.660	-3.947	15.913	1.00	34.63	O
HETATM	63	O	HOH	75	46.929	5.343	4.550	1.00	23.14	O
HETATM	64	O	HOH	76	73.475	12.039	28.412	1.00	27.26	O
HETATM	65	O	HOH	77	46.297	-6.982	30.032	1.00	43.41	O
HETATM	66	O	HOH	78	68.528	-3.422	40.869	1.00	38.47	O
HETATM	67	O	HOH	79	62.080	-1.448	42.803	1.00	24.60	O
HETATM	68	O	HOH	80	65.330	18.150	40.726	1.00	41.00	O
HETATM	69	O	HOH	81	51.775	16.128	37.607	1.00	25.11	O
HETATM	70	O	HOH	83	54.266	28.682	43.313	1.00	27.61	O
HETATM	71	O	HOH	85	73.291	-15.479	20.603	1.00	37.54	O
HETATM	72	O	HOH	86	34.760	21.479	28.544	1.00	43.87	O
HETATM	73	O	HOH	87	37.326	24.131	29.677	1.00	24.47	O
HETATM	74	O	HOH	88	65.168	20.148	6.735	1.00	26.10	O
HETATM	75	O	HOH	89	59.196	12.089	13.630	1.00	25.24	O
HETATM	76	O	HOH	91	66.576	-6.235	40.279	1.00	43.11	O
HETATM	77	O	HOH	93	37.339	29.394	25.515	1.00	27.56	O
HETATM	78	O	HOH	94	52.339	-17.014	42.271	1.00	48.96	O
HETATM	79	O	HOH	95	40.511	32.927	31.717	1.00	22.46	O
HETATM	80	O	HOH	96	78.580	13.121	34.138	1.00	27.98	O
HETATM	81	O	HOH	97	65.090	15.704	34.876	1.00	18.96	O
HETATM	82	O	HOH	99	84.562	2.951	27.181	1.00	35.92	O
HETATM	83	O	HOH	100	50.386	9.761	9.646	1.00	23.18	O
HETATM	84	O	HOH	101	67.649	-0.851	38.764	1.00	24.99	O
HETATM	85	O	HOH	102	44.001	4.293	34.315	1.00	31.13	O
HETATM	86	O	HOH	103	59.386	-5.071	26.211	1.00	29.10	O
HETATM	87	O	HOH	104	77.364	4.745	41.506	1.00	35.32	O
HETATM	88	O	HOH	105	59.034	21.201	32.414	1.00	23.43	O
HETATM	89	O	HOH	106	42.463	34.698	14.327	1.00	38.86	O
HETATM	90	O	HOH	107	70.217	14.292	20.864	1.00	42.39	O
HETATM	91	O	HOH	108	76.999	8.130	25.862	1.00	32.91	O
HETATM	92	O	HOH	109	49.766	29.937	22.173	1.00	42.52	O
HETATM	93	O	HOH	110	72.473	13.536	38.823	1.00	33.32	O
HETATM	94	O	HOH	111	64.328	-12.084	38.608	1.00	37.99	O
HETATM	95	O	HOH	112	60.161	16.382	42.682	1.00	35.68	O
HETATM	96	O	HOH	113	47.602	13.639	27.016	1.00	26.01	O
HETATM	97	O	HOH	115	64.606	11.644	40.107	1.00	30.33	O
HETATM	98	O	HOH	116	61.231	-15.137	27.255	1.00	38.76	O
HETATM	99	O	HOH	117	65.324	-11.223	35.098	1.00	30.45	O

```
HETATM  100  O   HOH   119      56.602  17.219  44.932  1.00 36.53           O
HETATM  101  O   HOH   120      37.564  19.860  23.135  1.00 31.27           O
HETATM  102  O   HOH   121      64.845   5.057  21.132  1.00 45.57           O
HETATM  103  O   HOH   123      63.391  16.801  26.898  1.00 38.46           O
HETATM  104  O   HOH   124      42.567   6.134  32.635  1.00 31.56           O
HETATM  105  O   HOH   125      72.485  13.236  35.059  1.00 29.61           O
HETATM  106  O   HOH   126      65.229   3.650  44.032  1.00 36.86           O
HETATM  107  O   HOH   127      37.089   7.148  31.083  1.00 39.58           O
HETATM  108  O   HOH   128      73.327  10.546  12.123  1.00 34.97           O
HETATM  109  O   HOH   129      74.450  10.299  26.598  1.00 30.80           O
HETATM  110 AO5* NDP A   1      67.524  13.055  26.692  1.00 36.42           O
HETATM  111 AC5* NDP A   1      68.089  12.297  25.614  1.00  9.30           C
HETATM  112 AC4* NDP A   1      69.601  12.124  25.858  1.00 27.73           C
HETATM  113 AO4* NDP A   1      70.193  11.258  24.848  1.00 22.87           O
HETATM  114 AC3* NDP A   1      70.484  13.390  25.873  1.00 17.83           C
HETATM  115 AO3* NDP A   1      71.192  13.436  27.066  1.00 16.11           O
HETATM  116 AC2* NDP A   1      71.373  13.220  24.626  1.00 11.46           C
HETATM  117 AO2* NDP A   1      72.623  13.886  24.655  1.00 31.96           O
HETATM  118 AC1* NDP A   1      71.510  11.702  24.656  1.00 19.02           C
HETATM  119  O3   NDP A   1      65.336  13.590  26.129  1.00 20.59           O
HETATM  120 NO5* NDP A   1      63.536  11.943  26.448  1.00 28.99           O
HETATM  121 NC5* NDP A   1      64.328  10.843  25.957  1.00 24.89           C
HETATM  122 NC4* NDP A   1      63.467   9.646  25.686  1.00 31.79           C
HETATM  123 NO4* NDP A   1      62.837   9.337  26.908  1.00 28.82           O
HETATM  124 NC3* NDP A   1      62.340   9.837  24.665  1.00 11.50           C
HETATM  125 NO3* NDP A   1      62.891   9.402  23.461  1.00 28.60           O
HETATM  126 NC2* NDP A   1      61.152   8.996  25.138  1.00 28.11           C
HETATM  127 NO2* NDP A   1      60.881   7.662  24.715  1.00 24.30           O
HETATM  128 NC1* NDP A   1      61.547   8.875  26.580  1.00 35.35           C
HETATM  129 AP2* NDP A   1      73.104  15.069  23.823  1.00 32.96           P
HETATM  130 AOP1 NDP A   1      74.500  15.308  24.308  1.00 37.84           O
HETATM  131 AOP2 NDP A   1      72.797  14.925  22.348  1.00 36.66           O
HETATM  132 AOP3 NDP A   1      72.163  16.217  23.958  1.00 31.97           O
HETATM  133  AP  NDP A   1      66.660  14.257  26.393  1.00 26.17          XX
HETATM  134  AO1 NDP A   1      66.886  14.795  25.047  1.00 15.31          XX
HETATM  135  AO2 NDP A   1      66.439  15.207  27.521  1.00 34.39          XX
HETATM  136  AN9 NDP A   1      71.820  11.224  23.353  1.00 13.63          XX
HETATM  137  AC8 NDP A   1      71.104  11.316  22.200  1.00 12.41          XX
HETATM  138  AN7 NDP A   1      71.758  10.835  21.161  1.00 15.71          XX
HETATM  139  AC5 NDP A   1      72.933  10.313  21.710  1.00 16.17          XX
HETATM  140  AC6 NDP A   1      74.053   9.657  21.140  1.00 31.35          XX
HETATM  141  AN6 NDP A   1      74.165   9.464  19.819  1.00 12.59          XX
HETATM  142  AN1 NDP A   1      75.078   9.280  21.942  1.00 17.56          XX
HETATM  143  AC2 NDP A   1      74.971   9.578  23.251  1.00 15.44          XX
HETATM  144  AN3 NDP A   1      74.027  10.302  23.889  1.00 24.82          XX
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HETATM  145  AC4 NDP A   1      73.036  10.653  23.047  1.00 17.48           XX
HETATM  146  NP  NDP A   1      64.183  13.106  27.191  1.00 25.47           N
HETATM  147  NO1 NDP A   1      63.142  14.169  27.253  1.00 28.69           N
HETATM  148  NO2 NDP A   1      64.837  12.643  28.492  1.00 24.32           N
HETATM  149  NN1 NDP A   1      60.598   9.775  27.109  1.00 23.63           N
HETATM  150  NC2 NDP A   1      60.143  10.905  26.442-99.00 78.36           N
HETATM  151  NC3 NDP A   1      59.070  11.648  27.007-99.00100.00           N
HETATM  152  NC7 NDP A   1      58.497  13.017  26.528-99.00100.00           N
HETATM  153  NO7 NDP A   1      59.358  13.703  25.972-99.00100.00           N
HETATM  154  NN7 NDP A   1      57.207  13.400  26.912-99.00 84.38           N
HETATM  155  NC4 NDP A   1      58.442  11.146  28.137-99.00100.00           N
HETATM  156  NC5 NDP A   1      58.912   9.963  28.754-99.00100.00           N
HETATM  157  NC6 NDP A   1      59.951   9.266  28.147-99.00100.00           N
ATOM    158  N   LYS A   3      76.227  -5.632  44.315  1.00 61.49           N
ATOM    159  CA  LYS A   3      76.152  -4.302  43.684  1.00 58.00           C
ATOM    160  C   LYS A   3      75.985  -4.421  42.171  1.00 52.79           C
ATOM    161  O   LYS A   3      76.921  -4.737  41.419  1.00 44.76           O
ATOM    162  CB  LYS A   3      77.359  -3.417  44.030  1.00 59.74           C
ATOM    163  CG  LYS A   3      77.011  -1.944  44.314  1.00 50.87           C
ATOM    164  CD  LYS A   3      78.208  -1.161  44.894  1.00 61.21           C
ATOM    165  CE  LYS A   3      77.855  -0.377  46.186  1.00100.00           C
ATOM    166  NZ  LYS A   3      78.857  -0.401  47.343  1.00 70.61           N
ATOM    167  N   GLN A   4      74.746  -4.242  41.747  1.00 45.15           N
ATOM    168  CA  GLN A   4      74.408  -4.326  40.347  1.00 37.18           C
ATOM    169  C   GLN A   4      74.983  -3.166  39.561  1.00 34.93           C
ATOM    170  O   GLN A   4      75.127  -2.050  40.087  1.00 28.48           O
ATOM    171  CB  GLN A   4      72.915  -4.445  40.221  1.00 34.65           C
ATOM    172  CG  GLN A   4      72.456  -5.854  40.584  1.00 31.82           C
ATOM    173  CD  GLN A   4      72.570  -6.788  39.405  1.00 79.25           C
ATOM    174  OE1 GLN A   4      72.165  -6.452  38.286  1.00100.00           O
ATOM    175  NE2 GLN A   4      73.206  -7.925  39.623  1.00 80.24           N
ATOM    176  N   ARG A   5      75.475  -3.495  38.375  1.00 27.16           N
ATOM    177  CA  ARG A   5      76.146  -2.546  37.483  1.00 39.16           C
ATOM    178  C   ARG A   5      75.191  -2.018  36.433  1.00 38.22           C
ATOM    179  O   ARG A   5      74.938  -2.698  35.438  1.00 32.44           O
ATOM    180  CB  ARG A   5      77.398  -3.163  36.826  1.00 41.76           C
ATOM    181  CG  ARG A   5      78.692  -2.954  37.663  1.00 37.34           C
ATOM    182  CD  ARG A   5      80.015  -3.236  36.876  1.00 32.99           C
ATOM    183  NE  ARG A   5      81.036  -2.203  37.125  1.00 25.71           N
ATOM    184  CZ  ARG A   5      81.617  -1.488  36.169  1.00 32.53           C
ATOM    185  NH1 ARG A   5      81.293  -1.704  34.904  1.00 40.07           N
ATOM    186  NH2 ARG A   5      82.516  -0.551  36.474  1.00100.00           N
ATOM    187  N   VAL A   6      74.743  -0.773  36.659  1.00 32.08           N
ATOM    188  CA  VAL A   6      73.715  -0.082  35.881  1.00 28.89           C
ATOM    189  C   VAL A   6      74.161   1.021  34.897  1.00 29.37           C
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ATOM	190	O	VAL	A	6	74.745	2.041	35.274	1.00 22.50	O
ATOM	191	CB	VAL	A	6	72.577	0.378	36.813	1.00 23.52	C
ATOM	192	CG1	VAL	A	6	71.366	0.960	36.006	1.00 20.29	C
ATOM	193	CG2	VAL	A	6	72.108	-0.852	37.644	1.00 18.45	C
ATOM	194	N	PHE	A	7	73.948	0.749	33.615	1.00 22.92	N
ATOM	195	CA	PHE	A	7	74.267	1.710	32.573	1.00 27.15	C
ATOM	196	C	PHE	A	7	72.975	2.423	32.192	1.00 20.24	C
ATOM	197	O	PHE	A	7	71.994	1.788	31.815	1.00 20.71	O
ATOM	198	CB	PHE	A	7	74.864	1.004	31.374	1.00 18.98	C
ATOM	199	CG	PHE	A	7	74.916	1.836	30.115	1.00 21.83	C
ATOM	200	CD1	PHE	A	7	75.521	3.087	30.108	1.00 19.36	C
ATOM	201	CD2	PHE	A	7	74.483	1.284	28.886	1.00 23.50	C
ATOM	202	CE1	PHE	A	7	75.614	3.828	28.902	1.00 27.52	C
ATOM	203	CE2	PHE	A	7	74.548	1.996	27.685	1.00 19.33	C
ATOM	204	CZ	PHE	A	7	75.128	3.255	27.673	1.00 18.59	C
ATOM	205	N	ILE	A	8	72.959	3.727	32.454	1.00 18.75	N
ATOM	206	CA	ILE	A	8	71.844	4.588	32.112	1.00 14.25	C
ATOM	207	C	ILE	A	8	72.337	5.351	30.909	1.00 11.22	C
ATOM	208	O	ILE	A	8	73.259	6.165	30.998	1.00 17.76	O
ATOM	209	CB	ILE	A	8	71.507	5.605	33.212	1.00 14.15	C
ATOM	210	CG1	ILE	A	8	71.356	4.949	34.582	1.00 8.24	C
ATOM	211	CG2	ILE	A	8	70.183	6.342	32.874	1.00 16.85	C
ATOM	212	CD1	ILE	A	8	71.091	5.961	35.707	1.00 10.32	C
ATOM	213	N	ALA	A	9	71.896	4.906	29.752	1.00 16.42	N
ATOM	214	CA	ALA	A	9	72.256	5.559	28.513	1.00 18.74	C
ATOM	215	C	ALA	A	9	71.530	6.913	28.511	1.00 28.45	C
ATOM	216	O	ALA	A	9	70.411	7.032	29.045	1.00 22.39	O
ATOM	217	CB	ALA	A	9	71.808	4.731	27.311	1.00 14.43	C
ATOM	218	N	GLY	A	10	72.199	7.922	27.940	1.00 20.06	N
ATOM	219	CA	GLY	A	10	71.706	9.284	27.911	1.00 18.62	C
ATOM	220	C	GLY	A	10	71.407	9.819	29.305	1.00 16.40	C
ATOM	221	O	GLY	A	10	70.379	10.448	29.481	1.00 17.36	O
ATOM	222	N	HIS	A	11	72.295	9.581	30.272	1.00 10.32	N
ATOM	223	CA	HIS	A	11	72.068	9.966	31.688	1.00 13.90	C
ATOM	224	C	HIS	A	11	72.008	11.504	31.916	1.00 21.52	C
ATOM	225	O	HIS	A	11	71.700	11.994	32.983	1.00 13.22	O
ATOM	226	CB	HIS	A	11	73.153	9.350	32.581	1.00 14.88	C
ATOM	227	CG	HIS	A	11	74.502	9.948	32.326	1.00 23.73	C
ATOM	228	ND1	HIS	A	11	75.239	9.648	31.197	1.00 24.90	N
ATOM	229	CD2	HIS	A	11	75.167	10.952	32.956	1.00 16.35	C
ATOM	230	CE1	HIS	A	11	76.317	10.407	31.170	1.00 22.54	C
ATOM	231	NE2	HIS	A	11	76.271	11.240	32.197	1.00 17.56	N
ATOM	232	N	ARG	A	12	72.310	12.288	30.908	1.00 22.31	N
ATOM	233	CA	ARG	A	12	72.147	13.693	31.122	1.00 18.90	C
ATOM	234	C	ARG	A	12	70.851	14.244	30.495	1.00 26.34	C

ATOM	235	O	ARG A	12	70.572	15.426	30.604	1.00	25.37	O
ATOM	236	CB	ARG A	12	73.352	14.418	30.587	1.00	25.93	C
ATOM	237	CG	ARG A	12	74.582	13.943	31.279	1.00	53.87	C
ATOM	238	CD	ARG A	12	75.757	14.619	30.699	1.00	32.53	C
ATOM	239	NE	ARG A	12	76.359	15.576	31.605	1.00	69.90	N
ATOM	240	CZ	ARG A	12	76.971	16.675	31.178	1.00	100.00	C
ATOM	241	NH1	ARG A	12	77.001	16.948	29.867	1.00	100.00	N
ATOM	242	NH2	ARG A	12	77.526	17.508	32.056	1.00	100.00	N
ATOM	243	N	GLY A	13	70.078	13.420	29.800	1.00	18.25	N
ATOM	244	CA	GLY A	13	68.802	13.904	29.258	1.00	16.50	C
ATOM	245	C	GLY A	13	67.849	14.144	30.428	1.00	18.88	C
ATOM	246	O	GLY A	13	68.202	13.902	31.624	1.00	14.04	O
ATOM	247	N	MET A	14	66.653	14.632	30.103	1.00	16.00	N
ATOM	248	CA	MET A	14	65.688	14.981	31.128	1.00	13.49	C
ATOM	249	C	MET A	14	65.293	13.760	31.901	1.00	14.02	C
ATOM	250	O	MET A	14	65.408	13.713	33.145	1.00	17.06	O
ATOM	251	CB	MET A	14	64.442	15.605	30.524	1.00	11.57	C
ATOM	252	CG	MET A	14	63.320	15.628	31.559	1.00	20.77	C
ATOM	253	SD	MET A	14	61.926	16.766	31.110	1.00	29.16	S
ATOM	254	CE	MET A	14	62.527	17.108	29.574	1.00	30.68	C
ATOM	255	N	VAL A	15	64.798	12.769	31.158	1.00	25.23	N
ATOM	256	CA	VAL A	15	64.439	11.468	31.738	1.00	20.90	C
ATOM	257	C	VAL A	15	65.654	10.713	32.378	1.00	17.26	C
ATOM	258	O	VAL A	15	65.590	10.239	33.524	1.00	18.41	O
ATOM	259	CB	VAL A	15	63.752	10.550	30.680	1.00	23.25	C
ATOM	260	CG1	VAL A	15	63.330	9.253	31.310	1.00	15.71	C
ATOM	261	CG2	VAL A	15	62.528	11.193	30.183	1.00	13.40	C
ATOM	262	N	GLY A	16	66.784	10.642	31.665	1.00	20.39	N
ATOM	263	CA	GLY A	16	67.941	9.904	32.186	1.00	19.54	C
ATOM	264	C	GLY A	16	68.522	10.432	33.492	1.00	29.29	C
ATOM	265	O	GLY A	16	68.896	9.659	34.434	1.00	16.91	O
ATOM	266	N	SER A	17	68.642	11.755	33.499	1.00	12.53	N
ATOM	267	CA	SER A	17	69.154	12.460	34.650	1.00	21.93	C
ATOM	268	C	SER A	17	68.209	12.214	35.818	1.00	13.35	C
ATOM	269	O	SER A	17	68.677	11.957	36.915	1.00	24.19	O
ATOM	270	CB	SER A	17	69.378	13.942	34.333	1.00	15.52	C
ATOM	271	OG	SER A	17	68.153	14.619	34.372	1.00	22.95	O
ATOM	272	N	ALA A	18	66.896	12.143	35.590	1.00	17.52	N
ATOM	273	CA	ALA A	18	65.991	11.828	36.729	1.00	13.14	C
ATOM	274	C	ALA A	18	66.220	10.393	37.307	1.00	19.29	C
ATOM	275	O	ALA A	18	66.149	10.150	38.522	1.00	16.94	O
ATOM	276	CB	ALA A	18	64.460	12.046	36.334	1.00	14.33	C
ATOM	277	N	ILE A	19	66.484	9.432	36.430	1.00	20.80	N
ATOM	278	CA	ILE A	19	66.705	8.078	36.900	1.00	18.08	C
ATOM	279	C	ILE A	19	67.975	8.090	37.730	1.00	16.09	C

ATOM	280	O	ILE	A	19	68.018	7.530	38.820	1.00 20.73	O
ATOM	281	CB	ILE	A	19	66.804	7.079	35.710	1.00 17.58	C
ATOM	282	CG1	ILE	A	19	65.444	6.812	35.162	1.00 10.09	C
ATOM	283	CG2	ILE	A	19	67.309	5.666	36.133	1.00 21.60	C
ATOM	284	CD1	ILE	A	19	65.528	6.361	33.741	1.00 19.05	C
ATOM	285	N	ARG	A	20	68.984	8.771	37.198	1.00 18.13	N
ATOM	286	CA	ARG	A	20	70.286	8.897	37.836	1.00 20.25	C
ATOM	287	C	ARG	A	20	70.231	9.491	39.242	1.00 30.62	C
ATOM	288	O	ARG	A	20	70.957	9.091	40.129	1.00 33.00	O
ATOM	289	CB	ARG	A	20	71.201	9.743	36.957	1.00 11.71	C
ATOM	290	CG	ARG	A	20	72.610	9.781	37.449	1.00 23.79	C
ATOM	291	CD	ARG	A	20	72.881	11.107	38.060	1.00 36.76	C
ATOM	292	NE	ARG	A	20	74.297	11.443	38.062	1.00 48.34	N
ATOM	293	CZ	ARG	A	20	74.990	11.841	36.988	1.00100.00	C
ATOM	294	NH1	ARG	A	20	74.393	11.931	35.808	1.00100.00	N
ATOM	295	NH2	ARG	A	20	76.289	12.139	37.076	1.00100.00	N
ATOM	296	N	ARG	A	21	69.368	10.461	39.439	1.00 22.10	N
ATOM	297	CA	ARG	A	21	69.216	11.052	40.750	1.00 17.45	C
ATOM	298	C	ARG	A	21	68.721	10.007	41.730	1.00 26.71	C
ATOM	299	O	ARG	A	21	69.147	10.001	42.885	1.00 30.27	O
ATOM	300	CB	ARG	A	21	68.142	12.144	40.708	1.00 17.93	C
ATOM	301	CG	ARG	A	21	68.682	13.522	40.321	1.00 27.57	C
ATOM	302	CD	ARG	A	21	67.586	14.599	40.130	1.00 23.02	C
ATOM	303	NE	ARG	A	21	67.619	15.000	38.743	1.00 55.12	N
ATOM	304	CZ	ARG	A	21	66.538	15.103	37.995	1.00 10.55	C
ATOM	305	NH1	ARG	A	21	65.343	14.974	38.552	1.00 29.80	N
ATOM	306	NH2	ARG	A	21	66.665	15.435	36.715	1.00 61.45	N
ATOM	307	N	GLN	A	22	67.713	9.223	41.345	1.00 27.48	N
ATOM	308	CA	GLN	A	22	67.167	8.257	42.313	1.00 24.79	C
ATOM	309	C	GLN	A	22	68.137	7.127	42.547	1.00 31.37	C
ATOM	310	O	GLN	A	22	68.394	6.724	43.685	1.00 27.47	O
ATOM	311	CB	GLN	A	22	65.818	7.706	41.894	1.00 17.11	C
ATOM	312	CG	GLN	A	22	64.921	8.745	41.243	1.00 66.14	C
ATOM	313	CD	GLN	A	22	63.425	8.456	41.397	1.00 41.27	C
ATOM	314	OE1	GLN	A	22	63.002	7.329	41.762	1.00 29.34	O
ATOM	315	NE2	GLN	A	22	62.610	9.464	41.046	1.00 20.12	N
ATOM	316	N	LEU	A	23	68.697	6.652	41.448	1.00 27.99	N
ATOM	317	CA	LEU	A	23	69.649	5.575	41.500	1.00 24.48	C
ATOM	318	C	LEU	A	23	70.828	5.971	42.334	1.00 28.87	C
ATOM	319	O	LEU	A	23	71.288	5.218	43.165	1.00 30.79	O
ATOM	320	CB	LEU	A	23	70.036	5.107	40.089	1.00 22.72	C
ATOM	321	CG	LEU	A	23	68.966	4.072	39.658	1.00 26.16	C
ATOM	322	CD1	LEU	A	23	69.271	3.083	38.481	1.00 24.80	C
ATOM	323	CD2	LEU	A	23	68.427	3.284	40.835	1.00 22.91	C
ATOM	324	N	GLU	A	24	71.279	7.192	42.153	1.00 28.77	N

ATOM	325	CA	GLU	A	24	72.419	7.675	42.909	1.00	33.79	C
ATOM	326	C	GLU	A	24	72.363	7.388	44.412	1.00	35.94	C
ATOM	327	O	GLU	A	24	73.381	7.140	45.031	1.00	39.07	O
ATOM	328	CB	GLU	A	24	72.647	9.165	42.653	1.00	36.21	C
ATOM	329	CG	GLU	A	24	74.068	9.482	42.243	1.00	42.54	C
ATOM	330	CD	GLU	A	24	74.158	10.689	41.333	1.00	89.51	C
ATOM	331	OE1	GLU	A	24	73.386	11.663	41.549	1.00	43.21	O
ATOM	332	OE2	GLU	A	24	74.994	10.646	40.398	1.00	66.28	O
ATOM	333	N	GLN	A	25	71.182	7.422	45.000	1.00	45.70	N
ATOM	334	CA	GLN	A	25	71.039	7.152	46.432	1.00	47.57	C
ATOM	335	C	GLN	A	25	70.887	5.669	46.740	1.00	67.34	C
ATOM	336	O	GLN	A	25	70.285	5.286	47.726	1.00	74.06	O
ATOM	337	CB	GLN	A	25	69.783	7.842	46.905	1.00	51.85	C
ATOM	338	CG	GLN	A	25	69.500	9.084	46.109	1.00	44.91	C
ATOM	339	CD	GLN	A	25	68.419	9.913	46.742	1.00	100.00	C
ATOM	340	OE1	GLN	A	25	68.271	9.947	47.972	1.00	100.00	O
ATOM	341	NE2	GLN	A	25	67.624	10.602	45.911	1.00	100.00	N
ATOM	342	N	ARG	A	26	71.322	4.831	45.825	1.00	75.37	N
ATOM	343	CA	ARG	A	26	71.182	3.407	46.026	1.00	74.87	C
ATOM	344	C	ARG	A	26	72.568	2.791	46.147	1.00	74.08	C
ATOM	345	O	ARG	A	26	73.440	2.997	45.289	1.00	77.00	O
ATOM	346	CB	ARG	A	26	70.390	2.790	44.885	1.00	52.44	C
ATOM	347	CG	ARG	A	26	68.916	2.927	45.070	1.00	43.51	C
ATOM	348	CD	ARG	A	26	68.428	1.752	45.864	1.00	40.70	C
ATOM	349	NE	ARG	A	26	67.200	1.176	45.338	1.00	42.33	N
ATOM	350	CZ	ARG	A	26	67.126	0.508	44.196	1.00	32.07	C
ATOM	351	NH1	ARG	A	26	68.215	0.324	43.486	1.00	44.02	N
ATOM	352	NH2	ARG	A	26	65.968	0.017	43.771	1.00	77.32	N
ATOM	353	N	GLY	A	27	72.778	2.114	47.266	1.00	46.30	N
ATOM	354	CA	GLY	A	27	74.060	1.531	47.549	1.00	46.82	C
ATOM	355	C	GLY	A	27	74.140	0.165	46.923	1.00	55.45	C
ATOM	356	O	GLY	A	27	75.204	-0.453	46.877	1.00	64.43	O
ATOM	357	N	ASP	A	28	73.017	-0.315	46.428	1.00	40.98	N
ATOM	358	CA	ASP	A	28	73.016	-1.647	45.861	1.00	40.35	C
ATOM	359	C	ASP	A	28	73.266	-1.536	44.400	1.00	39.55	C
ATOM	360	O	ASP	A	28	73.109	-2.518	43.654	1.00	48.80	O
ATOM	361	CB	ASP	A	28	71.680	-2.335	46.127	1.00	47.80	C
ATOM	362	CG	ASP	A	28	70.503	-1.373	46.064	1.00	35.34	C
ATOM	363	OD1	ASP	A	28	70.705	-0.140	46.095	1.00	39.23	O
ATOM	364	OD2	ASP	A	28	69.383	-1.870	45.872	1.00	69.86	O
ATOM	365	N	VAL	A	29	73.651	-0.329	43.996	1.00	31.03	N
ATOM	366	CA	VAL	A	29	73.881	-0.050	42.591	1.00	28.44	C
ATOM	367	C	VAL	A	29	75.166	0.676	42.281	1.00	28.00	C
ATOM	368	O	VAL	A	29	75.505	1.699	42.892	1.00	34.83	O
ATOM	369	CB	VAL	A	29	72.696	0.760	42.000	1.00	30.68	C

ATOM	370	CG1	VAL	A	29	72.935	1.088	40.549	1.00	23.65	C
ATOM	371	CG2	VAL	A	29	71.416	-0.028	42.156	1.00	27.95	C
ATOM	372	N	GLU	A	30	75.824	0.219	41.230	1.00	30.76	N
ATOM	373	CA	GLU	A	30	76.995	0.924	40.736	1.00	28.38	C
ATOM	374	C	GLU	A	30	76.678	1.471	39.332	1.00	31.03	C
ATOM	375	O	GLU	A	30	76.368	0.720	38.397	1.00	26.64	O
ATOM	376	CB	GLU	A	30	78.199	0.006	40.722	1.00	31.84	C
ATOM	377	CG	GLU	A	30	79.355	0.539	41.533	1.00	89.26	C
ATOM	378	CD	GLU	A	30	80.667	0.264	40.858	1.00	100.00	C
ATOM	379	OE1	GLU	A	30	81.082	-0.922	40.872	1.00	88.94	O
ATOM	380	OE2	GLU	A	30	81.202	1.206	40.219	1.00	100.00	O
ATOM	381	N	LEU	A	31	76.665	2.789	39.207	1.00	22.24	N
ATOM	382	CA	LEU	A	31	76.269	3.391	37.945	1.00	29.37	C
ATOM	383	C	LEU	A	31	77.404	3.507	36.941	1.00	25.79	C
ATOM	384	O	LEU	A	31	78.485	3.969	37.256	1.00	29.41	O
ATOM	385	CB	LEU	A	31	75.632	4.760	38.191	1.00	30.20	C
ATOM	386	CG	LEU	A	31	74.329	4.763	38.994	1.00	29.37	C
ATOM	387	CD1	LEU	A	31	73.841	6.143	39.240	1.00	23.43	C
ATOM	388	CD2	LEU	A	31	73.275	3.962	38.281	1.00	23.04	C
ATOM	389	N	VAL	A	32	77.146	3.100	35.711	1.00	21.94	N
ATOM	390	CA	VAL	A	32	78.143	3.265	34.685	1.00	25.48	C
ATOM	391	C	VAL	A	32	77.535	4.242	33.669	1.00	38.76	C
ATOM	392	O	VAL	A	32	76.429	3.999	33.180	1.00	29.70	O
ATOM	393	CB	VAL	A	32	78.517	1.902	34.055	1.00	34.25	C
ATOM	394	CG1	VAL	A	32	79.587	2.079	32.970	1.00	30.56	C
ATOM	395	CG2	VAL	A	32	79.003	0.950	35.139	1.00	25.27	C
ATOM	396	N	LEU	A	33	78.219	5.375	33.457	1.00	30.19	N
ATOM	397	CA	LEU	A	33	77.732	6.463	32.621	1.00	22.71	C
ATOM	398	C	LEU	A	33	78.727	6.979	31.645	1.00	29.55	C
ATOM	399	O	LEU	A	33	79.896	7.152	31.988	1.00	30.09	O
ATOM	400	CB	LEU	A	33	77.423	7.635	33.514	1.00	19.75	C
ATOM	401	CG	LEU	A	33	76.729	7.200	34.779	1.00	19.38	C
ATOM	402	CD1	LEU	A	33	76.814	8.344	35.762	1.00	27.24	C
ATOM	403	CD2	LEU	A	33	75.271	6.913	34.444	1.00	22.07	C
ATOM	404	N	ARG	A	34	78.239	7.421	30.496	1.00	15.09	N
ATOM	405	CA	ARG	A	34	79.154	8.008	29.541	1.00	26.04	C
ATOM	406	C	ARG	A	34	78.469	9.173	28.916	1.00	36.57	C
ATOM	407	O	ARG	A	34	77.288	9.130	28.651	1.00	38.59	O
ATOM	408	CB	ARG	A	34	79.486	7.048	28.398	1.00	22.89	C
ATOM	409	CG	ARG	A	34	80.579	6.081	28.706	1.00	23.29	C
ATOM	410	CD	ARG	A	34	81.370	6.575	29.860	1.00	52.06	C
ATOM	411	NE	ARG	A	34	81.783	5.458	30.711	1.00	80.25	N
ATOM	412	CZ	ARG	A	34	82.646	4.530	30.323	1.00	41.94	C
ATOM	413	NH1	ARG	A	34	83.173	4.596	29.104	1.00	53.02	N
ATOM	414	NH2	ARG	A	34	82.983	3.547	31.148	1.00	25.56	N

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ATOM    415  N   THR A  35      79.248  10.156  28.539  1.00 31.58           N
ATOM    416  CA  THR A  35      78.703  11.282  27.833  1.00 29.33           C
ATOM    417  C   THR A  35      78.719  10.951  26.340  1.00 32.53           C
ATOM    418  O   THR A  35      79.350   9.944  25.962  1.00 28.08           O
ATOM    419  CB  THR A  35      79.527  12.527  28.145  1.00 37.49           C
ATOM    420  OG1 THR A  35      80.844  12.429  27.560  1.00 31.91           O
ATOM    421  CG2 THR A  35      79.627  12.642  29.651  1.00 19.38           C
ATOM    422  N   ARG A  36      78.032  11.780  25.529  1.00 30.02           N
ATOM    423  CA  ARG A  36      78.002  11.639  24.056  1.00 29.37           C
ATOM    424  C   ARG A  36      79.406  11.765  23.503  1.00 31.46           C
ATOM    425  O   ARG A  36      79.772  11.012  22.591  1.00 36.56           O
ATOM    426  CB  ARG A  36      77.054  12.650  23.354  1.00 37.34           C
ATOM    427  CG  ARG A  36      76.937  12.465  21.846-99.00 49.47           C
ATOM    428  CD  ARG A  36      76.020  13.515  21.232-99.00 63.09           C
ATOM    429  NE  ARG A  36      75.528  13.124  19.915-99.00 75.23           N
ATOM    430  CZ  ARG A  36      74.381  13.549  19.391-99.00 91.44           C
ATOM    431  NH1 ARG A  36      73.605  14.375  20.079-99.00 79.32           N
ATOM    432  NH2 ARG A  36      74.009  13.144  18.185-99.00 78.73           N
ATOM    433  N   ASP A  37      80.217  12.677  24.063  1.00 41.30           N
ATOM    434  CA  ASP A  37      81.606  12.710  23.601  1.00 44.91           C
ATOM    435  C   ASP A  37      82.410  11.481  24.043  1.00 24.99           C
ATOM    436  O   ASP A  37      83.211  10.978  23.261  1.00 42.22           O
ATOM    437  CB  ASP A  37      82.347  14.048  23.718-99.00 47.07           C
ATOM    438  CG  ASP A  37      81.881  14.887  24.876-99.00 62.99           C
ATOM    439  OD1 ASP A  37      80.679  14.839  25.204-99.00 64.45           O
ATOM    440  OD2 ASP A  37      82.711  15.638  25.429-99.00 69.84           O
ATOM    441  N   GLU A  38      82.129  10.950  25.235  1.00 19.39           N
ATOM    442  CA  GLU A  38      82.790   9.717  25.682  1.00 27.84           C
ATOM    443  C   GLU A  38      82.203   8.527  24.901  1.00 37.14           C
ATOM    444  O   GLU A  38      82.873   7.511  24.699  1.00 35.04           O
ATOM    445  CB  GLU A  38      82.691   9.435  27.207  1.00 25.18           C
ATOM    446  CG  GLU A  38      83.116  10.549  28.183  1.00 37.45           C
ATOM    447  CD  GLU A  38      82.807  10.212  29.655  1.00 21.13           C
ATOM    448  OE1 GLU A  38      81.623   9.997  30.014  1.00 55.97           O
ATOM    449  OE2 GLU A  38      83.757   9.978  30.419  1.00 98.78           O
ATOM    450  N   LEU A  39      80.948   8.610  24.478  1.00 25.52           N
ATOM    451  CA  LEU A  39      80.440   7.483  23.739  1.00 18.17           C
ATOM    452  C   LEU A  39      79.291   7.764  22.825  1.00 20.34           C
ATOM    453  O   LEU A  39      78.152   7.810  23.259  1.00 26.35           O
ATOM    454  CB  LEU A  39      80.123   6.313  24.657  1.00 14.56           C
ATOM    455  CG  LEU A  39      79.410   5.075  24.058  1.00 19.52           C
ATOM    456  CD1 LEU A  39      80.205   4.392  22.994  1.00 18.84           C
ATOM    457  CD2 LEU A  39      78.890   4.051  25.084  1.00 17.41           C
ATOM    458  N   ASN A  40      79.598   7.880  21.543  1.00 16.73           N
ATOM    459  CA  ASN A  40      78.548   7.971  20.540  1.00 21.55           C
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ATOM	460	C	ASN A	40	77.798	6.649	20.308	1.00	24.53	C
ATOM	461	O	ASN A	40	78.328	5.720	19.688	1.00	19.96	O
ATOM	462	CB	ASN A	40	79.130	8.367	19.216	1.00	18.45	C
ATOM	463	CG	ASN A	40	78.054	8.727	18.225	1.00	42.19	C
ATOM	464	OD1	ASN A	40	78.327	9.093	17.080	1.00	38.89	O
ATOM	465	ND2	ASN A	40	76.827	8.730	18.697	1.00	23.71	N
ATOM	466	N	LEU A	41	76.543	6.622	20.754	1.00	21.08	N
ATOM	467	CA	LEU A	41	75.649	5.465	20.650	1.00	15.03	C
ATOM	468	C	LEU A	41	75.225	5.068	19.213	1.00	18.22	C
ATOM	469	O	LEU A	41	74.681	3.971	18.980	1.00	15.72	O
ATOM	470	CB	LEU A	41	74.426	5.705	21.532	1.00	15.85	C
ATOM	471	CG	LEU A	41	74.822	6.029	22.974	1.00	21.90	C
ATOM	472	CD1	LEU A	41	73.604	6.413	23.749	1.00	20.59	C
ATOM	473	CD2	LEU A	41	75.481	4.796	23.609	1.00	17.97	C
ATOM	474	N	LEU A	42	75.542	5.916	18.238	1.00	12.45	N
ATOM	475	CA	LEU A	42	75.256	5.607	16.831	1.00	15.99	C
ATOM	476	C	LEU A	42	76.290	4.680	16.280	1.00	26.18	C
ATOM	477	O	LEU A	42	76.066	4.039	15.257	1.00	22.41	O
ATOM	478	CB	LEU A	42	75.282	6.873	15.984	1.00	17.85	C
ATOM	479	CG	LEU A	42	74.180	7.854	16.399	1.00	30.70	C
ATOM	480	CD1	LEU A	42	74.318	9.184	15.704	1.00	24.31	C
ATOM	481	CD2	LEU A	42	72.764	7.241	16.208	1.00	31.13	C
ATOM	482	N	ASP A	43	77.462	4.705	16.911	1.00	26.87	N
ATOM	483	CA	ASP A	43	78.579	3.875	16.486	1.00	19.29	C
ATOM	484	C	ASP A	43	78.583	2.519	17.163	1.00	13.33	C
ATOM	485	O	ASP A	43	79.051	2.348	18.297	1.00	18.75	O
ATOM	486	CB	ASP A	43	79.870	4.580	16.776	1.00	31.06	C
ATOM	487	CG	ASP A	43	81.083	3.758	16.380	1.00	30.68	C
ATOM	488	OD1	ASP A	43	80.971	2.551	16.082	1.00	32.36	O
ATOM	489	OD2	ASP A	43	82.187	4.308	16.499	1.00	37.83	O
ATOM	490	N	SER A	44	78.139	1.544	16.377	1.00	16.89	N
ATOM	491	CA	SER A	44	77.978	0.173	16.789	1.00	17.67	C
ATOM	492	C	SER A	44	79.237	-0.463	17.392	1.00	20.40	C
ATOM	493	O	SER A	44	79.206	-1.126	18.444	1.00	26.27	O
ATOM	494	CB	SER A	44	77.504	-0.617	15.581	1.00	13.85	C
ATOM	495	OG	SER A	44	76.800	-1.740	16.063	1.00	43.83	O
ATOM	496	N	ARG A	45	80.335	-0.301	16.682	1.00	15.63	N
ATOM	497	CA	ARG A	45	81.616	-0.788	17.154	1.00	19.94	C
ATOM	498	C	ARG A	45	81.910	-0.225	18.521	1.00	29.48	C
ATOM	499	O	ARG A	45	82.244	-0.937	19.457	1.00	27.65	O
ATOM	500	CB	ARG A	45	82.684	-0.261	16.203	1.00	27.46	C
ATOM	501	CG	ARG A	45	83.463	-1.338	15.495	1.00	92.03	C
ATOM	502	CD	ARG A	45	84.854	-1.418	16.077	1.00	100.00	C
ATOM	503	NE	ARG A	45	85.636	-2.533	15.527	1.00	100.00	N
ATOM	504	CZ	ARG A	45	86.092	-3.570	16.236	1.00	100.00	C

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ATOM    505  NH1 ARG A  45      85.791  -3.695  17.547  1.00100.00           N
ATOM    506  NH2 ARG A  45      86.773  -4.544  15.642  1.00100.00           N
ATOM    507  N   ALA A  46      81.772   1.090  18.629  1.00 31.04           N
ATOM    508  CA  ALA A  46      82.045   1.743  19.881  1.00 24.72           C
ATOM    509  C   ALA A  46      81.111   1.176  20.899  1.00 17.73           C
ATOM    510  O   ALA A  46      81.512   0.825  22.027  1.00 22.73           O
ATOM    511  CB  ALA A  46      81.839   3.221  19.751  1.00 27.16           C
ATOM    512  N   VAL A  47      79.835   1.119  20.531  1.00 17.54           N
ATOM    513  CA  VAL A  47      78.878   0.608  21.508  1.00 21.41           C
ATOM    514  C   VAL A  47      79.262  -0.812  21.914  1.00 30.25           C
ATOM    515  O   VAL A  47      79.192  -1.202  23.097  1.00 15.85           O
ATOM    516  CB  VAL A  47      77.470   0.668  20.989  1.00 18.59           C
ATOM    517  CG1 VAL A  47      76.503   0.042  22.012  1.00 16.88           C
ATOM    518  CG2 VAL A  47      77.115   2.096  20.756  1.00 16.28           C
ATOM    519  N   HIS A  48      79.692  -1.585  20.920  1.00 21.00           N
ATOM    520  CA  HIS A  48      80.028  -2.969  21.192  1.00 20.17           C
ATOM    521  C   HIS A  48      81.268  -3.079  22.117  1.00 32.98           C
ATOM    522  O   HIS A  48      81.289  -3.850  23.102  1.00 28.20           O
ATOM    523  CB  HIS A  48      80.063  -3.801  19.855  1.00 14.93           C
ATOM    524  CG  HIS A  48      78.686  -4.172  19.338  1.00 26.67           C
ATOM    525  ND1 HIS A  48      78.085  -5.394  19.600  1.00 28.83           N
ATOM    526  CD2 HIS A  48      77.758  -3.448  18.659  1.00 25.56           C
ATOM    527  CE1 HIS A  48      76.887  -5.430  19.043  1.00 20.08           C
ATOM    528  NE2 HIS A  48      76.660  -4.260  18.475  1.00 25.22           N
ATOM    529  N   ASP A  49      82.217  -2.170  21.902  1.00 22.62           N
ATOM    530  CA  ASP A  49      83.455  -2.169  22.674  1.00 24.23           C
ATOM    531  C   ASP A  49      83.171  -1.899  24.122  1.00 38.72           C
ATOM    532  O   ASP A  49      83.708  -2.551  25.027  1.00 35.44           O
ATOM    533  CB  ASP A  49      84.396  -1.112  22.127  1.00 30.29           C
ATOM    534  CG  ASP A  49      84.991  -1.503  20.775  1.00 52.45           C
ATOM    535  OD1 ASP A  49      85.007  -2.726  20.449  1.00 42.67           O
ATOM    536  OD2 ASP A  49      85.416  -0.587  20.029  1.00 73.76           O
ATOM    537  N   PHE A  50      82.294  -0.929  24.324  1.00 32.19           N
ATOM    538  CA  PHE A  50      81.902  -0.550  25.649  1.00 29.76           C
ATOM    539  C   PHE A  50      81.299  -1.765  26.359  1.00 30.31           C
ATOM    540  O   PHE A  50      81.715  -2.124  27.449  1.00 29.22           O
ATOM    541  CB  PHE A  50      80.892   0.610  25.576  1.00 23.82           C
ATOM    542  CG  PHE A  50      80.137   0.843  26.859  1.00 19.13           C
ATOM    543  CD1 PHE A  50      80.740   1.515  27.931  1.00 20.14           C
ATOM    544  CD2 PHE A  50      78.835   0.360  27.018  1.00 13.99           C
ATOM    545  CE1 PHE A  50      80.034   1.742  29.129  1.00 25.81           C
ATOM    546  CE2 PHE A  50      78.114   0.553  28.212  1.00 22.84           C
ATOM    547  CZ  PHE A  50      78.698   1.276  29.259  1.00 23.40           C
ATOM    548  N   PHE A  51      80.280  -2.367  25.768  1.00 21.75           N
ATOM    549  CA  PHE A  51      79.655  -3.451  26.457  1.00 22.61           C
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ATOM	550	C	PHE	A	51	80.646	-4.603	26.612	1.00	34.01	C
ATOM	551	O	PHE	A	51	80.550	-5.401	27.590	1.00	25.28	O
ATOM	552	CB	PHE	A	51	78.389	-3.898	25.751	1.00	22.63	C
ATOM	553	CG	PHE	A	51	77.158	-3.140	26.170	1.00	27.58	C
ATOM	554	CD1	PHE	A	51	76.426	-3.525	27.280	1.00	21.78	C
ATOM	555	CD2	PHE	A	51	76.663	-2.100	25.380	1.00	19.55	C
ATOM	556	CE1	PHE	A	51	75.267	-2.796	27.662	1.00	28.34	C
ATOM	557	CE2	PHE	A	51	75.492	-1.403	25.734	1.00	14.47	C
ATOM	558	CZ	PHE	A	51	74.797	-1.744	26.878	1.00	14.55	C
ATOM	559	N	ALA	A	52	81.576	-4.706	25.659	1.00	26.43	N
ATOM	560	CA	ALA	A	52	82.587	-5.793	25.714	1.00	29.44	C
ATOM	561	C	ALA	A	52	83.687	-5.560	26.768	1.00	43.76	C
ATOM	562	O	ALA	A	52	84.502	-6.446	27.022	1.00	40.33	O
ATOM	563	CB	ALA	A	52	83.228	-6.049	24.344	1.00	24.25	C
ATOM	564	N	SER	A	53	83.702	-4.382	27.385	1.00	31.96	N
ATOM	565	CA	SER	A	53	84.705	-4.090	28.377	1.00	21.06	C
ATOM	566	C	SER	A	53	84.196	-3.625	29.709	1.00	26.41	C
ATOM	567	O	SER	A	53	84.985	-3.492	30.611	1.00	36.12	O
ATOM	568	CB	SER	A	53	85.709	-3.088	27.843	1.00	14.22	C
ATOM	569	OG	SER	A	53	85.140	-1.807	27.790	1.00	56.90	O
ATOM	570	N	GLU	A	54	82.892	-3.431	29.874	1.00	22.38	N
ATOM	571	CA	GLU	A	54	82.380	-2.893	31.139	1.00	17.27	C
ATOM	572	C	GLU	A	54	81.584	-3.735	32.118	1.00	26.32	C
ATOM	573	O	GLU	A	54	81.229	-3.281	33.191	1.00	37.43	O
ATOM	574	CB	GLU	A	54	81.677	-1.563	30.906	1.00	27.30	C
ATOM	575	CG	GLU	A	54	82.573	-0.543	30.262	1.00	44.77	C
ATOM	576	CD	GLU	A	54	83.669	-0.142	31.194	1.00	86.31	C
ATOM	577	OE1	GLU	A	54	83.392	-0.232	32.428	1.00	50.11	O
ATOM	578	OE2	GLU	A	54	84.785	0.198	30.692	1.00	50.99	O
ATOM	579	N	ARG	A	55	81.268	-4.971	31.804	1.00	29.63	N
ATOM	580	CA	ARG	A	55	80.636	-5.748	32.854	1.00	33.32	C
ATOM	581	C	ARG	A	55	79.347	-5.149	33.378	1.00	38.45	C
ATOM	582	O	ARG	A	55	79.214	-4.897	34.576	1.00	40.18	O
ATOM	583	CB	ARG	A	55	81.621	-5.875	34.045	1.00	57.61	C
ATOM	584	CG	ARG	A	55	82.666	-7.028	33.960	1.00	100.00	C
ATOM	585	CD	ARG	A	55	82.805	-7.805	35.305	1.00	100.00	C
ATOM	586	NE	ARG	A	55	82.838	-9.270	35.146	1.00	100.00	N
ATOM	587	CZ	ARG	A	55	83.206	-10.129	36.102	1.00	100.00	C
ATOM	588	NH1	ARG	A	55	83.583	-9.681	37.301	1.00	100.00	N
ATOM	589	NH2	ARG	A	55	83.208	-11.440	35.855	1.00	100.00	N
ATOM	590	N	ILE	A	56	78.367	-5.029	32.491	1.00	42.25	N
ATOM	591	CA	ILE	A	56	77.064	-4.434	32.794	1.00	25.49	C
ATOM	592	C	ILE	A	56	75.982	-5.474	33.244	1.00	20.18	C
ATOM	593	O	ILE	A	56	75.897	-6.579	32.704	1.00	24.74	O
ATOM	594	CB	ILE	A	56	76.672	-3.512	31.531	1.00	26.89	C

ATOM	595	CG1	ILE	A	56	77.643	-2.301	31.442	1.00	18.30	C
ATOM	596	CG2	ILE	A	56	75.214	-3.016	31.549	1.00	19.84	C
ATOM	597	CD1	ILE	A	56	77.998	-1.936	30.026	1.00	60.42	C
ATOM	598	N	ASP	A	57	75.166	-5.133	34.237	1.00	16.84	N
ATOM	599	CA	ASP	A	57	74.040	-5.999	34.630	1.00	16.33	C
ATOM	600	C	ASP	A	57	72.676	-5.451	34.123	1.00	28.40	C
ATOM	601	O	ASP	A	57	71.836	-6.198	33.657	1.00	25.50	O
ATOM	602	CB	ASP	A	57	74.009	-6.194	36.164	1.00	16.94	C
ATOM	603	CG	ASP	A	57	75.369	-6.720	36.703	1.00	34.27	C
ATOM	604	OD1	ASP	A	57	75.875	-7.729	36.141	1.00	31.76	O
ATOM	605	OD2	ASP	A	57	76.040	-6.007	37.499	1.00	28.36	O
ATOM	606	N	GLN	A	58	72.443	-4.152	34.220	1.00	28.91	N
ATOM	607	CA	GLN	A	58	71.183	-3.590	33.755	1.00	25.68	C
ATOM	608	C	GLN	A	58	71.425	-2.364	32.881	1.00	23.21	C
ATOM	609	O	GLN	A	58	72.403	-1.620	33.067	1.00	18.16	O
ATOM	610	CB	GLN	A	58	70.342	-3.151	34.946	1.00	33.14	C
ATOM	611	CG	GLN	A	58	69.798	-4.241	35.807	1.00	30.00	C
ATOM	612	CD	GLN	A	58	69.226	-3.712	37.105	1.00	27.18	C
ATOM	613	OE1	GLN	A	58	68.722	-2.601	37.161	1.00	31.20	O
ATOM	614	NE2	GLN	A	58	69.455	-4.436	38.186	1.00	16.89	N
ATOM	615	N	VAL	A	59	70.496	-2.138	31.961	1.00	18.35	N
ATOM	616	CA	VAL	A	59	70.562	-0.998	31.045	1.00	15.59	C
ATOM	617	C	VAL	A	59	69.238	-0.240	31.039	1.00	26.28	C
ATOM	618	O	VAL	A	59	68.178	-0.820	30.762	1.00	19.51	O
ATOM	619	CB	VAL	A	59	70.707	-1.456	29.601	1.00	15.32	C
ATOM	620	CG1	VAL	A	59	70.477	-0.274	28.649	1.00	11.93	C
ATOM	621	CG2	VAL	A	59	72.080	-2.111	29.364	1.00	15.83	C
ATOM	622	N	TYR	A	60	69.306	1.064	31.293	1.00	21.71	N
ATOM	623	CA	TYR	A	60	68.113	1.927	31.197	1.00	21.40	C
ATOM	624	C	TYR	A	60	68.289	2.756	29.928	1.00	18.69	C
ATOM	625	O	TYR	A	60	69.250	3.532	29.796	1.00	15.51	O
ATOM	626	CB	TYR	A	60	68.021	2.817	32.413	1.00	17.24	C
ATOM	627	CG	TYR	A	60	67.493	2.131	33.658	1.00	19.71	C
ATOM	628	CD1	TYR	A	60	68.345	1.583	34.586	1.00	21.14	C
ATOM	629	CD2	TYR	A	60	66.154	2.223	33.991	1.00	20.16	C
ATOM	630	CE1	TYR	A	60	67.835	1.080	35.794	1.00	19.11	C
ATOM	631	CE2	TYR	A	60	65.648	1.698	35.163	1.00	10.77	C
ATOM	632	CZ	TYR	A	60	66.476	1.094	36.054	1.00	20.07	C
ATOM	633	OH	TYR	A	60	65.921	0.585	37.248	1.00	16.04	O
ATOM	634	N	LEU	A	61	67.491	2.452	28.916	1.00	17.46	N
ATOM	635	CA	LEU	A	61	67.685	3.053	27.585	1.00	20.17	C
ATOM	636	C	LEU	A	61	67.003	4.412	27.409	1.00	23.36	C
ATOM	637	O	LEU	A	61	65.925	4.526	26.799	1.00	14.86	O
ATOM	638	CB	LEU	A	61	67.267	2.060	26.485	1.00	14.78	C
ATOM	639	CG	LEU	A	61	68.117	2.142	25.208	1.00	15.52	C

ATOM	640	CD1	LEU	A	61	67.815	1.010	24.109	1.00	7.75	C
ATOM	641	CD2	LEU	A	61	68.087	3.541	24.580	1.00	15.20	C
ATOM	642	N	ALA	A	62	67.656	5.434	27.956	1.00	20.35	N
ATOM	643	CA	ALA	A	62	67.120	6.784	27.963	1.00	18.55	C
ATOM	644	C	ALA	A	62	67.779	7.739	26.949	1.00	18.57	C
ATOM	645	O	ALA	A	62	67.455	8.924	26.920	1.00	24.31	O
ATOM	646	CB	ALA	A	62	67.071	7.377	29.439	1.00	11.69	C
ATOM	647	N	ALA	A	63	68.681	7.231	26.101	1.00	14.09	N
ATOM	648	CA	ALA	A	63	69.249	8.095	25.052	1.00	12.84	C
ATOM	649	C	ALA	A	63	68.310	8.005	23.877	1.00	27.00	C
ATOM	650	O	ALA	A	63	67.845	6.916	23.511	1.00	24.51	O
ATOM	651	CB	ALA	A	63	70.665	7.660	24.634	1.00	4.89	C
ATOM	652	N	ALA	A	64	68.076	9.148	23.262	1.00	21.05	N
ATOM	653	CA	ALA	A	64	67.202	9.286	22.086	1.00	13.50	C
ATOM	654	C	ALA	A	64	67.435	10.664	21.416	1.00	28.08	C
ATOM	655	O	ALA	A	64	67.987	11.600	22.021	1.00	26.63	O
ATOM	656	CB	ALA	A	64	65.642	9.171	22.518	1.00	7.63	C
ATOM	657	N	LYS	A	65	66.953	10.781	20.182	1.00	23.98	N
ATOM	658	CA	LYS	A	65	66.966	12.012	19.409	1.00	20.47	C
ATOM	659	C	LYS	A	65	65.488	12.443	19.551	1.00	24.37	C
ATOM	660	O	LYS	A	65	64.594	11.807	18.976	1.00	20.29	O
ATOM	661	CB	LYS	A	65	67.317	11.658	17.951	1.00	25.59	C
ATOM	662	CG	LYS	A	65	66.808	12.630	16.923	1.00	27.54	C
ATOM	663	CD	LYS	A	65	67.518	13.926	17.169	1.00	21.08	C
ATOM	664	CE	LYS	A	65	67.316	14.905	16.029	1.00	55.15	C
ATOM	665	NZ	LYS	A	65	67.876	16.263	16.392	1.00	81.63	N
ATOM	666	N	VAL	A	66	65.228	13.362	20.485	1.00	22.47	N
ATOM	667	CA	VAL	A	66	63.873	13.850	20.755	1.00	18.99	C
ATOM	668	C	VAL	A	66	63.711	15.343	20.394	1.00	31.44	C
ATOM	669	O	VAL	A	66	64.665	16.107	20.460	1.00	34.61	O
ATOM	670	CB	VAL	A	66	63.440	13.623	22.204	1.00	16.66	C
ATOM	671	CG1	VAL	A	66	64.269	12.623	22.869	1.00	15.01	C
ATOM	672	CG2	VAL	A	66	63.379	14.904	22.950	1.00	19.21	C
ATOM	673	N	GLY	A	67	62.514	15.755	19.994	1.00	18.03	N
ATOM	674	CA	GLY	A	67	62.298	17.149	19.614	1.00	14.90	C
ATOM	675	C	GLY	A	67	60.792	17.518	19.585	1.00	32.35	C
ATOM	676	O	GLY	A	67	59.922	16.666	19.888	1.00	18.88	O
ATOM	677	N	GLY	A	68	60.503	18.787	19.256	1.00	23.21	N
ATOM	678	CA	GLY	A	68	59.132	19.288	19.183	1.00	23.83	C
ATOM	679	C	GLY	A	68	58.540	19.137	17.771	1.00	19.31	C
ATOM	680	O	GLY	A	68	59.165	18.550	16.870	1.00	30.64	O
ATOM	681	N	ILE	A	69	57.343	19.684	17.588	1.00	15.20	N
ATOM	682	CA	ILE	A	69	56.595	19.632	16.317	1.00	16.80	C
ATOM	683	C	ILE	A	69	57.387	20.153	15.112	1.00	19.33	C
ATOM	684	O	ILE	A	69	57.425	19.519	14.061	1.00	14.66	O

ATOM	685	CB	ILE	A	69	55.257	20.432	16.480	1.00 30.11	C
ATOM	686	CG1	ILE	A	69	54.271	19.683	17.385	1.00 24.27	C
ATOM	687	CG2	ILE	A	69	54.610	20.749	15.181	1.00 47.53	C
ATOM	688	CD1	ILE	A	69	53.259	20.608	18.056	1.00 85.71	C
ATOM	689	N	VAL	A	70	58.010	21.327	15.269	1.00 23.03	N
ATOM	690	CA	VAL	A	70	58.797	21.913	14.183	1.00 19.34	C
ATOM	691	C	VAL	A	70	59.983	21.011	13.840	1.00 24.42	C
ATOM	692	O	VAL	A	70	60.335	20.829	12.662	1.00 24.14	O
ATOM	693	CB	VAL	A	70	59.304	23.404	14.467	1.00 21.37	C
ATOM	694	CG1	VAL	A	70	60.137	23.907	13.281	1.00 17.79	C
ATOM	695	CG2	VAL	A	70	58.136	24.410	14.678	1.00 15.74	C
ATOM	696	N	ALA	A	71	60.621	20.450	14.861	1.00 19.68	N
ATOM	697	CA	ALA	A	71	61.782	19.617	14.572	1.00 16.57	C
ATOM	698	C	ALA	A	71	61.427	18.289	13.910	1.00 23.36	C
ATOM	699	O	ALA	A	71	61.980	17.923	12.849	1.00 21.84	O
ATOM	700	CB	ALA	A	71	62.685	19.439	15.805	1.00 9.36	C
ATOM	701	N	ASN	A	72	60.463	17.598	14.511	1.00 16.80	N
ATOM	702	CA	ASN	A	72	59.998	16.357	13.923	1.00 18.84	C
ATOM	703	C	ASN	A	72	59.608	16.539	12.440	1.00 23.87	C
ATOM	704	O	ASN	A	72	59.919	15.696	11.593	1.00 21.52	O
ATOM	705	CB	ASN	A	72	58.835	15.806	14.738	1.00 8.60	C
ATOM	706	CG	ASN	A	72	59.309	15.013	15.911	1.00 23.75	C
ATOM	707	OD1	ASN	A	72	59.558	13.809	15.810	1.00 23.98	O
ATOM	708	ND2	ASN	A	72	59.572	15.701	16.996	1.00 9.96	N
ATOM	709	N	ASN	A	73	58.931	17.647	12.138	1.00 23.07	N
ATOM	710	CA	ASN	A	73	58.521	17.971	10.761	1.00 26.05	C
ATOM	711	C	ASN	A	73	59.665	18.454	9.817	1.00 26.95	C
ATOM	712	O	ASN	A	73	59.613	18.276	8.569	1.00 22.13	O
ATOM	713	CB	ASN	A	73	57.383	19.001	10.800	1.00 14.86	C
ATOM	714	CG	ASN	A	73	56.015	18.349	10.987	1.00 19.88	C
ATOM	715	OD1	ASN	A	73	55.620	17.468	10.217	1.00 27.02	O
ATOM	716	ND2	ASN	A	73	55.322	18.732	12.051	1.00 20.78	N
ATOM	717	N	THR	A	74	60.710	19.029	10.419	1.00 18.69	N
ATOM	718	CA	THR	A	74	61.845	19.540	9.657	1.00 10.07	C
ATOM	719	C	THR	A	74	62.968	18.548	9.375	1.00 21.00	C
ATOM	720	O	THR	A	74	63.537	18.561	8.289	1.00 11.75	O
ATOM	721	CB	THR	A	74	62.411	20.746	10.306	1.00 29.10	C
ATOM	722	OG1	THR	A	74	61.370	21.714	10.457	1.00 23.24	O
ATOM	723	CG2	THR	A	74	63.541	21.299	9.452	1.00 21.63	C
ATOM	724	N	TYR	A	75	63.230	17.636	10.310	1.00 17.10	N
ATOM	725	CA	TYR	A	75	64.267	16.620	10.112	1.00 9.07	C
ATOM	726	C	TYR	A	75	63.733	15.203	10.318	1.00 6.17	C
ATOM	727	O	TYR	A	75	64.143	14.542	11.267	1.00 15.58	O
ATOM	728	CB	TYR	A	75	65.302	16.825	11.188	1.00 11.89	C
ATOM	729	CG	TYR	A	75	65.779	18.234	11.252	1.00 27.12	C

ATOM	730	CD1	TYR	A	75	66.712	18.696	10.321	1.00	28.46	C
ATOM	731	CD2	TYR	A	75	65.234	19.151	12.173	1.00	24.83	C
ATOM	732	CE1	TYR	A	75	67.117	20.045	10.305	1.00	28.34	C
ATOM	733	CE2	TYR	A	75	65.652	20.523	12.180	1.00	21.00	C
ATOM	734	CZ	TYR	A	75	66.593	20.940	11.234	1.00	45.42	C
ATOM	735	OH	TYR	A	75	67.066	22.230	11.215	1.00	35.37	O
ATOM	736	N	PRO	A	76	62.759	14.775	9.532	1.00	13.30	N
ATOM	737	CA	PRO	A	76	62.185	13.438	9.742	1.00	14.64	C
ATOM	738	C	PRO	A	76	63.209	12.264	9.618	1.00	14.40	C
ATOM	739	O	PRO	A	76	63.157	11.335	10.409	1.00	20.54	O
ATOM	740	CB	PRO	A	76	61.055	13.366	8.709	1.00	7.83	C
ATOM	741	CG	PRO	A	76	61.447	14.388	7.617	1.00	12.61	C
ATOM	742	CD	PRO	A	76	62.068	15.504	8.455	1.00	11.18	C
ATOM	743	N	ALA	A	77	64.163	12.339	8.681	1.00	15.25	N
ATOM	744	CA	ALA	A	77	65.206	11.312	8.538	1.00	6.79	C
ATOM	745	C	ALA	A	77	66.053	11.166	9.820	1.00	17.22	C
ATOM	746	O	ALA	A	77	66.306	10.069	10.292	1.00	18.74	O
ATOM	747	CB	ALA	A	77	66.097	11.601	7.330	1.00	9.04	C
ATOM	748	N	ASP	A	78	66.466	12.267	10.424	1.00	10.92	N
ATOM	749	CA	ASP	A	78	67.256	12.191	11.659	1.00	11.87	C
ATOM	750	C	ASP	A	78	66.572	11.486	12.827	1.00	16.09	C
ATOM	751	O	ASP	A	78	67.212	10.741	13.601	1.00	18.07	O
ATOM	752	CB	ASP	A	78	67.578	13.609	12.088	1.00	19.16	C
ATOM	753	CG	ASP	A	78	68.424	14.325	11.068	1.00	26.82	C
ATOM	754	OD1	ASP	A	78	68.836	13.694	10.044	1.00	33.93	O
ATOM	755	OD2	ASP	A	78	68.673	15.514	11.316	1.00	32.06	O
ATOM	756	N	PHE	A	79	65.279	11.771	12.975	1.00	14.70	N
ATOM	757	CA	PHE	A	79	64.471	11.192	14.044	1.00	20.69	C
ATOM	758	C	PHE	A	79	64.224	9.707	13.876	1.00	20.22	C
ATOM	759	O	PHE	A	79	64.269	8.987	14.862	1.00	22.37	O
ATOM	760	CB	PHE	A	79	63.144	11.933	14.219	1.00	27.38	C
ATOM	761	CG	PHE	A	79	63.264	13.218	14.990	1.00	28.59	C
ATOM	762	CD1	PHE	A	79	63.137	13.230	16.386	1.00	27.49	C
ATOM	763	CD2	PHE	A	79	63.509	14.415	14.325	1.00	28.20	C
ATOM	764	CE1	PHE	A	79	63.281	14.413	17.109	1.00	21.76	C
ATOM	765	CE2	PHE	A	79	63.625	15.593	15.037	1.00	31.48	C
ATOM	766	CZ	PHE	A	79	63.509	15.582	16.439	1.00	26.31	C
ATOM	767	N	ILE	A	80	63.942	9.249	12.650	1.00	10.79	N
ATOM	768	CA	ILE	A	80	63.828	7.795	12.410	1.00	18.12	C
ATOM	769	C	ILE	A	80	65.197	7.052	12.432	1.00	10.97	C
ATOM	770	O	ILE	A	80	65.406	6.090	13.195	1.00	8.92	O
ATOM	771	CB	ILE	A	80	62.944	7.408	11.148	1.00	17.41	C
ATOM	772	CG1	ILE	A	80	62.651	5.886	11.105	1.00	10.16	C
ATOM	773	CG2	ILE	A	80	63.583	7.888	9.901	1.00	17.46	C
ATOM	774	CD1	ILE	A	80	61.722	5.410	9.980	1.00	7.30	C

ATOM	775	N	TYR	A	81	66.151	7.539	11.658	1.00 11.18	N
ATOM	776	CA	TYR	A	81	67.488	6.902	11.630	1.00 15.06	C
ATOM	777	C	TYR	A	81	68.237	6.782	12.959	1.00 16.83	C
ATOM	778	O	TYR	A	81	68.714	5.702	13.383	1.00 16.74	O
ATOM	779	CB	TYR	A	81	68.384	7.599	10.616	1.00 9.43	C
ATOM	780	CG	TYR	A	81	69.749	6.966	10.541	1.00 22.54	C
ATOM	781	CD1	TYR	A	81	69.963	5.824	9.747	1.00 22.37	C
ATOM	782	CD2	TYR	A	81	70.818	7.466	11.299	1.00 18.07	C
ATOM	783	CE1	TYR	A	81	71.202	5.163	9.746	1.00 15.02	C
ATOM	784	CE2	TYR	A	81	72.080	6.893	11.201	1.00 17.37	C
ATOM	785	CZ	TYR	A	81	72.255	5.698	10.472	1.00 24.27	C
ATOM	786	OH	TYR	A	81	73.491	5.063	10.409	1.00 19.57	O
ATOM	787	N	GLN	A	82	68.385	7.918	13.612	1.00 11.39	N
ATOM	788	CA	GLN	A	82	69.193	7.930	14.810	1.00 12.23	C
ATOM	789	C	GLN	A	82	68.544	7.089	15.834	1.00 14.18	C
ATOM	790	O	GLN	A	82	69.180	6.415	16.631	1.00 11.35	O
ATOM	791	CB	GLN	A	82	69.280	9.354	15.291	1.00 18.73	C
ATOM	792	CG	GLN	A	82	69.986	10.209	14.250	1.00 13.54	C
ATOM	793	CD	GLN	A	82	70.285	11.617	14.736	1.00 26.00	C
ATOM	794	OE1	GLN	A	82	70.410	11.850	15.927	1.00 22.99	O
ATOM	795	NE2	GLN	A	82	70.404	12.561	13.808	1.00 16.59	N
ATOM	796	N	ASN	A	83	67.235	7.181	15.869	1.00 11.35	N
ATOM	797	CA	ASN	A	83	66.549	6.408	16.860	1.00 13.71	C
ATOM	798	C	ASN	A	83	66.623	4.902	16.557	1.00 21.43	C
ATOM	799	O	ASN	A	83	66.831	4.101	17.463	1.00 12.10	O
ATOM	800	CB	ASN	A	83	65.132	6.945	17.074	1.00 13.51	C
ATOM	801	CG	ASN	A	83	65.131	8.245	17.871	1.00 28.91	C
ATOM	802	OD1	ASN	A	83	65.628	8.263	18.990	1.00 22.28	O
ATOM	803	ND2	ASN	A	83	64.756	9.354	17.237	1.00 20.17	N
ATOM	804	N	MET	A	84	66.592	4.517	15.290	1.00 15.63	N
ATOM	805	CA	MET	A	84	66.704	3.101	15.007	1.00 15.66	C
ATOM	806	C	MET	A	84	68.054	2.588	15.348	1.00 14.66	C
ATOM	807	O	MET	A	84	68.148	1.514	15.902	1.00 11.45	O
ATOM	808	CB	MET	A	84	66.418	2.815	13.563	1.00 17.59	C
ATOM	809	CG	MET	A	84	64.911	2.894	13.220	1.00 14.40	C
ATOM	810	SD	MET	A	84	64.638	2.811	11.387	1.00 15.99	S
ATOM	811	CE	MET	A	84	65.164	1.105	10.952	1.00 8.90	C
ATOM	812	N	MET	A	85	69.098	3.338	15.024	1.00 11.20	N
ATOM	813	CA	MET	A	85	70.468	2.879	15.321	1.00 11.67	C
ATOM	814	C	MET	A	85	70.779	2.831	16.774	1.00 13.04	C
ATOM	815	O	MET	A	85	71.359	1.893	17.265	1.00 15.26	O
ATOM	816	CB	MET	A	85	71.525	3.798	14.693	1.00 15.07	C
ATOM	817	CG	MET	A	85	71.530	3.726	13.173	1.00 32.01	C
ATOM	818	SD	MET	A	85	71.918	2.027	12.487	1.00 37.79	S
ATOM	819	CE	MET	A	85	73.379	1.801	13.320	1.00 15.94	C

ATOM	820	N	ILE	A	86	70.471	3.892	17.481	1.00	13.92	N
ATOM	821	CA	ILE	A	86	70.760	3.893	18.912	1.00	12.58	C
ATOM	822	C	ILE	A	86	70.159	2.662	19.591	1.00	21.61	C
ATOM	823	O	ILE	A	86	70.813	1.981	20.362	1.00	18.68	O
ATOM	824	CB	ILE	A	86	70.225	5.189	19.606	1.00	11.84	C
ATOM	825	CG1	ILE	A	86	70.978	6.429	19.119	1.00	19.78	C
ATOM	826	CG2	ILE	A	86	70.435	5.132	21.112	1.00	6.59	C
ATOM	827	CD1	ILE	A	86	70.505	7.694	19.772	1.00	20.37	C
ATOM	828	N	GLU	A	87	68.893	2.383	19.316	1.00	18.78	N
ATOM	829	CA	GLU	A	87	68.263	1.237	19.930	1.00	14.00	C
ATOM	830	C	GLU	A	87	68.797	-0.116	19.454	1.00	15.93	C
ATOM	831	O	GLU	A	87	69.017	-0.991	20.268	1.00	11.04	O
ATOM	832	CB	GLU	A	87	66.734	1.324	19.900	1.00	14.89	C
ATOM	833	CG	GLU	A	87	66.085	1.327	18.538	1.00	28.96	C
ATOM	834	CD	GLU	A	87	64.635	1.922	18.544	1.00	11.12	C
ATOM	835	OE1	GLU	A	87	64.307	2.801	19.376	1.00	25.46	O
ATOM	836	OE2	GLU	A	87	63.845	1.547	17.663	1.00	29.87	O
ATOM	837	N	SER	A	88	69.054	-0.259	18.155	1.00	16.18	N
ATOM	838	CA	SER	A	88	69.650	-1.482	17.569	1.00	19.52	C
ATOM	839	C	SER	A	88	71.029	-1.792	18.160	1.00	22.54	C
ATOM	840	O	SER	A	88	71.313	-2.929	18.592	1.00	13.80	O
ATOM	841	CB	SER	A	88	69.815	-1.326	16.023	1.00	14.61	C
ATOM	842	OG	SER	A	88	68.551	-1.201	15.355	1.00	15.41	O
ATOM	843	N	ASN	A	89	71.884	-0.773	18.143	1.00	22.63	N
ATOM	844	CA	ASN	A	89	73.227	-0.869	18.693	1.00	27.23	C
ATOM	845	C	ASN	A	89	73.195	-1.363	20.134	1.00	21.34	C
ATOM	846	O	ASN	A	89	73.795	-2.384	20.476	1.00	23.68	O
ATOM	847	CB	ASN	A	89	73.980	0.487	18.597	1.00	13.71	C
ATOM	848	CG	ASN	A	89	74.440	0.825	17.168	1.00	20.40	C
ATOM	849	OD1	ASN	A	89	74.305	-0.006	16.255	1.00	14.93	O
ATOM	850	ND2	ASN	A	89	74.937	2.067	16.960	1.00	13.32	N
ATOM	851	N	ILE	A	90	72.488	-0.646	20.979	1.00	16.55	N
ATOM	852	CA	ILE	A	90	72.437	-1.014	22.398	1.00	21.51	C
ATOM	853	C	ILE	A	90	71.876	-2.421	22.729	1.00	26.50	C
ATOM	854	O	ILE	A	90	72.384	-3.159	23.590	1.00	19.71	O
ATOM	855	CB	ILE	A	90	71.670	0.070	23.233	1.00	13.32	C
ATOM	856	CG1	ILE	A	90	72.539	1.299	23.401	1.00	11.05	C
ATOM	857	CG2	ILE	A	90	71.371	-0.445	24.637	1.00	7.54	C
ATOM	858	CD1	ILE	A	90	71.749	2.597	23.668	1.00	20.71	C
ATOM	859	N	ILE	A	91	70.755	-2.733	22.114	1.00	14.98	N
ATOM	860	CA	ILE	A	91	70.047	-3.953	22.442	1.00	21.33	C
ATOM	861	C	ILE	A	91	70.927	-5.098	21.994	1.00	26.27	C
ATOM	862	O	ILE	A	91	71.211	-6.011	22.751	1.00	26.56	O
ATOM	863	CB	ILE	A	91	68.556	-3.930	21.814	1.00	20.39	C
ATOM	864	CG1	ILE	A	91	67.692	-2.886	22.552	1.00	13.51	C

ATOM	865	CG2	ILE	A	91	67.841	-5.316	21.845	1.00	11.31	C
ATOM	866	CD1	ILE	A	91	66.320	-2.648	21.907	1.00	16.23	C
ATOM	867	N	HIS	A	92	71.446	-4.983	20.785	1.00	24.12	N
ATOM	868	CA	HIS	A	92	72.293	-6.015	20.243	1.00	26.71	C
ATOM	869	C	HIS	A	92	73.609	-6.251	21.071	1.00	29.30	C
ATOM	870	O	HIS	A	92	73.983	-7.366	21.443	1.00	18.58	O
ATOM	871	CB	HIS	A	92	72.561	-5.682	18.775	1.00	22.23	C
ATOM	872	CG	HIS	A	92	73.366	-6.720	18.077	1.00	26.32	C
ATOM	873	ND1	HIS	A	92	72.798	-7.711	17.307	1.00	27.19	N
ATOM	874	CD2	HIS	A	92	74.699	-6.978	18.106	1.00	21.95	C
ATOM	875	CE1	HIS	A	92	73.755	-8.487	16.826	1.00	23.66	C
ATOM	876	NE2	HIS	A	92	74.918	-8.062	17.296	1.00	17.36	N
ATOM	877	N	ALA	A	93	74.328	-5.187	21.333	1.00	15.66	N
ATOM	878	CA	ALA	A	93	75.530	-5.301	22.110	1.00	11.88	C
ATOM	879	C	ALA	A	93	75.222	-5.900	23.512	1.00	28.78	C
ATOM	880	O	ALA	A	93	75.912	-6.790	24.037	1.00	25.23	O
ATOM	881	CB	ALA	A	93	76.139	-3.959	22.221	1.00	6.30	C
ATOM	882	N	ALA	A	94	74.142	-5.442	24.113	1.00	18.82	N
ATOM	883	CA	ALA	A	94	73.777	-5.971	25.399	1.00	15.61	C
ATOM	884	C	ALA	A	94	73.593	-7.503	25.301	1.00	28.39	C
ATOM	885	O	ALA	A	94	74.133	-8.263	26.099	1.00	21.67	O
ATOM	886	CB	ALA	A	94	72.449	-5.279	25.911	1.00	18.46	C
ATOM	887	N	HIS	A	95	72.814	-7.966	24.329	1.00	26.35	N
ATOM	888	CA	HIS	A	95	72.551	-9.396	24.271	1.00	24.89	C
ATOM	889	C	HIS	A	95	73.845	-10.176	24.140	1.00	22.81	C
ATOM	890	O	HIS	A	95	74.077	-11.136	24.865	1.00	21.44	O
ATOM	891	CB	HIS	A	95	71.571	-9.778	23.129	1.00	22.39	C
ATOM	892	CG	HIS	A	95	71.554	-11.250	22.831	1.00	28.73	C
ATOM	893	ND1	HIS	A	95	70.979	-12.182	23.682	1.00	22.83	N
ATOM	894	CD2	HIS	A	95	72.159	-11.964	21.845	1.00	25.22	C
ATOM	895	CE1	HIS	A	95	71.171	-13.397	23.196	1.00	22.72	C
ATOM	896	NE2	HIS	A	95	71.911	-13.296	22.101	1.00	24.80	N
ATOM	897	N	GLN	A	96	74.709	-9.658	23.281	1.00	19.97	N
ATOM	898	CA	GLN	A	96	75.960	-10.299	22.917	1.00	22.27	C
ATOM	899	C	GLN	A	96	76.877	-10.353	24.086	1.00	26.58	C
ATOM	900	O	GLN	A	96	77.836	-11.093	24.088	1.00	24.17	O
ATOM	901	CB	GLN	A	96	76.642	-9.492	21.818	1.00	23.38	C
ATOM	902	CG	GLN	A	96	77.043	-10.299	20.596	1.00	61.06	C
ATOM	903	CD	GLN	A	96	78.033	-9.557	19.675	1.00	75.83	C
ATOM	904	OE1	GLN	A	96	78.999	-8.941	20.131	1.00	56.89	O
ATOM	905	NE2	GLN	A	96	77.815	-9.668	18.366	1.00	100.00	N
ATOM	906	N	ASN	A	97	76.652	-9.500	25.060	1.00	22.15	N
ATOM	907	CA	ASN	A	97	77.537	-9.536	26.208	1.00	14.74	C
ATOM	908	C	ASN	A	97	76.732	-10.022	27.387	1.00	29.78	C
ATOM	909	O	ASN	A	97	77.049	-9.762	28.564	1.00	27.09	O

ATOM	910	CB	ASN	A	97	78.241	-8.201	26.462	1.00	12.93	C
ATOM	911	CG	ASN	A	97	79.260	-7.897	25.407	1.00	24.91	C
ATOM	912	OD1	ASN	A	97	80.331	-8.518	25.375	1.00	57.17	O
ATOM	913	ND2	ASN	A	97	78.839	-7.135	24.392	1.00	34.88	N
ATOM	914	N	ASP	A	98	75.666	-10.732	27.055	1.00	27.98	N
ATOM	915	CA	ASP	A	98	74.907	-11.361	28.089	1.00	29.25	C
ATOM	916	C	ASP	A	98	74.400	-10.379	29.164	1.00	37.53	C
ATOM	917	O	ASP	A	98	74.505	-10.634	30.367	1.00	36.42	O
ATOM	918	CB	ASP	A	98	75.791	-12.450	28.700	1.00	36.37	C
ATOM	919	CG	ASP	A	98	75.016	-13.712	29.053	1.00	88.62	C
ATOM	920	OD1	ASP	A	98	73.775	-13.749	28.877	1.00	82.53	O
ATOM	921	OD2	ASP	A	98	75.656	-14.670	29.542	1.00	100.00	O
ATOM	922	N	VAL	A	99	73.879	-9.235	28.730	1.00	27.13	N
ATOM	923	CA	VAL	A	99	73.157	-8.351	29.635	1.00	21.57	C
ATOM	924	C	VAL	A	99	71.706	-8.868	29.530	1.00	16.15	C
ATOM	925	O	VAL	A	99	71.159	-9.088	28.422	1.00	19.47	O
ATOM	926	CB	VAL	A	99	73.264	-6.900	29.206	1.00	24.18	C
ATOM	927	CG1	VAL	A	99	72.517	-6.015	30.198	1.00	14.58	C
ATOM	928	CG2	VAL	A	99	74.720	-6.515	29.225	1.00	30.10	C
ATOM	929	N	ASN	A	100	71.149	-9.262	30.662	1.00	17.39	N
ATOM	930	CA	ASN	A	100	69.852	-9.925	30.613	1.00	25.77	C
ATOM	931	C	ASN	A	100	68.648	-9.034	30.910	1.00	24.95	C
ATOM	932	O	ASN	A	100	67.498	-9.377	30.582	1.00	20.88	O
ATOM	933	CB	ASN	A	100	69.846	-11.157	31.527	1.00	14.98	C
ATOM	934	CG	ASN	A	100	68.724	-12.112	31.180	1.00	20.38	C
ATOM	935	OD1	ASN	A	100	68.737	-12.709	30.100	1.00	29.59	O
ATOM	936	ND2	ASN	A	100	67.716	-12.240	32.076	1.00	16.35	N
ATOM	937	N	LYS	A	101	68.941	-7.923	31.584	1.00	17.91	N
ATOM	938	CA	LYS	A	101	67.970	-6.916	31.994	1.00	25.43	C
ATOM	939	C	LYS	A	101	68.107	-5.510	31.323	1.00	25.29	C
ATOM	940	O	LYS	A	101	69.151	-4.850	31.377	1.00	19.88	O
ATOM	941	CB	LYS	A	101	67.996	-6.807	33.521	1.00	29.28	C
ATOM	942	CG	LYS	A	101	67.464	-8.054	34.205	1.00	9.31	C
ATOM	943	CD	LYS	A	101	67.218	-7.719	35.668	1.00	38.93	C
ATOM	944	CE	LYS	A	101	66.206	-6.569	35.885	1.00	13.38	C
ATOM	945	NZ	LYS	A	101	64.750	-7.006	35.825	1.00	15.26	N
ATOM	946	N	LEU	A	102	67.013	-5.043	30.732	1.00	22.22	N
ATOM	947	CA	LEU	A	102	67.003	-3.744	30.092	1.00	15.40	C
ATOM	948	C	LEU	A	102	65.612	-3.115	30.156	1.00	18.55	C
ATOM	949	O	LEU	A	102	64.590	-3.811	30.102	1.00	18.92	O
ATOM	950	CB	LEU	A	102	67.465	-3.898	28.636	1.00	11.23	C
ATOM	951	CG	LEU	A	102	67.553	-2.711	27.651	1.00	15.51	C
ATOM	952	CD1	LEU	A	102	68.628	-2.985	26.559	1.00	9.65	C
ATOM	953	CD2	LEU	A	102	66.162	-2.407	26.995	1.00	13.10	C
ATOM	954	N	LEU	A	103	65.595	-1.798	30.318	1.00	17.05	N

ATOM	955	CA	LEU A 103	64.356	-1.036	30.265	1.00 16.23		C
ATOM	956	C	LEU A 103	64.346	-0.072	29.046	1.00 19.65		C
ATOM	957	O	LEU A 103	65.215	0.789	28.875	1.00 19.68		O
ATOM	958	CB	LEU A 103	64.099	-0.289	31.562	1.00 12.28		C
ATOM	959	CG	LEU A 103	62.686	0.259	31.594	1.00 14.13		C
ATOM	960	CD1	LEU A 103	61.645	-0.822	31.902	1.00 10.31		C
ATOM	961	CD2	LEU A 103	62.646	1.360	32.601	1.00 12.30		C
ATOM	962	N	PHE A 104	63.417	-0.333	28.140	1.00 16.41		N
ATOM	963	CA	PHE A 104	63.215	0.486	26.956	1.00 18.32		C
ATOM	964	C	PHE A 104	62.126	1.546	27.249	1.00 21.85		C
ATOM	965	O	PHE A 104	61.168	1.271	27.992	1.00 18.36		O
ATOM	966	CB	PHE A 104	62.796	-0.386	25.793	1.00 9.86		C
ATOM	967	CG	PHE A 104	62.732	0.348	24.508	1.00 16.81		C
ATOM	968	CD1	PHE A 104	63.894	0.714	23.840	1.00 25.04		C
ATOM	969	CD2	PHE A 104	61.511	0.795	24.005	1.00 22.59		C
ATOM	970	CE1	PHE A 104	63.836	1.448	22.619	1.00 31.26		C
ATOM	971	CE2	PHE A 104	61.449	1.535	22.814	1.00 15.59		C
ATOM	972	CZ	PHE A 104	62.625	1.895	22.139	1.00 11.67		C
ATOM	973	N	LEU A 105	62.341	2.762	26.734	1.00 20.33		N
ATOM	974	CA	LEU A 105	61.416	3.897	26.904	1.00 18.10		C
ATOM	975	C	LEU A 105	60.711	4.237	25.634	1.00 17.04		C
ATOM	976	O	LEU A 105	61.315	4.680	24.665	1.00 18.83		O
ATOM	977	CB	LEU A 105	62.178	5.146	27.214	1.00 17.49		C
ATOM	978	CG	LEU A 105	62.434	5.544	28.644	1.00 27.17		C
ATOM	979	CD1	LEU A 105	62.630	4.349	29.574	1.00 19.16		C
ATOM	980	CD2	LEU A 105	63.688	6.347	28.529	1.00 23.59		C
ATOM	981	N	GLY A 106	59.407	4.153	25.652	1.00 20.66		N
ATOM	982	CA	GLY A 106	58.679	4.536	24.455	1.00 21.03		C
ATOM	983	C	GLY A 106	58.080	5.935	24.597	1.00 17.32		C
ATOM	984	O	GLY A 106	58.690	6.858	25.113	1.00 26.89		O
ATOM	985	N	SER A 107	56.831	6.047	24.219	1.00 22.05		N
ATOM	986	CA	SER A 107	56.177	7.317	24.288	1.00 22.12		C
ATOM	987	C	SER A 107	54.686	7.212	23.923	1.00 19.06		C
ATOM	988	O	SER A 107	54.314	6.545	22.963	1.00 27.42		O
ATOM	989	CB	SER A 107	56.882	8.232	23.300	1.00 20.99		C
ATOM	990	OG	SER A 107	55.947	9.133	22.776	1.00 42.85		O
ATOM	991	N	SER A 108	53.826	7.890	24.671	1.00 27.42		N
ATOM	992	CA	SER A 108	52.382	7.947	24.339	1.00 26.43		C
ATOM	993	C	SER A 108	52.144	8.259	22.842	1.00 30.97		C
ATOM	994	O	SER A 108	51.242	7.709	22.217	1.00 33.46		O
ATOM	995	CB	SER A 108	51.710	9.072	25.144	1.00 19.87		C
ATOM	996	OG	SER A 108	52.495	10.266	25.071	1.00 70.88		O
ATOM	997	N	CYS A 109	52.927	9.180	22.278	1.00 24.73		N
ATOM	998	CA	CYS A 109	52.728	9.549	20.880	1.00 25.61		C
ATOM	999	C	CYS A 109	52.970	8.482	19.815	1.00 21.29		C

ATOM	1000	O	CYS A 109	52.967	8.737	18.623	1.00	31.31	O
ATOM	1001	CB	CYS A 109	53.369	10.899	20.544	1.00	39.55	C
ATOM	1002	SG	CYS A 109	55.153	11.077	20.847	1.00	49.24	S
ATOM	1003	N	ILE A 110	53.101	7.264	20.258	1.00	18.31	N
ATOM	1004	CA	ILE A 110	53.329	6.150	19.379	1.00	28.10	C
ATOM	1005	C	ILE A 110	51.977	5.489	19.082	1.00	15.38	C
ATOM	1006	O	ILE A 110	51.895	4.592	18.268	1.00	16.52	O
ATOM	1007	CB	ILE A 110	54.154	5.153	20.206	1.00	40.45	C
ATOM	1008	CG1	ILE A 110	55.604	5.510	20.136	1.00	39.02	C
ATOM	1009	CG2	ILE A 110	53.879	3.715	19.875	1.00	61.33	C
ATOM	1010	CD1	ILE A 110	56.429	4.338	20.549	1.00	82.74	C
ATOM	1011	N	TYR A 111	50.951	5.842	19.854	1.00	14.91	N
ATOM	1012	CA	TYR A 111	49.630	5.227	19.678	1.00	13.96	C
ATOM	1013	C	TYR A 111	48.956	5.831	18.459	1.00	20.40	C
ATOM	1014	O	TYR A 111	49.302	6.933	18.056	1.00	11.71	O
ATOM	1015	CB	TYR A 111	48.763	5.468	20.921	1.00	9.63	C
ATOM	1016	CG	TYR A 111	49.117	4.550	22.065	1.00	14.94	C
ATOM	1017	CD1	TYR A 111	48.985	3.159	21.938	1.00	9.73	C
ATOM	1018	CD2	TYR A 111	49.755	5.038	23.216	1.00	14.96	C
ATOM	1019	CE1	TYR A 111	49.344	2.273	23.014	1.00	6.53	C
ATOM	1020	CE2	TYR A 111	50.146	4.155	24.272	1.00	13.66	C
ATOM	1021	CZ	TYR A 111	49.873	2.787	24.171	1.00	17.86	C
ATOM	1022	OH	TYR A 111	50.266	1.927	25.157	1.00	11.37	O
ATOM	1023	N	PRO A 112	47.974	5.145	17.872	1.00	22.56	N
ATOM	1024	CA	PRO A 112	47.279	5.743	16.721	1.00	23.44	C
ATOM	1025	C	PRO A 112	46.589	7.111	16.988	1.00	17.82	C
ATOM	1026	O	PRO A 112	46.197	7.453	18.115	1.00	19.72	O
ATOM	1027	CB	PRO A 112	46.290	4.644	16.252	1.00	15.69	C
ATOM	1028	CG	PRO A 112	46.895	3.343	16.769	1.00	22.83	C
ATOM	1029	CD	PRO A 112	47.593	3.733	18.086	1.00	16.10	C
ATOM	1030	N	LYS A 113	46.418	7.866	15.915	1.00	19.48	N
ATOM	1031	CA	LYS A 113	45.793	9.167	15.994	1.00	23.50	C
ATOM	1032	C	LYS A 113	44.396	9.077	16.655	1.00	34.28	C
ATOM	1033	O	LYS A 113	44.046	9.887	17.524	1.00	46.14	O
ATOM	1034	CB	LYS A 113	45.675	9.735	14.593	1.00	30.04	C
ATOM	1035	CG	LYS A 113	46.219	11.124	14.477	1.00	43.78	C
ATOM	1036	CD	LYS A 113	45.381	11.941	13.515	1.00	100.00	C
ATOM	1037	CE	LYS A 113	44.361	12.836	14.250	1.00	100.00	C
ATOM	1038	NZ	LYS A 113	43.480	13.625	13.304	1.00	100.00	N
ATOM	1039	N	LEU A 114	43.591	8.103	16.250	1.00	26.33	N
ATOM	1040	CA	LEU A 114	42.267	7.957	16.833	1.00	20.65	C
ATOM	1041	C	LEU A 114	42.083	6.792	17.760	1.00	18.44	C
ATOM	1042	O	LEU A 114	41.002	6.278	17.918	1.00	34.04	O
ATOM	1043	CB	LEU A 114	41.194	8.002	15.780	1.00	24.37	C
ATOM	1044	CG	LEU A 114	41.587	9.122	14.830	1.00	40.86	C

ATOM	1045	CD1	LEU	A	114	40.991	8.797	13.504	1.00 49.29	C
ATOM	1046	CD2	LEU	A	114	41.139	10.512	15.300	1.00 26.85	C
ATOM	1047	N	ALA	A	115	43.103	6.473	18.527	1.00 29.00	N
ATOM	1048	CA	ALA	A	115	42.920	5.446	19.528	1.00 25.66	C
ATOM	1049	C	ALA	A	115	41.722	5.727	20.454	1.00 28.76	C
ATOM	1050	O	ALA	A	115	41.364	6.855	20.682	1.00 24.12	O
ATOM	1051	CB	ALA	A	115	44.177	5.272	20.326	1.00 16.86	C
ATOM	1052	N	LYS	A	116	41.137	4.675	20.998	1.00 30.21	N
ATOM	1053	CA	LYS	A	116	40.036	4.792	21.928	1.00 25.85	C
ATOM	1054	C	LYS	A	116	40.668	5.248	23.195	1.00 14.18	C
ATOM	1055	O	LYS	A	116	41.750	4.781	23.535	1.00 23.51	O
ATOM	1056	CB	LYS	A	116	39.369	3.415	22.116	1.00 22.05	C
ATOM	1057	CG	LYS	A	116	39.053	3.032	23.524	1.00 55.38	C
ATOM	1058	CD	LYS	A	116	37.963	1.955	23.549	1.00100.00	C
ATOM	1059	CE	LYS	A	116	37.120	1.953	24.835	1.00100.00	C
ATOM	1060	NZ	LYS	A	116	35.767	1.310	24.630	1.00100.00	N
ATOM	1061	N	GLN	A	117	40.021	6.208	23.856	1.00 18.23	N
ATOM	1062	CA	GLN	A	117	40.456	6.757	25.180	1.00 21.01	C
ATOM	1063	C	GLN	A	117	39.695	6.178	26.383	1.00 30.96	C
ATOM	1064	O	GLN	A	117	38.483	6.009	26.345	1.00 27.66	O
ATOM	1065	CB	GLN	A	117	40.215	8.263	25.179	1.00 11.32	C
ATOM	1066	CG	GLN	A	117	40.849	8.912	23.948	1.00 12.12	C
ATOM	1067	CD	GLN	A	117	42.404	8.823	23.954	1.00 24.10	C
ATOM	1068	OE1	GLN	A	117	43.041	8.628	22.896	1.00 47.88	O
ATOM	1069	NE2	GLN	A	117	43.001	8.953	25.131	1.00 14.24	N
ATOM	1070	N	PRO	A	118	40.374	5.992	27.499	1.00 30.02	N
ATOM	1071	CA	PRO	A	118	41.826	6.194	27.655	1.00 26.44	C
ATOM	1072	C	PRO	A	118	42.450	5.050	26.899	1.00 24.37	C
ATOM	1073	O	PRO	A	118	41.792	4.027	26.726	1.00 25.34	O
ATOM	1074	CB	PRO	A	118	42.055	5.994	29.167	1.00 23.89	C
ATOM	1075	CG	PRO	A	118	40.847	5.240	29.654	1.00 23.20	C
ATOM	1076	CD	PRO	A	118	39.695	5.519	28.709	1.00 15.79	C
ATOM	1077	N	MET	A	119	43.684	5.228	26.432	1.00 16.00	N
ATOM	1078	CA	MET	A	119	44.372	4.215	25.644	1.00 10.80	C
ATOM	1079	C	MET	A	119	45.062	3.083	26.444	1.00 23.61	C
ATOM	1080	O	MET	A	119	46.013	3.281	27.209	1.00 18.02	O
ATOM	1081	CB	MET	A	119	45.384	4.894	24.791	1.00 13.52	C
ATOM	1082	CG	MET	A	119	44.801	6.014	23.989	1.00 18.52	C
ATOM	1083	SD	MET	A	119	46.157	7.054	23.271	1.00 26.27	S
ATOM	1084	CE	MET	A	119	46.264	6.524	21.845	1.00 33.79	C
ATOM	1085	N	ALA	A	120	44.559	1.875	26.271	1.00 26.64	N
ATOM	1086	CA	ALA	A	120	45.177	0.712	26.884	1.00 29.17	C
ATOM	1087	C	ALA	A	120	46.356	0.308	25.984	1.00 23.21	C
ATOM	1088	O	ALA	A	120	46.439	0.759	24.833	1.00 20.19	O
ATOM	1089	CB	ALA	A	120	44.169	-0.419	26.944	1.00 26.02	C

ATOM	1090	N	GLU A 121	47.238	-0.553	26.507	1.00	12.30	N
ATOM	1091	CA	GLU A 121	48.427	-1.009	25.788	1.00	9.45	C
ATOM	1092	C	GLU A 121	48.070	-1.697	24.450	1.00	11.68	C
ATOM	1093	O	GLU A 121	48.828	-1.670	23.450	1.00	14.84	O
ATOM	1094	CB	GLU A 121	49.321	-1.883	26.715	1.00	16.74	C
ATOM	1095	CG	GLU A 121	50.132	-1.122	27.763	1.00	18.14	C
ATOM	1096	CD	GLU A 121	49.458	-1.000	29.137	1.00	13.00	C
ATOM	1097	OE1	GLU A 121	48.252	-1.294	29.276	1.00	20.79	O
ATOM	1098	OE2	GLU A 121	50.123	-0.521	30.080	1.00	17.86	O
ATOM	1099	N	SER A 122	46.887	-2.273	24.409	1.00	11.79	N
ATOM	1100	CA	SER A 122	46.427	-2.977	23.218	1.00	12.16	C
ATOM	1101	C	SER A 122	46.030	-2.058	22.100	1.00	11.70	C
ATOM	1102	O	SER A 122	45.717	-2.529	21.010	1.00	13.91	O
ATOM	1103	CB	SER A 122	45.186	-3.781	23.568	1.00	21.50	C
ATOM	1104	OG	SER A 122	44.143	-2.908	23.976	1.00	28.52	O
ATOM	1105	N	GLU A 123	46.041	-0.754	22.341	1.00	14.65	N
ATOM	1106	CA	GLU A 123	45.783	0.202	21.243	1.00	17.15	C
ATOM	1107	C	GLU A 123	46.959	0.313	20.240	1.00	11.48	C
ATOM	1108	O	GLU A 123	46.821	0.844	19.141	1.00	11.19	O
ATOM	1109	CB	GLU A 123	45.481	1.600	21.805	1.00	21.66	C
ATOM	1110	CG	GLU A 123	44.127	1.694	22.523	1.00	24.68	C
ATOM	1111	CD	GLU A 123	42.984	1.374	21.585	1.00	35.56	C
ATOM	1112	OE1	GLU A 123	43.019	1.865	20.426	1.00	41.73	O
ATOM	1113	OE2	GLU A 123	42.158	0.497	21.940	1.00	100.00	O
ATOM	1114	N	LEU A 124	48.134	-0.185	20.618	1.00	14.02	N
ATOM	1115	CA	LEU A 124	49.296	-0.082	19.740	1.00	15.32	C
ATOM	1116	C	LEU A 124	49.082	-0.754	18.458	1.00	17.76	C
ATOM	1117	O	LEU A 124	48.752	-1.917	18.445	1.00	18.91	O
ATOM	1118	CB	LEU A 124	50.564	-0.680	20.362	1.00	18.07	C
ATOM	1119	CG	LEU A 124	51.922	-0.222	19.803	1.00	21.52	C
ATOM	1120	CD1	LEU A 124	52.080	1.258	20.117	1.00	20.35	C
ATOM	1121	CD2	LEU A 124	53.042	-0.919	20.550	1.00	14.07	C
ATOM	1122	N	LEU A 125	49.514	-0.071	17.409	1.00	18.44	N
ATOM	1123	CA	LEU A 125	49.445	-0.564	16.052	1.00	19.92	C
ATOM	1124	C	LEU A 125	48.034	-0.754	15.509	1.00	25.56	C
ATOM	1125	O	LEU A 125	47.854	-1.188	14.364	1.00	18.26	O
ATOM	1126	CB	LEU A 125	50.355	-1.800	15.840	1.00	20.79	C
ATOM	1127	CG	LEU A 125	51.890	-1.511	15.778	1.00	17.21	C
ATOM	1128	CD1	LEU A 125	52.744	-2.649	16.316	1.00	19.95	C
ATOM	1129	CD2	LEU A 125	52.334	-1.219	14.338	1.00	5.81	C
ATOM	1130	N	GLN A 126	47.027	-0.327	16.276	1.00	21.97	N
ATOM	1131	CA	GLN A 126	45.652	-0.504	15.790	1.00	19.97	C
ATOM	1132	C	GLN A 126	45.213	0.447	14.724	1.00	28.31	C
ATOM	1133	O	GLN A 126	44.076	0.391	14.293	1.00	47.49	O
ATOM	1134	CB	GLN A 126	44.652	-0.404	16.911	1.00	19.87	C

ATOM	1135	CG	GLN A 126	44.949	-1.312	18.048	1.00	18.39	C
ATOM	1136	CD	GLN A 126	44.319	-2.626	17.835	1.00	66.80	C
ATOM	1137	OE1	GLN A 126	44.064	-3.376	18.792	1.00	40.75	O
ATOM	1138	NE2	GLN A 126	44.015	-2.952	16.565	1.00	71.74	N
ATOM	1139	N	GLY A 127	46.080	1.330	14.270	1.00	28.29	N
ATOM	1140	CA	GLY A 127	45.627	2.260	13.252	1.00	23.31	C
ATOM	1141	C	GLY A 127	46.662	3.315	12.953	1.00	22.90	C
ATOM	1142	O	GLY A 127	47.755	3.254	13.474	1.00	25.30	O
ATOM	1143	N	THR A 128	46.311	4.219	12.046	1.00	19.51	N
ATOM	1144	CA	THR A 128	47.149	5.314	11.588	1.00	22.12	C
ATOM	1145	C	THR A 128	47.705	6.219	12.695	1.00	22.60	C
ATOM	1146	O	THR A 128	47.061	6.461	13.731	1.00	18.58	O
ATOM	1147	CB	THR A 128	46.392	6.182	10.544	1.00	35.98	C
ATOM	1148	OG1	THR A 128	46.533	5.594	9.239	1.00	58.05	O
ATOM	1149	CG2	THR A 128	46.942	7.639	10.542	1.00	43.41	C
ATOM	1150	N	LEU A 129	48.907	6.715	12.425	1.00	18.32	N
ATOM	1151	CA	LEU A 129	49.674	7.534	13.356	1.00	16.76	C
ATOM	1152	C	LEU A 129	49.504	8.959	12.967	1.00	4.89	C
ATOM	1153	O	LEU A 129	49.232	9.260	11.814	1.00	16.14	O
ATOM	1154	CB	LEU A 129	51.205	7.191	13.261	1.00	17.91	C
ATOM	1155	CG	LEU A 129	51.769	5.804	13.752	1.00	18.21	C
ATOM	1156	CD1	LEU A 129	53.132	5.379	13.193	1.00	12.12	C
ATOM	1157	CD2	LEU A 129	51.683	5.532	15.251	1.00	3.89	C
ATOM	1158	N	GLU A 130	49.816	9.827	13.917	1.00	10.23	N
ATOM	1159	CA	GLU A 130	49.912	11.268	13.691	1.00	13.22	C
ATOM	1160	C	GLU A 130	51.128	11.544	12.775	1.00	23.44	C
ATOM	1161	O	GLU A 130	52.249	11.162	13.090	1.00	21.23	O
ATOM	1162	CB	GLU A 130	50.150	11.979	15.035	1.00	18.48	C
ATOM	1163	CG	GLU A 130	50.754	13.376	14.886	1.00	77.44	C
ATOM	1164	CD	GLU A 130	49.833	14.328	14.121	1.00	100.00	C
ATOM	1165	OE1	GLU A 130	48.588	14.205	14.340	1.00	36.19	O
ATOM	1166	OE2	GLU A 130	50.347	15.161	13.295	1.00	21.03	O
ATOM	1167	N	PRO A 131	50.920	12.219	11.648	1.00	21.35	N
ATOM	1168	CA	PRO A 131	52.023	12.409	10.731	1.00	14.78	C
ATOM	1169	C	PRO A 131	53.201	13.132	11.265	1.00	14.98	C
ATOM	1170	O	PRO A 131	54.325	12.847	10.853	1.00	20.99	O
ATOM	1171	CB	PRO A 131	51.413	13.154	9.552	1.00	14.76	C
ATOM	1172	CG	PRO A 131	50.071	13.485	9.949	1.00	20.99	C
ATOM	1173	CD	PRO A 131	49.641	12.626	11.047	1.00	17.25	C
ATOM	1174	N	THR A 132	52.986	14.095	12.159	1.00	18.77	N
ATOM	1175	CA	THR A 132	54.131	14.838	12.689	1.00	16.44	C
ATOM	1176	C	THR A 132	55.102	13.951	13.408	1.00	21.91	C
ATOM	1177	O	THR A 132	56.317	14.088	13.234	1.00	24.17	O
ATOM	1178	CB	THR A 132	53.716	15.907	13.606	1.00	23.45	C
ATOM	1179	OG1	THR A 132	52.976	16.883	12.850	1.00	31.15	O

ATOM	1180	CG2	THR	A	132	54.969	16.519	14.341	1.00 9.28	C
ATOM	1181	N	ASN	A	133	54.551	12.970	14.122	1.00 28.59	N
ATOM	1182	CA	ASN	A	133	55.359	12.007	14.875	1.00 26.38	C
ATOM	1183	C	ASN	A	133	55.666	10.682	14.207	1.00 14.85	C
ATOM	1184	O	ASN	A	133	56.446	9.884	14.755	1.00 18.67	O
ATOM	1185	CB	ASN	A	133	54.661	11.699	16.168	1.00 23.70	C
ATOM	1186	CG	ASN	A	133	54.480	12.894	16.968	1.00 50.55	C
ATOM	1187	OD1	ASN	A	133	53.354	13.272	17.252	1.00 40.07	O
ATOM	1188	ND2	ASN	A	133	55.568	13.638	17.163	1.00 40.36	N
ATOM	1189	N	GLU	A	134	55.100	10.469	13.022	1.00 9.98	N
ATOM	1190	CA	GLU	A	134	55.237	9.210	12.365	1.00 9.66	C
ATOM	1191	C	GLU	A	134	56.648	8.530	12.274	1.00 13.86	C
ATOM	1192	O	GLU	A	134	56.814	7.388	12.706	1.00 22.89	O
ATOM	1193	CB	GLU	A	134	54.448	9.200	11.070	1.00 17.55	C
ATOM	1194	CG	GLU	A	134	54.750	7.930	10.227	1.00 20.89	C
ATOM	1195	CD	GLU	A	134	53.926	7.868	8.970	1.00 13.59	C
ATOM	1196	OE1	GLU	A	134	52.678	7.738	9.085	1.00 35.28	O
ATOM	1197	OE2	GLU	A	134	54.497	8.048	7.869	1.00 13.44	O
ATOM	1198	N	PRO	A	135	57.680	9.222	11.789	1.00 15.72	N
ATOM	1199	CA	PRO	A	135	59.014	8.600	11.699	1.00 18.91	C
ATOM	1200	C	PRO	A	135	59.544	8.174	13.073	1.00 18.68	C
ATOM	1201	O	PRO	A	135	60.072	7.069	13.271	1.00 15.69	O
ATOM	1202	CB	PRO	A	135	59.896	9.755	11.169	1.00 13.84	C
ATOM	1203	CG	PRO	A	135	59.036	10.514	10.350	1.00 9.78	C
ATOM	1204	CD	PRO	A	135	57.594	10.395	10.908	1.00 14.43	C
ATOM	1205	N	TYR	A	136	59.449	9.117	13.994	1.00 8.64	N
ATOM	1206	CA	TYR	A	136	59.873	8.915	15.324	1.00 13.27	C
ATOM	1207	C	TYR	A	136	59.056	7.728	15.907	1.00 16.84	C
ATOM	1208	O	TYR	A	136	59.578	6.903	16.658	1.00 12.90	O
ATOM	1209	CB	TYR	A	136	59.604	10.234	16.100	1.00 15.51	C
ATOM	1210	CG	TYR	A	136	59.912	10.168	17.614	1.00 18.26	C
ATOM	1211	CD1	TYR	A	136	61.200	10.062	18.072	1.00 20.53	C
ATOM	1212	CD2	TYR	A	136	58.904	10.150	18.568	1.00 17.38	C
ATOM	1213	CE1	TYR	A	136	61.484	9.959	19.440	1.00 30.44	C
ATOM	1214	CE2	TYR	A	136	59.184	10.084	19.953	1.00 9.85	C
ATOM	1215	CZ	TYR	A	136	60.476	9.949	20.377	1.00 20.65	C
ATOM	1216	OH	TYR	A	136	60.792	9.873	21.734	1.00 24.41	O
ATOM	1217	N	ALA	A	137	57.760	7.687	15.638	1.00 7.19	N
ATOM	1218	CA	ALA	A	137	56.923	6.633	16.227	1.00 12.68	C
ATOM	1219	C	ALA	A	137	57.345	5.265	15.737	1.00 15.21	C
ATOM	1220	O	ALA	A	137	57.425	4.272	16.488	1.00 14.58	O
ATOM	1221	CB	ALA	A	137	55.517	6.849	15.871	1.00 11.40	C
ATOM	1222	N	ILE	A	138	57.567	5.213	14.447	1.00 8.93	N
ATOM	1223	CA	ILE	A	138	57.954	3.971	13.831	1.00 11.77	C
ATOM	1224	C	ILE	A	138	59.246	3.494	14.492	1.00 16.20	C

ATOM	1225	O	ILE A 138	59.307	2.377	14.970	1.00	13.79		O
ATOM	1226	CB	ILE A 138	58.064	4.172	12.316	1.00	17.85		C
ATOM	1227	CG1	ILE A 138	56.680	4.473	11.757	1.00	28.21		C
ATOM	1228	CG2	ILE A 138	58.674	2.986	11.602	1.00	9.81		C
ATOM	1229	CD1	ILE A 138	55.695	3.376	11.970	1.00	18.17		C
ATOM	1230	N	ALA A 139	60.243	4.361	14.625	1.00	11.54		N
ATOM	1231	CA	ALA A 139	61.494	3.937	15.288	1.00	13.22		C
ATOM	1232	C	ALA A 139	61.256	3.364	16.675	1.00	18.73		C
ATOM	1233	O	ALA A 139	61.791	2.318	17.031	1.00	20.44		O
ATOM	1234	CB	ALA A 139	62.434	5.073	15.390	1.00	13.62		C
ATOM	1235	N	LYS A 140	60.397	4.033	17.448	1.00	16.36		N
ATOM	1236	CA	LYS A 140	60.083	3.600	18.815	1.00	15.14		C
ATOM	1237	C	LYS A 140	59.392	2.262	18.824	1.00	15.18		C
ATOM	1238	O	LYS A 140	59.824	1.346	19.475	1.00	21.42		O
ATOM	1239	CB	LYS A 140	59.193	4.606	19.525	1.00	17.86		C
ATOM	1240	CG	LYS A 140	59.925	5.806	20.152	1.00	21.11		C
ATOM	1241	CD	LYS A 140	61.208	5.478	20.958	1.00	16.75		C
ATOM	1242	CE	LYS A 140	61.664	6.735	21.835	1.00	10.06		C
ATOM	1243	NZ	LYS A 140	62.688	6.496	22.921	1.00	14.40		N
ATOM	1244	N	ILE A 141	58.356	2.116	18.027	1.00	11.49		N
ATOM	1245	CA	ILE A 141	57.703	0.828	17.977	1.00	17.92		C
ATOM	1246	C	ILE A 141	58.729	-0.282	17.577	1.00	13.46		C
ATOM	1247	O	ILE A 141	58.730	-1.374	18.148	1.00	13.92		O
ATOM	1248	CB	ILE A 141	56.497	0.925	17.019	1.00	22.59		C
ATOM	1249	CG1	ILE A 141	55.466	1.906	17.557	1.00	17.61		C
ATOM	1250	CG2	ILE A 141	55.863	-0.411	16.700	1.00	10.49		C
ATOM	1251	CD1	ILE A 141	54.530	2.327	16.449	1.00	13.43		C
ATOM	1252	N	ALA A 142	59.637	0.028	16.650	1.00	10.29		N
ATOM	1253	CA	ALA A 142	60.657	-0.931	16.228	1.00	7.15		C
ATOM	1254	C	ALA A 142	61.456	-1.301	17.456	1.00	16.58		C
ATOM	1255	O	ALA A 142	61.839	-2.454	17.621	1.00	13.04		O
ATOM	1256	CB	ALA A 142	61.604	-0.288	15.130	1.00	4.44		C
ATOM	1257	N	GLY A 143	61.703	-0.307	18.316	1.00	9.56		N
ATOM	1258	CA	GLY A 143	62.448	-0.525	19.527	1.00	5.15		C
ATOM	1259	C	GLY A 143	61.770	-1.555	20.430	1.00	16.36		C
ATOM	1260	O	GLY A 143	62.392	-2.482	20.967	1.00	14.11		O
ATOM	1261	N	ILE A 144	60.476	-1.418	20.564	1.00	20.33		N
ATOM	1262	CA	ILE A 144	59.725	-2.314	21.407	1.00	15.35		C
ATOM	1263	C	ILE A 144	59.706	-3.732	20.859	1.00	19.84		C
ATOM	1264	O	ILE A 144	59.836	-4.700	21.608	1.00	17.93		O
ATOM	1265	CB	ILE A 144	58.317	-1.819	21.559	1.00	10.60		C
ATOM	1266	CG1	ILE A 144	58.311	-0.610	22.516	1.00	9.80		C
ATOM	1267	CG2	ILE A 144	57.410	-2.928	22.122	1.00	9.60		C
ATOM	1268	CD1	ILE A 144	57.022	0.076	22.517	1.00	18.32		C
ATOM	1269	N	LYS A 145	59.520	-3.841	19.556	1.00	7.20		N

ATOM	1270	CA	LYS A 145	59.459	-5.139	18.926	1.00	7.64	C
ATOM	1271	C	LYS A 145	60.840	-5.788	18.931	1.00	15.32	C
ATOM	1272	O	LYS A 145	60.923	-6.989	18.981	1.00	14.76	O
ATOM	1273	CB	LYS A 145	58.891	-5.001	17.516	1.00	11.25	C
ATOM	1274	CG	LYS A 145	57.414	-4.581	17.489	1.00	12.13	C
ATOM	1275	CD	LYS A 145	56.642	-5.434	18.495	1.00	25.23	C
ATOM	1276	CE	LYS A 145	55.189	-4.995	18.692	1.00	13.64	C
ATOM	1277	NZ	LYS A 145	54.441	-6.111	19.392	1.00	11.94	N
ATOM	1278	N	LEU A 146	61.934	-5.011	18.986	1.00	26.98	N
ATOM	1279	CA	LEU A 146	63.261	-5.642	19.167	1.00	19.72	C
ATOM	1280	C	LEU A 146	63.262	-6.316	20.542	1.00	18.20	C
ATOM	1281	O	LEU A 146	63.590	-7.511	20.703	1.00	19.86	O
ATOM	1282	CB	LEU A 146	64.398	-4.618	19.150	1.00	13.56	C
ATOM	1283	CG	LEU A 146	64.895	-4.258	17.759	1.00	21.84	C
ATOM	1284	CD1	LEU A 146	65.672	-2.945	17.817	1.00	17.94	C
ATOM	1285	CD2	LEU A 146	65.745	-5.397	17.102	1.00	16.10	C
ATOM	1286	N	CYS A 147	62.931	-5.523	21.548	1.00	7.91	N
ATOM	1287	CA	CYS A 147	62.875	-6.064	22.893	1.00	9.14	C
ATOM	1288	C	CYS A 147	62.072	-7.378	22.945	1.00	22.72	C
ATOM	1289	O	CYS A 147	62.568	-8.401	23.383	1.00	16.90	O
ATOM	1290	CB	CYS A 147	62.232	-5.058	23.809	1.00	12.63	C
ATOM	1291	SG	CYS A 147	63.411	-3.823	24.316	1.00	15.02	S
ATOM	1292	N	GLU A 148	60.823	-7.352	22.508	1.00	20.03	N
ATOM	1293	CA	GLU A 148	60.016	-8.555	22.567	1.00	16.09	C
ATOM	1294	C	GLU A 148	60.685	-9.715	21.802	1.00	22.61	C
ATOM	1295	O	GLU A 148	60.651	-10.888	22.226	1.00	12.05	O
ATOM	1296	CB	GLU A 148	58.597	-8.268	22.046	1.00	14.66	C
ATOM	1297	CG	GLU A 148	57.864	-7.189	22.840	1.00	11.45	C
ATOM	1298	CD	GLU A 148	56.471	-6.821	22.277	1.00	11.75	C
ATOM	1299	OE1	GLU A 148	56.117	-7.055	21.080	1.00	11.65	O
ATOM	1300	OE2	GLU A 148	55.728	-6.231	23.081	1.00	22.56	O
ATOM	1301	N	SER A 149	61.368	-9.377	20.715	1.00	15.57	N
ATOM	1302	CA	SER A 149	61.938	-10.428	19.887	1.00	10.21	C
ATOM	1303	C	SER A 149	63.040	-11.245	20.502	1.00	15.83	C
ATOM	1304	O	SER A 149	63.102	-12.458	20.291	1.00	12.72	O
ATOM	1305	CB	SER A 149	62.270	-9.936	18.488	1.00	9.44	C
ATOM	1306	OG	SER A 149	61.053	-9.650	17.782	1.00	15.91	O
ATOM	1307	N	TYR A 150	63.910	-10.546	21.224	1.00	18.44	N
ATOM	1308	CA	TYR A 150	65.065	-11.100	21.948	1.00	20.50	C
ATOM	1309	C	TYR A 150	64.514	-11.848	23.158	1.00	21.87	C
ATOM	1310	O	TYR A 150	64.939	-12.949	23.486	1.00	31.39	O
ATOM	1311	CB	TYR A 150	66.005	-9.950	22.425	1.00	13.71	C
ATOM	1312	CG	TYR A 150	66.994	-9.509	21.365	1.00	14.13	C
ATOM	1313	CD1	TYR A 150	66.611	-8.673	20.317	1.00	14.64	C
ATOM	1314	CD2	TYR A 150	68.288	-10.000	21.360	1.00	18.32	C

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ATOM   1315  CE1 TYR A 150      67.487  -8.390  19.278  1.00 11.91           C
ATOM   1316  CE2 TYR A 150      69.198  -9.682  20.345  1.00 11.10           C
ATOM   1317  CZ  TYR A 150      68.804  -8.900  19.326  1.00 20.95           C
ATOM   1318  OH  TYR A 150      69.739  -8.685  18.333  1.00 27.73           O
ATOM   1319  N   ASN A 151      63.536 -11.249  23.801  1.00 14.83           N
ATOM   1320  CA  ASN A 151      62.903 -11.889  24.937  1.00 23.62           C
ATOM   1321  C   ASN A 151      62.417 -13.244  24.410  1.00 28.53           C
ATOM   1322  O   ASN A 151      62.630 -14.248  25.072  1.00 25.89           O
ATOM   1323  CB  ASN A 151      61.655 -11.113  25.439  1.00 20.95           C
ATOM   1324  CG  ASN A 151      61.988  -9.867  26.284  1.00 15.07           C
ATOM   1325  OD1 ASN A 151      61.126  -9.020  26.466  1.00 26.72           O
ATOM   1326  ND2 ASN A 151      63.231  -9.709  26.700  1.00  6.31           N
ATOM   1327  N   ARG A 152      61.731 -13.249  23.259  1.00 19.91           N
ATOM   1328  CA  ARG A 152      61.129 -14.465  22.687  1.00 17.62           C
ATOM   1329  C   ARG A 152      62.090 -15.523  22.188  1.00 21.34           C
ATOM   1330  O   ARG A 152      61.959 -16.687  22.542  1.00 15.44           O
ATOM   1331  CB  ARG A 152      60.086 -14.148  21.610  1.00 15.30           C
ATOM   1332  CG  ARG A 152      58.672 -13.754  22.157  1.00 17.22           C
ATOM   1333  CD  ARG A 152      57.652 -13.297  21.049  1.00  9.11           C
ATOM   1334  NE  ARG A 152      57.161 -14.419  20.241  1.00 21.05           N
ATOM   1335  CZ  ARG A 152      57.159 -14.447  18.912  1.00 28.61           C
ATOM   1336  NH1 ARG A 152      57.590 -13.387  18.221  1.00 21.98           N
ATOM   1337  NH2 ARG A 152      56.717 -15.528  18.262  1.00 26.11           N
ATOM   1338  N   GLN A 153      63.098 -15.104  21.434  1.00 16.54           N
ATOM   1339  CA  GLN A 153      64.044 -16.036  20.842  1.00  9.74           C
ATOM   1340  C   GLN A 153      65.082 -16.443  21.807  1.00 16.70           C
ATOM   1341  O   GLN A 153      65.529 -17.545  21.763  1.00 24.35           O
ATOM   1342  CB  GLN A 153      64.789 -15.372  19.714  1.00  8.99           C
ATOM   1343  CG  GLN A 153      65.935 -16.225  19.116  1.00  4.63           C
ATOM   1344  CD  GLN A 153      66.315 -15.637  17.762  1.00 14.17           C
ATOM   1345  OE1 GLN A 153      65.611 -14.763  17.254  1.00 12.53           O
ATOM   1346  NE2 GLN A 153      67.466 -16.024  17.228  1.00 13.38           N
ATOM   1347  N   TYR A 154      65.566 -15.518  22.608  1.00 14.35           N
ATOM   1348  CA  TYR A 154      66.677 -15.839  23.483  1.00 12.16           C
ATOM   1349  C   TYR A 154      66.323 -15.930  24.954  1.00 19.06           C
ATOM   1350  O   TYR A 154      67.185 -16.207  25.777  1.00 25.59           O
ATOM   1351  CB  TYR A 154      67.829 -14.816  23.326  1.00 16.89           C
ATOM   1352  CG  TYR A 154      68.418 -14.733  21.943  1.00 17.53           C
ATOM   1353  CD1 TYR A 154      69.259 -15.726  21.467  1.00 18.91           C
ATOM   1354  CD2 TYR A 154      68.080 -13.712  21.091  1.00 13.97           C
ATOM   1355  CE1 TYR A 154      69.782 -15.686  20.190  1.00 10.98           C
ATOM   1356  CE2 TYR A 154      68.621 -13.639  19.806  1.00 23.81           C
ATOM   1357  CZ  TYR A 154      69.488 -14.634  19.380  1.00 23.08           C
ATOM   1358  OH  TYR A 154      70.002 -14.619  18.118  1.00 23.87           O
ATOM   1359  N   GLY A 155      65.080 -15.686  25.313  1.00 12.08           N
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ATOM	1360	CA	GLY A 155	64.747	-15.702	26.731	1.00	15.80		C
ATOM	1361	C	GLY A 155	65.323	-14.498	27.580	1.00	33.97		C
ATOM	1362	O	GLY A 155	65.491	-14.640	28.789	1.00	25.76		O
ATOM	1363	N	ARG A 156	65.564	-13.318	26.981	1.00	25.91		N
ATOM	1364	CA	ARG A 156	66.066	-12.146	27.734	1.00	14.13		C
ATOM	1365	C	ARG A 156	64.971	-11.486	28.581	1.00	16.23		C
ATOM	1366	O	ARG A 156	63.802	-11.919	28.583	1.00	22.61		O
ATOM	1367	CB	ARG A 156	66.601	-11.124	26.750	1.00	13.16		C
ATOM	1368	CG	ARG A 156	67.875	-11.570	26.099	1.00	15.18		C
ATOM	1369	CD	ARG A 156	68.930	-11.418	27.121	1.00	26.42		C
ATOM	1370	NE	ARG A 156	70.200	-11.912	26.633	1.00	21.25		N
ATOM	1371	CZ	ARG A 156	71.092	-12.555	27.386	1.00	42.25		C
ATOM	1372	NH1	ARG A 156	70.870	-12.795	28.679	1.00	20.02		N
ATOM	1373	NH2	ARG A 156	72.221	-12.966	26.843	1.00	20.88		N
ATOM	1374	N	ASP A 157	65.343	-10.446	29.321	1.00	16.00		N
ATOM	1375	CA	ASP A 157	64.370	-9.749	30.166	1.00	16.20		C
ATOM	1376	C	ASP A 157	64.444	-8.245	29.841	1.00	19.20		C
ATOM	1377	O	ASP A 157	64.865	-7.429	30.650	1.00	10.71		O
ATOM	1378	CB	ASP A 157	64.609	-10.061	31.652	1.00	16.50		C
ATOM	1379	CG	ASP A 157	63.489	-9.560	32.566	1.00	26.45		C
ATOM	1380	OD1	ASP A 157	62.433	-9.060	32.108	1.00	26.82		O
ATOM	1381	OD2	ASP A 157	63.673	-9.653	33.784	1.00	21.88		O
ATOM	1382	N	TYR A 158	64.038	-7.921	28.620	1.00	19.41		N
ATOM	1383	CA	TYR A 158	64.099	-6.564	28.083	1.00	18.96		C
ATOM	1384	C	TYR A 158	62.688	-5.977	28.127	1.00	22.62		C
ATOM	1385	O	TYR A 158	61.854	-6.296	27.282	1.00	10.12		O
ATOM	1386	CB	TYR A 158	64.562	-6.661	26.631	1.00	16.34		C
ATOM	1387	CG	TYR A 158	65.982	-7.166	26.484	1.00	12.04		C
ATOM	1388	CD1	TYR A 158	66.789	-7.415	27.621	1.00	13.76		C
ATOM	1389	CD2	TYR A 158	66.544	-7.349	25.218	1.00	16.35		C
ATOM	1390	CE1	TYR A 158	68.135	-7.786	27.482	1.00	8.18		C
ATOM	1391	CE2	TYR A 158	67.886	-7.732	25.060	1.00	13.73		C
ATOM	1392	CZ	TYR A 158	68.676	-7.942	26.186	1.00	24.45		C
ATOM	1393	OH	TYR A 158	69.993	-8.338	25.997	1.00	14.36		O
ATOM	1394	N	ARG A 159	62.423	-5.200	29.175	1.00	23.53		N
ATOM	1395	CA	ARG A 159	61.105	-4.603	29.483	1.00	21.15		C
ATOM	1396	C	ARG A 159	60.930	-3.172	28.878	1.00	23.55		C
ATOM	1397	O	ARG A 159	61.911	-2.566	28.424	1.00	18.12		O
ATOM	1398	CB	ARG A 159	60.891	-4.608	31.034	1.00	21.68		C
ATOM	1399	CG	ARG A 159	60.986	-6.029	31.722	1.00	16.41		C
ATOM	1400	CD	ARG A 159	61.135	-6.052	33.233	1.00	18.10		C
ATOM	1401	NE	ARG A 159	61.305	-7.402	33.772	1.00	19.25		N
ATOM	1402	CZ	ARG A 159	61.164	-7.720	35.058	1.00	36.67		C
ATOM	1403	NH1	ARG A 159	60.886	-6.776	35.962	1.00	15.32		N
ATOM	1404	NH2	ARG A 159	61.309	-8.986	35.448	1.00	11.79		N

ATOM	1405	N	SER A 160	59.689	-2.661	28.859	1.00	24.44		N
ATOM	1406	CA	SER A 160	59.312	-1.393	28.200	1.00	21.59		C
ATOM	1407	C	SER A 160	58.242	-0.577	28.950	1.00	25.07		C
ATOM	1408	O	SER A 160	57.257	-1.127	29.454	1.00	17.02		O
ATOM	1409	CB	SER A 160	58.719	-1.747	26.797	1.00	13.05		C
ATOM	1410	OG	SER A 160	59.782	-1.897	25.885	1.00	37.57		O
ATOM	1411	N	VAL A 161	58.378	0.742	28.927	1.00	21.01		N
ATOM	1412	CA	VAL A 161	57.369	1.644	29.509	1.00	9.70		C
ATOM	1413	C	VAL A 161	57.068	2.747	28.504	1.00	16.77		C
ATOM	1414	O	VAL A 161	57.955	3.149	27.729	1.00	16.33		O
ATOM	1415	CB	VAL A 161	57.806	2.248	30.862	1.00	17.94		C
ATOM	1416	CG1	VAL A 161	57.873	1.185	31.984	1.00	16.16		C
ATOM	1417	CG2	VAL A 161	59.137	2.992	30.750	1.00	21.10		C
ATOM	1418	N	MET A 162	55.794	3.147	28.443	1.00	22.46		N
ATOM	1419	CA	MET A 162	55.296	4.185	27.513	1.00	19.23		C
ATOM	1420	C	MET A 162	54.880	5.312	28.397	1.00	25.19		C
ATOM	1421	O	MET A 162	53.788	5.269	28.961	1.00	18.35		O
ATOM	1422	CB	MET A 162	53.979	3.796	26.850	1.00	15.55		C
ATOM	1423	CG	MET A 162	54.013	2.630	25.949	1.00	37.79		C
ATOM	1424	SD	MET A 162	54.354	3.100	24.235	1.00	52.07		S
ATOM	1425	CE	MET A 162	56.193	3.134	24.410	1.00	36.30		C
ATOM	1426	N	PRO A 163	55.730	6.313	28.521	1.00	18.43		N
ATOM	1427	CA	PRO A 163	55.390	7.472	29.337	1.00	17.76		C
ATOM	1428	C	PRO A 163	54.300	8.384	28.667	1.00	21.23		C
ATOM	1429	O	PRO A 163	54.208	8.448	27.433	1.00	15.20		O
ATOM	1430	CB	PRO A 163	56.727	8.196	29.423	1.00	11.43		C
ATOM	1431	CG	PRO A 163	57.352	7.874	28.031	1.00	13.99		C
ATOM	1432	CD	PRO A 163	57.086	6.401	27.949	1.00	12.24		C
ATOM	1433	N	THR A 164	53.478	9.060	29.478	1.00	13.95		N
ATOM	1434	CA	THR A 164	52.581	10.121	28.963	1.00	25.82		C
ATOM	1435	C	THR A 164	53.406	11.441	28.781	1.00	19.67		C
ATOM	1436	O	THR A 164	54.633	11.393	28.868	1.00	13.97		O
ATOM	1437	CB	THR A 164	51.373	10.391	29.903	1.00	25.51		C
ATOM	1438	OG1	THR A 164	50.470	11.321	29.267	1.00	14.77		O
ATOM	1439	CG2	THR A 164	51.818	10.886	31.298	1.00	9.06		C
ATOM	1440	N	ASN A 165	52.751	12.589	28.556	1.00	14.99		N
ATOM	1441	CA	ASN A 165	53.448	13.901	28.481	1.00	7.83		C
ATOM	1442	C	ASN A 165	54.167	14.064	29.824	1.00	11.21		C
ATOM	1443	O	ASN A 165	53.554	13.929	30.894	1.00	17.66		O
ATOM	1444	CB	ASN A 165	52.434	15.061	28.416	1.00	14.48		C
ATOM	1445	CG	ASN A 165	51.492	14.941	27.262	1.00	23.70		C
ATOM	1446	OD1	ASN A 165	51.939	14.800	26.129	1.00	22.37		O
ATOM	1447	ND2	ASN A 165	50.173	14.925	27.539	1.00	27.22		N
ATOM	1448	N	LEU A 166	55.418	14.490	29.777	1.00	8.23		N
ATOM	1449	CA	LEU A 166	56.187	14.604	30.994	1.00	14.40		C

ATOM	1450	C	LEU A 166	56.629	16.017	31.120	1.00	25.05	C
ATOM	1451	O	LEU A 166	56.624	16.718	30.125	1.00	25.09	O
ATOM	1452	CB	LEU A 166	57.460	13.743	30.870	1.00	17.48	C
ATOM	1453	CG	LEU A 166	57.423	12.218	30.652	1.00	16.63	C
ATOM	1454	CD1	LEU A 166	58.837	11.639	31.000	1.00	22.52	C
ATOM	1455	CD2	LEU A 166	56.336	11.539	31.514	1.00	7.46	C
ATOM	1456	N	TYR A 167	57.146	16.391	32.300	1.00	19.78	N
ATOM	1457	CA	TYR A 167	57.678	17.760	32.511	1.00	18.58	C
ATOM	1458	C	TYR A 167	58.534	17.763	33.767	1.00	15.53	C
ATOM	1459	O	TYR A 167	58.474	16.852	34.575	1.00	16.71	O
ATOM	1460	CB	TYR A 167	56.509	18.778	32.665	1.00	18.33	C
ATOM	1461	CG	TYR A 167	55.671	18.561	33.931	1.00	14.23	C
ATOM	1462	CD1	TYR A 167	54.624	17.618	33.977	1.00	13.35	C
ATOM	1463	CD2	TYR A 167	55.984	19.258	35.106	1.00	16.52	C
ATOM	1464	CE1	TYR A 167	53.889	17.446	35.146	1.00	21.17	C
ATOM	1465	CE2	TYR A 167	55.302	19.084	36.264	1.00	8.26	C
ATOM	1466	CZ	TYR A 167	54.228	18.203	36.296	1.00	23.56	C
ATOM	1467	OH	TYR A 167	53.526	18.078	37.504	1.00	22.81	O
ATOM	1468	N	GLY A 168	59.334	18.797	33.952	1.00	16.59	N
ATOM	1469	CA	GLY A 168	60.158	18.817	35.152	1.00	18.21	C
ATOM	1470	C	GLY A 168	61.534	19.428	34.880	1.00	13.69	C
ATOM	1471	O	GLY A 168	61.746	20.028	33.837	1.00	16.52	O
ATOM	1472	N	PRO A 169	62.473	19.263	35.817	1.00	20.33	N
ATOM	1473	CA	PRO A 169	63.801	19.822	35.656	1.00	16.07	C
ATOM	1474	C	PRO A 169	64.430	19.353	34.387	1.00	27.18	C
ATOM	1475	O	PRO A 169	64.305	18.186	33.981	1.00	21.23	O
ATOM	1476	CB	PRO A 169	64.595	19.206	36.805	1.00	17.28	C
ATOM	1477	CG	PRO A 169	63.649	18.919	37.830	1.00	19.89	C
ATOM	1478	CD	PRO A 169	62.263	18.772	37.189	1.00	22.47	C
ATOM	1479	N	HIS A 170	65.226	20.235	33.829	1.00	19.48	N
ATOM	1480	CA	HIS A 170	65.952	19.877	32.638	1.00	25.56	C
ATOM	1481	C	HIS A 170	65.096	19.707	31.428	1.00	29.15	C
ATOM	1482	O	HIS A 170	65.553	19.091	30.479	1.00	29.71	O
ATOM	1483	CB	HIS A 170	66.783	18.600	32.845	1.00	28.94	C
ATOM	1484	CG	HIS A 170	67.703	18.671	34.034	1.00	33.88	C
ATOM	1485	ND1	HIS A 170	68.975	19.203	33.969	1.00	25.46	N
ATOM	1486	CD2	HIS A 170	67.518	18.298	35.326	1.00	34.77	C
ATOM	1487	CE1	HIS A 170	69.531	19.151	35.166	1.00	25.63	C
ATOM	1488	NE2	HIS A 170	68.673	18.603	36.008	1.00	31.72	N
ATOM	1489	N	ASP A 171	63.881	20.245	31.440	1.00	21.52	N
ATOM	1490	CA	ASP A 171	63.041	20.267	30.218	1.00	28.63	C
ATOM	1491	C	ASP A 171	63.630	21.459	29.359	1.00	41.94	C
ATOM	1492	O	ASP A 171	64.534	22.171	29.835	1.00	29.69	O
ATOM	1493	CB	ASP A 171	61.552	20.558	30.602	1.00	26.40	C
ATOM	1494	CG	ASP A 171	60.552	20.097	29.540	1.00	22.32	C

ATOM	1495	OD1	ASP	A	171	60.890	20.067	28.325	1.00 32.03	O
ATOM	1496	OD2	ASP	A	171	59.427	19.719	29.916	1.00 42.13	O
ATOM	1497	N	ASN	A	172	63.141	21.712	28.137	1.00 42.08	N
ATOM	1498	CA	ASN	A	172	63.616	22.893	27.388	1.00 35.95	C
ATOM	1499	C	ASN	A	172	62.665	24.056	27.674	1.00 33.71	C
ATOM	1500	O	ASN	A	172	61.586	24.102	27.104	1.00 32.69	O
ATOM	1501	CB	ASN	A	172	63.632	22.667	25.869	1.00 41.60	C
ATOM	1502	CG	ASN	A	172	63.807	23.987	25.086	1.00 39.09	C
ATOM	1503	OD1	ASN	A	172	62.973	24.347	24.259	1.00 83.94	O
ATOM	1504	ND2	ASN	A	172	64.855	24.740	25.418	1.00 65.07	N
ATOM	1505	N	PHE	A	173	63.021	24.953	28.583	1.00 31.93	N
ATOM	1506	CA	PHE	A	173	62.082	26.030	28.944	1.00 48.24	C
ATOM	1507	C	PHE	A	173	61.989	27.260	28.045	1.00 69.01	C
ATOM	1508	O	PHE	A	173	62.278	28.395	28.465	1.00 58.79	O
ATOM	1509	CB	PHE	A	173	62.225	26.459	30.390	1.00 43.43	C
ATOM	1510	CG	PHE	A	173	61.867	25.399	31.356	1.00 34.19	C
ATOM	1511	CD1	PHE	A	173	62.810	24.488	31.751	1.00 24.68	C
ATOM	1512	CD2	PHE	A	173	60.621	25.354	31.925	1.00 24.84	C
ATOM	1513	CE1	PHE	A	173	62.524	23.548	32.682	1.00 23.64	C
ATOM	1514	CE2	PHE	A	173	60.305	24.366	32.804	1.00 31.32	C
ATOM	1515	CZ	PHE	A	173	61.263	23.457	33.192	1.00 24.30	C
ATOM	1516	N	HIS	A	174	61.510	27.036	26.831	1.00 68.16	N
ATOM	1517	CA	HIS	A	174	61.401	28.109	25.871	1.00 64.53	C
ATOM	1518	C	HIS	A	174	59.973	28.221	25.400	1.00 71.58	C
ATOM	1519	O	HIS	A	174	59.309	27.186	25.249	1.00 73.20	O
ATOM	1520	CB	HIS	A	174	62.418	27.870	24.736	1.00 71.71	C
ATOM	1521	CG	HIS	A	174	63.835	27.868	25.229	1.00 92.29	C
ATOM	1522	ND1	HIS	A	174	64.921	27.539	24.440	1.00100.00	N
ATOM	1523	CD2	HIS	A	174	64.338	28.133	26.463	1.00100.00	C
ATOM	1524	CE1	HIS	A	174	66.032	27.628	25.160	1.00100.00	C
ATOM	1525	NE2	HIS	A	174	65.705	27.981	26.393	1.00100.00	N
ATOM	1526	N	PRO	A	175	59.469	29.461	25.262	1.00 65.71	N
ATOM	1527	CA	PRO	A	175	58.109	29.658	24.770	1.00 55.72	C
ATOM	1528	C	PRO	A	175	58.233	29.297	23.267	1.00 75.83	C
ATOM	1529	O	PRO	A	175	57.224	29.226	22.554	1.00 69.59	O
ATOM	1530	CB	PRO	A	175	57.866	31.142	25.026	1.00 49.14	C
ATOM	1531	CG	PRO	A	175	59.258	31.790	24.901	1.00 42.23	C
ATOM	1532	CD	PRO	A	175	60.286	30.695	25.109	1.00 49.59	C
ATOM	1533	N	SER	A	176	59.480	28.954	22.879	1.00 85.09	N
ATOM	1534	CA	SER	A	176	59.954	28.474	21.548	1.00 81.18	C
ATOM	1535	C	SER	A	176	59.660	26.965	21.343	1.00 73.90	C
ATOM	1536	O	SER	A	176	59.617	26.458	20.213	1.00 57.03	O
ATOM	1537	CB	SER	A	176	61.493	28.666	21.447	1.00 71.32	C
ATOM	1538	OG	SER	A	176	62.048	29.349	22.578	1.00 51.93	O
ATOM	1539	N	ASN	A	177	59.520	26.276	22.480	1.00 66.23	N

ATOM	1540	CA	ASN A 177	59.274	24.847	22.619	1.00	56.41	C
ATOM	1541	C	ASN A 177	57.810	24.497	22.353	1.00	60.91	C
ATOM	1542	O	ASN A 177	56.914	25.215	22.811	1.00	55.58	O
ATOM	1543	CB	ASN A 177	59.619	24.469	24.065	1.00	50.45	C
ATOM	1544	CG	ASN A 177	59.562	22.970	24.319	1.00	66.57	C
ATOM	1545	OD1	ASN A 177	59.095	22.216	23.476	1.00	100.00	O
ATOM	1546	ND2	ASN A 177	60.099	22.546	25.464	1.00	35.61	N
ATOM	1547	N	SER A 178	57.583	23.387	21.627	1.00	57.10	N
ATOM	1548	CA	SER A 178	56.234	22.853	21.279	1.00	50.50	C
ATOM	1549	C	SER A 178	55.557	22.159	22.491	1.00	76.24	C
ATOM	1550	O	SER A 178	54.575	21.400	22.304	1.00	99.63	O
ATOM	1551	CB	SER A 178	56.316	21.800	20.118	1.00	10.17	C
ATOM	1552	OG	SER A 178	57.397	22.112	19.217	1.00	71.69	O
ATOM	1553	N	HIS A 179	56.134	22.284	23.694	1.00	37.39	N
ATOM	1554	CA	HIS A 179	55.569	21.587	24.855	1.00	30.96	C
ATOM	1555	C	HIS A 179	54.961	22.616	25.767	1.00	21.93	C
ATOM	1556	O	HIS A 179	55.641	23.598	26.138	1.00	25.17	O
ATOM	1557	CB	HIS A 179	56.634	20.683	25.575	1.00	36.20	C
ATOM	1558	CG	HIS A 179	56.973	19.419	24.835	1.00	42.90	C
ATOM	1559	ND1	HIS A 179	56.973	19.335	23.457	1.00	49.52	N
ATOM	1560	CD2	HIS A 179	57.323	18.190	25.278	1.00	52.42	C
ATOM	1561	CE1	HIS A 179	57.283	18.109	23.084	1.00	44.78	C
ATOM	1562	NE2	HIS A 179	57.500	17.393	24.168	1.00	50.49	N
ATOM	1563	N	VAL A 180	53.661	22.454	26.038	1.00	19.14	N
ATOM	1564	CA	VAL A 180	52.886	23.449	26.789	1.00	29.03	C
ATOM	1565	C	VAL A 180	53.373	23.890	28.142	1.00	31.29	C
ATOM	1566	O	VAL A 180	53.348	25.075	28.447	1.00	19.55	O
ATOM	1567	CB	VAL A 180	51.403	23.115	26.914	1.00	35.47	C
ATOM	1568	CG1	VAL A 180	50.630	24.399	27.217	1.00	35.84	C
ATOM	1569	CG2	VAL A 180	50.923	22.550	25.663	1.00	36.11	C
ATOM	1570	N	ILE A 181	53.684	22.935	29.005	1.00	26.57	N
ATOM	1571	CA	ILE A 181	54.138	23.285	30.360	1.00	24.49	C
ATOM	1572	C	ILE A 181	55.371	24.213	30.361	1.00	16.51	C
ATOM	1573	O	ILE A 181	55.326	25.315	30.909	1.00	24.42	O
ATOM	1574	CB	ILE A 181	54.285	22.018	31.264	1.00	20.20	C
ATOM	1575	CG1	ILE A 181	52.878	21.428	31.528	1.00	18.22	C
ATOM	1576	CG2	ILE A 181	55.014	22.315	32.581	1.00	13.37	C
ATOM	1577	CD1	ILE A 181	52.867	20.086	32.286	1.00	8.03	C
ATOM	1578	N	PRO A 182	56.452	23.779	29.718	1.00	22.21	N
ATOM	1579	CA	PRO A 182	57.664	24.605	29.640	1.00	22.07	C
ATOM	1580	C	PRO A 182	57.379	25.852	28.828	1.00	24.18	C
ATOM	1581	O	PRO A 182	57.811	26.949	29.210	1.00	18.35	O
ATOM	1582	CB	PRO A 182	58.682	23.725	28.890	1.00	24.97	C
ATOM	1583	CG	PRO A 182	57.925	22.473	28.471	1.00	25.77	C
ATOM	1584	CD	PRO A 182	56.727	22.359	29.401	1.00	18.23	C

ATOM	1585	N	ALA A 183	56.628	25.707	27.729	1.00	21.45	N
ATOM	1586	CA	ALA A 183	56.261	26.896	26.943	1.00	21.66	C
ATOM	1587	C	ALA A 183	55.464	27.900	27.811	1.00	26.10	C
ATOM	1588	O	ALA A 183	55.773	29.091	27.856	1.00	19.50	O
ATOM	1589	CB	ALA A 183	55.473	26.513	25.703	1.00	13.26	C
ATOM	1590	N	LEU A 184	54.472	27.389	28.543	1.00	23.34	N
ATOM	1591	CA	LEU A 184	53.642	28.215	29.401	1.00	19.05	C
ATOM	1592	C	LEU A 184	54.312	28.693	30.655	1.00	21.91	C
ATOM	1593	O	LEU A 184	54.017	29.771	31.158	1.00	19.71	O
ATOM	1594	CB	LEU A 184	52.309	27.553	29.715	1.00	14.41	C
ATOM	1595	CG	LEU A 184	51.342	27.595	28.525	1.00	23.42	C
ATOM	1596	CD1	LEU A 184	49.918	27.244	28.928	1.00	31.06	C
ATOM	1597	CD2	LEU A 184	51.380	28.896	27.690	1.00	21.73	C
ATOM	1598	N	LEU A 185	55.178	27.879	31.213	1.00	18.39	N
ATOM	1599	CA	LEU A 185	55.833	28.332	32.417	1.00	16.39	C
ATOM	1600	C	LEU A 185	56.669	29.528	31.985	1.00	23.67	C
ATOM	1601	O	LEU A 185	56.681	30.590	32.644	1.00	29.38	O
ATOM	1602	CB	LEU A 185	56.723	27.233	33.015	1.00	15.05	C
ATOM	1603	CG	LEU A 185	56.021	26.348	34.041	1.00	15.56	C
ATOM	1604	CD1	LEU A 185	56.819	25.022	34.301	1.00	21.06	C
ATOM	1605	CD2	LEU A 185	55.722	27.113	35.321	1.00	11.02	C
ATOM	1606	N	ARG A 186	57.337	29.397	30.852	1.00	17.09	N
ATOM	1607	CA	ARG A 186	58.137	30.523	30.429	1.00	18.82	C
ATOM	1608	C	ARG A 186	57.308	31.752	30.069	1.00	29.00	C
ATOM	1609	O	ARG A 186	57.629	32.880	30.476	1.00	23.91	O
ATOM	1610	CB	ARG A 186	59.026	30.146	29.281	1.00	22.06	C
ATOM	1611	CG	ARG A 186	59.653	31.365	28.652	1.00	38.46	C
ATOM	1612	CD	ARG A 186	60.825	31.804	29.462	1.00	83.66	C
ATOM	1613	NE	ARG A 186	62.012	31.861	28.631	1.00	70.77	N
ATOM	1614	CZ	ARG A 186	63.058	32.622	28.904	1.00	91.68	C
ATOM	1615	NH1	ARG A 186	63.053	33.386	29.995	1.00	56.56	N
ATOM	1616	NH2	ARG A 186	64.098	32.639	28.082	1.00	100.00	N
ATOM	1617	N	ARG A 187	56.234	31.544	29.310	1.00	20.96	N
ATOM	1618	CA	ARG A 187	55.361	32.662	28.941	1.00	19.32	C
ATOM	1619	C	ARG A 187	54.765	33.453	30.142	1.00	28.41	C
ATOM	1620	O	ARG A 187	54.823	34.700	30.193	1.00	17.23	O
ATOM	1621	CB	ARG A 187	54.270	32.223	27.957	1.00	17.05	C
ATOM	1622	CG	ARG A 187	54.813	31.546	26.720	1.00	61.42	C
ATOM	1623	CD	ARG A 187	53.696	31.244	25.757	1.00	44.57	C
ATOM	1624	NE	ARG A 187	53.033	32.472	25.354	1.00	29.47	N
ATOM	1625	CZ	ARG A 187	51.831	32.534	24.790	1.00	17.82	C
ATOM	1626	NH1	ARG A 187	51.136	31.427	24.544	1.00	24.95	N
ATOM	1627	NH2	ARG A 187	51.341	33.716	24.447	1.00	37.77	N
ATOM	1628	N	PHE A 188	54.192	32.734	31.101	1.00	23.48	N
ATOM	1629	CA	PHE A 188	53.604	33.399	32.259	1.00	21.24	C

ATOM	1630	C	PHE A 188	54.638	34.080	33.095	1.00	21.39	C
ATOM	1631	O	PHE A 188	54.394	35.126	33.626	1.00	23.90	O
ATOM	1632	CB	PHE A 188	52.723	32.466	33.077	1.00	19.95	C
ATOM	1633	CG	PHE A 188	51.389	32.215	32.435	1.00	22.28	C
ATOM	1634	CD1	PHE A 188	50.440	33.229	32.375	1.00	19.42	C
ATOM	1635	CD2	PHE A 188	51.144	31.038	31.734	1.00	23.82	C
ATOM	1636	CE1	PHE A 188	49.191	33.026	31.742	1.00	24.77	C
ATOM	1637	CE2	PHE A 188	49.936	30.826	31.057	1.00	20.17	C
ATOM	1638	CZ	PHE A 188	48.945	31.815	31.068	1.00	23.14	C
ATOM	1639	N	HIS A 189	55.831	33.513	33.118	1.00	24.15	N
ATOM	1640	CA	HIS A 189	56.933	34.122	33.837	1.00	28.79	C
ATOM	1641	C	HIS A 189	57.303	35.506	33.315	1.00	28.58	C
ATOM	1642	O	HIS A 189	57.480	36.463	34.083	1.00	20.07	O
ATOM	1643	CB	HIS A 189	58.148	33.268	33.641	1.00	31.38	C
ATOM	1644	CG	HIS A 189	59.364	33.844	34.290	1.00	29.98	C
ATOM	1645	ND1	HIS A 189	59.548	33.833	35.658	1.00	31.00	N
ATOM	1646	CD2	HIS A 189	60.449	34.464	33.766	1.00	21.79	C
ATOM	1647	CE1	HIS A 189	60.722	34.371	35.945	1.00	24.04	C
ATOM	1648	NE2	HIS A 189	61.257	34.815	34.821	1.00	19.53	N
ATOM	1649	N	GLU A 190	57.539	35.561	32.006	1.00	28.43	N
ATOM	1650	CA	GLU A 190	57.876	36.816	31.324	1.00	27.72	C
ATOM	1651	C	GLU A 190	56.725	37.829	31.437	1.00	32.56	C
ATOM	1652	O	GLU A 190	56.949	38.995	31.717	1.00	27.06	O
ATOM	1653	CB	GLU A 190	58.122	36.529	29.849	1.00	28.55	C
ATOM	1654	CG	GLU A 190	59.150	35.461	29.614	1.00	35.29	C
ATOM	1655	CD	GLU A 190	60.553	35.941	29.892	1.00	99.81	C
ATOM	1656	OE1	GLU A 190	60.913	36.037	31.085	1.00	86.56	O
ATOM	1657	OE2	GLU A 190	61.293	36.167	28.910	1.00	100.00	O
ATOM	1658	N	ALA A 191	55.493	37.391	31.196	1.00	32.67	N
ATOM	1659	CA	ALA A 191	54.349	38.286	31.311	1.00	25.30	C
ATOM	1660	C	ALA A 191	54.287	38.795	32.742	1.00	36.20	C
ATOM	1661	O	ALA A 191	53.920	39.924	33.014	1.00	27.52	O
ATOM	1662	CB	ALA A 191	53.055	37.563	31.000	1.00	16.48	C
ATOM	1663	N	THR A 192	54.549	37.927	33.693	1.00	29.39	N
ATOM	1664	CA	THR A 192	54.395	38.386	35.041	1.00	19.08	C
ATOM	1665	C	THR A 192	55.420	39.494	35.298	1.00	44.78	C
ATOM	1666	O	THR A 192	55.094	40.550	35.839	1.00	40.58	O
ATOM	1667	CB	THR A 192	54.515	37.235	35.983	1.00	18.99	C
ATOM	1668	OG1	THR A 192	53.410	36.348	35.755	1.00	34.36	O
ATOM	1669	CG2	THR A 192	54.461	37.738	37.425	1.00	21.15	C
ATOM	1670	N	ALA A 193	56.617	39.312	34.757	1.00	48.58	N
ATOM	1671	CA	ALA A 193	57.705	40.286	34.905	1.00	50.59	C
ATOM	1672	C	ALA A 193	57.496	41.613	34.145	1.00	54.42	C
ATOM	1673	O	ALA A 193	57.952	42.698	34.553	1.00	48.28	O
ATOM	1674	CB	ALA A 193	59.047	39.640	34.496	1.00	51.78	C

ATOM	1675	N	GLN A 194	56.810	41.530	33.022	1.00	43.16	N
ATOM	1676	CA	GLN A 194	56.586	42.722	32.242	1.00	38.03	C
ATOM	1677	C	GLN A 194	55.264	43.389	32.576	1.00	40.85	C
ATOM	1678	O	GLN A 194	54.830	44.284	31.845	1.00	51.20	O
ATOM	1679	CB	GLN A 194	56.599	42.358	30.750	1.00	35.96	C
ATOM	1680	CG	GLN A 194	57.910	41.692	30.290	1.00	100.00	C
ATOM	1681	CD	GLN A 194	57.715	40.661	29.158	1.00	100.00	C
ATOM	1682	OE1	GLN A 194	56.619	40.546	28.579	1.00	100.00	O
ATOM	1683	NE2	GLN A 194	58.782	39.904	28.848	1.00	100.00	N
ATOM	1684	N	GLY A 195	54.583	42.949	33.630	1.00	32.29	N
ATOM	1685	CA	GLY A 195	53.236	43.464	33.864	1.00	36.26	C
ATOM	1686	C	GLY A 195	52.299	43.332	32.593	1.00	45.33	C
ATOM	1687	O	GLY A 195	51.515	44.242	32.346	1.00	45.16	O
ATOM	1688	N	GLY A 196	52.405	42.245	31.788	1.00	36.33	N
ATOM	1689	CA	GLY A 196	51.515	41.965	30.608	1.00	19.06	C
ATOM	1690	C	GLY A 196	50.037	41.958	31.117	1.00	22.49	C
ATOM	1691	O	GLY A 196	49.724	41.479	32.223	1.00	33.09	O
ATOM	1692	N	PRO A 197	49.144	42.657	30.431	1.00	29.22	N
ATOM	1693	CA	PRO A 197	47.790	42.732	30.953	1.00	25.29	C
ATOM	1694	C	PRO A 197	47.091	41.413	30.674	1.00	24.64	C
ATOM	1695	O	PRO A 197	46.192	40.991	31.411	1.00	24.75	O
ATOM	1696	CB	PRO A 197	47.162	43.911	30.176	1.00	26.31	C
ATOM	1697	CG	PRO A 197	48.188	44.407	29.252	1.00	26.56	C
ATOM	1698	CD	PRO A 197	49.307	43.454	29.203	1.00	30.25	C
ATOM	1699	N	ASP A 198	47.572	40.723	29.658	1.00	16.88	N
ATOM	1700	CA	ASP A 198	47.067	39.418	29.405	1.00	21.65	C
ATOM	1701	C	ASP A 198	48.046	38.522	28.677	1.00	31.28	C
ATOM	1702	O	ASP A 198	49.062	38.978	28.172	1.00	34.57	O
ATOM	1703	CB	ASP A 198	45.739	39.507	28.669	1.00	32.80	C
ATOM	1704	CG	ASP A 198	45.868	40.055	27.256	1.00	46.13	C
ATOM	1705	OD1	ASP A 198	46.982	40.230	26.725	1.00	57.45	O
ATOM	1706	OD2	ASP A 198	44.817	40.271	26.640	1.00	67.61	O
ATOM	1707	N	VAL A 199	47.713	37.234	28.614	1.00	38.67	N
ATOM	1708	CA	VAL A 199	48.499	36.226	27.901	1.00	27.79	C
ATOM	1709	C	VAL A 199	47.462	35.469	27.065	1.00	25.88	C
ATOM	1710	O	VAL A 199	46.460	35.023	27.598	1.00	24.22	O
ATOM	1711	CB	VAL A 199	49.163	35.229	28.905	1.00	24.37	C
ATOM	1712	CG1	VAL A 199	49.874	34.047	28.160	1.00	20.28	C
ATOM	1713	CG2	VAL A 199	50.121	35.942	29.835	1.00	22.25	C
ATOM	1714	N	VAL A 200	47.661	35.386	25.757	1.00	23.72	N
ATOM	1715	CA	VAL A 200	46.701	34.694	24.903	1.00	23.99	C
ATOM	1716	C	VAL A 200	47.167	33.286	24.499	1.00	22.85	C
ATOM	1717	O	VAL A 200	48.321	33.108	24.188	1.00	29.77	O
ATOM	1718	CB	VAL A 200	46.358	35.548	23.680	1.00	23.11	C
ATOM	1719	CG1	VAL A 200	45.561	34.737	22.598	1.00	16.25	C

ATOM	1720	CG2	VAL	A	200	45.652	36.823	24.130	1.00	27.86	C
ATOM	1721	N	VAL	A	201	46.296	32.278	24.632	1.00	27.39	N
ATOM	1722	CA	VAL	A	201	46.588	30.893	24.265	1.00	9.63	C
ATOM	1723	C	VAL	A	201	45.653	30.529	23.165	1.00	19.63	C
ATOM	1724	O	VAL	A	201	44.452	30.755	23.312	1.00	17.61	O
ATOM	1725	CB	VAL	A	201	46.306	29.952	25.426	1.00	19.95	C
ATOM	1726	CG1	VAL	A	201	46.703	28.519	25.054	1.00	20.85	C
ATOM	1727	CG2	VAL	A	201	47.086	30.439	26.661	1.00	16.73	C
ATOM	1728	N	TRP	A	202	46.210	30.080	22.030	1.00	14.36	N
ATOM	1729	CA	TRP	A	202	45.422	29.693	20.865	1.00	18.97	C
ATOM	1730	C	TRP	A	202	44.495	28.572	21.313	1.00	36.22	C
ATOM	1731	O	TRP	A	202	44.934	27.694	22.057	1.00	31.46	O
ATOM	1732	CB	TRP	A	202	46.292	29.055	19.823	1.00	19.14	C
ATOM	1733	CG	TRP	A	202	47.243	29.894	19.066	1.00	33.65	C
ATOM	1734	CD1	TRP	A	202	48.391	29.463	18.429	1.00	35.28	C
ATOM	1735	CD2	TRP	A	202	47.126	31.282	18.772	1.00	39.90	C
ATOM	1736	NE1	TRP	A	202	48.941	30.481	17.693	1.00	37.86	N
ATOM	1737	CE2	TRP	A	202	48.228	31.624	17.922	1.00	38.35	C
ATOM	1738	CE3	TRP	A	202	46.206	32.281	19.138	1.00	39.39	C
ATOM	1739	CZ2	TRP	A	202	48.380	32.884	17.367	1.00	36.15	C
ATOM	1740	CZ3	TRP	A	202	46.356	33.542	18.578	1.00	39.60	C
ATOM	1741	CH2	TRP	A	202	47.428	33.828	17.684	1.00	40.99	C
ATOM	1742	N	GLY	A	203	43.245	28.564	20.842	1.00	25.59	N
ATOM	1743	CA	GLY	A	203	42.332	27.483	21.169	1.00	13.09	C
ATOM	1744	C	GLY	A	203	41.260	27.813	22.193	1.00	21.12	C
ATOM	1745	O	GLY	A	203	41.340	28.815	22.886	1.00	22.86	O
ATOM	1746	N	SER	A	204	40.270	26.919	22.262	1.00	16.88	N
ATOM	1747	CA	SER	A	204	39.163	26.979	23.192	1.00	18.36	C
ATOM	1748	C	SER	A	204	39.561	26.664	24.659	1.00	22.07	C
ATOM	1749	O	SER	A	204	38.888	27.096	25.604	1.00	34.39	O
ATOM	1750	CB	SER	A	204	38.053	25.998	22.740	1.00	9.99	C
ATOM	1751	OG	SER	A	204	38.237	24.695	23.291	1.00	16.37	O
ATOM	1752	N	GLY	A	205	40.562	25.813	24.854	1.00	12.42	N
ATOM	1753	CA	GLY	A	205	40.963	25.411	26.208	1.00	11.64	C
ATOM	1754	C	GLY	A	205	40.208	24.178	26.711	1.00	19.49	C
ATOM	1755	O	GLY	A	205	40.422	23.723	27.838	1.00	13.59	O
ATOM	1756	N	THR	A	206	39.292	23.683	25.881	1.00	15.38	N
ATOM	1757	CA	THR	A	206	38.432	22.594	26.281	1.00	10.80	C
ATOM	1758	C	THR	A	206	39.056	21.221	26.154	1.00	26.39	C
ATOM	1759	O	THR	A	206	38.564	20.267	26.737	1.00	23.28	O
ATOM	1760	CB	THR	A	206	37.124	22.562	25.460	1.00	12.86	C
ATOM	1761	OG1	THR	A	206	37.438	22.395	24.082	1.00	13.12	O
ATOM	1762	CG2	THR	A	206	36.348	23.840	25.620	1.00	10.62	C
ATOM	1763	N	PRO	A	207	40.101	21.083	25.354	1.00	21.10	N
ATOM	1764	CA	PRO	A	207	40.658	19.743	25.175	1.00	18.15	C

ATOM	1765	C	PRO A 207	41.316	19.181	26.423	1.00	21.75		C
ATOM	1766	O	PRO A 207	41.951	19.925	27.215	1.00	20.65		O
ATOM	1767	CB	PRO A 207	41.638	19.909	24.013	1.00	17.51		C
ATOM	1768	CG	PRO A 207	41.146	21.213	23.307	1.00	21.45		C
ATOM	1769	CD	PRO A 207	40.698	22.062	24.431	1.00	23.44		C
ATOM	1770	N	MET A 208	41.112	17.876	26.624	1.00	15.60		N
ATOM	1771	CA	MET A 208	41.694	17.167	27.775	1.00	22.94		C
ATOM	1772	C	MET A 208	43.058	16.427	27.579	1.00	21.90		C
ATOM	1773	O	MET A 208	43.248	15.677	26.633	1.00	23.16		O
ATOM	1774	CB	MET A 208	40.645	16.273	28.386	1.00	32.86		C
ATOM	1775	CG	MET A 208	39.630	17.057	29.223	1.00	46.17		C
ATOM	1776	SD	MET A 208	38.301	15.990	29.826	1.00	57.85		S
ATOM	1777	CE	MET A 208	37.999	15.028	28.343	1.00	58.23		C
ATOM	1778	N	ARG A 209	44.022	16.681	28.456	1.00	17.75		N
ATOM	1779	CA	ARG A 209	45.318	16.042	28.324	1.00	19.88		C
ATOM	1780	C	ARG A 209	45.871	15.534	29.639	1.00	16.92		C
ATOM	1781	O	ARG A 209	45.433	15.946	30.697	1.00	16.58		O
ATOM	1782	CB	ARG A 209	46.340	16.963	27.658	1.00	21.07		C
ATOM	1783	CG	ARG A 209	45.980	17.478	26.275	1.00	22.57		C
ATOM	1784	CD	ARG A 209	45.833	16.357	25.282	1.00	28.26		C
ATOM	1785	NE	ARG A 209	45.586	16.819	23.906	1.00	23.15		N
ATOM	1786	CZ	ARG A 209	44.420	16.742	23.267	1.00	34.52		C
ATOM	1787	NH1	ARG A 209	43.336	16.267	23.890	1.00	18.03		N
ATOM	1788	NH2	ARG A 209	44.339	17.175	22.012	1.00	29.78		N
ATOM	1789	N	GLU A 210	46.878	14.675	29.547	1.00	20.87		N
ATOM	1790	CA	GLU A 210	47.530	14.079	30.720	1.00	17.37		C
ATOM	1791	C	GLU A 210	49.031	14.490	30.851	1.00	20.96		C
ATOM	1792	O	GLU A 210	49.748	14.622	29.841	1.00	22.44		O
ATOM	1793	CB	GLU A 210	47.400	12.562	30.571	1.00	16.26		C
ATOM	1794	CG	GLU A 210	47.807	11.785	31.809	1.00	19.91		C
ATOM	1795	CD	GLU A 210	48.057	10.304	31.531	1.00	27.81		C
ATOM	1796	OE1	GLU A 210	48.111	9.919	30.343	1.00	17.29		O
ATOM	1797	OE2	GLU A 210	48.268	9.540	32.494	1.00	21.63		O
ATOM	1798	N	PHE A 211	49.504	14.712	32.084	1.00	14.02		N
ATOM	1799	CA	PHE A 211	50.887	15.159	32.353	1.00	17.48		C
ATOM	1800	C	PHE A 211	51.458	14.414	33.531	1.00	33.62		C
ATOM	1801	O	PHE A 211	50.716	14.031	34.443	1.00	27.96		O
ATOM	1802	CB	PHE A 211	50.933	16.677	32.644	1.00	17.78		C
ATOM	1803	CG	PHE A 211	50.303	17.490	31.541	1.00	21.49		C
ATOM	1804	CD1	PHE A 211	51.009	17.676	30.320	1.00	17.36		C
ATOM	1805	CD2	PHE A 211	48.933	17.844	31.618	1.00	15.09		C
ATOM	1806	CE1	PHE A 211	50.399	18.334	29.237	1.00	16.37		C
ATOM	1807	CE2	PHE A 211	48.288	18.491	30.533	1.00	9.61		C
ATOM	1808	CZ	PHE A 211	49.053	18.756	29.344	1.00	12.71		C
ATOM	1809	N	LEU A 212	52.761	14.161	33.495	1.00	23.76		N

ATOM	1810	CA	LEU	A	212	53.405	13.448	34.603	1.00 21.24	C
ATOM	1811	C	LEU	A	212	54.772	14.053	34.898	1.00 14.00	C
ATOM	1812	O	LEU	A	212	55.519	14.398	33.985	1.00 13.99	O
ATOM	1813	CB	LEU	A	212	53.548	11.954	34.294	1.00 21.52	C
ATOM	1814	CG	LEU	A	212	54.033	11.039	35.406	1.00 21.09	C
ATOM	1815	CD1	LEU	A	212	52.866	10.634	36.280	1.00 20.84	C
ATOM	1816	CD2	LEU	A	212	54.768	9.829	34.832	1.00 13.18	C
ATOM	1817	N	HIS	A	213	55.023	14.302	36.175	1.00 9.60	N
ATOM	1818	CA	HIS	A	213	56.290	14.864	36.555	1.00 13.66	C
ATOM	1819	C	HIS	A	213	57.380	13.828	36.293	1.00 20.37	C
ATOM	1820	O	HIS	A	213	57.238	12.614	36.542	1.00 16.08	O
ATOM	1821	CB	HIS	A	213	56.280	15.250	38.002	1.00 18.72	C
ATOM	1822	CG	HIS	A	213	57.491	16.017	38.408	1.00 21.22	C
ATOM	1823	ND1	HIS	A	213	58.703	15.406	38.656	1.00 24.29	N
ATOM	1824	CD2	HIS	A	213	57.716	17.353	38.499	1.00 23.67	C
ATOM	1825	CE1	HIS	A	213	59.615	16.331	38.917	1.00 19.13	C
ATOM	1826	NE2	HIS	A	213	59.041	17.523	38.847	1.00 21.99	N
ATOM	1827	N	VAL	A	214	58.459	14.295	35.698	1.00 21.07	N
ATOM	1828	CA	VAL	A	214	59.532	13.383	35.361	1.00 19.23	C
ATOM	1829	C	VAL	A	214	60.067	12.523	36.551	1.00 27.20	C
ATOM	1830	O	VAL	A	214	60.604	11.444	36.359	1.00 22.23	O
ATOM	1831	CB	VAL	A	214	60.625	14.125	34.566	1.00 11.84	C
ATOM	1832	CG1	VAL	A	214	61.390	15.199	35.485	1.00 8.52	C
ATOM	1833	CG2	VAL	A	214	61.560	13.097	33.902	1.00 12.39	C
ATOM	1834	N	ASP	A	215	59.893	12.984	37.790	1.00 25.29	N
ATOM	1835	CA	ASP	A	215	60.406	12.228	38.936	1.00 18.19	C
ATOM	1836	C	ASP	A	215	59.530	11.023	39.230	1.00 13.85	C
ATOM	1837	O	ASP	A	215	59.988	9.981	39.666	1.00 17.44	O
ATOM	1838	CB	ASP	A	215	60.575	13.129	40.155	1.00 16.27	C
ATOM	1839	CG	ASP	A	215	61.859	13.979	40.068	1.00 30.73	C
ATOM	1840	OD1	ASP	A	215	62.782	13.614	39.308	1.00 23.02	O
ATOM	1841	OD2	ASP	A	215	61.957	15.029	40.730	1.00 26.00	O
ATOM	1842	N	ASP	A	216	58.276	11.136	38.863	1.00 20.08	N
ATOM	1843	CA	ASP	A	216	57.378	10.017	39.016	1.00 18.78	C
ATOM	1844	C	ASP	A	216	57.761	9.083	37.894	1.00 23.56	C
ATOM	1845	O	ASP	A	216	57.715	7.880	38.026	1.00 20.79	O
ATOM	1846	CB	ASP	A	216	55.912	10.457	38.821	1.00 17.18	C
ATOM	1847	CG	ASP	A	216	55.193	10.757	40.162	1.00 38.03	C
ATOM	1848	OD1	ASP	A	216	55.503	10.119	41.223	1.00 26.02	O
ATOM	1849	OD2	ASP	A	216	54.249	11.587	40.124	1.00 25.41	O
ATOM	1850	N	MET	A	217	58.092	9.653	36.755	1.00 18.11	N
ATOM	1851	CA	MET	A	217	58.394	8.785	35.636	1.00 22.41	C
ATOM	1852	C	MET	A	217	59.572	7.942	35.992	1.00 27.54	C
ATOM	1853	O	MET	A	217	59.579	6.752	35.710	1.00 20.86	O
ATOM	1854	CB	MET	A	217	58.637	9.592	34.345	1.00 21.24	C

ATOM	1855	CG	MET	A	217	59.478	8.918	33.287	1.00 16.37	C
ATOM	1856	SD	MET	A	217	58.962	7.412	32.473	1.00 30.51	S
ATOM	1857	CE	MET	A	217	57.465	7.608	32.391	1.00 19.57	C
ATOM	1858	N	ALA	A	218	60.561	8.562	36.623	1.00 19.09	N
ATOM	1859	CA	ALA	A	218	61.774	7.841	37.002	1.00 13.65	C
ATOM	1860	C	ALA	A	218	61.436	6.778	38.028	1.00 22.61	C
ATOM	1861	O	ALA	A	218	61.934	5.670	37.967	1.00 19.36	O
ATOM	1862	CB	ALA	A	218	62.809	8.780	37.579	1.00 12.23	C
ATOM	1863	N	ALA	A	219	60.605	7.109	39.000	1.00 19.34	N
ATOM	1864	CA	ALA	A	219	60.310	6.105	40.023	1.00 18.01	C
ATOM	1865	C	ALA	A	219	59.630	4.901	39.413	1.00 23.57	C
ATOM	1866	O	ALA	A	219	59.781	3.777	39.898	1.00 22.71	O
ATOM	1867	CB	ALA	A	219	59.387	6.678	41.083	1.00 10.11	C
ATOM	1868	N	ALA	A	220	58.753	5.174	38.454	1.00 18.99	N
ATOM	1869	CA	ALA	A	220	57.905	4.158	37.855	1.00 14.12	C
ATOM	1870	C	ALA	A	220	58.753	3.213	37.034	1.00 25.33	C
ATOM	1871	O	ALA	A	220	58.584	2.006	37.114	1.00 20.63	O
ATOM	1872	CB	ALA	A	220	56.796	4.798	37.023	1.00 8.53	C
ATOM	1873	N	SER	A	221	59.770	3.772	36.379	1.00 23.92	N
ATOM	1874	CA	SER	A	221	60.702	3.011	35.556	1.00 18.38	C
ATOM	1875	C	SER	A	221	61.537	1.989	36.353	1.00 20.90	C
ATOM	1876	O	SER	A	221	61.683	0.799	35.983	1.00 19.84	O
ATOM	1877	CB	SER	A	221	61.604	3.985	34.804	1.00 10.67	C
ATOM	1878	OG	SER	A	221	60.847	4.744	33.867	1.00 15.61	O
ATOM	1879	N	ILE	A	222	62.083	2.476	37.463	1.00 18.12	N
ATOM	1880	CA	ILE	A	222	62.866	1.644	38.381	1.00 21.56	C
ATOM	1881	C	ILE	A	222	62.020	0.554	39.068	1.00 29.10	C
ATOM	1882	O	ILE	A	222	62.504	-0.566	39.307	1.00 19.03	O
ATOM	1883	CB	ILE	A	222	63.467	2.516	39.432	1.00 24.56	C
ATOM	1884	CG1	ILE	A	222	64.465	3.473	38.765	1.00 32.13	C
ATOM	1885	CG2	ILE	A	222	64.129	1.671	40.500	1.00 28.26	C
ATOM	1886	CD1	ILE	A	222	64.973	4.585	39.649	1.00 15.61	C
ATOM	1887	N	HIS	A	223	60.772	0.907	39.384	1.00 19.34	N
ATOM	1888	CA	HIS	A	223	59.829	-0.031	39.996	1.00 20.46	C
ATOM	1889	C	HIS	A	223	59.599	-1.097	38.964	1.00 24.82	C
ATOM	1890	O	HIS	A	223	59.723	-2.283	39.270	1.00 24.66	O
ATOM	1891	CB	HIS	A	223	58.465	0.637	40.359	1.00 19.53	C
ATOM	1892	CG	HIS	A	223	57.373	-0.333	40.759	1.00 28.64	C
ATOM	1893	ND1	HIS	A	223	57.021	-0.564	42.082	1.00 24.16	N
ATOM	1894	CD2	HIS	A	223	56.497	-1.062	40.004	1.00 30.39	C
ATOM	1895	CE1	HIS	A	223	55.983	-1.399	42.112	1.00 30.39	C
ATOM	1896	NE2	HIS	A	223	55.652	-1.727	40.869	1.00 28.13	N
ATOM	1897	N	VAL	A	224	59.354	-0.684	37.725	1.00 22.06	N
ATOM	1898	CA	VAL	A	224	59.111	-1.657	36.652	1.00 19.15	C
ATOM	1899	C	VAL	A	224	60.350	-2.490	36.333	1.00 25.89	C

ATOM	1900	O	VAL A 224	60.282	-3.709	36.250	1.00	22.37	O
ATOM	1901	CB	VAL A 224	58.559	-1.022	35.377	1.00	22.59	C
ATOM	1902	CG1	VAL A 224	58.512	-2.050	34.231	1.00	22.61	C
ATOM	1903	CG2	VAL A 224	57.161	-0.491	35.650	1.00	23.44	C
ATOM	1904	N	MET A 225	61.499	-1.838	36.255	1.00	27.83	N
ATOM	1905	CA	MET A 225	62.710	-2.577	36.004	1.00	23.69	C
ATOM	1906	C	MET A 225	62.896	-3.678	37.071	1.00	31.95	C
ATOM	1907	O	MET A 225	63.290	-4.805	36.785	1.00	24.33	O
ATOM	1908	CB	MET A 225	63.902	-1.604	36.056	1.00	21.34	C
ATOM	1909	CG	MET A 225	65.295	-2.296	35.999	1.00	17.83	C
ATOM	1910	SD	MET A 225	65.750	-2.958	34.306	1.00	23.33	S
ATOM	1911	CE	MET A 225	67.080	-1.896	33.785	1.00	16.46	C
ATOM	1912	N	GLU A 226	62.644	-3.319	38.316	1.00	19.54	N
ATOM	1913	CA	GLU A 226	62.988	-4.161	39.428	1.00	21.58	C
ATOM	1914	C	GLU A 226	61.999	-5.200	39.918	1.00	30.77	C
ATOM	1915	O	GLU A 226	62.308	-6.012	40.780	1.00	29.39	O
ATOM	1916	CB	GLU A 226	63.613	-3.323	40.547	1.00	20.47	C
ATOM	1917	CG	GLU A 226	64.937	-2.673	40.122	1.00	23.03	C
ATOM	1918	CD	GLU A 226	65.504	-1.809	41.208	1.00	32.62	C
ATOM	1919	OE1	GLU A 226	64.721	-1.455	42.122	1.00	26.12	O
ATOM	1920	OE2	GLU A 226	66.711	-1.479	41.152	1.00	17.67	O
ATOM	1921	N	LEU A 227	60.837	-5.248	39.295	1.00	34.11	N
ATOM	1922	CA	LEU A 227	59.883	-6.296	39.642	1.00	35.26	C
ATOM	1923	C	LEU A 227	60.537	-7.644	39.320	1.00	27.91	C
ATOM	1924	O	LEU A 227	61.291	-7.766	38.340	1.00	19.89	O
ATOM	1925	CB	LEU A 227	58.693	-6.236	38.678	1.00	36.48	C
ATOM	1926	CG	LEU A 227	57.381	-5.569	38.955	1.00	40.30	C
ATOM	1927	CD1	LEU A 227	57.697	-4.194	39.382	1.00	42.04	C
ATOM	1928	CD2	LEU A 227	56.610	-5.577	37.647	1.00	46.21	C
ATOM	1929	N	ALA A 228	60.026	-8.688	39.955	1.00	27.15	N
ATOM	1930	CA	ALA A 228	60.425	-10.051	39.616	1.00	25.26	C
ATOM	1931	C	ALA A 228	59.801	-10.435	38.279	1.00	27.93	C
ATOM	1932	O	ALA A 228	58.624	-10.093	37.934	1.00	31.26	O
ATOM	1933	CB	ALA A 228	60.003	-11.052	40.703	1.00	22.05	C
ATOM	1934	N	HIS A 229	60.624	-11.160	37.539	1.00	27.05	N
ATOM	1935	CA	HIS A 229	60.275	-11.605	36.222	1.00	24.42	C
ATOM	1936	C	HIS A 229	58.905	-12.260	36.184	1.00	21.74	C
ATOM	1937	O	HIS A 229	58.015	-11.851	35.398	1.00	22.22	O
ATOM	1938	CB	HIS A 229	61.351	-12.520	35.698	1.00	17.71	C
ATOM	1939	CG	HIS A 229	61.284	-12.701	34.220	1.00	27.24	C
ATOM	1940	ND1	HIS A 229	61.060	-11.650	33.350	1.00	34.38	N
ATOM	1941	CD2	HIS A 229	61.292	-13.821	33.465	1.00	31.45	C
ATOM	1942	CE1	HIS A 229	60.992	-12.113	32.115	1.00	30.50	C
ATOM	1943	NE2	HIS A 229	61.124	-13.427	32.159	1.00	35.23	N
ATOM	1944	N	GLU A 230	58.681	-13.161	37.140	1.00	20.24	N

ATOM	1945	CA	GLU A 230	57.425	-13.895	37.209	1.00	29.41	C
ATOM	1946	C	GLU A 230	56.181	-13.051	37.341	1.00	22.20	C
ATOM	1947	O	GLU A 230	55.159	-13.359	36.679	1.00	17.78	O
ATOM	1948	CB	GLU A 230	57.464	-14.997	38.274	1.00	38.51	C
ATOM	1949	CG	GLU A 230	58.085	-14.582	39.567	1.00	63.09	C
ATOM	1950	CD	GLU A 230	57.036	-14.473	40.661	1.00	100.00	C
ATOM	1951	OE1	GLU A 230	55.859	-14.872	40.400	1.00	100.00	O
ATOM	1952	OE2	GLU A 230	57.409	-14.003	41.768	1.00	81.48	O
ATOM	1953	N	VAL A 231	56.272	-12.004	38.182	1.00	16.53	N
ATOM	1954	CA	VAL A 231	55.202	-11.029	38.356	1.00	20.23	C
ATOM	1955	C	VAL A 231	55.009	-10.164	37.102	1.00	24.45	C
ATOM	1956	O	VAL A 231	53.864	-9.834	36.705	1.00	21.00	O
ATOM	1957	CB	VAL A 231	55.541	-10.057	39.426	1.00	28.61	C
ATOM	1958	CG1	VAL A 231	54.362	-9.098	39.610	1.00	29.78	C
ATOM	1959	CG2	VAL A 231	55.881	-10.757	40.677	1.00	28.96	C
ATOM	1960	N	TRP A 232	56.133	-9.798	36.486	1.00	17.17	N
ATOM	1961	CA	TRP A 232	56.052	-9.044	35.262	1.00	21.52	C
ATOM	1962	C	TRP A 232	55.388	-9.844	34.156	1.00	20.53	C
ATOM	1963	O	TRP A 232	54.588	-9.306	33.380	1.00	24.31	O
ATOM	1964	CB	TRP A 232	57.438	-8.644	34.801	1.00	29.88	C
ATOM	1965	CG	TRP A 232	57.430	-7.843	33.500	1.00	27.65	C
ATOM	1966	CD1	TRP A 232	57.184	-6.464	33.356	1.00	25.42	C
ATOM	1967	CD2	TRP A 232	57.714	-8.336	32.169	1.00	27.75	C
ATOM	1968	NE1	TRP A 232	57.325	-6.095	32.033	1.00	22.53	N
ATOM	1969	CE2	TRP A 232	57.655	-7.203	31.279	1.00	25.11	C
ATOM	1970	CE3	TRP A 232	58.037	-9.603	31.640	1.00	22.72	C
ATOM	1971	CZ2	TRP A 232	57.917	-7.316	29.879	1.00	17.23	C
ATOM	1972	CZ3	TRP A 232	58.238	-9.720	30.223	1.00	25.97	C
ATOM	1973	CH2	TRP A 232	58.154	-8.581	29.368	1.00	22.07	C
ATOM	1974	N	LEU A 233	55.749	-11.121	34.018	1.00	23.80	N
ATOM	1975	CA	LEU A 233	55.141	-11.949	32.937	1.00	24.78	C
ATOM	1976	C	LEU A 233	53.652	-12.118	33.122	1.00	24.51	C
ATOM	1977	O	LEU A 233	52.865	-12.075	32.163	1.00	28.50	O
ATOM	1978	CB	LEU A 233	55.765	-13.348	32.820	1.00	26.20	C
ATOM	1979	CG	LEU A 233	57.250	-13.505	32.503	1.00	19.39	C
ATOM	1980	CD1	LEU A 233	57.745	-14.850	33.023	1.00	19.90	C
ATOM	1981	CD2	LEU A 233	57.561	-13.287	31.017	1.00	16.01	C
ATOM	1982	N	GLU A 234	53.298	-12.343	34.372	1.00	25.45	N
ATOM	1983	CA	GLU A 234	51.929	-12.523	34.822	1.00	30.04	C
ATOM	1984	C	GLU A 234	51.128	-11.319	34.367	1.00	35.69	C
ATOM	1985	O	GLU A 234	49.926	-11.390	34.052	1.00	28.25	O
ATOM	1986	CB	GLU A 234	52.007	-12.468	36.344	1.00	37.30	C
ATOM	1987	CG	GLU A 234	50.908	-13.133	37.118	1.00	45.39	C
ATOM	1988	CD	GLU A 234	51.112	-12.881	38.601	1.00	100.00	C
ATOM	1989	OE1	GLU A 234	52.240	-13.137	39.104	1.00	99.09	O

ATOM	1990	OE2	GLU	A	234	50.211	-12.257	39.211	1.00100.00	O
ATOM	1991	N	ASN	A	235	51.802	-10.184	34.364	1.00 25.04	N
ATOM	1992	CA	ASN	A	235	51.109	-8.986	33.992	1.00 26.17	C
ATOM	1993	C	ASN	A	235	51.280	-8.494	32.571	1.00 30.46	C
ATOM	1994	O	ASN	A	235	50.824	-7.393	32.259	1.00 22.90	O
ATOM	1995	CB	ASN	A	235	51.427	-7.895	34.981	1.00 29.23	C
ATOM	1996	CG	ASN	A	235	50.878	-8.197	36.342	1.00 39.27	C
ATOM	1997	OD1	ASN	A	235	49.722	-7.882	36.628	1.00 29.06	O
ATOM	1998	ND2	ASN	A	235	51.653	-8.934	37.140	1.00 40.22	N
ATOM	1999	N	THR	A	236	51.935	-9.268	31.708	1.00 20.97	N
ATOM	2000	CA	THR	A	236	52.108	-8.795	30.344	1.00 22.30	C
ATOM	2001	C	THR	A	236	51.867	-9.943	29.419	1.00 29.74	C
ATOM	2002	O	THR	A	236	51.551	-11.033	29.895	1.00 21.23	O
ATOM	2003	CB	THR	A	236	53.545	-8.306	30.161	1.00 22.73	C
ATOM	2004	OG1	THR	A	236	54.422	-9.325	30.636	1.00 21.23	O
ATOM	2005	CG2	THR	A	236	53.801	-7.048	31.041	1.00 19.69	C
ATOM	2006	N	GLN	A	237	52.003	-9.699	28.109	1.00 22.23	N
ATOM	2007	CA	GLN	A	237	52.097	-10.783	27.122	1.00 16.69	C
ATOM	2008	C	GLN	A	237	53.335	-10.507	26.331	1.00 21.02	C
ATOM	2009	O	GLN	A	237	53.729	-9.362	26.204	1.00 22.19	O
ATOM	2010	CB	GLN	A	237	50.913	-10.999	26.189	1.00 8.23	C
ATOM	2011	CG	GLN	A	237	49.639	-11.096	26.904	1.00 21.04	C
ATOM	2012	CD	GLN	A	237	48.907	-9.862	26.606	1.00 62.07	C
ATOM	2013	OE1	GLN	A	237	48.437	-9.712	25.460	1.00 59.32	O
ATOM	2014	NE2	GLN	A	237	49.220	-8.847	27.388	1.00 37.82	N
ATOM	2015	N	PRO	A	238	54.002	-11.579	25.917	1.00 28.76	N
ATOM	2016	CA	PRO	A	238	55.275	-11.438	25.246	1.00 30.28	C
ATOM	2017	C	PRO	A	238	55.194	-10.643	23.958	1.00 29.08	C
ATOM	2018	O	PRO	A	238	56.181	-10.029	23.600	1.00 15.95	O
ATOM	2019	CB	PRO	A	238	55.733	-12.879	25.011	1.00 22.54	C
ATOM	2020	CG	PRO	A	238	54.898	-13.710	25.886	1.00 18.92	C
ATOM	2021	CD	PRO	A	238	53.626	-12.998	26.068	1.00 11.75	C
ATOM	2022	N	MET	A	239	54.041	-10.635	23.286	1.00 17.26	N
ATOM	2023	CA	MET	A	239	53.924	-9.807	22.104	1.00 17.85	C
ATOM	2024	C	MET	A	239	53.109	-8.509	22.362	1.00 18.63	C
ATOM	2025	O	MET	A	239	52.792	-7.741	21.419	1.00 16.82	O
ATOM	2026	CB	MET	A	239	53.460	-10.588	20.881	1.00 15.22	C
ATOM	2027	CG	MET	A	239	54.536	-11.534	20.261	1.00 12.90	C
ATOM	2028	SD	MET	A	239	53.994	-12.534	18.808	1.00 17.49	S
ATOM	2029	CE	MET	A	239	54.350	-11.357	17.422	1.00 13.12	C
ATOM	2030	N	LEU	A	240	52.847	-8.252	23.646	1.00 18.55	N
ATOM	2031	CA	LEU	A	240	52.159	-7.037	24.131	1.00 16.68	C
ATOM	2032	C	LEU	A	240	52.774	-6.733	25.493	1.00 11.82	C
ATOM	2033	O	LEU	A	240	52.124	-6.803	26.549	1.00 13.84	O
ATOM	2034	CB	LEU	A	240	50.645	-7.249	24.240	1.00 16.91	C

ATOM	2035	CG	LEU A 240	49.646	-6.120	23.852	1.00	22.29	C
ATOM	2036	CD1	LEU A 240	48.968	-5.488	25.033	1.00	25.51	C
ATOM	2037	CD2	LEU A 240	50.070	-5.059	22.815	1.00	28.07	C
ATOM	2038	N	SER A 241	54.076	-6.467	25.456	1.00	13.09	N
ATOM	2039	CA	SER A 241	54.842	-6.315	26.682	1.00	24.20	C
ATOM	2040	C	SER A 241	54.947	-4.938	27.377	1.00	30.52	C
ATOM	2041	O	SER A 241	55.363	-4.854	28.547	1.00	17.02	O
ATOM	2042	CB	SER A 241	56.247	-6.900	26.495	1.00	14.04	C
ATOM	2043	OG	SER A 241	57.062	-6.144	25.598	1.00	13.95	O
ATOM	2044	N	HIS A 242	54.661	-3.861	26.659	1.00	17.87	N
ATOM	2045	CA	HIS A 242	54.894	-2.548	27.221	1.00	13.55	C
ATOM	2046	C	HIS A 242	53.990	-2.254	28.373	1.00	13.70	C
ATOM	2047	O	HIS A 242	52.974	-2.885	28.539	1.00	13.29	O
ATOM	2048	CB	HIS A 242	54.826	-1.430	26.130	1.00	16.05	C
ATOM	2049	CG	HIS A 242	53.595	-1.504	25.272	1.00	18.88	C
ATOM	2050	ND1	HIS A 242	52.591	-0.553	25.326	1.00	23.24	N
ATOM	2051	CD2	HIS A 242	53.165	-2.461	24.413	1.00	13.19	C
ATOM	2052	CE1	HIS A 242	51.629	-0.887	24.483	1.00	17.44	C
ATOM	2053	NE2	HIS A 242	51.962	-2.031	23.901	1.00	19.54	N
ATOM	2054	N	ILE A 243	54.310	-1.203	29.095	1.00	15.84	N
ATOM	2055	CA	ILE A 243	53.492	-0.809	30.192	1.00	19.10	C
ATOM	2056	C	ILE A 243	53.336	0.714	30.191	1.00	23.23	C
ATOM	2057	O	ILE A 243	54.312	1.406	30.385	1.00	12.10	O
ATOM	2058	CB	ILE A 243	54.166	-1.273	31.482	1.00	24.62	C
ATOM	2059	CG1	ILE A 243	54.014	-2.783	31.576	1.00	25.60	C
ATOM	2060	CG2	ILE A 243	53.497	-0.665	32.735	1.00	17.37	C
ATOM	2061	CD1	ILE A 243	54.725	-3.365	32.714	1.00	14.82	C
ATOM	2062	N	ASN A 244	52.112	1.217	30.013	1.00	16.43	N
ATOM	2063	CA	ASN A 244	51.824	2.689	30.038	1.00	18.99	C
ATOM	2064	C	ASN A 244	52.252	3.292	31.348	1.00	18.83	C
ATOM	2065	O	ASN A 244	51.965	2.727	32.405	1.00	19.58	O
ATOM	2066	CB	ASN A 244	50.304	2.987	29.910	1.00	15.67	C
ATOM	2067	CG	ASN A 244	49.768	2.702	28.517	1.00	14.57	C
ATOM	2068	OD1	ASN A 244	50.546	2.583	27.580	1.00	13.64	O
ATOM	2069	ND2	ASN A 244	48.443	2.491	28.393	1.00	10.16	N
ATOM	2070	N	VAL A 245	52.800	4.499	31.326	1.00	13.50	N
ATOM	2071	CA	VAL A 245	53.159	5.134	32.602	1.00	13.49	C
ATOM	2072	C	VAL A 245	52.528	6.566	32.644	1.00	16.25	C
ATOM	2073	O	VAL A 245	52.786	7.405	31.770	1.00	15.20	O
ATOM	2074	CB	VAL A 245	54.754	5.163	32.810	1.00	21.07	C
ATOM	2075	CG1	VAL A 245	55.154	6.085	33.937	1.00	15.08	C
ATOM	2076	CG2	VAL A 245	55.280	3.817	33.143	1.00	15.82	C
ATOM	2077	N	GLY A 246	51.696	6.843	33.649	1.00	14.03	N
ATOM	2078	CA	GLY A 246	51.027	8.136	33.707	1.00	16.87	C
ATOM	2079	C	GLY A 246	50.146	8.203	34.939	1.00	26.95	C

ATOM	2080	O	GLY A 246	50.323	7.401	35.850	1.00	23.04	O
ATOM	2081	N	THR A 247	49.207	9.161	34.963	1.00	21.44	N
ATOM	2082	CA	THR A 247	48.232	9.276	36.063	1.00	21.39	C
ATOM	2083	C	THR A 247	46.868	8.677	35.673	1.00	24.08	C
ATOM	2084	O	THR A 247	46.069	8.306	36.508	1.00	21.03	O
ATOM	2085	CB	THR A 247	47.988	10.730	36.404	1.00	22.24	C
ATOM	2086	OG1	THR A 247	47.409	11.389	35.265	1.00	18.62	O
ATOM	2087	CG2	THR A 247	49.275	11.378	36.724	1.00	18.99	C
ATOM	2088	N	GLY A 248	46.583	8.651	34.384	1.00	24.95	N
ATOM	2089	CA	GLY A 248	45.319	8.143	33.924	1.00	22.61	C
ATOM	2090	C	GLY A 248	44.223	9.160	34.226	1.00	21.42	C
ATOM	2091	O	GLY A 248	43.059	8.866	34.137	1.00	25.70	O
ATOM	2092	N	VAL A 249	44.615	10.386	34.521	1.00	30.72	N
ATOM	2093	CA	VAL A 249	43.673	11.464	34.827	1.00	26.09	C
ATOM	2094	C	VAL A 249	43.747	12.596	33.786	1.00	32.70	C
ATOM	2095	O	VAL A 249	44.853	13.006	33.387	1.00	26.92	O
ATOM	2096	CB	VAL A 249	44.020	12.085	36.214	1.00	38.59	C
ATOM	2097	CG1	VAL A 249	43.225	13.324	36.470	1.00	36.11	C
ATOM	2098	CG2	VAL A 249	43.782	11.083	37.306	1.00	41.30	C
ATOM	2099	N	ASP A 250	42.581	13.125	33.397	1.00	27.95	N
ATOM	2100	CA	ASP A 250	42.488	14.232	32.439	1.00	20.64	C
ATOM	2101	C	ASP A 250	42.611	15.581	33.155	1.00	27.63	C
ATOM	2102	O	ASP A 250	42.188	15.783	34.308	1.00	26.23	O
ATOM	2103	CB	ASP A 250	41.075	14.302	31.827	1.00	23.89	C
ATOM	2104	CG	ASP A 250	40.768	13.180	30.850	1.00	39.52	C
ATOM	2105	OD1	ASP A 250	41.283	13.184	29.688	1.00	39.96	O
ATOM	2106	OD2	ASP A 250	39.767	12.501	31.153	1.00	45.34	O
ATOM	2107	N	CYS A 251	43.029	16.566	32.388	1.00	20.12	N
ATOM	2108	CA	CYS A 251	42.962	17.906	32.851	1.00	27.20	C
ATOM	2109	C	CYS A 251	42.918	18.779	31.577	1.00	26.47	C
ATOM	2110	O	CYS A 251	43.699	18.560	30.633	1.00	19.45	O
ATOM	2111	CB	CYS A 251	44.148	18.157	33.778	1.00	34.86	C
ATOM	2112	SG	CYS A 251	45.129	19.619	33.453	1.00	29.47	S
ATOM	2113	N	THR A 252	41.932	19.673	31.494	1.00	14.85	N
ATOM	2114	CA	THR A 252	41.834	20.588	30.335	1.00	21.21	C
ATOM	2115	C	THR A 252	42.999	21.592	30.236	1.00	20.53	C
ATOM	2116	O	THR A 252	43.657	21.926	31.249	1.00	15.24	O
ATOM	2117	CB	THR A 252	40.506	21.407	30.329	1.00	32.08	C
ATOM	2118	OG1	THR A 252	40.460	22.304	31.447	1.00	19.26	O
ATOM	2119	CG2	THR A 252	39.309	20.495	30.372	1.00	13.91	C
ATOM	2120	N	ILE A 253	43.228	22.095	29.024	1.00	14.81	N
ATOM	2121	CA	ILE A 253	44.264	23.118	28.812	1.00	16.90	C
ATOM	2122	C	ILE A 253	43.934	24.383	29.627	1.00	23.41	C
ATOM	2123	O	ILE A 253	44.834	25.012	30.247	1.00	15.27	O
ATOM	2124	CB	ILE A 253	44.404	23.452	27.302	1.00	24.05	C

ATOM 2125 CG1 ILE A 253 44.862 22.200 26.561 1.00 27.33 C
ATOM 2126 CG2 ILE A 253 45.473 24.479 27.077 1.00 9.22 C
ATOM 2127 CD1 ILE A 253 45.662 21.276 27.452 1.00 49.56 C
ATOM 2128 N ARG A 254 42.637 24.709 29.707 1.00 19.56 N
ATOM 2129 CA ARG A 254 42.228 25.865 30.522 1.00 19.41 C
ATOM 2130 C ARG A 254 42.712 25.713 31.970 1.00 18.10 C
ATOM 2131 O ARG A 254 43.311 26.616 32.515 1.00 13.89 O
ATOM 2132 CB ARG A 254 40.704 26.101 30.480 1.00 15.98 C
ATOM 2133 CG ARG A 254 40.282 27.378 31.255 1.00 9.96 C
ATOM 2134 CD ARG A 254 38.809 27.702 31.218 1.00 24.79 C
ATOM 2135 NE ARG A 254 38.498 28.414 29.997 1.00 29.42 N
ATOM 2136 CZ ARG A 254 38.693 29.723 29.794 1.00 59.85 C
ATOM 2137 NH1 ARG A 254 39.194 30.527 30.732 1.00 42.58 N
ATOM 2138 NH2 ARG A 254 38.377 30.245 28.620 1.00 18.44 N
ATOM 2139 N ASP A 255 42.406 24.564 32.586 1.00 20.22 N
ATOM 2140 CA ASP A 255 42.795 24.205 33.974 1.00 16.48 C
ATOM 2141 C ASP A 255 44.321 24.372 34.069 1.00 22.43 C
ATOM 2142 O ASP A 255 44.868 24.897 35.060 1.00 18.53 O
ATOM 2143 CB ASP A 255 42.478 22.686 34.157 1.00 19.17 C
ATOM 2144 CG ASP A 255 42.144 22.246 35.610 1.00 47.08 C
ATOM 2145 OD1 ASP A 255 41.780 23.090 36.429 1.00 49.66 O
ATOM 2146 OD2 ASP A 255 42.020 21.016 35.880 1.00 48.12 O
ATOM 2147 N LEU A 256 45.014 23.809 33.078 1.00 15.98 N
ATOM 2148 CA LEU A 256 46.465 23.844 33.069 1.00 21.76 C
ATOM 2149 C LEU A 256 47.020 25.275 33.076 1.00 16.79 C
ATOM 2150 O LEU A 256 47.825 25.697 33.946 1.00 15.24 O
ATOM 2151 CB LEU A 256 46.967 23.056 31.859 1.00 23.33 C
ATOM 2152 CG LEU A 256 48.491 23.100 31.765 1.00 26.80 C
ATOM 2153 CD1 LEU A 256 49.171 22.334 32.984 1.00 17.13 C
ATOM 2154 CD2 LEU A 256 49.040 22.724 30.346 1.00 15.42 C
ATOM 2155 N ALA A 257 46.520 26.048 32.140 1.00 13.77 N
ATOM 2156 CA ALA A 257 46.938 27.436 32.025 1.00 12.70 C
ATOM 2157 C ALA A 257 46.656 28.237 33.267 1.00 10.73 C
ATOM 2158 O ALA A 257 47.451 29.073 33.672 1.00 20.33 O
ATOM 2159 CB ALA A 257 46.208 28.073 30.834 1.00 13.34 C
ATOM 2160 N GLN A 258 45.470 28.080 33.835 1.00 12.40 N
ATOM 2161 CA GLN A 258 45.102 28.911 34.981 1.00 8.39 C
ATOM 2162 C GLN A 258 45.879 28.480 36.166 1.00 13.48 C
ATOM 2163 O GLN A 258 46.178 29.281 37.029 1.00 22.96 O
ATOM 2164 CB GLN A 258 43.614 28.761 35.305 1.00 16.12 C
ATOM 2165 CG GLN A 258 42.674 29.096 34.130 1.00 30.19 C
ATOM 2166 CD GLN A 258 42.574 30.585 33.781 1.00 37.29 C
ATOM 2167 OE1 GLN A 258 42.911 31.471 34.610 1.00 21.24 O
ATOM 2168 NE2 GLN A 258 42.021 30.876 32.572 1.00 15.94 N
ATOM 2169 N THR A 259 46.179 27.182 36.232 1.00 16.21 N

ATOM	2170	CA	THR A 259	46.982	26.678	37.336	1.00	16.85	C
ATOM	2171	C	THR A 259	48.410	27.186	37.233	1.00	20.56	C
ATOM	2172	O	THR A 259	49.002	27.621	38.214	1.00	21.44	O
ATOM	2173	CB	THR A 259	47.066	25.192	37.361	1.00	27.56	C
ATOM	2174	OG1	THR A 259	45.752	24.620	37.509	1.00	20.92	O
ATOM	2175	CG2	THR A 259	47.936	24.796	38.545	1.00	12.85	C
ATOM	2176	N	ILE A 260	48.952	27.170	36.028	1.00	19.96	N
ATOM	2177	CA	ILE A 260	50.292	27.704	35.839	1.00	23.01	C
ATOM	2178	C	ILE A 260	50.313	29.180	36.225	1.00	31.73	C
ATOM	2179	O	ILE A 260	51.211	29.627	36.993	1.00	25.90	O
ATOM	2180	CB	ILE A 260	50.835	27.456	34.390	1.00	22.46	C
ATOM	2181	CG1	ILE A 260	51.153	25.940	34.232	1.00	24.12	C
ATOM	2182	CG2	ILE A 260	52.099	28.361	34.106	1.00	13.47	C
ATOM	2183	CD1	ILE A 260	51.501	25.443	32.810	1.00	12.58	C
ATOM	2184	N	ALA A 261	49.280	29.910	35.764	1.00	15.35	N
ATOM	2185	CA	ALA A 261	49.177	31.355	36.048	1.00	16.00	C
ATOM	2186	C	ALA A 261	49.316	31.604	37.550	1.00	20.58	C
ATOM	2187	O	ALA A 261	50.104	32.443	37.987	1.00	16.09	O
ATOM	2188	CB	ALA A 261	47.832	31.958	35.487	1.00	13.65	C
ATOM	2189	N	LYS A 262	48.551	30.843	38.323	1.00	11.50	N
ATOM	2190	CA	LYS A 262	48.578	30.905	39.770	1.00	10.13	C
ATOM	2191	C	LYS A 262	49.968	30.460	40.296	1.00	28.08	C
ATOM	2192	O	LYS A 262	50.503	31.084	41.205	1.00	29.37	O
ATOM	2193	CB	LYS A 262	47.453	30.032	40.335	1.00	12.50	C
ATOM	2194	CG	LYS A 262	47.332	29.962	41.888	1.00	16.51	C
ATOM	2195	CD	LYS A 262	46.092	29.092	42.371	1.00	46.61	C
ATOM	2196	CE	LYS A 262	46.344	27.555	42.661	1.00	99.70	C
ATOM	2197	NZ	LYS A 262	45.157	26.703	43.200	1.00	36.59	N
ATOM	2198	N	VAL A 263	50.589	29.443	39.705	1.00	17.44	N
ATOM	2199	CA	VAL A 263	51.915	29.039	40.171	1.00	18.72	C
ATOM	2200	C	VAL A 263	52.997	30.170	39.997	1.00	32.12	C
ATOM	2201	O	VAL A 263	53.871	30.412	40.834	1.00	21.18	O
ATOM	2202	CB	VAL A 263	52.389	27.709	39.476	1.00	16.35	C
ATOM	2203	CG1	VAL A 263	53.920	27.518	39.647	1.00	11.83	C
ATOM	2204	CG2	VAL A 263	51.646	26.522	40.093	1.00	14.99	C
ATOM	2205	N	VAL A 264	52.913	30.899	38.909	1.00	21.75	N
ATOM	2206	CA	VAL A 264	53.917	31.877	38.653	1.00	19.81	C
ATOM	2207	C	VAL A 264	53.719	33.208	39.377	1.00	35.79	C
ATOM	2208	O	VAL A 264	54.632	34.032	39.482	1.00	28.99	O
ATOM	2209	CB	VAL A 264	54.059	32.014	37.175	1.00	24.27	C
ATOM	2210	CG1	VAL A 264	54.728	33.269	36.822	1.00	33.58	C
ATOM	2211	CG2	VAL A 264	54.840	30.808	36.674	1.00	23.01	C
ATOM	2212	N	GLY A 265	52.550	33.378	39.969	1.00	25.30	N
ATOM	2213	CA	GLY A 265	52.241	34.620	40.636	1.00	24.14	C
ATOM	2214	C	GLY A 265	51.730	35.694	39.632	1.00	35.03	C

ATOM	2215	O	GLY A 265	51.773	36.911	39.962	1.00 33.71		O
ATOM	2216	N	TYR A 266	51.294	35.257	38.428	1.00 26.25		N
ATOM	2217	CA	TYR A 266	50.698	36.151	37.373	1.00 26.55		C
ATOM	2218	C	TYR A 266	49.364	36.745	37.818	1.00 31.01		C
ATOM	2219	O	TYR A 266	48.532	36.067	38.456	1.00 27.99		O
ATOM	2220	CB	TYR A 266	50.501	35.463	36.008	1.00 24.31		C
ATOM	2221	CG	TYR A 266	49.994	36.381	34.884	1.00 28.64		C
ATOM	2222	CD1	TYR A 266	50.670	37.582	34.542	1.00 35.05		C
ATOM	2223	CD2	TYR A 266	48.860	36.038	34.118	1.00 22.60		C
ATOM	2224	CE1	TYR A 266	50.212	38.434	33.472	1.00 20.73		C
ATOM	2225	CE2	TYR A 266	48.428	36.859	33.012	1.00 20.91		C
ATOM	2226	CZ	TYR A 266	49.088	38.062	32.735	1.00 23.85		C
ATOM	2227	OH	TYR A 266	48.622	38.851	31.710	1.00 33.40		O
ATOM	2228	N	LYS A 267	49.217	38.043	37.604	1.00 25.72		N
ATOM	2229	CA	LYS A 267	47.988	38.697	38.009	1.00 30.77		C
ATOM	2230	C	LYS A 267	47.217	39.280	36.798	1.00 28.85		C
ATOM	2231	O	LYS A 267	46.179	39.894	36.949	1.00 31.17		O
ATOM	2232	CB	LYS A 267	48.279	39.741	39.092	1.00 27.13		C
ATOM	2233	CG	LYS A 267	48.728	39.128	40.403	1.00 23.18		C
ATOM	2234	CD	LYS A 267	48.420	40.096	41.562	1.00 30.98		C
ATOM	2235	CE	LYS A 267	47.933	39.358	42.820	1.00 48.52		C
ATOM	2236	NZ	LYS A 267	47.005	38.208	42.505	1.00100.00		N
ATOM	2237	N	GLY A 268	47.716	39.054	35.594	1.00 22.67		N
ATOM	2238	CA	GLY A 268	47.019	39.518	34.394	1.00 21.38		C
ATOM	2239	C	GLY A 268	45.856	38.568	34.085	1.00 31.03		C
ATOM	2240	O	GLY A 268	45.455	37.728	34.911	1.00 19.71		O
ATOM	2241	N	ARG A 269	45.387	38.645	32.849	1.00 30.40		N
ATOM	2242	CA	ARG A 269	44.263	37.846	32.399	1.00 26.47		C
ATOM	2243	C	ARG A 269	44.680	36.705	31.489	1.00 22.35		C
ATOM	2244	O	ARG A 269	45.378	36.926	30.524	1.00 22.75		O
ATOM	2245	CB	ARG A 269	43.297	38.753	31.626	1.00 22.65		C
ATOM	2246	CG	ARG A 269	42.201	39.390	32.463	1.00 24.21		C
ATOM	2247	CD	ARG A 269	40.936	39.465	31.568	1.00 83.45		C
ATOM	2248	NE	ARG A 269	40.113	40.676	31.762	1.00100.00		N
ATOM	2249	CZ	ARG A 269	38.808	40.751	31.431	1.00100.00		C
ATOM	2250	NH1	ARG A 269	38.201	39.691	30.921	1.00 99.93		N
ATOM	2251	NH2	ARG A 269	38.094	41.865	31.663	1.00100.00		N
ATOM	2252	N	VAL A 270	44.195	35.494	31.758	1.00 19.87		N
ATOM	2253	CA	VAL A 270	44.468	34.389	30.856	1.00 24.82		C
ATOM	2254	C	VAL A 270	43.319	34.456	29.824	1.00 22.51		C
ATOM	2255	O	VAL A 270	42.145	34.501	30.181	1.00 25.79		O
ATOM	2256	CB	VAL A 270	44.436	32.979	31.571	1.00 24.03		C
ATOM	2257	CG1	VAL A 270	44.576	31.861	30.533	1.00 20.72		C
ATOM	2258	CG2	VAL A 270	45.506	32.849	32.639	1.00 11.27		C
ATOM	2259	N	VAL A 271	43.660	34.409	28.554	1.00 25.18		N

ATOM	2260	CA	VAL A 271	42.666	34.492	27.487	1.00	28.32	C
ATOM	2261	C	VAL A 271	42.819	33.370	26.442	1.00	24.89	C
ATOM	2262	O	VAL A 271	43.923	33.115	25.980	1.00	21.98	O
ATOM	2263	CB	VAL A 271	42.901	35.813	26.736	1.00	29.25	C
ATOM	2264	CG1	VAL A 271	42.256	35.773	25.370	1.00	31.91	C
ATOM	2265	CG2	VAL A 271	42.421	36.989	27.565	1.00	18.72	C
ATOM	2266	N	PHE A 272	41.716	32.758	26.019	1.00	26.14	N
ATOM	2267	CA	PHE A 272	41.752	31.747	24.963	1.00	24.34	C
ATOM	2268	C	PHE A 272	41.236	32.266	23.623	1.00	28.95	C
ATOM	2269	O	PHE A 272	40.155	32.826	23.582	1.00	22.01	O
ATOM	2270	CB	PHE A 272	40.960	30.506	25.391	1.00	20.97	C
ATOM	2271	CG	PHE A 272	41.764	29.570	26.243	1.00	21.77	C
ATOM	2272	CD1	PHE A 272	41.940	29.842	27.610	1.00	14.60	C
ATOM	2273	CD2	PHE A 272	42.504	28.550	25.656	1.00	22.19	C
ATOM	2274	CE1	PHE A 272	42.763	29.041	28.434	1.00	17.89	C
ATOM	2275	CE2	PHE A 272	43.336	27.726	26.454	1.00	27.64	C
ATOM	2276	CZ	PHE A 272	43.478	27.979	27.851	1.00	25.14	C
ATOM	2277	N	ASP A 273	42.012	32.114	22.542	1.00	29.45	N
ATOM	2278	CA	ASP A 273	41.557	32.536	21.214	1.00	22.33	C
ATOM	2279	C	ASP A 273	40.896	31.365	20.493	1.00	25.67	C
ATOM	2280	O	ASP A 273	41.539	30.570	19.793	1.00	17.81	O
ATOM	2281	CB	ASP A 273	42.672	33.114	20.343	1.00	21.45	C
ATOM	2282	CG	ASP A 273	42.131	33.626	18.990	1.00	26.89	C
ATOM	2283	OD1	ASP A 273	40.975	33.249	18.598	1.00	27.76	O
ATOM	2284	OD2	ASP A 273	42.838	34.421	18.327	1.00	30.06	O
ATOM	2285	N	ALA A 274	39.589	31.284	20.649	1.00	15.59	N
ATOM	2286	CA	ALA A 274	38.932	30.128	20.128	1.00	23.75	C
ATOM	2287	C	ALA A 274	38.853	30.168	18.653	1.00	32.30	C
ATOM	2288	O	ALA A 274	38.284	29.256	18.029	1.00	29.37	O
ATOM	2289	CB	ALA A 274	37.567	29.905	20.777	1.00	18.87	C
ATOM	2290	N	SER A 275	39.372	31.243	18.081	1.00	21.10	N
ATOM	2291	CA	SER A 275	39.343	31.288	16.631	1.00	26.90	C
ATOM	2292	C	SER A 275	40.390	30.300	16.116	1.00	43.37	C
ATOM	2293	O	SER A 275	40.421	29.949	14.927	1.00	46.32	O
ATOM	2294	CB	SER A 275	39.547	32.683	16.074	1.00	15.19	C
ATOM	2295	OG	SER A 275	40.904	33.070	16.078	1.00	28.71	O
ATOM	2296	N	LYS A 276	41.192	29.780	17.037	1.00	22.98	N
ATOM	2297	CA	LYS A 276	42.178	28.791	16.638	1.00	23.28	C
ATOM	2298	C	LYS A 276	41.645	27.405	16.976	1.00	29.73	C
ATOM	2299	O	LYS A 276	40.992	27.206	18.010	1.00	25.10	O
ATOM	2300	CB	LYS A 276	43.544	29.051	17.275	1.00	19.19	C
ATOM	2301	CG	LYS A 276	43.957	30.496	17.218	1.00	32.11	C
ATOM	2302	CD	LYS A 276	44.062	30.852	15.798	1.00	22.43	C
ATOM	2303	CE	LYS A 276	44.930	32.067	15.570	1.00	23.18	C
ATOM	2304	NZ	LYS A 276	45.454	32.117	14.152	1.00	29.42	N

ATOM	2305	N	PRO A 277	41.892	26.476	16.055	1.00	36.04	N
ATOM	2306	CA	PRO A 277	41.446	25.087	16.170	1.00	35.93	C
ATOM	2307	C	PRO A 277	42.022	24.332	17.363	1.00	29.30	C
ATOM	2308	O	PRO A 277	43.103	24.650	17.885	1.00	30.54	O
ATOM	2309	CB	PRO A 277	41.975	24.453	14.878	1.00	39.65	C
ATOM	2310	CG	PRO A 277	43.249	25.261	14.566	1.00	42.90	C
ATOM	2311	CD	PRO A 277	42.787	26.670	14.892	1.00	37.84	C
ATOM	2312	N	ASP A 278	41.273	23.339	17.809	1.00	22.35	N
ATOM	2313	CA	ASP A 278	41.745	22.501	18.903	1.00	22.16	C
ATOM	2314	C	ASP A 278	42.184	21.189	18.272	1.00	19.66	C
ATOM	2315	O	ASP A 278	41.905	20.917	17.117	1.00	23.49	O
ATOM	2316	CB	ASP A 278	40.636	22.241	19.971	1.00	15.09	C
ATOM	2317	CG	ASP A 278	40.216	23.503	20.702	1.00	22.86	C
ATOM	2318	OD1	ASP A 278	41.113	24.254	21.096	1.00	25.18	O
ATOM	2319	OD2	ASP A 278	38.999	23.787	20.812	1.00	39.55	O
ATOM	2320	N	GLY A 279	42.846	20.355	19.044	1.00	30.65	N
ATOM	2321	CA	GLY A 279	43.229	19.034	18.546	1.00	33.78	C
ATOM	2322	C	GLY A 279	42.115	18.099	18.944	1.00	38.10	C
ATOM	2323	O	GLY A 279	40.963	18.517	19.068	1.00	47.52	O
ATOM	2324	N	THR A 280	42.419	16.839	19.177	1.00	29.44	N
ATOM	2325	CA	THR A 280	41.328	15.990	19.587	1.00	26.68	C
ATOM	2326	C	THR A 280	40.889	16.439	20.972	1.00	23.52	C
ATOM	2327	O	THR A 280	41.670	17.067	21.713	1.00	23.62	O
ATOM	2328	CB	THR A 280	41.695	14.492	19.540	1.00	40.78	C
ATOM	2329	OG1	THR A 280	42.889	14.272	20.296	1.00	25.56	O
ATOM	2330	CG2	THR A 280	41.893	14.054	18.095	1.00	37.71	C
ATOM	2331	N	PRO A 281	39.672	16.063	21.346	1.00	25.54	N
ATOM	2332	CA	PRO A 281	39.129	16.454	22.628	1.00	25.72	C
ATOM	2333	C	PRO A 281	39.776	15.778	23.800	1.00	26.02	C
ATOM	2334	O	PRO A 281	39.752	16.314	24.915	1.00	22.68	O
ATOM	2335	CB	PRO A 281	37.650	15.990	22.559	1.00	28.89	C
ATOM	2336	CG	PRO A 281	37.417	15.540	21.201	1.00	29.39	C
ATOM	2337	CD	PRO A 281	38.761	15.138	20.646	1.00	26.82	C
ATOM	2338	N	ARG A 282	40.281	14.567	23.587	1.00	27.88	N
ATOM	2339	CA	ARG A 282	40.806	13.817	24.720	1.00	34.08	C
ATOM	2340	C	ARG A 282	41.977	12.918	24.384	1.00	27.62	C
ATOM	2341	O	ARG A 282	41.913	12.182	23.425	1.00	23.83	O
ATOM	2342	CB	ARG A 282	39.676	13.017	25.405	1.00	20.89	C
ATOM	2343	CG	ARG A 282	40.035	12.467	26.775	1.00	22.81	C
ATOM	2344	CD	ARG A 282	38.762	11.925	27.442	1.00	26.77	C
ATOM	2345	NE	ARG A 282	38.963	11.345	28.781	1.00	36.48	N
ATOM	2346	CZ	ARG A 282	38.518	10.139	29.164	1.00	37.74	C
ATOM	2347	NH1	ARG A 282	37.813	9.360	28.346	1.00	28.45	N
ATOM	2348	NH2	ARG A 282	38.754	9.700	30.384	1.00	27.25	N
ATOM	2349	N	LYS A 283	43.016	12.963	25.223	1.00	28.91	N

ATOM	2350	CA	LYS A 283	44.217	12.171	25.051	1.00	24.32	C
ATOM	2351	C	LYS A 283	44.796	11.766	26.404	1.00	29.57	C
ATOM	2352	O	LYS A 283	45.262	12.626	27.138	1.00	33.16	O
ATOM	2353	CB	LYS A 283	45.226	13.008	24.287	1.00	21.93	C
ATOM	2354	CG	LYS A 283	46.111	12.251	23.316	1.00	32.38	C
ATOM	2355	CD	LYS A 283	46.526	13.171	22.143	1.00	95.77	C
ATOM	2356	CE	LYS A 283	45.710	12.937	20.836	1.00	100.00	C
ATOM	2357	NZ	LYS A 283	46.418	13.332	19.535	1.00	100.00	N
ATOM	2358	N	LEU A 284	44.747	10.467	26.734	1.00	23.37	N
ATOM	2359	CA	LEU A 284	45.327	9.905	27.997	1.00	16.08	C
ATOM	2360	C	LEU A 284	45.463	8.386	28.047	1.00	20.46	C
ATOM	2361	O	LEU A 284	44.679	7.655	27.446	1.00	25.45	O
ATOM	2362	CB	LEU A 284	44.641	10.387	29.284	1.00	16.30	C
ATOM	2363	CG	LEU A 284	43.334	9.700	29.714	1.00	25.97	C
ATOM	2364	CD1	LEU A 284	42.881	10.089	31.152	1.00	22.11	C
ATOM	2365	CD2	LEU A 284	42.203	9.953	28.693	1.00	23.92	C
ATOM	2366	N	LEU A 285	46.453	7.939	28.820	1.00	18.51	N
ATOM	2367	CA	LEU A 285	46.792	6.527	29.003	1.00	16.77	C
ATOM	2368	C	LEU A 285	45.880	5.865	30.006	1.00	30.75	C
ATOM	2369	O	LEU A 285	45.576	6.439	31.058	1.00	22.02	O
ATOM	2370	CB	LEU A 285	48.229	6.389	29.585	1.00	15.85	C
ATOM	2371	CG	LEU A 285	49.307	6.970	28.672	1.00	21.51	C
ATOM	2372	CD1	LEU A 285	50.703	6.705	29.122	1.00	15.15	C
ATOM	2373	CD2	LEU A 285	49.051	6.368	27.330	1.00	16.94	C
ATOM	2374	N	ASP A 286	45.565	4.599	29.734	1.00	26.62	N
ATOM	2375	CA	ASP A 286	44.945	3.726	30.698	1.00	10.90	C
ATOM	2376	C	ASP A 286	46.128	3.055	31.498	1.00	20.54	C
ATOM	2377	O	ASP A 286	46.991	2.372	30.938	1.00	23.38	O
ATOM	2378	CB	ASP A 286	44.073	2.702	29.970	1.00	14.65	C
ATOM	2379	CG	ASP A 286	43.409	1.699	30.943	1.00	24.60	C
ATOM	2380	OD1	ASP A 286	43.932	1.437	32.083	1.00	24.60	O
ATOM	2381	OD2	ASP A 286	42.316	1.231	30.583	1.00	26.03	O
ATOM	2382	N	VAL A 287	46.230	3.317	32.791	1.00	15.44	N
ATOM	2383	CA	VAL A 287	47.354	2.816	33.556	1.00	15.58	C
ATOM	2384	C	VAL A 287	46.973	1.695	34.521	1.00	16.48	C
ATOM	2385	O	VAL A 287	47.613	1.473	35.572	1.00	16.63	O
ATOM	2386	CB	VAL A 287	48.101	4.006	34.260	1.00	29.84	C
ATOM	2387	CG1	VAL A 287	48.534	5.085	33.224	1.00	18.39	C
ATOM	2388	CG2	VAL A 287	47.173	4.670	35.258	1.00	37.79	C
ATOM	2389	N	THR A 288	45.904	0.992	34.152	1.00	22.27	N
ATOM	2390	CA	THR A 288	45.428	-0.152	34.956	1.00	19.34	C
ATOM	2391	C	THR A 288	46.561	-1.177	35.227	1.00	27.47	C
ATOM	2392	O	THR A 288	46.778	-1.586	36.365	1.00	24.87	O
ATOM	2393	CB	THR A 288	44.288	-0.909	34.244	1.00	22.86	C
ATOM	2394	OG1	THR A 288	43.120	-0.096	34.106	1.00	24.84	O

ATOM	2395	CG2	THR	A 288	43.916	-2.113	35.024	1.00 25.08	C
ATOM	2396	N	ARG	A 289	47.290	-1.585	34.179	1.00 26.08	N
ATOM	2397	CA	ARG	A 289	48.428	-2.506	34.319	1.00 16.92	C
ATOM	2398	C	ARG	A 289	49.405	-2.037	35.408	1.00 22.96	C
ATOM	2399	O	ARG	A 289	49.847	-2.790	36.275	1.00 23.03	O
ATOM	2400	CB	ARG	A 289	49.208	-2.607	32.976	1.00 12.43	C
ATOM	2401	CG	ARG	A 289	48.934	-3.804	32.103	1.00 29.39	C
ATOM	2402	CD	ARG	A 289	50.016	-4.102	31.037	1.00 25.88	C
ATOM	2403	NE	ARG	A 289	49.441	-4.996	30.020	1.00 17.26	N
ATOM	2404	CZ	ARG	A 289	50.053	-5.459	28.930	1.00 38.82	C
ATOM	2405	NH1	ARG	A 289	51.306	-5.153	28.660	1.00 13.51	N
ATOM	2406	NH2	ARG	A 289	49.400	-6.262	28.096	1.00 37.68	N
ATOM	2407	N	LEU	A 290	49.815	-0.786	35.306	1.00 26.60	N
ATOM	2408	CA	LEU	A 290	50.809	-0.254	36.219	1.00 25.42	C
ATOM	2409	C	LEU	A 290	50.324	-0.376	37.656	1.00 24.17	C
ATOM	2410	O	LEU	A 290	51.072	-0.759	38.574	1.00 19.94	O
ATOM	2411	CB	LEU	A 290	51.000	1.219	35.876	1.00 24.66	C
ATOM	2412	CG	LEU	A 290	52.281	2.019	36.066	1.00 24.67	C
ATOM	2413	CD1	LEU	A 290	51.992	3.479	36.504	1.00 29.25	C
ATOM	2414	CD2	LEU	A 290	53.450	1.335	36.788	1.00 15.82	C
ATOM	2415	N	HIS	A 291	49.093	0.075	37.868	1.00 30.10	N
ATOM	2416	CA	HIS	A 291	48.513	0.074	39.212	1.00 34.17	C
ATOM	2417	C	HIS	A 291	48.411	-1.367	39.730	1.00 43.41	C
ATOM	2418	O	HIS	A 291	48.621	-1.654	40.929	1.00 38.81	O
ATOM	2419	CB	HIS	A 291	47.113	0.674	39.143	1.00 28.01	C
ATOM	2420	CG	HIS	A 291	47.097	2.153	38.984	1.00 29.68	C
ATOM	2421	ND1	HIS	A 291	48.242	2.921	39.015	1.00 35.63	N
ATOM	2422	CD2	HIS	A 291	46.068	3.024	38.855	1.00 31.18	C
ATOM	2423	CE1	HIS	A 291	47.926	4.197	38.845	1.00 24.20	C
ATOM	2424	NE2	HIS	A 291	46.612	4.289	38.747	1.00 21.92	N
ATOM	2425	N	GLN	A 292	48.048	-2.260	38.821	1.00 30.71	N
ATOM	2426	CA	GLN	A 292	47.950	-3.654	39.181	1.00 34.82	C
ATOM	2427	C	GLN	A 292	49.287	-4.197	39.622	1.00 36.93	C
ATOM	2428	O	GLN	A 292	49.323	-5.040	40.510	1.00 27.56	O
ATOM	2429	CB	GLN	A 292	47.322	-4.487	38.069	1.00 28.23	C
ATOM	2430	CG	GLN	A 292	45.798	-4.405	38.171	1.00 81.15	C
ATOM	2431	CD	GLN	A 292	45.023	-4.954	36.963	1.00100.00	C
ATOM	2432	OE1	GLN	A 292	45.597	-5.410	35.951	1.00 99.65	O
ATOM	2433	NE2	GLN	A 292	43.687	-4.895	37.073	1.00 40.86	N
ATOM	2434	N	LEU	A 293	50.375	-3.658	39.058	1.00 31.75	N
ATOM	2435	CA	LEU	A 293	51.750	-4.072	39.383	1.00 22.67	C
ATOM	2436	C	LEU	A 293	52.238	-3.323	40.613	1.00 28.64	C
ATOM	2437	O	LEU	A 293	53.420	-3.377	41.017	1.00 22.27	O
ATOM	2438	CB	LEU	A 293	52.665	-3.769	38.205	1.00 25.57	C
ATOM	2439	CG	LEU	A 293	52.497	-4.703	37.016	1.00 35.11	C

ATOM	2440	CD1	LEU	A	293	53.306	-4.170	35.836	1.00 28.25	C
ATOM	2441	CD2	LEU	A	293	52.965	-6.110	37.439	1.00 47.81	C
ATOM	2442	N	GLY	A	294	51.316	-2.510	41.111	1.00 33.08	N
ATOM	2443	CA	GLY	A	294	51.488	-1.793	42.347	1.00 24.90	C
ATOM	2444	C	GLY	A	294	52.272	-0.512	42.326	1.00 29.31	C
ATOM	2445	O	GLY	A	294	53.070	-0.249	43.223	1.00 25.25	O
ATOM	2446	N	TRP	A	295	52.000	0.347	41.368	1.00 27.83	N
ATOM	2447	CA	TRP	A	295	52.687	1.623	41.385	1.00 19.45	C
ATOM	2448	C	TRP	A	295	51.684	2.731	41.081	1.00 25.79	C
ATOM	2449	O	TRP	A	295	50.765	2.527	40.297	1.00 20.43	O
ATOM	2450	CB	TRP	A	295	53.961	1.614	40.524	1.00 12.85	C
ATOM	2451	CG	TRP	A	295	54.750	2.911	40.618	1.00 23.04	C
ATOM	2452	CD1	TRP	A	295	55.897	3.161	41.368	1.00 23.68	C
ATOM	2453	CD2	TRP	A	295	54.415	4.159	39.979	1.00 20.72	C
ATOM	2454	NE1	TRP	A	295	56.258	4.493	41.244	1.00 18.67	N
ATOM	2455	CE2	TRP	A	295	55.389	5.113	40.373	1.00 20.95	C
ATOM	2456	CE3	TRP	A	295	53.406	4.550	39.102	1.00 21.47	C
ATOM	2457	CZ2	TRP	A	295	55.338	6.439	39.958	1.00 17.58	C
ATOM	2458	CZ3	TRP	A	295	53.403	5.873	38.632	1.00 21.57	C
ATOM	2459	CH2	TRP	A	295	54.368	6.787	39.058	1.00 19.45	C
ATOM	2460	N	TYR	A	296	51.709	3.797	41.884	1.00 25.17	N
ATOM	2461	CA	TYR	A	296	50.720	4.883	41.731	1.00 24.90	C
ATOM	2462	C	TYR	A	296	51.517	6.178	41.857	1.00 30.85	C
ATOM	2463	O	TYR	A	296	52.363	6.272	42.745	1.00 21.27	O
ATOM	2464	CB	TYR	A	296	49.654	4.813	42.840	1.00 25.18	C
ATOM	2465	CG	TYR	A	296	48.685	3.651	42.744	1.00 23.04	C
ATOM	2466	CD1	TYR	A	296	49.078	2.343	43.088	1.00 31.62	C
ATOM	2467	CD2	TYR	A	296	47.380	3.853	42.289	1.00 26.02	C
ATOM	2468	CE1	TYR	A	296	48.203	1.268	42.935	1.00 24.42	C
ATOM	2469	CE2	TYR	A	296	46.493	2.770	42.127	1.00 24.81	C
ATOM	2470	CZ	TYR	A	296	46.902	1.483	42.464	1.00 39.41	C
ATOM	2471	OH	TYR	A	296	45.984	0.434	42.337	1.00 66.19	O
ATOM	2472	N	HIS	A	297	51.324	7.123	40.924	1.00 20.95	N
ATOM	2473	CA	HIS	A	297	52.130	8.343	40.938	1.00 26.86	C
ATOM	2474	C	HIS	A	297	51.947	9.175	42.210	1.00 35.01	C
ATOM	2475	O	HIS	A	297	50.885	9.132	42.874	1.00 26.92	O
ATOM	2476	CB	HIS	A	297	51.819	9.192	39.733	1.00 25.77	C
ATOM	2477	CG	HIS	A	297	50.489	9.842	39.803	1.00 31.16	C
ATOM	2478	ND1	HIS	A	297	49.314	9.145	39.633	1.00 34.21	N
ATOM	2479	CD2	HIS	A	297	50.135	11.094	40.167	1.00 25.83	C
ATOM	2480	CE1	HIS	A	297	48.290	9.972	39.776	1.00 24.14	C
ATOM	2481	NE2	HIS	A	297	48.761	11.164	40.087	1.00 23.35	N
ATOM	2482	N	GLU	A	298	52.983	9.926	42.554	1.00 24.98	N
ATOM	2483	CA	GLU	A	298	52.957	10.683	43.798	1.00 27.65	C
ATOM	2484	C	GLU	A	298	52.831	12.187	43.741	1.00 36.86	C

ATOM	2485	O	GLU A 298	52.433	12.792	44.718	1.00	43.61	O
ATOM	2486	CB	GLU A 298	54.153	10.319	44.686	1.00	22.02	C
ATOM	2487	CG	GLU A 298	54.004	8.943	45.285	1.00	36.42	C
ATOM	2488	CD	GLU A 298	54.999	8.664	46.406	1.00	100.00	C
ATOM	2489	OE1	GLU A 298	56.223	8.561	46.152	1.00	44.79	O
ATOM	2490	OE2	GLU A 298	54.526	8.470	47.547	1.00	100.00	O
ATOM	2491	N	ILE A 299	53.232	12.800	42.639	1.00	23.49	N
ATOM	2492	CA	ILE A 299	53.268	14.244	42.562	1.00	13.25	C
ATOM	2493	C	ILE A 299	52.016	14.848	41.906	1.00	27.05	C
ATOM	2494	O	ILE A 299	51.681	14.530	40.757	1.00	26.73	O
ATOM	2495	CB	ILE A 299	54.586	14.711	41.862	1.00	15.93	C
ATOM	2496	CG1	ILE A 299	55.836	14.183	42.606	1.00	23.83	C
ATOM	2497	CG2	ILE A 299	54.596	16.213	41.541	1.00	17.37	C
ATOM	2498	CD1	ILE A 299	57.232	14.221	41.787	1.00	21.32	C
ATOM	2499	N	SER A 300	51.323	15.716	42.648	1.00	18.55	N
ATOM	2500	CA	SER A 300	50.177	16.449	42.091	1.00	19.58	C
ATOM	2501	C	SER A 300	50.714	17.415	41.042	1.00	17.29	C
ATOM	2502	O	SER A 300	51.824	17.941	41.178	1.00	21.06	O
ATOM	2503	CB	SER A 300	49.542	17.307	43.181	1.00	16.78	C
ATOM	2504	OG	SER A 300	50.548	17.969	43.923	1.00	75.80	O
ATOM	2505	N	LEU A 301	49.870	17.755	40.075	1.00	16.13	N
ATOM	2506	CA	LEU A 301	50.246	18.675	39.014	1.00	17.70	C
ATOM	2507	C	LEU A 301	50.689	19.964	39.646	1.00	20.11	C
ATOM	2508	O	LEU A 301	51.714	20.568	39.303	1.00	20.46	O
ATOM	2509	CB	LEU A 301	48.990	18.981	38.197	1.00	17.92	C
ATOM	2510	CG	LEU A 301	49.182	20.030	37.112	1.00	25.15	C
ATOM	2511	CD1	LEU A 301	50.233	19.552	36.086	1.00	18.82	C
ATOM	2512	CD2	LEU A 301	47.854	20.177	36.436	1.00	25.88	C
ATOM	2513	N	GLU A 302	49.845	20.398	40.554	1.00	27.01	N
ATOM	2514	CA	GLU A 302	50.053	21.636	41.280	1.00	37.72	C
ATOM	2515	C	GLU A 302	51.410	21.618	41.996	1.00	29.99	C
ATOM	2516	O	GLU A 302	52.245	22.514	41.798	1.00	27.15	O
ATOM	2517	CB	GLU A 302	48.899	21.841	42.275	1.00	43.10	C
ATOM	2518	CG	GLU A 302	49.061	23.061	43.174	1.00	90.85	C
ATOM	2519	CD	GLU A 302	48.451	24.324	42.580	1.00	100.00	C
ATOM	2520	OE1	GLU A 302	47.566	24.209	41.706	1.00	100.00	O
ATOM	2521	OE2	GLU A 302	48.808	25.432	43.036	1.00	64.50	O
ATOM	2522	N	ALA A 303	51.646	20.591	42.801	1.00	8.72	N
ATOM	2523	CA	ALA A 303	52.937	20.455	43.459	1.00	15.03	C
ATOM	2524	C	ALA A 303	54.102	20.355	42.450	1.00	19.85	C
ATOM	2525	O	ALA A 303	55.104	21.090	42.553	1.00	22.24	O
ATOM	2526	CB	ALA A 303	52.938	19.258	44.410	1.00	18.97	C
ATOM	2527	N	GLY A 304	53.953	19.472	41.467	1.00	13.05	N
ATOM	2528	CA	GLY A 304	54.970	19.321	40.448	1.00	8.94	C
ATOM	2529	C	GLY A 304	55.239	20.621	39.695	1.00	20.31	C

ATOM	2530	O	GLY A 304	56.394	20.900	39.322	1.00 14.30	O
ATOM	2531	N	LEU A 305	54.191	21.383	39.361	1.00 10.76	N
ATOM	2532	CA	LEU A 305	54.483	22.622	38.611	1.00 20.29	C
ATOM	2533	C	LEU A 305	55.281	23.669	39.456	1.00 28.92	C
ATOM	2534	O	LEU A 305	56.194	24.385	38.974	1.00 17.69	O
ATOM	2535	CB	LEU A 305	53.202	23.245	38.033	1.00 24.03	C
ATOM	2536	CG	LEU A 305	52.357	22.647	36.880	1.00 27.66	C
ATOM	2537	CD1	LEU A 305	50.975	23.384	36.789	1.00 13.44	C
ATOM	2538	CD2	LEU A 305	53.079	22.724	35.543	1.00 18.39	C
ATOM	2539	N	ALA A 306	54.904	23.757	40.724	1.00 19.94	N
ATOM	2540	CA	ALA A 306	55.544	24.660	41.655	1.00 24.79	C
ATOM	2541	C	ALA A 306	57.035	24.380	41.743	1.00 27.51	C
ATOM	2542	O	ALA A 306	57.852	25.280	41.662	1.00 29.68	O
ATOM	2543	CB	ALA A 306	54.937	24.471	43.002	1.00 17.87	C
ATOM	2544	N	SER A 307	57.378	23.137	42.011	1.00 18.46	N
ATOM	2545	CA	SER A 307	58.793	22.756	42.162	1.00 16.31	C
ATOM	2546	C	SER A 307	59.547	22.885	40.832	1.00 22.66	C
ATOM	2547	O	SER A 307	60.742	23.212	40.786	1.00 28.47	O
ATOM	2548	CB	SER A 307	58.851	21.304	42.622	1.00 20.47	C
ATOM	2549	OG	SER A 307	58.517	20.454	41.526	1.00 29.03	O
ATOM	2550	N	THR A 308	58.849	22.631	39.735	1.00 27.31	N
ATOM	2551	CA	THR A 308	59.458	22.738	38.413	1.00 22.89	C
ATOM	2552	C	THR A 308	59.757	24.216	38.107	1.00 26.06	C
ATOM	2553	O	THR A 308	60.819	24.546	37.591	1.00 29.89	O
ATOM	2554	CB	THR A 308	58.536	22.115	37.318	1.00 18.72	C
ATOM	2555	OG1	THR A 308	58.356	20.714	37.545	1.00 20.17	O
ATOM	2556	CG2	THR A 308	59.094	22.330	35.923	1.00 12.37	C
ATOM	2557	N	TYR A 309	58.846	25.118	38.453	1.00 28.20	N
ATOM	2558	CA	TYR A 309	59.110	26.549	38.241	1.00 31.09	C
ATOM	2559	C	TYR A 309	60.383	27.059	39.045	1.00 16.31	C
ATOM	2560	O	TYR A 309	61.179	27.858	38.577	1.00 16.91	O
ATOM	2561	CB	TYR A 309	57.819	27.373	38.533	1.00 31.19	C
ATOM	2562	CG	TYR A 309	57.944	28.895	38.392	1.00 14.57	C
ATOM	2563	CD1	TYR A 309	58.397	29.457	37.224	1.00 17.51	C
ATOM	2564	CD2	TYR A 309	57.575	29.757	39.442	1.00 24.99	C
ATOM	2565	CE1	TYR A 309	58.527	30.801	37.100	1.00 18.41	C
ATOM	2566	CE2	TYR A 309	57.744	31.129	39.351	1.00 19.04	C
ATOM	2567	CZ	TYR A 309	58.212	31.641	38.164	1.00 29.13	C
ATOM	2568	OH	TYR A 309	58.300	33.004	37.966	1.00 28.22	O
ATOM	2569	N	GLN A 310	60.560	26.579	40.260	1.00 15.41	N
ATOM	2570	CA	GLN A 310	61.705	26.964	41.087	1.00 22.35	C
ATOM	2571	C	GLN A 310	63.001	26.492	40.446	1.00 31.46	C
ATOM	2572	O	GLN A 310	64.009	27.191	40.442	1.00 33.42	O
ATOM	2573	CB	GLN A 310	61.587	26.335	42.482	1.00 17.67	C
ATOM	2574	CG	GLN A 310	62.579	26.921	43.461	1.00 57.58	C

ATOM	2575	CD	GLN A 310	62.287	28.370	43.782	1.00	65.14	C
ATOM	2576	OE1	GLN A 310	61.134	28.754	44.000	1.00	41.94	O
ATOM	2577	NE2	GLN A 310	63.330	29.194	43.801	1.00	99.09	N
ATOM	2578	N	TRP A 311	62.957	25.321	39.830	1.00	28.76	N
ATOM	2579	CA	TRP A 311	64.146	24.822	39.163	1.00	26.29	C
ATOM	2580	C	TRP A 311	64.474	25.769	38.040	1.00	17.91	C
ATOM	2581	O	TRP A 311	65.599	26.193	37.880	1.00	22.89	O
ATOM	2582	CB	TRP A 311	63.938	23.383	38.643	1.00	27.53	C
ATOM	2583	CG	TRP A 311	65.176	22.784	38.119	1.00	17.82	C
ATOM	2584	CD1	TRP A 311	66.132	22.090	38.826	1.00	20.21	C
ATOM	2585	CD2	TRP A 311	65.652	22.881	36.784	1.00	17.99	C
ATOM	2586	NE1	TRP A 311	67.197	21.776	37.992	1.00	20.39	N
ATOM	2587	CE2	TRP A 311	66.933	22.284	36.746	1.00	19.57	C
ATOM	2588	CE3	TRP A 311	65.141	23.461	35.621	1.00	20.26	C
ATOM	2589	CZ2	TRP A 311	67.686	22.236	35.599	1.00	14.25	C
ATOM	2590	CZ3	TRP A 311	65.901	23.446	34.501	1.00	18.59	C
ATOM	2591	CH2	TRP A 311	67.169	22.831	34.494	1.00	16.86	C
ATOM	2592	N	PHE A 312	63.469	26.109	37.256	1.00	17.47	N
ATOM	2593	CA	PHE A 312	63.665	27.064	36.179	1.00	20.14	C
ATOM	2594	C	PHE A 312	64.224	28.371	36.733	1.00	18.33	C
ATOM	2595	O	PHE A 312	65.080	29.024	36.104	1.00	24.76	O
ATOM	2596	CB	PHE A 312	62.328	27.318	35.458	1.00	29.51	C
ATOM	2597	CG	PHE A 312	62.328	28.544	34.603	1.00	28.52	C
ATOM	2598	CD1	PHE A 312	62.883	28.508	33.338	1.00	30.53	C
ATOM	2599	CD2	PHE A 312	61.825	29.758	35.104	1.00	29.31	C
ATOM	2600	CE1	PHE A 312	62.936	29.660	32.554	1.00	34.73	C
ATOM	2601	CE2	PHE A 312	61.900	30.904	34.362	1.00	38.40	C
ATOM	2602	CZ	PHE A 312	62.432	30.860	33.063	1.00	40.73	C
ATOM	2603	N	LEU A 313	63.697	28.787	37.876	1.00	22.46	N
ATOM	2604	CA	LEU A 313	64.170	30.025	38.516	1.00	28.47	C
ATOM	2605	C	LEU A 313	65.627	29.827	38.898	1.00	37.53	C
ATOM	2606	O	LEU A 313	66.452	30.693	38.629	1.00	34.20	O
ATOM	2607	CB	LEU A 313	63.375	30.410	39.783	1.00	20.44	C
ATOM	2608	CG	LEU A 313	61.955	30.897	39.555	1.00	16.29	C
ATOM	2609	CD1	LEU A 313	61.499	31.399	40.871	1.00	15.94	C
ATOM	2610	CD2	LEU A 313	61.959	31.961	38.524	1.00	14.44	C
ATOM	2611	N	GLU A 314	65.953	28.685	39.508	1.00	30.70	N
ATOM	2612	CA	GLU A 314	67.353	28.432	39.875	1.00	24.15	C
ATOM	2613	C	GLU A 314	68.291	28.149	38.703	1.00	36.34	C
ATOM	2614	O	GLU A 314	69.485	28.047	38.890	1.00	43.10	O
ATOM	2615	CB	GLU A 314	67.459	27.366	40.947	1.00	19.90	C
ATOM	2616	CG	GLU A 314	66.634	27.754	42.141	1.00	27.37	C
ATOM	2617	CD	GLU A 314	66.450	26.666	43.182	1.00	31.09	C
ATOM	2618	OE1	GLU A 314	67.157	25.648	43.085	1.00	59.60	O
ATOM	2619	OE2	GLU A 314	65.634	26.872	44.125	1.00	46.20	O

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ATOM   2620  N   ASN A 315      67.778  28.114  37.479  1.00 40.17           N
ATOM   2621  CA  ASN A 315      68.637  27.802  36.343  1.00 37.76           C
ATOM   2622  C   ASN A 315      68.383  28.578  35.112  1.00 43.75           C
ATOM   2623  O   ASN A 315      68.591  28.001  34.047  1.00 39.15           O
ATOM   2624  CB  ASN A 315      68.425  26.360  35.884  1.00 33.74           C
ATOM   2625  CG  ASN A 315      69.028  25.383  36.801  1.00 53.18           C
ATOM   2626  OD1 ASN A 315      68.456  25.087  37.835  1.00 49.13           O
ATOM   2627  ND2 ASN A 315      70.239  24.926  36.479  1.00 97.72           N
ATOM   2628  N   GLN A 316      67.852  29.803  35.197  1.00 49.87           N
ATOM   2629  CA  GLN A 316      67.627  30.550  33.957  1.00 77.90           C
ATOM   2630  C   GLN A 316      68.797  31.448  33.525  1.00100.00           C
ATOM   2631  O   GLN A 316      69.272  31.387  32.375  1.00 51.33           O
ATOM   2632  CB  GLN A 316      66.280  31.276  33.902  1.00 75.89           C
ATOM   2633  CG  GLN A 316      65.683  31.589  35.231  1.00 80.97           C
ATOM   2634  CD  GLN A 316      65.233  33.036  35.350  1.00 54.58           C
ATOM   2635  OE1 GLN A 316      64.881  33.699  34.367  1.00 46.46           O
ATOM   2636  NE2 GLN A 316      65.257  33.538  36.566  1.00 33.46           N
TER    2637       GLN A 316
CONECT  110  111
CONECT  111  110  112
CONECT  112  111  113  114
CONECT  113  112  118
CONECT  114  112  115  116
CONECT  115  114
CONECT  116  114  117  118
CONECT  117  116  129
CONECT  118  113  116
CONECT  120  121
CONECT  121  120  122
CONECT  122  121  123  124
CONECT  123  122  128
CONECT  124  122  125  126
CONECT  125  124
CONECT  126  124  127  128
CONECT  127  126
CONECT  128  123  126
CONECT  129  117  130  131  132
CONECT  130  129
CONECT  131  129
CONECT  132  129
MASTER      208    0    1   13   10    0    3    6 2636    1   22   25
END
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While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. However, it is to be expressly understood that such modifications and adaptations are within the spirit and scope of the present invention, as set forth in the following claims.

What is claimed:

1. A method for producing ascorbic acid or esters thereof in a microorganism, comprising culturing a microorganism having a genetic modification to increase the action of an enzyme selected from the group consisting of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-D-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and L-galactono-γ-lactone dehydrogenase; and recovering said ascorbic acid or esters thereof.

2. A method, as claimed in Claim 1, wherein said genetic modification is a genetic modification to increase the action of an enzyme selected from the group consisting of GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and L-galactono-γ-lactone dehydrogenase.

3. A method, as claimed in Claim 1, wherein said genetic modification is a genetic modification to increase the action of an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose.

4. A method, as claimed in Claim 3, wherein said genetic modification is a genetic modification to increase the action of GDP-D-mannose:GDP-L-galactose epimerase.

5. The method of Claim 3, wherein said genetic modification comprises transformation of said microorganism with a recombinant nucleic acid molecule that expresses said epimerase.

6. The method of Claim 5, wherein said epimerase has a tertiary structure that substantially conforms to the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

7. The method of Claim 5, wherein said epimerase has a structure having an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

8. The method of Claim 5, wherein said epimerase has a tertiary structure having an average root mean square deviation of less than about 1 Å over at least about 25% of Cα positions of the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

9. The method of Claim 5, wherein said epimerase comprises a substrate binding site having a tertiary structure that substantially conforms to the tertiary structure of the substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

10. The method of Claim 9, wherein said substrate binding site has a tertiary structure with an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a substrate binding site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

11. The method of Claim 5, wherein said epimerase comprises a catalytic site having a tertiary structure that substantially conforms to the tertiary structure of the catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

12. The method of Claim 11, wherein said catalytic site has a tertiary structure with an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a catalytic site of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

13. The method of Claim 11, wherein said catalytic site comprises the amino acid residues serine, tyrosine and lysine.

14. The method of Claim 13, wherein tertiary structure positions of said amino acid residues serine, tyrosine and lysine substantially conform to tertiary structure positions of residues Ser107, Tyr136 and Lys140, respectively, as represented by atomic coordinates in Brookhaven Protein Data Bank Accession Code 1bws.

15. The method of Claim 5, wherein said epimerase binds NADPH.

16. The method of Claim 5, wherein said epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a CLUSTAL alignment program, wherein amino acid residues in said amino acid sequence align with 100% identity with at least about 50% of non-Xaa residues in SEQ ID NO:11.

17. The method of Claim 5, wherein said epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a CLUSTAL alignment program, wherein amino acid residues in said amino acid sequence align with 100% identity with at least about 75% of non-Xaa residues in SEQ ID NO:11.

18. The method of Claim 5, wherein said epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a CLUSTAL alignment program, wherein amino acid residues in said amino acid sequence align with 100% identity with at least about 90% of non-Xaa residues in SEQ ID NO:11.

19. The method of Claim 5, wherein said epimerase comprises an amino acid sequence having at least 4 contiguous amino acid residues that are 100% identical to at least 4 contiguous amino acid residues of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10.

20. The method of Claim 5, wherein said recombinant nucleic acid molecule comprises a nucleic acid sequence comprising at least about 12 contiguous nucleotides having 100% identity with at least about 12 contiguous nucleotides of a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9.

21. The method of Claim 5, wherein said epimerase comprises an amino acid sequence having a motif: Gly-Xaa-Xaa-Gly-Xaa-Xaa-Gly.

22. The method of Claim 5, wherein said recombinant nucleic acid molecule comprises a nucleic acid sequence that is at least about 15% identical to a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, as determined using a Lipman-Pearson method with Lipman-Pearson standard default parameters.

23. The method of Claim 5, wherein said recombinant nucleic acid molecule comprises a nucleic acid sequence that is at least about 20% identical to a nucleic acid

sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, as determined using a Lipman-Pearson method with Lipman-Pearson standard default parameters.

24. The method of Claim 5, wherein said recombinant nucleic acid molecule comprises a nucleic acid sequence that is at least about 25% identical to a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, as determined using a Lipman-Pearson method with Lipman-Pearson standard default parameters.

25. The method of Claim 5, wherein said recombinant nucleic acid molecule comprises a nucleic acid sequence that hybridizes under stringent hybridization conditions to a nucleic acid sequence encoding a GDP-4-keto-6-deoxy-D-mannose epimerase/ reductase.

26. The method of Claim 25, wherein said nucleic acid sequence encoding said GDP-4-keto-6-deoxy-D-mannose epimerase/reductase is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.

27. The method of Claim 25, wherein said GDP-4-keto-6-deoxy-D-mannose epimerase/reductase comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.

28. A method, as claimed in Claim 1, wherein said microorganism is selected from the group consisting of bacteria, fungi and microalgae.

29. A method, as claimed in Claim 1, wherein said microorganism is acid-tolerant.

30. A method, as claimed in Claim 1, wherein said microorganism is a bacterium.

31. A method, as claimed in Claim 30, wherein said bacterium is selected from the group consisting of *Azotobacter* and *Pseudomonas*.

32. A method, as claimed in Claim 1, wherein said microorganism is a fungus.

33. A method, as claimed in Claim 32, wherein said microorganism is a yeast.

34. A method, as claimed in Claim 33, wherein said yeast is selected from the group consisting of *Saccharomyces* yeast.

35. A method, as claimed in Claim 1, wherein said microorganism is a microalga.

36. A method, as claimed in Claim 35, wherein said microalga is selected from the group consisting of microalgae of the genera *Prototheca* and *Chlorella*.

37. A method, as claimed in Claim 36, wherein said microalga is selected from the genus *Prototheca*.

38. A method, as claimed in Claim 1, wherein said microorganism further comprises a genetic modification to decrease the action of an enzyme having GDP-D-mannose as a substrate, other than GDP-D-mannose:GDP-L-galactose epimerase.

39. A method, as claimed in Claim 38, wherein said genetic modification to decrease the action of an enzyme having GDP-D-mannose as a substrate, other than GDP-D-mannose:GDP-L-galactose epimerase is a genetic modification to decrease the action of GDP-D-mannose-dehydrogenase.

40. A method, as claimed in Claim 1, wherein said microorganism is acid-tolerant and said step of culturing is conducted at a pH of less than about 6.0.

41. A method, as claimed in Claim 1, wherein said microorganism is acid-tolerant and said step of culturing is conducted at a pH of less than about 5.5.

42. A method, as claimed in Claim 1, wherein said microorganism is acid-tolerant and said step of culturing is conducted at a pH of less than about 5.0.

43. A method, as claimed in Claim 1, wherein said step of culturing is conducted in a fermentation medium that is magnesium (Mg) limited.

44. A method, as claimed in Claim 1, wherein said step of culturing is conducted in a fermentation medium that is Mg limited during a cell growth phase.

45. A method, as claimed in Claim 1, wherein said step of culturing is conducted in a fermentation medium that comprises less than about 0.5 g/L of Mg during a cell growth phase.

46. A method, as claimed in Claim 1, wherein said step of culturing is conducted in a fermentation medium that comprises less than about 0.2 g/L of Mg during a cell growth phase.

47. A method, as claimed in Claim 1, wherein said step of culturing is conducted in a fermentation medium that comprises less than about 0.1 g/L of Mg during a cell growth phase.

48. A method, as claimed in Claim 1, wherein said step of culturing is conducted in a fermentation medium that comprises a carbon source other than D-mannose.

49. A method, as claimed in Claim 1, wherein said step of culturing is conducted in a fermentation medium that comprises glucose as a carbon source.

50. A microorganism for producing ascorbic acid or esters thereof, wherein said microorganism has a genetic modification to increase the action of an enzyme selected from the group consisting of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-D-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and L-galactono-γ-lactone dehydrogenase.

51. A microorganism, as claimed in Claim 50, wherein said genetic modification is a genetic modification to increase the action of an enzyme selected from the group consisting of GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and L-galactono-γ-lactone dehydrogenase.

52. A microorganism, as claimed in Claim 50, wherein said genetic modification is a genetic modification to increase the action of GDP-D-mannose:GDP-L-galactose epimerase.

53. A microorganism, as claimed in Claim 50, wherein said microorganism has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein said epimerase has a tertiary structure having an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a GDP-4-keto-6-deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

54. A microorganism, as claimed in Claim 50, wherein said microorganism is selected from the group consisting of bacteria, fungi and microalgae.

55. A microorganism, as claimed in Claim 50, wherein said microorganism is a bacterium.

56. A microorganism, as claimed in Claim 55, wherein said bacterium is selected from the group consisting of *Azotobacter* and *Pseudomonas*.

57. A microorganism, as claimed in Claim 50, wherein said microorganism is a fungus.

58. A microorganism, as claimed in Claim 57, wherein said microorganism is a yeast.

59. A microorganism, as claimed in Claim 58, wherein said yeast is selected from the group consisting of *Saccharomyces* yeast.

60. A plant for producing ascorbic acid or esters thereof, wherein said plant has a genetic modification to increase the action of an enzyme selected from the group consisting of hexokinase, glucose phosphate isomerase, phosphomannose isomerase, phosphomannomutase, GDP-D-mannose pyrophosphorylase, GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and L-galactono-γ-lactone dehydrogenase.

61. A plant, as claimed in Claim 60, wherein said genetic modification is a genetic modification to increase the action of an enzyme selected from the group consisting of GDP-D-mannose:GDP-L-galactose epimerase, GDP-L-galactose phosphorylase, L-galactose-1-P-phosphatase, L-galactose dehydrogenase, and L-galactono-γ-lactone dehydrogenase.

62. A plant, as claimed in Claim 60, wherein said genetic modification is a genetic modification to increase the action of GDP-D-mannose:GDP-L-galactose epimerase.

63. A plant, as claimed in Claim 60, wherein said plant has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein said epimerase has a tertiary structure having an average root mean square deviation of less than about 2.5 Å over at least about 25% of Cα positions of the tertiary structure of a GDP-4-keto-6-

deoxy-D-mannose epimerase/reductase represented by atomic coordinates having Brookhaven Protein Data Bank Accession Code 1bws.

64. A plant, as claimed in Claim 60, wherein said plant further comprises a genetic modification to decrease the action of an enzyme having GDP-D-mannose as a substrate other than GDP-D-mannose:GDP-L-galactose epimerase.

65. A plant, as claimed in Claim 60, wherein said genetic modification to decrease the action of an enzyme having GDP-D-mannose as a substrate other than GDP-D-mannose:GDP-L-galactose epimerase is a genetic modification to decrease the action of GDP-D-mannose-dehydrogenase.

66. A plant, as claimed in Claim 60, wherein said plant is a microalga.

67. A plant, as claimed in Claim 66, wherein said plant is selected from the group consisting of microalgae of the genera *Prototheca* and *Chlorella*.

68. A plant, as claimed in Claim 66, wherein said microalga is selected from the genus *Prototheca*.

69. A plant, as claimed in Claim 60, wherein said plant is a higher plant.

70. A plant, as claimed in Claim 60, wherein said plant is a consumable higher plant.

71. A microorganism for producing ascorbic acid or esters thereof, wherein said microorganism has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein said epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a CLUSTAL alignment program, wherein amino acid residues in said amino acid sequence align with 100% identity with at least about 50% of non-Xaa residues in SEQ ID NO:11.

72. A plant for producing ascorbic acid or esters thereof, wherein said plant has been genetically modified to express a recombinant nucleic acid molecule encoding an epimerase that catalyzes conversion of GDP-D-mannose to GDP-L-galactose, wherein said epimerase comprises an amino acid sequence that aligns with SEQ ID NO:11 using a CLUSTAL alignment program, wherein amino acid residues in said amino acid sequence align with 100% identity with at least about 50% of non-Xaa residues in SEQ ID NO:11.

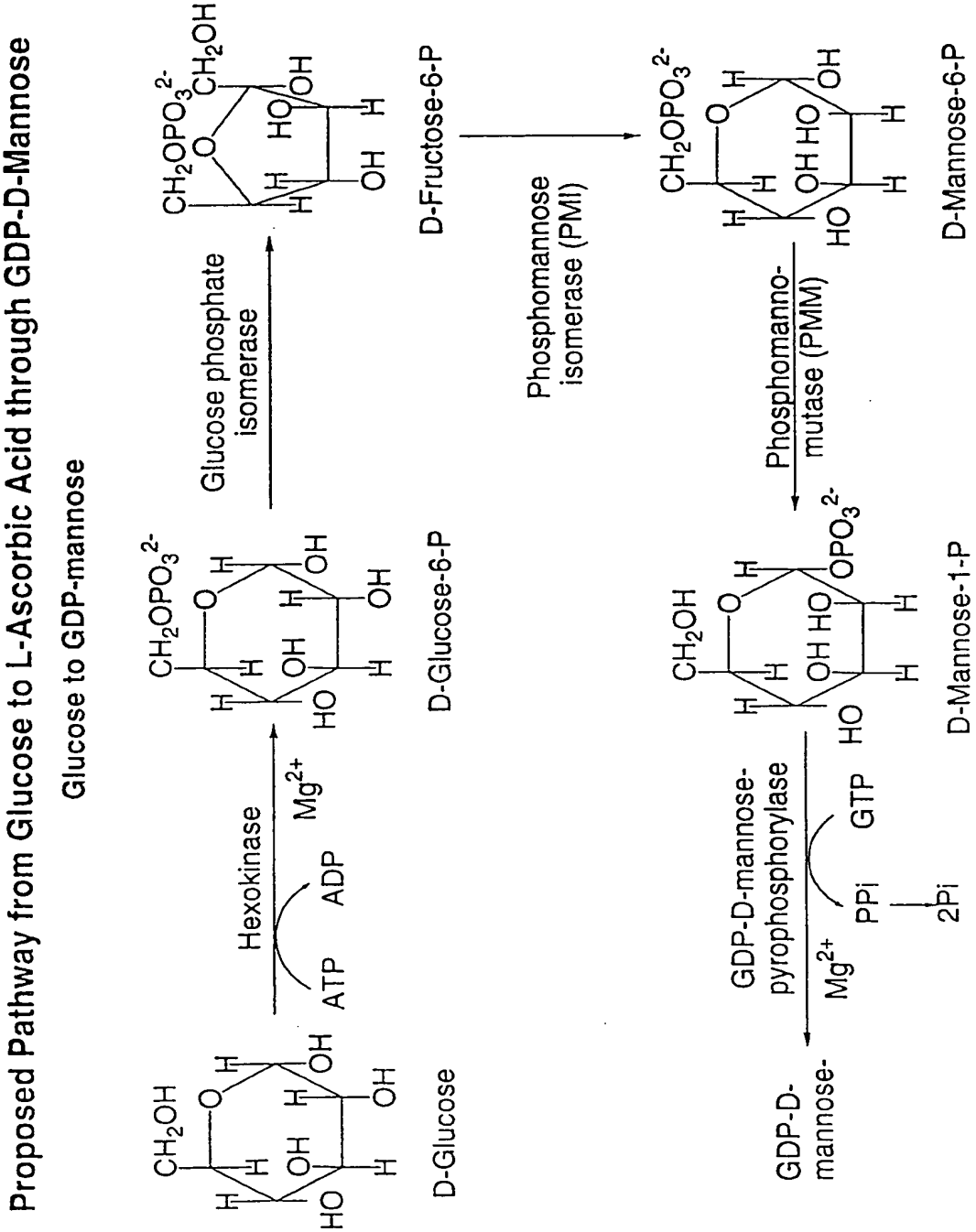
Proposed Pathway from Glucose to L-Ascorbic Acid through GDP-D-Mannose
Glucose to GDP-mannose
D-Glucose
Hexokinase
ATP
ADP
Mg^{2+}
D-Glucose-6-P
Glucose phosphate isomerase
D-Fructose-6-P
Phosphomannose isomerase (PMI)
D-Mannose-6-P
Phosphomanno-mutase (PMM)
D-Mannose-1-P
GDP-D-mannose-pyrophosphorylase
GTP
PPi
2Pi
Mg^{2+}
GDP-D-mannose-

FIG. 1A

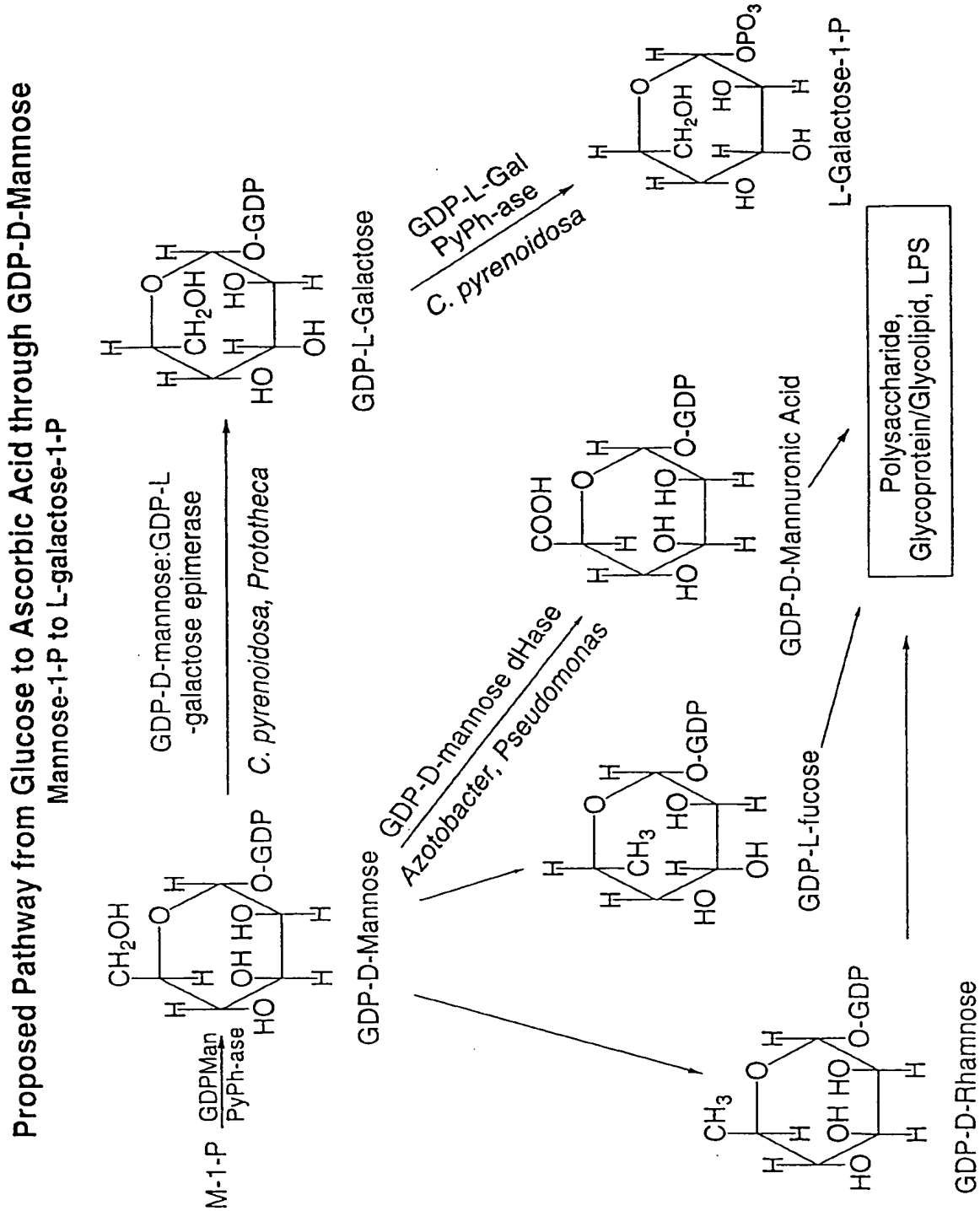
Proposed Pathway from Glucose to Ascorbic Acid through GDP-D-Mannose
Mannose-1-P to L-galactose-1-P
M-1-P
GDPMan
PyPh-ase
GDP-D-Mannose
GDP-D-mannose:GDP-L
-galactose epimerase
C. pyrenoidosa, Prototheca
GDP-L-Galactose
GDP-L-Gal
PyPh-ase
C. pyrenoidosa
L-Galactose-1-P
GDP-D-mannose dHase
Azotobacter, Pseudomonas
GDP-D-Mannuronic Acid
GDP-L-fucose
Polysaccharide,
Glycoprotein/Glycolipid, LPS
GDP-D-Rhamnose

FIG. 1B

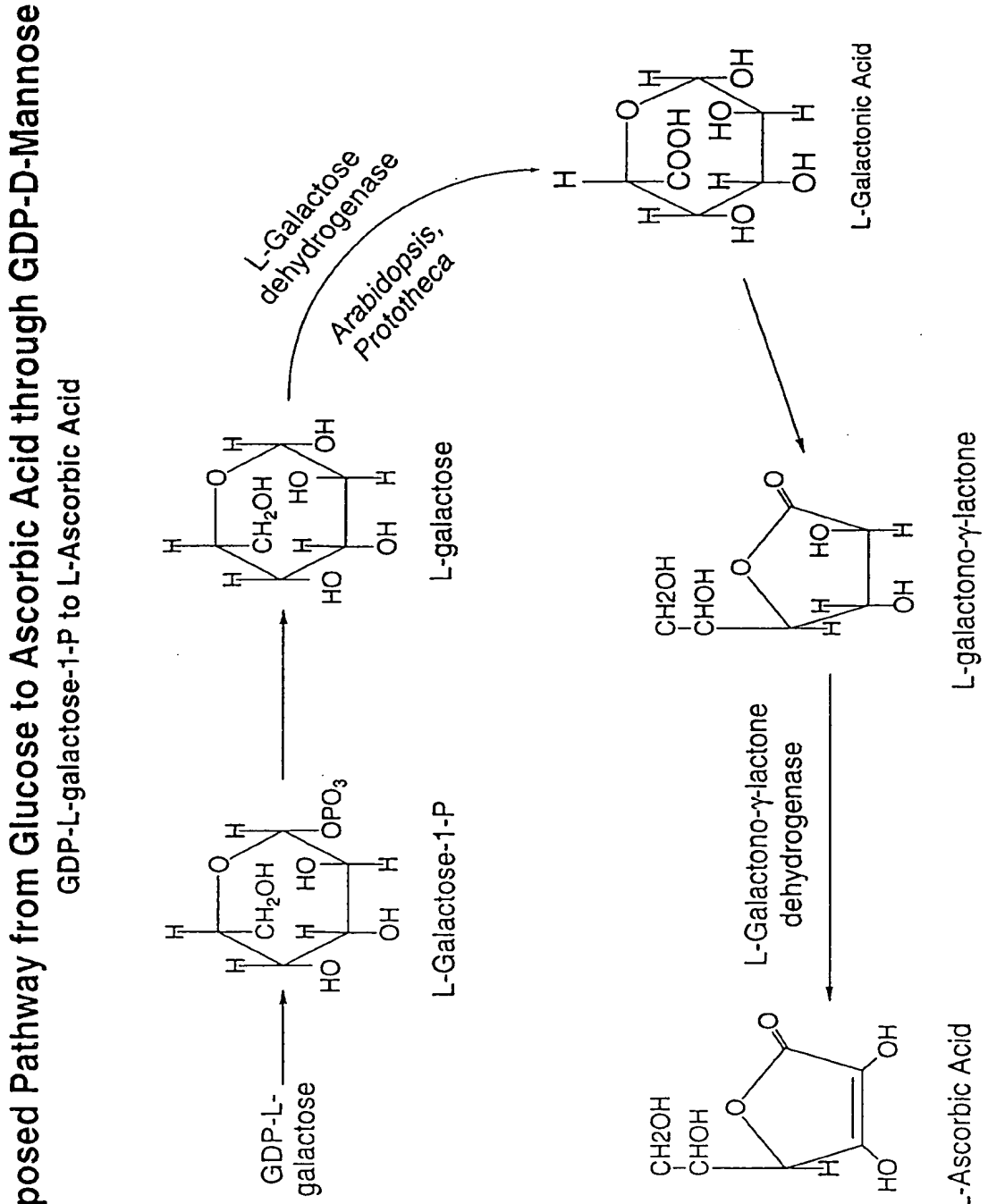
Proposed Pathway from Glucose to Ascorbic Acid through GDP-D-Mannose
GDP-L-galactose-1-P to L-Ascorbic Acid
GDP-L-galactose
L-Galactose-1-P
L-galactose
L-Galactose dehydrogenase
Arabidopsis, Prototheca
L-Galactonic Acid
L-Galactono-γ-lactone dehydrogenase
L-galactono-γ-lactone
L-Ascorbic Acid

FIG. 1C

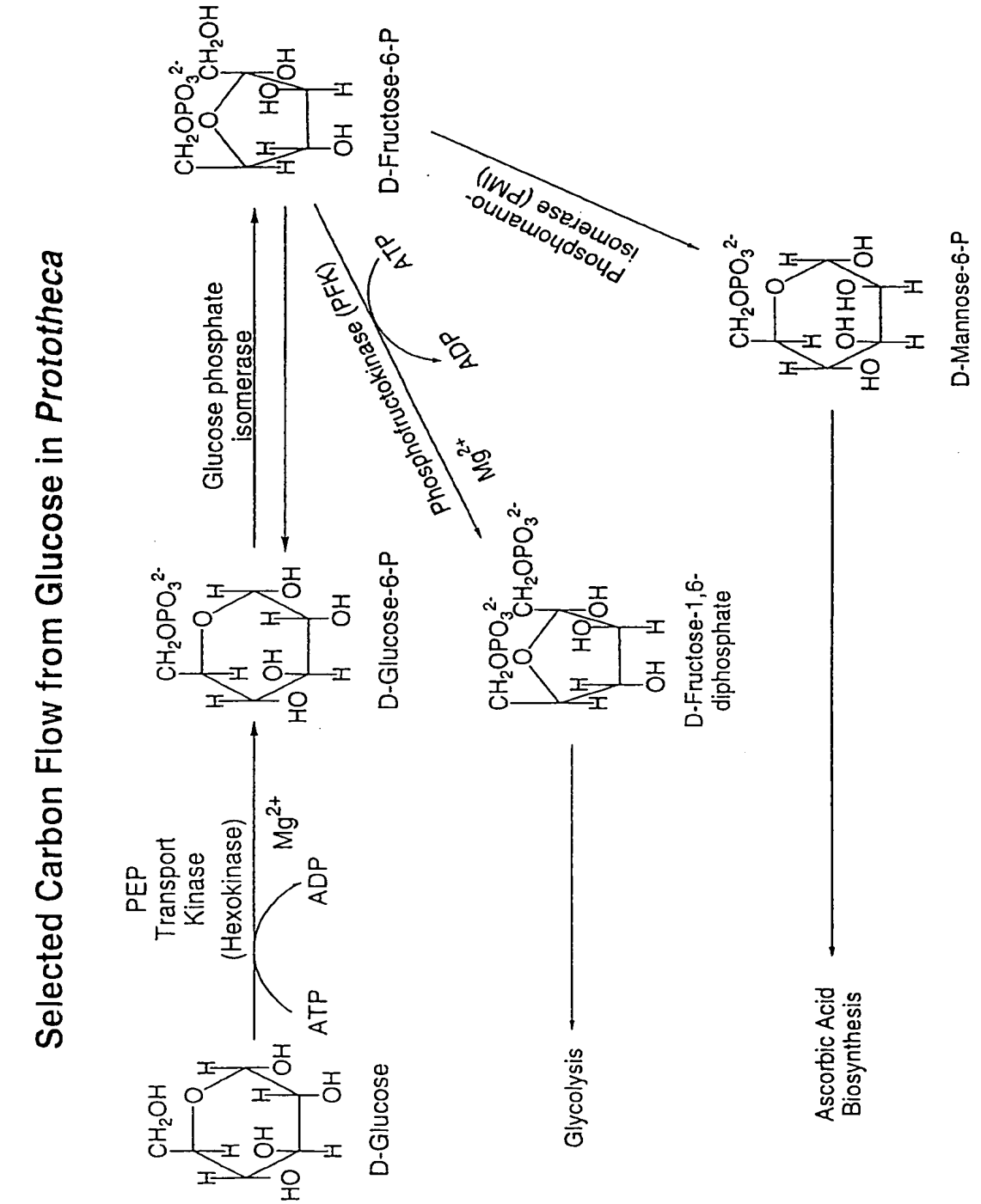
Selected Carbon Flow from Glucose in *Prototheca*
D-Glucose
PEP
Transport
Kinase
(Hexokinase)
ATP
ADP
Mg^{2+}
D-Glucose-6-P
Glucose phosphate isomerase
D-Fructose-6-P
Phosphofructokinase (PFK)
ATP
ADP
Mg^{2+}
D-Fructose-1,6-diphosphate
Glycolysis
Phosphomanno-isomerase (PMI)
D-Mannose-6-P
Ascorbic Acid Biosynthesis

FIG. 2A

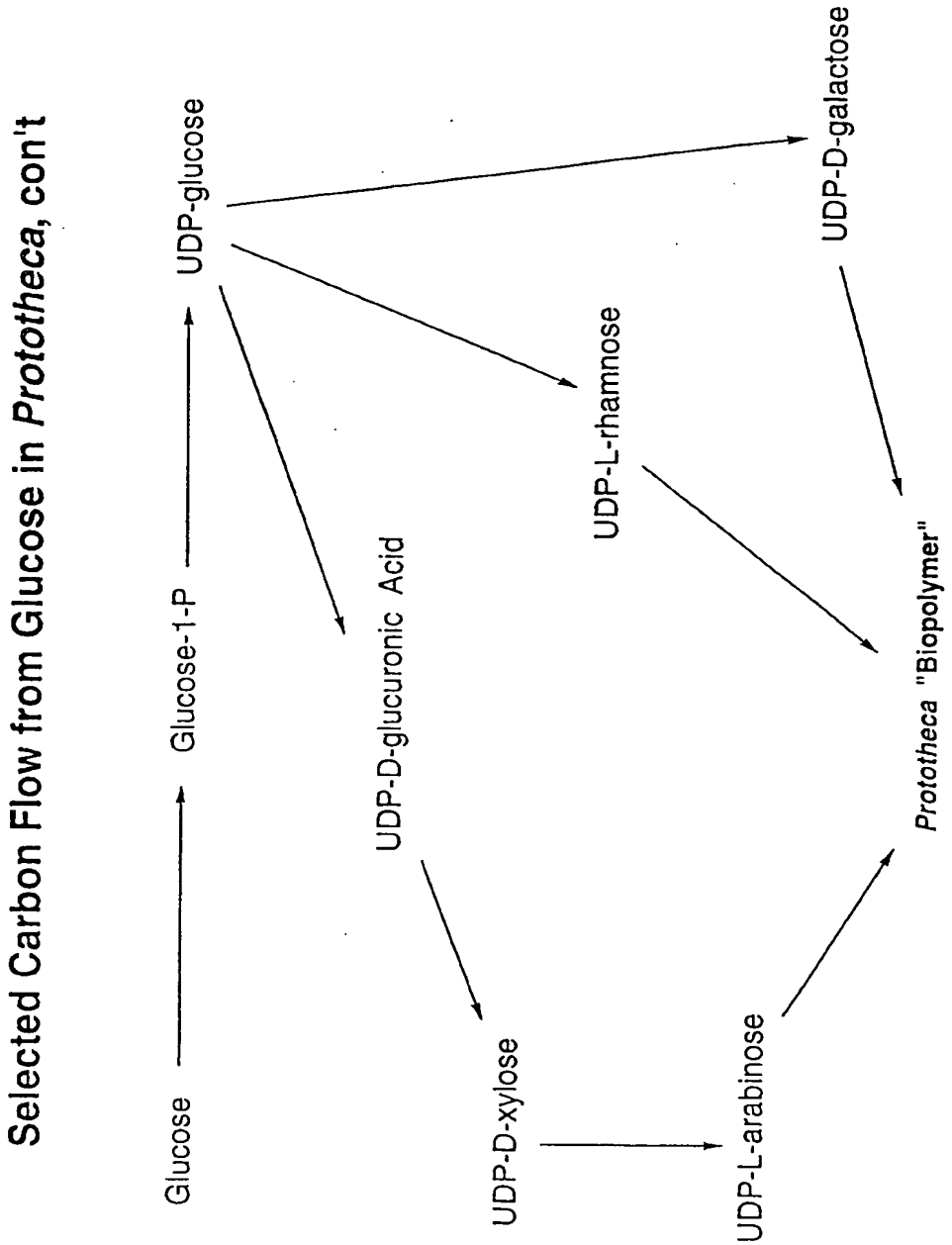
Selected Carbon Flow from Glucose in *Prototheca*, con't
Glucose
Glucose-1-P
UDP-glucose
UDP-D-galactose
UDP-L-rhamnose
UDP-D-glucuronic Acid
UDP-D-xylose
UDP-L-arabinose
Prototheca "Biopolymer"

FIG. 2B

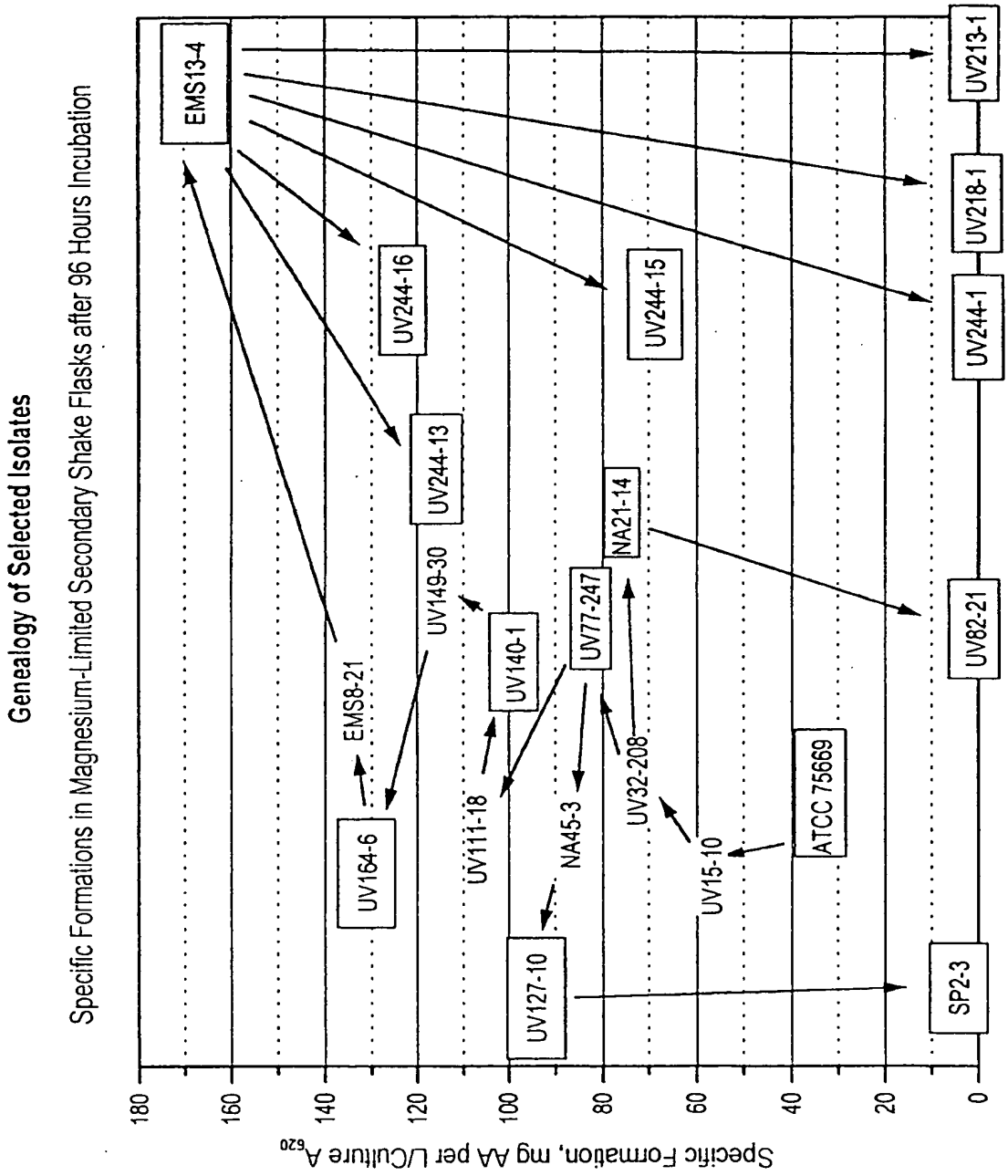
Genealogy of Selected Isolates
Specific Formations in Magnesium-Limited Secondary Shake Flasks after 96 Hours Incubation
Specific Formation, mg AA per L/Culture A_{620}
180
160
140
120
100
80
60
40
20
0
EMS13-4
UV213-1
UV218-1
UV244-1
UV82-21
SP2-3
UV244-16
UV244-13
UV244-15
NA21-14
UV149-30
UV140-1
UV77-247
EMS8-21
UV164-6
UV111-18
NA45-3
UV32-208
UV15-10
ATCC 75669
UV127-10

FIG. 3

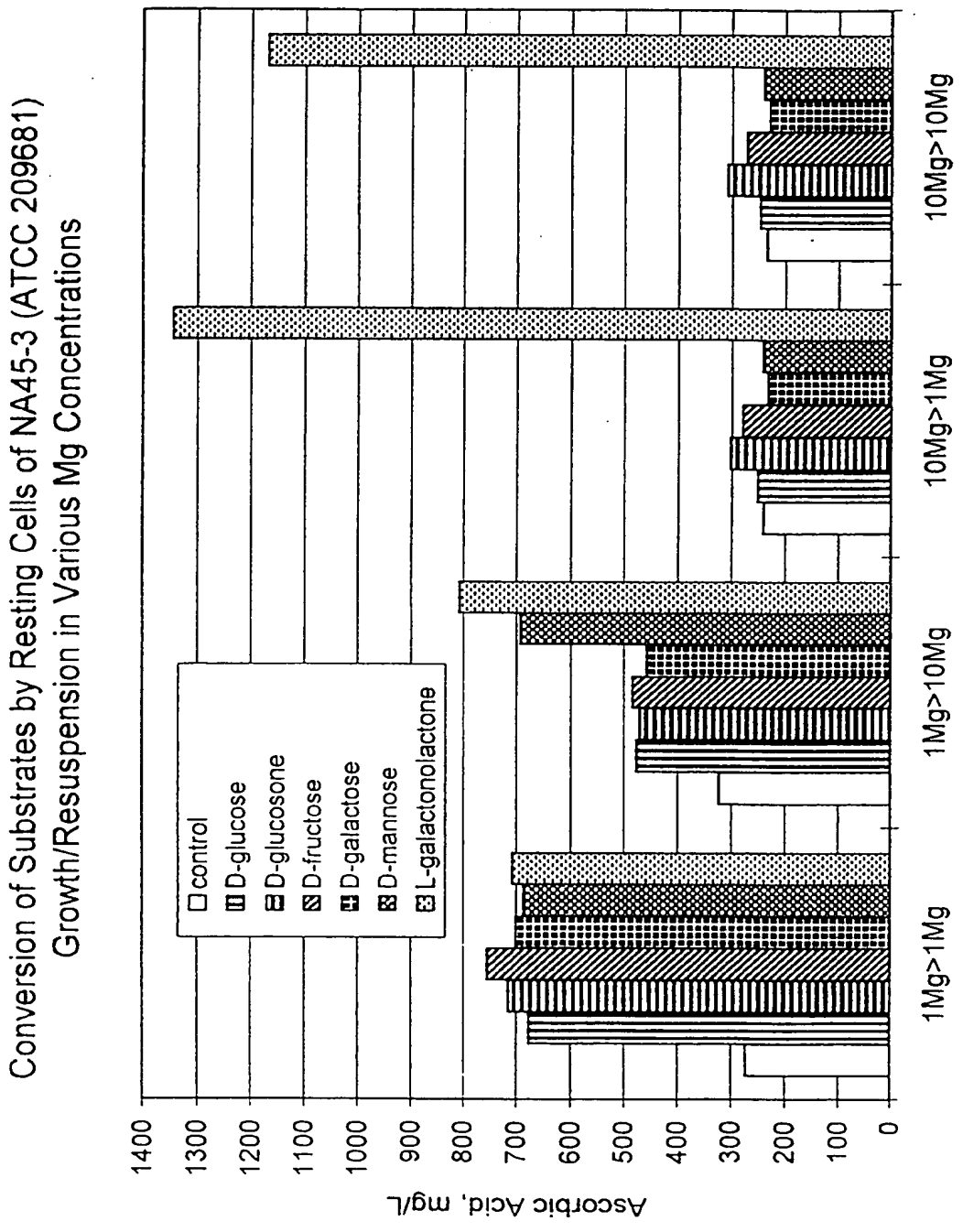
Conversion of Substrates by Resting Cells of NA45-3 (ATCC 209681)
Growth/Resuspension in Various Mg Concentrations
control
D-glucose
D-glucosone
D-fructose
D-galactose
D-mannose
L-galactonolactone
Ascorbic Acid, mg/L
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1Mg>1Mg
1Mg>10Mg
10Mg>1Mg
10Mg>10Mg
Growth>Resuspnsion [Mg]

Fig. 4

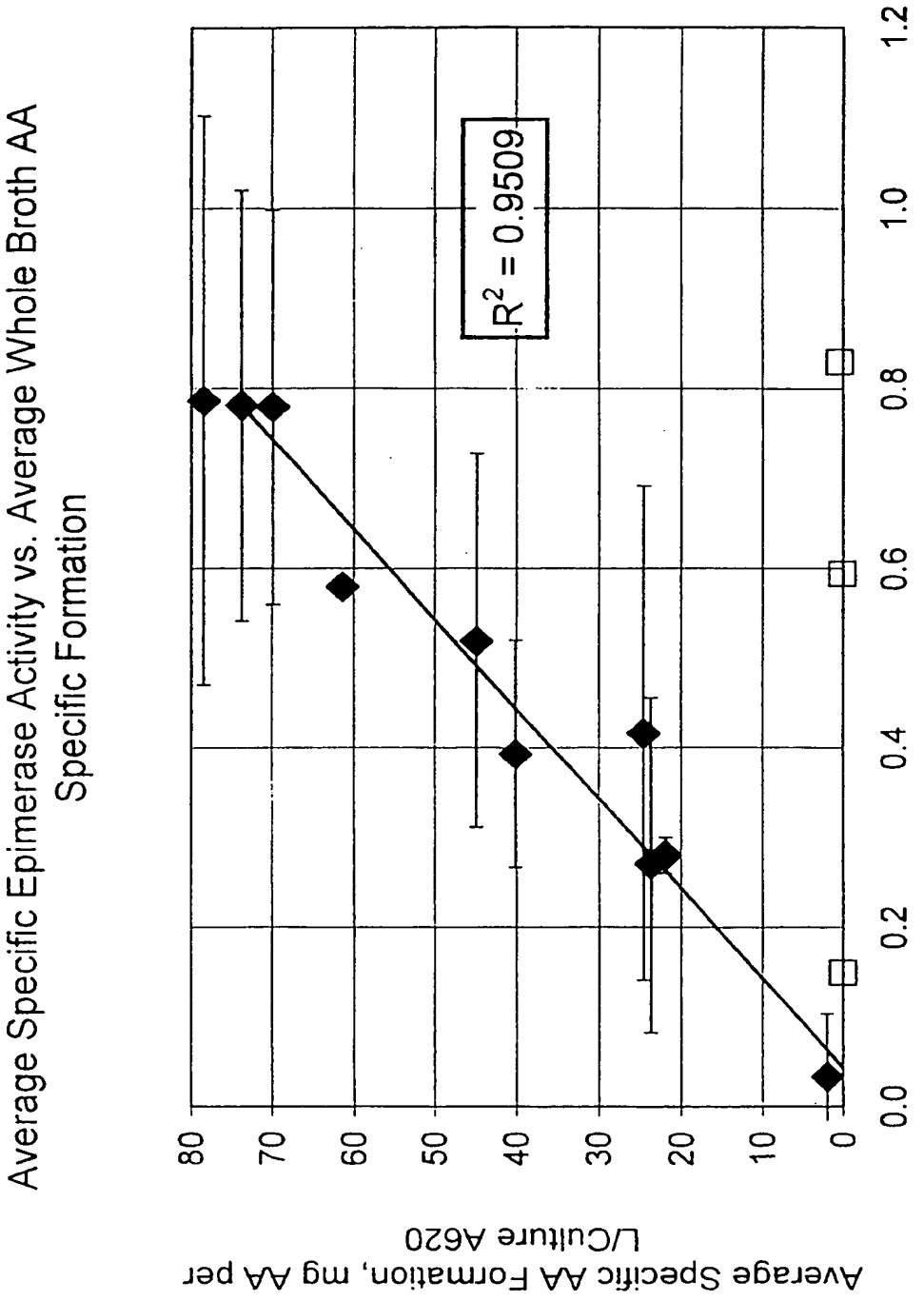
Average Specific Epimerase Activity vs. Average Whole Broth AA Specific Formation
R^2 = 0.9509
Average Specific AA Formation, mg AA per L/Culture A620
0
10
20
30
40
50
60
70
80
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Average Specific Epimerase Activity, mU

Fig. 5

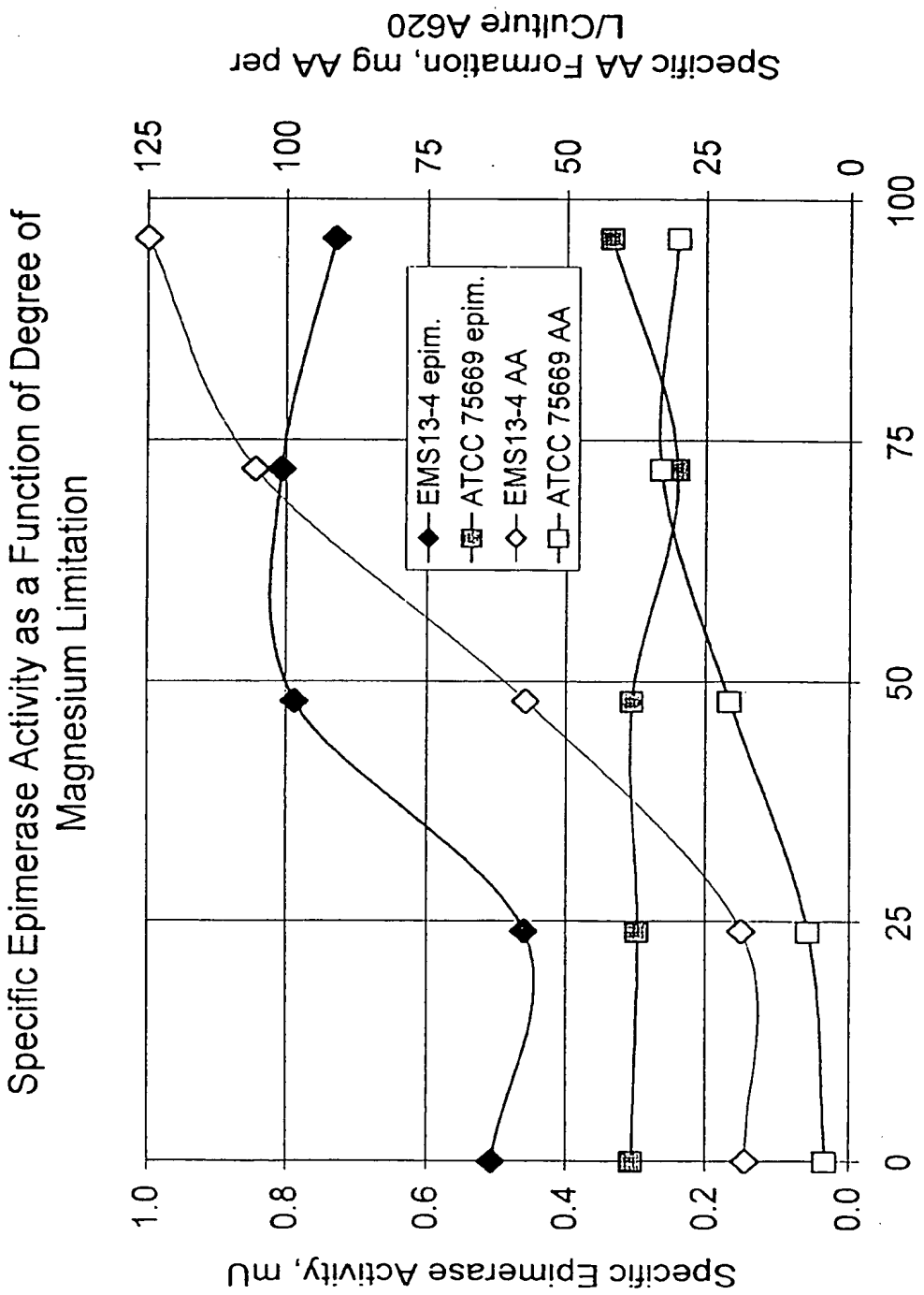
Specific Epimerase Activity as a Function of Degree of Magnesium Limitation
Specific Epimerase Activity, mU
1.0
0.8
0.6
0.4
0.2
0.0
Specific AA Formation, mg AA per L/Culture A620
125
100
75
50
25
0
Hours of Mg-limiting Culture
0
25
50
75
100
EMS13-4 epim.
ATCC 75669 epim.
EMS13-4 AA
ATCC 75669 AA

Fig. 6