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(54) Title: BI- AND MONOSPECIFIC, ASYMMETRIC ANTIBODIES AND METHODS OF GENERATING THE SAME

(57) Abstract: An antibody is provided. The antibody comprises an Fc region and a Fab region, wherein: (i) the Fc region comprises two non-identical heavy chains, wherein at least one of the two non-identical heavy chains comprises an amino acid modification so as to form complementation between the two non-identical heavy chains thereby increasing the probability of forming heterodimers of the non-identical heavy chains and decreasing the probability of forming homodimers of identical heavy chains; and (ii) the Fab region comprises a first covalent link between a first heavy chain and a first light chain of the Fab region and a second covalent link between a second heavy chain and a second light chain of said Fab region, wherein a position of the first covalent link relative to the first heavy chain is different to a position of the second covalent link relative to the second heavy chain. Methods of generating same and uses thereof are also provided.

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BI- AND MONOSPECIFIC, ASYMMETRIC ANTIBODIES AND METHODS SAME OF GENERATING THE

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to bispecific antibodies, monospecific, asymmetric antibodies and methods of generating same.

Bispecific antibodies (BsAbs) are antibodies with two binding sites, each directed against a different target antigen, to which they can bind simultaneously (Baeuerle and Reinhardt, 2009). This property enables the development of therapeutic strategies that are not possible with conventional monoclonal antibodies. The primary applications of bispecific antibodies include a) simultaneous inhibition of two targets (e.g. receptors of soluble ligands, a receptor and a ligand or two different ligands), b) retargeting, where one binding specificity is directed against a target cell (usually a tumor cell) whereas the other binding site is used to recruit a toxic activity or moiety to the target cell (T or NK cells; enzyme for prodrug activation; cytokine, radionuclide, virus, toxin), c) increased specificity, when strong binding mediated by simultaneous engagement of both antibody arms can only occur on cells expressing both antigens (Fischer and Leger, 2007; Amann et al., 2009; Lutterbuese et al., 2010). Since bispecific antibodies are regarded as promising therapeutic agents, several bispecific modalities have been developed, but their utility is limited due to problems with stability and manufacturing complexity. Several strategies for the creation of bispecific antibodies have been proposed over the past 20 years but despite numerous attempts and various proposed antibody formats, the BsAbs suffer from lack of product homogeneity and challenging production problems (Fischer and Leger, 2007; Chames and Baty, 2009).

Initially, attempts were made to produce bispecific antibodies by fusing two hybridomas, each producing a different antibody, resulting in what was referred to as "quadromas" or hybrid hybridomas. However, quadromas suffered from genetic instability and yielded heterogeneous mixes of the heavy and light chains. It was found that on average an at random association of L chains with H chains was found of the two antibodies, and only a tiny fraction were the desired bispecific antibodies (De Lau et al., 1991; Massino et al., 1997). If one considers creating a bispecific antibody from

two monospecific antibodies, A and B, efficient assembly of a bispecific antibody in an IgG format has two basic requirements, one is that each heavy chain associates with the heavy chain of the second antibody (heavy chain A associates with heavy chain B) and no homoassociation (A+A or B+B) occurs. The second requirement is that each light chain associates with its cognate heavy chain (light chain A with heavy chain A, and not light chain B with heavy chain A or light chain A with heavy chain B). The random association of antibody chains in quadromas could not meet those requirements.

Efficient generation of bispecific antibodies was made possible by advances in antibody engineering. Advanced antibody engineering enabled the creation of new recombinant antibody formats like tandem single-chain variable fragment (scFv) (Robinson et al., 2008), diabodies (Hudson and Kortt, 1999), tandem diabodies (Kipriyanov, 2009), two-in-one antibody (Bostrom et al., 2009), and dual variable domain antibodies (Wu et al., 2007). These new antibody formats solved some of the manufacturing issues, providing homogeneous preparations. However, most of these scaffolds, due to their small size, suffer from poor pharmacokinetics and therefore require frequent dosing or conjugation to larger carrier molecules to improve half-life (Constantinou et al., 2009).

Ridgway et al., 1996 provided a solution to one of the two criteria for making bispecific antibodies making it possible to re-consider IgG-based bispecific antibodies technically feasible. They described an engineering approach termed "knobs into holes" which allows only heterodimerization between the heavy chains of "antibodies A and B" to form, disallowing homodimerization. While studying the rules for heavy chain association, the authors postulated that it is primarily dependent on interfacial interactions between the C_H3 domains of the two heavy chains. When protein domains or subdomain interact, a knob is a bulky side chains that protrudes into the opposite domain where it is aligned with a small side chain that makes such invasion possible. In their approach, knob and hole variants were anticipated to heterodimerize by virtue of the knob inserting into an appropriately designed hole on the partner C_H3 domain. Knobs were constructed by replacing small side chains with the largest side chains, tyrosine or tryptophan. Holes of identical or similar size to the knobs were created by replacing large side chains with the smaller ones, in this case alanine or threonine. This way, two heavy chains that are knob variants can not homoassociate because of side

chain clashes, and the homoassociation of two hole variants is less favored because of the absence of a stabilizing side-chain interaction. Subsequently, this group engineered a disulfide bond near the c-terminus of the CH3 domain to further stabilize the assembled bispecific antibodies (Merchant et al., 1998).

U.S. Patent No. 7,183,076 teaches a method of generating bifunctional antibodies using the knob and hole approach.

However, the knobs into holes approach provided a solution only for the heteroassociation of the heavy chain and did not provide one for the correct pairing of each heavy chain with its cognate light chain. Therefore, in that study, a bispecific IgG capable of simultaneously binding to the human receptors HER3 and cMpl was prepared by coexpressing a common light chain and the corresponding remodeled heavy chains followed by protein A chromatography. The engineered heavy chains retain their ability to support antibody-dependent cell-mediated cytotoxicity as demonstrated with an anti-HER2 antibody (Merchant et al., 1998).

International application 2010/115589 teaches trivalent bispecific antibodies in which to a monospecific IgG carrying knobs into holes mutations, a V_H and V_L of a second specificity are fused at the C-terminus of the two CH3 domains.

Similar molecules are described in U.S. patent application US 2010/0256340.

Disulfide-stabilized Fvs were first described by the group of Andreas Plückthun (Glockshuber et al., 1990) and later by the group of Ira Pastan (Brinkmann et al., 1993; Reiter et al., 1994a; Reiter et al., 1994b; Reiter et al., 1995). The Pastan group did extensive work on dsFvs, and used molecular modeling to identify positions in conserved framework regions of antibody Fv fragments (Fvs) that are distant from CDRs, and potentially can be used to make recombinant Fv fragments in which the unstable V_H and V_L heterodimer is stabilized by an engineered interchain disulfide bond inserted between structurally conserved framework positions. A disulfide bond was introduced at one of these positions, V_H44-V_L105 or V_H111-V_L48 was shown to stabilize various Fvs that retain full binding and specificity.

U.S. Patent Nos. 5,747,654, 6,147,203 and 6,558,672 teach disulfide-stabilized Fvs, wherein the Fvs are engineered to introduce additional disulfide bonds between the light and heavy chains.

Additional background art includes Jackman et al., Journal of Biological Chemistry Vol 285, No.27, pp. 20850-20859, July 2, 2010 and Schaefer et al., Proc Natl Acad Sci U S A. 2011 July 5; 108(27): 11187–11192.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an antibody comprising an Fc region and a Fab region, wherein:

(i) the Fc region comprises two non-identical heavy chains, wherein at least one of the two non-identical heavy chains comprises an amino acid modification so as to form complementation between the two non-identical heavy chains thereby increasing the probability of forming heterodimers of the non-identical heavy chains and decreasing the probability of forming homodimers of identical heavy chains; and

(ii) the Fab region comprises a first covalent link between a first heavy chain and a first light chain of the Fab region and a second covalent link between a second heavy chain and a second light chain of the Fab region, wherein a position of the first covalent link relative to the first heavy chain is different to a position of the second covalent link relative to the second heavy chain.

According to an aspect of some embodiments of the present invention there is provided a method of preparing an antibody, comprising:

(a) providing a first nucleic acid molecule encoding the first heavy chain;

(b) providing a second nucleic acid molecule encoding the second heavy chain;

(c) providing a third nucleic acid molecule encoding the first light chain;

(d) providing a fourth nucleic acid molecule encoding the second light chain;

(e) culturing host cells comprising the first, second, third and fourth nucleic acid molecules under conditions that permit expression of the nucleic acid molecules; and

(f) recovering the antibody.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising as an active agent the antibody disclosed herein and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the present invention there is provided an antibody for treating an infection or inflammatory disease or disorder.

According to an aspect of some embodiments of the present invention there is provided a method of treating an infection or an inflammatory disease or disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the antibody disclosed herein, thereby treating the infection or inflammatory disease or disorder.

According to some embodiments of the invention, the antibody is a bispecific antibody.

According to some embodiments of the invention, the antibody is an asymmetric, monospecific antibody.

According to some embodiments of the invention, the complementation comprises a steric complementation.

According to some embodiments of the invention, the complementation comprises a charge complementation.

According to some embodiments of the invention, the Fc region comprises a protuberance of one heavy chain of the Fc region and a sterically compensatory cavity on a second heavy chain of the Fc region, the protuberance protruding into the compensatory cavity.

According to some embodiments of the invention, the protuberance is generated by substituting an amino acid at one position on a CH3 domain of the one heavy chain with another amino acid having a larger side chain volume than the original amino acid.

According to some embodiments of the invention, the compensatory cavity is generated by substituting an amino acid at one position on a CH3 domain of the second heavy chain with another amino acid having a smaller side chain volume than the original amino acid.

According to some embodiments of the invention, the first covalent link is between a CH1 domain of the one heavy chain and a CL domain of the one light chain; and the second covalent link is between a V_H domain of the second heavy chain and a V_L domain of the second light chain.

According to some embodiments of the invention, the first and the second covalent links are disulfide bonds.

According to some embodiments of the invention, the amino acid having a larger side chain volume than the original amino acid is selected from the group consisting of tyrosine, arginine, phenylalanine, isoleucine and tryptophan.

According to some embodiments of the invention, the amino acid having a smaller side chain volume than the original amino acid is selected from the group consisting of alanine, glycine, valine and threonine.

According to some embodiments of the invention, the antibody is selected from the group consisting of a chimeric antibody, a humanized antibody and a fully human antibody.

According to some embodiments of the invention, the CH3 domain of the first heavy chain is covalently linked to the CH3 domain of the second heavy chain.

According to some embodiments of the invention, the first antigen binding site of the antibody binds a first epitope of an antigen and the second antigen binding site of the antibody binds a second epitope of the antigen.

According to some embodiments of the invention, the first antigen binding site of the antibody binds an epitope of a first antigen and the second antigen binding site of the antibody binds an epitope of a second antigen.

According to some embodiments of the invention, each light chain is linked to its cognate heavy chain via a single disulfide bond.

According to some embodiments of the invention, the antibody is an intact antibody.

According to some embodiments of the invention, the antibody is selected from the group consisting of IgA, IgD, IgE and IgG.

According to some embodiments of the invention, the IgG comprises IgG1, IgG2, IgG3 or IgG4.

According to some embodiments of the invention, the first heavy chain comprises a T366W mutation; and the second heavy chain comprises T366S, L368A, Y407V mutations.

According to some embodiments of the invention, the first heavy chain comprises an S354C mutation and the second heavy chain comprises a Y349C mutation.

According to some embodiments of the invention, the first antigen binding site binds CD30 and the second antigen binding site binds erbB2.

According to some embodiments of the invention, the first antigen binding site binds CD30 and the second antigen binding site binds Pseudomonas Exotoxin (PE).

According to some embodiments of the invention, the first antigen binding site binds CD30 and the second antigen binding site binds strepavidin

According to some embodiments of the invention, at least one of the heavy chains is attached to a therapeutic moiety.

According to some embodiments of the invention, at least one of the heavy chains is attached to an identifiable moiety.

According to some embodiments of the invention, the antibody is selected from the group consisting of a primate antibody, a porcine antibody, a murine antibody, a bovine antibody, a goat antibody and an equine antibody.

According to some embodiments of the invention, the host cells comprise bacterial cells.

According to some embodiments of the invention, the host cells comprise mammalian cells.

According to some embodiments of the invention, the expression takes place in inclusion bodies of the bacterial cells.

According to some embodiments of the invention, each of the nucleic acid molecules are transfected into different host cells.

According to some embodiments of the invention, each of the nucleic acid molecules are transfected into the same host cell.

According to some embodiments of the invention, the bacterial cells comprise gram negative bacterial cells.

According to some embodiments of the invention, the method further comprises purifying the antibody on Protein A/G/L following step (f).

According to some embodiments of the invention, the inflammatory disorder is cancer.

According to some embodiments of the invention, the inflammatory disease or disorder is cancer.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-B are schematic structure of a novel strategy for production of bispecific antibodies. (A) scheme of an IgG antibody produced by the knobs into holes approach, there are two different heavy chains but a common light chain. (B) scheme of a bispecific antibody prepared according to embodiments of the present invention. There are two different heavy chains, each paired to its cognate light chain. The "knob" mutation corresponds to T366W, the "hole" mutations correspond to T366S, L368A Y407V replacements. Cysteine replacement mutations S354C and Y349C at CH3 region of "knob" or "hole" heavy chain, respectively, provide 95 % heterodimerization (Merchant et al., 1998).

FIGs. 2A-H are schematic representation of pHAK-IgH- and pHAK-IgL-based plasmid maps for expression of mono- and bispecific antibodies in *E. coli*: pHAK-IgL for expression of antibodies with human κ or light chain, pHAK-LC-Cys for expression of light chains containing dsFv-like intrachain disulfide bond, pHAK-IgH for expression of antibodies with human γ1 heavy chain, pHAK-HC-knob for expression of heavy chain containing S354C and T366W "knob" mutations in the constant region, pHAK-HC-hole for expression of heavy chain containing Y349C,

T366S, L368A and Y407V "hole" mutation in constant region, pHAK-HC-hole-PE38 for expression of heavy chain containing "hole" mutations fused to a truncated form of *Pseudomonas* exotoxin A (PE38), pHAK-HC-Cys for expression of heavy chain containing dsFv-like disulfide intrachain bond, pHAK-HC-Cys-knob for expression of heavy chain containing "knob" mutations in constant region and dsFv-like intrachain disulfide bond.

FIG. 3 is a photograph of SDS-PAGE analysis of heavy and light chains purification. The expressed proteins were collected as inclusion bodies, purified by sequential centrifugation steps and dissolved in a 6M guanidinium hydrochloride buffered solution. (1) T427 IgL, (2) T427 IgH, (3) T427-IgH-knob, (4) T427-IgH-PE38, (5) T427-IgH-hole-PE38. "Knob" mutations correspond to S354C:T366W. "Hole" mutations correspond to Y349C:T366S:L368A:Y407V.

FIGs. 4A-B provide analysis of bispecific IgG-like proteins. (A) The schematic structures of IgG heavy and light chains and the theoretically possible IgG molecules that can be formed. Each variant can be easily detected according the significant differences in molecular weight. (B) SDS-PAGE (10% acrylamide) analysis of protein A purified products: wt T427 antibody displaying PE38 on heavy chain (1), "knobs-into-holes" version (2) of T427 antibody (S354C:T366W/Y349C:T366S:L368A:Y407V mutations), wt FRP5 antibody (3).

FIGs. 5A-B provide SDS-PAGE (10% acrylamide) analysis of protein A purified products. (1) T427 "knob-knob" version (IgL + IgH-knob S354C:T366W mutations). (2) "Knobs-into-holes" version of T427 antibody (S354C:T366W/Y349C:T366S:L368A:Y407V mutations). (3) T427 "hole-hole" version (IgL + IgH-hole-PE38 Y349C:T366S:L368A:Y407V mutations). (4) wt T427 antibody displaying PE38 on heavy chain. (M) Marker.

FIG. 6 shows a gel filtration analysis of IgG and IgG-like proteins. T427 IgG antibody (150 kDa) elutes the Sephadex 200 gel filtration column at 11.5 min. The IgG-like T427 heterodimer (2 IgL + IgH-knob + IgH-hole-PE38), 190 kDa elutes at 10.3 min. The small fraction of knob-knob homodimer (150 kDa) elutes at 11.5 min. The hole-hole homodimer (230 kDa) probably elutes at void volume (6.5 min (not shown)).

FIGs. 7A-C illustrate SDS-PAGE (7.5% acrylamide) and density analysis of protein A purified products. (A) SDS-PAGE analysis of T427 IgG wt (1), T427-knob-

hole-PE38 (2) and T427-PE38 (3) proteins. (B) Protein band density analysis of SDS-PAGE by ImageMaster 1D scanning laser densitometry. (C) The pie chart of the heterodimerization yield was measured according the pixel intensity at band position. T427-knob-hole-PE38 (2) consists of 2IgL+IgH-knob+IgH-hole-PE38. T427-PE38 (3) consists of 2IgL+2IgH-PE38. "Knob" mutations correspond to S354C:T366W. "Hole" mutations correspond to Y349C:T366S:L368A:Y407V.

FIGs. 8A-B illustrate ELISA analysis of IgG and IgG-like proteins. The binding ability of FRP5 IgG and bispecific FRP5-T437-PE38 (PE38 fused to T427 heavy chain). (A) The ELISA plate was coated with erbB2 (antigen of FRP5 antibody) and antibodies were detected with anti-human secondary antibody. (B) The ELISA plate was coated with erbB2 (antigen of FRP5 antibody) and antibodies were detected with anti-PE secondary antibody (detection of bispecific antibodies).

The FRP5-T427-PE38 antibody consists of IgL-FRP5+IgL-T427+IgH-FRP5-knob+IgH-T427-hole-PE38 proteins. "Knobs-into-holes" mutations: S354C:T366W/Y349C:T366S:L368A:Y407V.

FIGs. 9A-B illustrate SDS-PAGE (12% and 6% acrylamide) analyses of protein A purified IgG and IgG-like proteins. (1) FRP5 IgG wt. (2) T427 IgG wt. (3) T427 IgG-Cys (IgH-Cys44:Cys222Ala+IgL-Cys104:Cys218del). (4) Bispecific T427-FRP5 IgG (IgH-FRP5-hole+IgL-FRP5+IgH-T427-khob-Cys44:Cys222Ala+IgL-T427-Cys104:Cys218del IgG. "Knob" mutations correspond to S354C:T366W. "Hole" mutations correspond to Y349C:T366S:L368A:Y407V.

FIG. 10 is an SDS-PAGE (10% acrylamide) analysis of protein A purified IgG and IgG-like proteins. (1) T427 IgG wt. (2) Anti-Tac IgG wt. (3) T427 IgG-Cys control A (IgH wt + IgL-Cys104:Cys218del). (4) T427 IgG-Cys control B (IgH-Cys44:Cys222Ala+IgL wt).

FIG. 11 is an SDS-PAGE analysis of heavy and light chains of αPE (B11), T427 and αSA antibodies purified as inclusion bodies and resuspended in 6 M guanidinium hydrochloride. The samples were separated in reducing condition on 12 % acrylamide gel.

FIGs.12A-B is ELISA analysis of αSA (anti-streptavidin) antibodies. The T427, αSA (monoclonal) and T427-αSA (bispecific) protein A purified antibodies were analyzed for their ability to bind CD30 (A). The binding was detected using goat-anti-

human HRP conjugated antibodies. Coating with bovine serum albumin (BSA) served as a control (B).

FIGs. 13A-C is an ELISA analysis of αPE (anti-*Pseudomonas exotoxin* 38kDa fragment) antibodies. The T427, αPE B11 clone (monoclonal) and T427-αPE (bispecific) protein A purified antibodies were analyzed for their ability to bind avitag-PE38 (A) and dsFv-PE38 (B) antigens. The binding was detected using goat-anti-human HRP conjugated antibodies. Coating with bovine serum albumin (BSA) served as a control (C).

FIG. 14 is a schematic presentation of pDual vector system. pDual vectors are bi-cistronic, CMV promoter-based plasmids for the expression of IgGs in mammalian cells. They were constructed by combining heavy and light chain expression cassettes from the pMAZ vectors (Mazor Y, J Immunol Methods. 2007 Apr 10;321(1-2):41-59).

FIGs. 15A-B illustrate analyses of secreted IgG in medium of $CaCl_2$ transfected 293 Trex cells. (A) Western blot analysis of cell media transfected with pDual wt, pDual L(Cys)+H(wt) or pMAZ-IgL + pMAZ-IgH vectors systems. The antibodies were detected with goat-anti-human HRP conjugated secondary antibody. The antibody concentration in media was determined in comparison to the secondary dilutions of Erbitux antibody (B).

FIG.16 illustrates a Western blot analysis of 293 Trex cells transfected with pDual mono-specific and bispecific vectors or combination of vectors. The antibodies were detected with goat-anti-human HRP conjugated antibody.

FIG.17 illustrates exemplary results from a Dot blot analysis of antibody secreting clones. The cell media were absorbed to nitrocellulose membrane and antibodies were detected with goat-anti-human HRP conjugated antibody. The secretion level was determined relatively to other clones. The cell media of non-treated cells served as control.

FIGs. 18A-B illustrate validation of binding activity of bispecific clones. The cell media were incubated with either erbB2 (18A) or CD30 (18B) antigens. The binding was detected with goat-anti-human HRP conjugated antibody. The marked clones demonstrated the binding ability to both antigens.

FIGs. 19A-B illustrates an SDS-PAGE analysis of IgGs produced in HEK 293 T-REx™ mammalian cells followed by protein A purification. The proteins were

separated in unreduced conditions on 10 % acrylamide gel in order to evaluate the 150 kDa IgGs (A) and in reduced conditions on 12 % acrylamide gel (B) in order to evaluate the minimal differences between T427 and FRP5 heavy chains and light chains and determine the double bands in bispecific T427-FRP5 molecules.

FIGs. 20A-B illustrate ELISA analysis of protein A purified IgGs produced in mammalian cells. A5 is a control cell line transfected with four plasmids, two encoding the monospecific T427 antibody and two encoding the monospecific FRP5 antibody. Bispecific T427-FRP5 represents bispecific antibody secreting stable clone D3 with mono-valent binding ability to each antigen (erbB2 and CD30). The binding was detected using goat-anti-human HRP conjugated secondary antibody.

FIGs. 21A-B are graphs illustrating ELISA analysis of protein A purified IgGs produced in mammalian cells. T427 and FRP5 represent mono-specific antibodies with bi-valent binding activity. Bispecific T427-FRP5 represents bispecific antibody-secreting stable clone D3 with mono-valent binding ability to each antigen (erbB2 and CD30). Erbitux served as a negative control. The binding was detected using goat-anti-human HRP conjugated secondary antibody

FIG. 22 is a graph illustrating cell-ELISA analysis of binding ability of B3 clone secreting T427-FRP5 bispecific antibody, (protein A-purified from conditioned medium of the stable clone) to A431/CD30 and SKBR3 (erbB2+) cells. The binding was detected using goat-anti-human HRP conjugated secondary antibody.

FIG. 23 is a schematic representation of the monospecific antibody of embodiments of the present invention.

FIG. 24 is ELISA analysis of T427 KIH. The binding ability of T427 IgG and T427-PE38 (PE38 fused to heavy chain) in comparison to knobs-into-holes (KIH) version of T427 antibody (2×IgL+IgH-knob+IgH-hole-PE38).

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to bispecific antibodies, monospecific antibodies, asymmetric antibodies and methods of generating same.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

In past years, both laboratory and early clinical studies have demonstrated that bispecific antibodies (BsAbs) may have significant potential application in cancer therapy either by targeting tumor cells with cytotoxic agents including effector cells, radionuclides, drugs, and toxins, or by simultaneously blocking two relevant tumor targets, that is, growth factor receptors or their ligands, thus neutralizing multiple receptor activation and downstream signal transduction pathways. A major obstacle in the development of BsAb has been the difficulty of producing the materials in sufficient quality and quantity by traditional technologies such as the hybrid hybridoma and chemical conjugation methods. Thus, it is believed that the development of IgG-like BsAbs as therapeutic agents will depend heavily on the advances made in the design of recombinant BsAb constructs and production efficiency.

In order to ensure heterodimerization between the heavy chains of antibodies "A" and "B" to form, and to prevent homodimerization of antibody "A" to antibody "A" and antibody "B" to antibody "B", a knob and hole approach has been suggested, as disclosed in U.S. Patent No. 7,183,076. However, the knobs into holes approach provides a solution only for the heteroassociation of the heavy chains and does not provide one for the correct pairing of each heavy chain with its cognate light chain.

The present invention relates to an approach of efficient assembly of bispecific antibodies in an IgG format. The approach involves heterodimerization of the two heavy chains by applying the knobs into holes approach, combined with facilitation of pairing of each heavy chain with only its cognate light chain.

The present inventors suggest pairing the heavy and light chains of the same antibody using one native CH1-CL binding disulfide bond and one non-native V_H- V_L binding dsFv-like di-sulfide bond (as illustrated in Figure 1B). This way one antibody branch would stay molecularly untouched while the other antibody branch would acquire a new disulfide covalent bond in variable region instead of the wt S-S bond. The mis-paired light and heavy chains would not form the S-S stabilized interface and would

not produce stable IgG molecule. Thus this strategy supposes the conversion of one antibody branch into dsFv-like molecule without any affinity or stability loss.

Whilst reducing the present invention to practice, the present inventors generated a bispecific antibody by combining an anti-CD30 (T427) and an anti-erbB2 (FRP5) antibody. In the erbB2 antibody, heavy – light chain association was facilitated by the natural disulfide bond that covalently links the C_H1 domain of the heavy chain with the C_L domain of the light chain. In the anti-CD30 antibody, the cysteine in C_H1 was mutated to alanine and the C-terminal cysteine of C_L was deleted, preventing the formation of the native H-L disulfide bond. Instead of the eliminated disulfide bond, two cysteines, one in the variable domain of the heavy chain and one in the variable domain of the light chain were introduced according the rules of disulfide-stabilized Fv fragments (dsFvs). As a result, the heavy and light chains of the anti-CD30 antibody associated covalently via a disulfide bond that forms between V_H and V_L through these two cysteine residues. Thus, the present invention contemplates both the generation of a novel disulfide bridge between the heavy chain and its cognate light chain on one arm of the bifunctional antibody and so as to further enhance correct assembly, deletion of the naturally occurring disulfide bridge between the same heavy chain with its cognate light chain.

As illustrated in Figures 9A-B, using this approach, full-length bifunctional antibodies were generated in bacterial cells. When the heavy and light chains of the anti-CD30 antibody were not mutated as described above, full length bifunctional antibodies were not generated (Figure 10).

Further, using bispecific vectors, the present inventors showed that the generation of full-length bifunctional antibodies in mammalian cells was promoted by applying the knobs into holes approach, combined with facilitation of pairing of each heavy chain with only its cognate light chain (as illustrated in Figures 17-22)

Thus, according to an aspect of the present invention there is provided an antibody comprising an Fc region and a Fab region, wherein:

(i) the Fc region comprises two non-identical heavy chains, wherein at least one of the two non-identical heavy chains comprises an amino acid modification so as to form complementation between the two non-identical heavy chains thereby increasing

the probability of forming heterodimers of the non-identical heavy chains and decreasing the probability of forming homodimers of identical heavy chains; and

(ii) the Fab region comprises a first covalent link between a first heavy chain and a first light chain of the Fab region and a second covalent link between a second heavy chain and a second light chain of the Fab region, wherein a position of the first covalent link relative to the first heavy chain is different to a position of the second covalent link relative to the second heavy chain.

An antibody is characterized by a centrally placed disulfide bridge that stabilizes a series of antiparallel beta strands into an immunoglobulin-like fold. An antibody heavy or light chain has an N-terminal (NH_2) variable region (V), and a C-terminal (--COOH) constant region (C). The heavy chain variable region is referred to as V_H, and the light chain variable region is referred to as V_L. V_H and V_L fragments together are referred to as "Fv". The variable region is the part of the molecule that binds to the antibody's cognate antigen, while the constant region determines the antibody's effector function (e.g., complement fixation, opsonization). Full-length immunoglobulin or antibody "light chains" (generally about 25 kilodaltons (Kd), about 214 amino acids) are encoded by a variable region gene at the N-terminus (generally about 110 amino acids) and a constant region gene at the COOH-terminus. Full-length immunoglobulin or antibody "heavy chains" (generally about 50 Kd, about 446 amino acids), are similarly encoded by a variable region gene (generally encoding about 116 amino acids) and one of the constant region genes (encoding about 330 amino acids). An antibody light or heavy chain variable region comprises three hypervariable regions, also called complementarity determining regions or CDRs, flanked by four relatively conserved framework regions or FRs.

According to one embodiment of this aspect of the present invention the antibody is a bispecific antibody.

As used herein, the term "bispecific antibody" refers to an antibody which comprises two antigen binding sites, each binding to a different epitope of an antigen. The bispecific antibodies of this aspect of the present invention do not share common light chains nor common heavy chains.

According to one embodiment, the two antigen binding sites each bind to different epitopes of an identical antigen. According to another embodiment, the two antigen binding sites each bind to different epitopes on different antigens.

According to another embodiment of this aspect of the present invention, the antibody is a monospecific, asymmetric antibody.

The monospecific antibodies of this aspect of the present invention have the same paratope on both arms which bind an identical antigen. However, unlike conventional monoclonal antibodies which are symmetric assemblies of two identical heavy chains and two identical light chains, monospecific antibodies described herein are asymmetric assemblies of two non-identical heavy chains and two non-identical light chains. The differences between the two heavy chains and between the two light chains are in the constant domains and in framework regions of the variable domains that allow heterodimerization of the chains. Accordingly, the CDR loops of the variable domains and supporting variable domain residues that may comprise the paratope are identical in the chain pairs – see Figure 23.

According to a particular embodiment, the monospecific antibody is an IgG4.

Preferably, the affinity of each of the antigen binding sites of the antibody for its target is not substantially reduced as compared with one arm of its corresponding monoclonal antibody for the identical target. According to a specific embodiment, the affinity is not reduced more than 100 fold, more preferably is not reduced more than 50 fold, more preferably is not reduced more than 20 fold, more preferably is not reduced more than 10 fold and even more preferably is not reduced more than 5 fold.

Examples of bispecific antibodies include those with one antigen binding site directed against a first growth factor ligand and a second antigen binding site directed against a second growth factor ligand; one antigen binding site directed against a first growth factor receptor and a second antigen binding site directed against a second growth factor receptor; one antigen binding site directed against a first cytokine and a second antigen binding site directed against a second cytokine; one antigen binding site directed against a first cytokine receptor and a second antigen binding site directed against a second cytokine receptor; one antigen binding site directed against a growth factor receptor and a second antigen binding site directed against a growth factor ligand; one antigen binding site directed against a cytokine receptor and a second antigen

binding site directed against a cytokine ligand. Additional combinations of growth factors, growth factor receptors, cytokines and cytokine receptors are also contemplated.

According to another embodiment, the bispecific antibody block two pathways of angiogenesis, one antigen binding site is directed towards a receptor or ligand associated with the first pathway and the other antigen binding site is directed towards a receptor or ligand associated with the second pathway.

According to a specific embodiment, the bispecific antibody comprises one antigen binding site directed against a tumor cell antigen and the other antigen binding site directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD 3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell adhesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3.

Bispecific antibodies with one antigen binding site binding specifically to a tumor antigen and one antigen binding site binding to a toxin include for example anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α (IFN- α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid.

Other contemplated bispecific antibodies include those for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol).

Other contemplated bispecific antibodies include those which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA).

Additional contemplated bispecific antibodies include those for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, FcγRII or FcγRIII).

Additional contemplated bispecific antibodies include those for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell

receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV. Further bispecific antibodies for tumor detection in vitro or in vivo include anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-p185HER2/anti-hapten.

Bispecific antibodies may be used as vaccine adjuvants (see Fanger et al., Critical Reviews in Immunology 12(3,4):101-124 (1992)).

Bispecific antibodies may be used as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase.

Additional contemplated bispecific antibodies include ones where the first antigen binding site binds CD30 and the second antigen binding site binds erbB2; ones where the first antigen binding site binds CD30 and the second antigen binding site binds Pseudomonas Exotoxin (PE); ones where the first antigen binding site binds CD30 and the second antigen binding site binds Streptavidin.

Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37.

The Fc region of the antibodies of the present invention may be may be obtained from any antibody, such as IgG_1, IgG_2, IgG_3, or IgG_4 subtypes, IgA, IgE, IgD or IgM.

According to one embodiment, the Fc region is an IgG Fc region.

As mentioned, the Fc region of the antibodies described herein comprises two non-identical heavy chains (e.g. that differ in the sequence of the variable domains), wherein at least one of the two non-identical heavy chains comprises an amino acid modification so as to increase the probability of forming a stable heterodimer of the non-identical heavy chains and decrease the probability of forming a stable homodimer of identical heavy chains.

According to one embodiment, at least one heavy chain is genetically modified such that an altered charge polarity across the interface is created. As a consequence, a stable heterodimer between electrostatically matched Fc chains is promoted, and unwanted Fc homodimer formation is suppressed due to unfavorable repulsive charge interactions.

Determination of which amino acids to modify and to which amino acids is further explained in Gunasekaran K, Pentony M, Shen M, Garrett L, Forte C, Woodward A, Ng SB, Born T, Retter M, Manchulenko K, Sweet H, Foltz IN, Wittekind

M, Yan W. Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem. 2010 Jun 18;285(25):19637-46. Epub 2010 Apr 16, incorporated herein by reference.

According to one embodiment, the amino acid modifications (that affect charge complementarity) are effected at the rim of the interface between the two heavy chains and not in structurally conserved buried residues at the hydrophobic core of the interface.

According to another embodiment, at least one heavy chain is genetically modified, to generate a heavy chain with a 3D structure which binds more efficiently to the non-identical heavy chain (i.e. a heterodimer) as opposed to an identical heavy chain (i.e. a homodimer). The generation of heterodimers is encouraged due to steric complementation and the generation of homodimers is discouraged due to steric hindrance.

According to this embodiment, one heavy chain is genetically modified to generate a protuberance and the second heavy chain is genetically modified to generate a sterically compensatory cavity, the protuberance protruding into the compensatory cavity.

"Proturbances" are constructed by replacing small amino acid side chains from the interface of the first heavy chains with larger side chains (e.g. tyrosine, arginine, phenylalanine, isoleucine, leucine or tryptophan). Compensatory "cavities" of identical or similar size to the protuberances are optionally created on the interface of the second heavy chain by replacing large amino acid side chains with smaller ones (e.g. alanine, glycine, serine, valine, or threonine).

The protuberance or cavity can be "introduced" into the interface of the first or second heavy chain by synthetic means, e.g. by recombinant techniques, in vitro peptide synthesis, those techniques for introducing non-naturally occurring amino acid residues previously described, by enzymatic or chemical coupling of peptides or some combination of these techniques. According, the protuberance, or cavity which is "introduced" is "non-naturally occurring" or "non-native", which means that it does not exist in nature or in the original polypeptide (e.g. a humanized monoclonal antibody).

Preferably the import amino acid residue for forming the protuberance has a relatively small number of "rotamers" (e.g. about 3 6). A "rotamer" is an energetically

favorable conformation of an amino acid side chain. The number of rotamers of the various amino acid residues are reviewed in Ponders and Richards, J. Mol. Biol. 193:775 791 (1987).

As a first step to selecting original residues for forming the protuberance and/or cavity, the three-dimensional structure of the antibodies are obtained using techniques which are well known in the art such as X-ray crystallography or NMR. Based on the three-dimensional structure, those skilled in the art will be able to identify the interface residues.

The preferred interface is the C_{H3} domain of an immunoglobulin constant domain. It is preferable to select "buried" residues to be replaced. The interface residues of the CH3 domains of IgG, IgA, IgD, IgE and IgM have been identified (see, for example, PCT/US96/01598, herein incorporated by reference in its entirety), including those which are optimal for replacing with import residues; as were the interface residues of various IgG subtypes and "buried" residues. The preferred C_{H3} domain is derived from an IgG antibody, such as an human IgG_1.

The C_{H3}/C_{H3} interface of human IgG_1 involves sixteen residues on each domain located on four anti-parallel β-strands which buries 1090 ANG^2 from each surface. Mutations are preferably targeted to residues located on the two central anti-parallel β-strands. The aim is to minimize the risk that the protuberances which are created can be accommodated by protruding into surrounding solvent rather than by compensatory cavities in the partner C_{H3} domain. Methods of selection particular sites on the heavy chains have been disclosed in U.S. Patent No. 7,183,076, incorporated herein by reference.

According to a specific embodiment, the first heavy chain comprises a T366W mutation (i.e. threonine to tryptophan); and the second heavy chain comprises T366S, L368A, Y407V mutations (i.e. threonine to serine; leucine to alanine; and tyrosine to valine).

According to one embodiment, the amino acid modifications (that affect structural complementarity) are effected at structurally conserved buried residues at the hydrophobic core of the interface, and not in at the rim of the interface between the two heavy chains.

The effect of replacing residues on the heavy chains can be studied using a molecular graphics modeling program such as the Insight™ program (Biosym Technologies).

Once the preferred original/import residues are identified by molecular modeling, the amino acid replacements may be introduced into the heavy chains using techniques which are well known in the art.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution variants of the DNA encoding the first or second heavy chain. This technique is well known in the art as described by Adelman et al., DNA, 2:183 (1983). Briefly, first or second polypeptide-coding DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of heteromultimer. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the heteromultimer DNA.

Cassette mutagenesis can be performed as described Wells et al. Gene 34:315 (1985) by replacing a region of the DNA of interest with a synthetic mutant fragment generated by annealing complimentary oligonucleotides. PCR mutagenesis is also suitable for making variants of the first or second polypeptide DNA. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, Science, 252:1643 1650 (1991), the chapter by R. Higuchi, p. 61 70).

Additional modifications are also contemplated to further enhance the specificity of interaction between the two heavy chains. Accordingly, the present invention incorporates a covalent link between the two heavy chains (e.g. on the CH3 domains).

Examples of covalent links contemplated by the present invention include amide links and disulfide links.

Thus, for example the present invention contemplates introduction of a free thiol which forms an intermolecular disulfide bond between the two heavy chains of the antibody. The free thiol may be introduced into the interface of one of the heavy chains by substituting a naturally occurring residue of the heavy chain with, for example, a

cysteine at a position allowing for the formation of a disulfide bond between the heavy chains.

The phrase "free thiol-containing compound" as used herein refers to a compound that can be incorporated into or reacted with an amino acid of a polypeptide interface of the invention such that the free thiol moiety of the compound is positioned to interact with a free thiol of moiety at the interface of additional polypeptide of the invention to form a disulfide bond. Preferably, the free thiol-containing compound is cysteine.

According to a specific embodiment, the first heavy chain comprises a S354C mutation (i.e. serine to cysteine); and the second heavy chain comprises a Y349C mutation (tyrosine to cysteine).

As well as having modifications in their heavy chains, at least one light chain of the antibodies described herein is also modified such that there is a first covalent link between a first heavy chain and a first light chain and a second covalent link between a second heavy chain and a second light chain, wherein a position of the first covalent link relative to the first heavy chain is different to a position of the second covalent link relative to the second heavy chain.

The positioning of the first and second covalent link is selected such that pairing between a heavy chain with its cognate light chain is facilitated, whilst the specificity and stability of the antibody is not reduced by more than 20 % or preferably by more than 10 % or even more preferably by more than 5 % as compared to the individual antibodies from which it is generated.

According to another embodiment, the covalent link between the first heavy chain to its cognate light chain is positioned between the C_{H1} and the C_L region and the covalent link between the second heavy chain to its cognate light chain is positioned between the V_H and the V_L region.

Examples of covalent links contemplated by the present invention include for example amide links, disulfide links and additional forms of covalent bonds occurring between site-specifically inserted amino acid residues, including non-natural amino acids (see Wu, X., Schultz, P.G. "Synthesis at the Interface of Chemistry and Biology." J. Am. Chem. Soc., 131(35):12497-515, 2009; Hutchins BM, Kazane SA, Staflin K, Forsyth JS, Felding-Habermann B, Schultz PG, Smider VV. Site-specific coupling and

sterically controlled formation of multimeric antibody fab fragments with unnatural amino acids J Mol Biol. 2011 Mar 4;406(4):595-603. Epub 2011 Jan 13; Liu CC, Schultz PG. Adding new chemistries to the genetic code. Annu Rev Biochem. 2010;79:413-44. Review, all of which are incorporated herein by reference).

Accordingly, the present invention contemplates mutating at least one of the heavy chains and its cognate light chain such that at least one naturally occurring (i.e. native) disulfide bond that connects the two molecules can no longer be generated. Typically, this is effected by deleting (or substituting) the cysteines at the positions described herein above.

As used herein, the phrase "native disulfide bond" refers to the interchain disulfide bond that connects a heavy chain to its cognate light chain (typically between the constant region of the light chain and the CH1 region of the heavy chain) encoded in a naturally occurring germline antibody gene.

Substitution of the cysteine is typically effected by replacing the amino acid with one similar in size and charge (i.e. a conservative amino acid, such as cysteine to alanine).

The present invention contemplates that the first covalent link is a naturally occurring disulfide bond and the second covalent link is a non-naturally occurring covalent bond, (e.g. an engineered disulfide bond), wherein at least one cysteine amino acid residue has been inserted into the chain – i.e. an engineered cysteine.

The term "engineered cysteine" as used herein, refers to a cysteine which has been introduced into the antibody fragment sequence at a position where a cysteine does not occur in the natural germline antibody sequence.

Alternatively, both the first and second covalent links may be non-naturally occurring and the cysteines (which in the non-modified antibody serve as amino acid residues to generate disulfide bonds) may be replaced by other amino acids that are not capable of serving as amino acid residues to generate covalent bonds.

Information regarding the antibody of interest is required in order to produce proper placement of the disulfide bond. The amino acid sequences of the variable regions that are of interest are compared by alignment with those analogous sequences in the well-known publication by Kabat and Wu [Sequences of Proteins of Immunological Interest," E. Kabat, et al., U.S. Government Printing Office, NIH

Publication No. 91-3242 (1991)], incorporated herein by reference, to determine which sequences can be mutated so that cysteine is encoded for in the proper position of each heavy and light chain variable region to provide a disulfide bond in the framework regions of the desired antibody.

After the sequences are aligned, the amino acid positions in the sequence of interest that align with the following positions in the numbering system used by Kabat and Wu are identified: positions 43, 44, 45, 46, and 47 (group 1) and positions 103, 104, 105, and 106 (group 2) of the heavy chain variable region; and positions 42, 43, 44, 45, and 46 (group 3) and positions 98, 99, 100, and 101 (group 4) of the light chain variable region. In some cases, some of these positions may be missing, representing a gap in the alignment.

Then, the nucleic acid sequences encoding the amino acids at two of these identified positions are changed such that these two amino acids are mutated to cysteine residues. Contemplated pairs of amino acids to be selected are,: V_H44-V_L100, V_H105-V_L43, V_H105-V_L42, V_H44-V_L101, V_H106-V_L43, V_H104-V_L43, V_H44-V_L99, V_H45-V_L98, V_H46-V_L98, V_H103-V_L43, V_H103-V_L44, V_H103-V_L45.

Most preferably, substitutions of cysteine are made at the positions: V_H44-V_L100; or V_H105-V_L43. (The notation V_H44-V_L100, for example, refers to a polypeptide with a V_H having a cysteine at position 44 and a cysteine in V_L at position 100; the positions being in accordance with the numbering given by Kabat and Wu.)

Note that with the assignment of positions according to Kabat and Wu, the numbering of positions refers to defined conserved residues and not to actual sequentially numbered amino acid positions in a given antibody. For example, CysL100 (of Kabat and Wu) which is used to generate ds(Fv)B3 as described in the example below, actually corresponds to position 105 of B3(V_L).

According to one embodiment, selection of which amino acid to mutate may be effected according to the rules set out in U.S. Patent No. 5,747,654, incorporated herein by reference. The sites of mutation to the cysteine residues can be identified by review of either the actual antibody or the model antibody of interest as exemplified below. Computer programs to create models of proteins such as antibodies are generally available and well-known to those skilled in the art (see Kabat and Wu; Loew, et al., Int. J. Quant. Chem., Quant. Biol. Symp., 15:55-66 (1988); Bruccoleri, et al., Nature,

335:564-568 (1988); Chothia, et al., Science, 233:755-758 (1986), all of which are incorporated herein by reference. Commercially available computer programs can be used to display these models on a computer monitor, to calculate the distance between atoms, and to estimate the likelihood of different amino acids interacting (see, Ferrin, et al., J. Mol. Graphics, 6:13-27 (1988), incorporated by reference herein). For example, computer models can predict charged amino acid residues that are accessible and relevant in binding and then conformationally restricted organic molecules can be synthesized. See, for example, Saragovi, et al., Science, 253:792 (1991), incorporated by referenced herein. In other cases, an experimentally determined actual structure of the antibody may be available.

According to one embodiment, a pair of suitable amino acid residues should (1) have a C_α-C_α distance between the two residues less than or equal to 8 ANG, preferably less than or equal to 6.5 ANG (determined from the crystal structure of antibodies which are available such as those from the Brookhaven Protein Data Bank) and (2) be as far away from the CDR region as possible. Once they are identified, they can be substituted with cysteins.

Modifications of the genes to encode cysteine at the target point may be readily accomplished by well-known techniques, such as oligonucleotides-directed mutagenesis (as described herein above), site-directed mutagenesis (see, Gillman and Smith, Gene, 8:81-97 (1979) and Roberts, S., et al, Nature, 328:731-734 (1987), both of which are incorporated herein by reference), by the method described in Kunkel, Proc. Natl. Acad. Sci. USA 82:488-492 (1985), incorporated by reference herein, by total gene synthesis (Hughes, R.A. et al, , Methods in Enzymology, Volume 498 p. 277-309 (2011)) or by any other means known in the art.

Antibodies of some embodiments of the present invention may be from any mammalian origin including human, porcine, murine, bovine, goat, equine, canine, feline, ovine and the like. The antibody may be a heterologous antibody.

As used herein a "heterologous antibody" is defined in relation to a transgenic host such as a plant expressing the antibody.

According to some embodiments of the invention, the antibody is an isolated intact antibody (i.e., substantially free of cellular material other antibodies having different antigenic specificities and/or other chemicals).

As used herein "recombinant antibody" refers to intact antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., mouse) that is transgenic for immunoglobulin genes (e.g., human immunoglobulin genes) or hybridoma prepared therefrom; (b) antibodies isolated from a host cell transformed to express the antibody; (c) antibodies isolated from a recombinant antibody library; and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of immunoglobulin gene sequences to other DNA sequences. In certain embodiments immunoglobulin of the present invention may have variable and constant regions derived from human germline immunoglobulin sequences. In other embodiments, such recombinant human antibodies can be subjected to in vitro mutagenesis and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies comprise sequences that while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire in vivo.

The following exemplary embodiments of antibodies are encompassed by the scope of the invention.

A used herein "human antibody" refers to intact antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences as described, for example, by Kabat et al. (see Kabat 1991, Sequences of proteins of immunological Interest, 5th Ed. NIH Publication No. 91-3242). The constant region of the human antibody is also described from human germline immunoglobulin sequences. The human antibodies may include amino residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site directed mutagenesis in vitro or somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

As used herein, a "chimeric antibody" refers to an intact antibody in which the variable regions derive from a first species and the constant regions are derived from a second species. Chimeric immunoglobulins can be constructed by genetic engineering from immunoglobulin gene segments belonging to different species (e.g., VH and VL domains from a mouse antibody with constant domains of human origin).

As used herein "humanized immunoglobulin" refers to an intact antibody in which the minimum mouse part from a non-human (e.g., murine) antibody is transplanted onto a human antibody; generally humanized antibodies are 5-10 % mouse and 90-95 % human.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be

made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10,: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

The antibodies of the present invention may be conjugated to a functional moiety such as a detectable or a therapeutic moiety.

Various types of detectable or reporter moieties may be conjugated to the antibody of the invention. These include, but not are limited to, a radioactive isotope (such as $^{[125]}$iodine), a phosphorescent chemical, a chemiluminescent chemical, a fluorescent chemical (fluorophore), an enzyme, a fluorescent polypeptide, an affinity tag, and molecules (contrast agents) detectable by Positron Emission Tomagraphy (PET) or Magnetic Resonance Imaging (MRI).

Examples of suitable fluorophores include, but are not limited to, phycoerythrin (PE), fluorescein isothiocyanate (FITC), Cy-chrome, rhodamine, green fluorescent protein (GFP), blue fluorescent protein (BFP), Texas red, PE-Cy5, and the like. For additional guidance regarding fluorophore selection, methods of linking fluorophores to various types of molecules see Richard P. Haugland, "Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals 1992–1994", 5th ed., Molecular Probes, Inc. (1994); U.S. Pat. No. 6,037,137 to Oncoimmunin Inc.; Hermanson, "Bioconjugate Techniques", Academic Press New York, N.Y. (1995); Kay M. *et al.*, 1995. Biochemistry 34:293; Stubbs *et al.*, 1996. Biochemistry 35:937; Gakamsky D. *et al.*, "Evaluating Receptor Stoichiometry by Fluorescence Resonance Energy Transfer," in "Receptors: A Practical Approach," 2nd ed., Stanford C. and Horton R. (eds.), Oxford University Press, UK. (2001); U.S. Pat. No. 6,350,466 to Targesome, Inc.]. Fluorescence detection methods which can be used to detect the antibody when conjugated to a fluorescent detectable moiety include, for example, fluorescence

activated flow cytometry (FACS), immunofluorescence confocal microscopy, fluorescence *in-situ* hybridization (FISH) and fluorescence resonance energy transfer (FRET).

Numerous types of enzymes may be attached to the antibody of the invention [e.g., horseradish peroxidase (HRP), beta-galactosidase, and alkaline phosphatase (AP)] and detection of enzyme-conjugated antibodies can be performed using ELISA (e.g., in solution), enzyme-linked immunohistochemical assay (e.g., in a fixed tissue), enzyme-linked chemiluminescence assay (e.g., in an electrophoretically separated protein mixture) or other methods known in the art [see e.g., Khatkhatay MI. and Desai M., 1999. J Immunoassay 20:151-83; Wisdom GB., 1994. Methods Mol Biol. 32:433-40; Ishikawa E. *et al.*, 1983. J Immunoassay 4:209-327; Oellerich M., 1980. J Clin Chem Clin Biochem. 18:197-208; Schuurs AH. and van Weemen BK., 1980. J Immunoassay 1:229-49).

The affinity tag (or a member of a binding pair) can be an antigen identifiable by a corresponding antibody [e.g., digoxigenin (DIG) which is identified by an anti-DIG antibody) or a molecule having a high affinity towards the tag [e.g., streptavidin and biotin]. The antibody or the molecule which binds the affinity tag can be fluorescently labeled or conjugated to enzyme as described above.

Various methods, widely practiced in the art, may be employed to attach a streptavidin or biotin molecule to the antibody of the invention. For example, a biotin molecule may be attached to the antibody of the invention via the recognition sequence of a biotin protein ligase (e.g., BirA) as described in the Examples section which follows and in Denkberg, G. *et al.*, 2000. Eur. J. Immunol. 30:3522-3532. Alternatively, a streptavidin molecule may be attached to an antibody fragment, such as a single chain Fv, essentially as described in Cloutier SM. *et al.*, 2000. Molecular Immunology 37:1067-1077; Dubel S. *et al.*, 1995. J Immunol Methods 178:201; Huston JS. *et al.*, 1991. Methods in Enzymology 203:46; Kipriyanov SM. *et al.*, 1995. Hum Antibodies Hybridomas 6:93; Kipriyanov SM. *et al.*, 1996. Protein Engineering 9:203; Pearce LA. *et al.*, 1997. Biochem Molec Biol Intl 42:1179-1188).

Functional moieties, such as fluorophores, conjugated to streptavidin are commercially available from essentially all major suppliers of immunofluorescence flow cytometry reagents (for example, Pharmingen or Becton-Dickinson).

According to some embodiments of the invention, biotin conjugated antibodies are bound to a streptavidin molecule to form a multivalent composition (e.g., a dimmer or tetramer form of the antibody).

Table 1 provides non-limiting examples of identifiable moieties which can be conjugated to the antibody of the invention.

Table 1

Identifiable Moiety	***Amino Acid sequence (GenBank Accession No.)***	***Nucleic Acid sequence (GenBank Accession No.)***
Green Fluorescent protein	AAL33912	AF435427
Alkaline phosphatase	AAK73766	AY042185
Peroxidase	CAA00083	A00740
Histidine tag	Amino acids 264-269 of GenBank Accession No. AAK09208	Nucleotides 790-807 of GenBank Accession No. AF329457
Myc tag	Amino acids 273-283 of GenBank Accession No. AAK09208	Nucleotides 817-849 of GenBank Accession No. AF329457
Biotin lygase tag	LHHILDAQ**K**MVWNHR /	
orange fluorescent protein	AAL33917	AF435432
Beta galactosidase	ACH42114	EU626139
Streptavidin	AAM49066	AF283893

Table 1.

As mentioned, the antibody may be conjugated to a therapeutic moiety. The therapeutic moiety can be, for example, a cytotoxic moiety, a toxic moiety, a cytokine moiety and a second antibody moiety comprising a different specificity to the antibodies of the invention.

Non-limiting examples of therapeutic moieties which can be conjugated to the antibody of the invention are provided in Table 2, hereinbelow.

Table 2

Therapeutic moiety	***Amino acid sequence (GenBank Accession No.)***	***Nucleic acid sequence (GenBank Accession No.)***
Pseudomonas exotoxin	ABU63124 – SEQ ID NO:42	EU090068 – SEQ ID NO:51
Diphtheria toxin	AAV70486 – SEQ ID NO:43	AY820132.1 – SEQ ID NO:52
interleukin 2	CAA00227 – SEQ ID NO:44	A02159 – SEQ ID NO:53
CD3	P07766 – SEQ ID NO:45	X03884 – SEQ ID NO:54
CD16	NP_000560.5 – SEQ ID NO:46	NM_000569.6 – SEQ ID NO:55
interleukin 4	NP_000580.1 – SEQ ID NO:47	NM_000589.2 – SEQ ID NO:56
HLA-A2	P01892 – SEQ ID NO:48	K02883 – SEQ ID NO:57
interleukin 10	P22301 – SEQ ID NO:49	M57627 – SEQ ID NO:58
Ricin toxin	EEF27734 – SEQ ID NO:50	EQ975183 – SEQ ID NO:59

The functional moiety may be conjugated to the V_H or the V_L sequence at either the N- or C-terminus or be inserted into other protein sequences in a suitable position. For example, for Pseudomonas exotoxin (PE) derived fusion proteins, either V_H or V_L should be linked to the N-terminus of the toxin or be inserted into domain III of PE. For Diphtheria toxin-derived antibodies, V_H or V_L is preferably linked to the C-terminus of the toxin.

It will be appreciated that such fusions can also be effected using chemical conjugation (i.e., not by recombinant DNA technology).

The V_H and V_L sequences for application in this invention can be obtained from antibodies produced by any one of a variety of techniques known in the art.

Methods of producing polyclonal and monoclonal antibodies are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Typically, antibodies are provided by immunization of a non-human animal, preferably a mouse, with an immunogen comprising a desired antigen or immunogen. Alternatively, antibodies may be provided by selection of combinatorial libraries of immunoglobulins, as disclosed for instance in Ward et al (Nature 341 (1989) 544). Thus any method of antibody production is envisaged according to the present teachings as long as an immunoglobulin antibody is finally expressed in the bacterial host.

The step of immunizing a non-human mammal with an antigen may be carried out in any manner well known in the art for stimulating the production of antibodies in a mouse (see, for example, E. Harlow and D. Lane, Antibodies: A Laboratory Manual., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988)). In a preferred embodiment, the non-human animal is a mammal, such as a rodent (e.g., mouse, rat, etc.), bovine, porcine, horse, rabbit, goat, sheep, etc. As mentioned, the non-human mammal may be genetically modified or engineered to produce "human" antibodies, such as the Xenomouse™ (Abgenix) or HuMAb-Mouse™ (Medarex). Typically, the immunogen is suspended or dissolved in a buffer, optionally with an adjuvant, such as complete Freund's adjuvant. Methods for determining the amount of immunogen, types of buffers and amounts of adjuvant are well known to those of skill in the art and are not limiting in any way on the present invention. These parameters may be different for different immunogens, but are easily elucidated.

Similarly, the location and frequency of immunization sufficient to stimulate the production of antibodies is also well known in the art. In a typical immunization protocol, the non-human animals are injected intraperitoneally with antigen on day 1 and again about a week later. This is followed by recall injections of the antigen around day 20, optionally with adjuvant such as incomplete Freund's adjuvant. The recall injections are performed intravenously or intraperitoneally and may be repeated for several consecutive days. This is followed by a booster injection at day 40, either intravenously or intraperitoneally, typically without adjuvant. This protocol results in

the production of antigen-specific antibody-producing B cells after about 40 days. Other protocols may also be utilized as long as they result in the production of B cells expressing an antibody directed to the antigen used in immunization.

In an alternate embodiment, lymphocytes from a non-immunized non-human mammal are isolated, grown in vitro, and then exposed to the immunogen in cell culture. The lymphocytes are then harvested and the fusion step described below is carried out.

For monoclonal antibodies, the next step is the isolation of splenocytes from the immunized non-human mammal and the subsequent fusion of those splenocytes with an immortalized cell in order to form an antibody-producing hybridoma. The isolation of splenocytes from a non-human mammal is well-known in the art and typically involves removing the spleen from an anesthetized non-human mammal, cutting it into small pieces and squeezing the splenocytes from the splenic capsule and through a nylon mesh of a cell strainer into an appropriate buffer so as to produce a single cell suspension. The cells are washed, centrifuged and re-suspended in a buffer that lyses any red blood cells. The solution is again centrifuged and remaining lymphocytes in the pellet are finally re-suspended in fresh buffer.

Once isolated and present in single cell suspension, the lymphocytes are fused to an immortal cell line. This is typically a mouse myeloma cell line, although many other immortal cell lines useful for creating hybridomas are known in the art. Preferred murine myeloma lines include, but are not limited to, those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. U.S.A., X63 Ag8653 and SP-2 cells available from the American Type Culture Collection, Rockville, Md. U.S.A. The fusion is effected using polyethylene glycol or the like. The resulting hybridomas are then grown in selective media that contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

The hybridomas are typically grown on a feeder layer of macrophages. The macrophages are preferably from littermates of the non-human mammal used to isolate splenocytes and are typically primed with incomplete Freund's adjuvant or the like several days before plating the hybridomas. Fusion methods are described in (Goding, "Monoclonal Antibodies: Principles and Practice," pp. 59-103 (Academic Press, 1986

The cells are allowed to grow in the selection media for sufficient time for colony formation and antibody production. This is usually between 7 and 14 days. The hybridoma colonies are then assayed for the production of antibodies that bind the immunogen/antigen. The assay is typically a colorimetric ELISA-type assay, although any assay may be employed that can be adapted to the wells that the hybridomas are grown in. Other assays include immunoprecipitation and radioimmunoassay. The wells positive for the desired antibody production are examined to determine if one or more distinct colonies are present. If more than one colony is present, the cells may be re-cloned and grown to ensure that only a single cell has given rise to the colony producing the desired antibody. Positive wells with a single apparent colony are typically recloned and re-assayed to insure only one monoclonal antibody is being detected and produced.

Hybridomas that are confirmed to be producing a monoclonal antibody are then grown up in larger amounts in an appropriate medium, such as DMEM or RPMI-1640. Alternatively, the hybridoma cells can be grown in vivo as ascites tumors in an animal.

After sufficient growth to produce the desired monoclonal antibody, the growth media containing monoclonal antibody (or the ascites fluid) is separated away from the cells and the monoclonal antibody present therein is purified. Purification is typically achieved by gel electrophoresis, dialysis, chromatography using protein A or protein G-Sepharose, or an anti-mouse Ig linked to a solid support such as agarose or Sepharose beads (all described, for example, in the Antibody Purification Handbook, Amersham Biosciences, publication No. 18-1037-46, Edition AC, the disclosure of which is hereby incorporated by reference). The bound antibody is typically eluted from protein A, protein G or protein L columns by using low pH buffers (glycine or acetate buffers of pH 3.0 or less) with immediate neutralization of antibody-containing fractions. These fractions are pooled, dialyzed, and concentrated as needed.

DNA encoding the heavy and light chains of the antibody may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide

probes that are capable of binding specifically to genes encoding the heavy and light chains of antibodies such as murine or human). Once isolated, the DNA can be ligated into expression vectors, which are then transfected into host cells.

The antibodies according to the invention are typically produced by recombinant means.

The DNA sequences encoding the immunoglobulin light chain and heavy chain polypeptides may be independently inserted into separate recombinant vectors or one single vector, which may be any vector, which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced.

Methods for recombinant production are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody and usually purification to a pharmaceutically acceptable purity.

For the expression of the antibodies as aforementioned in a host cell, nucleic acids encoding the respective modified light and heavy chains are inserted into expression vectors by standard methods.

The procedures used to ligate the DNA sequences coding for the polypeptides, the promoter (e.g., constitutive or inducible) and optionally the terminator sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (see, for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y., 1989).

Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NSO cells, SP2/0 cells, HEK293 cells, COS cells, PER.C6 cells, yeast, or bacterial cells, and the antibody is recovered from the cells (supernatant or cells after lysis).

The present invention contemplates expressing each component of the antibody in its own individual host cell, or various combinations of the antibody components in their own host cells. Thus for example, the light chains may be expressed in one host cell and the heavy chains in another host cell. Alternatively, one light chain and one heavy chain is expressed in one host cell and the second light chain and the second

heavy chain is expressed in another host cell. Still alternatively, both the heavy chains and both the light chains may be expressed in the same host cell.

It will be appreciated that when both the heavy chains and both the light chains are expressed in the same host cell, in vitro assembly of the chains is not necessary and only purification of the antibodies form the conditioned medium i.e. by protein A chromatography is required (See for example: Jackman J, J Biol Chem. 2010 Jul 2;285(27):20850-9. Epub 2010 May 5).

When at least one of the chains is expressed in a different host cell to the other three chains, in vitro assembly of the chains is required.

According to a specific embodiment, the host cell comprises bacterial cells.

According to another embodiment the antibodies are generated as inclonals as described in WO2009/107129 incorporated herein by reference.

The bacterial host may be selected capable of producing the recombinant proteins (i.e., heavy and light chains) as inclusion bodies (i.e., nuclear or cytoplasmic aggregates of stainable substances).

The host cells (e.g., first host cell and second host cell) used can be of identical species or different species.

According to specific embodiments of the present invention the host cells are selected from a Gram-negative bacterium/bacteria.

As used herein "Gram negative bacteria" refers to bacteria having characteristic staining properties under the microscope, where they either do not stain or are decolorized by alcohol during Gram's method of staining. Gram negative bacteria generally have the following characteristics: (i) their cell wall comprises only a few layers of peptidoglycans (which is present in much higher levels in Gram positive bacteria); (ii) the cells are surrounded by an outer membrane containing lipopolysaccharide (which consists of Lipid A, core polysaccharide, and O-polysaccharide) outside the peptidoglycan layer; (iii) porins exist in the outer membrane, which act like pores for particular molecules; (iv) there is a space between the layers of peptidoglycan and the secondary cell membrane called the periplasmic space; (v) the S-layer is directly attached to the outer membrane, rather than the peptidoglycan (vi) lipoproteins are attached to the polysaccharide backbone, whereas in Gram positive bacteria no lipoproteins are present.

Examples of Gram-negative bacteria which can be used in accordance with the present teachings include, but are not limited to, *Escherichia coli Pseudomonas*, *erwinia* and *Serratia*. It should be noted that the use of such Gram-negative bacteria other than *E. coli* such as *Pseudomonas* as a host cell would provide great economic value owing to both the metabolic and physiologic properties of pseudomonas. Under certain conditions, pseudomonas, for example, can be grown to higher cell culture densities than *E. coli* thus providing potentially greater product yields.

Examples of bacterial expression vectors suitable for use in accordance with the present teachings include, but are not limited to, pET™ systems, the T7 systems and the pBAD™ system, which are well known in the art.

Methods of introducing expression vectors into bacterial host cells are well known in the art and mainly depend on the host system used.

The host cells can either be co-cultured in the same medium, or cultured separately.

Host cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant heavy and light chain. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit recombinant protein production. An effective medium refers to any medium in which a bacterium is cultured to produce the recombinant protein of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Bacterial hosts of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates, dependent on the desired amount. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant host. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Once appropriate expression levels of immunoglobulin heavy and light chains are obtained, the polypeptides are recovered from the inclusion bodies. Methods of recovering recombinant proteins from bacterial inclusion bodies are well known in the art and typically involve cell lysis followed by solubilization in denaturant [e.g., De Bernardez-Clark and Georgiou, "Inclusion bodies and recovery of proteins from the

aggregated state" Protein Refolding Chapter 1:1-20 (1991). See also Examples section which follows, under *"Expression of Inclonals in E. coli"*].

Briefly, the inclusion bodies can be separated from the bulk of cytoplasmic proteins by simple centrifugation giving an effective purification strategy. They can then be solubilized by strong denaturing agents like urea (e.g., 8 M) or guanidinium hydrochloride and sometimes with extremes of pH or temperature. The denaturant concentration, time and temperature of exposure should be standardized for each protein. Before complete solubilization, inclusion bodies can be washed with diluted solutions of denaturant and detergent to remove some of the contaminating proteins.

Finally, the solubilized inclusion bodies can be directly subjected to further purification through chromatographic techniques under denaturing conditions or the heavy and light chains may be refolded to native conformation before purification.

Thus, further purification of the reconstituted/refolded heavy and light chain polypeptides (i.e., solubilized reduced polypeptides) can be effected prior to, and alternatively or additionally, following refolding.

Methods of antibody purification are well known in the art and are described hereinabove and in the Examples section which follows. Other methods for purification of IgG are described in "Purification of IgG and insulin on supports grafted by sialic acid developing "thiophilic-like" interactions Hamid Lakhiaria and Daniel Mullerb, Journal of Chromatography B Volume 818, Issue 1, 15 April 2005, Pages 53-59.

Alternatively or additionally, purification can be affinity-based through the identifiable or therapeutic moiety (e.g., using affinity columns which bind PE38 to purify antibodies that are fused to PE38).

Further purification of antibodies may be performed in order to eliminate cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987). Different methods are well established and widespread used for protein purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino

ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M.A., Appl. Biochem. Biotech. 75 (1998) 93-102).

To improve the refolding yield, the reconstituted heavy chains and reconstituted light chains are provided at a ratio selected to maximize the formation of an intact antibody. To this end, a heavy to light chain molar ratio of about 1:1 to 1:3, 1:1.5 to 1:3, 1:2 to 1:3 is. In an exemplary embodiment the heavy to light chain molar ratio is about 1:1.

When desired the immunoglobulin may be subjected to directed *in vitro* glycosylation, which can be done according to the method described by Isabelle Meynial-salles and Didier Combes. In vitro glycosylation of proteins: An enzymatic approach. Journal of Biotechnology Volume 46, Issue 1, 18 April 1996, Pages 1-14.

One aspect of the invention is a pharmaceutical composition comprising an antibody according to the invention. Another aspect of the invention is the use of an antibody according to the invention for the manufacture of a pharmaceutical composition. A further aspect of the invention is a method for the manufacture of a pharmaceutical composition comprising an antibody according to the invention. In another aspect, the present invention provides a composition, e.g. a pharmaceutical composition, containing an antibody according to the present invention, formulated together with a pharmaceutical carrier.

Antibodies and compositions (e.g., pharmaceutical composition) comprising same may be used in diagnostic and therapeutic applications and as such may be included in therapeutic or diagnostic kits.

Thus, compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient i.e., antibody. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser

may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

One use of the antibody according to the invention is for the treatment of diseases related to inflammation and infections.

As used herein the term "inflammation" refers to any medical condition which comprises an inflammatory response in which migration of cells (e.g. to the lymph nodes) contributes to inflammation onset or progression.

A number of diseases and conditions, which involve an inflammatory response, can be treated using the methodology described hereinabove including both chronic inflammatory diseases and acute inflammatory diseases.

Examples of such diseases include inflammatory diseases associated with hypersensitivity.

Examples of hypersensitivity include, but are not limited to, Type I hypersensitivity, Type II hypersensitivity, Type III hypersensitivity, Type IV hypersensitivity, immediate hypersensitivity, antibody mediated hypersensitivity, immune complex mediated hypersensitivity, T lymphocyte mediated hypersensitivity and DTH.

Other types of inflammatory diseases which may be treated with the bifunctional antibodies disclosed herein are autoimmune diseases, infectious diseases, graft rejection diseases, allergic diseases and cancerous diseases.

The term "cancer" as used herein refers to proliferative diseases including by not limited to carcinoma, lymphoma, blastoma, sarcoma, and leukemia. Particular examples of cancerous diseases but are not limited to: Myeloid leukemia such as Chronic myelogenous leukemia. Acute myelogenous leukemia with maturation. Acute promyelocytic leukemia, Acute nonlymphocytic leukemia with increased basophils,

Acute monocytic leukemia. Acute myelomonocytic leukemia with eosinophilia; Malignant lymphoma, such as Birkitt's Non-Hodgkin's; Lymphocytic leukemia, such as Acute lumphoblastic leukemia. Chronic lymphocytic leukemia; Myeloproliferative diseases, such as Solid tumors Benign Meningioma, Mixed tumors of salivary gland, Colonic adenomas; Adenocarcinomas, such as Small cell lung cancer, Kidney, Uterus, Prostate, Bladder, Ovary, Colon, Sarcomas, Liposarcoma, myxoid, Synovial sarcoma, Rhabdomyosarcoma (alveolar), Extraskeletel myxoid chonodrosarcoma, Ewing's tumor; other include Testicular and ovarian dysgerminoma, Retinoblastoma, Wilms' tumor, Neuroblastoma, Malignant melanoma, Mesothelioma, breast, skin, prostate, and ovarian.

Treatment of diseases may be effected by administering the antibody alone, or together with a carrier as a pharmaceutical composition.

As used herein, "pharmaceutical carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g. by injection or infusion).

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent.

Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intra-

arterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intra-articular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions.

In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier preferably is an isotonic buffered saline solution.

Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of

surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition.

Other contemplated uses of the bispecific antibodies of the present invention include purification of analytes; in immunohistochemistry and enzyme immunoassays; for radioimaging and radioimmunotherapy and for drug delivery

Other contemplated uses are set forth in Cao Y, Suresh MR. Bispecific antibodies as novel bioconjugates. Bioconjug Chem. 1998 Nov-Dec;9(6):635-44, incorporated herein by reference.

As used herein the term "about" refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various

embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins

S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

MATERIALS AND METHODS FOR EXAMPLES 1-4

Construction of expression vectors for heavy and light chains: The vector for production of antibody heavy and light chains in *E. coli* were constructed on the backbone of pHAK vectors (Hakim and Benhar, 2009). The heavy chains vectors were modified at the CH2-CH3 constant regions using Kunkel mutagenesis (Kunkel, 1985) to contain heavy-heavy heterodimer-preferable mutations according to "knobs-into-holes" approach (Merchant et al., 1998). To that end, DNA of the pHAK-IgH vector was prepared in *E. coli* CJ236 strain, infected with M13KO7 helper phage and released single-stranded uracil-containing plasmid DNA was collected the next day using phenol-chloroform purification. The DNA samples were incubated with either primer 1 (for introduction of "knob" mutations) or the mixture of primer 2, primer 3 and primer 4 (for introduction of "hole" mutations) (Table 3, herein below) in TM buffer (0.01 M $MgCl_2$, 0.05 M Tris pH 7.5). In the next step, the DNA samples were incubated in presence of T7 polymerase and T4 ligase enzymes (supplied by 0.4 mM ATP, 0.4 mM dNTPs, 6mM DTT) and transformed into DH5α *E. coli* bacteria. The resulted constructs were named pHAK-HC-knob (carrying mutations T366W, S354C) and pHAK-HC-hole (carrying mutations T366S, L368A, Y407V, Y349C). The mutation-containing regions were subcloned using *NsiI-NdeI* restriction enzymes into pHAK-IgH-PE38 vector (Hakim and Benhar, 2009) that resulted in pHAK-HC-knob-PE38 and pHAK-HC-hole-PE38 vectors. The above constructs provided expression of antibody heavy chain fused to PE38 toxin.

Table 3

Primer name	*Sequence 5' to 3'*	*Notes*
Primer1 S→C:T →W	GAAGCCTTTGACCAGGCAccaCAG GCTGACCTGGTTCTTGGTCATCTC CTCCCGGcATGGGGGCAGGGTGT ACAC – SEQ ID NO: 1	Reverse primer for Kunkel mutagenesis that provides S354C and T366W replacements on pHAK-IgH vector.
Primer2 Y→C	GGATGGGGGCAGGGTGcACACCT GTGGTTCTCGG – SEQ ID NO: 2	Reverse primer for Kunkel mutagenesis that provides Y349C replacement on pHAK-IgH vector.
Primer3 T→S:L →A	GGATAGAAGCCTTTGACCgcGC AGcTCAGGCTGACCTGGTTCTTG – SEQ ID NO: 3	Reverse primer for Kunkel mutagenesis that provides L368A and T366S replacements on pHAK-IgH vector.
Primer4 Y→V	GTCCACGGTGAGCTTGCTAacG AGGAAGAAGGAGCCGTC – SEQ ID NO: 4	Reverse primer for Kunkel mutagenesis that provides Y407 replacement on pHAK-IgH vector.
Primer5	ATATA**CATATG**GACATTGTGCTG *NdeI* – SEQ ID NO: 5	Forward primer for PCR amplification of variable region of light chain on pHAK-T427-IgL vector
Primer6	tatata**cgtacg**TTTGATTTCCAGTTTGG *BsiWI* TGCCgcaACCGAACGTCCGAGG – SEQ ID NO: 6	Reverse primer for A104C replacement in variable domain of T427-IgL.
Primer7	tatata**GAATTC**TTACTCTCCCCTGTT *EcoRI* GAAGCTCTTTGTG – SEQ ID NO: 7	Reverse primer for removal of C218 amino acid codon from light chain sequence.
Primer8	AAACAG**AGGCCT**GGACAGtGTC *StuI* TGGAATGGATTG	Forward primer for G44C replacement in variable domain of T427-IgH.

	– SEQ ID NO: 8	
Primer9	tatata**GCTAGC**GGAGGAGACTGTG *NheI* AG – SEQ ID NO: 9	Reverse primer for PCR amplification of variable region of heavy chain on pHAK-T427-IgH vector.
Primer10	GCCCAAATCTgccGACAAAACTCA CACATGCCCACC – SEQ ID NO: 10	Forward primer for C222A replacement in heavy chain constant region on pHAK-IgH vector.
Primer11	TGTGTGAGTTTTGTCggcAGATTTG GGCTCAACTCTCTTG – SEQ ID NO: 11	Reverse primer for C222A replacement in heavy chain constant region on pHAK-IgH vector.
Primer12	GAGGAGATGACCAAGAACCAGGT – SEQ ID NO: 12	Reverse primer for amplification of heavy chain constant region of pHAK-IgH vector.
Primer13	atata**CATATG**CAGGTCAAACTGC *NdeI* – SEQ ID NO: 13	Forward primer for amplification of heavy chain variable region of pHAK-T427-IgH vector.

To provide for efficient pairing of the heavy-light chains, the native interchain di-sulfide bond was replaced with an engineered bond at an alternative position in one IgH/IgL pair. The mutations that were inserted in pHAK-LC-Cys were A104C in V_L, and a C218del in C-Kappa. The mutations that were inserted in pHAK-HC-Cys were A44C in V_H and C222A in CH1. The construction of pHAK-LC-Cys vector included two sequential cloning steps. First, the light chain variable domain of the selected antibody was amplified with primer 5 and primer 6, digested with *NdeI-BsiWI* restriction enzymes and cloned to pHAK-IgL previously digested with the same enzymes. The resulted vector served as a template for amplification of IgL with primer 5 and primer 7, which was digested with *Nde*I-*Eco*RI enzymes and cloned to pHAK-IgL (*NdeI-EcoRI* digested). In order to construct pHAK-HC-Cys (A44C), the heavy chain variable region of selected antibody was amplified with primer 8 and primer 9,

following *StuI-NheI* digestion and cloning into pHAK-IgH vector. The insertion of the C222A mutations into CH1 was carried out by amplification of two of the two PCR fragments that were generated by either 10 and 12 primers or 11 and 13 primers, followed by assembly PCR with primer 12 and primer 13. The assembled DNA fragment was digested with *NdeI* and *BsrGI* restriction enzymes and cloned into previously constructed pHAK-HC-Cys (A44C) vector.

The combined pHAK-HC-Cys-knob vector (A44C, C222A, T366W, and S354C) was constructed by insertion of *NdeI-SacII* digested region of pHAK-HC-Cys to pHAK-HC-knob vector. The light or heavy variable regions of desired antibody were cloned on either pHAK-LC-based vector (using *NdeI-BsiWI* subcloning) or pHAK-HC-based vector (using *NdeI-NheI* subcloning).

***IgG production in E. coli*:** Heavy and light chains constructs based on pHAK-IgH and pHAK-IgL, respectively, were expressed in separate *E. coli* BL21 (DE3) pUBS500 bacterial cultures as inclusion bodies. The inclusion bodies were purified, denatured, mixed and refolded according to the Inclonals IgG production method (Hakim and Benhar, 2009). For bispecific IgG production the complement heavy chains were added at 1:1 molar ratio. The same rule was applied for the light chains.

Protein A purification: Following the refolding process IgG and IgG-based fusion proteins were loaded on a protein A affinity column and separated from bacterial contaminants and not efficiently refolded proteins. The proteins were eluted with 0.1 mM citric acid neutralized with 1M Tris (HCl) pH 8.5 followed by dialysis against 20 mM phosphate buffer solution (PBS) pH 7.4. The protein final concentration was determined by absorbance at 280 nm.

Gel filtration chromatography: Gel filtration analysis was carried out on Amersham Pharmacia ÄKTA FPLC System to determine the molecular mass of the purified antibodies. The protein A purified proteins were applied to a Superdex 200 column, previously equilibrated with PBS (pH 7.4), and separated using the same buffer at a flow rate of 0.5 ml/min. The molecular weight of examined IgG-like proteins was determined by comparing its elution volume with that of standard IgG (150 kDa) and IgG-based immunotoxin IgG-PE38 (225 kDa).

SDS-PAGE analysis: Polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (Laemmli, 1970) 1/5 volume of 5x sample buffer was

added to the protein samples followed by boiling for 5 min prior to the loading onto the gel. 7.5 %, 10 % and 12 % mini-gels were run at 120 V. For evaluation of full length IgG, the non-reduced samples (without β-mercaptoethanol) were loaded, while the reduced protein samples separated into heavy and light chains components. Gels were stained with Coomassic blue solution (0.05 % Coomassic R-250, 20 % ethanol, 10 % glacial acetic acid) for 2 hours and washed in destain solution (20 % ethanol, 10 % glacial acetic acid) until protein bands could be clearly seen. The protein band density was analyzed by ImageMaster 1D scanning laser densitometer (Pharmacia, Sweden). Gels that were stained were loaded with 20μg of protein per lane for non-purified fraction or 3-5 μg for purified proteins. Gels that were further processed by immunoblotting were loaded with 1/10 that quantity.

Western blot analysis: Proteins resolved by SDS-PAGE were electro-transferred onto the nitrocellulose membrane according to (Towbin et al., 1992). The membrane was blocked for at least 1 hour with PBS containing 5 % non-fat milk powder at room temperature with slow agitation. The membrane was washed with PBS followed by incubation HRP conjugated goat-anti-human secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). After three washes with PBS containing 0.05 % Tween-20 (PBST) and one wash with PBS the nitrocellulose filter was developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) as described by the vendor.

ELISA analysis: The antigen binding by mono- and bispecific IgGs was determined as follows: the 96-well ELISA plate was coated with 5 μg/ml of pure antigen in PBS 100 μl/well for overnight at 4 °C and blocked with 3 % skim milk (in PBS) for 1 hour at 37 °C. All subsequent steps were carried out at room temperature (25°C). Protein A purified proteins were applied onto the plates in a three-fold dilution series in PBST for 1 hour incubation and washed with PBST for three times. Following the 1 hour incubation with HRP conjugated secondary antibodies (1:5000 dilution in PBST, 100 μl/well), the plates were washed in PBST and developed using chromogenic HRP substrate TMB and colour development was terminated with 1M H_2SO_4. The plated were read at 450 nm.

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EXAMPLE 1

Production of full-length IgG in E. coli using Inclonals method

The Inclonals method for production of full-length IgG in *E. coli* bacteria (Hakim and Benhar, 2009) includes using pHAK-IgH and pHAK-IgL vectors for production of IgH and IgL, respectively in separate bacterial cultures. The variable regions of heavy and light chains define the antibody specificity while the constant region is common for each vector. The protein expression, purification and refolding was carried out according to the Inclonals protocol and the purified proteins were evaluated using SDS-PAGE, Western blot, size exclusion chromatography and antigen binding analysis. As opposed to mono-specific antibody, the bispecific IgGs consists of 2 different heavy chains and 2 different light chains, thus expression and refolding steps include concomitant work with 4 proteins.

EXAMPLE 2

Construction and evaluation of heavy-heavy chain heterodimers

The "knobs-into-holes" approach (Ridgway et al., 1996) was implemented as a solution to preferable heterodimerization of different heavy chains for bispecific IgG production in *E. coli*. It was previously demonstrated that introduction of 4 mutations (T366W in "knob" heavy chain and T366S, L368A, Y407V in "hole" heavy chain) and the asymmetric disulfide bond (S354C and Y349C on complement heavy chains) provided high (>95%) heterodimerization level of heavy chains in IgG produced in mammalian cells (Merchant et al., 1998), (Figure 1A). The above mutations were used for construction of pHAK-HC-knob and pHAK-HC-hole vectors that were used for expression and examination of heavy chains, while the common unmodified light chain served for all IgG constructs (Figure 2A-H). T427 (anti-CD30) and FRP5 (anti-erbB2) antibodies were used as model IgGs for method evaluation (Harwerth et al., 1992; Nagata et al., 2004). The antibody heavy and light chains were expressed as inclusion bodies, purified by centrifugation and analyzed by SDS-PAGE (Figure 3). The refolding of 4 antibody chains together followed by protein A purification according to Inclonals protocol enabled production of full-length IgG.

For detailed characterization of heterodimerization yield the "hole-heavy" chain was expressed as fusion protein with PE38 toxin (Kreitman et al., 1992) that provided

additional 38 kDa to protein molecular weight. As illustrated in Figures 4A-B, using SDS-PAGE analysis it was possible to distinguish between the homodimer of "knob" heavy chains (150 kDa), the homodimer of "hole" toxin-fused heavy chains (230 kDa) and the heterodimer of two different heavy chains (190 kDa). Figure 4B demonstrates that Inclonals' produced T427 "knobs-into-holes" antibody migrated as 190 kDa band on a non-reducing polyacrylamide gel and could be separated to 3 components (IgL, IgH and IgH-PE38) under reducing conditions.

The attempt to produce "knob-knob" and "hole-hole" versions of IgG by supplying the refolding solution with only one heavy chain type (either "knob" or "hole") resulted in assembly failure of IgG and performance of partial-sized molecules (Figures 5A-B).

The evaluation of bispecific inclonals "knobs-into-holes" antibodies using size-exclusion chromatography demonstrated that protein majority migrated as a 190 kDa molecules while only small protein fraction represented homodimers (Figure 6). Density analysis SDS-PAGE of the Inclonals "knobs-into-holes" antibody concluded that >90 % of *E. coli* produced IgGs underwent heavy chains heterodimerization (Figures 7A-C).

In order to evaluate the binding activity of bispecific molecules the "knobs-into-holes" bispecific T427-FRP5 antibody was constructed. This IgG consisted of 4 different chains: FRP5-knob and T427-hole-PE38 heavy chains, and FRP5 wt and T427 wt light chains. The PE38 toxin in this construct was used as a detection signal for T427 heavy chain presence. The mono-specific T427 and FRP5 IgGs served as controls. Using indirect ELISA the present inventors demonstrated the antibodies' binding ability to each one of its antigens (erbB2 for FRP5 (Figure 8A) and CD30 for T427 (not shown)). The special ELISA (Figure 8B) analysis examines the antibody binding to FRP5 antigen while T427-PE38 chain was detected. This assay demonstrated the presence of T427-FRP5 heterodimer that was able to bind its' two antigens.

EXAMPLE 3

Construction and evaluation of heavy-light chains specific pairing

In order to introduce the disulfide bond between the two variable domains to replace the native heavy-light interchain S-S bond, the T427 antibody was used. This antibody has been extensively studied and its' cysteine positions for dsFv have been

well defined (Nagata et al., 2004). Vectors pHAK-HC-Cys and pHAK-LC-Cys were constructed by replacement of conventional cysteine position by dsFv defined. The production of dsFv-like modified mono-specific IgG demonstrated the efficient formation of full length IgG stabilized by a single dsFv-like heavy-light interchain S-S bond (Figure 9, lane 3).

The construction of pHAK-HC-Cys-knob enabled the production of fully bispecific full length T427-FRP5 IgG (Figure 9, lane 4). Heterodimerization of heavy chains was provided by "knobs-into-holes" strategy and heavy-light pair matching was ensured by asymmetric interchain disulfide bond. Further, the IgG refolding solutions provided with unpaired heavy and light chains did not generate complete IgG molecules (Figure 10).

EXAMPLE 4

Two additional bispecific antibodies were produced, purified and evaluated, as described above in the materials and methods.

1. T427-αSA IgG: binding to CD30 and streptavidin (SA). The bispecific antibody consisted of 4 chains: IgL-T427-Cys (Cys104:Cys218del), IgH-T427-knob-Cys (Cys44:Cys222Ala + S354C:T366W), IgL-αSA and IgH-αSA-hole (Y349C:T366S:L368A:Y407V).
2. T427-αPE (B11 clone) IgG: binding to CD30 and PE38 (*Pseudomonas exotoxin* 38kDa fragment). The bispecific antibody consisted of 4 chains: IgL-T427-Cys (Cys104:Cys218del), IgH-T427-knob-Cys (Cys44:Cys222Ala + S354C:T366W), IgL-αPE and IgH-αPE-hole (Y349C:T366S:L368A:Y407V).

The anti-streptavidin (αSA) and anti-PE B11 clone (αPE) antibodies were isolated as scFvs by affinity selecting the "Ronit1" antibody phage display library (Azriel-Rosenfeld et al., 2004, J Mol Biol 335, 177-92). The heavy and light domains were cloned into the pHAK-IgH-hole and pHAK-IgL, respectively (as mentioned above). The chains were produced in *E. coli* bacteria as inclusion bodies, purified by centrifugation and analyzed by SDS-PAGE electrophoresis (Figure 11). The appropriate heavy and light chains were mixed, refolded and purified by Protein A affinity purification for production of mono-specific T427, αSA, αPE and bispecific T427-αSA

and T427-αPE antibodies according to Inclonals protocol (Hakim and Benhar, 2009). The antibodies were analyzed by ELISA for binding activity to each antigen (Figures 12 and 13). As shown, these bispecific antibodies were successfully produced and bound the two antigens according to the specificities of the two arms. Specificity was demonstrated by negligible binding to bovine serum albumin (BSA).

MATERIALS AND METHODS FOR EXAMPLES 5-9

Construction of pDual vectors for expression of IgGs in mammalian cells: The vector for production of antibody heavy and light chains in *E. coli* were constructed on the backbone of pMAZ vectors (Mazor Y., et al. J Immunol Methods. 2007 Apr 10;321(1-2):41-59.). Two bi-cistronic pMAZ vectors were constructed - pMAZ-IgH that carried the heavy chain and a Neomycin selection marker; and pMAZ-IgL that carried the light chain and a hygromycin selection marker. IgG expression was mediated by co-transfection of the two vectors, followed by double drug selection for obtaining stable transfectants.

The pDual vector was based on pMAZ-IgH vector that was previously mutated using IgH-*Apa*del-*Nhe*I-For and IgH-*BsrG*I-Rev primers in order to delete the *Apa*I restriction site in the constant region. The next step was the construction of pCMV-IgL-term cassette and cloning it between the *Kpn*I-*EcoR*I restriction sites of pMAZ-IgH-*Apa*del vector. The pCMV-IgL-term cassette was built by assembly of three PCR products that included: 1) amplification of pCMV promoter using pCMV-*Kpn*I-For and pCMV-Rev primers that provided the replacement of *BssH*I by *Apa*I restriction site (this *Apa*I site will be unique in the plasmid since the *Apa*I site that was present in the Fc coding region was mutated); 2) amplification of the T427 light chain antibody (VL+LC) using T427L-For and T427L-Rev primers that provided the replacement of *Xba*I by *Not*I restriction site; 3) amplification of the BGH polyadenylation site using BGH-polyA-For and BGH-polyA-*EcoR*I-Rev primers. The above PCR products were assembled into pCMV-IgL-term cassette by overlap-extension polymerase chain reaction (assembly PCR) followed by digestion with *Kpn*I and *EcoR*I restriction enzymes and cloning into pMAZ-IgH-*Apa*del vector as described above. The resulted vector was named pDual that was further used for cloning of variable domains of different antibodies using *Apa*I-*BsiW*I restriction sites for VL (kappa light chains, for

lambda light chains, a separate vector is required that carries a lambda light chain, into which V-lambda variable domain should be cloned as *Apa*I-*Avr*II restriction fragments) and *BssH*I-*Nhe*I restriction sites for VH.

A similar pDual vector was constructed that carries the hygromycin selection marker.

The list of primers used for generating the above described pDual vectors is summarized in Table 4, herein below.

Table 4

Primer name	Sequence 5' to 3'	Notes
IgH-*Apa*del-NheI-For	tcctcaGCTAGCaccaa**ggg**A**cc**atcggtcttccccctg *Nhe*I SEQ ID NO: 60	Forward primer for removal of ApaI restriction site at IgH constant domain by silent mutation
IgH-*BsrG*I-Rev	gcagggTGTACAcctgtggttc *BsrG*I SEQ ID NO: 61	Reverse primer for IgH-*Apa*del-NheI-For
pCMV-*Kpn*I-For	actgaaccttggagtcaGGTACCacattgat *Kpn*I Tattgagtagttattaatag SEQ ID NO: 62	Forward primer for amplification of CMV promoter
pCMV-Rev	GGGCCCctgtggagagaaaggcaaagtggatg *Apa*I SEQ ID NO: 63	Reverse primer for amplification of CMV promoter and insertion of ApaI restriction site between ER secretion signal and VL antibody region.
T427L-For	ctttgcctttctctccacagGGGCCCactccgac *Apa*I attgtgctgacccaatc SEQ ID NO: 64	Forward primer for amplification of T427 IgL and assembly with pCMV fragment.

T427L-Rev	cggtttaaaaaacgggacctctggaGCGGCCGCtt *Not*I attaacactctcccctgttgaagctctttgtg SEQ ID NO: 65	Reverse primer for amplification of T427 IgL that allows the replacement of XbaI by NotI restriction site.
BGH-polyA-For	tccagaggtcccgtttttttaaaccggtttttttaaaccgctg atcagcctcg SEQ ID NO: 66	Forward primer for amplification of polyadenylation site and assembly with T427 IgL fragment.
BGH-polyA-*EcoR*I-Rev	tagctcgatccgtcgagaGAATTCccccagcat *EcoR*I gcctgctattg SEQ ID NO: 67	Reverse primer for amplification of polyadenylation site.

Transfection of HEK293 T-REx™ cells: The calcium-phosphate transfection method was applied for introducing 1 μg of the pDual or pMAZ plasmids into T-REx 293 cells, seeded 3×10^5 cells/well on 6-well plate 24 hours before transfection. For transient transfection, the medium samples were collected 24, 48 and 72 hours post transfection. In order to obtain the stable transfectants, the cells were harvested 24 hours post transfection and seeded on DMEM supplemented with appropriate antibiotics (1.2 mg/ml G418 and 0.2 mg/ml Hygromycin). The stable clones were collected and their media were evaluated for the presence of antibody.

IgG production in HEK293 T-REx™ cells: The previously obtained stable clones were transferred to tissue culture flasks (250 cm^3) in DMEM supplement with 0.9 mg/ml G418 and 0.15 mg/ml Hygromycin (75 % of the regular concentration). The next day (or when the cells reached 80% confluence) the medium was changed to 50 % DMEM (+L-Glu, PNS and bovine serum) and 50 % DCCM1 (+L-Glu, PNS, serum free) + 75% of antibiotics concentration (0.9 mg/ml G418 and 0.15 mg/ml of Hygromycin) for 24 hours. The next day the medium was changed to 100 % serum free DCCM1 (+L-Glu, PNS). The DCCM1 media from cells were collected every 2-4 days

and gently changed to new serum-free media. It was possible to collect up to 4 harvests from the flask.

Protein A purification of IgG produced in mammalian cells: The collected DCCM1 medium from antibody secreting cells was centrifuged at 5500 rpm for 15 minutes and filtered using 0.45μm filtrap. The medium was diluted 1:20 with ×20 concentration phosphate buffer (400mM) to final concentration of 20 mM Na_2HPO_4 and 20 mM NaH_2PO_4 and the mixture was loaded onto protein A column at a flow rate of 1 ml/min. The proteins were eluted with 0.1 mM citric acid (pH 3), neutralized with 1M Tris (HCl) pH 8.5 which was followed by dialysis against 20 mM phosphate buffer solution (PBS) pH 7.4. The protein final concentration was determined by absorbance at 280 nm.

ELISA analysis: The antigen binding by mono- and bispecific IgGs was determined as follows: 96-well ELISA plates were coated with 5 μg/ml of pure antigen in PBS 100 μl/well for overnight at 4 °C and blocked with 3 % milk (in PBS) for 1 hr at 37°C. All subsequent steps were carried out at room temperature (25 °C). Protein-A purified proteins (or conditioned media) were applied onto the plates in a three-fold dilution series in PBST for 1 hour incubation and washed with PBST for three times. Following the 1 hour incubation with HRP-conjugated secondary antibody (1:5000 dilution in PBST, 100 μl/well), the plates were washed in PBST and developed using chromogenic HRP substrate TMB and colour development was terminated with 1 M H_2SO_4. The plates were read at 450 nm.

Cell ELISA analysis: The A431/CD30 (expressing CD30, target antigen for T427) and SKBR3 (expressing ErbB2, target antigen for FRP5) cell lines were maintained in DMEM supplemented by 10 % fetal calf serum, 1 % L-glutamine and 1 % penicillin-streptomycin and grown at 37 °C with 5 % CO_2. The cells (2×10^4/well) were seeded onto 96-well tissue culture plates in 100 μl medium and grown at 37 °C for overnight. Following the overnight growth the medium was gently poured out and the cells were fixed with 3 % glutaraldehyde solution in water for 15 minutes at room temperature. The cells were washed with PBS and blocked with 5 % BSA in PBS for 2 hours at 37 °C. All subsequent steps were carried out according the regular ELISA protocol at room temperature (25 °C).

Dot blot analysis: The 100 µl samples of 72 hours post-transfection cell conditioned media were diluted in an equal volume of PBS and applied via a vacuum manifold onto a nitrocellulose membrane filter using a dot–blot apparatus (Schleicher and Schuell, USA). After blocking the membranes with 3 % (v/v) non-fat milk in PBS for 1 hour at 37°C, the membrane was washed briefly with PBS followed by incubation with goat-anti-human HRP conjugated secondary antibody for 1 hour at room temperature. After three washes with PBS the membrane was developed with the ECL reagent (Pierce, USA).

SDS-PAGE analysis: Polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (Laemmli, 1970) 1/5 volume of 5x sample buffer was added to the protein samples followed by boiling for 5 minutes prior to loading onto the gel. 7.5 %, 10 % and 12 % mini-gels were run at 120 V. For evaluation of full length IgG, non-reduced samples (without β-mercaptoethanol) were loaded, while the reduced protein samples separated into heavy and light chains components. Gels were stained with Coomassie blue solution (0.05 % Coomassie R-250, 20 % ethanol, 10 % glacial acetic acid) for 2 hours and washed in destain solution (20 % ethanol, 10 % glacial acetic acid) until protein bands could be clearly seen. The protein band density was analyzed by ImageMaster 1D scanning laser densitometer (Pharmacia, Sweeden). Gels that were stained were loaded with 20 µg of protein per lane for non-purified fraction or 3-5µg for purified proteins. Gels that were further processed by immunoblotting were loaded with 1/10 that quantity.

Western blot analysis: Proteins resolved by SDS-PAGE were electro-transferred onto the nitrocellulose membrane according to (Towbin et al., 1992). The membrane was blocked for at least 1 hour with PBS containing 5 % non-fat milk powder at room temperature with slow agitation. The membrane was washed with PBS followed by incubation HRP conjugated goat-anti-human secondary antibodies (Jackson Laboratories, West Grove, PA). After three washes with PBS containing 0.05 % Tween-20 (PBST) and one wash with PBS the nitrocellulose filter was developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) as described by the vendor.

EXAMPLE 5

The production of IgG in mammalian cells

The vector system used for the production of IgG in mammalian cells for production of bispecific antibodies was based on pMAZ vectors for production of monoclonal antibodies in mammalian cell culture (Mazor Y et al, 2007). Vector pMAZ-IgH was designed for human γ1 heavy chain expression and pMAZ-IgL for human κ light chain expression. The variable domains of light and heavy chains were introduced to the appropriate vector, co-transfected into HEK293 cells and stable antibody secreting clones were identified and kept. The starvation of cell clones to serum resulted in secretion of the desired antibody at a total yield of up to 20 mg per liter of culture.

EXAMPLE 6

The construction of dual vector for production of IgG molecules in mammalian cells

The pDual vector was constructed by fusion of DNA fragments derived from the pMAZ-IgL and pMAZ-IgH vectors (Mazor Y et al, 2007) that were previously used for production of antibodies' light and heavy chains independently (Figure 14), in order to build chimeric construct for production of light and heavy antibody chains using the same vector. The IgL and IgH were constructed in pDual vector as separate cassettes under the control of separate CMV promoters. The replacement of *BssH*I by *Apa*I restriction site in the light chain cassette simplified the following cloning of variable light and heavy domains into dual vector: *Apa*I-*BsiW*I were used for cloning of VL and *BssH*I-*Nhe*I were used for cloning of VH.

EXAMPLE 7

The construction of bispecific vectors for transfection to mammalian cells

In order to construct the pDual-based vectors for production of bispecific molecules, the "knob", "hole" and "Cys" related mutations were cloned from the pHAK vectors into the pDual system. The cloning process resulted in the series of constructs listed in Table 5. The replacement of Neo^R with Hygro resistance cassette in pDual-

T427 vectors was carried out by subcloning of the cassette from pMAZ-IgL vector using *AvrII* and *KpnI* restriction enzymes. The resulted pDual-Neo^R and pDual-Hygro^R vectors' pair can be used for transfection in mammalian cells and selection of stable clones that produce 4 different antibody chains: two heavy and two light chains within one cell line.

Table 5

Vector name	***Product***
pDual-T427 wt Neo^R	IgL-T427 + IgH-T427
pDual-FRP5 wt Neo^R	IgL-FRP5 + IgH-FRP5
pDual-T427-L(wt)-H(knob) Neo^R	IgL-T427 + IgH-T427(knob)
pDual-FRP5-L(wt)-H(hole) Neo^R	IgL-FRP5 + IgH-FRP5(hole)
pDual-T427-L(Cys)-H(wt) Neo^R	IgL-T427(Cys) + IgH-T427
pDual-T427-L(Cys)-H(knob) Neo^R	IgL-T427(Cys) + IgH-T427(knob)
pDual-T427-L(wt)-H(Cys-knob) Neo^R	IgL-T427 + IgH-T427(Cys-knob)
pDual-T427-L(Cys)-H(Cys-knob) Neo^R	IgL-T427(Cys) + IgH-T427(Cys-knob)
pDual-T427 wt Hygro^R	IgL-T427 + IgH-T427
pDual-T427-L(Cys)-H(Cys-knob) Hygro^R	IgL-T427(Cys) + IgH-T427(Cys-knob)

EXAMPLE 8

The production and evaluation of bispecific IgG molecules in transient transfected HEK293 T-REx™ cells

The following example demonstrates the importance of the S-S bridge between the light and heavy chain of the antibody in IgG secretion system and proves that the "alternative Cysteine" theory for coupling the appropriate light and heavy chain of bispecific antibody is relevant in mammalian production system as well as it had been demonstrated in the *E. coli* produced bispecific "Inclonals". The HEK293 T-REx™ cell line was used for this study. The cells were transiently transfected with either pDual-T427 wt (encoding wt IgG), pDual-T427-L(Cys)-H(wt) (encoding wt heavy chain and light chain that lacks the C-kappa cysteine and does contain the engineerd cysteine in VL, this should be a pair of chains that should not form an IgG) or with pMAZ-

IgL+pMAZ-IgH (previous system for wt IgG production) pair as control. The evaluation of post-transfection medium by Western blot analysis showed that no full-size antibodies were detected in media of pDual-T427-L(Cys)-H(wt) transfected cells, while the transfection of pDual wt construct produced the detectable levels of the secreted antibody (up to 1 μg/ml in comparison to Erbitux dilutions) (Figures 15A and B).

The secretion of bispecific IgG molecules was also demonstrated and the preference of bispecific IgG formation using "knobs-into-holes" and "alternative Cysteine" (also called "disulfide stabilization") approaches was also estimated. The pDual vectors were transiently transfected to HEK293 TRex cells and the secreted antibodies were detected by Western blot analysis of conditioned media. The cells that were transfected with four pMAZ vectors (pMAZ-T427-IgL, pMAZ-T427-IgH, pMAZ-FRP5-IgL and pMAZ-FRP5-IgH) served as a control. The analysis of experiment demonstrated: 1) the "knobs-into-holes" approach is a solution for efficient heterodimerization of different heavy chains, 2) "alternative Cys" approach provide the solution for coupling of light and heavy chains of the same antibody arm, 3) the combination of the two above approaches provides the secretion of full-length bispecific IgG antibodies in mammalian cells production systems. As shown in Figure 16, when "wrong" combinations of chains are used, no full size IgG can be seen in the immunoblot analysis of conditioned media. Intact IgG can be observed in cells that express monospecific IgG (lanes 1 and 2), in cells that express two monospecific IgGs (lanes 5, two pDual vectors and lane 4 four pMAZ vectors) and in cells that express a bispecific IgG (lane 7).

EXAMPLE 9

The production and evaluation bispecific IgG molecules in stable transfected HEK293 T-REx™ cells and evaluation of antigen binding

In order to obtain stable antibody-secreting clones, the present inventors co-transfected HEK293 T-REx™ cells with pDual-FRP5-L(wt)-H(hole) Neo^R and pDual-T427-L(Cys)-H(Cys-knob) Hygro^R vectors. The resultant Neo + Hygro resistant clones were verified for their ability: 1) to secrete antibody, 2) to bind both antigens, 3) to

secret full-length IgG for further purification. The preliminary antibody secretion test was performed using Dot blot analysis (the test example demonstrated in Figure 17) and identified the low, medium and high secreting clones. The clones marked as medium and high secretors were examined for their antigen binding activity. ELISA was carried out to evaluate the binding level of each clone to erbB2 (FRP5) and CD30 (T427) recombinant antibodies (Figure 18). The several clones that were able to bind each of the antigens continued to the third step and were purified on protein A affinity column (Figure 19). The purified antibodies were analyzed to determine their size, purity and binding activity to either recombinant antigens or antigen-presenting cells (Figures 20 and 21). As shown in Figure 18, the binding signal in ELISA correlated to the secretion level of these clones. As shown in Figures 20 and 21, a protein A-purified bispecific IgG bound to both CD30 and ErbB2 antigens. In such an ELISA (Figure 21) it was expected that the monospecific IgGs (which are bi-valent) will show a more intense binding signal, each on its cognate antigen, due to avidity effect. A preliminary cell-ELISA (Figure 22) shows that the bispecific antibody secreted by clone D3 stains antigen-positive cells.

EXAMPLE 10

Construction of Monospecific antibodies

First, a mono-specific antibody (T427 IgG) was generated that comprised the knobs into hole (KIH) mutation. In order to evaluate the binding activity of KIH T427 IgG molecules, ELISA was carried out. The ELISA plate was coated with MBP-CD30 and incubated with T427 KIH IgG (fused to PE38). It was demonstrated that the binding ability of T427 KIH molecule was similar to the binding of unmodified T427 IgG and T427-PE38 IgG-PE38 (Figure 24).

Subsequently, a mono-specific antibody (T427 IgG) was generated that comprised both the KIH mutation and the cysteine mutations in the light chains as described herein above.

In order to produce the mono-specific T427 antibody, 4 chains were constructed: IgL-PE38, IgH-knob, IgH-Cys-hole and IgL-Cys. The presence of 38 kDa PE38 fused to VL- unmodified light chain provided the possibility to analyze the pairing of the

appropriate heavy and light chains (analogous to KIH heterodimerization analysis) and the formation of the full-sized mono bi-specific molecule (Figure not shown).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. An antibody comprising an Fc region and a Fab region, wherein:

(i) said Fc region comprises two non-identical heavy chains, wherein at least one of said two non-identical heavy chains comprises an amino acid modification so as to form complementation between said two non-identical heavy chains thereby increasing the probability of forming heterodimers of said non-identical heavy chains and decreasing the probability of forming homodimers of identical heavy chains; and

(ii) said Fab region comprises a first covalent link between a first heavy chain and a first light chain of said Fab region and a second covalent link between a second heavy chain and a second light chain of said Fab region, wherein a position of said first covalent link relative to said first heavy chain is different to a position of said second covalent link relative to said second heavy chain.

2. The antibody of claim 1 being a bispecific antibody.

3. The antibody of claim 1 being an asymmetric, monospecific antibody.

4. The antibody of claim 1, wherein said complementation comprises a steric complementation.

5. The antibody of claim 1, wherein said complementation comprises a charge complementation.

6. The antibody of claim 4, wherein said Fc region comprises a protuberance of one heavy chain of said Fc region and a sterically compensatory cavity on a second heavy chain of said Fc region, said protuberance protruding into said compensatory cavity.

7. The antibody of claim 4, wherein said protuberance is generated by substituting an amino acid at one position on a CH3 domain of said one heavy chain with another amino acid having a larger side chain volume than the original amino acid.

8. The antibody of claim 7, wherein said compensatory cavity is generated by substituting an amino acid at one position on a CH3 domain of said second heavy chain with another amino acid having a smaller side chain volume than the original amino acid.

9. The antibody of claim 1, wherein said first covalent link is between a CH1 domain of said one heavy chain and a CL domain of said one light chain; and said second covalent link is between a V_H domain of said second heavy chain and a V_L domain of said second light chain.

10. The antibody of claim 1, wherein said first and said second covalent links are disulfide bonds.

11. The antibody of claim 7, wherein said amino acid having a larger side chain volume than the original amino acid is selected from the group consisting of tyrosine, arginine, phenylalanine, isoleucine and tryptophan.

12. The antibody of claim 8, wherein said amino acid having a smaller side chain volume than the original amino acid is selected from the group consisting of alanine, glycine, valine and threonine.

13. The antibody of claim 1, being selected from the group consisting of a chimeric antibody, a humanized antibody and a fully human antibody.

14. The antibody of claim 1, wherein said CH3 domain of said first heavy chain is covalently linked to said CH3 domain of said second heavy chain.

15. The antibody of claim 2, wherein a first antigen binding site of the antibody binds a first epitope of an antigen and a second antigen binding site of the antibody binds a second epitope of said antigen.

16. The antibody of claim 2, wherein a first antigen binding site of the antibody binds an epitope of a first antigen and a second antigen binding site of the antibody binds an epitope of a second antigen.

17. The antibody of claim 1, wherein each light chain is linked to its cognate heavy chain via a single disulfide bond.

18. The antibody of claim 1 being an intact antibody.

19. The antibody of claim 1, wherein the antibody is selected from the group consisting of IgA, IgD, IgE and IgG.

20. The antibody of claim 1, wherein said IgG comprises IgG1, IgG2, IgG3 or IgG4.

21. The antibody of claim 1, wherein said first heavy chain comprises a T366W mutation; and said second heavy chain comprises T366S, L368A, Y407V mutations.

22. The antibody of claim 21, wherein said first heavy chain comprises an S354C mutation and said second heavy chain comprises a Y349C mutation.

23. The antibody of claim 16, wherein said first antigen binding site binds CD30 and said second antigen binding site binds erbB2.

24. The antibody of claim 16, wherein said first antigen binding site binds CD30 and said second antigen binding site binds Pseudomonas Exotoxin (PE).

25. The antibody of claim 16, wherein said first antigen binding site binds CD30 and said second antigen binding site binds strepavidin.

26. The antibody of claim 1, wherein at least one of said heavy chains is attached to a therapeutic moiety.

27. The antibody of claim 1, wherein at least one of said heavy chains is attached to an identifiable moiety.

28. The antibody of claim 1, being selected from the group consisting of a primate antibody, a porcine antibody, a murine antibody, a bovine antibody, a goat antibody and an equine antibody.

29. A method of preparing the antibody of claim 1, comprising:

(a) providing a first nucleic acid molecule encoding said first heavy chain;

(b) providing a second nucleic acid molecule encoding said second heavy chain;

(c) providing a third nucleic acid molecule encoding said first light chain;

(d) providing a fourth nucleic acid molecule encoding said second light chain;

(e) culturing host cells comprising said first, second, third and fourth nucleic acid molecules under conditions that permit expression of the nucleic acid molecules; and

(f) recovering the antibody of claim 1.

30. The method of claim 29, wherein said host cells comprise bacterial cells.

31. The method of claim 29, wherein said host cells comprise mammalian cells.

32. The method of claim 30, wherein said expression takes place in inclusion bodies of said bacterial cells.

33. The method of claim 29, wherein each of said nucleic acid molecules are transfected into different host cells.

34. The method of claim 27, wherein each of said nucleic acid molecules are transfected into the same host cell.

35. The method of claim 30, wherein said bacterial cells comprise gram negative bacterial cells.

36. The method of claim 29, further comprising purifying the antibody on Protein A/G/L following step (f).

37. A pharmaceutical composition comprising as an active agent the antibody of claim 1 and a pharmaceutically acceptable carrier.

38. The antibody of claim 1 for treating an infection or inflammatory disease or disorder.

39. The antibody of claim 38, wherein said inflammatory disorder is cancer.

40. A method of treating an infection or an inflammatory disease or disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the antibody of claim 1, thereby treating the infection or inflammatory disease or disorder.

41. The method of claim 40 wherein the inflammatory disease or disorder is cancer.

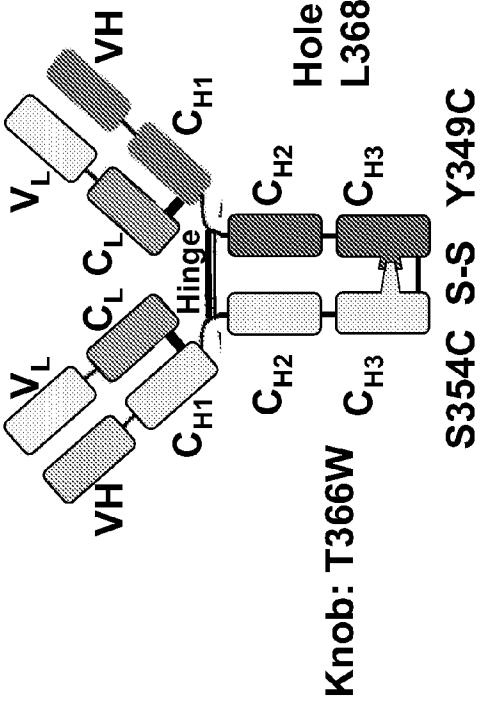

VL
VH
CL
CH1
Hinge
CH2
CH3
Hole: T366S,
L368A, Y407V
Knob: T366W
S354C S-S Y349C

FIG. 1A

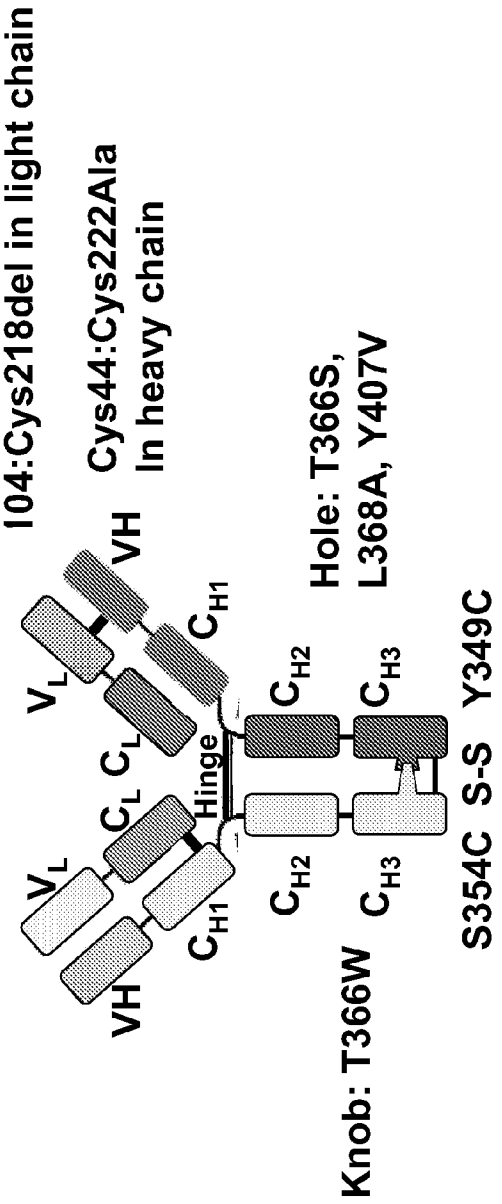

I04:Cys218del in light chain
Cys44:Cys222Ala
In heavy chain
VL
VH
CL
CH1
Hinge
CH2
CH3
Hole: T366S,
L368A, Y407V
Knob: T366W
S354C S-S Y349C

FIG. 1B

FIG. 2A

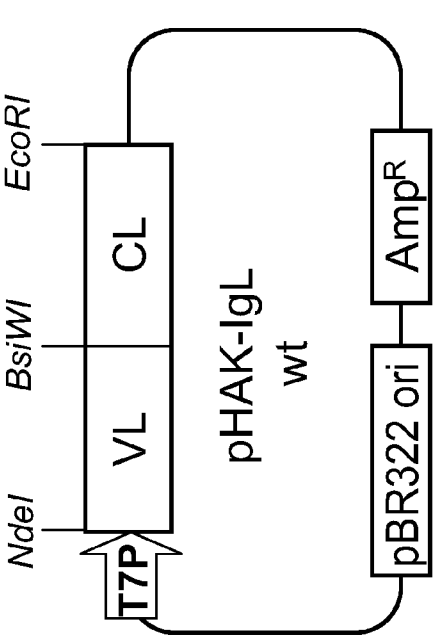


FIG. 2B

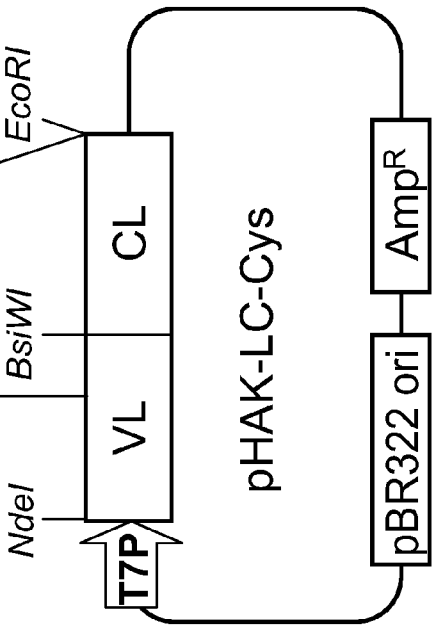


FIG. 2C

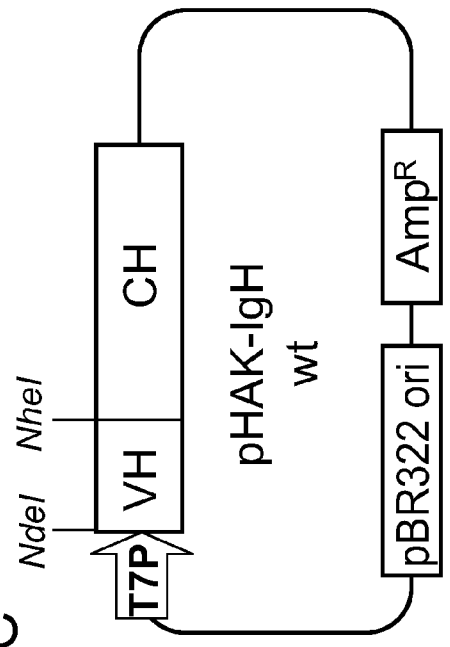


FIG. 2D

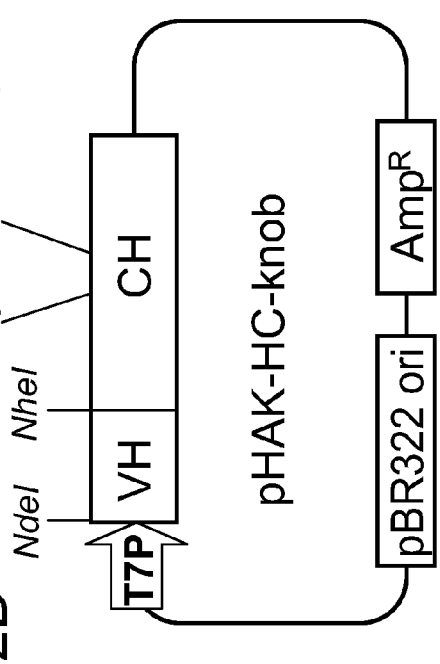


FIG. 2E

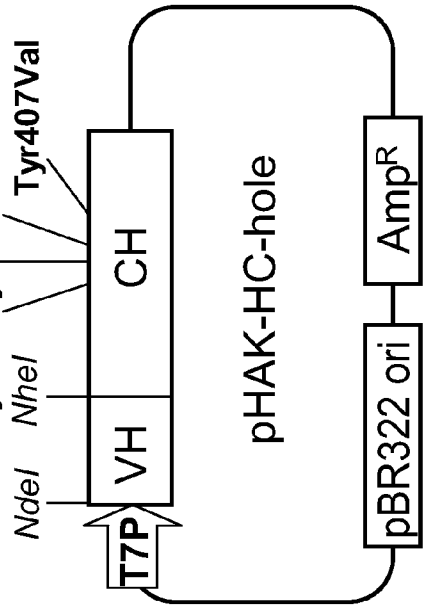


FIG. 2F

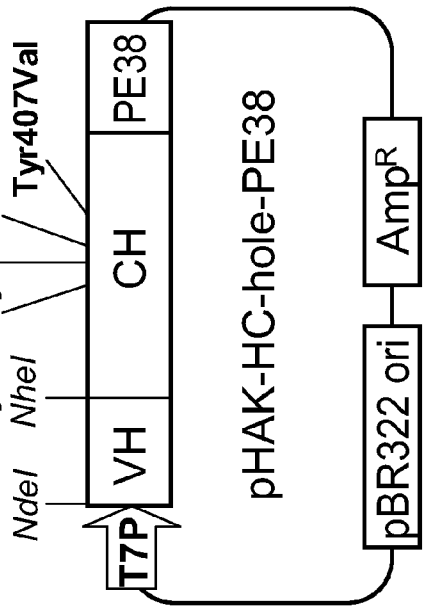


FIG. 2G

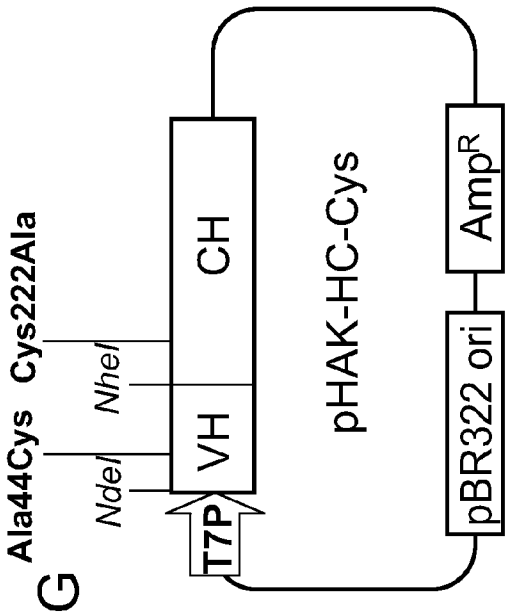


FIG. 2H

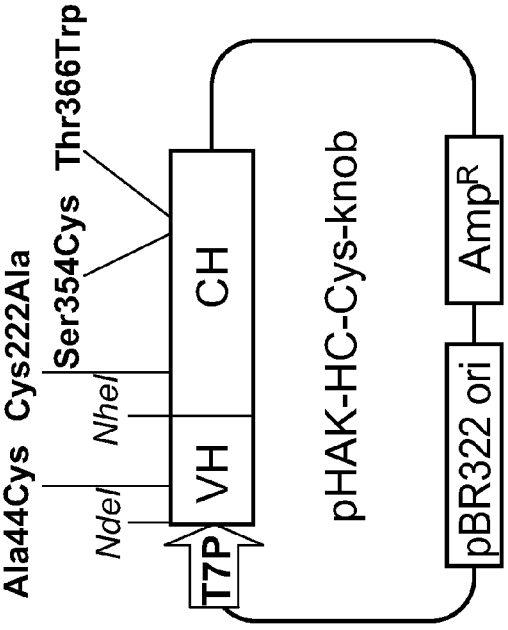


FIG. 3

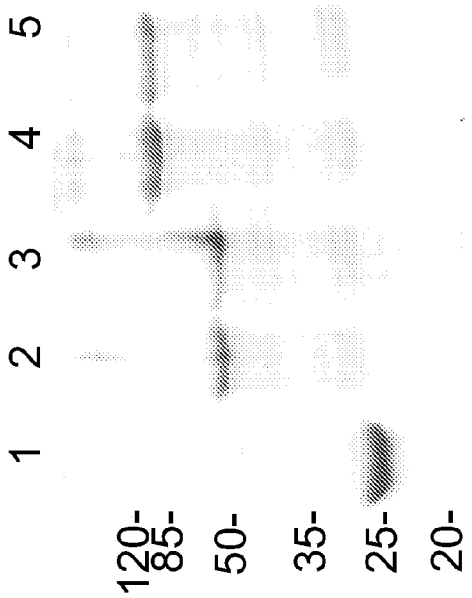


FIG. 4A

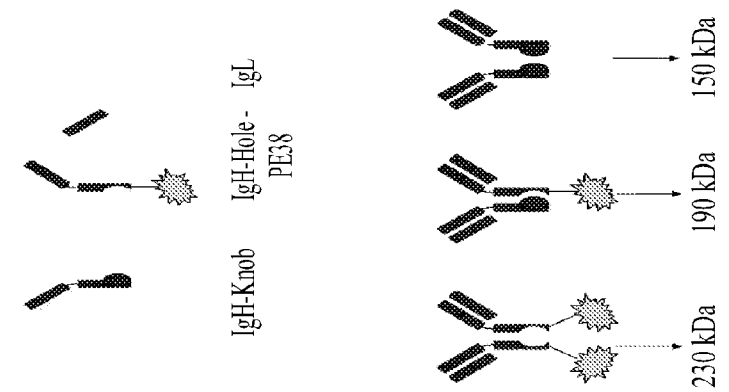

IgH-Knob
IgH-Hole-PE38
IgL
230 kDa
190 kDa
150 kDa

FIG. 4B

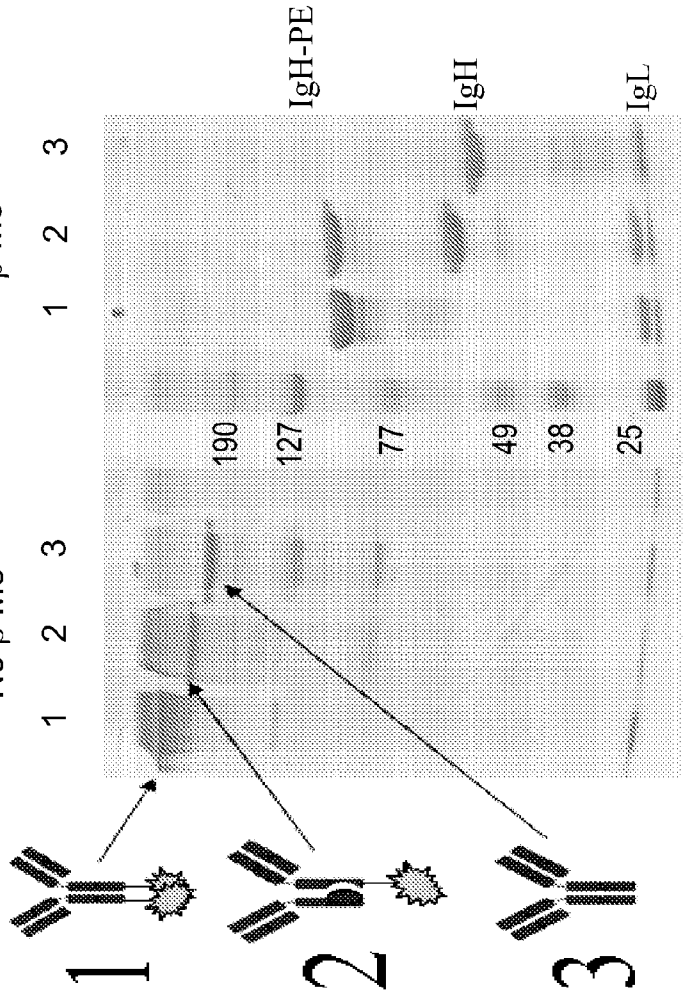

No β-Me
+ β-Me
1 2 3
1 2 3
190
127
77
49
38
25
IgH-PE
IgH
IgL
1
2
3

FIG. 5A

FIG. 5B

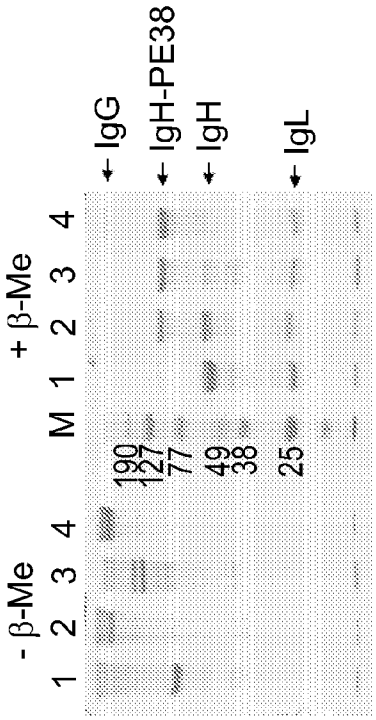


FIG. 6

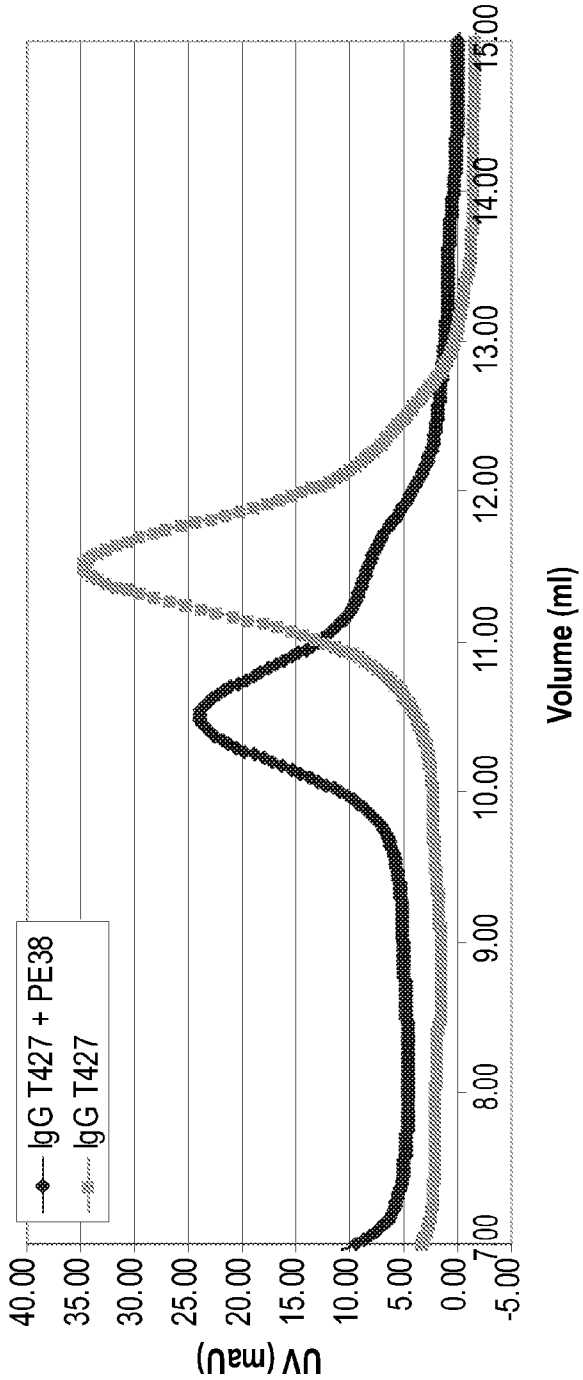

IgG T427 + PE38
IgG T427
UV (mAU)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
14.00
15.00
Volume (ml)

FIG. 7A

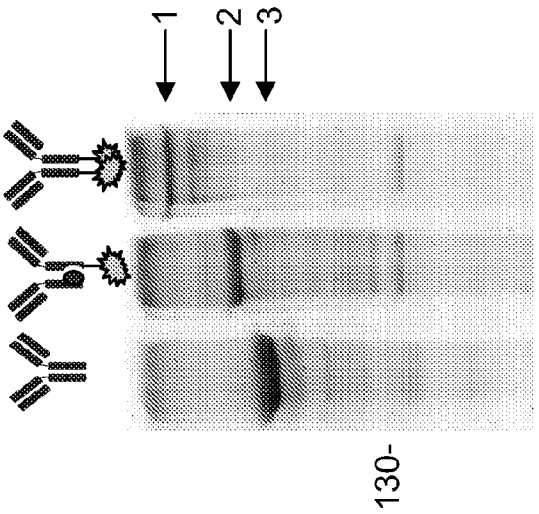


FIG. 7B

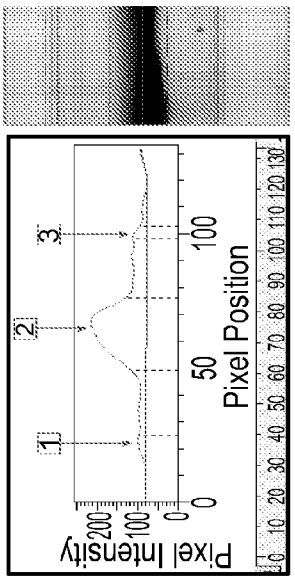
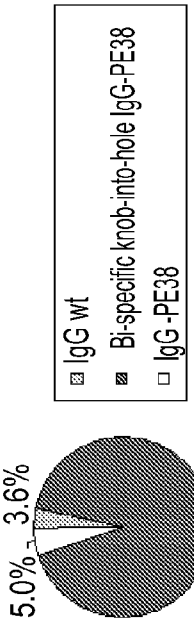


FIG. 7C



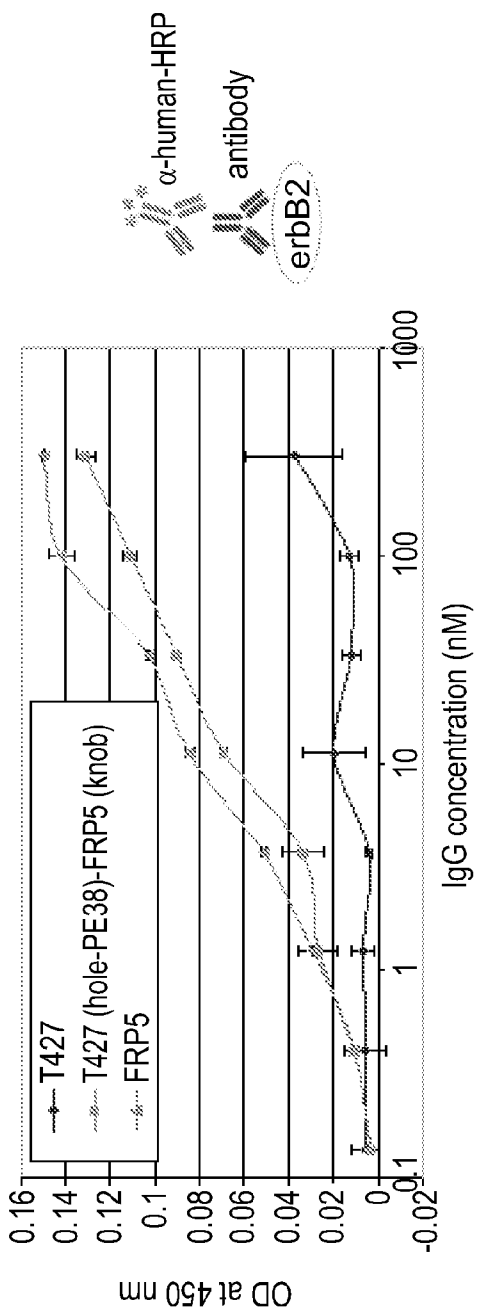

α-human-HRP
antibody
erbB2
T427
T427 (hole-PE38)-FRP5 (knob)
FRP5
OD at 450 nm
IgG concentration (nM)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
0.1
1
10
100
1000

FIG. 8A

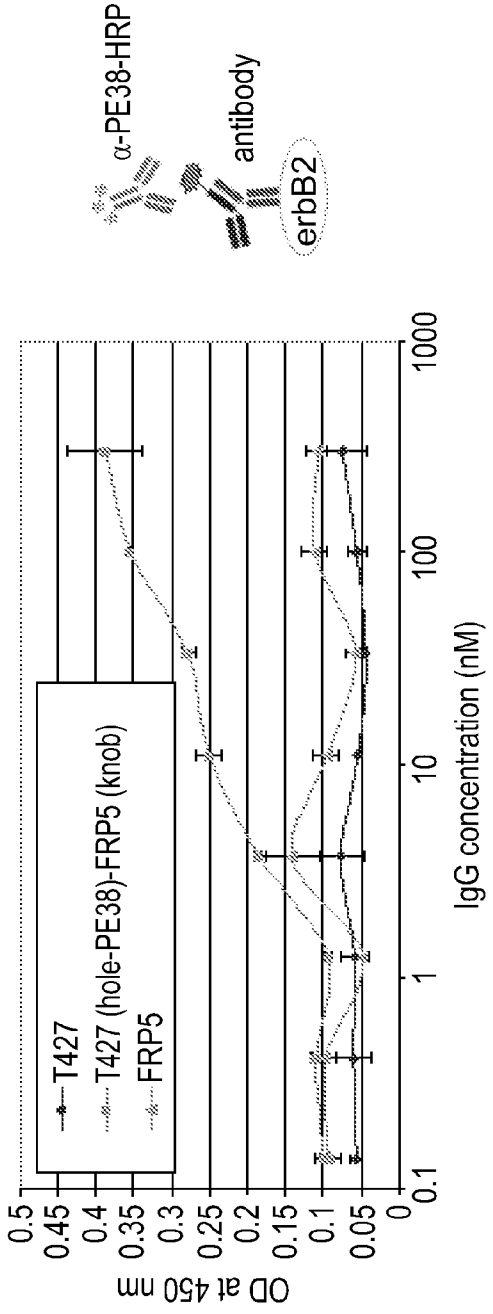

α-PE38-HRP
antibody
erbB2
T427
T427 (hole-PE38)-FRP5 (knob)
FRP5
OD at 450 nm
IgG concentration (nM)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.1
1
10
100
1000

FIG. 8B

FIG. 9A

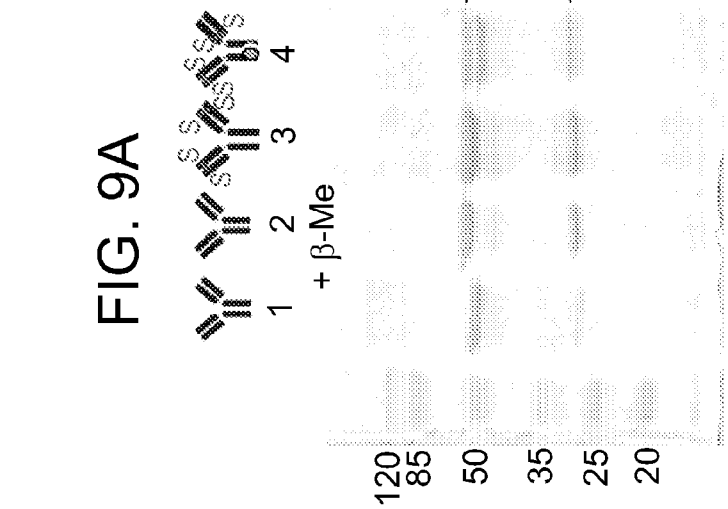


FIG. 9B

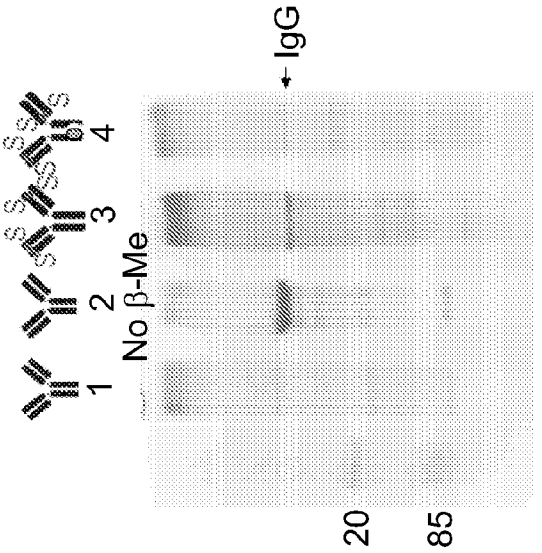


FIG. 10

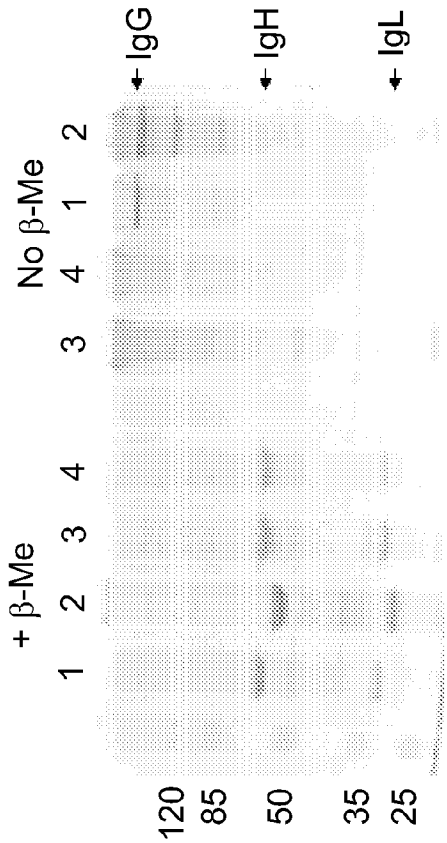


FIG. 11

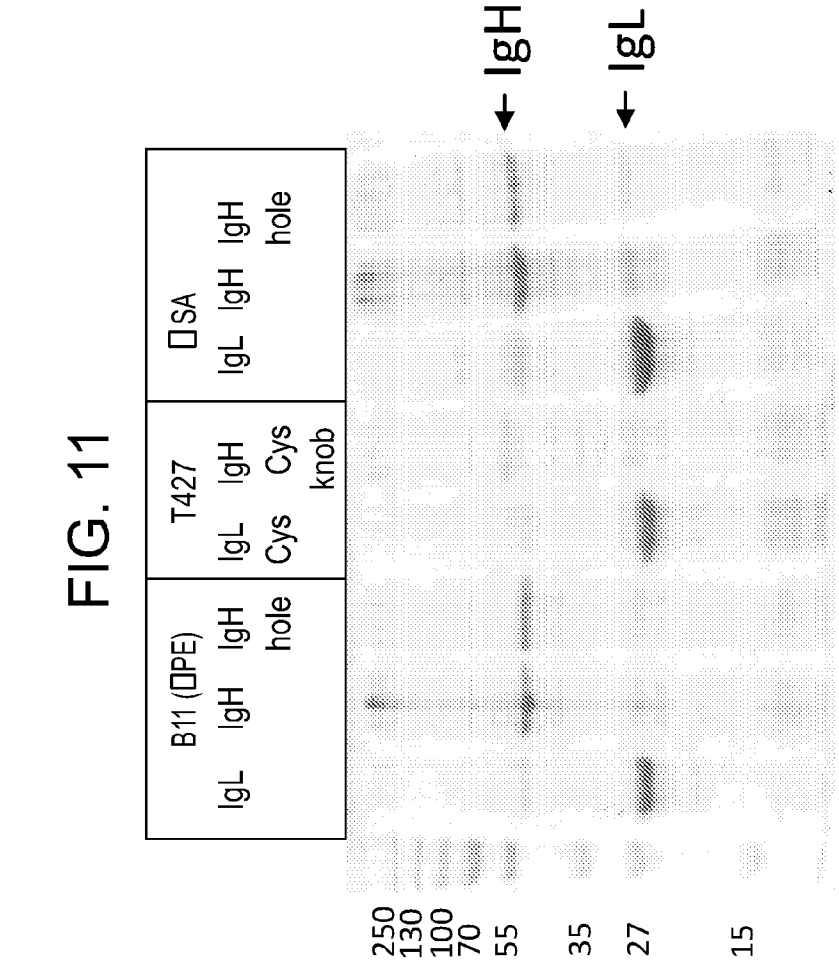

B11 (PE)
IgL IgH IgH hole
T427
IgL Cys IgH Cys knob
SA
IgL IgH IgH hole
IgH
IgL
250
130
100
70
55
35
27
15

FIG. 12A

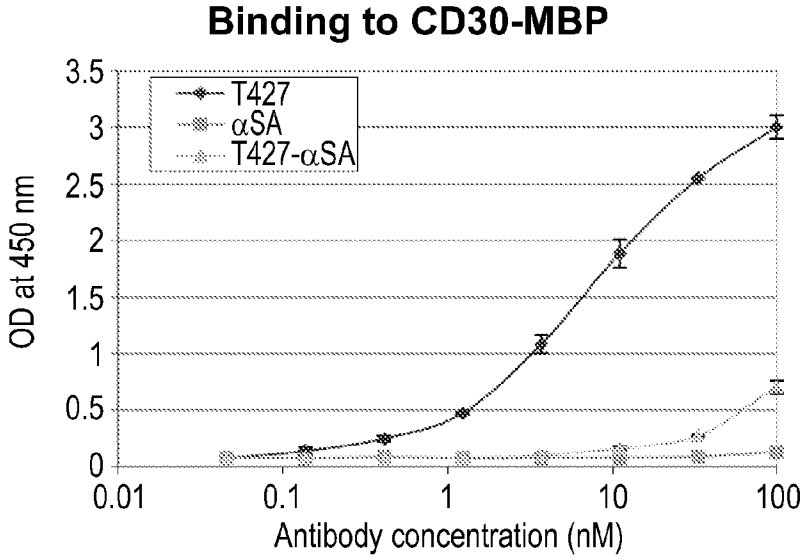


FIG. 12B

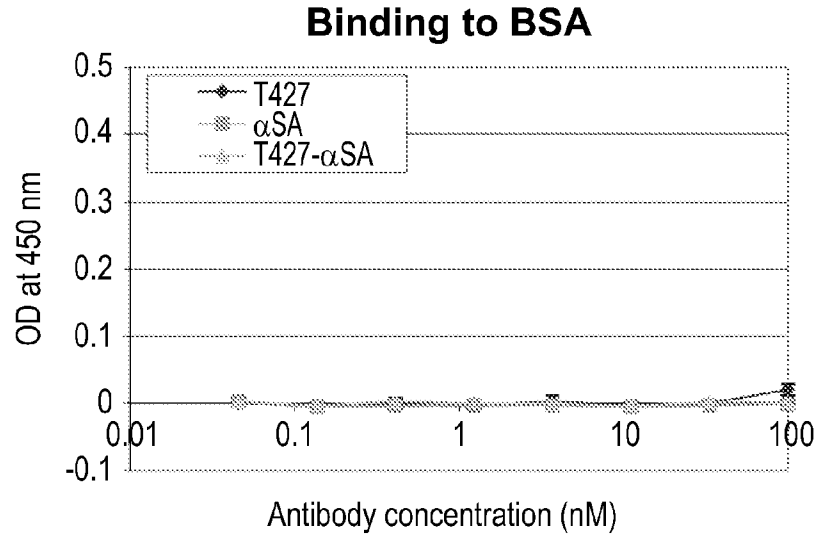


FIG. 13A
Binding to avitag-PE38

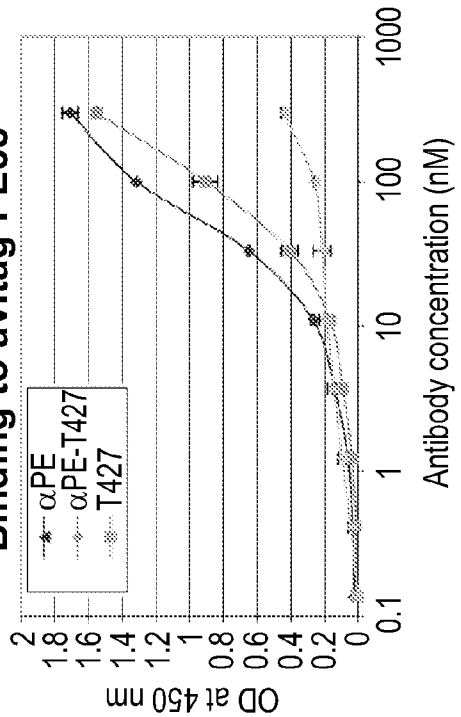


FIG. 13B
B11-T427 on dsFv-PE38

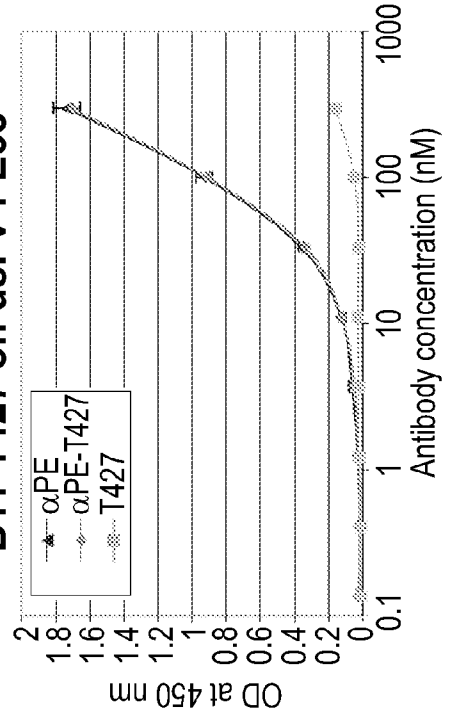
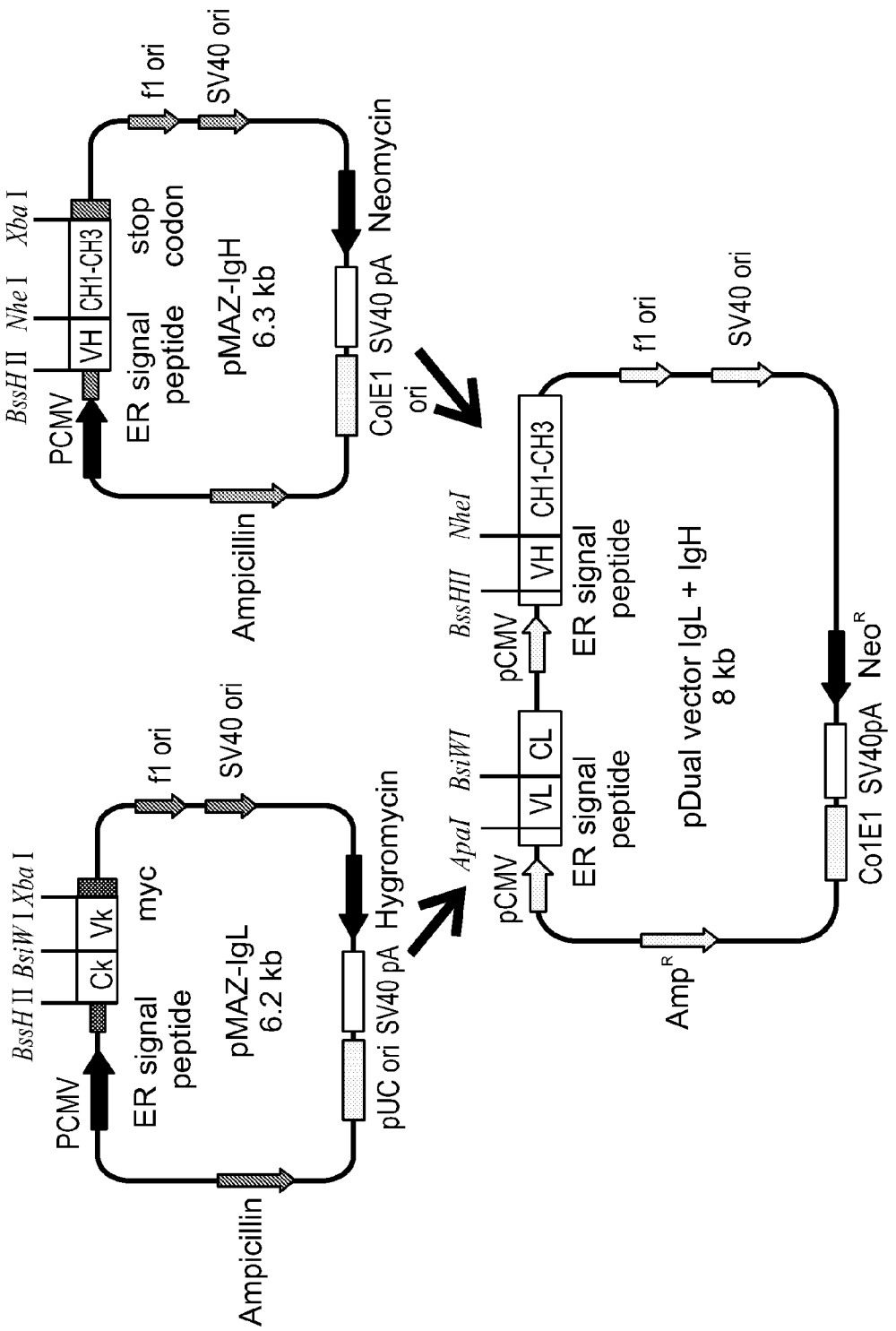


FIG. 13C
Binding to BSA

FIG. 14


BssH II BsiW I Xba I
Ck
Vk
ER signal peptide
myc
pMAZ-IgL 6.2 kb
PCMV
Ampicillin
f1 ori
SV40 ori
pUC ori SV40 pA Hygromycin
BssH II Nhe I Xba I
VH
CH1-CH3
ER signal peptide
stop codon
pMAZ-IgH 6.3 kb
PCMV
Ampicillin
f1 ori
SV40 ori
ColE1 SV40 pA Neomycin
ApaI BsiWI
VL
CL
ER signal peptide
pCMV
BssHII NheI
VH
CH1-CH3
ER signal peptide
pCMV
pDual vector IgL + IgH 8 kb
Amp^R
f1 ori
SV40 ori
Co1E1 SV40pA Neo^R

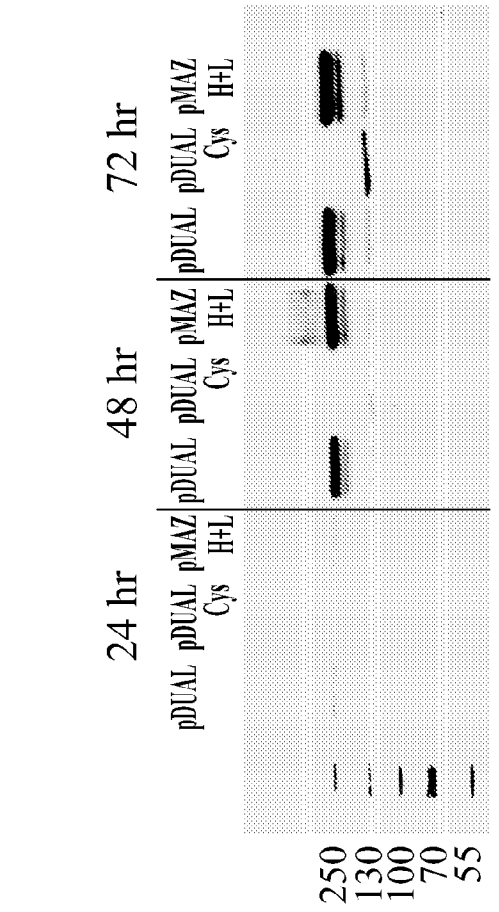
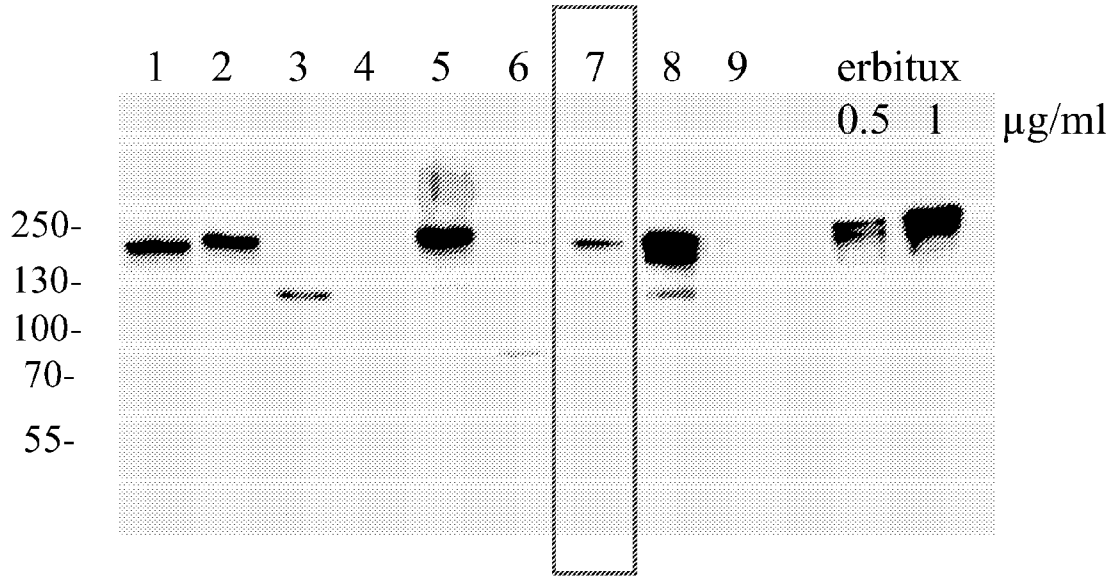


FIG. 15A

FIG. 15B

FIG. 16



Mono	1- pDual-T427 L+H wt
Mono	2- pDual-FRP5 L+H wt
	3- pDual-T427 L(Cys) + H(wt)
	4- pDual-T427 L(Cys) + H(Cys-knob)
Bis-control	5- pDual-T427 L+H wt + pDual-FRP5 L+H wt
	6- pDual-FRP5 L(wt) + H(hole)
Bi-specific	7- pDual-T427 L(Cys) + H(Cys-knob) +pDual-FRP5 L(wt) + H(hole)
	8- pMAZ-IgL-T427 + pMAZ-IgH-T427 + pMAZ-IgL-FRP5 + pMAZ-IgH-FRP5
	9- pDual-T427 L(wt) + H(Cys-knob) + pDual-FRP5 L(wt) + H(Cys-hole)

FIG. 17

A3-c	A3-c	B2-c	B2-c	C2-b	C2-b	D2-b	D2-b	A2-b	A3-b
A4-c	A4-c	B3-c	B3-c	C3-b	C3-b	D3-b	D3-b	293	293
A5-c	A5-c	B4-b	B4-b	C4-b	C4-b	D4-b	D4-b		
A6-c	A6-c	B5-b	B5-b	C5-b	C5-b	D5-b	D5-b		
B1-c	B1-c	B6-b	B6-b	C6-b	C6-b	D6-b	D6-b		
293	293	C1-b	C1-b	D1-b	D1-b	A1-b	A1-b		

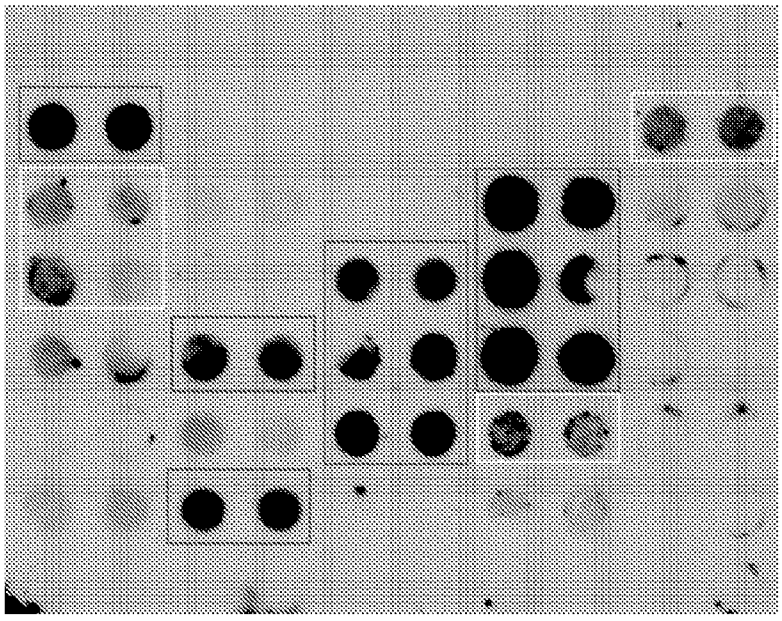


FIG. 18A

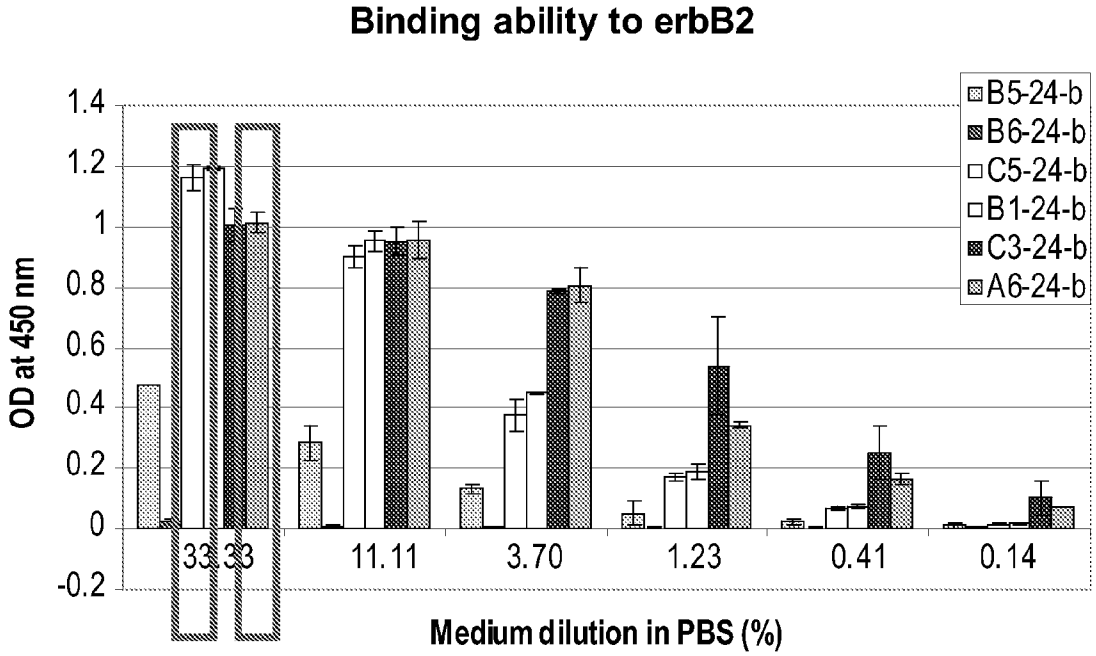
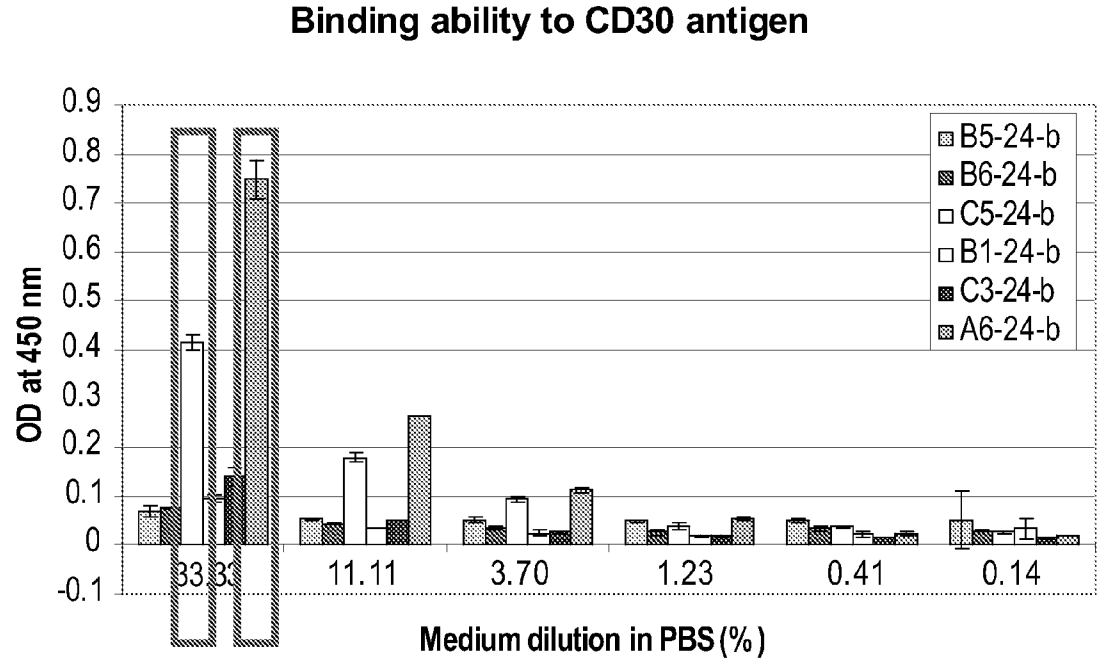


FIG. 18B



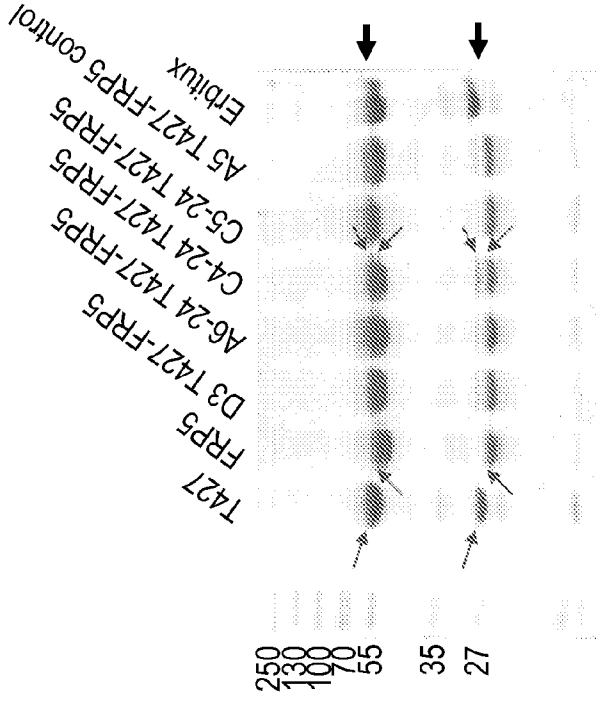


FIG. 19B

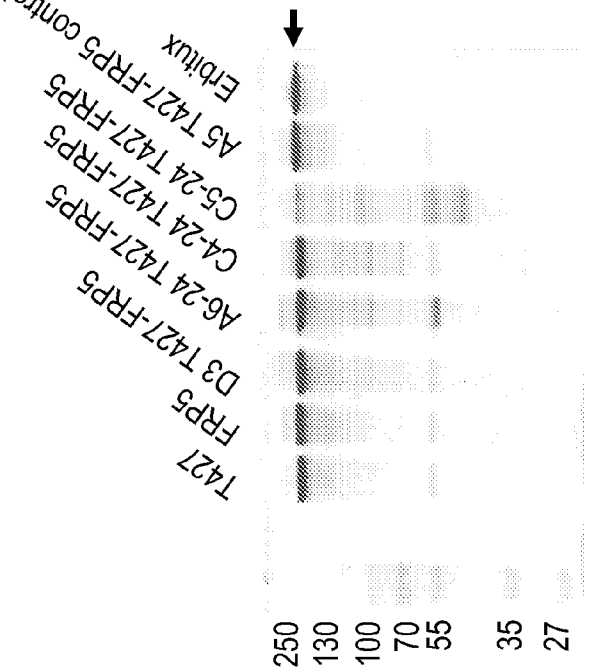
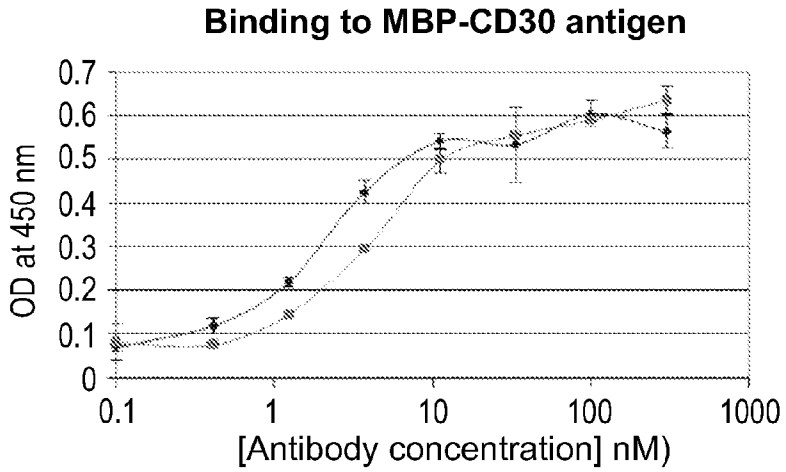
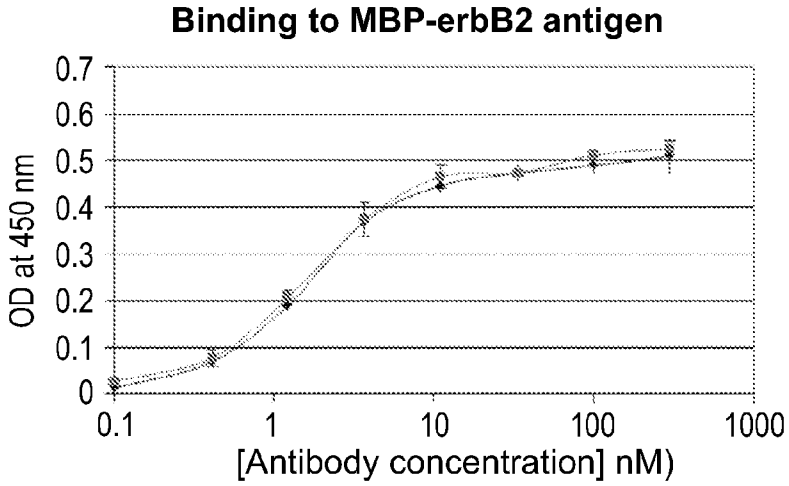


FIG. 19A



A5: 4 mixed chains of monospecific FRP5 and T427

FIG. 20A



D3: bi-specific

FIG. 20B

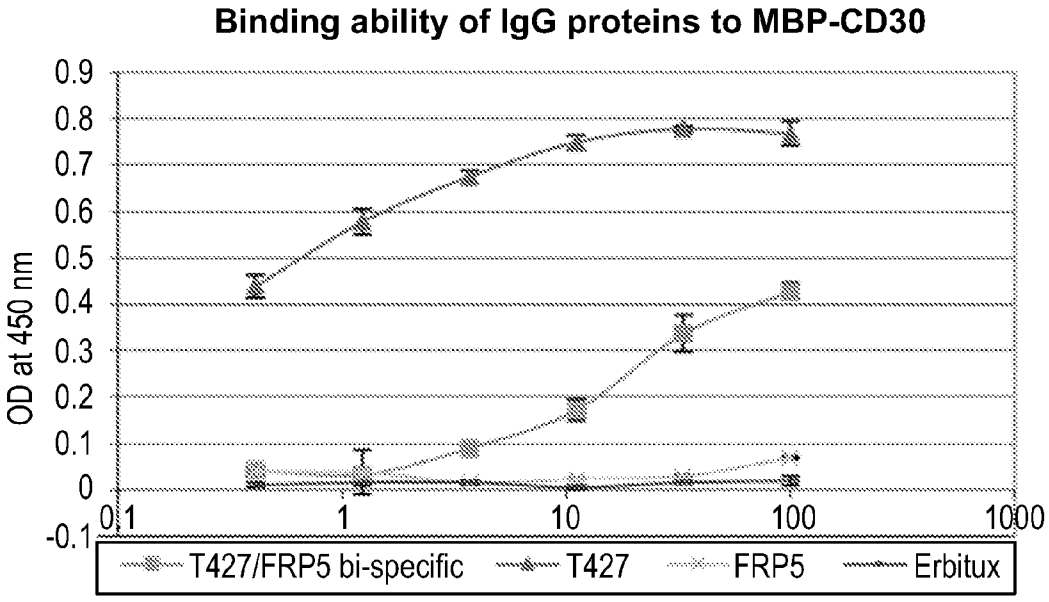


FIG. 21A

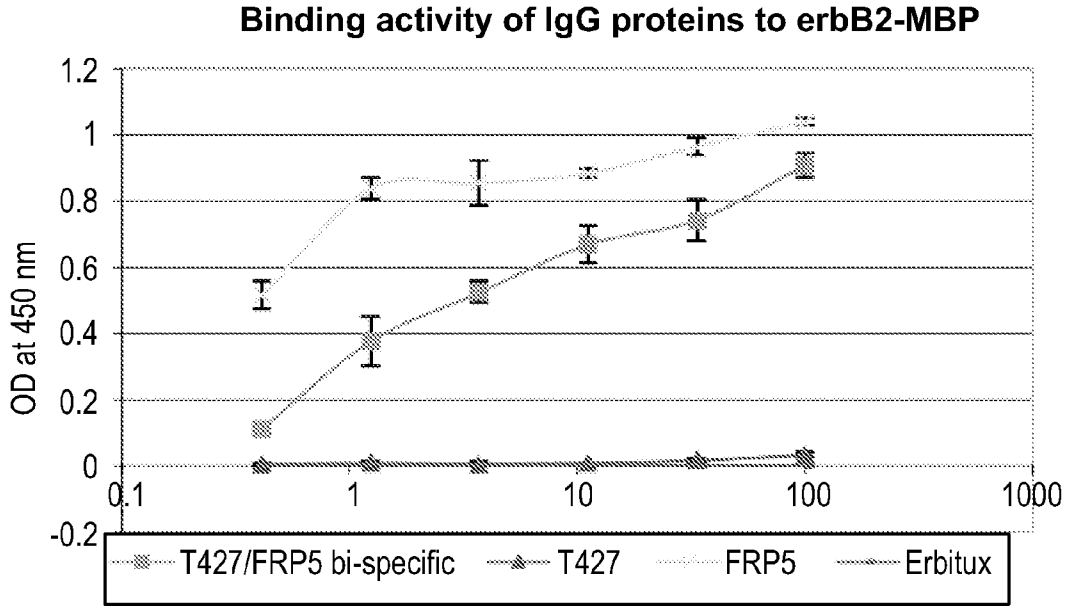


FIG. 21B

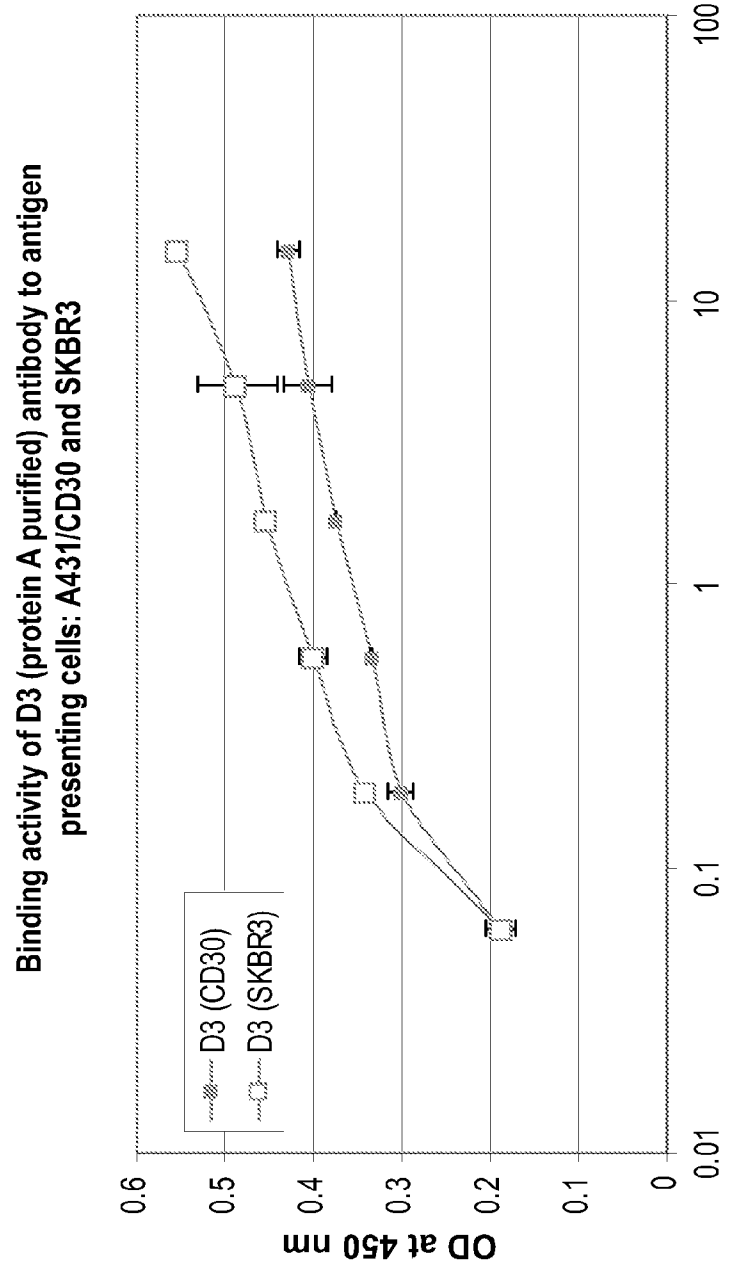

Binding activity of D3 (protein A purified) antibody to antigen presenting cells: A431/CD30 and SKBR3
D3 (CD30)
D3 (SKBR3)
OD at 450 nm
0
0.1
0.2
0.3
0.4
0.5
0.6
Antibody concentration (microgr/ml)
0.01
0.1
1
10
100

FIG. 22

FIG.23

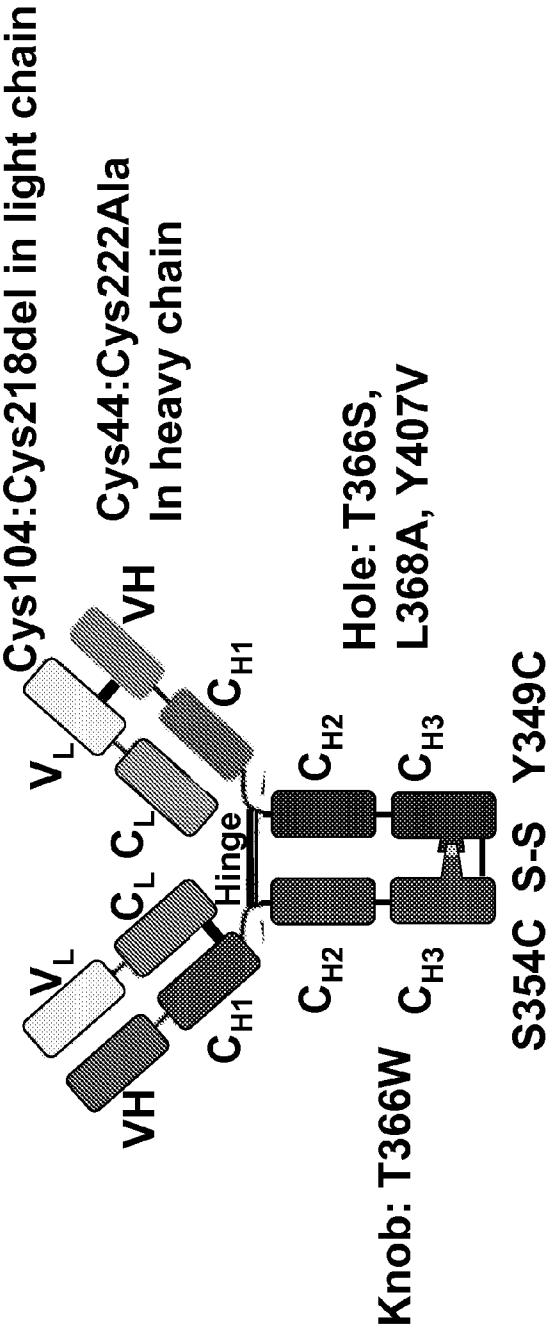
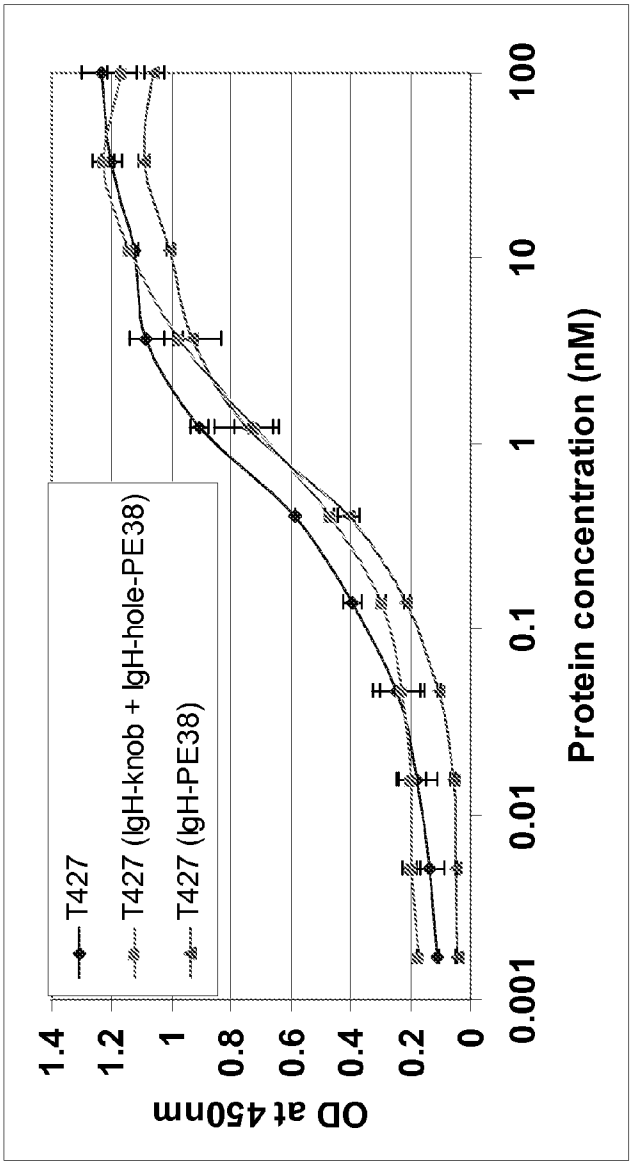

Cys104:Cys218del in light chain
Cys44:Cys222Ala
In heavy chain
VH
VL
CH1
CL
Hinge
CH2
CH3
Hole: T366S,
L368A, Y407V
Knob: T366W
S354C S-S Y349C

FIG.24


α-human-HRP
T427 antibody
CD30
OD at 450nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
Protein concentration (nM)
T427
T427 (lgH-knob + lgH-hole-PE38)
T427 (lgH-PE38)

<110> Ramot at Tel-Aviv University Ltd.
Benhar, Itai
Vaks, Lilach

<120> BISPECIFIC AND MONOSPECIFIC, ASYMMETRIC ANTIBODIES AND METHODS OF GENERATING SAME

<130> 52585

<150> US 61/453,591
<151> 2011-03-17

<160> 75

<170> PatentIn version 3.5

<210> 1
<211> 75
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 1
gaagcctttg accaggcacc acaggctgac ctggttcttg gtcatctcct cccggcatgg 60

gggcagggtg tacac 75

<210> 2
<211> 34
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 2
ggatgggggc agggtgcaca cctgtggttc tcgg 34

<210> 3
<211> 45
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 3
ggatagaagc ctttgaccgc gcagctcagg ctgacctggt tcttg 45

<210> 4
<211> 39
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 4
gtccacggtg agcttgctaa cgaggaagaa ggagccgtc 39

<210> 5
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 5
atatacatat ggacattgtg ctg 23

<210> 6
<211> 51
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 6
tatatacgta cgtttgattt ccagtttggt gccgcaaccg aacgtccgag g 51

<210> 7
<211> 40
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 7
tatatagaat tcttactctc ccctgttgaa gctctttgtg 40

<210> 8
<211> 34
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 8
aaacagaggc ctggacagtg tctggaatgg attg 34

<210> 9
<211> 27
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 9
tatatagcta gcggaggaga ctgtgag 27

<210> 10
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 10
gcccaaatct gccgacaaaa ctcacacatg cccacc 36

<210> 11
<211> 40
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 11
tgtgtgagtt ttgtcggcag atttgggctc aactctcttg 40

<210> 12
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 12
gaggagatga ccaagaacca ggt 23

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

<220>
<223> Single strand DNA oligonucleotide

<400> 13
atatacatat gcaggtcaaa ctgc 24

<210> 14
<211> 660
<212> DNA
<213> Artificial sequence

<220>
<223> Light chain T427 (wt IgG) coding sequence

<400> 14
atggacattg tgctgaccca atctccaact tctttggctg tgtctctagg gcagagggcc 60

accatatcct gcagagccag tgaaagtgtt gatagttatg gcaatagttt tatgcactgg 120

ttccagcaga aaccaggaca gccacccaaa ctcctcatct atcgtgcatc caacctagaa 180

tctgggatcc ctgccaggtt cagtggcagt gggtcttgga cagacttcac cctcaccatt 240

aatcctgtgg aggctgatga tgttgcaacc tattactgtc agcaaagtaa tgaggatcct 300

cggacgttcg gtggaggcac caaactggaa atcaaacgta cggtggctgc accatctgtc 360

ttcatcttcc cgccatctga tgagcagttg aaatctggaa ctgcctctgt tgtgtgcctg 420

ctgaataact tctatcccag agaggccaaa gtacagtgga aggtggataa cgccctccaa 480

tcgggtaact cccaggagag tgtcacagag caggacagca aggacagcac ctacagcctc 540

agcagcaccc tgacgctgag caaagcagac tacgagaaac acaaagtcta cgcctgcgaa 600

gtcacccatc agggcctgag ctcgcccgtc acaaagagct tcaacagggg agagtgttaa 660

<210> 15
<211> 219
<212> PRT
<213> Artificial sequence

<220>
<223> Light chain T427 (wt IgG)

<400> 15

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

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

Tyr Gly Asn Ser Phe Met His Trp Phe Gln Gln Lys Pro Gly Gln Pro
35 40 45

Pro Lys Leu Leu Ile Tyr Arg Ala Ser Asn Leu Glu Ser Gly Ile Pro
50 55 60

Ala Arg Phe Ser Gly Ser Gly Ser Trp Thr Asp Phe Thr Leu Thr Ile
65 70 75 80

Asn Pro Val Glu Ala Asp Asp Val Ala Thr Tyr Tyr Cys Gln Gln Ser
85 90 95

Asn Glu Asp Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105 110

Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
115 120 125

Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
130 135 140

Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
145 150 155 160

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
165 170 175

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

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

Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
210 215

<210> 16
<211> 648
<212> DNA
<213> Artificial sequence

<220>
<223> Light chain FRP5 (wt IgG) coding sequence

<400> 16
atggacatcc agctgaccca gtctcacaaa ttcctgtcca cttcagtagg agacagggtc 60

agcatcacct gcaaggccag tcaggatgtg tataatgctg ttgcctggta tcaacagaaa 120

ccaggacaat ctcctaaact tctgatttac tcggcatcct cccggtacac tggagtccct 180

tctcgcttca ctggcagtgg ctctgggccg gatttcactt tcaccatcag cagtgtgcag 240

gctgaagacc tggcagttta tttctgtcag caacattttc gtactccatt cacgttcggc 300

tcggggacaa aattggagat caaacgtacg gtggctgcac catctgtctt catcttcccg 360

ccatctgatg agcagttgaa atctggaact gcctctgttg tgtgcctgct gaataacttc 420

tatcccagag aggccaaagt acagtggaag gtggataacg ccctccaatc gggtaactcc 480

caggagagtg tcacagagca ggacagcaag gacagcacct acagcctcag cagcaccctg 540

acgctgagca aagcagacta cgagaaacac aaagtctacg cctgcgaagt cacccatcag 600

ggcctgagct cgcccgtcac aaagagcttc aacaggggag agtgttaa 648

<210> 17
<211> 215
<212> PRT
<213> Artificial sequence

<220>
<223> Light chain FRP5 (wt IgG)

<400> 17

Met Asp Ile Gln Leu Thr Gln Ser His Lys Phe Leu Ser Thr Ser Val
1 5 10 15

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

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

Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val Pro Ser Arg Phe Thr
50 55 60

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

Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln His Phe Arg Thr Pro
85 90 95

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

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

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

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

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

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

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

Ser Phe Asn Arg Gly Glu Cys
210 215

<210> 18
<211> 645
<212> DNA
<213> Artificial sequence

<220>
<223> Light chain anti-Tac (wt IgG) coding sequence

<400> 18
atgcaaattg ttctcaccca gtctccagca atcatgtctg catctccagg ggagaaggtc 60

accataacct gcagtgccag ctcaagtata agttacatgc actggttcca gcagaagcca 120

ggcacttctc ccaaactctg gatttatacc acatccaacc tggcttctgg agtccctgct 180

cgcttcagtg gcagtggatc tgggacctct tactctctca caatcagccg aatggaggct 240

gaagatgctg ccacttatta ctgccatcaa aggagtactt acccactcac gttcggttct 300

gggaccaagc tggaaataaa acgtacggtg gctgcaccat ctgtcttcat cttcccgcca 360

tctgatgagc agttgaaatc tggaactgcc tctgttgtgt gcctgctgaa taacttctat 420

cccagagagg ccaaagtaca gtggaaggtg gataacgccc tccaatcggg taactcccag 480

gagagtgtca cagagcagga cagcaaggac agcacctaca gcctcagcag caccctgacg 540

ctgagcaaag cagactacga gaaacacaaa gtctacgcct gcgaagtcac ccatcagggc 600

ctgagctcgc ccgtcacaaa gagcttcaac aggggagagt gttaa 645

<210> 19
<211> 214
<212> PRT
<213> Artificial sequence

<220>
<223> Light chain anti-Tac (wt IgG)

<400> 19

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

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

Met His Trp Phe Gln Gln Lys Pro Gly Thr Ser Pro Lys Leu Trp Ile
35 40 45

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

Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys His Gln Arg Ser Thr Tyr Pro Leu
85 90 95

Thr Phe Gly Ser Gly Thr Lys Leu 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> 20
<211> 657
<212> DNA
<213> Artificial sequence

<220>

<223> Light chain T427A104C:Cys218del (Cys) coding sequence

<400> 20
atggacattg tgctgaccca atctccaact tctttggctg tgtctctagg gcagagggcc 60

accatatcct gcagagccag tgaaagtgtt gatagttatg gcaatagttt tatgcactgg 120

ttccagcaga aaccaggaca gccacccaaa ctcctcatct atcgtgcatc caacctagaa 180

tctgggatcc ctgccaggtt cagtggcagt gggtcttgga cagacttcac cctcaccatt 240

aatcctgtgg aggctgatga tgttgcaacc tattactgtc agcaaagtaa tgaggatcct 300

cggacgttcg gttgtggcac caaactggaa atcaaacgta cggtggctgc accatctgtc 360

ttcatcttcc cgccatctga tgagcagttg aaatctggaa ctgcctctgt tgtgtgcctg 420

ctgaataact tctatcccag agaggccaaa gtacagtgga aggtggataa cgccctccaa 480

tcgggtaact cccaggagag tgtcacagag caggacagca aggacagcac ctacagcctc 540

agcagcaccc tgacgctgag caaagcagac tacgagaaac acaaagtcta cgcctgcgaa 600

gtcacccatc agggcctgag ctcgcccgtc acaaagagct tcaacagggg agagtaa 657

<210> 21
<211> 218
<212> PRT
<213> Artificial sequence

<220>
<223> Light chain T427A104C:Cys218del (Cys)

<400> 21

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

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

Tyr Gly Asn Ser Phe Met His Trp Phe Gln Gln Lys Pro Gly Gln Pro
35 40 45

Pro Lys Leu Leu Ile Tyr Arg Ala Ser Asn Leu Glu Ser Gly Ile Pro
50 55 60

Ala Arg Phe Ser Gly Ser Gly Ser Trp Thr Asp Phe Thr Leu Thr Ile
65 70 75 80

Asn Pro Val Glu Ala Asp Asp Val Ala Thr Tyr Tyr Cys Gln Gln Ser
85 90 95

Asn Glu Asp Pro Arg Thr Phe Gly Cys Gly Thr Lys Leu Glu Ile Lys
100 105 110

Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
115 120 125

Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
130 135 140

Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
145 150 155 160

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
165 170 175

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

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

Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210 215

<210> 22
<211> 1353
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain T427 (wt IgG human gamma 1) coding sequence

<400> 22
atgcaggtcc aactgcagca gccggggact gaactggtga ggcctggagc ttcagtgaag 60

ctgtcctgca aggcttctgg cttctccttc accagttact ggatgaactg ggtgaagcag 120

aggcctggac aaggccttga gtggattggc atgattcatc cttccgatag tgaaactagg 180

ttaaatcaga agttcaagga cagggccaca ttgactgtag acaaatcctc cagcacagcc 240

tacatgcaac tcagcagccc gacatctgag gactctgcgg tctattactg tgcaagtgag 300

atggattatt actttgctat ggactactgg ggtcaaggaa cctcagtcac cgtctcctca 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 720

ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 780

gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 840

tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 900

agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 960

gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 1020

aaagccaaag ggcagccccg agaaccacag gtgtacaccc tgcccccatc ccgggaggag 1080

atgaccaaga accaggtcag cctgacctgc ctggtcaaag gcttctatcc cagcgacatc 1140

gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 1200

ctggactccg acggctcctt cttcctctat agcaagctca ccgtggacaa gagcaggtgg 1260

cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1320

cagaagagcc tctccctgtc cccgggtaaa tga 1353

<210> 23
<211> 450
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain T427 (wt IgG human gamma 1)

<400> 23

Met Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly
1 5 10 15

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

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

Ile Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Leu Asn Gln Lys
50 55 60

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

Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Tyr
85 90 95

Cys Ala Ser Glu Met Asp Tyr Tyr Phe Ala Met Asp Tyr Trp Gly Gln
100 105 110

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

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

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

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

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

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

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275 280 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
325 330 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
340 345 350

Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
355 360 365

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
370 375 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
405 410 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
420 425 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
435 440 445

Gly Lys
450

<210> 24
<211> 1353
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain FRP5 (wt IgG human gamma 1) coding sequence

<400> 24
atgcaggtac aactgcagca gtctggacct gaactgaaga agcctggaga gacagtcaag 60

atctcctgca aggcctctgg gtatcctttc acaaactatg gaatgaactg ggtgaagcag 120

gctccaggac agggtttaaa gtggatgggc tggattaaca cctccactgg agagtcaaca 180

tttgctgatg acttcaaggg acggtttgac tttctctttg gaaacctctgc caacactgcc 240

tatttgcaga tcaacaacct caaaagtgaa gacatggcta catatttctg tgcaagatgg 300

gaggtttacc acggctacgt tcctttactgg ggccaaggga ccacggtcac cgttttcctct 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 720

ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 780

gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 840

tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 900

agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 960

gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 1020

aaagccaaag ggcagccccg agaaccacag gtgtacaccc tgcccccatc ccgggaggag 1080

atgaccaaga accaggtcag cctgacctgc ctggtcaaag gcttctatcc cagcgacatc 1140

gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 1200

ctggactccg acggctcctt ctttcctcta tagcaagctca ccgtggacaa gagcaggtgg 1260

cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1320

cagaagagcc tctccctgtc cccgggtaaa tga 1353

<210> 25
<211> 450
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain FRP5 (wt IgG human gamma 1)

<400> 25

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

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

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

Met Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp
50 55 60

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

Tyr Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe
85 90 95

Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln
100 105 110

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

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

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

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

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

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

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

```
Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
        275                 280                 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
    290                 295                 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305                 310                 315                 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
                325                 330                 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
            340                 345                 350

Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
        355                 360                 365

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
    370                 375                 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385                 390                 395                 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
                405                 410                 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
            420                 425                 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
        435                 440                 445

Gly Lys
    450
```

<210> 26
<211> 1344
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain anti-Tac (wt IgG human gamma 1) coding sequence

<400> 26

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atgcaggtca aactgcagga gtctggggct gaactggcaa aacctggggc tcagtgaag      60

atgtcctgca aggcttctgg ctacaccttt actagctaca ggatgcactg ggtaaaacag     120

aggcctggac agggtctgga atggattgga tatattaatc ctagcactgg gtatactgaa     180

tacaatcaga agttcaagga caaggccaca ttgactgcag acaaatcctc cagcacagcc     240

tacatgcaac tgagcagcct gacatttgag gactctgcag tctattactg tgcaagaggg     300

ggggggtct ttgactactg gggccaagga accactctca cagtctcctc cgctagcacc     360
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aagggcccat cggtcttccc cctggcaccc tcctccaaga gcacctctgg gggcacagcg 420

gccctgggct gcctggtcaa ggactacttc cccgaaccgg tgacggtgtc gtggaactca 480

ggcgccctga ccagcggcgt gcacaccttc ccggctgtcc tacagtcctc aggactctac 540

tccctcagca gcgtggtgac cgtgccctcc agcagcttgg gcacccagac ctacatctgc 600

aacgtgaatc acaagcccag caacaccaag gtggacaaga aagttgagcc caaatcttgt 660

gacaaaactc acacatgccc accgtgccca gcacctgaac tcctgggggg accgtcagtc 720

ttcctcttcc ccccaaaacc caaggacacc ctcatgatct cccggacccc tgaggtcaca 780

tgcgtggtgg tggacgtgag ccacgaagac cctgaggtca agttcaactg gtacgtggac 840

ggcgtggagg tgcataatgc caagacaaag ccgcgggagg agcagtacaa cagcacgtac 900

cgtgtggtca gcgtcctcac cgtcctgcac caggactggc tgaatggcaa ggagtacaag 960

tgcaaggtct ccaacaaagc cctcccagcc cccatcgaga aaaccatctc caaagccaaa 1020

gggcagcccc gagaaccaca ggtgtacacc ctgcccccat cccgggagga gatgaccaag 1080

aaccaggtca gcctgacctg cctggtcaaa ggcttctatc ccagcgacat cgccgtggag 1140

tgggagagca atgggcagcc ggagaacaac tacaagacca cgcctcccgt gctggactcc 1200

gacggctcct tcttcctcta tagcaagctc accgtggaca agagcaggtg gcagcagggg 1260

aacgtcttct catgctccgt gatgcatgag gctctgcaca accactacac gcagaagagc 1320

ctctccctgt ccccgggtaa atga 1344

<210> 27
<211> 447
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain anti-Tac (wt IgG human gamma 1)

<400> 27

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

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

Tyr Arg Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp
35 40 45

Ile Gly Tyr Ile Asn Pro Ser Thr Gly Tyr Thr Glu Tyr Asn Gln Lys
50 55 60

Phe Lys Asp Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala
65 70 75 80

Tyr Met Gln Leu Ser Ser Leu Thr Phe Glu Asp Ser Ala Val Tyr Tyr
85 90 95

Cys Ala Arg Gly Gly Gly Val Phe Asp Tyr Trp Gly Gln Gly Thr Thr
100 105 110

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

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

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

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

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

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

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His
210 215 220

Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val
225 230 235 240

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
245 250 255

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
260 265 270

Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
275 280 285

Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
290 295 300

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
305 310 315 320

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
325 330 335

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
340 345 350

Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
355 360 365

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
370 375 380

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
385 390 395 400

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
405 410 415

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
420 425 430

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
435 440 445

<210> 28
<211> 1353
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain T427 T366W S354C (knob) coding sequence

<400> 28
atgcaggtcc aactgcagca gccggggact gaactggtga ggcctggagc ttcagtgaag 60

ctgtcctgca aggcttctgg cttctccttc accagttact ggatgaactg ggtgaagcag 120

aggcctggac aaggccttga gtggattggc atgattcatc cttccgatag tgaaactagg 180

ttaaatcaga agttcaagga cagggccaca ttgactgtag acaaatcctc cagcacagcc 240

tacatgcaac tcagcagccc gacatctgag gactctgcgg tctattactg tgcaagtgag 300

atggattatt actttgctat ggactactgg ggtcaaggaa cctcagtcac cgtctcctca 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 720

ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 780

gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 840

tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 900

agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 960

gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 1020

aaagccaaag ggcagccccg agaaccacag gtgtacaccc tgccccatg ccgggaggag 1080

atgaccaaga accaggtcag cctgtggtgc ctggtcaaag gcttctatcc cagcgacatc 1140

gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 1200

ctggactccg acggctcctt ctttcctctat agcaagctca ccgtggacaa gagcaggtgg 1260

cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1320

cagaagagcc tctccctgtc cccgggtaaa tga 1353

<210> 29
<211> 465
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain T427 T366W/S354C (knob) coding sequence

<400> 29

Met Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly
1 5 10 15

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

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

Ile Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Leu Asn Gln Lys
50 55 60

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

Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Tyr
85 90 95

Cys Ala Ser Glu Met Asp Tyr Tyr Phe Ala Met Asp Tyr Trp Gly Gln
100 105 110

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

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

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

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

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

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

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Glu Pro
245 250 255

Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Met Ile Ser
260 265 270

Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
275 280 285

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn
290 295 300

Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val
305 310 315 320

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu
325 330 335

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys
340 345 350

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr
355 360 365

Leu Pro Pro Cys Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Trp
370 375 380

Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu
385 390 395 400

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
405 410 415

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys
420 425 430

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
435 440 445

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
450 455 460

Lys
465

<210> 30
<211> 1352
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain T427 T366S:L368A:Y407V:Y349C (hole) coding sequence

<400> 30
atgcaggtcc aactgcagca gccggggact gaactggtga ggcctggagc ttcagtgaag 60

ctgtcctgca aggcttctgg cttctcctac accagttact ggatgaactg ggtgaagcag 120

aggcctggac aaggccttga gtggattggc atgattcatc ctccgatagt gaaactagg 180

ttaaatcaga agttcaagga cagggccaca ttgactgtag acaaatcctc cagcacagcc 240

tacatgcaac tcagcagccc gacatctgag gactctgcgg tctattactg tgcaagtgag 300

atggattatt actttgctat ggactactgg ggtcaaggaa cctcagtcac cgtctcctca 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact gaacaaggac 720

cgtcagtctt cctcttcccc ccaaaaccca aggacaccct catgatctcc cggacccctg 780

aggtcacatg cgtggtggtg gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt 840

acgtggacgg cgtggaggtg cataatgcca agacaaagcc gcgggaggag cagtacaaca 900

gcacgtaccg tgtggtcagc gtcctcaccg tcctgcacca ggactggctg aatggcaagg 960

agtacaagtg caaggtctcc aacaaagccc tcccagcccc catcgagaaa accatctcca 1020

aagccaaagg gcagccccga gaaccacagg tgtgcaccct gcccccatcc cgggaggaga 1080

tgaccaagaa ccaggtcagc ctgagctgcg cggtcaaagg cttctatccc agcgacatcg 1140

ccgtggagtg ggagagcaat gggcagccgg agaacaacta caagaccacg cctcccgtgc 1200

tggactccga cggctccttc ttcctcgtta gcaagctcac cgtggacaag agcaggtggc 1260

agcaggggaa cgtcttctca tgctccgtga tgcatgaggc tctgcacaac cactacacgc 1320

agaagagcct ctccctgtcc ccgggtaaat ga 1352

<210> 31
<211> 450
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain T427 T366S:L368A:Y407V:Y349C (hole)

<400> 31

Met Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly
1 5 10 15

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

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

Ile Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Leu Asn Gln Lys
50 55 60

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

Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Tyr
85 90 95

Cys Ala Ser Glu Met Asp Tyr Tyr Phe Ala Met Asp Tyr Trp Gly Gln
100 105 110

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

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

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

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

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

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

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275 280 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
325 330 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Cys
340 345 350

Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
355 360 365

Ser Cys Ala Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
370 375 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Val Ser Lys Leu Thr Val Asp
405 410 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
420 425 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
435 440 445

Gly Lys
450

<210> 32
<211> 2406
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain T427-PE38 coding sequence

<400> 32
atgcaggtcc aactgcagca gccggggact gaactggtga ggcctggagc ttcagtgaag 60

ctgtcctgca aggcttctgg cttctccttc accagttact ggatgaactg ggtgaagcag 120

aggcctggac aaggccttga gtggattggc atgattcatc cttccgatag tgaaactagg 180

ttaaatcaga agttcaagga cagggccaca ttgactgtag acaaatcctc cagcacagcc 240

tacatgcaac tcagcagccc gacatctgag gactctgcgg tctattactg tgcaagtgag 300

at ggat t at t act t t gct at ggact act gg ggt caaggaa cct cagt cac cgt ct cct ca 360

gct agcacca agggcccat c ggt ct t cccc ct ggcaccct cct ccaagag cacct ct ggg 420

ggcacagcgg ccct gggct g cct ggt caag gact act t cc ccgaaccggt gacggt gt cg 480

t ggaact cag gcgccct gac cagcggcgt g cacacct t cc cggct gt cct acagt cct ca 540

ggact ct act ccct cagcag cgt ggt gacc gt gccct cca gcagct t ggg cacccagacc 600

t acat ct gca acgt gaat ca caagcccagc aacaccaagg t ggacaagaa agt t gagccc 660

aaat ct t gt g acaaaact ca cacat gccca ccgt gcccag cacct gaact cct gggggga 720

ccgt cagt ct t cct ct t ccc cccaaaaccc aaggacaccc t cat gat ct c ccggacccct 780

gaggt cacat gcgt ggt ggt ggacgt gagc cacgaagacc ct gaggt caa gt t caact gg 840

t acgt ggacg gcgt ggaggt gcat aat gcc aagacaaagc cgcgggagga gcagt acaac 900

agcacgt acc gt gt ggt cag cgt cct cacc gt cct gcacc aggact ggct gaat ggcaag 960

gagt acaagt gcaaggt ct c caacaaagcc ct cccagccc ccat cgagaa aaccat ct cc 1020

aaagccaaag ggcagccccg agaaccacag gt gt acaccc t gcccccat c ccgggaggag 1080

at gaccaaga accaggt cag cct gacct gc ct ggt caaag gct t ct at cc cagcgacat c 1140

gccgt ggagt gggagagcaa t gggcagccg gagaacaact acaagaccac gcct cccgt g 1200

ct ggact ccg acggct cct t ct t cct ct at agcaagct ca ccgt ggacaa gagcaggt gg 1260

cagcagggga acgt ct t ct c at gct ccgt g at gcat gagg ct ct gcacaa ccact acacg 1320

cagaagagcc t ct ccct gt c cccgggt aaa gct t ccggag gt cccgaggg cggcagcct g 1380

gccgcgct ga ccgcgcacca ggct t gccac ct gccgct gg agact t t cac ccgt cat cgc 1440

cagccgcgcg gct gggaaca act ggagcag t gcggct at c cggt gcagcg gct ggt cgcc 1500

ct ct acct gg cggcgcggct gt cgt ggaac caggt cgacc aggt gat ccg caacgccct g 1560

gccagccccg gcagcggcgg cgacct gggc gaagcgat cc gcgagcagcc ggagcaggcc 1620

cgt ct ggccc t gaccct ggc cgccgccgag agcgagcgct t cgt ccggca gggcaccggc 1680

aacgacgagg ccggcgcggc caacggcccg gcggacagcg gcgacgccct gct ggagcgc 1740

aact at ccca ct ggcgcgga gt t cct cggc gacggcggcg acgt cagct t cagcacccgc 1800

ggcacgcaga act ggacggt ggagcggct g ct ccaggcgc accgccaact ggaggagcgc 1860

ggct at gt gt t cgt cggct a ccacggcacc t t cct cgaag cggcgcaaag cat cgt ct t c 1920

ggcggggt gc gcgcgcgcag ccaggacct c gacgcgat ct ggcgcggt t t ct at at cgcc 1980

ggcgat ccgg cgct ggcct a cggct acgcc caggaccagg aacccgacgc acgcggccgg 2040

at ccgcaacg gt gccct gct gcgggt ct at gt gccgcgct cgagcct gcc gggct t ct ac 2100

cgcaccagcc t gaccct ggc cgcgccggag gcggcgggcg aggt cgaacg gct gat cggc 2160

cat ccgct gc cgct gcgcct ggacgccat c accggccccg aggaggaagg cgggcgcct g 2220

gagaccat t c t cggct ggcc gct ggccgag cgcaccgt gg t gat t ccct c ggcgat cccc 2280

accgacccgc gcaacgt cgg cggcgacct c gacccgt cca gcat ccccga caaggaacag 2340

gcgatcagcg ccctgccgga ctacgccagc cagcccggca aaccgccgcg cgaggacctg 2400

aagtaa 2406

<210> 33
<211> 800
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain T427-PE38

<400> 33

Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly Ala
1 5 10 15

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

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

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Leu Asn Gln Lys Phe
50 55 60

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

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

Ala Ser Glu Met Asp Tyr Tyr Phe Ala Met Asp Tyr Trp Gly Gln Gly
100 105 110

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

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

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser
245 250 255

Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
260 265 270

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn
275 280 285

Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val
290 295 300

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu
305 310 315 320

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys
325 330 335

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr
340 345 350

Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr
355 360 365

Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu
370 375 380

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
385 390 395 400

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys
405 410 415

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
420 425 430

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
435 440 445

Lys Ala Ser Gly Gly Pro Glu Gly Gly Ser Leu Ala Ala Leu Thr Ala
450 455 460

His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr Arg His Arg Gln
465 470 475 480

Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg
485 490 495

Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp
500 505 510

Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu
515 520 525

Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu Thr
530 535 540

Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn
545 550 555 560

Asp Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser Gly Asp Ala Leu
565 570 575

Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly
580 585 590

Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg
595 600 605

Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val
610 615 620

Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly
625 630 635 640

Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe
645 650 655

Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln
660 665 670

Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val
675 680 685

Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr
690 695 700

Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His
705 710 715 720

Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly
725 730 735

Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val
740 745 750

Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp
755 760 765

Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu
770 775 780

Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
785 790 795 800

<210> 34
<211> 2406
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain T427-PE38 T366S:L368A:Y407V:Y349C (hole) coding sequence

<400> 34
atgcaggtcc aactgcagca gccggggact gaactggtga ggcctggagc ttcagtgaag 60

ctgtcctgca aggcttctgg cttctccttc accagttact ggatgaactg ggtgaagcag 120

aggcctggac aaggccttga gtggattggc atgattcatc cttccgatag tgaaactagg 180

ttaaatcaga agttcaagga cagggccaca ttgactgtag acaaaatcct cagcacagcc 240

tacatgcaac tcagcagccc gacatctgag gactctgcgg tctattactg tgcaagtgag 300

atggattatt actttgctat ggactactgg ggtcaaggaa cctcagtcac cgtctcctca 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 720

ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 780

gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 840

tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 900

agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 960

gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 1020

aaagccaaag ggcagccccg agaaccacag gtgtgcaccc tgccccatc ccgggaggag 1080

atgaccaaga accaggtcag cctgagctgc gcggtcaaag gcttctatcc cagcgacatc 1140

gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 1200

ctggactccg acggctcctt ctttcctcgt tagcaagctca ccgtggacaa gagcaggtgg 1260

cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1320

cagaagagcc tctccctgtc cccgggtaaa gcttccggag gtcccgaggg cggcagcctg 1380

gccgcgctga ccgcgcacca ggcttgccac ctgccgctgg agacttttcac ccgtcatcgc 1440

cagccgcgcg gctgggaaca actggagcag tgcggctatc cggtgcagcg gctggtcgcc 1500

ctctacctgg cggcgcggct gtcgtggaac caggtcgacc aggtgatccg caacgccctg 1560

gccagccccg gcagcggcgg cgacctgggc gaagcgatcc gcgagcagcc ggagcaggcc 1620

cgtctggccc tgaccctggc cgccgccgag agcgagcgct tcgtccggca gggcaccggc 1680

aacgacgagg ccggcgcggc caacggcccg gcggacagcg gcgacgccct gctggagcgc 1740

aactatccca ctggcgcgga gttcctcggc gacggcggcg acgtcagctt cagcacccgc 1800

ggcacgcaga actggacggt ggagcggctg ctccaggcgc accgccaact ggaggagcgc 1860

ggctatgtgt tcgtcggcta ccacggcacc ttcctcgaag cggcgcaaag catcgtcttc 1920

ggcggggtgc gcgcgcgcag ccaggacctc gacgcgatct ggcgcggttt ctatatcgcc 1980

ggcgatccgg cgctggccta cggctacgcc caggaccagg aacccgacgc acgcggccgg 2040

atccgcaacg gtgccctgct gcgggtctat gtgccgcgct cgagcctgcc gggcttctac 2100

cgcaccagcc tgaccctggc cgcgccggag gcggcgggcg aggtcgaacg gctgatcggc 2160

catccgctgc cgctgcgcct ggacgccatc accggccccg aggaggaagg cgggcgcctg 2220

gagaccattc tcggctggcc gctggccgag cgcaccgtgg tgattccctc ggcgatcccc 2280

accgacccgc gcaacgtcgg cggcgacctc gacccgtcca gcatccccga caaggaacag 2340

gcgatcagcg ccctgccgga ctacgccagc cagcccggca aaccgccgcg cgaggacctg 2400

aagtaa 2406

<210> 35
<211> 800
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain T427-PE38 T366S:L368A:Y407V:Y349C (hole)

<400> 35

Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly Ala
1 5 10 15

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

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

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Leu Asn Gln Lys Phe
50 55 60

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

Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys

85 90 95

Ala Ser Glu Met Asp Tyr Tyr Phe Ala Met Asp Tyr Trp Gly Gln Gly
100 105 110

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

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

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser
245 250 255

Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
260 265 270

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn
275 280 285

Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val
290 295 300

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu
305 310 315 320

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys
325 330 335

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Cys Thr
340 345 350

Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Ser

355 360 365

Cys Ala Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu
370 375 380

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
385 390 395 400

Asp Ser Asp Gly Ser Phe Phe Leu Val Ser Lys Leu Thr Val Asp Lys
405 410 415

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
420 425 430

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
435 440 445

Lys Ala Ser Gly Gly Pro Glu Gly Gly Ser Leu Ala Ala Leu Thr Ala
450 455 460

His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr Arg His Arg Gln
465 470 475 480

Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg
485 490 495

Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp
500 505 510

Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu
515 520 525

Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu Thr
530 535 540

Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn
545 550 555 560

Asp Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser Gly Asp Ala Leu
565 570 575

Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly
580 585 590

Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg
595 600 605

Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val
610 615 620

Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly

625 630 635 640

Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe
645 650 655

Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln
660 665 670

Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val
675 680 685

Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr
690 695 700

Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His
705 710 715 720

Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly
725 730 735

Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val
740 745 750

Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp
755 760 765

Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu
770 775 780

Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
785 790 795 800

<210> 36
<211> 1353
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain FRP5 T366W.S354C (knob) coding sequence

<400> 36
atgcaggtac aactgcagca gtctggacct gaactgaaga agcctggaga gacagtcaag 60

atctcctgca aggcctctgg gtatcctttc acaaactatg gaatgaactg ggtgaagcag 120

gctccaggac agggtttaaa gtggatgggc tggattaaca cctccactgg agagtcaaca 180

tttgctgatg acttcaaggg acggtttgac ttctctttgg aaacctctgc caacactgcc 240

tatttgcaga tcaacaacct caaaagtgaa gacatggcta catatttctg tgcaagatgg 300

gaggtttacc acggctacgt tccttactgg ggccaaggga ccacggtcac cgttcctct 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacacctt cc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagctt ggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 720

ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 780

gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 840

tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 900

agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 960

gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 1020

aaagccaaag ggcagccccg agaaccacag gtgtacaccc tgccccatg ccgggaggag 1080

atgaccaaga accaggtcag cctgtggtgc ctggtcaaag gcttctatcc cagcgacatc 1140

gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 1200

ctggactccg acggctcctt ctttcctcta tagcaagctca ccgtggacaa gagcaggtgg 1260

cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1320

cagaagagcc tctccctgtc cccgggtaaa tga 1353

<210> 37
<211> 450
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain FRP5 T366W S354C (knob)

<400> 37

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

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

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

Met Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp
50 55 60

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

Tyr Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe
85 90 95

Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln
100 105 110

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

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

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

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

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

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

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275 280 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
325 330 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
340 345 350

Thr Leu Pro Pro Cys Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
355 360 365

Trp Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
370 375 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
405 410 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
420 425 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
435 440 445

Gly Lys
450

<210> 38
<211> 1353
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain FRP5 T366S:L368A:Y407V:Y349C (hole) coding sequence

<400> 38
atgcaggtac aactgcagca gtctggacct gaactgaaga agcctggaga gacagtcaag 60

atctcctgca aggcctctgg gtatcctttc acaaactatg gaatgaactg ggtgaagcag 120

gctccaggac agggtttaaa gtggatgggc tggattaaca cctccactgg agagtcaaca 180

tttgctgatg acttcaaggg acggtttgac tttctctttg gaaacctctgc caacactgcc 240

tatttgcaga tcaacaacct caaaagtgaa gacatggcta catatttctg tgcaagatgg 300

gaggtttacc acggctacgt tcctttactgg ggccaaggga ccacggtcac cgttcctct 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 720

ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 780

gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 840

tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 900

agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 960

gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 1020

aaagccaaag ggcagccccg agaaccacag gtgtgcaccc tgcccccatc ccgggaggag 1080

atgaccaaga accaggtcag cctgagctgc gcggtcaaag gcttctatcc cagcgacatc 1140

gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 1200

ctggactccg acggctcctt ctttcctcgtt agcaagctca ccgtggacaa gagcaggtgg 1260

cagcagggga acgtctttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1320

cagaagagcc tctccctgtc cccgggtaaa tga 1353

<210> 39
<211> 450
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain FRP5 T366S:L368A:Y407V:Y349C (hole)

<400> 39

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

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

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

Met Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp
50 55 60

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

Tyr Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe
85 90 95

Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln
100 105 110

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

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

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

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

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

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys

195 200 205

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275 280 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
325 330 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Cys
340 345 350

Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
355 360 365

Ser Cys Ala Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
370 375 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Val Ser Lys Leu Thr Val Asp
405 410 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
420 425 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
435 440 445

Gly Lys
450

<210> 40

<211> 1353
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain T427 T366W S354C (knob) and A44C:C222A (Cys) coding sequence

<400> 40
atgcaggtcc aactgcagca gccggggact gaactggtga ggcctggagc ttcagtgaag 60

ctgtcctgca aggcttctgg cttctccttc accagttact ggatgaactg ggtgaagcag 120

aggcctggac aatgccttga gtggattggc atgattcatc cttccgatag tgaaactagg 180

ttaaatcaga agttcaagga cagggccaca ttgactgtag acaaatcctc cagcacagcc 240

tacatgcaac tcagcagccc gacatctgag gactctgcgg tctattactg tgcaagtgag 300

atggattatt actttgctat ggactactgg ggtcaaggaa cctcagtcac cgtctcctca 360

gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 420

ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480

tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 540

ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 600

tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agttgagccc 660

aaatctgccg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 720

ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggaccccct 780

gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 840

tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 900

agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 960

gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 1020

aaagccaaag ggcagccccg agaaccacag gtgtacaccc tgccccatg ccgggaggag 1080

atgaccaaga accaggtcag cctgtggtgc ctggtcaaag gcttctatcc cagcgacatc 1140

gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 1200

ctggactccg acggctcctt ctttcctcta tagcaagctca ccgtggacaa gagcaggtgg 1260

cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1320

cagaagagcc tctccctgtc cccgggtaaa tga 1353

<210> 41
<211> 450
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain T427 T366W S354C (knob) and A44C:C222A (Cys)

<400> 41

Met Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly
1 5 10 15

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

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

Ile Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Leu Asn Gln Lys
50 55 60

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

Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Tyr
85 90 95

Cys Ala Ser Glu Met Asp Tyr Tyr Phe Ala Met Asp Tyr Trp Gly Gln
100 105 110

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

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

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

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

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

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

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Ala Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275 280 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
325 330 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
340 345 350

Thr Leu Pro Pro Cys Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
355 360 365

Trp Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
370 375 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
405 410 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
420 425 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
435 440 445

Gly Lys
450

<210> 42
<211> 216
<212> PRT
<213> Pseudomonas aeruginosa

<400> 42

Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly
1 5 10 15

Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu
20 25 30

Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu
35 40 45

Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp
50 55 60

Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu
65 70 75 80

Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile
85 90 95

Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro
100 105 110

Gly Phe Tyr Arg Thr Gly Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly
115 120 125

Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala
130 135 140

Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly
145 150 155 160

Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr
165 170 175

Asp Pro Arg Asn Val Gly Gly Asp Leu Ala Pro Ser Ser Ile Pro Asp
180 185 190

Gln Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly
195 200 205

Lys Pro Ser Arg Glu Asp Leu Lys
210 215

<210> 43
<211> 536
<212> PRT
<213> Corynebacterium diphtheriae

<400> 43

Met Gly Ala Asp Asp Val Val Asp Ser Ser Lys Ser Phe Val Met Glu
1 5 10 15

Asn Phe Ser Ser Tyr His Gly Thr Lys Pro Gly Tyr Val Asp Ser Ile
20 25 30

Gln Lys Gly Ile Gln Lys Pro Lys Ser Gly Thr Gln Gly Asn Tyr Asp
35 40 45

Asp Asp Trp Lys Gly Phe Tyr Ser Thr Asp Asn Lys Tyr Asp Ala Ala
50 55 60

Gly Tyr Ser Val Asp Asn Glu Asn Pro Leu Ser Gly Lys Ala Gly Gly
65 70 75 80

Val Val Lys Val Thr Tyr Pro Gly Leu Thr Lys Val Leu Ala Leu Lys
85 90 95

Val Asp Asn Ala Glu Thr Ile Lys Lys Glu Leu Gly Leu Ser Leu Thr
100 105 110

Glu Pro Leu Met Glu Gln Val Gly Thr Glu Glu Phe Ile Lys Arg Phe
115 120 125

Gly Asp Gly Ala Ser Arg Val Val Leu Ser Leu Pro Phe Ala Glu Gly
130 135 140

Ser Ser Ser Val Glu Tyr Ile Asn Asn Trp Glu Gln Ala Lys Ala Leu
145 150 155 160

Ser Val Glu Leu Glu Ile Asn Phe Glu Thr Arg Gly Lys Arg Gly Gln
165 170 175

Asp Ala Met Tyr Glu Tyr Met Ala Gln Ala Cys Ala Gly Asn Arg Val
180 185 190

Arg Arg Ser Val Gly Ser Ser Leu Ser Cys Ile Asn Leu Asp Trp Asp
195 200 205

Val Ile Arg Asp Lys Thr Lys Thr Lys Ile Glu Ser Leu Lys Glu His
210 215 220

Gly Pro Ile Lys Asn Lys Met Ser Glu Ser Pro Asn Lys Thr Val Ser
225 230 235 240

Glu Glu Lys Ala Lys Gln Tyr Leu Glu Glu Phe His Gln Thr Ala Leu
245 250 255

Glu His Pro Glu Leu Ser Glu Leu Lys Thr Val Thr Gly Thr Asn Pro
260 265 270

Val Phe Ala Gly Ala Asn Tyr Ala Ala Trp Ala Val Asn Val Ala Gln
275 280 285

Val Ile Asp Ser Glu Thr Ala Asp Asn Leu Glu Lys Thr Thr Ala Ala
290 295 300

Leu Ser Ile Leu Pro Gly Ile Gly Ser Val Met Gly Ile Ala Asp Gly
305 310 315 320

Ala Val His His Asn Thr Glu Glu Ile Val Ala Gln Ser Ile Ala Leu
325 330 335

Ser Ser Leu Met Val Ala Gln Ala Ile Pro Leu Val Gly Glu Leu Val
340 345 350

Asp Ile Gly Phe Ala Ala Tyr Asn Phe Val Glu Ser Ile Ile Asn Leu
355 360 365

Phe Gln Val Val His Asn Ser Tyr Asn Arg Pro Ala Tyr Ser Pro Gly
370 375 380

His Lys Thr Gln Pro Phe Leu His Asp Gly Tyr Ala Val Ser Trp Asn
385 390 395 400

Thr Val Glu Asp Ser Ile Ile Arg Thr Gly Phe Gln Gly Glu Ser Gly
405 410 415

His Asp Ile Lys Ile Thr Ala Glu Asn Thr Pro Leu Pro Ile Ala Gly
420 425 430

Val Leu Leu Pro Thr Ile Pro Gly Lys Leu Asp Val Asn Lys Ser Lys
435 440 445

Thr His Ile Ser Val Asn Gly Arg Lys Ile Arg Met Arg Cys Arg Ala
450 455 460

Ile Asp Gly Asp Val Thr Phe Cys Arg Pro Lys Ser Pro Val Tyr Val
465 470 475 480

Gly Asn Gly Val His Ala Asn Leu His Val Ala Phe His Arg Ser Ser
485 490 495

Ser Glu Lys Ile His Ser Asn Glu Ile Ser Ser Asp Ser Ile Gly Val
500 505 510

Leu Gly Tyr Gln Lys Thr Val Asp His Thr Lys Val Asn Ser Lys Leu
515 520 525

Ser Leu Phe Phe Glu Ile Lys Ser
530 535

<210> 44
<211> 127
<212> PRT
<213> Artificial sequence

<220>
<223> interleukin 2

<400> 44

Thr Lys Lys Thr Gln Leu Gln Leu Glu His Leu Leu Leu Asp Leu Gln
1 5 10 15

Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg
20 25 30

Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys
35 40 45

His Leu Gln Cys Leu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu
50 55 60

Asn Leu Ala Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile
65 70 75 80

Ser Asn Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr
85 90 95

Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu
100 105 110

Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr
115 120 125

<210> 45
<211> 207
<212> PRT
<213> Homo sapiens

<400> 45

Met Gln Ser Gly Thr His Trp Arg Val Leu Gly Leu Cys Leu Leu Ser
1 5 10 15

Val Gly Val Trp Gly Gln Asp Gly Asn Glu Glu Met Gly Gly Ile Thr
20 25 30

Gln Thr Pro Tyr Lys Val Ser Ile Ser Gly Thr Thr Val Ile Leu Thr
35 40 45

Cys Pro Gln Tyr Pro Gly Ser Glu Ile Leu Trp Gln His Asn Asp Lys
50 55 60

Asn Ile Gly Gly Asp Glu Asp Asp Lys Asn Ile Gly Ser Asp Glu Asp
65 70 75 80

His Leu Ser Leu Lys Glu Phe Ser Glu Leu Glu Gln Ser Gly Tyr Tyr
85 90 95

Val Cys Tyr Pro Arg Gly Ser Lys Pro Glu Asp Ala Asn Phe Tyr Leu
100 105 110

Tyr Leu Arg Ala Arg Val Cys Glu Asn Cys Met Glu Met Asp Val Met
115 120 125

Ser Val Ala Thr Ile Val Ile Val Asp Ile Cys Ile Thr Gly Gly Leu
130 135 140

Leu Leu Leu Val Tyr Tyr Trp Ser Lys Asn Arg Lys Ala Lys Ala Lys
145 150 155 160

Pro Val Thr Arg Gly Ala Gly Ala Gly Gly Arg Gln Arg Gly Gln Asn
165 170 175

Lys Glu Arg Pro Pro Pro Val Pro Asn Pro Asp Tyr Glu Pro Ile Arg
180 185 190

Lys Gly Gln Arg Asp Leu Tyr Ser Gly Leu Asn Gln Arg Arg Ile
195 200 205

<210> 46
<211> 290
<212> PRT
<213> Homo sapiens

<400> 46

Met Gly Gly Gly Ala Gly Glu Arg Leu Phe Thr Ser Ser Cys Leu Val
1 5 10 15

Gly Leu Val Pro Leu Gly Leu Arg Ile Ser Leu Val Thr Cys Pro Leu
20 25 30

Gln Cys Gly Ile Met Trp Gln Leu Leu Leu Pro Thr Ala Leu Leu Leu
35 40 45

Leu Val Ser Ala Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val
50 55 60

Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr
65 70 75 80

Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp
85 90 95

Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile
100 105 110

Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn
115 120 125

Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp
130 135 140

Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile
145 150 155 160

His Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr
165 170 175

Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp
180 185 190

Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys
195 200 205

Arg Gly Leu Phe Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile
210 215 220

Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro
225 230 235 240

Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala
245 250 255

Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser
260 265 270

Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln
275 280 285

Asp Lys
290

<210> 47
<211> 153
<212> PRT
<213> Homo sapiens

<400> 47

Met Gly Leu Thr Ser Gln Leu Leu Pro Pro Leu Phe Phe Leu Leu Ala
1 5 10 15

Cys Ala Gly Asn Phe Val His Gly His Lys Cys Asp Ile Thr Leu Gln
20 25 30

Glu Ile Ile Lys Thr Leu Asn Ser Leu Thr Glu Gln Lys Thr Leu Cys
35 40 45

Thr Glu Leu Thr Val Thr Asp Ile Phe Ala Ala Ser Lys Asn Thr Thr
50 55 60

Glu Lys Glu Thr Phe Cys Arg Ala Ala Thr Val Leu Arg Gln Phe Tyr
65 70 75 80

Ser His His Glu Lys Asp Thr Arg Cys Leu Gly Ala Thr Ala Gln Gln
85 90 95

Phe His Arg His Lys Gln Leu Ile Arg Phe Leu Lys Arg Leu Asp Arg
100 105 110

Asn Leu Trp Gly Leu Ala Gly Leu Asn Ser Cys Pro Val Lys Glu Ala
115 120 125

Asn Gln Ser Thr Leu Glu Asn Phe Leu Glu Arg Leu Lys Thr Ile Met

130 135 140

Arg Glu Lys Tyr Ser Lys Cys Ser Ser
145 150

<210> 48
<211> 365
<212> PRT
<213> Homo sapiens

<400> 48

Met Ala Val Met Ala Pro Arg Thr Leu Val Leu Leu Leu Ser Gly Ala
1 5 10 15

Leu Ala Leu Thr Gln Thr Trp Ala Gly Ser His Ser Met Arg Tyr Phe
20 25 30

Phe Thr Ser Val Ser Arg Pro Gly Arg Gly Glu Pro Arg Phe Ile Ala
35 40 45

Val Gly Tyr Val Asp Asp Thr Gln Phe Val Arg Phe Asp Ser Asp Ala
50 55 60

Ala Ser Gln Arg Met Glu Pro Arg Ala Pro Trp Ile Glu Gln Glu Gly
65 70 75 80

Pro Glu Tyr Trp Asp Gly Glu Thr Arg Lys Val Lys Ala His Ser Gln
85 90 95

Thr His Arg Val Asp Leu Gly Thr Leu Arg Gly Tyr Tyr Asn Gln Ser
100 105 110

Glu Ala Gly Ser His Thr Val Gln Arg Met Tyr Gly Cys Asp Val Gly
115 120 125

Ser Asp Trp Arg Phe Leu Arg Gly Tyr His Gln Tyr Ala Tyr Asp Gly
130 135 140

Lys Asp Tyr Ile Ala Leu Lys Glu Asp Leu Arg Ser Trp Thr Ala Ala
145 150 155 160

Asp Met Ala Ala Gln Thr Thr Lys His Lys Trp Glu Ala Ala His Val
165 170 175

Ala Glu Gln Leu Arg Ala Tyr Leu Glu Gly Thr Cys Val Glu Trp Leu
180 185 190

Arg Arg Tyr Leu Glu Asn Gly Lys Glu Thr Leu Gln Arg Thr Asp Ala
195 200 205

Pro Lys Thr His Met Thr His His Ala Val Ser Asp His Glu Ala Thr
210 215 220

Leu Arg Cys Trp Ala Leu Ser Phe Tyr Pro Ala Glu Ile Thr Leu Thr
225 230 235 240

Trp Gln Arg Asp Gly Glu Asp Gln Thr Gln Asp Thr Glu Leu Val Glu
245 250 255

Thr Arg Pro Ala Gly Asp Gly Thr Phe Gln Lys Trp Ala Ala Val Val
260 265 270

Val Pro Ser Gly Gln Glu Gln Arg Tyr Thr Cys His Val Gln His Glu
275 280 285

Gly Leu Pro Lys Pro Leu Thr Leu Arg Trp Glu Pro Ser Ser Gln Pro
290 295 300

Thr Ile Pro Ile Val Gly Ile Ile Ala Gly Leu Val Leu Phe Gly Ala
305 310 315 320

Val Ile Thr Gly Ala Val Val Ala Ala Val Met Trp Arg Arg Lys Ser
325 330 335

Ser Asp Arg Lys Gly Gly Ser Tyr Ser Gln Ala Ala Ser Ser Asp Ser
340 345 350

Ala Gln Gly Ser Asp Val Ser Leu Thr Ala Cys Lys Val
355 360 365

<210> 49
<211> 178
<212> PRT
<213> Homo sapiens

<400> 49

Met His Ser Ser Ala Leu Leu Cys Cys Leu Val Leu Leu Thr Gly Val
1 5 10 15

Arg Ala Ser Pro Gly Gln Gly Thr Gln Ser Glu Asn Ser Cys Thr His
20 25 30

Phe Pro Gly Asn Leu Pro Asn Met Leu Arg Asp Leu Arg Asp Ala Phe
35 40 45

Ser Arg Val Lys Thr Phe Phe Gln Met Lys Asp Gln Leu Asp Asn Leu
50 55 60

Leu Leu Lys Glu Ser Leu Leu Glu Asp Phe Lys Gly Tyr Leu Gly Cys
65 70 75 80

Gln Ala Leu Ser Glu Met Ile Gln Phe Tyr Leu Glu Glu Val Met Pro
85 90 95

```
Gln Ala Glu Asn Gln Asp Pro Asp Ile Lys Ala His Val Asn Ser Leu
            100                 105                 110

Gly Glu Asn Leu Lys Thr Leu Arg Leu Arg Leu Arg Arg Cys His Arg
        115                 120                 125

Phe Leu Pro Cys Glu Asn Lys Ser Lys Ala Val Glu Gln Val Lys Asn
    130                 135                 140

Ala Phe Asn Lys Leu Gln Glu Lys Gly Ile Tyr Lys Ala Met Ser Glu
145                 150                 155                 160

Phe Asp Ile Phe Ile Asn Tyr Ile Glu Ala Tyr Met Thr Met Lys Ile
                165                 170                 175

Arg Asn
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<210> 50
<211> 576
<212> PRT
<213> Ricinus communis

<400> 50

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Met Lys Pro Gly Gly Asn Thr Ile Val Ile Trp Met Tyr Ala Val Ala
1               5                   10                  15

Thr Trp Leu Cys Phe Gly Ser Thr Ser Gly Trp Ser Phe Thr Leu Glu
            20                  25                  30

Asp Asn Asn Ile Phe Pro Lys Gln Tyr Pro Ile Ile Asn Phe Thr Thr
        35                  40                  45

Ala Gly Ala Thr Val Gln Ser Tyr Thr Asn Phe Ile Arg Ala Val Arg
    50                  55                  60

Gly Arg Leu Thr Thr Gly Ala Asp Val Arg His Glu Ile Pro Val Leu
65                  70                  75                  80

Pro Asn Arg Val Gly Leu Pro Ile Asn Gln Arg Phe Ile Leu Val Glu
                85                  90                  95

Leu Ser Asn His Ala Glu Leu Ser Val Thr Leu Ala Leu Asp Val Thr
            100                 105                 110

Asn Ala Tyr Val Val Gly Tyr Arg Ala Gly Asn Ser Ala Tyr Phe Phe
        115                 120                 125

His Pro Asp Asn Gln Glu Asp Ala Glu Ala Ile Thr His Leu Phe Thr
    130                 135                 140
```

Asp Val Gln Asn Arg Tyr Thr Phe Ala Phe Gly Gly Asn Tyr Asp Arg
145 150 155 160

Leu Glu Gln Leu Ala Gly Asn Leu Arg Glu Asn Ile Glu Leu Gly Asn
165 170 175

Gly Pro Leu Glu Glu Ala Ile Ser Ala Leu Tyr Tyr Tyr Ser Thr Gly
180 185 190

Gly Thr Gln Leu Pro Thr Leu Ala Arg Ser Phe Ile Ile Cys Ile Gln
195 200 205

Met Ile Ser Glu Ala Ala Arg Phe Gln Tyr Ile Glu Gly Glu Met Arg
210 215 220

Thr Arg Ile Arg Tyr Asn Arg Arg Ser Ala Pro Asp Pro Ser Val Ile
225 230 235 240

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

Asn Gln Gly Ala Phe Ala Ser Pro Ile Gln Leu Gln Arg Arg Asn Gly
260 265 270

Ser Lys Phe Ser Val Tyr Asp Val Ser Ile Leu Ile Pro Ile Ile Ala
275 280 285

Leu Met Val Tyr Arg Cys Ala Pro Pro Pro Ser Ser Gln Phe Ser Leu
290 295 300

Leu Ile Arg Pro Val Val Pro Asn Phe Asn Ala Asp Val Cys Met Asp
305 310 315 320

Pro Glu Pro Ile Val Arg Ile Val Gly Arg Asn Gly Leu Cys Val Asp
325 330 335

Val Arg Asp Gly Arg Phe His Asn Gly Asn Ala Ile Gln Leu Trp Pro
340 345 350

Cys Lys Ser Asn Thr Asp Ala Asn Gln Leu Trp Thr Leu Lys Arg Asp
355 360 365

Asn Thr Ile Arg Ser Asn Gly Lys Cys Leu Thr Thr Tyr Gly Tyr Ser
370 375 380

Pro Gly Val Tyr Val Met Ile Tyr Asp Cys Asn Thr Ala Ala Thr Asp
385 390 395 400

Ala Thr Arg Trp Gln Ile Trp Asp Asn Gly Thr Ile Ile Asn Pro Arg
405 410 415

Ser Ser Leu Val Leu Ala Ala Thr Ser Gly Asn Ser Gly Thr Thr Leu
420 425 430

Thr Val Gln Thr Asn Ile Tyr Ala Val Ser Gln Gly Trp Leu Pro Thr
435 440 445

Asn Asn Thr Gln Pro Phe Val Thr Thr Ile Val Gly Leu Tyr Gly Leu
450 455 460

Cys Leu Gln Ala Asn Ser Gly Gln Val Trp Ile Glu Asp Cys Ser Ser
465 470 475 480

Glu Lys Ala Glu Gln Gln Trp Ala Leu Tyr Ala Asp Gly Ser Ile Arg
485 490 495

Pro Gln Gln Asn Arg Asp Asn Cys Leu Thr Ser Asp Ser Asn Ile Arg
500 505 510

Glu Thr Val Val Lys Ile Leu Ser Cys Gly Pro Ala Ser Ser Gly Gln
515 520 525

Arg Trp Met Phe Lys Asn Asp Gly Thr Ile Leu Asn Leu Tyr Ser Gly
530 535 540

Leu Val Leu Asp Val Arg Ala Ser Asp Pro Ser Leu Lys Gln Ile Ile
545 550 555 560

Leu Tyr Pro Leu His Gly Asp Pro Asn Gln Ile Trp Leu Pro Leu Phe
565 570 575

<210> 51
<211> 651
<212> DNA
<213> Pseudomonas aeruginosa

<400> 51
gcggagttcc tcggcgacgg cggcgacgtc agcttcagca cccgcggcac gcagaactgg 60

acggtggagc ggctgctcca ggcgcaccgc caactggagg agcgcggcta tgtgttcgtc 120

ggctaccacg gcaccttcct cgaagcggcg caaagcatcg tcttcggcgg ggtgcgcgcg 180

cgcagccagg accttgacgc gatctggcgc ggttttctat atcgccggcga tccggcgctg 240

gcctacggct acgcccagga ccaggaaccc gacgcgcgcg gccggatccg caacggtgcc 300

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catctctaatg aaatttcatc ggattccata ggcgttcttg ggtaccagaa aacagtagat 1560

cacaccaagg ttaattctaa gctatcgcta tttttttgaaa tcaaaagctg a 1611

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<213> Artificial sequence

<220>

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<223> n is a, c, g, or t

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<213> Homo sapiens

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<211> 2406
<212> DNA
<213> Homo sapi ens

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t gccagact t cagact gaga agt cagat ga agt t t caaga aaaggaaat t ggt gggt gac 180

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gact ct gaag t gccagggag cct act cccc t gaggacaat t ccacacagt ggt t t cacaa 480

t gagagcct c at ct caagcc aggcct cgag ct act t cat t gacgct gcca cagt cgacga 540

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aggact at at t t ct ct gt ga agacaaacat t cgaagct ca acaagagact ggaaggacca 1020

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<211> 921
<212> DNA
<213> Homo sapiens

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ttcctattgg tctgatttca caggaacatt ttacctgttt gtgaggcatt ttttctcctg 180

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<210> 57
<211> 4000
<212> DNA
<213> Homo sapi ens

<400> 57
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ccatt gggt g t cgggt t t cc agagaagcca at cagt gt cg t cgcggt cgc ggt t ct aaag 480

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<211> 1601
<212> DNA
<213> Homo sapiens

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<223> Light chain of anti-streptavidin (anti-SA)

<400> 69

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

Gln Arg Val Thr Leu Ser Cys Thr Gly Ser Ser Ser Asn Ile Gly Ser
20 25 30

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

Leu Ile Tyr Asp Asn Asn Lys Arg Pro Ser Gly Val Ser Asp Arg Phe
50 55 60

Ser Gly Ser Lys Ser Gly Thr Ser Ala Ser Leu Ala Ile Ser Gly Leu
65 70 75 80

Arg Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gln Ser Tyr Asp Ser Ser
85 90 95

Leu Thr Gly Ser Val Val Phe Gly Gly Gly Thr Gln Leu Thr Val Leu
100 105 110

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

Glu Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp
130 135 140

Phe Tyr Pro Gly Ala Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro
145 150 155 160

Val Lys Ala Gly Val Glu Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn
165 170 175

Lys Tyr Ala Ala Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys
180 185 190

Ser His Arg Ser Tyr Ser Cys Gln Val Thr His Glu Gly Ser Thr Val
195 200 205

Glu Lys Thr Val Ala Pro Ala Glu Cys Ser
210 215

<210> 70
<211> 657
<212> DNA
<213> Artificial sequence

<220>
<223> Light chain of anti-PE clone B11

<400> 70
atggatatcg tgctgactca gccaccctca gcgtctggga cccccgggca gagggtcacc 60

atctcttgct ctggaaccag cagtgatgtt gggaattcta accttgtctc ttggtaccag 120

cagctcccag gaacggctcc caaactcctc atttatggga acagcaatcg gccctcaggg 180

gtccctgacc ggttctctgg ctccaagtct ggcacctcag cctccctggc catcagtggg 240

ctgcggtccg aggatgaggc tgattattac tgctgctcat atgcaggtac tggctccct 300

gatgtcttcg gaactggcac caagctgacc gtcctaggtc agcccaaggc tgccccctcg 360

gtcactctgt tcccgccctc ctctgaggag cttcaagcca acaaggccac actggtgtgt 420

ctcataagtg acttctaccc gggagccgtg acagtggcct ggaaggcaga tagcagcccc 480

gtcaaggcgg gagtggagac caccacaccc tccaaacaaa gcaacaacaa gtacgcggcc 540

agcagctatc tgagcctgac gcctgagcag tggaagtccc acagaagcta cagctgccag 600

gtcacgcatg aagggagcac cgtggagaag acagtggccc ctgcagaatg ttcttaa 657

<210> 71
<211> 218
<212> PRT
<213> Artificial sequence

<220>
<223> Light chain of anti-PE clone B11

<400> 71

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

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

Ser Asn Leu Val Ser Trp Tyr Gln Gln Leu Pro Gly Thr Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Gly Asn Ser Asn Arg Pro Ser Gly Val Pro Asp Arg
50 55 60

Phe Ser Gly Ser Lys Ser Gly Thr Ser Ala Ser Leu Ala Ile Ser Gly
65 70 75 80

Leu Arg Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Cys Ser Tyr Ala Gly
85 90 95

Thr Gly Ser Pro Asp Val Phe Gly Thr Gly Thr Lys Leu Thr Val Leu
100 105 110

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

Glu Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp
130 135 140

Phe Tyr Pro Gly Ala Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro
145 150 155 160

Val Lys Ala Gly Val Glu Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn
165 170 175

Lys Tyr Ala Ala Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys
180 185 190

Ser His Arg Ser Tyr Ser Cys Gln Val Thr His Glu Gly Ser Thr Val
195 200 205

Glu Lys Thr Val Ala Pro Ala Glu Cys Ser
210 215

<210> 72
<211> 1347
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain of anti-streptavidin (anti-SA)

<400> 72
atggaggtgc agctgttgga gtctggggga ggcttggtac agcctggggg gtccctgaga 60

ctctcctgtg cagcctctgg attcaccttc agtggctact ggatgcactg ggtccgccag 120

gctccaggca aggggctgga gtgggtttca gaaattagtg gtagtggtga tagcacacac 180

tacggagact ccgtgaaggg ccggttcacc atctccagag acaattccaa gaacacgctg 240

tatctgcaaa tgaacagcct gagagccgag gacacggccg tgtattactg tgcaagagga 300

cggaacggat ccctcgacta ctggggccag ggcaccctgg tcacagtctc ctcagctagc 360

accaagggcc catcggtctt ccccctggca ccctcctcca agagcacctc tgggggcaca 420

gcggccctgg gctgcctggt caaggactac ttccccgaac cggtgacggt gtcgtggaac 480

tcaggcgccc tgaccagcgg cgtgcacacc ttcccggctg tcctacagtc ctcaggactc 540

tactccctca gcagcgtggt gaccgtgccc tccagcagct tgggcaccca gacctacatc 600

tgcaacgtga atcacaagcc cagcaacacc aaggtggaca agagagttga gcccaaatct 660

tgtgacaaaa ctcacacatg cccaccgtgc ccagcacctg aactcctggg gagaccgtca 720

gtcttcctct tccccccaaa acccaaggac accctcatga tctcccggac ccctgaggtc 780

acatgcgtgg tggtggacgt gagccacgaa gaccctgagg tcaagttcaa ctggtacgtg 840

gacggcgtgg aggtgcataa tgccaagaca aagccgcggg aggagcagta caacagcacg 900

taccgtgtgg tcagcgtcct caccgtcctg caccaggact ggctgaatgg caaggagtac 960

aagtgcaagg tctccaacaa agccctccca gcccccatcg agaaaaccat ctccaaagcc 1020

aaagggcagc cccgagaacc acaggtgtgc accctgcccc catcccggga ggagatgacc 1080

aagaaccagg tcagcctgag ctgcgcggtc aaaggcttct atcccagcga catcgccgtg 1140

gagtgggaga gcaatgggca gccggagaac aactacaaga ccacgcctcc cgtgctggac 1200

tccgacggct ccttcttcct cgttagcaag ctcaccgtgg acaagagcag gtggcagcag 1260

gggaacgtct tctcatgctc cgtgatgcat gaggctctgc acaaccacta cacgcagaag 1320

agcctctccc tgtccccggg taaatga 1347

<210> 73
<211> 448
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain of anti-streptavidin (anti-SA)

<400> 73

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

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

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

Val Ser Glu Ile Ser Gly Ser Gly Asp Ser Thr His Tyr Gly Asp Ser
50 55 60

Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu
65 70 75 80

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

Cys Ala Arg Gly Arg Asn Gly Ser Leu 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 Arg Val Glu Pro Lys Ser Cys Asp Lys Thr
210 215 220

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Arg Pro Ser
225 230 235 240

Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
245 250 255

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro
260 265 270

Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
275 280 285

Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val
290 295 300

Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
305 310 315 320

Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr
325 330 335

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Cys Thr Leu
340 345 350

Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Ser Cys
355 360 365

Ala Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
370 375 380

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
385 390 395 400

Ser Asp Gly Ser Phe Phe Leu Val Ser Lys Leu Thr Val Asp Lys Ser
405 410 415

Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
420 425 430

Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
435 440 445

<210> 74
<211> 1362
<212> DNA
<213> Artificial sequence

<220>
<223> Heavy chain of anti-PE clone B11

<400> 74
atggaggtgc agctgttgga gactggggga ggcttggtac agcctggggg gtccctgaga 60

ctctcctgtg cagcctctgg atttcacctt tgacaactat gccataaact ggtccgccag 120

gctccaggca aggggctgga gtgggtctca ggtattagtt ggaatagtgg tagcataggc 180

tatgcggact ctgtgaaggg ccgattcacc atctccagag acaattccaa gaacacgctg 240

tatctgcaaa tgaacagcct gagagccgag gacacggccg tatattactg tgcgagaggg 300

gccccccact acggtgggag gggggagttt gacttctggg gccagggcac cctggtcacg 360

gtctcctcag ctagcaccaa gggcccatcg gtcttccccc tggcaccctc ctccaagagc 420

acctctgggg gcacagcggc cctgggctgc ctggtcaagg actacttccc cgaaccggtg 480

acggtgtcgt ggaactcagg cgccctgacc agcggcgtgc acaccttccc ggctgtccta 540

cagtcctcag gactctactc cctcagcagc gtggtgaccg tgccctccag cagcttgggc 600

acccagacct acatctgcaa cgtgaatcac aagcccagca acaccaaggt ggacaagaga 660

gttgagccca aatcttgtga caaaactcac acatgcccac cgtgcccagc acctgaactc 720

ctggggagac cgtcagtctt cctcttcccc ccaaaaccca aggacaccct catgatctcc 780

cggacccctg aggtcacatg cgtggtggtg gacgtgagcc acgaagaccc tgaggtcaag 840

ttcaactggt acgtggacgg cgtggaggtg cataatgcca agacaaagcc gcgggaggag 900

cagtacaaca gcacgtaccg tgtggtcagc gtcctcaccg tcctgcacca ggactggctg 960

aatggcaagg agtacaagtg caaggtctcc aacaaagccc tcccagcccc catcgagaaa 1020

accatctcca aagccaaagg gcagccccga gaaccacagg tgtgcaccct gccccatcc 1080

cgggaggaga tgaccaagaa ccaggtcagc ctgagctgcg cggtcaaagg cttctatccc 1140

agcgacatcg ccgtggagtg ggagagcaat gggcagccgg agaacaacta caagaccacg 1200

cctcccgtgc tggactccga cggctccttc ttcctcgtta gcaagctcac cgtggacaag 1260

agcaggtggc agcaggggaa cgtcttctca tgctccgtga tgcatgaggc tctgcacaac 1320

cactacacgc agaagagcct ctccctgtcc ccgggtaaat ga 1362

<210> 75
<211> 453
<212> PRT
<213> Artificial sequence

<220>
<223> Heavy chain of anti-PE clone B11

<400> 75

Met Glu Val Gln Leu Leu Glu Thr Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

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

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

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

Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu
65 70 75 80

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

Cys Ala Arg Gly Ala Pro His Tyr Gly Gly Arg Gly Glu Phe Asp Phe
100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
115 120 125

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

Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
145 150 155 160

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
165 170 175

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
180 185 190

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

Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Pro Lys
210 215 220

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu
225 230 235 240

Leu Gly Arg Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
245 250 255

Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val
260 265 270

Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
275 280 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
290 295 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu
305 310 315 320

Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala
325 330 335

Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
340 345 350

Gln Val Cys Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln
355 360 365

Val Ser Leu Ser Cys Ala Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
370 375 380

Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
385 390 395 400

Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Val Ser Lys Leu
405 410 415

Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
420 425 430

Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
435 440 445

Leu Ser Pro Gly Lys
450