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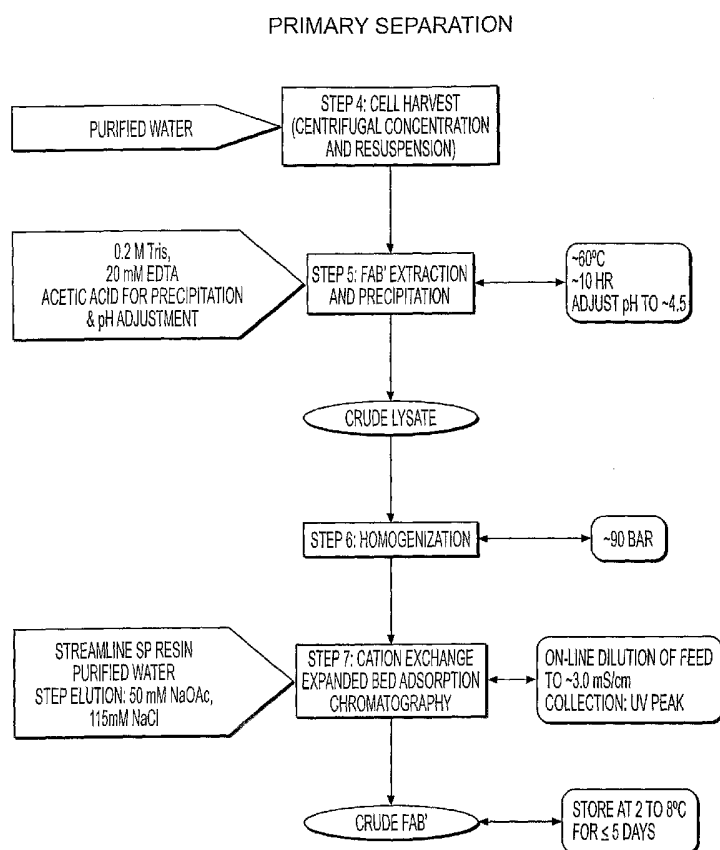
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(54) Title: METHOD FOR THE PREPARATION OF MOLECULES HAVING ANTIBODY ACTIVITY



(57) Abstract: The present invention provides a process for the preparation of an antibody, wherein the method comprises: fermenting a cell mixture comprising the cells and a supernatant solution, wherein the cells are capable of expressing the light chain and the heavy chain; separating the cells from the supernatant solution to form a cell pellet; allowing the cell pellet to stand for a hold time; and extracting the cell pellet with an extracting solution; thereby producing the antibody.



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METHOD FOR THE PREPARATION OF MOLECULES
HAVING ANTIBODY ACTIVITY

BACKGROUND OF THE INVENTION

5

Field of the Invention

This invention relates to the preparation of molecules having antibody activity.

10 Description of Related Art

Rheumatoid arthritis (herein, "RA") is a crippling autoimmune condition which is characterized by synovial membrane proliferation and degradation of articular cartilage and subchondral bone. The condition is further characterized by increased levels of TNF-alpha in both the synovial fluid and peripheral blood of the patient.

Inflammatory Bowel Disease (herein, "IBD") is a term which encompasses a variety of chronic inflammatory conditions of the large and small intestines. One such condition is Crohn's Disease, which can manifest symptoms such as inflammation of the bowel wall and can result in abscesses and fistulae (Goodman & Gilman's The Pharmacological Basis of Therapeutics, 10th Ed., J.G. Hardman and L.E. Limbird, eds., McGraw-Hill, New York, 2001, pp. 1047-8). TNF-alpha is expressed in the inflamed tissues associated with IBD.

RA and IBD have each been treated using methods designed to suppress or antagonize TNF-alpha. For example, infliximab is a monoclonal antibody to TNF-alpha which has been reported to be useful to treat either RA (id., p. 1475) or IBD (id., p. 1053). Another TNF-alpha monoclonal antibody which has been reported to be useful for the treatment of RA and IBD is etanercept.

35 A drawback to the therapeutic use of etanercept is that it requires a relatively frequent (every two weeks)

subcutaneous injection. A drawback to the therapeutic use of infliximab is that its use requires intravenous administration at a physician's office. In addition, because both infliximab and etanercept are large
5 proteins, their manufacture is difficult and expensive.

PCT Patent Application No. WO 01/94585 describes a molecule having antibody activity which is specific for human TNF-alpha and wherein the molecule comprises a TNF-alpha monoclonal antibody fragment (Fab') and
10 further comprising a methoxypolyethyleneglycolated (PEGylated) portion covalently attached to the Fab' by a linker moiety. That application further describes methods of preparation and methods of using such a molecule. Such molecules are useful for the treatment
15 or prevention of RA and of IBD.

The continued interest in the therapeutic uses of TNF-alpha antibodies indicates a strong need for discovery of new TNF-alpha molecules and for new methods to economically manufacture them.

20

Summary of the Invention

Among the several embodiments of the present invention may be noted the provision of a process for the preparation of an antibody or an antibody fragment
25 or a dimer or adduct thereof wherein the method comprises: fermenting a cell mixture comprising the cells and a supernatant solution, wherein the cells are capable of expressing the light chain and the heavy chain; separating the cells from the supernatant
30 solution to form a cell pellet; allowing the cell pellet to stand for a hold time; and extracting the cell pellet with an extracting solution; thereby producing

the antibody or antibody fragment or a dimer or an adduct thereof.

Further scope of the applicability of the present
5 invention will become apparent from the detailed
description provided below. However, it should be
understood that the following detailed description and
examples, while indicating embodiments of the invention,
are given by way of illustration only since various
10 changes and modifications within the spirit and scope of
the invention will become apparent to those skilled in
the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1. Comparison of framework regions of light
chain of antibody hTNF-40 and human group 1 consensus
sequences.

Figure 2. Comparison of framework regions of heavy
20 chain of antibody hTNF-40 and human group 1 and group 3
consensus sequences.

Figure 3. Sequence of CDRs of hTNF-40.

25 Figure 4. Schematic of vector pMR 15.1.

Figure 5. Schematic of vector pMR14.

Figure 6. The nucleotide and predicted amino acid
30 sequence of the murine hTNF40VI (SEQ ID NO: 99).

Figure 7. The nucleotide and predicted amino acid
sequence of the murine hTNF40Vh (SEQ ID NO: 100).

Figure 8. The nucleotide and predicted amino acid sequence of hTNF46- gLI (SEQ ID NO: 8).

5 Figure 9. The nucleotide and predicted amino acid sequence of hTNF40-gL2 (SEQ ID NO:9).

Figure 10. The nucleotide and predicted amino acid sequence of ghlhTNF40.4 (SEQ ID NO: 10).

10

Figure 11. The nucleotide and predicted amino acid sequence of gh3hTNF40.4 (SEQ ID NO: 11).

Figure 12. Schematic of vector CTIL5-gL6.

15

Figure 13. The structure of a compound called CDP-870 comprising a modified Fab' fragment derived from antibody hTNF40 covalently linked via a cysteine residue to a lysyl-maleimide linker wherein each amino group on
20 the lysyl residue has covalently attached to it a methoxy PEG residue wherein n is about 420.

Figure 14. Schematic of vector pTTQ9.

25 Figure 15. The sequence of the OmpA oligonucleotide adapter (SEQ ID NO: 10 1).

Figure 16. Schematic of vector pACYC 184.

30 Figure 17. Schematic of vector pTTO-1.

Figure 18. Schematic of vector pTTO-2.

Figure 19. Schematic of vector pDNAbEng-G1.

Figure 20. The oligonucleotide cassettes encoding
different intergenic sequences for E. coli modified Fab'
5 expression (SEQ ID NOS: 102 to 105).

Figure 21. Graph of the periplasmic modified Fab'
accumulation of IGS variants.

10 Figure 22. Schematic of vector pTTO(CDP-870).

Figure 23. Flow chart for the fermentation step in the
synthesis of CDP-870 Fab'-PEG.

15 Figure 24. Flow chart for the primary separation step
in the synthesis of CDP-870 Fab'-PEG.

Figure 25. Effect of pellet hold time on Fab' recovery.

20

DETAILED DESCRIPTION

The following detailed description is provided to
aid those skilled in the art in practicing the present
invention. Even so, this detailed description should
25 not be construed to unduly limit the present invention
as modifications and variations in the embodiments
discussed herein can be made by those of ordinary skill
in the art without departing from the spirit or scope of
the present inventive discovery.

30 The contents of each of the references cited
herein, including the contents of the references cited
within these primary references, are herein incorporated
by reference in their entirety.

a. Definitions

The following definitions are provided in order to aid the reader in understanding the detailed description of the present invention:

"AEX" as used herein means anion exchange chromatography.

The term "antibody" as used herein means an antigen-binding protein and includes complete antibody molecules, antibody fragments, chimeric antibodies, chimeric antibody fragments, and chemically modified antibodies or antibody fragments (e.g., PEGylated).

"CDP-870 Fab'-PEG" as used herein is used interchangeably with the term "CDP-870" and means the CDP-870 molecule described in PCT Patent Application No. WO 01/94585.

"CDR" means complementary determining region.

"CEX" as used herein means cation exchange chromatography.

"EBA" as used herein means expanded bed adsorption or expanded bed adsorption chromatography.

"EDTA" as used herein means ethylenediaminetetraacetic acid.

"Fab'" as used herein means an antibody fragment having a heavy chain and a light chain. When used in the context of CDP-870, "Fab'" means a TNF-alpha antibody wherein the heavy chain of the antibody 5' part has the sequence given as SEQ ID NO: 115 and the light chain has the sequence given in SEQ ID NO: 113. In the context of CDP-870, this term is used interchangeably with the term "CDP-870 Fab'."

"F(ab')₂" means a dimer of Fab'.

"Fab' Species" as used herein means any or all of Fab', F(ab')₂, Fab' adducts, and any other species that can be reduced to form Fab'

"HCP" as used herein means host cell proteins.

5 "HIC" as used herein means hydrophobic interaction chromatography.

"NaOAc" as used herein means sodium acetate.

"OD₆₀₀" as used herein means optical density at 600 nanometers.

10 "PEG" as used herein means polyethylene glycol in which one terminal hydroxyl group is optionally capped with a methyl (i.e., methoxy(polyethyleneglycol)).

"PEGylated" as used herein means having a chemical moiety containing PEG attached thereto.

15 "Tris" as used herein means tris(hydroxymethyl)-aminomethane.

b. Process Details

20

In accordance with the present invention, a process has been discovered for economically producing an antibody (including Fab' and F(ab')₂). In one embodiment, the present invention provides a method for
25 the production of a molecule having antibody activity which is specific for human TNF-alpha. For example, the present invention provides methods for the production of antibody molecules wherein the molecule comprises a TNF-alpha monoclonal antibody fragment (Fab') and further
30 comprising a methoxypolyethyleneglycolated (PEGylated) portion covalently attached to the Fab' by a linker moiety. Particularly, the present application describes a method for the preparation of such a molecule known as

CDP-870. CDP-870 is described in PCT Patent Application No. WO 01/94585 (U.S. Patent Application No. 09/875,221, herein incorporated by reference).

In one embodiment, the present invention provides a method of preparing an antibody molecule or antibody molecule fragment having specificity for TNF-alpha, wherein the molecule or fragment comprises a heavy chain having a variable domain comprising a CDR having the sequence given as H1 in Figure 3 (SEQ ID NO: 1) for CDRH1, as H2' in Figure 3 (SEQ ID NO:2) or as H2 in Figure 3 (SEQ ID NO:7) for CDRH2 or as H3 in Figure 3 (SEQ ID NO:3) for CDRH3. The antibody molecule or fragment comprises at least one CDR selected from H 1, H2' or H2 and H3 (SEQ ID NO: 1; SEQ ID NO:2 or SEQ ID NO:7 and SEQ ID NO:3) for the heavy chain variable domain. In one embodiment, the antibody molecule comprises at least two or all three CDRs in the heavy chain variable domain.

The present invention further provides a method of preparing an antibody molecule having specificity for human TNF-alpha, wherein the antibody molecule comprises a light chain having a variable domain which comprises a CDR having the sequence given as L1 in Figure 3 (SEQ ID NO:4) for CDRL1, L2 in Figure 3 (SEQ ID NO:5) for CDRL2 or L3 in Figure 3 (SEQ ID NO:6) for CDRL3. This antibody molecule or fragment comprises at least one CDR selected from L1, L2 and L3 (SEQ ID NO:4 to SEQ ID NO:6) for the light chain variable domain. In one embodiment, the antibody molecule comprises at least two or all three CDRs in the light chain variable domain.

In one embodiment, the antibody molecules or fragments have a complementary light chain or a complementary heavy chain, respectively.

In one embodiment, the antibody molecule or
5 fragments comprise a heavy chain wherein the variable domain comprises a CDR having the sequence given as H1 in Figure (SEQ ID NO: 1) for CDRH1, as H2' or H2 in Figure 3 (SEQ ID NO:2 or SEQ ID NO:7) for CDRH2 or as H3 in Figure 3 (SEQ ID NO:3) for CDRH3 and a light chain
10 wherein the variable domain comprises a CDR having the sequence given as LI in Figure 3 (SEQ ID NO:4) for CDRL1, as L2 in Figure 3 (SEQ ID NO:5) for CDRL2 or as L3 in Figure 3 (SEQ ID NO:6) for CDRL3.

The CDRs given in SEQ IDS NOS: 1 and 3 to 7 and in
15 Figure 3 referred to above are derived from a mouse monoclonal antibody hTNF40. However, SEQ ID NO:2 comprises a hybrid CDR. The hybrid CDR comprises part of heavy chain CDR2 from mouse monoclonal antibody hTNF40 (SEQ ID NO:7) and part of heavy chain CDR2 from a human
20 group 3 germline V region sequence.

The complete sequences of the variable domains of the mouse hTNF40 antibody 10 are shown in Figures 6 (light chain) (SEQ ID NO:99) and Figure 7 (heavy chain) (SEQ ID NO: 100). This mouse antibody is referred to
25 below as "the donor antibody".

In an alternative embodiment, the present invention provides a method of preparing a mouse monoclonal antibody hTNF40 having the light and heavy chain variable domain sequences shown in Figure 6 (SEQ ID
30 NO:99) and Figure 7 (SEQ ID NO: 100), respectively. The

light chain constant region of hTNF40 is kappa and the heavy chain constant region is IgG2a.

In another embodiment, the antibody is a chimeric mouse/human antibody molecule or fragment, referred to
5 herein as the chimeric hTNF40 antibody molecule. The chimeric antibody molecule comprises the variable domains of the mouse monoclonal antibody hTNF40 (SEQ ID NOS:99 and 100) and human constant domains. In another embodiment, the chimeric hTNF40 antibody molecule
10 comprises the human C kappa domain (Hieter et al., Cell, 22, 197-207, 1980; Genbank accession number J00241) in the light chain and the human gamma 4 domains (Flanagan et al., Nature, 300, 709-713, 1982) in the heavy chain.

In another embodiment, the antibody is a CDR-
15 grafted antibody molecule or fragment. The term "a CDR-grafted antibody molecule" as used herein refers to an antibody molecule wherein the heavy and/or light chain contains one or more CDRs (including, if desired, a hybrid CDR) from the donor antibody (e.g. a murine
20 monoclonal antibody) grafted into a heavy and/or light chain variable region framework of an acceptor antibody (e.g. a human antibody).

In one embodiment, such a CDR-grafted antibody has a variable domain comprising human acceptor framework
25 regions as well as one or more of the donor CDRs referred to above.

When the CDRs are grafted, any appropriate acceptor variable region framework sequence may be used having regard to the class or type of the donor antibody from
30 which the CDRs are derived, including mouse, primate and human framework regions. Examples of human frameworks

which can be used in the present invention are KOL, NEWM, REI, EU, TUR, TEI, LAY and POM. For example, KOL and NEWM can be used for the heavy chain, REI can be used for the light chain and EU, LAY and POM can be used
5 for both the heavy chain and the light chain. Useful framework regions for the light chain include the human group I framework regions shown in Figure 1 (SEQ ID NOS:83, 85, 87 and 89). Useful framework regions for the heavy chain include the human group 1 and group 3
10 framework regions shown in Figure 2 (SEQ ID NOS:91, 93, 95 and 97 and SEQ ID NOS: 106, 107, 108 and 109), respectively.

In a CDR-grafted antibody, it is possible to use an acceptor antibody having chains which are homologous to
15 the chains of the donor antibody. The acceptor heavy and light chains do not necessarily need to be derived from the same antibody and may, if desired, comprise composite chains having framework regions derived from different chains.

20 In the CDR-grafted antibody molecule of the present invention, if the acceptor heavy chain has human group I framework regions (shown in Figure 2) (SEQ ID NOS:91, 93, 95 and 97), then the acceptor framework regions of the heavy chain comprise, in addition to one or more
25 donor CDRs, donor residues at positions 28, 69 and 71.

Alternatively, if the acceptor heavy chain has group I framework regions, then the acceptor framework regions of the heavy chain comprise, in addition to one or more donor CDRs, donor residues at positions 28, 38,
30 46, 67, 69 and 71.

Alternatively, in the CDR-grafted antibody molecule, if the acceptor heavy chain has human group 3 framework regions (shown in Figure 2) (SEQ ID NOS:106, 107, 108 and 109), then the acceptor framework regions of the heavy chain comprise, in addition to one or more donor CDRs, donor residues at positions 27, 28, 30, 48, 49, 69, 71, 73, 76 and 78.

Alternatively, in a CDR-grafted antibody molecule, if the acceptor light chain has human group I framework regions (shown in Figure 1) (SEQ ID NOS:83, 85, 87 and 89) then the acceptor framework regions of the light chain comprise donor residues at positions 46 and 60.

Donor residues are residues from the donor antibody, i.e. the antibody from which the CDRs were originally derived.

The antibody molecule (whether a TNF-alpha antibody or another antibody) made by the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, modified Fab, Fab', F(ab')₂ or Fv fragment; a light chain or heavy chain monomer or dimer; a single chain antibody, e.g. a single chain Fv in which the heavy and light chain variable domains are joined by a peptide linker. Similarly, the heavy and light chain variable regions may be combined with other antibody domains as appropriate.

In one embodiment the antibody molecule made by the present method is a Fab' (i.e., an antibody fragment).

For example the Fab' can comprise a heavy chain having the sequence given as SEQ ID NO: 111 and a light chain having the sequence given as SEQ ID NO: 113. The

amino acid sequences given in SEQ ID NO: 111 and SEQ ID NO: 113 can be encoded by the nucleotide sequences given in SEQ ID NO: 110 and SEQ ID NO: 112, respectively.

Alternatively, the antibody molecule made by the present invention can comprise a modified Fab' fragment wherein the modification is the addition to the C-terminal end of its heavy chain one or more amino acids to allow the attachment of an effector or reporter molecule. In one embodiment, the additional amino acids form a modified hinge region containing one or two cysteine residue to which the effector or reporter molecule may be attached. In one embodiment, such a modified Fab' fragment may have has a heavy chain having the sequence given as SEQ ID NO: 115 and the light chain having the sequence given as SEQ ID NO: 113. The amino acid sequence given in SEQ ID NO: 115 can be encoded by the nucleotide sequence given in SEQ ID NO: 114.

A useful effector group is a polymer molecule, which may be attached to the modified Fab' fragment to increase its half-life in vivo.

The polymer molecule may, in general, be a synthetic or a naturally occurring polymer, for example an optionally substituted straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymer or a branched or unbranched polysaccharide, e.g. a homo- or hetero- polysaccharide.

Particular optional substituents which may be present on the above-mentioned synthetic polymers include one or more hydroxy, methyl or methoxy groups. Particular examples of synthetic polymers include optionally substituted straight or branched chain

poly(ethyleneglycol), poly(propyleneglycol)
poly(vinylalcohol) or derivatives thereof, especially
optionally substituted poly(ethyleneglycol) such as
methoxypoly(ethyleneglycol) or derivatives thereof.

5 Particular naturally occurring polymers include amylose,
dextran, glycogen or derivatives thereof. Alternatively,
a monomer such as lactose can be used. "Derivatives" as
used herein is intended to include reactive derivatives,
for example thiol-selective reactive groups such as
10 maleimides and the like. The reactive group may be
linked directly or through a linker segment to the
polymer. It will be appreciated that the residue of such
a group will in some instances form part of the product
as the linking group between the antibody fragment and
15 the polymer.

The size of the polymer may be varied as desired,
but will generally be in an average molecular weight
range from 500 Da to 50,000 Da, for example from 5000 to
40,000 Da and in another embodiment from 25,000 to
20 40,000 Da. The polymer size may in particular be
selected on the basis of the intended use of the
product. Thus, for example, where the product is
intended to leave the circulation and penetrate tissue,
for example for use in the treatment of a tumor, it may
25 be advantageous to use a small molecular weight polymer,
for example with a molecular weight of around 5000 Da.
For applications where the product remains in the
circulation, it may be advantageous to use a higher
molecular weight polymer, for example having a molecular
30 weight in the range from 25,000 Da to 40,000 Da.
Particularly useful polymers include a polyalkylene
polymer, such as a poly(ethyleneglycol) or, especially,
a methoxypoly(ethyleneglycol) or a derivative thereof,

and especially with a molecular weight in the range from about 25,000 Da to about 40,000 Da. Each polymer molecule attached to the modified antibody fragment may be covalently linked to the sulphur atom of a cysteine
5 residue located in the fragment. The covalent linkage will generally be a disulphide bond or, in particular, a sulphur-carbon bond.

Where desired, the antibody fragment may have one or more effector or reporter molecules attached to it.
10 The effector or reporter molecules may be attached to the antibody fragment through any available amino acid side-chain or terminal amino acid functional group located in the fragment, for example any free amino, imino, hydroxyl or carboxyl group.

15 An activated polymer may be used as the starting material in the preparation of polymer-modified antibody fragments as described above. The activated polymer may be any polymer containing a thiol reactive group such as an α -halocarboxylic acid or ester, e.g. iodoacetamide,
20 an imide, e.g. maleimide, a vinyl sulphone or a disulphide. Such starting materials may be obtained commercially (for example from Nektar Therapeutics, Huntsville, AL, USA) or may be prepared from commercially available starting materials using
25 conventional chemical procedures.

Where it is desired to obtain an antibody fragment linked to an effector or reporter molecule, this may be prepared by standard chemical or recombinant DNA
procedures in which the antibody fragment is linked
30 either directly or via a coupling agent to the effector or reporter molecule either before or after reaction with the activated polymer as appropriate. Particular

chemical procedures include for example, those described in each of the following individual references:

WO 93/62331.

WO 92/22583.

5 WO 90/00195.

WO 89/01476.

Alternatively, where the effector or reporter molecule is a protein or polypeptide the linkage may be achieved using recombinant DNA procedures, for example
10 as described in WO 86/01533. Another example is described in EP-A 0392745.

In one embodiment, the modified Fab' fragment of the present invention is PEGylated (i.e. has PEG (poly(ethyleneglycol)) or methyl-capped
15 (poly(ethyleneglycol)) covalently attached thereto). A method for covalently attaching PEG is described in EP-A-0948544. In one embodiment the antibody molecule of the present invention is a PEGylated modified Fab' fragment as shown in Figure 13. In Figure 13, the
20 modified Fab' fragment has a maleimide group covalently linked to a single thiol group in a modified hinge region. A lysine residue is covalently linked to the maleimide group. To each of the amine groups on the lysine residue is attached a methoxypoly(ethyleneglycol)
25 polymer having a molecular weight of approximately 20,000 Da. The total molecular weight of the entire effector molecule is therefore approximately 40,000 Da.

In one embodiment, in the compound shown in Figure 13, the heavy chain of the antibody 5' part has the
30 sequence given as SEQ ID NO: 115 and the light chain has

the sequence given in SEQ ID NO: 113. This compound is referred to herein as CDP-870.

In another embodiment, the present method provides an E. coli expression vector comprising a DNA sequence
5 of the present invention. In another embodiment the expression vector is PTTO(CDP-870) as shown schematically in Figure 22.

The present invention also provides vector pDNAbEng-G1 as shown in Figure 19.

10 DNA sequences coding for part or all of the antibody heavy and light chains may be synthesized as desired from the determined DNA sequences or on the basis of the corresponding amino acid sequences.

Standard techniques of molecular biology may be
15 used to prepare DNA sequences coding for the antibody molecule made by the present invention. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR)
20 techniques may be used as appropriate.

Any suitable host cell/vector system may be used for expression of the DNA sequences encoding the antibody molecule made by the present invention. Bacterial, for example E. coli, and other microbial
25 systems may be used, in part, for expression of antibody fragments such as Fab' and F(ab')₂ fragments, and especially Fv fragments and single chain antibody fragments, for example, single chain Fvs. Eukaryotic, e.g. mammalian, host cell expression systems may be used
30 for production of larger antibody molecules, including

complete antibody molecules. Suitable mammalian host cells include CHO, myeloma. or hybridoma cells.

The present invention also provides a process for the production of an antibody molecule wherein the
5 process comprises culturing a host cell comprising a vector of the present invention, wherein the culturing is performed under conditions suitable for leading to expression of protein from DNA encoding the antibody molecule of the present invention, and isolating the
10 antibody molecule.

The process for the production of the antibody molecule made by the present invention can comprise culturing E. coli bacteria wherein the bacteria comprise
15 an E. coli expression vector comprising the DNA sequence of the present invention, and wherein the culturing is performed under conditions suitable for leading to expression of protein from the DNA sequence and isolating the antibody molecule. The antibody molecule
20 may be secreted from the cell or targeted to the periplasm by suitable signal sequences. Alternatively, the antibody molecules may accumulate within the cell's cytoplasm. In one embodiment the antibody molecule is targeted to the periplasm. Depending on the antibody
25 molecule being produced and the process used, it is desirable to allow the antibody molecules to refold and adopt a functional conformation. Procedures for allowing antibody molecules to refold are well known to those skilled in the art.

30 The antibody molecule may comprise only a heavy or light chain polypeptide, in which case only a heavy

chain or light chain polypeptide coding sequence needs to be used to transfect the host cells. For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, a first
5 vector encoding a light chain polypeptide and a second vector encoding a heavy chain polypeptide. Alternatively, a single vector may be used, the vector including sequences encoding light chain and heavy chain polypeptides.

10

The antibody molecules made by the present invention can be prepared, for example, by the methods exemplified below. In these methods, CDP-870 Fab'-PEG is used as an example, but other antibody molecules can
15 be prepared by similar methods. CDP-870 Fab'-PEG is prepared using a recombinant *E. coli* expression system, W3110 pTTOCDP-870. The system utilizes the lacIq gene encoding the lacI repressor to control the activity of the tac promoter driving transcription of the antibody
20 genes. A DNA sequence encoding the OmpA leader sequence is placed directly upstream of the structural genes for both antibody chains. This polypeptide sequence directs translocation of the polypeptides to the *E. coli* periplasm and is cleaved from the N-terminal of the
25 antibody chains during this process.

The recombinant *E. coli* can be grown on a medium containing salts, trace metals, and a source of carbon and metabolic energy, and then induced to express CDP-870 Fab' and other Fab' species. The source of carbon
30 and metabolic energy can vary widely. For example the source of carbon and metabolic energy can be a carbohydrate. The carbohydrate can be, for example,

glycerol or a saccharide. When the carbohydrate is a saccharide, it can be a monosaccharide or it can be a polysaccharide (where a polysaccharide contains two or more saccharide subunits). When the carbohydrate is a
5 saccharide, it is advantageously a monosaccharide such as glucose. In one useful embodiment the source of carbon and metabolic energy is glycerol. When the optical density (OD₆₀₀) of the growth medium reaches approximately 40 - 50 (conveniently about 45), a protein
10 production inducer (herein, "inducer") can be added to the fermentation. The inducer can vary widely in nature. For example, the inducer can include lactose or IPTG. The start of induction is defined as the point at which the source of carbon and metabolic energy (e.g.,
15 glycerol) is depleted from the medium. Inducer (e.g., lactose) levels can be maintained and CDP-870 Fab' may be allowed to accumulate for a period of time. The period of time during which the CDP-870 Fab' accumulates can be varied depending on the desired results. It is
20 convenient to allow the CDP-870 Fab' to accumulate for about 1 to about 50 hours, alternatively for about 10 to about 40 hours, alternatively for about 20 to about 35 hours, alternatively for about 30 hours. After the accumulation time, the fermentation broth can be cooled
25 and the cells harvested by an appropriate method. Useful cell harvest methods include, but are not limited to, centrifugation or filtration. The periplasmic Fab' can be extracted by resuspension of the cell slurry in extraction buffer and incubation. Incubation can be
30 carried out at a variety of temperatures, conveniently at a temperature less than about 65°C, alternatively about 50 to about 62°C, alternatively about 55 to about 62°C, alternatively at about 60°C. Extraction time can

vary widely. It is useful to extract, for example, for 0.1 to about 48 hours, alternatively 0.5 to about 36 hours, alternatively about 1 to about 24 hours, alternatively 5 to about 15 hours, alternatively about 10 hours. After the extraction, the suspension can be cooled and/or pH adjusted, if desired. If the pH is adjusted, a variety of acids or bases can be used. Commonly it is necessary to lower the pH. For this purpose, a variety of acids can be used, for example organic acids or mineral acids. It is convenient to use an organic acid for this purpose. Useful organic acids include carbonic acid or a carboxylic acid. When a carboxylic acid is used, it can conveniently be a C₁ to about C₁₀ carboxylic acid, alternatively a C₁ to about C₅ carboxylic acid, alternatively a C₁ to about C₃ carboxylic acid, and alternatively acetic acid. If the pH is adjusted, it is convenient to adjust it to about 4 to about 5, alternatively about 4.5. If any cell debris, precipitated proteins, or aggregates are present, they can be conveniently removed by a variety of methods. Convenient methods for the removal of cell debris, precipitated proteins, or aggregates include chromatographic methods, for example through the use of an ion exchange chromatographic method. Useful ion exchange chromatographic methods include anionic exchange chromatography and cationic exchange chromatography. Conveniently, one can use Cation Exchange Expanded Bed Adsorption Chromatography for separating cell debris, precipitated proteins, or aggregates. The CDP-870 Fab' can be further purified by one or more additional chromatography steps. Such additional chromatography steps include anion exchange chromatography (herein, "AEX"), cation exchange

chromatography (herein, "CEX"), hydrophobic interaction chromatography (herein, "HIX"), and others. It is convenient to use two or more column chromatography steps, such as Anion Exchange Chromatography (AEX) and
5 Hydrophobic Interaction Chromatography (HIC). Ultrafiltration/diafiltration (herein, "UF/DF") can be used before or after the additional chromatography step(s) for further purification of the CDP-870 Fab'. For example, UF/DF can be used before an AEX step and
10 after an HIC step (where AEX and HIC are used sequentially) to adjust CDP-870 Fab' concentration or to exchange buffers.

The CDP-870 Fab' can be treated with a reductant to activate the single Fab' hinge thiol. Such reduction
15 can conveniently be performed after a purification procedure as described above.

c. Detailed Preparative Methods

The starting materials for use in the methods of
20 preparation of the invention are known or can be prepared by conventional methods known to a skilled person or in an analogous manner to processes described in the art.

Generally, the process methods of the present
25 invention are exemplified as follows.

Example 1. CDP-870 Fab'

Fermentation

Conveniently, the fermentation stage can comprise
30 steps including: Seed Shake Fermentation, Inoculum Fermentation, and Production Fermentation. An example

of the Fermentation Stage is shown schematically in Figure 23.

A purpose of Seed Shake Fermentation (Step 1) is to propagate cells. This step can begin when shake flasks containing Phytone, Yeast Extract, and sodium chloride, with Tetracycline Solution (about 5 micrograms/mL of tetracycline) added as a selective agent, are inoculated with a seed vial (i.e., a vial containing approximately 1 ml of cell culture to inoculate the seed flask to start the fermentation process). The flasks can be incubated at a temperature of about 25 to about 35°C, alternatively at about 30°C, with agitation until OD₆₀₀ of the medium reaches about 2 to about 5, for example about 4. A total of approximately three liter of seed shake culture is pooled from five flasks and transferred to the inoculum fermenter.

A purpose of Inoculum Fermentation (Step 2) is to further propagate cells to a concentration that is sufficient for inoculating the production fermentor. This step can begin when Nutrient Solution (defined below) is inoculated with the seed shake culture. Tetracycline Solution is added to the Nutrient Solution as a selective agent, Antifoam (e.g., PPG 2000) can be added to prevent foaming, and Ammonium Hydroxide Solution can be added to control pH. The culture parameters of temperature, pH, agitation, backpressure, and aeration are controlled. For examples of useful culture parameters, see Figure 23. The culture is harvested when the OD₆₀₀ reaches about 30 to about 40, alternatively about 34.

Nutrient Solution:

Component	Concentration
Citric acid	0.005Kg/L
Magnesium sulfate . 7 H ₂ O	0.001Kg/L
Sodium dihydrogen phosphate . 2 H ₂ O	0.005Kg/L
Ammonium sulfate	0.005Kg/L
Potassium chloride	0.004Kg/L
Calcium chloride . 2 H ₂ O	0.291g/L
Glycerol	0.097Kg/L
Zinc sulfate . 7 H ₂ O	0.020g/L
Manganese sulfate . 1 H ₂ O	0.020g/L
Copper sulfate . 5 H ₂ O	0.008g/L
Cobalt sulfate . 7 H ₂ O	0.004g/L
Iron (III) chloride hexahydrate	0.100g/L
Boric acid	0.0003g/L
Sodium molybdate . 2 H ₂ O	0.0002g/L
Foam inhibitor PPG	0.250mL/L

A purpose of Production Fermentation (Step 3) is to propagate cells to a desired concentration where they are induced to synthesize CDP-870 Fab'. This step begins when approximately 300 L of the seeding bioreactor is used to inoculate Nutrient Solution (see Step 2) in the production bioreactor. The culture parameters of temperature, pH, agitation, backpressure, and aeration are controlled. For examples of useful culture parameters for this step, see Figure 23. Antifoam (e.g., PPG 2000) can be added to control foaming, Magnesium Solution and Lactose Solution are added to supplement the medium. Ammonium Hydroxide Solution and sulfuric acid (if necessary) are added to

control pH. The culture is stopped (e.g., the production bioreactor or fermenter is harvested) when the time post-induction is about 10 to about 60 hours, alternatively about 20 to 40 hours, alternatively about
5 30 hours.

Primary Separation of CDP-870 Fab' can include the following four steps: Cell Harvest, Fab' Extraction and Precipitation, Homogenization, and Chromatographic Separation. An example of the Primary Separation stage
10 is shown schematically in Figure 24.

A purpose of Cell Harvest (Step 4) is to separate cells containing CDP-870 Fab' from the fermentation medium. After fermentation, the cells are separated for example by filtration or centrifugation. The
15 supernatant is discarded. The wet cells can be collected and diluted with purified water to ~50% of the original harvest broth volume. Alternatively, it is now reported that holding the cell paste (either as an isolated pellet, or in the presence of supernatant or
20 other liquid) for a period of time prior to the extraction step will result in an increase in the amount of Fab' extracted into solution relative to the situation in which the cell paste is extracted immediately after Cell Harvest.

25 The example in Table 1, discussed in more detail below, shows over 30% increase in extraction yield was achieved by holding the cell paste at ~20°C for about 30 hrs or longer prior to carrying out the typical extraction process described above.

A purpose of Fab' Extraction and Precipitation (Step 5) is to release CDP-870 Fab' from the cells. An extracting solution (e.g., a buffer solution such as 0.2 M Tris and 20 mM EDTA) is added to the harvested and
5 diluted cells and the solution is heated to about 50 to about 65°C, alternatively about 60°C for approximately 2 to about 30 hours, alternatively about 5 to about 20 hours, alternatively about 10 hours. The solution can then be cooled, e.g., to about 10 to about 50°C,
10 alternatively about 10 to about 30°C, alternatively about 15 to about 20°C, alternatively about 19 to about 20°C. The pH during this process, if desired, can be higher than about 3, alternatively higher than about 4.5, alternatively higher than about 7, and
15 alternatively higher than about 8. A convenient buffer system to use for pH adjustment is acetate buffer. If desired, the extraction can be performed in the presence of a chaotropic agent (e.g., urea) or a detergent.

A purpose of Homogenization (Step 6) is to prepare
20 the process stream for the following chromatography step. The extraction solution is homogenized at low pressure to create a homogeneous slurry.

A purpose of Cation Exchange Expanded Bed Adsorption Chromatography (Step 7) is to separate CDP-
25 870 Fab' from the cells and debris and to remove host cell proteins (herein, "HCP") and endotoxin. The homogenized crude extract can be, if desired, diluted online with purified water (e.g., approximately 3.5 fold) during the load to a chromatography column. A
30 variety of chromatography columns can be used, for example an expanded bed chromatography column or a fixed bed chromatography column. It is convenient to use an

Expanded Bed Adsorption (herein, "EBA") chromatography column in expanded bed mode. A convenient resin is a cation exchanger, such as an agarose-derived resin. A useful cation exchanger is Streamline SP (Amersham).

5 After a washing step with equilibration buffer in both the expanded and fixed bed modes, bound Fab' is eluted with a 50 mM NaOAc, 115 mM NaCl buffer in fixed bed mode. The collected product can be either processed immediately, or held for a period of time (e.g., hours
10 or days).

When the collected product is held, it is convenient to hold it at sub-ambient temperature, for example at about 0 to about 15°C, alternatively about 2 to about 8°C.

Also, EBA pool lots may be combined for further
15 downstream processing. RP-HPLC and SDS-PAGE data can be used to evaluate the stability of the pool.

Parameters affecting the extraction process include extraction temperature, extraction time, extraction solution ionic strength, and pH. It is now reported
20 that holding the cell paste for a period of time prior to the extraction step will result in a significant increase in the amount of Fab' extracted into solution. Table 1 shows the effect of hold time on Fab' species (i.e., Fab', F(ab')₂, Fab' adducts, and any other
25 species that can be reduced to form Fab') extraction efficiency. For example at least a 30% increase in extraction yield was achieved with holding the cell paste at about 20°C for about 30 hrs or longer prior to carrying out the typical extraction process described
30 above. The data from Table 1 are plotted in Figure 25.

Table 1. Effect of Pellet Hold Time on Fab' Extraction Efficiency

Pellet Hold Time at 20°C, hr	Fab' Species Concentration in Supernatant (mg/ml)	Relative Amount of Extracted Fab'
4	0.20	100%
16	0.24	119%
28	0.26	131%
40	0.27	136%
52	0.26	130%

In addition to the length of hold time, the temperature at which the pellet is held can have an effect on the extraction yield. Table 2 shows the results obtained at 4, 20 and 37°C holding temperature. As can be seen from the data, extraction yield increased as a function of hold temperature.

10

Table 2. Effect of hold time and temperature on Fab' extraction efficiency

Pellet Hold Time, hr	Fab' Species Concentration in Supernatant (mg/ml)			
	0.1 L Extraction			1.5 L Extraction
	4°C Hold	20°C Hold	37°C Hold	20°C Hold
1		0.29		0.28
13		0.36		
25		0.41		
37		0.40		0.41
49	0.31	0.40	0.43	

The effect on the Fab' extraction efficiency of the amount of fermentation broth left in the cell paste during the hold is shown in Table 8.

5 Table 8. Effects of Broth Level in Cell Pellet
 on Fab Extraction

Sample	Pellet Treatment (20°C)		Extraction Vol, L	Extracted Fab' + DiFab' in Super- natant, mg/ml
	Hold Time, hr	% Fermen- tation Wt		
40% Broth Cell Paste - (small volume)	2.5	40	0.1	0.24
	50.5	40	0.1	0.32
~20% Broth Cell Paste - (small volume)	2.5	~20	0.1	0.26
	50.5	~20	0.1	0.35
40% Broth Cell Paste - (larger volume)	38.5	40	1.5	0.32
Whole Broth Extraction	0	100	0.1	0.29

The extraction can be performed at a variety of pH's. For example, Table 9 shows the effect of extraction pH on the amount of Fab' extracted based on RP-HPLC analysis (over 40% increase based on Protein G assay). It is useful to perform the extraction at a pH of about 7 or higher, alternatively about 8 or higher, in another embodiment about 9 or higher, and in another embodiment about 9 to about 11. In one embodiment, protein degradation reactions such as deamidation will be more extensive at higher pH. A pH of about 8 to about 9 will be useful to obtain an increased yield without significant product degradation.

Table 9. Effects of pH on Fab' Extraction

Extraction conditions	Extracted Fab' (mg/L)	
Cntrl pH 7.4 extracted for 4 hr at 60 °C	Time = 0 hr (initial)	50
	4 hr extraction (60 °C)	230
pH 8 extracted for 4 hr at 60 °C	T= 0	90
	4 hr extraction (60 °C)	250
pH 9 extracted for 4 hr at 60 °C	T= 0	120
	4 hr extraction (60 °C)	320
pH 10 extracted for 4 hr at 60 °C	T= 0	170
	4 hr extraction (60 °C)	380

Studies were carried out to explore the effect of extracting Fab' from the cell paste using predetermined amounts of Tris and EDTA in the buffer solution without additional pH adjustment. This approach will eliminate the unnecessary salt addition from pH adjustment, which will simplify buffer preparation and subsequent column separation. All buffer combinations were prepared with a 1 M stock solution of Tris and a 200 mM stock solution of EDTA. The results in Table 10 show that unadjusted buffers with pH above 9 gave higher extraction efficiency over the control with lower solution conductivity.

Table 10. Fab' extraction using Tris-EDTA buffers without pH adjustment

Buffer Conc	Buffer pH	Initial Extraction pH	Final Extraction pH	Final conductivity (mS/cm)	Total Fab' (mg/L)
200mM Tris/ 20mM EDTA	7.4	7.08	5.67	12.78	270
	9.0	8.63	7.59	9.08	380
	11	9.08	8.10	8.47	460
	Unad-justed (pH 9.22)	8.78	7.86	8.28	460
600mM Tris/ 20mM EDTA	7.4	7.56	6.05	21.7	290
	9.0	8.94	8.14	9.68	490
	11	9.42	8.61	7.64	430
	Unad-justed (pH 9.67)	9.23	8.38	7.59	450

Extraction experiments were conducted with two main chaotropic reagents, urea and guanidine (using 1-mL 5 fermentation pellets in standard extraction buffer of 10 mM EDTA, 100 mM Tris pH 7.5). Shown in Table 11, addition of urea resulted in increases in Fab' extraction.

10 Table 11. Extraction of fermentation pellets
Extraction buffer: 100 mM Tris, 10 mM EDTA, pH 7.5;
extraction temperature = 60°C

Extraction Solution	Additive Concentration	Extraction Time, hr	% of Total Fab' Area (RP-HPLC)					Fab' + F(ab') ₂ mg/mL
			Light Chain	Heavy Chain	HC/LC Ratio	Fab' Species	F(ab') ₂ Species	
Fab' Reference			2.4%	5.0%	2.1	77.2%	15.4%	
Extraction Buffer	None	4	9.1%	8.3%	0.9	82.6%	0.0%	0.31
		8	7.3%	6.3%	0.9	86.4%	0.0%	0.30
+ Guanidine	1M	4	6.6%	25.6%	3.9	67.8%	0.0%	0.32
		8	6.3%	25.9%	4.1	67.8%	0.0%	0.31
	3M	4	6.7%	49.4%	7.4	43.9%	0.0%	0.33

		8	8.3%	49.7%	6.0	42.0%	0.0%	0.28
	6M	4	31.1%	43.5%	1.4	25.3%	0.0%	0.38
		8	29.3%	44.3%	1.5	26.4%	0.0%	0.21
	1M	4	5.6%	16.3%	2.9	78.1%	0.0%	0.36
		8	6.7%	7.7%	1.1	85.6%	0.0%	0.35
	3M	4	5.4%	27.4%	5.0	67.2%	0.0%	0.44
		8	4.9%	24.7%	5.0	70.4%	0.0%	0.43
	6M	4	13.9%	36.7%	2.6	49.4%	0.0%	0.37
		8	15.8%	35.0%	2.2	49.2%	0.0%	0.35

A study was carried out to test the effects of Tris and EDTA concentrations on the Fab' extraction yield. The results are shown in Tables 12 and 13. In this study, EDTA concentration was held at 20 mM with Tris level ranging from 200 to 600 mM and solution pH adjusted independently to cover a range of 7.4 to 11. Analysis of the obtained data indicates that both high Tris and high pH at moderate level of EDTA increase Fab' extraction.

In the fermentation, samples at the 30-hour time after induction were extracted with pH 7.4 buffer to produce 250 mg/L Fab'. Other samples at the 30-hour time after induction were extracted with pH 9.0 buffer to produce 340 mg/L Fab'.

Table 12. Effects of Tris & EDTA

Extraction:

All buffer combinations were prepared with a 1 M stock solution of Tris and a 200 mM stock solution of EDTA

Tris (mM)	EDTA (mM)	Final buffer pH	Extraction pH	Extracted Fab' (mg/L)
300	20	11.06	9.08	390

500	100	11.12	9.78	270
200	50	11.08	9.43	360
100	50	11.05	9.25	300
500	20	11.12	9.39	370
300	50	11.07	9.57	480
150	15	11.07	9.01	310
300	10	11.02	9.27	380
100	100	11.04	9.53	340
100	20	11.04	8.93	330
500	10	11.07	9.35	410
500	50	11.14	9.51	450
150	15	11.04	8.97	310
150	15	11.07	8.9	310
200	10	11.04	9.04	390
100	10	10.98	8.97	350
200	20	11.07	9.26	400
300	100	11.12	9.47	340
200	100	11.02	9.46	290
200	20	9.01	8.68	340

5 Table 13. Effects of Tris & pH on Fab' extraction

Tris Conc (mM)	EDTA Conc (mM)	Buffer pH	Extraction pH	Post Extraction pH	Total Fab' g/L
600	20	9	8.92	8.16	450
200	20	9	8.67	7.58	330
600	20	7.4	7.53	5.74	190
600	20	11	9.43	8.63	90?
400	20	7.4	7.28	5.57	220
600	20	11	9.41	8.59	550
400	20	9	8.85	7.95	410
300	20	8.2	8.84	7.97	400
200	20	7.4	6.72	5.52	160
200	20	9	8.66	7.47	380
200	20	11	9.08	8.17	310
600	20	7.4	7.49	5.60	170
400	20	9	8.87	7.98	300
400	20	11	9.30	8.52	310
600	20	9	8.97	8.19	350
400	20	7.4	7.06	5.50	150
300	20	8.2	8.82	7.96	290
200	20	7.4	6.44	5.48	150
200	20	11	9.08	8.13	270
400	20	11	9.28	8.39	360

The effects of various detergents, nonionic as well as ionic, on Fab' extraction are shown in Table 14. Most of the detergents tested at room temperature show significant increases in the amount of Fab' extracted relative to heated extraction. Useful detergents for extraction of Fab' include octoxynol detergents (e.g., octoxynol-1, octoxynol-3, octoxynol-5, octoxynol-8, octoxynol-9, octoxynol-13, and others for example sold under the tradename Triton); polysorbate detergents (e.g., polyethoxylated polysorbates such as Tween 20, Tween 21, Tween 40, Tween 61, Tween 81, Tween 85, and others); alkylsulfates (e.g., sodium dodecyl sulfate or "SDS"); alkoyl sarcosine detergents (e.g., lauroyl sarcosine, cocoyl sarcosine, or lauroyl sarcosinate, and others for example sold under the tradename Sarkosyl); benzalkonium chloride; or others.

Table 14. Effects of detergents on Fab' extraction

Sample ID17070-010	% of Total Area (RP-HPLC)		HC/LC Ratio	% of Total Fab' Area (RP-HPLC)		(Fab' + DiFab) mg/mL	
	Light Chain	Heavy Chain		Fab' Species	Di-Fab' Species	RP-HPLC	Protein G
Fab' Reference	2.5%	4.8%	2.0	77.2%	15.5%		
Heat Extraction: 100 mM Tris, 10 mM EDTA, pH 7.4, 60°C for 4 hr	9.1%	8.3%	0.9	82.6%	0.0%	0.31	
Extraction Buffer: 100 mM Tris, 10 mM EDTA, pH 7.4, 22°C, overnight							
+ 1% SDS	37.9%	34.5%	0.9	27.5%	0.0%	0.58	0.30
+ 1% sarkosyl	35.8%	31.7%	0.9	32.5%	0.0%	0.61	0.52
+ 1% deoxycholic acid	37.2%	29.1%	0.8	33.7%	0.0%	0.60	0.47

+1% benzalkonium Cl-	38.4%	10.7%	0.3	50.8%	0.0%	0.38	1.04
+ 1% Tween 20	36.1%	28.2%	0.8	35.6%	0.0%	0.38	0.50
+ 1% Tween 85	36.3%	29.1%	0.8	34.6%	0.0%	0.35	0.46
+ 1% Triton X- 114	36.8%	28.8%	0.8	34.4%	0.0%	0.44	0.40
+ 1% Triton X- 100	36.6%	25.9%	0.7	37.6%	0.0%	0.40	0.43
Extraction Buffer: 100 mM Tris, 10 mM Na ₂ EDTA, pH 9, 22°C, overnight							
+ 1% SDS	38.0%	38.5%	1.0	23.6%	0.0%	0.46	0.38
+ 1% sarkosyl	34.6%	35.7%	1.0	29.7%	0.0%	0.48	0.49
+ 1% deoxycholic acid	37.9%	28.8%	0.8	33.3%	0.0%	0.48	0.47
+ 1% benzalkonium Cl-	27.1%	6.5%	0.2	66.4%	0.0%	0.27	0.93
+ 1% Tween 20	35.0%	29.6%	0.8	35.4%	0.0%	0.51	0.51
+ 1% Tween 85	34.9%	28.6%	0.8	36.5%	0.0%	0.48	0.46
+ 1% Triton X- 114	34.9%	28.6%	0.8	36.5%	0.0%	0.48	0.44
+ 1% Triton X- 100	42.6%	27.6%	0.6	29.7%	0.0%	0.43	0.46

5 Without limiting the scope of the present invention
to any particular mechanism(s), there may be several
explanations for the present observations regarding
pellet hold time. One is that the cellular synthesis is
still going at the cell paste stage. Thus, holding the
10 cells could allow more time for the synthesis process to
occur, making more products. Another possible mechanism
is that the extra holding time results either in
breakdown of the cell outer membrane making it more
permeable, or results in additional transport of Fab'
15 from the cytoplasm into the periplasmic space where it
can be extracted. Another possible mechanism is that
the additional hold time may also allow the process of
refold and assembly of light chains and heavy chains in

the periplasm into Fab' to carry out to greater completion.

Example 2a.

- 5 A typical extraction and sampling procedure is as follows.

Pellet Preparation:

- Centrifuge four 500 mL centrifuge bottles (3000 RPM, 15
10 minutes), each containing 300 grams of production
fermentation broth (from step 3 of Figure 23). Decant
supernatant from all four bottles to 40% original weight
(120 g each). Combine three of the four bottles for
extraction usage (total pellet weight 360 gm) into 500
15 ml. Aliquot the gently stirred pellet slurry into eight
250 ml flasks, 40 g in each flask. Cover the flasks
with Parafilm. Randomize the flasks before the next
step to minimize systematic error due to the order of
pellet addition (e.g. solid settling out skewing the
20 results towards later samples). Mark the flasks #1
through 6, with three for #5: #5R (room temperature) ,
#5H (hot), #5C (cold). Store flasks #1 through 6 and
#5R on the bench (temperature ~20°C), #5C in the
refrigerator (~4°C), #5H in the 37°C incubator. Note:
25 Flasks #1 through 5 can be used for extraction
experiments, #6 is a spare if needed

Extraction Experiments:

- 30 After Pellet hold time:

Add the following to flask containing 40g pellet:
10mL Distilled water

50mL of Buffer (200 mM Tris, 20 mM EDTA, pH 7.5). Gently mix until mixture became homogeneous. Cover flask and placed in a shaker with temperature set at 60°C, 94 RPM.

5

Example 2a.

Alternative cell pellet preparation.

An appropriate number of 500 mL centrifuge bottles, each containing 400 mL of harvest fermentation broth, are centrifuged at 3000 rpm for 15 minutes. Post centrifugation supernatant solutions from all bottles are decanted and saved together in a separate container. Cell pellets are then resuspended with supernatant to 40% of the original starting weight in each bottle (~160 g each) and mixed well to form a slurry (to mimic the concentration effect which occurs during large scale centrifugation processing of harvest fermentation broth). All of the cell pellet slurries from each individual centrifuge bottle are then combined and mixed well to create a uniform mixture. A 40 mL aliquot of homogenous pellet slurry is transferred to each 250 ml flask. Ten mL of deionized water is then added to each 40 mL pellet slurry and mixed well. Additionally 50 mL of specified extraction buffer is added to cell slurry and mixed well. These 100 mL experiments are then incubated for 4 hrs at indicated temperature with 100 rpm mixing.

30 Example 3.

Gene Cloning and Expression of a Chimeric hTNF40
Antibody Molecule

RNA Preparation from hTNF40 Hybridoma Cells Total

RNA was prepared from 3×10^7 hTNF40 hybridoma cells as described below. Cells were washed in physiological saline and dissolved in RNazol (0.2 ml per 5 10^6 Cells). Chloroform (0.2 ml per 2 ml homogenate) was added, the mixture shaken vigorously for 15 seconds and then left on ice for 15 minutes. The resulting aqueous and organic phases were separated by centrifugation for 15 minutes in an Eppendorf centrifuge and RNA was 10 precipitated from the aqueous phase- by the addition of an equal volume of isopropanol. After 15 minutes on ice, the RNA was pelleted by centrifugation, washed with 70% ethanol, dried and dissolved in sterile, RNase free water. The yield of RNA was 400 micrograms.

15 PCR Cloning of hTNF40 Vh and VI

cDNA sequences coding for the variable domains of hTNF40 heavy and light chains were synthesised using reverse transcriptase to produce single stranded cDNA copies of the mRNA present in the total RNA, followed by 20 Polymerase Chain Reaction (PCR) on the cDNAs with specific oligonucleotide primers.

a) cDNA Synthesis

cDNA was synthesized in a 20 microliter reaction volume containing the following reagents: 50 mM Tris- 25 HCl. pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM each deoxyribonucleoside triphosphate, 20 units RNasin, 75 ng random hexanucleotide primer, 2 micrograms hTNF40 RNA and 200 units Moloney Murine Leukemia Virus reverse transcriptase. After incubation 30 at $42^\circ C$ for 60 minutes, the reaction was terminated by heating at $95^\circ C$ for 5 minutes.

b) PCR

Aliquots of the cDNA were subjected to PCR using combinations of primers specific for the heavy and light chains. The nucleotide sequences of the 5' primers for the heavy and light chains are shown in Tables 3 and 4 respectively. These sequences all contain, in order, a restriction site starting 7 nucleotides from their 5' ends, the sequence GCCGCCACC (SEQ ID NO: 12), to allow optimal translation of the resulting mRNAs, an initiation codon and 20-30 nucleotides based on the leader peptide sequences of known mouse antibodies.

The 3' primers are shown in Table 5. The light chain primer spans the J-C junction of the antibody and contains a restriction site for the enzyme *SplI* to facilitate cloning of the V_L PCR fragment. The heavy chain 3' primers are a mixture designed to span the J-C junction of the antibody. The 3' primer includes an *ApaI* restriction site to facilitate cloning. The 3' region of the primers contains a mixed sequence based on those found in known mouse antibodies.

The combinations of primers described above enable the PCR products for V_H and V_L to be cloned directly into an appropriate expression vector (see below) to produce chimeric (mouse-human) heavy and light chains and for these genes to be expressed in mammalian cells to produce chimeric antibodies of the desired isotype.

Incubations (100 microliters) for the PCR were set up as follows. Each reaction contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 10 pmoles 5' primer mix (Table 6), 10 pmoles 3' primer (CL12 (light

chain) or R2155 (heavy chain) (Table 5)), 1 microliter cDNA and 1 unit Taq polymerase. Reactions were incubated at 95°C for 5 minutes and then cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 5 30 cycles, aliquots of each reaction were analysed by electrophoresis on an agarose gel. Light chain reactions containing 5' primer mixes from light chain pools 1, 2 and 7 produced bands with sizes consistent with full length VI fragments while the reaction from heavy chain 10 reaction pool 3 produced a fragment with a size expected of a Vh gene. The band produced by the light chain pool 1 primers was not followed up as previous results had shown that this band corresponds to a light chain pseudogene produced by the hybridoma cell. The band 15 produced by the light chain pool 7 primers was weaker than the band from the pool 2 primers and therefore was not followed up. Only the band from light chain reaction pool 2, which was the strongest band, was followed up.

c) Molecular Cloning of the PCR Fragments

20 The DNA fragments produced in the light chain reaction pool 2 were digested with the enzymes BstBI and Sp1I, concentrated by ethanol precipitation, electrophoresed on a 1.4% agarose gel and DNA bands in the range of 400 base pairs recovered. These were cloned 25 by ligation into the vector pMR15.1 (Figure 4) that had been restricted with BstBI and Sp1I. After ligation, mixtures were transformed into E. coli LM 1035 and plasmids from the resulting bacterial colonies screened for inserts by digestion with BstBI and Sp1I. 30 Representatives with inserts from each ligation were analyzed further by nucleotide sequencing.

In a similar manner, the DNA fragments produced in heavy chain reaction pool 3 were digested with HindIII and ApaI and cloned into the vector pMR14 (Figure 5) that had been restricted with HindIII and
5 ApaI. Again, representative plasmids containing inserts were analysed by nucleotide sequencing.

d) Nucleotide Sequence Analysis

Plasmid DNA from a number of isolates containing Vh inserts was sequenced using the primers R1053 (see Table
10 7) (which primes in the 3' region of the HCMV promoter in pMR14) and R720 (see Table 7) (which primes in the 5' region of human C-gamma 4 and allows sequencing through the DNA insert on pMR14). It was found that the nucleotide sequences of the Vh insert in a number of
15 clones were identical, except for differences in the signal peptide and J regions. This indicated that the clones examined are independent isolates arising from the use of different primers from the mixture of oligonucleotides during the PCR stage. The determined
20 nucleotide sequence and predicted amino acid sequence of the variable domain of the heavy chain of antibody hTNF40 (hTNF40Vh) are given in Figure 7 (SEQ ID NO: 100).

To analyse the light chain clones, the sequence
25 derived from priming with R1053 (see Table 7) and R684 (SEQ ID NO:62) (which primes in the 5' region of human C-kappa and allows sequencing through the DNA insert on pMR15.1) was examined. The nucleotide sequence and predicted amino acid sequence of the VI genes arising
30 from reactions in pool 2 were similarly analysed. Again it was found that the nucleotide sequences of the VI insert in a number of clones were identical, except for

differences in the signal peptide and J regions, indicating that the clones examined were independent isolates arising from the use of different primers from the mixture of oligonucleotides used during the PCR stage. The determined nucleotide sequence and predicted amino acid sequence of the variable domain of the light chain of antibody hTNF40 (hTNF40V1) are given in Figure 6 (SEQ ID NO:99).

Table 3. Oligonucleotide primers for the 5' region of mouse heavy chains.

- CH1: 5' ATGAAATGCAGCTGGGTCAT (G, C) TTCTT3' (SEQ ID NO:13)
- CH2: 5' ATGGGATGGAGCT (A, G) TATCAT (C, G) (C, T) TCTT3' (SEQ ID NO: 14)
- 15 CH3: 5' ATGAAG (A, T) TGTGGTTAAACTGGGTTTT3' (SEQ ID NO: 15)
- CH4: 5' ATG (G, A) ACTTTGGG (T, C) TCAGCTTG (G, A) T3' (SEQ ID NO: 16)
- CH5: 5' ATGGACTCCAGGCTCAATTTAGTTTT3' (SEQ ID NO: 17)
- 20 CH6: 5' ATGGCTGTC (C, T) T (G, A) G (G, C) GCT (G, A) CTCTTCTG3' (SEQ ID NO: 18)
- CH7: 5' ATGG (G, A) ATGGAGC (G, T) GG (G, A) TCTTT (A, C) TCTT3' (SEQ ID NO: 19)
- CH8: 5' ATGAGAGTGCTGATTCTTTTGTG3' (SEQ ID NO:20)
- 25 CH9: 5' ATGG (C, A) TTGGGTGTGGA (A, C) CTTGCTATT3' (SEQ ID NO:21)
- CH10: 5' ATGGGCAGACTTACATTCTCATTCT3' (SEQ ID NO:22)

CH11: 5' ATGGATTTTGGGCTGATTTT TTTTATTG3' (SEQ ID NO:23)

CH12: 5' ATGATGGTGTTAAGTCTTCTGTACCT3' (SEQ ID NO:24)

Each of the above primers has the sequence

5 5' GCGCGCAAGCTTGCCGCCACC3' (SEQ ID NO:25) added to its 5' end.

Table 4. Oligonucleotide primers for the 5' region of mouse light chains.

10 CL1 5' ATGAAGTTGCCTGTTAGGCTGTTGGTGCT3' (SEQ ID NO:26)

CL2 5' ATGGAG (T, A) CAGACACACTCCTG (T, C) TATGGGT3' (SEQ ID NO:27)

CL3 5' ATGAGTGTGCTCACTCAGGTCCT3' (SEQ ID NO:28)

15 CL4 5' ATGAGG (G, A) CCCCTGCTCAG (A, T) TT (C, T) TTGG3' (SEQ ID NO:29)

CL5 5' ATGGATTT (T, A) CAGGTGCAGATT (T, A) TCAGCTT3' (SEQ ID NO:30)

CL5A 5' ATGGATTT (T, A) CA (A, G) GTGCAGATT (T, A) TCAGCTT3' 20 (SEQ ID NO:31)

CL6
5' ATGAGGT (T, G) C (T, C) (T, C) TG (T, C) T (G, C) AG (T, C) T (T, C)
CTG (A, G) G3' (SEQ ID NO:32)

CL7 5' ATGGGC (T, A) TCAAGATGGAGTCACA3' (SEQ ID NO:33)

25 CL8
5' ATGTGGGGA (T, C) CT (G, T) TTT (T, C) C (A, C) (A, C) TTTTTC AAT3'
(SEQ ID NO:34)

CL9 5' ATGGT (G, A) TCC (T, A) CA (G, C) CTCAGTTCCTT3' (SEQ ID NO:35)

CL10 5' ATGTATATATGTTTGTGTCTATTTTC3' (SEQ ID NO: 36)

CLI 'I 5' ATGGAAGCCCCAGCTCAGCTTCTCTT3' (SEQ IID NO:37)

5 CL12A

5' ATG (A, G) AGT (T, C) (A, T) CAGACCCAGGTCTT (T, C) (A, G) T3' (SEQ ID NO:38)

CL12B: 5' ATGGAGACACATTCTCAGGTCTTTGT3' (SEQ ID NO:39)

10 CL13 5' ATGGATTTCACAGGCCAGGTTCTTAT3' (SEQ ID NO:40)

CL 14 5' ATGATGAGTCCTGCCCAGTTCCTGTT3' (SEQ ID NO:41)

CL15 5' ATGAATTTGCCTGTTTCATCTCTTGGTGCT3' (SEQ ID NO:42)

15 CL16 5' ATGGATTTTCAATTGGTCCTCATCTCCTT3' (SEQ ID NO:43)

CL17A: 5' ATGAGGTGCCTA (A, G) CT (C, G) AGTTCCTG (A, G) G3' (SEQ ID NO:44)

CL17B: 5' ATGAAGTACTCTGCTCAGTTTCTAGG3' (SEQ ID NO:45)

20 CL17C: 5' ATGAGGCATTCTCTTCAATTCTTGGG3' (SEQ ID NO:46)

Each of the above primers has the sequence
5' GGACTGTTCGAAGCCGCCACC3' (SEQ ID NO:47) added to its 5' end.

Table 5. Oligonucleotide primers for the 3' ends of mouse Vh and VI genes.

Light chain (CL 12):

5'GGATACAGTTGGTGCAGCATCCGTACGTTT3' (SEQ ID NO:48)

5 Heavy chain (R2155):

5'GCAGATGGGCCCTTCGTTGAGGCTG(A,C)(A,G)GAGAC(G,T,A)GTGA3' (SEQ ID NO:49)

Table 6.

10 a) 5' Primer mixtures for light chain PCR reactions pool 1 CL2.

pool 2 CL7.

pool 3 CL13.

pool 4 CL6.

15 pool 5 CL5A, CL9, CL17A.

pool 6 CL8.

pool 7 CL12A.

pool 8 CLI, CL3, CL4, CL5, CLIO, CLI 1, CUB, CL14, CL15, CL16, CL17B, CL17C

20 b) 5' Primer mixtures for heavy chain PCR reactions

pool 1: CH 1, CH2, CH3, CH4.

pool 2: CH5, CH6, CH7, CH8.

pool 3: CH9, CHIO, CHII, CH12.

Table 7. Primers used in nucleotide sequence analysis

R1053: 5'GCTGACAGACTAACAGACTGTTCC3' (SEQ ID NO:50)

R720: 5'GCTCTCGGAGGTGCTCCT3' (SEQ ID NO: 5 1)

5

Evaluation of Activities of Chimeric Genes

The activities of the chimeric genes were evaluated by expressing them in mammalian cells and purifying and quantitating the newly synthesised antibodies. The methodology for this is described below, followed by a description of the biochemical and cell based assays used for the biological characterisation of the antibodies.

a) Production of Chimeric hTNF40 Antibody Molecule.

Chimeric antibody for biological evaluation was produced by transient expression of the appropriate heavy and light chain pairs after co-transfection into Chinese Hamster Ovary (CHO) cells using calcium phosphate precipitation.

On the day prior to transfection, semi-confluent flasks of CHO-L761 cells were trypsinised, the cells counted and T75 flasks set up each with 10^7 cells.

On the next day, the culture medium-was changed 3 hours before transfection. For transfection, the calcium phosphate precipitate was prepared by mixing 1. 25 ml of 0.25 M CaCl_2 containing 50 micrograms of each of heavy and light chain expression vectors with 1.25 ml of 2 x HBS (16.36 g NaCl, 11.0 g HEPES and 0.4 g Na_2HPO_4 in 1

liter water with the pH adjusted to 7.1 with NaOH) and adding immediately into the medium of the cells. After 3 hours at 37°C in a CO₂ incubator, the medium and precipitate were removed and the cells shocked by the addition of 15 ml 15% glycerol in phosphate buffered saline (PBS) for 1 minute. The glycerol was removed, the cells washed once with PBS and incubated for 48 to 96 hours in 25 ml medium containing 10 mM sodium butyrate. Antibody could be purified from the culture medium by binding to and elution from protein A-Sepharose.

b) ELISA.

For the ELISA, Nunc ELISA plates were coated overnight at 4°C with a F(ab')₂ fragment of a polyclonal goat anti-human Fc fragment specific antibody (Jackson ImmunoResearch, code 109-006-098) at 5 micrograms/mL in coating buffer (15 mM sodium carbonate, 35 mM sodium hydrogen carbonate, pH 6.9). Uncoated antibody was removed by washing 5 times with distilled water. Samples and purified standards to be quantitated were diluted to approximately 1 microgram/mL in conjugate buffer (0.1 M Tris-HCl, pH 7.0, 0.1 M NaCl, 0.2% v/v Tween 20, 0.2% w/v Harnmersten casein). The samples were titrated in the microtitre wells in 2-fold dilutions to give a final volume of 0.1 ml in each well and the plates incubated at room temperature for 1 hour with shaking. After the first incubation step the plates were washed 10 times with distilled water and then incubated for 1 hour as before with 0.1 ml of a mouse monoclonal anti-human kappa (clone GD12) peroxidase conjugated antibody (The Binding Site, code MP135) at a dilution of 1 in 700 in conjugate buffer. The plate was washed again and substrate solution (0.1 ml) added to each well.

Substrate solution contained 150 microliters N,N,N,N-tetramethylbenzidine (10 mg/mL in DMSO), 150 microliters hydrogen peroxide (30% solution) in 10 ml 0.1 M sodium acetate/sodium citrate, pH 6.0. The plate was developed
5 for 5-10 minutes until the absorbance at 630 nm was approximately 1.0 for the top standard. Absorbance at 630 nm was measured using a plate reader and the concentration of the sample determined by comparing the titration curves with those of the standard.

10 c) Determination of Affinity constants by BiaCore analysis.

The binding interaction between hTNF40 and human TNF was investigated using BIA technology. An affinity purified goat polyclonal antibody, directed against the
15 constant region of hTNF40, was immobilised on the dextran polymer sensor chip surface using standard NHS/EDC chemistry. Relatively low levels (200-500 RU) of hTNF40 were captured to ensure mass transport effects were minimised. Human TNF at different concentrations
20 was passed over the captured hTNF40 to allow assessment of the association kinetics. Following the injection of ligand, buffer was passed over the surface so that the dissociation could be measured. The association and dissociation rate constants for the interaction between
25 solid phase hTNF40 and human TNF were calculated, and a KD value was derived.

Example 4.

CDR-Grafting of hTNF40

The molecular cloning of genes for the variable regions of the heavy and light chains of the hTNF40 antibody and their use to produce chimeric (mouse-human) hTNF40 antibodies has been described above. The
5 nucleotide and amino acid sequences of the murine hTNF40 VI and Vh are shown in Figures 6 and 7 (SEQ ID NOS:99 and 100), respectively. This example describes the CDR-grafting of the hTNF40 antibody.

CDR-Grafting of hTNF40 Light Chain

10 Alignment of the framework regions of hTNF40 light chain with those of the four human light chain subgroups revealed that hTNF40 was most homologous to antibodies in human light chain subgroup 1. Consequently, for constructing the CDR-grafted light chain, the framework
15 regions chosen corresponded to those of the human group 1 consensus sequence.

A comparison of the amino acid sequences of the framework regions of murine hTNF40 and the consensus human group 1 light chains is given in Figure 1 and
20 shows that there are 22 differences (underlined) between the two sequences. Analysis of the contribution that any of these framework differences might have on antigen binding identified two residues for investigation; these are at positions 46 and 60. Based on this analysis, two
25 versions of the CDR-grafted light chain were constructed. In the first of these, hTNF40-gL1 (SEQ ID NO:8), residues 46 and 60 are derived from the hTNF40 light chain while in the second, hTNF40-gL2 (SEQ ID NO:9), all residues are human consensus except residue
30 number 60 which is from the hTNF40 light chain.

Construction of CDR-Grafted Light Chain hTNF40-gL1.

The construction of hTNF40-gLI is given below in detail. The following overlapping oligonucleotides (P7982-P7986) were used in the Polymerase Chain Reactions (PCR) to assemble a truncated grafted light chain. The assembled fragment lacks the antibody leader sequence and the first 17 amino acids of framework 1.

oligo 1 P7982:

5'GAATTCAGGGTCACCATCACTTGTAAGCCAGTCAGAACGTAGGTACTA
AC GTAGCCTGGTATCAGCAA3' (SEQ H) NO: 52)

10 oligo 2 P7983:

5'ATAGAGGAAAGAGGCACTGTAGATGAGGGCTTTTGGGGCTTTACCTGGT
TT TTGCTGATACCAGGCTACGT3' (SEQ ID NO:53)

oligo 3 P7984:

5'TACAGTGCCTCTTTCCTCTATAGTGGTGTACCATACAGGTTTCAGCGGATCCG
15 GTAGTGGTACTGATTTTCAC3' (SEQ ID NO: 54)

oligo 4 P7985

5'GACAGTAATAAGTGGCGAAATCTTCTGGCTGGAGGCTACTGATCGTGAGGGT
GAAATCAGTACCACTACCG3' (SEQ ID NO:55)

oligo 5 P7986:

20 5'ATTTCCGCACTTATTACTGTCAACAGTATAACATCTACCACTCACATTCGGT
CAGGGTACTAAAGTAGAAATCAAACGTACGGAATTC3' (SEQ ID NO:56)

Fwd P7981: 5'GAATTCAGGGTCACCATCACTTGTAAGCC3' (SEQ
ID NO: 57)

Bwd P7980 5'GAATTCCGTACGTTTGATTCTACTTTAGT3' (SEQ ID
25 NO: 58).

A PCR reaction, 100 microliters, was set up containing, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside

triphosphate, 2 pmoles of P7982, P7983, P7984, P7985, P7986, 10 pmoles of P7980, P7981 and 1 unit of Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 5 30 cycles, each reaction was analysed by electrophoresis on an agarose gel and the PCR fragment excised from the gel and recovered using a Mermaid Kit.

The recovered fragment was restricted with the enzymes BstEII and SphI in the appropriate buffer. The 10 resulting product was finally electrophoresed on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into vector CTIL57gL6 (Figure 12), that had previously been digested with the same enzymes. The above vector provides the missing 15 antibody leader sequence and the first 17 amino acids of framework 1.

The ligation mixture was used to transform E. coli strain LM1035 and resulting colonies analysed by PCR, restriction enzyme digests and nucleotide sequencing. 20 The nucleotide and amino acid sequence of the VI region of hTNF40-gL1 is shown in Figure 8 (SEQ ID NO:8).

Construction of CDR-Grafted Light Chain hTNF40-gL2.

hTNF40-gL2 (SEQ ID NO:9) was constructed using PCR. The following oligonucleotides were used to introduce 25 the amino acid changes:

R1053: 5'GCTGACAGACTAACAGACTGTTCC3' (SEQ ID NO:59)
25 R5350: 5'TCTAGATGGCACACCATCTGCTAAGTTTGATGCAGCATAGAT
CAGGAGCTTAGGAGC3' (SEQ ID NO: 60) R5349:
5'GCAGATGGTGTGCCATCTAGATTCAGTGGCAGTGGATCA 30
30 GGCACAGACTTTACCCTAAC3' (SEQ ID NO: 61) R684:
5'TTCAACTGCTCATCAGAT3' (SEQ ID NO:62).

Two reactions, each 20 microliters, were set up each containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 0.1 micrograms hTNF40-gL1, 6 pmoles of R1053/R5350 or R5349/R684 and 0.25 units Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, each reaction was analyzed by electrophoresis on an agarose gel and the PCR fragments excised from the gel and recovered using a Mermaid Kit.

Aliquots of these were then subjected to a second round of PCR. The reaction, 100 microliters, contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 1/5 of each of the PCR fragments from the first set of reactions, 30 pmoles of R1053 and R684 and 2.5 units Taq polymerase. Reaction temperatures were as above. After the PCR, the mixture was extracted with phenol/chloroform and then with chloroform and precipitated with ethanol. The ethanol precipitate was recovered by centrifugation, dissolved in the appropriate buffer and restricted with the enzymes BstEII and SphI. The resulting product was finally electrophoresed on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into the vector pMR15.1 (Figure 4) that had previously been digested with the same enzymes.

The ligation mixture was used to transform E. coli LM1035 and resulting colonies analyzed by PCR, restriction enzyme digests and nucleotide sequencing. The nucleotide and amino acid sequence of the VI region of hTNF40-gIL2 is shown in Figure 9 (SEQ ID NO:9).

CDR-Grafting of hTNF40 Heavy Chain CDR-grafting of hTNF40 heavy chain was accomplished using the same strategy as described for the light chain. hTNF40 heavy chain was found to be most homologous to human heavy chains belonging to subgroup I and therefore the consensus sequence of the human subgroup 1 frameworks was chosen to accept the hTNF40 heavy chain CDRs.

To investigate the requirement of a homologous human framework to act as an acceptor framework for CDR grafting, a second framework, human group 3, was selected to humanize hTNF40 heavy chain.

A comparison of hTNF40 with the two different frameworks region is shown in Figure 2 where it can be seen that hTNF40 differs from the human subgroup 1 consensus at 32 positions (underlined) and differs from the human subgroup 3 consensus at 40 positions (underlined). After analysis of the contribution that any of these might make to antigen binding, residues 28, 38, 46, 67, 69 and 71 were retained as donor in the CDR-grafted heavy chain ghlhTNF40.1, using the group 1 framework. Residues 27, 28, 30, 49, 49, 69, 71, 73, 76 and 78 were retained as donor in the CDR-grafted heavy chain, gh3hTNF40.4 using the group 3 framework. Residues 28, 69 and 71 were retained as donor in the CDR grafted heavy chain, ghlhTNF40.4 using the group I framework.

Construction of CDR-Grafted Heavy Chain ghlhTNF40.4

ghlhTNF40.4 (SEQ ID NO:10) was assembled by subjecting overlapping oligonucleotides to PCR in the presence of the appropriate primers. The following oligonucleotides were used in the PCR:

Group I graft

oligo I P7989:

5'GAAGCACCAGGCTTCTTAACCTCTGCTCCTGACTGGACCAGCTGCACCT
GAG AGTGCACGAATTC3' (SEQ ID NO: 63)

5 oligo 2 P7990:

5'GGTTAAGAAGCCTGGTGCTTCCGTCAAAGTTTCGTGTAAGGCCTCAGGC
TAC GTGTTACAGACTATGGTA3' (SEQ ID NO:64)

oligo 3 P7991:

5'CCAACCCATCCATTTCAAGGCCTTGTCCCGGGGCCTGCTTGACCCAATTC
10 ATAC CATAGTCTGTGAACACGT3' (SEQ ID NO:65)

oligo 4 P7995:

5'GGCCTGAAATGGATGGGTTGGATTAATACTTACATTGGAGAGCCTATTT
ATGT TGACGACTTCAAGGGCAGATTCACGTTTC3' (SEQ ID NO: 66)

oligo 5 P7992:

15 5'CCATGTATGCAGTGCGTTGTGGAGGTGTCTAGAGTGAACGTGAATCTGC
CCTT GAA3' (SEQ ID NO:67)

oligo 6 P7993:

5'CCACAAGCACTGCATACATGGAGCTGTCATCTCTGAGATCCGAGGACAC
CGC AGTGTACTAT3' (SEQ ID NO:68)

20 oligo 7 P7994:

5'GAATTCGGTACCCTGGCCCCAGTAGTCCATGGCATAAGATCTGTATCCT
CTAG CACAATAGTACACTGCGGTGTCCTC3' (SEQ ID NO:69)

Fwd: P7988:

5'GAATTCGTGCACTCTCAGGTGCAGCTGGTC3' (SEQ ID NO:70)

Bwd P7987:

5'GAATTCGGTACCCTGGCCCCAGTAGTCCAT3' (SEQ ED NO:71)

The assembly reaction, 100 microliters, contained 10 ml 4 Tris-HCl pH 8.3, 1.5 mM MgCl₂, mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 2 pmole of each of p7989, p7990, p7991, p7995, p7992, p7993 and p7994, 10 pmoles of each of p7988 and p7987 and 1 unit Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, the reaction was extracted with phenol/chloroform (1/1), then with chloroform and precipitated with ethanol. After centrifugation, the DNA was dissolved in the appropriate restriction buffer and digested with ApaI and KpnI. The resulting fragment was isolated from an agarose gel and ligated into pMR14 (Figure 5) that had previously been digested with the same enzymes. pMR14 contains the human gamma 4 heavy chain constant region when pMR14 is cleaved with ApaI and KpnI, the cleaved vector is able to receive the digested DNA such that the 3' end of the digested DNA joins in reading frame to the 5' end of the sequence encoding the gamma 4 constant region. Therefore, the heavy chain expressed from this vector will be a gamma 4 isotype. The ligation mixture was used to transform E. coli LM1035 and resulting bacterial colonies screened by restriction digest and nucleotide sequence analysis. In this way, a plasmid was identified containing the correct sequence for gh1hTNF40.4 (Figure 10) (SEQ ID NO: 10).

30

Construction of CDR-Grafted Heavy Chain gh3hTNF40.4

gh3hTNF40.4 (SEQ ID NO: 11) was assembled by subjecting overlapping oligonucleotides to PCR in the presence of the appropriate primers. The following 5' oligonucleotides were used in the PCR:

5 Group 3 graft

oligo, 1 P7999:

5' GATCCGCCAGGCTGCACGAGACCGCCTCCTGACTCGACCAGCTGAACCT
CAG AGTGCACGAATTC3' (SEQ ID NO:72)

oligo 2 P8000:

10 5' TCTCGTGCAGCCTGGCGGATCGCTGAGATTGTCCTGTGCTGCATCTGGT
TACG 15 TCTTCACAGACTATGGAA3' (SEQ ID NO:73)

oligo 3 P8001

5' CCAACCCATCCATTTTCAGGCCCTTTCCCGGGGCCTGCTTAACCCAATTCATTC
CATAGTCTGTGAAGACGT3' (SEQ ID NO:74)

15 oligo 4 P7995:

5' GGCCTGAAATGGATGGGTTGGATTAATACTTACATTGGAGAGCCTATTT
ATGT TGACGACTTCAAGGGCAGATTCACGTTC3' (SEQ ID NO: 66)

oligo 5 P7997:

5' GGAGGTATGCTGTTGACTTGGATGTGTCTAGAGAGAACGTGAATCTGCC
20 CTT GAA3' (SEQ ID NO:75)

oligo 6 P7998:

5' CCAAGTCAACAGCATACCTCCAAATGAATAGCCTGAGAGCAGAGGACAC
CGC AGTGTACTAT3' (SEQ ID NO:76)

oligo 7 P7993:

25 5' GAATTCGGTACCCTGGCCCCAGTAGTCCATGGCATAAGATCTGTATCCT
CTAG CACAATAGTACACTGCGGTGTCCTC3' (SEQ ID NO:77)

Fwd P7996:

5'GAATTCGTGCACTCTGAGGTTTCAGCTGGTC3' (SEQ ID NO:79)

Bwd P7987:

5'GAATTCGGTACCCTGGCCCCAGTAGTCCAT3' (SEQ ID NO:71)

5 The assembly reaction, 100 microliters, contained
10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01%
w/v gelatin, 0.25 mM each deoxyribonucleoside
triphosphate, 2 pmole of each of p7999, p8000, p8001,
p7995, p7997, p7998 and p7993, 10 pmoles of each of
10 p7996 and p7987 and 1 unit Taq polymerase. Reactions
were cycled through 94°C for 1 minute, 55°C for 1 minute
and 72°C for 1 minute. After 30 cycles, the reaction was
extracted with phenol/chloroform (1/1), then with
chloroform and precipitated with ethanol. After
15 centrifugation, the DNA was dissolved in the appropriate
restriction buffer and digested with ApaLI and KpnI. The
resulting fragment was isolated from an agarose gel and
ligated into pMR14 (Figure 5) that had previously been
digested with the same enzymes. pMR14 contained the
20 human gamma 4 heavy chain constant region. When pMR14 is
cleaved with ApaLI and KpnI, the cleaved vector is able
to receive the digested DNA such that the 3' end of the
digested DNA joins in reading frame to the 5' end of the
sequence encoding the gamma 4 constant region.
25 Therefore, the heavy chain expressed from this vector
will be a gamma 4 isotype. The ligation mixture was used
to transform E. coli LM1035 and resulting bacterial
colonies screened by restriction digestion and
nucleotide sequence analysis. In this way, a plasmid was
30 identified containing the correct sequence for
gh3hTNF40.4 (SEQ ID NO: 11) (Figure 11).

Production of CDR-Grafted Modified Fab' Fragment.

A CDR-grafted, modified Fab' fragment, based on antibody hTNF40, was constructed using the E. coli vector pTTO-1. The variable regions of antibody hTNF40 are sub-cloned into this vector and the intergenic sequence optimized to create pTTO(CDP-870). The pTTO expression vector is designed to give rise to soluble, periplasmic accumulation of recombinant proteins in E. coli. The main features of this plasmid are:

- (i) tetracycline resistance marker - antibiotic not inactivated by the product of resistance gene, hence selection for plasmid-containing cells is maintained;
- (ii) low copy number - origin of replication derived from plasmid p15A, which is compatible with plasmids containing colE1 derived replicons;
- (iii) strong, inducible tac promoter for transcription of cloned gene(s);
- (iv) lacI^q gene - gives constitutive expression of the lac repressor protein, maintaining the tac promoter in the repressed state until induction with IPTG / allolactose;
- (v) OmpA signal sequence - gives periplasmic secretion of cloned gene(s); and
- (vi) translational coupling of OmpA signal sequence to a short lacZ peptide, giving efficient initiation of translation.

The vector has been developed for expression of modified Fab' fragments from a dicistronic message by

the design of a method to select empirically the optimum intergenic sequence from a series of four purpose-built cassettes. The application of this in the construction of pTTO(CDP-870) is described.

5 Materials and Methods

DNA techniques

Standard procedures were used for protocols including DNA restriction, agarose gel electrophoresis, ligation and transformation. Restriction enzymes and DNA
10 modifying enzymes were obtained from New England Biolabs or Boehringer Mannheim, and were used according to the supplier's recommendations. DNA fragments were purified from agarose using the GeneClean protocol (BIO 101). Oligonucleotides were supplied by Oswel Oligonucleotide
15 Service and were synthesized at the 40 nm scale. Plasmid DNA was isolated using Plasmid DNA Mini/Midi kits from Qiagen. PCR was performed using Perkin Elmer 'Amplitaq' as recommended. DNA sequencing was performed using the Applied Biosystems Taq cycle sequencing kit.

20 Shake-flask induction

E. coli W3110 cultures were grown in L-broth supplemented with tetracycline (7.5 micrograms/mL). For inductions, fresh overnight cultures (grown at 30-C) were diluted to OD₆₀₀ Of 0.1 into 200 ml L-broth in a 2 L
25 baffled flask and were grown at 30°C in an orbital incubator. At OD₆₀₀ of 0.5, IPTG was added to 200 pM. Samples (normalized for OD) were taken at intervals.

Periplasmic Extraction

Culture samples were chilled on ice (5 minutes)
30 then cells were harvested by centrifugation. Following

resuspension in extraction buffer, (100 mM Tris.HCl, 10 MM EDTA, pH 7.4) samples were incubated overnight at 30-C, then clarified by centrifugation.

Assembly Assay

5 Modified Fab' concentrations were determined by ELISA. Plates were coated at 4°C overnight with anti-human Fd 6045 (2 micrograms/mL in coating buffer, physiological saline, 100 microliters per well). After washing, 100 pl of sample was loaded per well; purified
10 A5B7 gamma-1 Fab', initially at 2 micrograms/mL, was used as a standard. Samples were serially diluted 2-fold across the plate in sample conjugate buffer (per litre: 6.05 g trisaminomethane; 2.92 g NaCl; 0.1 Tween-20; 1 ml casein (0.2%)); plates were incubated for 1 hour at room
15 temperature, with agitation. Plates were washed and dried, then 100 microliters of anti-human C-kappa (GD12)-peroxidase was added (diluted in sample conjugate buffer). Incubation was carried out at room temperature for 1 hour with agitation. Plates were washed and dried,
20 then 100 microliters of substrate solution was added (10 ml sodium acetate/citrate solution (0.1 M pH 6); 100 microliters of H₂O₂ solution; 100 microliters tetramethylbenzidine solution (10 mg/ml in dimethylsulphoxide)). Absorbance at 630 nm was read 4 -
25 6 minutes after substrate addition.

Construction of Plasmid pTTO-1

(a) Replacement of the VTTQ9 Polylinker

Plasmid pTTQ9 was obtained from Amersham and is shown in Figure 14. An aliquot (2 micrograms) was
30 digested with restriction enzymes SalI and EcoRI, the digest was run on a 1% agarose gel and the large DNA

fragment (4520 bp) was purified. Two oligonucleotides were synthesized which, when annealed together, encode the OmpA polylinker region shown in Figure 15. This sequence has cohesive ends which are compatible with the
5 SalI and EcoRI ends generated by restriction of pTTQ9. By cloning this oligonucleotide, 'cassette' into the pTTQ9 vector, the SalI site is not regenerated, but the EcoRI site is maintained. The cassette encodes the first 13 amino acids of the signal sequence of the E. coli
10 outer-membrane protein Omp-A, preceded by the Shine Dalgarno ribosome binding site of the OmpA gene. In addition restriction sites for enzymes XbaI, MunI, StyI and Sp1I are present. The MunI and StyI sites are within the coding region of 5 the OmpA signal sequence and are
15 intended as the 5' cloning sites for insertion of genes. The two oligonucleotides which make up this cassette were annealed together by mixing at a concentration of 5 pmoles/microliter and heating in a waterbath to 95-C for 3 minutes, then slow cooling to room temperature. The
20 annealed sequence was then ligated into the SalI / EcoRI cut pTTQ9. The resulting plasmid intermediate, termed pTQOmp, was verified by DNA sequencing.

(b) Fragment Preparation and Ligation

Plasmid pTTO-1 was constructed by ligating one DNA
25 fragment from plasmid pACYC 184 to two fragments generated from pTQOmp. Plasmid pACYC 184 was obtained from New England Biolabs, and a restriction map is shown in Figure 16. An aliquot (2 microgram) was digested to completion with restriction enzyme StyI, then treated
30 with Mung Bean Nuclease; this treatment creates blunt ends by cutting back 5' base overhangs. Following phenol extraction and ethanol precipitation, the DNA was

restricted with enzyme PvuII, generating fragments of 2348, 1081, 412 and 403 bp. The 2348 bp fragment was purified after agarose gel electrophoresis. This fragment encodes the tetracycline resistance marker and
5 the p15A origin of replication. The fragment was then treated with calf intestinal alkaline phosphatase to remove 5' terminal phosphates, thereby preventing the self-ligation of this molecule.

An aliquot (2 micrograms) of plasmid pTQOmp was
10 digested with enzymes SspI and EcoRI, and the 2350 bp fragment was purified from unwanted fragments of 2040 bp and 170 bp following agarose gel electrophoresis; this fragment encodes the transcriptional terminator region and the lacIq gene. Another aliquot (2 micrograms) of
15 pTQOmp was digested with EcoRI and XmnI, generating fragments of 2289, 1670, 350 and 250 bp. The 350 bp fragment, encoding the tac promoter, OmpA signal sequence and multicloning site, was gel purified.

The three fragments were then ligated, using
20 approximately equimolar amounts of each fragment, to generate the plasmid pTTO-1. All cloning junctions were verified by DNA sequencing. The restriction map of this plasmid is shown in Figure 17. Plasmid pTTO-2 was then created by insertion of DNA encoding the human Ig light
25 chain kappa constant domain. This was obtained as a SphI - EcoRI restriction fragment from plasmid pHCl32, and inserted into the corresponding sites in pTTO-1. Plasmid pTTO- 2 is shown in Figure 18.

Insertion of humanized hTNF40 variable regions into PTTO-2

The variable light chain region hTNF40gL1 (SEQ ID NO:8) was obtained by PCR 'rescue' from the
5 corresponding vector for mammalian cell expression pMR10.1. The OmpA leader sequence replaces the native Ig leader. The sequence of the PCR primers is shown below:

5' primer:

CGCGCGGCAATTGCAGTGGCCTTGGCTGGTTTCGCTACCGTAGCGCAAG
10 CTGACATTCAAATGACCCAGAGCCC (SEQ ID NO:79)

3' primer:

TTCAACTGCTCATCAGATGG (SEQ ID NO:80)

Following PCR under standard conditions, the
product was purified, digested with enzymes MunI and
15 Sp1I then gel purified. The purified fragment was then inserted into the MunI / Sp1I sites of pTTO-2 to create the light chain intermediate pTTO(hTNF40L).

The variable heavy chain region of gh3hTNF40.4 was obtained in the same way from the vector pGamma-4. The
20 sequence of the PCR primers is shown below:

5' primer:

GCTATCGCAATTGCAGTGGCGCTAGCTGGTTTCGCCACCGTGGCGCAAG
CTGAGGTTTCAGCTGGTCGAGTCAGGAGGC (SEQ ID NO: 81)

3' primer:

25 GCCTGAGTTCCACGACAC (SEQ ID NO:82)

Following PCR the product was purified, digested with enzymes NheI and ApaI then sub-cloned into the

vector pDNAbEng-G1 (Figure 19). After verification by DNA sequencing, the heavy chain was restricted with enzyme EcoR1 and sub- cloned into the EcoRI site of pTTO(hTNF40L) to create the E. coli expression plasmid
5 pTTO(hTNF40).

Optimisation of Intergenic Sequence for Modified Fab' Expression

In the pTTO vector, modified Fab' expression occurs
10 from a dicistronic message encoding first light chain then heavy chain. The DNA sequence between the two genes (intergenic sequence, IGS) can influence the level of expression of the heavy chain by affecting the rate of translational initiation. For example, a short
15 intergenic sequence may result in translational coupling between the light and heavy chains, in that the translating ribosome may not fully dissociate from the mRNA after completing light chain synthesis before initiating heavy chain synthesis. The 'strength' of any
20 Shine Dalgarno (SD) ribosome binding site (homolgy to 16S rRNA) can also have an effect, as can the distance and sequence composition between the SD and the ATG start codon. The potential secondary structure of mRNA around the ATG can also have an effect; the ATG should be in a
25 'loop' and not constrained within a 'stem', while the reverse applies to the SD. Thus by modifying the composition and length of the IGS it is possible to modify the strength of translational initiation and therefore the level of heavy chain production. It is
30 possible that an optimum rate of translational initiation needs to be achieved to maximise expression of the heavy chain of a given modified Fab. For example,

with one modified Fab, a high level of expression may be tolerated, but for a different modified Fab' with different amino acid sequence, a high level of expression might prove toxic, perhaps because of
5 different efficiencies of secretion or folding. For this reason, a series of four intergenic sequences were designed (Figure 20), permitting the empirical determination of the optimum IGS for the hTNF40-based modified Fab. IGS1 and IGS2 have very short intergenic
10 sequences (-1 and +1 respectively) and might be expected to give closely coupled translation; the SD sequences (underlined) are subtly different. These two sequences will most likely confer a high level of translational initiation. IGS3 and IGS4 have a longer distance between
15 start and stop codons (+13) and differ in their sequence composition; IGS3 has a 'stronger' SD sequence. All sequences were studied for secondary structure (using m/fold program) and 'optimized' as far as possible; however, with tight coupling of translation of the two
20 chains the lack of ribosomal dissociation means that the mRNA may not be 'naked', preventing secondary structure formation.

Cloning of IGS variants

The IGS cassettes shown in Figure 20 have flanking
25 SacI and MunI cloning sites. They were built by annealing complementary oligonucleotide pairs. A vector fragment was prepared by digesting pTTO(hTNF40) with SacI and NotI, and a heavy chain fragment was prepared by digesting pDNAbEngG1(hTNF40H) with MunI and NotI.
30 Three-way ligations were then performed, using equimolar amounts of the two restriction fragments and approximately 0.05 pmoles of each annealed oligo

cassette. This created the four expression plasmids pTTO(hTNF40 IGS-1), pTTO(hTNF40 IGS-2), pTTO(hTNF40 IGS-3), pTTO(hTNF40 IGS-4).

5 Shake flask expression analysis

The four plasmids were transformed into E. coli strain W3110, along with the original expression construct, and then analysed for expression in shake flasks as described.

- 10 The results of a typical experiment are shown in Figure 21. The different intergenic sequences confer different expression profiles. IGS1 and IGS2 accumulate periplasmic modified Fab' rapidly with a peak at 1 hour post induction, after which the level recovered falls.
- 15 The peak is greater and the fall sharper for IGS1. These results are consistent with a high level of synthesis, as expected for close translational coupling for these constructs.

- IGS1 apparently confers a higher level of heavy
- 20 chain expression than does IGS2. In this instance, it appears that this high level of expression is poorly tolerated, since periplasmic expression levels fall after the 1 hour peak. This is seen on the growth profile of the IGS1 culture (not shown), which peaks at
- 25 hour post induction before falling, suggesting cell death and lysis. IGS3 accumulates modified Fab' more slowly but peaks at 2 hours post induction with a higher peak value (325 ng/ml/OD), before levels fall. The growth of this culture continued to 3 hours post
- 30 induction and reached a higher peak biomass (not shown). This is consistent with a lower level of heavy chain

synthesis. IGS4 accumulates material at a slower rate still and fails to reach the high peak of productivity of the other three constructs. All IGS variants out-perform the original vector significantly. The
5 hypothesis that the different IGS sequences confer different rates of translational initiation is supported by these experimental results. For, the hTNF40- based modified Fab' it appears that a high rate of heavy chain translational initiation is poorly tolerated and is
10 therefore not optimal. A slower rate, as conferred by IGS3, results in better growth characteristics and consequently a better yield accumulates over time.

Following comparison of productivity in the fermenter the IGS3 construct was selected as the highest
15 yielding and was termed pTTO(CDP-870) (see Figure 22).

The heavy chain encoded by the plasmid pTTO(CDP-870) has the sequence given in SEQ ID NO:115 and the light chain has the sequence given in SEQ ID NO:113.

Assays

20 Analytical Method for CDP-870 Fab'.

The analytical method used for CDP-870 Fab' assay is reverse phase HPLC using a C4 Vydac column and a gradient of A (4.5 M Guanidine, 5 mM Phosphate, pH 6.5) and B(3M Guanidine, 50% IPA, 5 mM Sodium Phosphate).

25 Assay for Reduction of Fab' Species.

Stachowicz, M. et.al., "Determination of total cysteamine in human serum by high-performance liquid chromatography with fluorescence detection" Journal of Pharmaceutical and Biomedical Analysis (1998), 17(4,5),
30 767-773.

Assay for Protein G.

The analytical method for Protein G is reverse phase HPLC using the conditions below:

- 5 Solutions and Buffer:
 20mM Sodium Phosphate, pH 7.2 (mobile phase A)
 20mM Sodium Phosphate, pH 2.5 (mobile phase B)
- 10 Column:
 1 mL HiTrap Protein G HP
 Cat. No. 170404-01
- 15 Method:
 Run time: 6 min
 Flow rate: 2 mL/min
 Absorbance: 220 nm
 Equilibrate the column with mobile phase A.
 Inject 10 microliters of sample neat.
 Elute with mobile phase B using a step elution.
- 20

The examples herein can be performed by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

The invention being thus described, it is apparent that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications and equivalents as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

CLAIMS

What is claimed is:

- 5 1. A method for the preparation of an antibody having a heavy chain and a light chain, wherein the method comprises:
- 10 fermenting a cell mixture comprising the cells and a supernatant solution, wherein the cells are capable of expressing the light chain and the heavy chain;
- separating the cells from the supernatant solution to form a cell pellet;
- 15 allowing the cell pellet to stand for a hold time; and
- extracting the cell pellet with an extracting solution,
- thereby producing the TNF-alpha antibody fragment or a dimer or an adduct thereof.
- 20
2. The method of Claim 1 wherein the antibody is a TNF-alpha antibody fragment or a dimer or an adduct thereof wherein the antibody or fragment has a 5' part having the sequence given as SEQ
- 25 ID NO: 115, and a light chain having the sequence given in SEQ ID NO: 113.
3. The method of Claim 2 wherein the hold time is about 1 to about 100 hours.
- 30
4. The method of Claim 3 wherein the hold time is about 5 to about 75 hours.

5. The method of Claim 4 wherein the hold time is about 10 to about 50 hours.
6. The method of Claim 5 wherein the hold time is about 20 to about 40 hours.
7. The method of Claim 6 wherein the hold time is about 25 to about 35 hours.
8. The method of Claims 1 or 2 wherein the cell pellet is allowed to stand for a hold time at a temperature in the range of about 5°C to about 50°C.
9. The method of Claim 8 wherein the temperature is about 10°C to about 40°C.
10. The method of Claim 9 wherein the temperature is about 15°C to about 40°C.
11. The method of Claim 10 wherein the temperature is about 20°C to about 37°C.
12. The method of Claims 1 or 2 wherein the extraction is performed at a pH greater than about 7.
13. The method of Claim 12 wherein the extraction is performed at a pH greater than about 9.
14. The method of Claim 13 wherein the extraction is performed at a pH of about 8 to about 9.

COMPARISONS OF FRAMEWORK REGIONS OF LIGHT CHAIN OF
ANTIBODY hTNF40 AND HUMAN GROUP 1 CONSENSUS SEQUENCES

Hu GROUP 1 CONSENSUS : DIQMTQSPSSLSASVGDRVITC (SEQ ID NO: 83)
hTNF40 : DIVMTQSQKFMSTSVGDRVSVTC (SEQ ID NO: 84)

Hu GROUP 1 CONSENSUS : WYQQKPGKAPKLLIY (SEQ ID NO: 85)
hTNF40 : WYQQKPGQSPKALIY (SEQ ID NO: 86)

Hu GROUP 1 CONSENSUS : GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC (SEQ ID NO: 87)
hTNF40 : GVPYRFTGSGSGTDFTLTISTVQSEDLAEYFC (SEQ ID NO: 88)

Hu GROUP 1 CONSENSUS : FGQGTKVEIKR (SEQ ID NO: 89)
hTNF40 : FGAGTKLELKR (SEQ ID NO: 90)

FIG. 1

SEQUENCE OF CDRs OF hTNF40

H1 DYGMN (SEQ ID NO: 1)
H2 WINTYIGEPIYVDDFKG (SEQ ID NO: 7)
H2' WINTYIGEPIYADSVKG (SEQ ID NO: 2)
H3 GYRSYAMDY (SEQ ID NO: 3)

L1 KASQNVGTNVA (SEQ ID NO: 4)
L2 SASFLYS (SEQ ID NO: 5)
L3 QQYNIYPLT (SEQ ID NO: 6)

FIG. 3

COMPARISONS OF FRAMEWORK REGIONS OF HEAVY CHAIN OF ANTIBODY
hTNF40 AND HUMAN GROUP 1 AND GROUP 3 CONSENSUS SEQUENCES

Hu GROUP 1 CONSENSUS : QVQLVQSGAEVKKPGASVKVSCKASGYTFT (SEQ ID NO: 91)
hTNF40 : QIQLVQSGPELKKPGETVKISCKASGYVFT (SEQ ID NO: 92)

Hu GROUP 1 CONSENSUS : WVRQAPGQGLEWMG (SEQ ID NO: 93)
hTNF40 : WVKQAPGKAFKWMG (SEQ ID NO: 94)

Hu GROUP 1 CONSENSUS : RVTITRDTSTSTAYMELSSLRSED TAVYYCAR (SEQ ID NO: 95)
hTNF40 : RFAFSLETSASTAFLOINNLKNEDTATYFCAR (SEQ ID NO: 96)

Hu GROUP 1 CONSENSUS : WGQGTTLTVSS (SEQ ID NO: 97)
hTNF40 : WGQGTTTLTVSS (SEQ ID NO: 98)

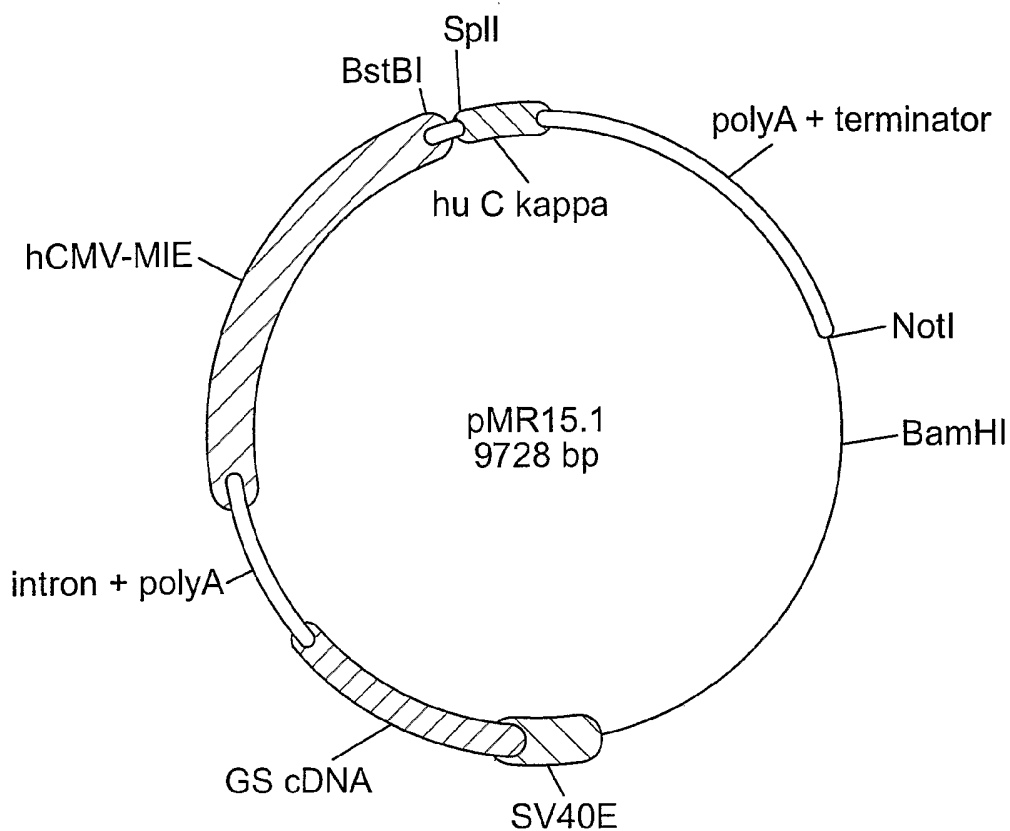
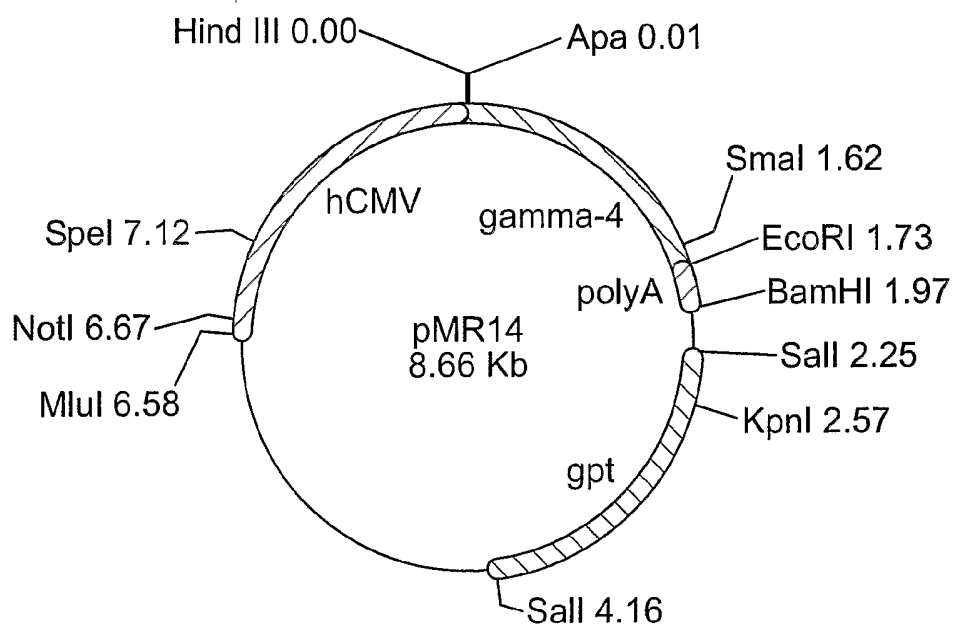
Hu GROUP 3 CONSENSUS : EVQLVESGGGLVQPGGSLRLSCAASGFTFS (SEQ ID NO: 106)
hTNF40 : QIQLVQSGPELKKPGETVKISCKASGYVFT (SEQ ID NO: 92)

Hu GROUP 3 CONSENSUS : WVRQAPGKGLEWVS (SEQ ID NO: 107)
hTNF40 : WVKQAPGKAFKWMG (SEQ ID NO: 94)

Hu GROUP 3 CONSENSUS : RFTISRDN SKNTLYLQMNSLRAEDTAVYYCAR (SEQ ID NO: 108)
hTNF40 : RFAFSLETSASTAFLOINNLKNEDTATYFCAR (SEQ ID NO: 96)

Hu GROUP 3 CONSENSUS : WGQGTTLTVSS (SEQ ID NO: 109)
hTNF40 : WGQGTTTLTVSS (SEQ ID NO: 98)

FIG. 2

**FIG. 4****FIG. 5**

MURINE VI SEQUENCE OF hTNF40 (SEQ ID NO: 99)

```

10  GAC ATT GTG ATG ACC CAG TCT CAA AAA TTC ATG TCC ACA TCA 40  GTA GGA GAC AGG
   CTG TAA TAC TGG TGC AAG GTC AGA S  Q  GTT Q  S  M  T  S  AGG CTG D  R>
20  10  GTC AGC TCG S  V  CAG V  GTC ACC TGC AAG GCC CGG A  S  AAT TTA CAC GTG GGT ACT AAT 100  GTA CAT V  TGG ACC W  TAT
   CAG V  S  TCG S  V  CAG V  GTC ACC TGC AAG GCC CGG A  S  AAT TTA CAC GTG GGT ACT AAT 100  GTA CAT V  TGG ACC W  TAT
60  60  GTC AGC TCG S  V  CAG V  GTC ACC TGC AAG GCC CGG A  S  AAT TTA CAC GTG GGT ACT AAT 100  GTA CAT V  TGG ACC W  TAT
   GTC AGC TCG S  V  CAG V  GTC ACC TGC AAG GCC CGG A  S  AAT TTA CAC GTG GGT ACT AAT 100  GTA CAT V  TGG ACC W  TAT
110 110  CAA CAG CAG AAA CCA GGA CCT G  G  CAA CAA TCT CCT AAA TTT CGT A  L  GCA TCC TTC 160  TCC CTA
   GTT GTC TTT K  P  GGT P  G  CCA GGA CCT G  G  CAA CAA TCT CCT AAA TTT CGT A  L  GCA TCC TTC 160  TCC CTA
120 120  CAA CAG CAG AAA CCA GGA CCT G  G  CAA CAA TCT CCT AAA TTT CGT A  L  GCA TCC TTC 160  TCC CTA
   GTT GTC TTT K  P  GGT P  G  CCA GGA CCT G  G  CAA CAA TCT CCT AAA TTT CGT A  L  GCA TCC TTC 160  TCC CTA
170 170  TAT AGT GGA GTC CCT CCA GGA CCT G  G  CAA CAA TCT CCT AAA TTT CGT A  L  GCA TCC TTC 160  TCC CTA
   ATA TCA CCT G  V  CAG V  GTC CCT CCA GGA CCT G  G  CAA CAA TCT CCT AAA TTT CGT A  L  GCA TCC TTC 160  TCC CTA
220 220  CTC ACC ATC AGC TCG TGA T  S  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC
   GAG TGG T  I  S  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC
270 270  CTC ACC ATC AGC TCG TGA T  S  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC
   GAG TGG T  I  S  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC
280 280  TAT AAC ATC TAG ATA Y  Y  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC
   TAT AAC ATC TAG ATA Y  Y  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC
320 320  TAT AAC ATC TAG ATA Y  Y  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC
   TAT AAC ATC TAG ATA Y  Y  CAG GTC Q  V  GTG CAC V  T  TCT AGA S  D  GAA GAC 240  TCT AGA S  D  GAA GAC

```

FIG. 6

CAG GTC Q	ATC TAG I	CAG GTC Q	GTG CAC V	10	TTC AAC L	CAG GTC Q	20	TCT AGA S	GGA CCT G	CCT GGA P	GAG CTC E	30	CTG GAC L	AAG TTC K	AAG TTC K	40	CCT GGA P	GGA CCT G	GAG CTC E	50	ACA TGT T	ACA TGT T	GTC CAG V
AAG TTC K	ATC TAG I	TCC AGG S	TGC ACG C	60	AAG TTC K	AAG GCT A	70	TCT AGA S	GGA CCT G	TAT ATA Y	GTT CAA V	80	TTC AAG F	ACA TGT T	ACA TGT T	90	TAT ATA Y	GGA CCT G	ATG TAC M	100	AAT TTA N	AAT TTA N	TGG ACC W
110	AAG TTC K	CAG GTC Q	CCA GGT P	120	GGA CCT G	AAG TTC K	130	AAG TTC K	GCT CGA A	TTC AAG F	AAG TTC K	140	TGG ACC W	ATG TAC M	ATG TAC M	150	TGG ACC W	ATA TAT I	AAC TTG N	160	ACC TGG T	ACC TGG T	TAC ATG Y
ATT TAA I	GGA CCT G	GAG CTC E	ATA TAT I	170	TAT ATA Y	GTT CAA V	180	GAT CTA D	GAC CTG D	TTC AAG F	TTC AAG F	190	TTC AAG F	GGA CCT G	GGA CCT G	200	TTT AAA F	GCC CGG A	TTC TTC F	210	TCT AGA S	TCT AGA S	TTG AAC L
220	ACC TGG T	TCT AGA S	GCC CGG A	230	AGC TCG S	ACT TGA T	240	TTC AAC L	CAG GTC Q	ATC TAG I	ATC TAG I	250	ATC TAG I	AAC TTG N	AAC TTG N	260	CTC GAG L	CTC GAG L	AAT TTA N	270	GAG CTC E	GAG CTC E	GTC CTG D
ACG TGC T	GCT CGA A	ACA TGT T	TAT ATA Y	280	TAT ATA Y	TGT ACA C	290	GCA CGT A	AGA TCT R	GGT CCA G	TAC ATG Y	300	CGG GCC R	TCC AGG S	TCC AGG S	310	TAT ATA Y	ATG CGA A	GAC CTG D	320	TAC ATG Y	TAC ATG Y	TGG ACC W
GGT CCA G	CAA GTT Q	GGA CCT G	ACC TGG T	330	TCA AGT S	TCA AGT S	340	TCA AGT S	GTC CAG V	TCT AGA S	TCA AGT S	350	GTC CAG V	ACC TGG T	ACC TGG T	360	TCT AGA S	TCT AGA S	TCT AGA S	370	TCT AGA S	TCT AGA S	TCT AGA S

FIG. 7

GRAFTED VI SEQUENCE OF hTNF40 (SEQ ID NO: 8)

```

10  GAC ATT CAA ATG ACC CAG AGC CCA TCC AGC CTG AGC GCA TCT 40  GTA GGA GAC CGG
   CTG TAA GTT TAC TAC GTC TCG TCG GGT AGG TCG GAC GAC TCG S A S AGA S 50  CCT CTG D R>
20  20  AGC CAG GTC TCG TCG S P S S L L S S 30  TCT TCT
30  30  TCC AGG TCG S S S S S S 40  TCT TCT
40  40  TCC AGG TCG S S S S S S 50  TCT TCT
50  50  TCC AGG TCG S S S S S S 60  TCT TCT
60  60  TCC AGG TCG S S S S S S 70  TCT TCT
70  70  TCC AGG TCG S S S S S S 80  TCT TCT
80  80  TCC AGG TCG S S S S S S 90  TCT TCT
90  90  TCC AGG TCG S S S S S S 100  TCT TCT
100 100  TCC AGG TCG S S S S S S 110  TCT TCT
110 110  TCC AGG TCG S S S S S S 120  TCT TCT
120 120  TCC AGG TCG S S S S S S 130  TCT TCT
130 130  TCC AGG TCG S S S S S S 140  TCT TCT
140 140  TCC AGG TCG S S S S S S 150  TCT TCT
150 150  TCC AGG TCG S S S S S S 160  TCT TCT
160 160  TCC AGG TCG S S S S S S 170  TCT TCT
170 170  TCC AGG TCG S S S S S S 180  TCT TCT
180 180  TCC AGG TCG S S S S S S 190  TCT TCT
190 190  TCC AGG TCG S S S S S S 200  TCT TCT
200 200  TCC AGG TCG S S S S S S 210  TCT TCT
210 210  TCC AGG TCG S S S S S S 220  TCT TCT
220 220  TCC AGG TCG S S S S S S 230  TCT TCT
230 230  TCC AGG TCG S S S S S S 240  TCT TCT
240 240  TCC AGG TCG S S S S S S 250  TCT TCT
250 250  TCC AGG TCG S S S S S S 260  TCT TCT
260 260  TCC AGG TCG S S S S S S 270  TCT TCT
270 270  TCC AGG TCG S S S S S S 280  TCT TCT
280 280  TCC AGG TCG S S S S S S 290  TCT TCT
290 290  TCC AGG TCG S S S S S S 300  TCT TCT
300 300  TCC AGG TCG S S S S S S 310  TCT TCT
310 310  TCC AGG TCG S S S S S S 320  TCT TCT
320 320  TCC AGG TCG S S S S S S 330  TCT TCT
330 330  TCC AGG TCG S S S S S S 340  TCT TCT
340 340  TCC AGG TCG S S S S S S 350  TCT TCT
350 350  TCC AGG TCG S S S S S S 360  TCT TCT
360 360  TCC AGG TCG S S S S S S 370  TCT TCT
370 370  TCC AGG TCG S S S S S S 380  TCT TCT
380 380  TCC AGG TCG S S S S S S 390  TCT TCT
390 390  TCC AGG TCG S S S S S S 400  TCT TCT
400 400  TCC AGG TCG S S S S S S 410  TCT TCT
410 410  TCC AGG TCG S S S S S S 420  TCT TCT
420 420  TCC AGG TCG S S S S S S 430  TCT TCT
430 430  TCC AGG TCG S S S S S S 440  TCT TCT
440 440  TCC AGG TCG S S S S S S 450  TCT TCT
450 450  TCC AGG TCG S S S S S S 460  TCT TCT
460 460  TCC AGG TCG S S S S S S 470  TCT TCT
470 470  TCC AGG TCG S S S S S S 480  TCT TCT
480 480  TCC AGG TCG S S S S S S 490  TCT TCT
490 490  TCC AGG TCG S S S S S S 500  TCT TCT
500 500  TCC AGG TCG S S S S S S 510  TCT TCT
510 510  TCC AGG TCG S S S S S S 520  TCT TCT
520 520  TCC AGG TCG S S S S S S 530  TCT TCT
530 530  TCC AGG TCG S S S S S S 540  TCT TCT
540 540  TCC AGG TCG S S S S S S 550  TCT TCT
550 550  TCC AGG TCG S S S S S S 560  TCT TCT
560 560  TCC AGG TCG S S S S S S 570  TCT TCT
570 570  TCC AGG TCG S S S S S S 580  TCT TCT
580 580  TCC AGG TCG S S S S S S 590  TCT TCT
590 590  TCC AGG TCG S S S S S S 600  TCT TCT
600 600  TCC AGG TCG S S S S S S 610  TCT TCT
610 610  TCC AGG TCG S S S S S S 620  TCT TCT
620 620  TCC AGG TCG S S S S S S 630  TCT TCT
630 630  TCC AGG TCG S S S S S S 640  TCT TCT
640 640  TCC AGG TCG S S S S S S 650  TCT TCT
650 650  TCC AGG TCG S S S S S S 660  TCT TCT
660 660  TCC AGG TCG S S S S S S 670  TCT TCT
670 670  TCC AGG TCG S S S S S S 680  TCT TCT
680 680  TCC AGG TCG S S S S S S 690  TCT TCT
690 690  TCC AGG TCG S S S S S S 700  TCT TCT
700 700  TCC AGG TCG S S S S S S 710  TCT TCT
710 710  TCC AGG TCG S S S S S S 720  TCT TCT
720 720  TCC AGG TCG S S S S S S 730  TCT TCT
730 730  TCC AGG TCG S S S S S S 740  TCT TCT
740 740  TCC AGG TCG S S S S S S 750  TCT TCT
750 750  TCC AGG TCG S S S S S S 760  TCT TCT
760 760  TCC AGG TCG S S S S S S 770  TCT TCT
770 770  TCC AGG TCG S S S S S S 780  TCT TCT
780 780  TCC AGG TCG S S S S S S 790  TCT TCT
790 790  TCC AGG TCG S S S S S S 800  TCT TCT
800 800  TCC AGG TCG S S S S S S 810  TCT TCT
810 810  TCC AGG TCG S S S S S S 820  TCT TCT
820 820  TCC AGG TCG S S S S S S 830  TCT TCT
830 830  TCC AGG TCG S S S S S S 840  TCT TCT
840 840  TCC AGG TCG S S S S S S 850  TCT TCT
850 850  TCC AGG TCG S S S S S S 860  TCT TCT
860 860  TCC AGG TCG S S S S S S 870  TCT TCT
870 870  TCC AGG TCG S S S S S S 880  TCT TCT
880 880  TCC AGG TCG S S S S S S 890  TCT TCT
890 890  TCC AGG TCG S S S S S S 900  TCT TCT
900 900  TCC AGG TCG S S S S S S 910  TCT TCT
910 910  TCC AGG TCG S S S S S S 920  TCT TCT
920 920  TCC AGG TCG S S S S S S 930  TCT TCT
930 930  TCC AGG TCG S S S S S S 940  TCT TCT
940 940  TCC AGG TCG S S S S S S 950  TCT TCT
950 950  TCC AGG TCG S S S S S S 960  TCT TCT
960 960  TCC AGG TCG S S S S S S 970  TCT TCT
970 970  TCC AGG TCG S S S S S S 980  TCT TCT
980 980  TCC AGG TCG S S S S S S 990  TCT TCT
990 990  TCC AGG TCG S S S S S S 1000 TCT TCT

```

FIG. 8

GRAFTED Vh SEQUENCE OF hTNF40 (SEQ ID NO: 10)

```

10  CAG GTG CAG CAG GTC CAG GCA GAG GTT AAG AAG CCT GGT GCT TCC GTC
    GTC CAC CAC GTC GTC AGT CCT GCGT CGT CTC CAA TTC K K P GGA CGA AGG S V>
    10  20  30  40  50

60  AAA GTT TCG TGT AAG GCC TCA GGC TAC GTG TTC ACA GAC TAT GGT ATG AAT TGG
    TTT CAA V TTT CAG GTC CCG AGT S GCGT AAG CAC V CAC TGT T TGA ACC TTA ACC
    K  V  K  A  A  G  S  G  Y  Y  V  V  F  T  D  Y  G  M  N  W>
    60  70  80  90  100

110 GTC AGA CAG CCG GGC CCG GCA CAA GGT CTG TGG ATT AAT ACT 160
    CAG TCT GTC CCG GGC CCG GCA CAA GGT CTG TGG ATT AAT ACT TGA ATG Y>
    V  R  Q  A  120  130  140  150  160

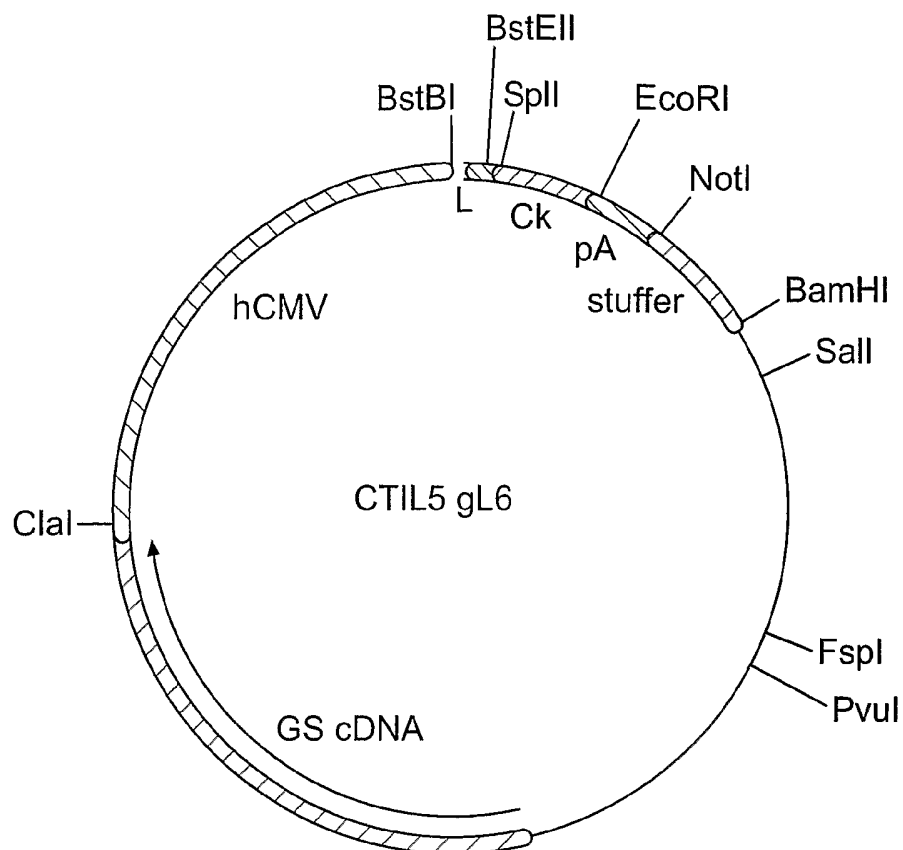
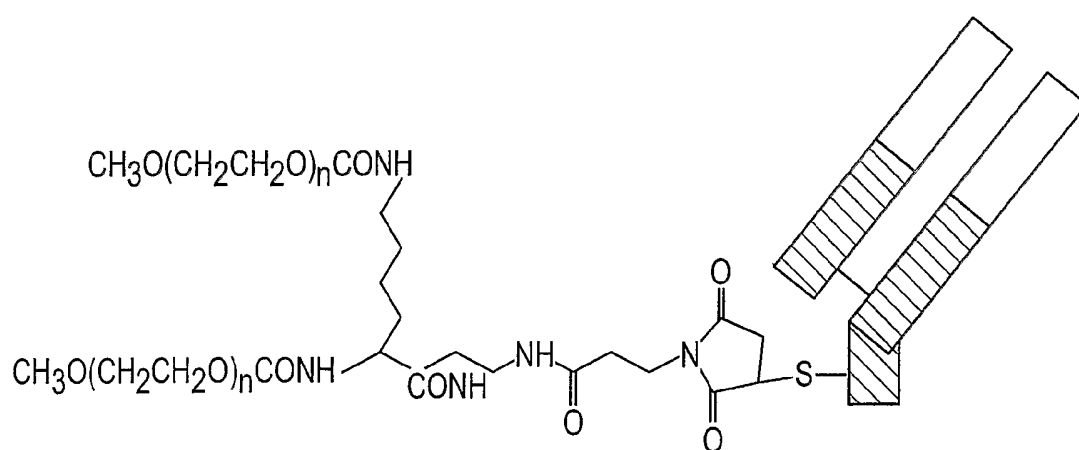
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    TAA CCT CTC GGA GGA ATA Y TTC K F GAG TTC CAG GTC CCG TCT R GTC CAG TGC
    I  G  E  P  180  190  200  210  220
    GAC ACC TCC ACA AGC ACT TGA TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG
    CTG TGG AGG TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT
    D  T  S  T  230  240  250  260  270
    GAC ACC TCC ACA AGC ACT TGA TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG
    CTG TGG AGG TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT
    280  290  300  310  320  330
    ACC GCA GTG TAC TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT
    TGG CGT CAC ATG ATG ATG ATG ATG ATG ATG ATG ATG ATG ATG ATG ATG ATG ATG
    T  A  V  Y  C  A  G  G  G  G  G  G  G  G  G  G  G  G  G  G  G  G  G  G  G  G
    340  350
    GGC CAG GGT ACC ACC CTA GTC ACA GTC TCC TCA TCA TCA TCA TCA TCA TCA TCA
    CCG GTC CCA G GGT CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG
    G  Q  360  370  380  390  400

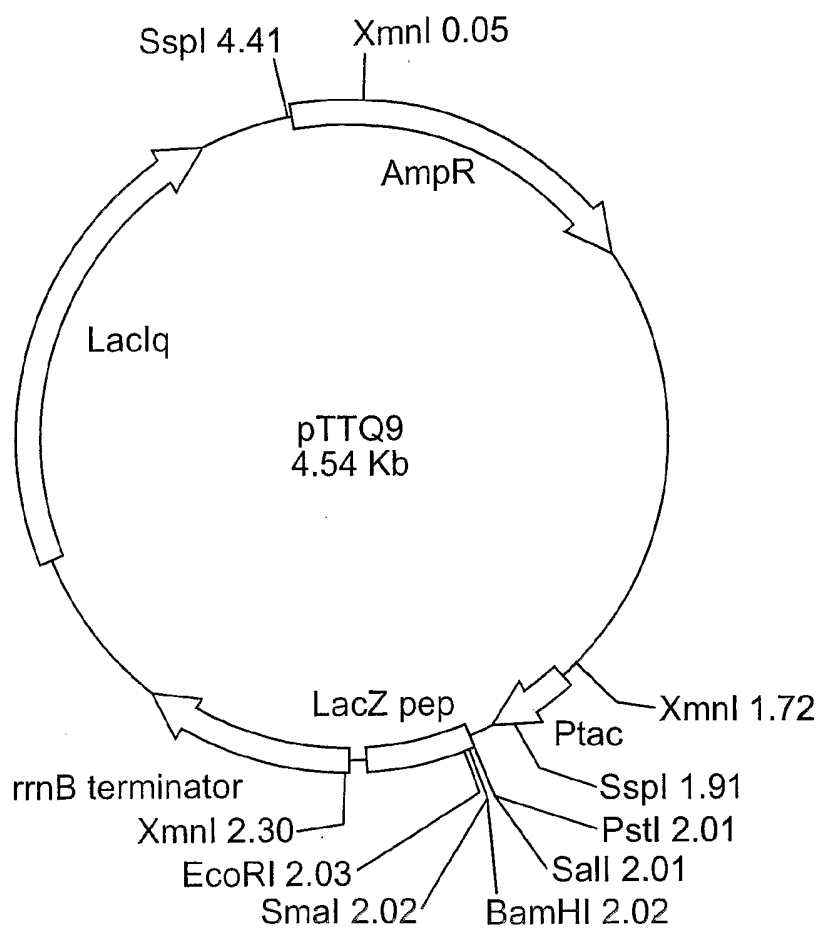
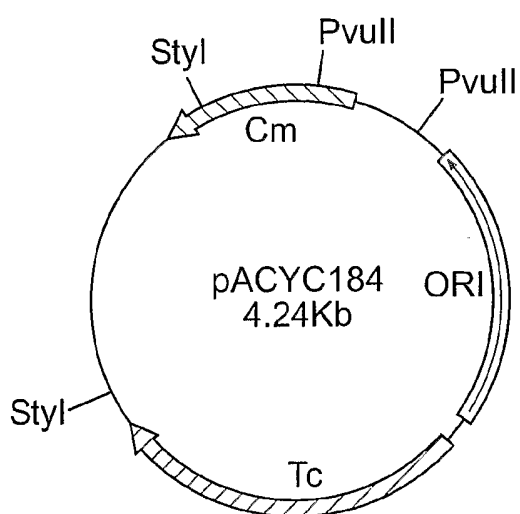
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FIG. 10

[illegible]

FIG. 11

**FIG. 12****FIG. 13**

**FIG. 14****FIG. 16**

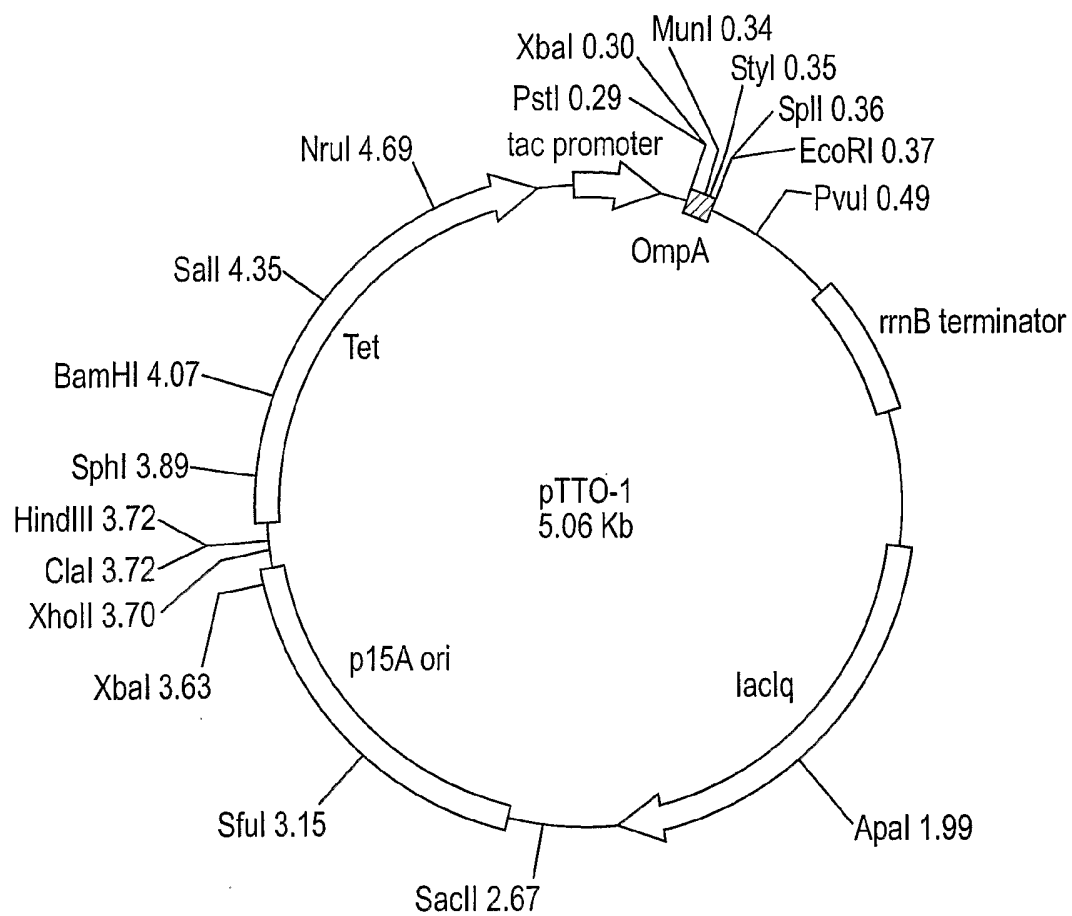
SEQUENCE OF OmpA OLIGONUCLEOTIDE ADAPTER (SEQ ID NO: 101)

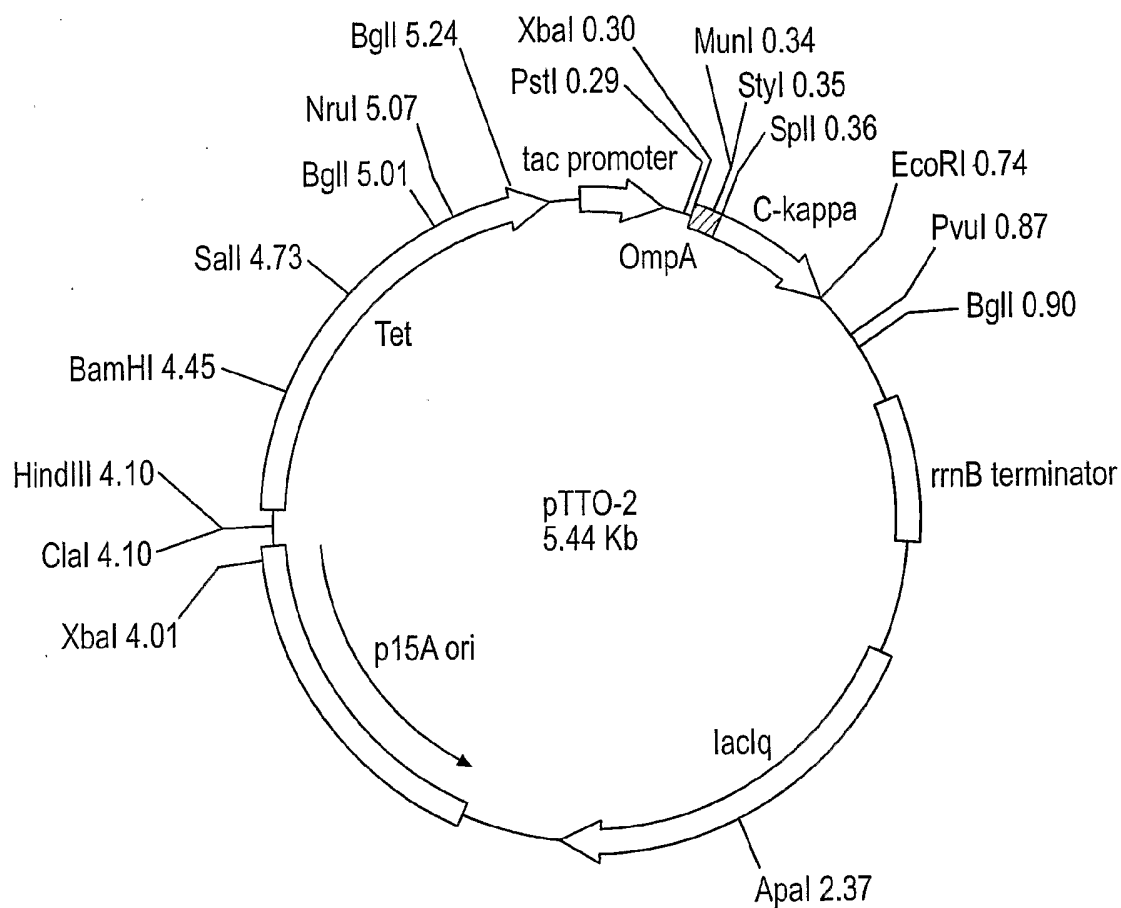
OmpA LEADER
→

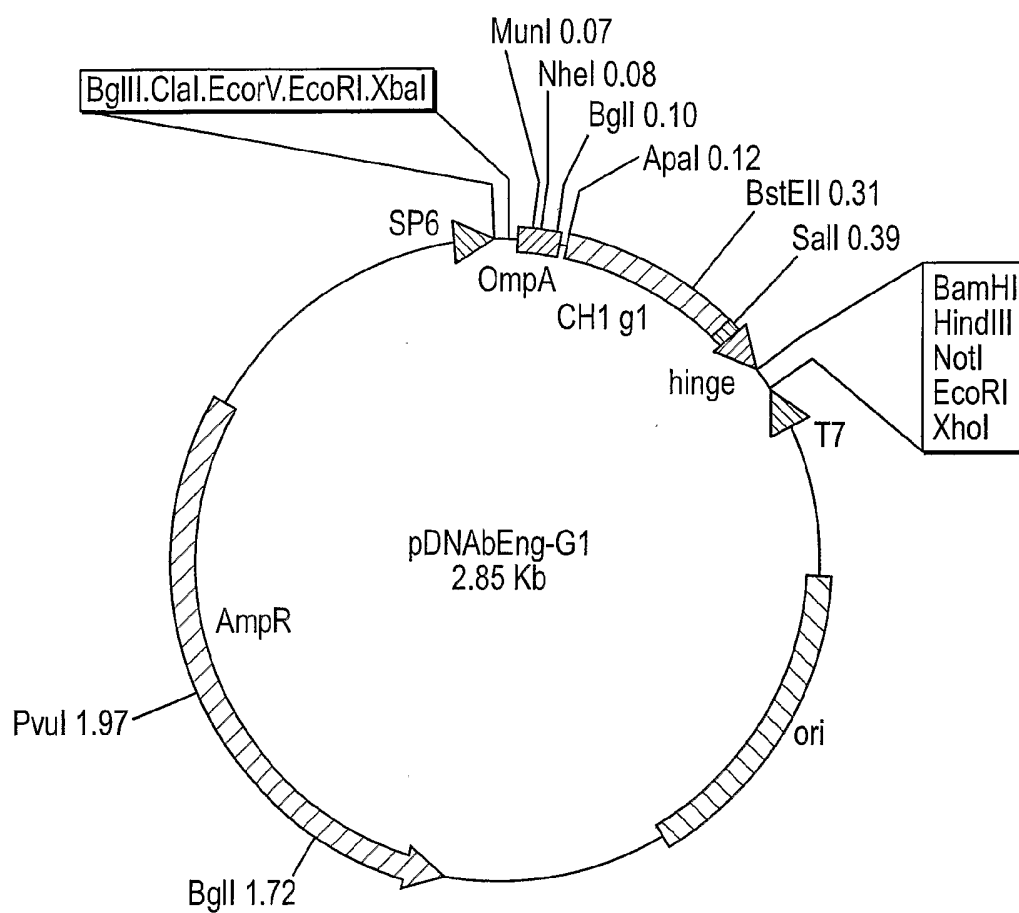
	10	20	30	40
	*	*	*	*
XhoI	XbaI	S.D.		
T CGA GTT CTA GAT AAC GAG GCG TAA AAA ATG AAA AAG ACA				
	CAA GAT CTA TTG CTC CGC ATT TTT TAC TTT TTC TGT			
			M K K T>	
	50	60	70	80
	*	*	*	*
	MunI	StyI	SplI	
GCT ATC GCA ATT GCA GTG GCC TTG GCT CTG ACG TAC GAG TCA				
CGA TAG CGT TAA CGT CAC CGG AAC CGA GAC TGC ATG CTC AGT				
A I A I A V A L A				
90				
*				
EcoRI				
GG				
CCT TAA				

- INTERNAL RESTRICTION SITES ARE SHOWN IN BOLD
- THE 5' XhoI COHESIVE AND LIGATES INTO THE VECTOR SalI SITE, BLOCKING IT
- S.D. REPRESENTS THE OmpA SHINE DALGARNO SEQUENCE

FIG. 15

**FIG. 17**

**FIG. 18**

**FIG. 19**

OLIGONUCLEOTIDE CASSETTES ENCODING DIFFERENT INTERGENIC SEQUENCES FOR E. Coli Fab' EXPRESSION

IGS CASSETTE-1: INTERGENIC SPACE = -1

G, AGC, TCA, CCA, GTA, ACA, AAA, AGT, TTT, AAT, AGA, GGA, GAG, TGT, TAATG, AAG, AAG, ACT, GCT, ATA, GCA, ATT, G (SEQ ID NO: 102)

S S P V T K S F N R G E C * M K K T A I A I

END OF c-KAPPA SEQUENCE ->

START OF OmpA SEQUENCE ->

IGS CASSETTE-2: INTERGENIC SPACE = +1

G, AGC, TCA, CCA, GTA, ACA, AAA, AGT, TTT, AAT, AGA, GGG, GAG, TGT, TAA AATG, AAG, AAG, ACT, GCT, ATA, GCA, ATT, G (SEQ ID NO: 103)

S S P V T K S F N R G E C * M K K T A I A I

IGS CASSETTE-3: INTERGENIC SPACE = +13

G, AGC, TCA, CCA, GTA, ACA, AAA, AGC, TTT, AAT, AGA, GGA, GAG, TGT, TGA GGAGGAAAAAAAAATG, AAG, AAA, ACT, GCT, ATA, GCA, ATT, G (SEQ ID NO: 104)

S S P V T K S F N R G E C * M K K T A I A I

IGS CASSETTE-4: INTERGENIC SPACE = +13

G, AGC, TCA, CCA, GTA, ACA, AAA, AGT, TTT, AAT, AGA, GGA, GAG, TGT, TGA CGAGGATTATATAATG, AAG, AAA, ACT, GCT, ATA, GCA, ATT, G (SEQ ID NO: 105)

S S P V T K S F N R G E C * M K K T A I A I

FIG. 20

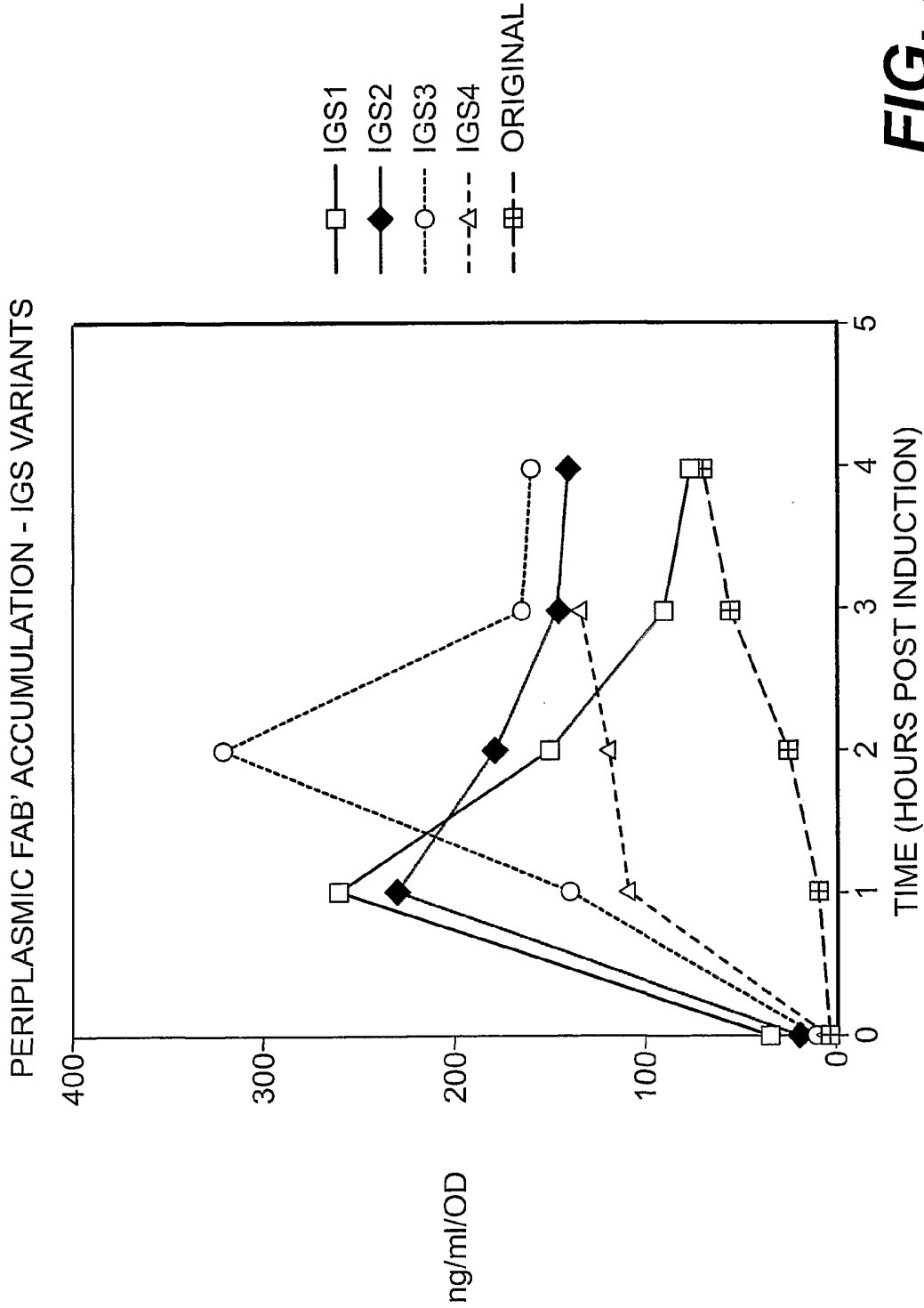


FIG. 21

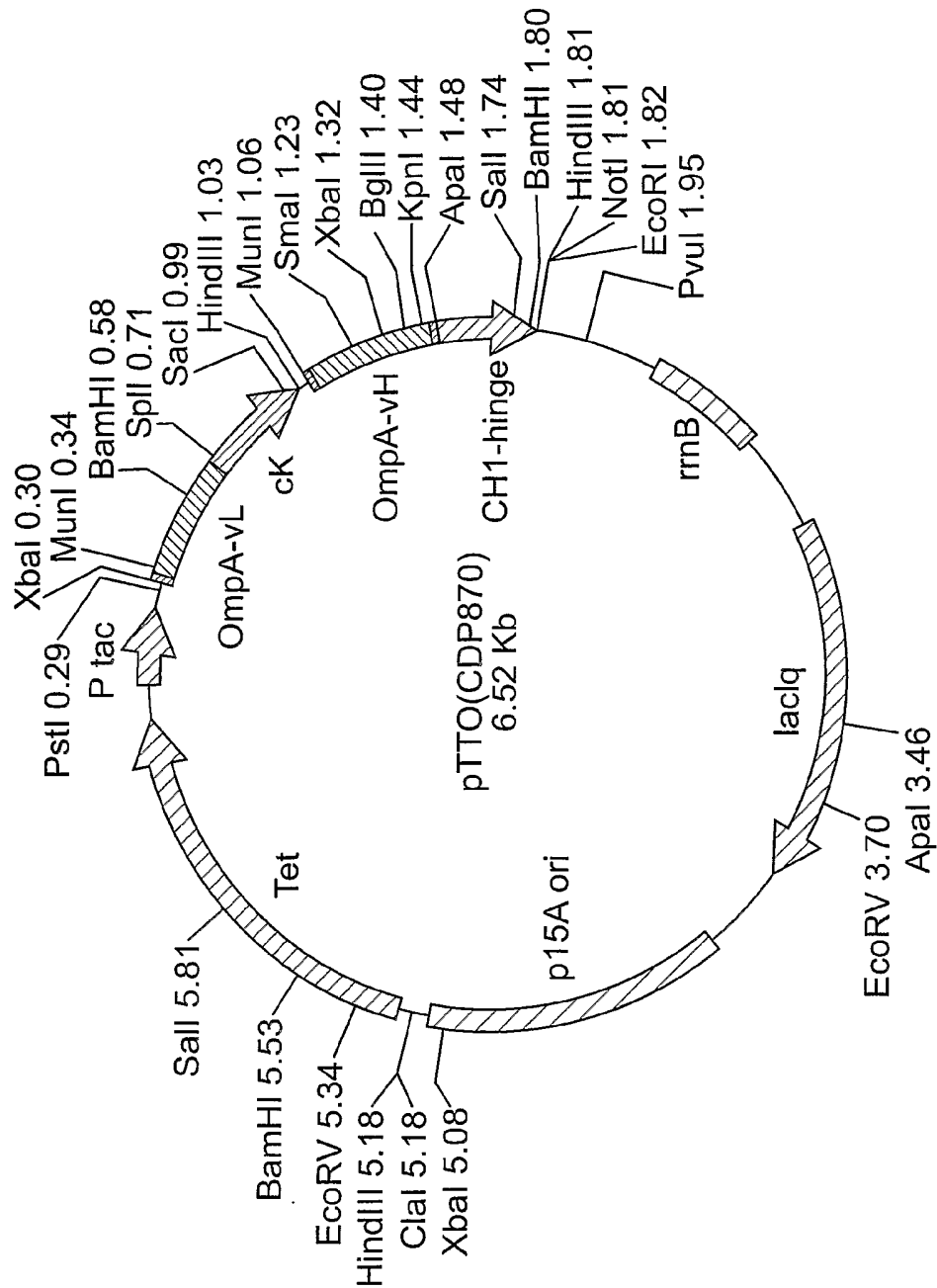
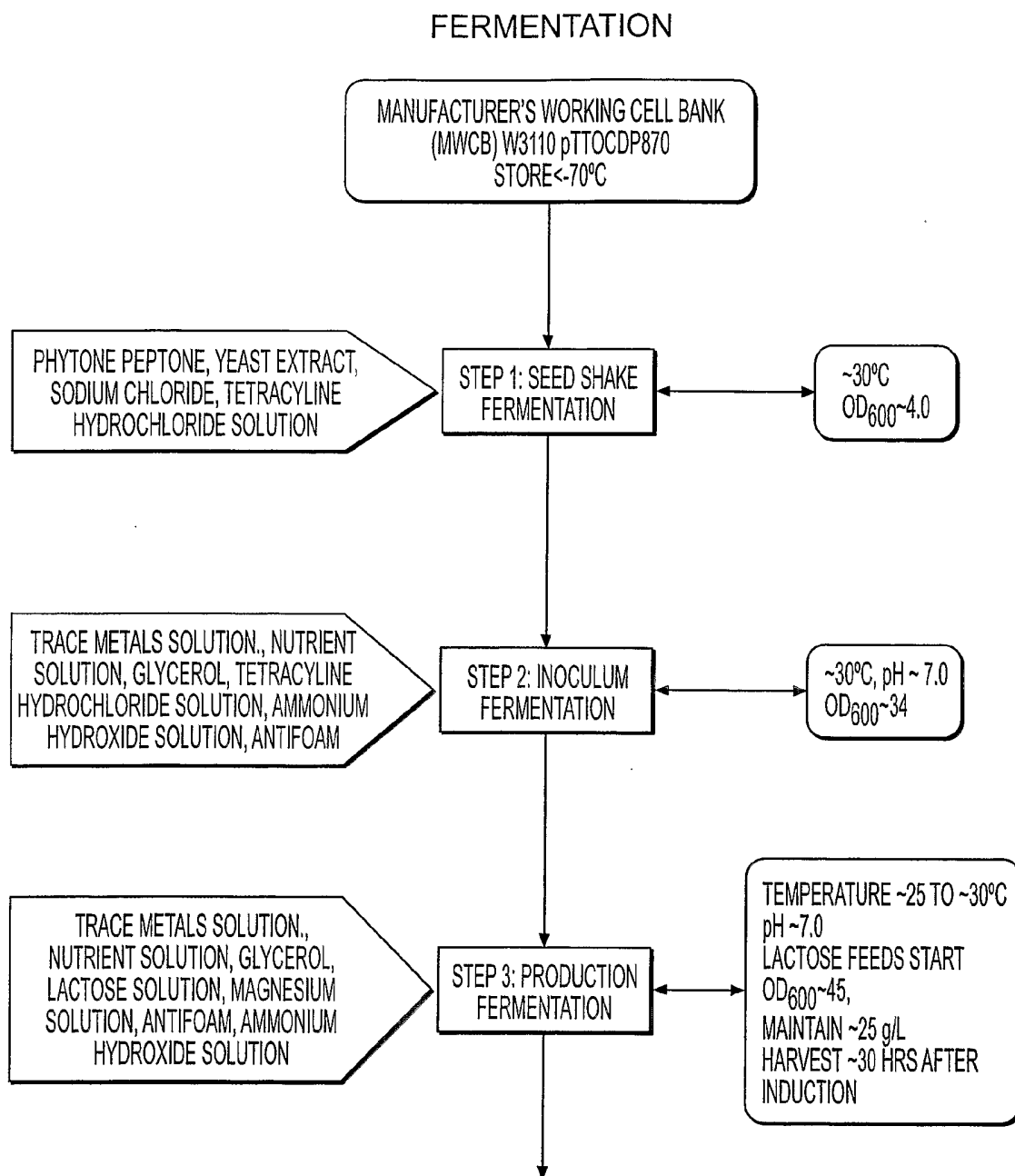
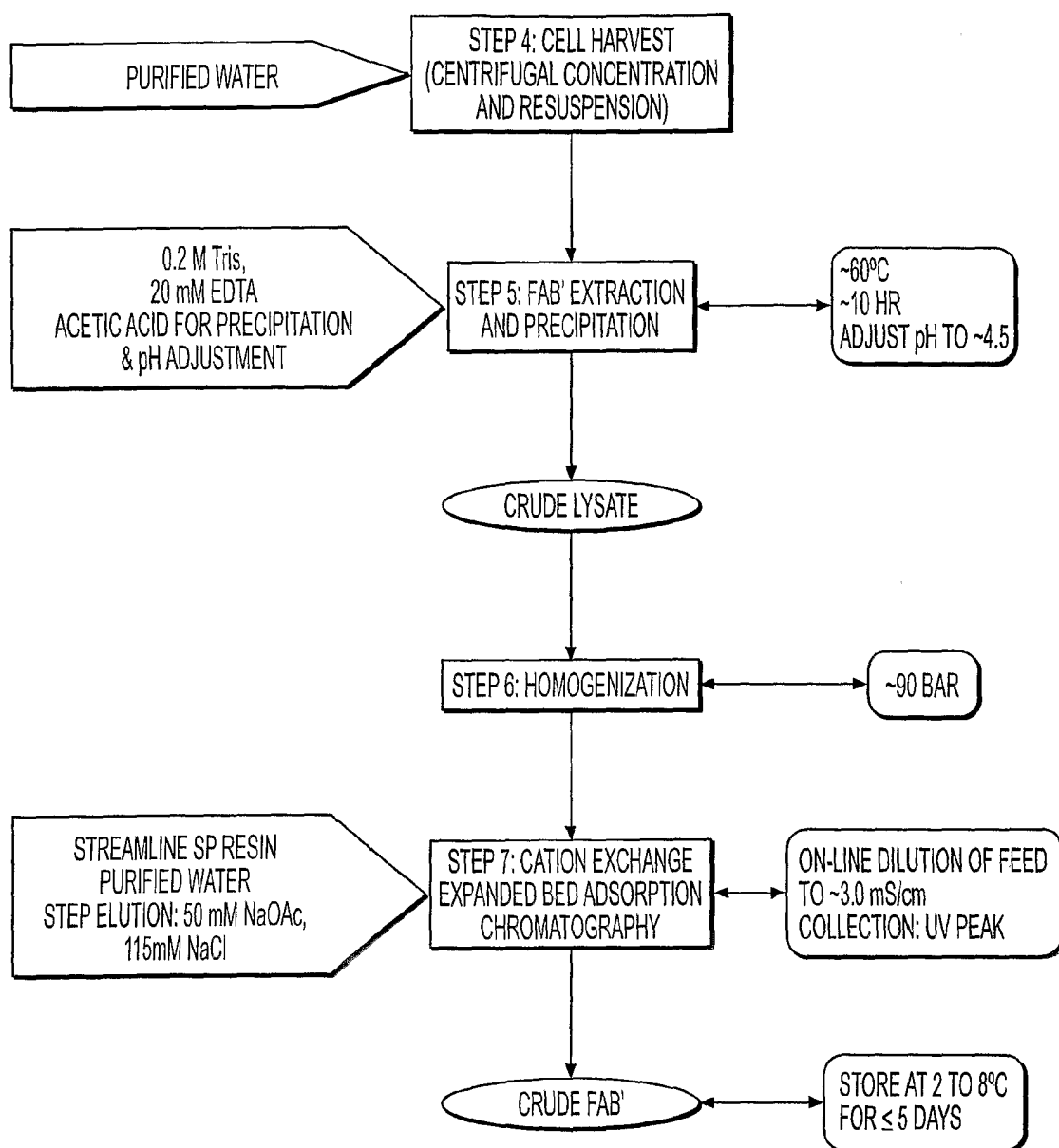


FIG. 22

**FIG. 23**

PRIMARY SEPARATION

**FIG. 24**

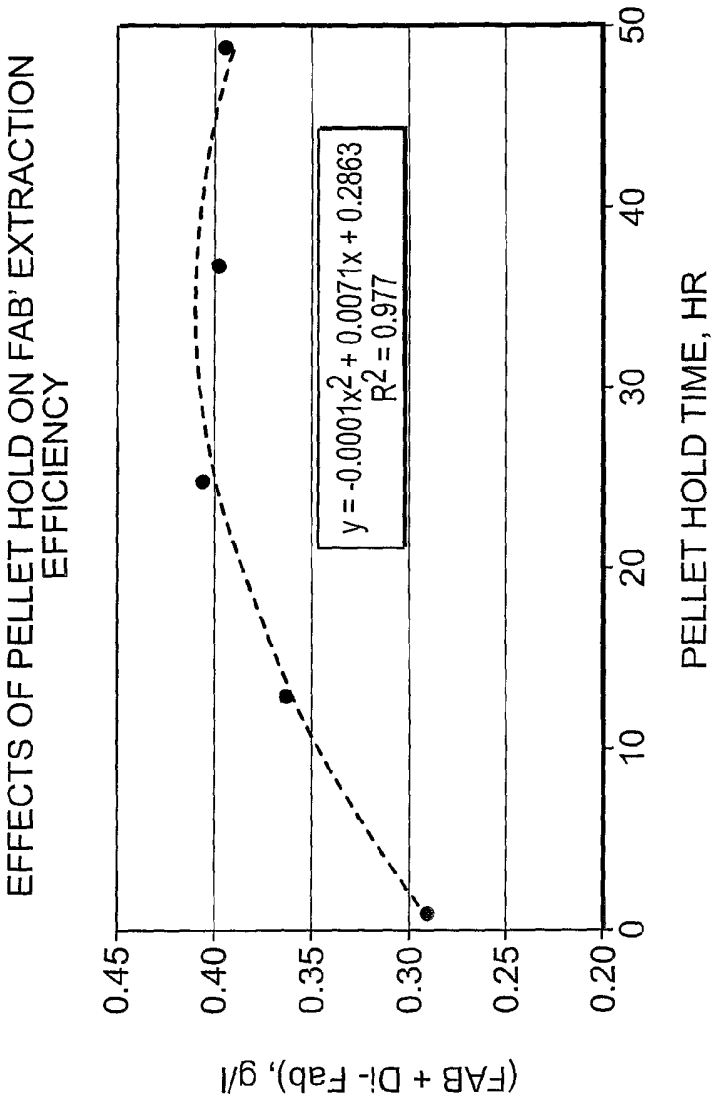


FIG. 25

SEQUENCE LISTING

<110> PHARMACIA CORPORATION

<120> METHOD FOR THE PREPARATION OF MOLECULES HAVING ANTIBODY ACTIVITY

<130> PHA-01686/1

<140>

<141>

<150> US 60/493,740

<151> 2003-08-08

<160> 115

<170> PatentIn Ver. 3.2

<210> 1

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 CDRH1

<400> 1

Asp Tyr Gly Met Asn
1 5

<210> 2

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40/human hybrid CDRH2

<400> 2

Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val Lys
1 5 10 15

Gly

<210> 3

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 CDRH3

<400> 3

Gly Tyr Arg Ser Tyr Ala Met Asp Tyr

1 5

<210> 4
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRL1

<400> 4
Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala
1 5 10

<210> 5
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRL2

<400> 5
Ser Ala Ser Phe Leu Tyr Ser
1 5

<210> 6
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRL3

<400> 6
Gln Gln Tyr Asn Ile Tyr Pro Leu Thr
1 5

<210> 7
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRH2

<400> 7
Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Val Asp Asp Phe Lys
1 5 10 15

Gly

<210> 8
<211> 321
<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(321)

<220>

<223> Description of Artificial Sequence: hTNF40-gL1

<400> 8

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly	
1 5 10 15	
gac cgg gtc acc atc act tgt aaa gcc agt cag aac gta ggt act aac	96
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn	
20 25 30	
gta gcc tgg tat cag caa aaa cca ggt aaa gcc cca aaa gcc ctc atc	144
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Ala Leu Ile	
35 40 45	
tac agt gcc tct ttc ctc tat agt ggt gta cca tac agg ttc agc gga	192
Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly	
50 55 60	
tcc ggt agt ggt act gat ttc acc ctc acg atc agt agc ctc cag cca	240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro	
65 70 75 80	
gaa gat ttc gcc act tat tac tgt caa cag tat aac atc tac cca ctc	288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu	
85 90 95	
aca ttc ggt cag ggt act aaa gta gaa atc aaa	321
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys	
100 105	

<210> 9

<211> 321

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(321)

<223> Description of Artificial Sequence: hTNF40-gL2

<400> 9

gac att caa atg acc cag agc cca tcc agc ctg agc gca tct gta gga	48
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly	
1 5 10 15	
gac cgg gtc acc atc act tgt aaa gcc agt cag aac gta ggt act aac	96
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn	
20 25 30	

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gta gcc tgg tat cag caa aaa cca ggt aaa gcc cca aaa ctc ctc atc      144
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
      35                      40                      45

tac agt gcc tct ttc ctc tat agt ggt gta cca tac agg ttc agc gga      192
Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly
      50                      55                      60

tcc ggt agt ggt act gat ttc acc ctc acg atc agt agc ctc cag cca      240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
      65                      70                      75                      80

gaa gat ttc gcc act tat tac tgt caa cag tat aac atc tac cca ctc      288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu
      85                      90                      95

aca ttc ggt cag ggt act aaa gta gaa atc aaa                        321
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
      100                      105

<210> 10
<211> 354
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)..(354)

<220>
<223> Description of Artificial Sequence:ghlhTNF40.4

<400> 10
cag gtg cag ctg gtc cag tca gga gca gag gtt aag aag cct ggt gct      48
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
      1                      5                      10                      15

tcc gtc aaa gtt tgc tgt aag gcc tca ggc tac gtg ttc aca gac tat      96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Val Phe Thr Asp Tyr
      20                      25                      30

ggt atg aat tgg gtc aga cag gcc ccg gga caa ggc ctg gaa tgg atg      144
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
      35                      40                      45

ggt tgg att aat act tac att gga gag cct att tat gct caa aag ttc      192
Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Gln Lys Phe
      50                      55                      60

cag ggc aga gtc acg ttc act cta gac acc tcc aca agc act gca tac      240
Gln Gly Arg Val Thr Phe Thr Leu Asp Thr Ser Thr Ser Thr Ala Tyr
      65                      70                      75                      80

atg gag ctg tca tct ctg aga tcc gag gac acc gca gtg tac tat tgt      288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys

```

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      85              90              95
gct aga gga tac aga tct tat gcc atg gac tac tgg ggc cag ggt acc 336
Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
      100              105              110

cta gtc aca gtc tcc tca 354
Leu Val Thr Val Ser Ser
      115

<210> 11
<211> 354
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)..(354)

<220>
<223> Description of Artificial Sequence: gh3hTNF40.4

<400> 11
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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
      1              5              10              15

tca ctg aga ttg tcc tgt gct gca tct ggt tac gtc ttc aca gac tat 96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr
      20              25              30

gga atg aat tgg gtt aga cag gcc ccg gga aag ggc ctg gaa tgg atg 144
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met
      35              40              45

ggt tgg att aat act tac att gga gag cct att tat gct gac agc gtc 192
Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val
      50              55              60

aag ggc aga ttc acg ttc tct cta gac aca tcc aag tca aca gca tac 240
Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
      65              70              75              80

ctc caa atg aat agc ctg aga gca gag gac acc gca gtg tac tat tgt 288
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
      85              90              95

gct aga gga tac aga tct tat gcc atg gac tac tgg ggc cag ggt acc 336
Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
      100              105              110

cta gtc aca gtc tcc tca 354
Leu Val Thr Val Ser Ser
      115

<210> 12
<211> 9

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<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:part of a primer sequence

<400> 12
gccgccacc

9

<210> 13
<211> 26
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer CHI

<400> 13
atgaaatgca gctgggtcat sttctt

26

<210> 14
<211> 26
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CH2

<400> 14
atgggatgga gctratatcat sytctt

26

<210> 15
<211> 26
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CH3

<400> 15
atgaagwtgt ggtaaactg ggtttt

26

<210> 16
<211> 23
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CH4

<400> 16
atgractttg ggytcagctt grt

23

<210> 17
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH5

<400> 17
atggactcca ggctcaattt agtttt

26

<210> 18
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH6

<400> 18
atggctgtcy trsggctrct cttctg

26

<210> 19
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH7

<400> 19
atggratgga gckggrtctt tmtctt

26

<210> 20
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH8

<400> 20
atgagagtgc tgattctttt gtg

23

<210> 21
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH9

<400> 21
atggmttggg tgtggamctt gctatt

26

<210> 22

<211> 26
<212> DNA
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<220>
<223> Description of Artificial Sequence:primer CH10

<400> 22
atgggcagac ttacattctc attcct 26

<210> 23
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<212> DNA
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<220>
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<400> 23
atggattttg ggctgatttt ttttattg 28

<210> 24
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer CH12

<400> 24
atgatggtgt taagtcttct gtacct 26

<210> 25
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 5'end

<400> 25
gcgcgcaagc ttgccgccac c 21

<210> 26
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL1

<400> 26
atgaagttgc ctgtaggct gttggtgct 29

<210> 27
<211> 29

<212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer CL2

<400> 27
 atggagwcag acacactcct gytatgggt 29

<210> 28
 <211> 23
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer CL3

<400> 28
 atgagtgtgc tcactcaggt cct 23

<210> 29
 <211> 26
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer CL4

<400> 29
 atgaggrccc ctgctcagwt tyttgg 26

<210> 30
 <211> 29
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer CL5

<400> 30
 atggatttwc aggtgcagat twtcagctt 29

<210> 31
 <211> 29
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer CL5A

<400> 31
 atggatttwc argtgcagat twtcagctt 29

<210> 32
 <211> 26
 <212> DNA
 <213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL6

<400> 32
atgaggtkcy ytgytsagyt yctgrg 26

<210> 33
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL7

<400> 33
atgggcwtca agatggagtc aca 23

<210> 34
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CLB

<400> 34
atgtggggay ctktttymmm tttttcaat 29

<210> 35
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL9

<400> 35
atggtrtccw casctcagtt cctt 24

<210> 36
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL10

<400> 36
atgtatatat gtttggtgtc tatttc 26

<210> 37
<211> 26
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer CL11

<400> 37

atggaagccc cagctcagct tctctt

26

<210> 38

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL12A

<400> 38

atgragtywc agacccaggt cttyrt

26

<210> 39

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL12B

<400> 39

atggagacac attctcaggt ctttgt

26

<210> 40

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL13

<400> 40

atggattcac aggcccaggt tcttat

26

<210> 41

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL14

<400> 41

atgatgagtc ctgcccagtt cctggt

26

<210> 42

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL15

<400> 42
atgaatttgc ctgttcatct cttggtgct 29

<210> 43
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL16

<400> 43
atgaatttgc aattggctct catctcctt 29

<210> 44
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL17A

<400> 44
atgaggtgcc tarctsagtt cctarg 26

<210> 45
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL17B

<400> 45
atgaagtact ctgctcagtt tctagg 26

<210> 46
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer CL17C

<400> 46
atgaggcatt ctcttcaatt cttggg 26

<210> 47
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer 5' end

<400> 47
ggactgttcg aagccgccac c 21

<210> 48
<211> 30
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL12

<400> 48
ggatacagtt ggtgcagcat ccgtacgttt

30

<210> 49
<211> 37
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer R2155

<400> 49
gcagatgggc ccttcgttga ggctgmrgag acdgtga

37

<210> 50
<211> 24
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer R1053

<400> 50
gctgacagac taacagactg ttcc

24

<210> 51

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

<220>

<223> Description of Artificial Sequence:primer R720

<400> 51
gctctcggag gtgctcct

18

<210> 52
<211> 70
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide P7982

<400> 52

gaattcaggg tcaccatcac ttgtaaagcc agtcagaacg taggtactaa cgtagcctgg 60
tatcagcaaa 70

<210> 53
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligonucleotide P7983

<400> 53
atagaggaaa gaggcactgt agatgagggc ttttggggct ttacctgggt tttgctgata 60
ccaggtacg t 71

<210> 54
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide P7984

<400> 54
tacagtgcct ctttcctcta tagtggtgta ccatacaggt tcagcggatc cggtagtgggt 60
actgatttca c 71

<210> 55
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide P7985

<400> 55
gacagtaata agtggcgaaa tcttctggct ggaggctact gatcgtgagg gtgaaatcag 60
taccactacc g 71

<210> 56
<211> 89
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide P7986

<400> 56
atttcgccac ttattactgt caacagtata acatctaccc actcacattc ggtcagggta 60
ctaaagtaga aatcaaactg acggaattc 89

<210> 57
<211> 30
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide P7981

<400> 57

gaattcaggg tcaccatcac ttgtaaaacc 30

<210> 58

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: oligonucleotide P7980

<400> 58

gaattccgta cgtttgattt ctactttagt 30

<210> 59

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R1053

<400> 59

gctgacagac taacagactg ttcc 24

<210> 60

<211> 57

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R5350

<400> 60

tctagatggc acaccatctg ctaagtttga tgcagcatag atcaggagct taggagc 57

<210> 61

<211> 59

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R5349

<400> 61

gcagatggtg tgccatctag attcagtggc agtggatcag gcacagactt taccctaac 59

<210> 62

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R684

<400> 62
ttcaactgct catcagat

18

<210> 63
<211> 65
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7989

<400> 63
gaagcaccag gcttcttaac ctctgctcct gactggacca gctgcacctg agagtgcacg 60
aattc 65

<210> 64
<211> 71
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7990

<400> 64
ggttaagaag cctggtgctt ccgtcaaagt ttcgtgtaag gcctcaggct acgtgttcac 60
agactatggt a 71

<210> 65
<211> 71
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7991

<400> 65
ccaaccatc catttcaggc cttgtcccgg ggcctgcttg acccaattca taccatagtc 60
tgtgaacacg t 71

<210> 66
<211> 81
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7995

<400> 66
ggcctgaaat ggatgggttg gattaatact tacattggag agcctattta tggtgacgac 60
ttcaagggca gattcacgtt c 81

<210> 67
<211> 56
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7992

<400> 67

ccatgtatgc agtgcgttgt ggaggtgtct agagtgaacg tgaatctgcc cttgaa 56

<210> 68

<211> 62

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7993

<400> 68

ccacaagcac tgcatacatg gagctgtcat ctctgagatc cgaggacacc gcagtgtact 60
at 62

<210> 69

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7994

<400> 69

gaattcggta ccctggcccc agtagtccat ggcataagat ctgtatcctc tagcacaata 60
gtacactgcg gtgtcctc 78

<210> 70

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7988

<400> 70

gaattcgtgc actctcaggt gcagctggtc 30

<210> 71

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7987

<400> 71

gaattcggta ccctggcccc agtagtccat 30

<210> 72

<211> 65

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7999

<400> 72

gatccgccag gctgcacgag accgcctcct gactcgacca gctgaacctc agagtgcacg	60
aattc	65

<210> 73

<211> 71

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P8000

<400> 73

tctcgtgcag cctggcggat cgctgagatt gtcctgtgct gcatctggtt acgtcttcac	60
agactatgga a	71

<210> 74

<211> 71

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P8001

<400> 74

ccaacccatc catttcaggc cctttcccgg ggctgctta acccaattca ttccatagtc	60
tgtgaagacg t	71

<210> 75

<211> 55

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7997

<400> 75

ggaggtatgc tgttgacttg gatgtgtcta gagagaacgt gaatctaccc ttgaa	55
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<210> 76

<211> 62

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7998

<400> 76

ccaagtcaac agcatacctc caaatgaata gcctgagagc agaggacacc gcagtgtact	60
at	62

<210> 77

<211> 78
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer P7993

<400> 77
 gaattcggta ccctggcccc agtagtccat ggcataagat ctgtatcctc tagcacaata 60
 gtacactgcg gtgtcctc 78

<210> 78
 <211> 30
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer P7996

<400> 78
 gaattcgtgc actctgaggt tcagctggtc 30

<210> 79
 <211> 74
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 5' primer

<400> 79
 cgcgcgga ttcagtggt cttggctggt ttgcgtaccg tagcgcaagc tgacattcaa 60
 atgaccaga gcc 74

<210> 80
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 3' primer

<400> 80
 ttcaactgct catcagatgg 20

<210> 81
 <211> 78
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 5' primer

<400> 81
 gctatcgcaa ttgcagtggt gctagctggt ttgccaccg tggcgcaagc tgagggttcag 60
 ctggctcagc caggaggc 78

<210> 82
 <211> 18
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: 3' primer

<400> 82
 gcctgagttc cacgacac 18

<210> 83
 <211> 23
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: human group I consensus framework L1

<400> 83
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15

Asp Arg Val Thr Ile Thr Cys
 20

<210> 84
 <211> 23
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: hTNF40 framework L1

<400> 84
 Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15

Asp Arg Val Ser Val Thr Cys
 20

<210> 85
 <211> 15
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: human group 1 consensus framework L2

<400> 85
 Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
 1 5 10 15

<210> 86
 <211> 15
 <212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: hTNF40 framework L2

<400> 86

Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ser	Pro	Lys	Ala	Leu	Ile	Tyr
1				5				10					15	

<210> 87

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: human group 1 consensus framework L3

<400> 87

Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr
1				5				10					15		

Leu	Thr	Ile	Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys
			20					25					30		

<210> 88

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: hTNF40 framework L3

<400> 88

Gly	Val	Pro	Tyr	Arg	Phe	Thr	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr
1				5				10					15		

Leu	Thr	Ile	Ser	Thr	Val	Gln	Ser	Glu	Asp	Leu	Ala	Glu	Tyr	Phe	Cys
			20					25					30		

<210> 89

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: human group 1 consensus framework L4

<400> 89

Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu	Ile	Lys	Arg
1				5				10		

<210> 90

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework L4

<400> 90

Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
1 5 10

<210> 91

<211> 30

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus framework H1

<400> 91

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
20 25 30

<210> 92

<211> 30

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework H1

<400> 92

Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
1 5 10 15

Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Val Phe Thr
20 25 30

<210> 93

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus framework H2

<400> 93

Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly
1 5 10

<210> 94

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework H2

<400> 94

Trp Val Lys Gln Ala Pro Gly Lys Ala Phe Lys Trp Met Gly
 1 5 10

<210> 95

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus framework H3

<400> 95

Arg Val Thr Ile Thr Arg Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu
 1 5 10 15

Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
 20 25 30

<210> 96

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework H3

<400> 96

Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Phe Leu Gln
 1 5 10 15

Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr Tyr Phe Cys Ala Arg
 20 25 30

<210> 97

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus framework H4

<400> 97

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 1 5 10

<210> 98

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework H4

<400> 98

Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
 1 5 10

<210> 99

<211> 324
 <212> DNA
 <213> murine

<220>
 <221> CDS
 <222> (1)..(324)
 <223> mouse hTNF40 light chain variable domain

<400> 99
 gac att gtg atg acc cag tct caa aaa ttc atg tcc aca tca gta gga 48
 Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15
 gac agg gtc agc gtc acc tgc aag gcc act cag aat gtg ggt act aat 96
 Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
 20 25 30
 gta gcc tgg tat caa cag aaa cca gga caa tct cct aaa gca ctg att 144
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile
 35 40 45
 tac tcg gca tcc ttc cta tat agt gga gtc cct tat cgc ttc aca ggc 192
 Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Thr Gly
 50 55 60
 agt gga tct ggg aca gat ttc act ctc acc atc agc act gtg cag tct 240
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Thr Val Gln Ser
 65 70 75 80
 gaa gac ttg gca gag tat ttc tgt cag caa tat aac atc tat cct ctc 288
 Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ile Tyr Pro Leu
 85 90 95
 acg ttc ggt gct ggg acc aag ctg gag ctg aaa cgt 324
 Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
 100 105

<210> 100
 <211> 354
 <212> DNA
 <213> murine

<220>
 <221> CDS
 <222> (1)..(354)
 <223> mouse hTNF40 heavy chain variable domain

<400> 100
 cag atc cag ttg gtg cag tct gga cct gag cta aag aag cct gga gag 48
 Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
 1 5 10 15
 aca gtc aag atc tcc tgc aag gct tct gga tat gtt ttc aca gac tat 96
 Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Val Phe Thr Asp Tyr
 20 25 30

gga atg aat tgg gtg aag cag gct cca gga aag gct ttc aag tgg atg 144
 Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Ala Phe Lys Trp Met
 35 40 45

ggc tgg ata aac acc tac att gga gag cca ata tat gtt gat gac ttc 192
 Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Val Asp Asp Phe
 50 55 60

aag gga cga ttt gcc ttc tct ttg gaa acc tct gcc agc act gcc ttt 240
 Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Phe
 65 70 75 80

ttg cag atc aac aac ctc aaa aat gag gac acg gct aca tat ttc tgt 288
 Leu Gln Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr Tyr Phe Cys
 85 90 95

gca aga ggt tac cgg tcc tat gct atg gac tac tgg ggt caa gga acc 336
 Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
 100 105 110

tca gtc acc gtc tct tca 354
 Ser Val Thr Val Ser Ser
 115

<210> 101
 <211> 84
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (29)..(67)

<223> Description of Artificial Sequence:OmpA oligonucleotide adaptor

<400> 101
 tcgagttcta gataacgagg cgtaaaaa atg aaa aag aca gct atc aca att 52
 Met Lys Lys Thr Ala Ile Ala Ile
 1 5

gca gtg gcc ttg gct ctgacgtacg agtcagg 84
 Ala Val Ala Leu Ala
 10

<210> 102
 <211> 67
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (2)..(40)

<220>
 <221> CDS
 <222> (43)..(66)

<220>

<223> Description of Artificial Sequence:IGS cassette-1

<400> 102

g	agc	tca	cca	gta	aca	aaa	agt	ttt	aat	aga	gga	gag	tgt	ta	atg	aag	48
Ser	Ser	Pro	Val	Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Xaa		Xaa	Lys		
1				5					10						15		

aag	act	gct	ata	gca	att	g											67
Lys	Thr	Ala	Ile	Ala	Ile												
				20													

<210> 103

<211> 69

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (2)..(43)

<220>

<221> CDS

<222> (45)..(68)

<220>

<223> Description of Artificial Sequence:IGS cassette-2

<400> 103

g	agc	tca	cca	gta	aca	aaa	agt	ttt	aat	aga	ggg	gag	tgt	taa	a	atg	47
Ser	Ser	Pro	Val	Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Cys				Met	
1				5					10							15	

aag	aag	act	gct	ata	gca	att	g										69
Lys	Lys	Thr	Ala	Ile	Ala	Ile											
				20													

<210> 104

<211> 81

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (2)..(43)

<220>

<221> CDS

<222> (57)..(80)

<220>

<223> Description of Artificial Sequence:IGS cassette-3

<400> 104

g	agc	tca	cca	gta	aca	aaa	agc	ttt	aat	aga	gga	gag	tgt	tga			43
Ser	Ser	Pro	Val	Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Cys					
1				5					10								

ggaggaaaaa aaa atg aag aaa act gct ata gca att g 81
 Met Lys Lys Thr Ala Ile Ala Ile
 15 20

<210> 105
 <211> 81
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (2)..(43)

<220>
 <221>CDS
 <222> (57)..(80)

<220>
 <223> Description of Artificial Sequence:IGS cassette-4

<400> 105
 g agc tca cca gta aca aaa agt ttt aat aga gga gag tgt tga 43
 Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
 1 5 10

cgaggattat ata atg aag aaa act gct ata gca att g 81
 Met Lys Lys Thr Ala Ile Ala Ile
 15 20

<210> 106
 <211> 30
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 3 consensus framework H1

<400> 106
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
 20 25 30

<210> 107
 <211> 14
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 3 consensus framework H2

<400> 107
 Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser

1 5 10

<210> 108
 <211> 32
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 3 consensus framework H3

<400> 108
 Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln
 1 5 10 15

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
 20 25 30

<210> 109
 <211> 11
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 3 consensus framework H4

<400> 109
 Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 1 5 10

<210> 110
 <211> 648
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Grafted Heavy Chain for Fab

<400> 110
 gaggttcagc tggctcgagtc aggaggcggt ctctgtgcagc ctggcggatc actgagattg 60
 tcctgtgctg catctgggta cgtcttcaca gactatggaa tgaattgggt tagacaggcc 120
 ccgggaaaagg gcctggaatg gatgggttggt attaatactt acattggaga gcctatttat 180
 gctgacagcg tcaagggcag attcacgttc tctctagaca catccaagtc aacagcatac 240
 ctccaaatga atagcctgag agcagaggac accgcagtgt actattgtgc tagaggatac 300
 agatcttatg ccatggacta ctggggccag ggtaccctag tcacagtctc ctcagcttcc 360
 accaagggcc catcgtgttt ccccttgga cctcctcca agagcacctc tgggggcaca 420
 gctgccttgg gctgcctggt caaggactac ttccccgaac cggtgacggt gtcgtggaac 480
 tcaggcgccc tgaccagcgg cgtgcacacc ttctcggctg tcctacagtc ctcaggactc 540
 tactccctca gcagcgtggt gaccgtgccc tccagcagct tgggcaccca gacctacatc 600
 tgcaacgtga atcacaagcc cagcaacacc aaggctcgaca agaaagtt 648

<210> 111
 <211> 216
 <212> PRT
 <213> Artificial Sequence

<220>

<223> Grafted Heavy Chain for Fab

<400> 111

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr
20 25 30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met
35 40 45

Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
100 105 110

Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro
115 120 125

Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
130 135 140

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn
145 150 155 160

Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln
165 170 175

Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser
180 185 190

Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
195 200 205

Asn Thr Lys Val Asp Lys Lys Val
210 215

<210> 112

<211> 642

<212> DNA

<213> Artificial Sequence

<220>

<223> Grafted Light Chain for Fab and Modified Fab

<400> 112

gacattcaaa tgaccagag cccatccagc ctgagcgcac ctgtaggaga ccgggtcacc 60
atcacttgta aagccagtca gaacgtaggc actaacgtac cctgggtatca gcaaaaacca 120
ggtaaagccc caaaagccct catctacagt gcctctttcc tctatagtgg tgtaccatac 180

```

aggttcagcg gatccggtag tggtagtgat ttcaccctca cgatcagtag cctccagcca 240
gaagatttcg ccacttatta ctgtcaacag tataacatct acccactcac attcgggtcag 300
ggtactaaag tagaaatcaa acgtacggta acggcccat ctgtcttcat cttcccgcca 360
tctgatgagc agttgaaatc tggaactgcc tctgttgtgt gcctgctgaa taacttctat 420
cccagagagg ccaaagtaca gtggaagggtg gataacgccc tccaatcggg taactcccag 480
gagagtgtca cagagcagga cagcaaggac agcacctaca gcctcagcag caccctgacg 540
ctgagcaaag cagactacga gaaacacaaa gtctacgcct gcgaagtcac ccatcagggc 600
ctgagctcac cagtaacaaa aagctttaat agaggagagt gt 642

```

<210> 113

<211> 214

<212> PRT

<213> Artificial Sequence

<220>

<223> Grafted Light Chain for Fab and Modified Fab

<400> 113

```

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
  1                      5                      10                      15

```

```

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
          20                      25                      30

```

```

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Ala Leu Ile
      35                      40                      45

```

```

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly
      50                      55                      60

```

```

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
      65                      70                      75                      80

```

```

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu
          85                      90                      95

```

```

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
      100                      105                      110

```

```

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
      115                      120                      125

```

```

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
      130                      135                      140

```

```

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
      145                      150                      155                      160

```

```

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
          165                      170                      175

```

```

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
          180                      185                      190

```

```

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
      195                      200                      205

```

Phe Asn Arg Gly Glu Cys
210

<210> 114

<211> 687

<212> DNA

<213> Artificial Sequence

<220>

<223> Grafted Heavy Chain for Modified Fab

<400> 114

```

gaggttcagc tggctcgagtc aggaggcggg ctcgtgcagc ctggcggatc actgagattg 60
tcctgtgctg catctgggta cgtcttcaca gactatggaa tgaattgggt tagacaggcc 120
ccgggaaagg gcctggaatg gatggggttgg attaatactt acattggaga gcctatattat 180
gctgacagcg tcaagggcag attcacgttc tctctagaca catccaagtc aacagcatac 240
ctccaaatga atagcctgag agcagaggac accgcagtgt actattgtgc tagaggatac 300
agatcttatg ccatggacta ctggggccag ggtaccctag tcacagtctc ctcagcttcc 360
accaagggcc catcggctct ccccttggca ccctcttcca agagcacctc tgggggcaca 420
gcggccctgg gctgcctggg caaggactac ttccccgaac cggtgacggg gtcgtggaac 480
tcaggcgccc tgaccagcgg cgtgcacacc ttcccggtg tcctacagtc ctcaggactc 540
tactccctca gcagcgtggg gaccgtgccc tccagcagct tgggcaccca gacctacatc 600
tgcaacgtga atcacaagcc catcaacacc aaggtcgaca agaaagttga gccc aaatct 660
tgtgacaaaa ctcacacatg cgccgcg                                     687

```

<210> 115

<211> 229

<212> PRT

<213> Artificial Sequence

<220>

<223> Grafted Heavy Chain for Modified Fab

<400> 115

```

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1             5             10             15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr
      20             25             30
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met
      35             40             45
Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val
      50             55             60
Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
      65             70             75             80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
      85             90             95
Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
      100            105            110
Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro

```

115	120	125
Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly		
130	135	140
Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn		
145	150	155
Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln		
165	170	175
Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser		
180	185	190
Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser		
195	200	205
Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr		
210	215	220
His Thr Cys Ala Ala		
225		