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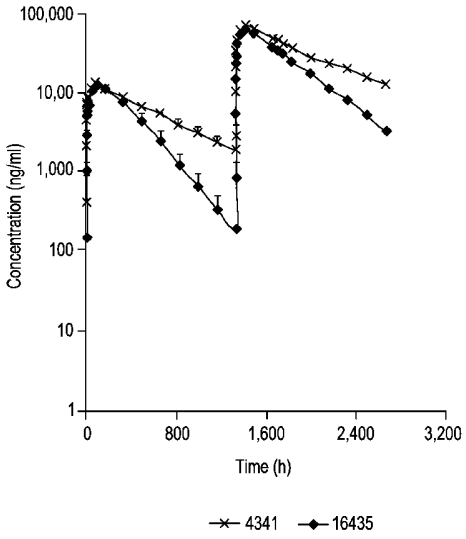
[Continued on next page]

(54) Title: CARRIER IMMUNOGLOBULINS AND USES THEREOF

FIG. 44



(57) Abstract: Disclosed is an isolated immunoglobulin. Also disclosed are pharmaceutical compositions and medicaments comprising the immunoglobulin, isolated nucleic acid encoding it, vectors, host cells, useful in methods of making it. In some embodiments the immunoglobulin comprises one to twenty-four pharmacologically active chemical moieties conjugated thereto, such as a pharmacologically active polypeptide.

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CARRIER IMMUNOGLOBULINS AND USES THEREOF

[0001] This application claims the benefit of U.S. Provisional Application No. 61/385,460, filed September 22, 2010, which is hereby incorporated by reference in its entirety.

[0002] The instant application contains an ASCII "txt" compliant sequence listing submitted via EFS-WEB on September 22, 2011, which serves as both the computer readable form (CRF) and the paper copy required by 37 C.F.R. Section 1.821(c) and 1.821(e), and is hereby incorporated by reference in its entirety. The name of the "txt" file created on September 22, 2011, is: A-1536-WO-PCTSeqList092211_ST25.txt, and is 501 kb in size.

[0003] Throughout this application various publications are referenced within parentheses or brackets. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

[0004] 1. Field of the Invention.

[0005] This invention relates to immunoglobulins to which one or more pharmacologically active chemical moieties can be conjugated for improved pharmacokinetic characteristics.

[0006] 2. Discussion of the Related Art.

[0007] A "carrier" moiety refers to a pharmacologically inactive molecule to which a pharmacologically active chemical moiety, such as a non-peptide organic moiety (i.e., "small molecule") or a polypeptide agent (e.g., the inventive immmunoglublins), can be covalently conjugated or fused. Effective carriers have

been sought to prevent or mitigate in vivo degradation of pharmacologically active moieties by proteolysis or other in vivo activity-diminishing chemical modifications of the pharmacologically active chemical moiety, or to reduce renal clearance, to enhance in vivo half-life or other pharmacokinetic properties of a therapeutic, such as increasing the rate of absorption, reducing toxicity or immunogenicity, improving solubility, and/or increasing manufacturability or storage stability, compared to an unconjugated form of the pharmacologically active moiety.

[0008] Examples of such carrier moieties that have been employed in the pharmaceutical industry include polyethylene glycol (see, e.g., Burg et al., Erythropoietin conjugates with polyethylene glycol, WO 01/02017), immunoglobulin Fc domain (see, e.g., Feige et al., Modified peptides as therapeutic agents, US Patent No. 6,660,843), human serum albumin (see, e.g., Rosen et al., Albumin fusion proteins, US Patent No. 6,926,898 and US 2005/0054051; Bridon et al., Protection of endogenous therapeutic peptides from peptidase activity through conjugation to blood components, US 6,887,470), transthyretin (see, e.g., Walker et al., Use of transthyretin peptide/protein fusions to increase the serum half-life of pharmacologically active peptides/proteins, US 2003/0195154 A1; 2003/0191056 A1), or thyroxine-binding globulin, or a combination such as immunoglobulin(light chain+heavy chain) and Fc domain (the heterotrimeric combination a so-called "hemibody"), for example as described in Sullivan et al., Toxin Peptide Therapeutic Agents, PCT/US2007/022831, published as WO 2008/088422. Pharmacologically active moieties have also been conjugated to a peptide or small molecule that has an affinity for a long half-life serum protein. (See, e.g., Blaney et al., Method and compositions for increasing the serum half-life of pharmacologically active agents by binding to transthyretin-selective ligands, US Patent. No. 5,714,142; Sato et al., Serum albumin binding moieties, US 2003/0069395 A1; Jones et al., Pharmaceutical active conjugates, US Patent No. 6,342,225).

[0009] Fischer et al. described a peptide-immunoglobulin-conjugate, in which the immunoglobulin consisted of two heavy chains or two heavy chains and two light

chains, in which the immunoglobulin was not a functionable immunoglobulin (Fischer et al., A peptide-immunoglobulin conjugate, WO 2007/045463 A1).

[0010] The present invention provides immunoglobulins yielding exceptional uniformity and efficiency of recombinant expression, in vitro stability and non-aggregation, resistance to photodegradation and oxidation, non-cross-reactivity with human antigens, and good pharmacokinetic properties.

SUMMARY OF THE INVENTION

[0011] The invention relates to immunoglobulins, which are useful as carrier moieties. These immunoglobulins, including antibodies and antibody fragments, have reliable expression and purification characteristics, resulting in products that are stable and relatively uniform, and have outstanding pharmacokinetic (PK) properties in rats and cynomolgous monkeys. The inventive immunoglobulins have not been detected to bind to human proteins, cells or tissues. These immunoglobulins can also be used for many purposes, including, but not limited to, quality control or analytical standards for antibody-based drugs and as controls for biologically relevant isotype-matched antibodies.

[0012] Certain embodiments of the invention include an isolated immunoglobulin, comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

> the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323 [VH10] and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 [VL4] or SEQ ID NO:190 [VL5]; or
> the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:321 [VH9] and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 [VL4] or SEQ ID NO:190 [VL5]; or
> the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325 [VH11] and the light chain variable region comprises the amino acid sequence of SEQ ID NO:182 [VL1], SEQ ID NO:188 [VL4], or SEQ ID NO:190 [VL5].

Examples include antibodies # 16435, 16436, 16438, 16439, 16440, 16441, and 16444, disclosed in Table 2C. Typically, the inventive immunoglobulin at 30 micromolar concentration does not significantly bind soluble human IL-17R (SEQ ID NO:89) at 30 nanomolar concentration in an aqueous solution incubated under physiological conditions, e.g., as measured by a surface plasmon resonance binding

assay, as described herein.

[0013] Other embodiments of the invention include an isolated immunoglobulin, comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

the light chain variable region comprises the amino acid sequence of SEQ ID NO:196 [VL8] and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335 [VH16], SEQ ID NO:349 [VH23], SEQ ID NO:351 [VH24], SEQ ID NO:353 [VH25], SEQ ID NO:355 [VH26], or SEQ ID NO:359 [VH28]; or

the light chain variable region comprises the amino acid sequence of SEQ ID NO:204 [VL12] and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349 [VH23] or SEQ ID NO:355 [VH26]; or

the light chain variable region comprises the amino acid sequence of SEQ ID NO:202 [VL11] and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349 [VH23]; or

the light chain variable region comprises the amino acid sequence of SEQ ID NO:192 [VL6] and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:357 [VH27], SEQ ID NO:359 [VH28], or SEQ ID NO:369 [VH33]; or

the light chain variable region comprises the amino acid sequence of SEQ ID NO:194 [VL7] and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335 [VH16], SEQ ID NO:349 [VH23], or SEQ ID NO:351 [VH24].

Examples include antibodies #1961, 1962, 1963, 1964, 1965, 1966, 2323, 2324 2330, 4241, 4341, 10182, 10183, 10184, and 10188, disclosed in Table 2C. Typically, the inventive immunoglobulin at 10 micromolar concentration does not significantly bind soluble human TR2 (SEQ ID NO:82) at 10 nanomolar concentration in an aqueous solution incubated under physiological conditions, e.g., as measured by a surface plasmon resonance binding assay, as described herein.

[0014] In some embodiments, the immunoglobulin of the present invention is used as a carrier for pharmacologically active chemical moieties, e.g., small molecules, peptides, and/or proteins to enhance their PK properties. The pharmacologically active moieties can be conjugated, i.e., covalently bound, to the inventive immunoglobulin by a chemical conjugation reaction, or through recombinant genetic expression, they can be fused to the immunoglobulin.

[0015] The invention also provides materials and methods for producing such inventive immunoglobulins, including isolated nucleic acids that encode them, vectors and isolated host cells. Also provided are isolated nucleic acids encoding any of the immunoglobulin heavy and/or light chain sequences and/or VH and/or VL sequences. In a related embodiment, an expression vector comprising any of the aforementioned nucleic acids is provided. In still another embodiment, a host cell is provided comprising any of the aforementioned nucleic acids or expression vectors.

[0016] The inventive immunoglobulin can be used in the manufacture of a pharmaceutical composition or medicament. The inventive pharmaceutical composition or medicament comprises the immunoglobulin conjugated with a pharmacologically active agent, and a pharmaceutically acceptable diluent, carrier or excipient.

[0017] Numerous methods are contemplated in the present invention. For example, a method is provided involving culturing the aforementioned host cell comprising the expression vector of the invention such that the encoded immunoglobulin is expressed. Such methods can also comprise the step of recovering the immunoglobulin from the host cell culture. In a related embodiment, an isolated immunoglobulin produced by the aforementioned method is provided.

[0018] The foregoing summary is not intended to define every aspect of the invention, and additional aspects are described in other sections, such as the Detailed Description of Embodiments. The entire document is intended to be related as a unified disclosure, and it should be understood that all combinations of features

described herein are contemplated, even if the combination of features are not found together in the same sentence, or paragraph, or section of this document.

[0019] In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations defined by specific paragraphs above. For example, certain aspects of the invention that are described as a genus, and it should be understood that every member of a genus is, individually, an aspect of the invention. Also, aspects described as a genus or selecting a member of a genus, should be understood to embrace combinations of two or more members of the genus. Although the applicant(s) invented the full scope of the invention described herein, the applicants do not intend to claim subject matter described in the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1A-N shows schematic structures of some embodiments of a composition of the invention that include one or more units of a pharmacologically active toxin peptide analog (squiggle) fused, via an optional peptidyl linker moiety such as but not limited to L5 or L10 described herein, with one or more domains of an immunoglobulin. These schematics show a more typical IgG1, although they are intended to apply as well to IgG2s, which will have 4 disulfide bonds in the hinge and a different arrangement of the disulfide bond linking the heavy and light chain, and IgG3s and IgG4s. Figure 1A represents a monovalent heterodimeric Fc-toxin peptide analog fusion with the toxin peptide analog fused to the C-terminal end of one of the immunoglobulin Fc domain monomers. Figure 1B represents a bivalent homodimeric Fc-toxin peptide analog fusion, with toxin peptide analogs fused to the C-terminal ends of both of the immunoglobulin Fc domain monomers. Figure 1C represents a monovalent heterodimeric toxin peptide analog-Fc fusion with the toxin peptide analog fused to the N-terminal end of one of the immunoglobulin Fc domain monomers. Figure 1D represents a bivalent homodimeric toxin peptide analog-Fc fusion, with toxin peptide analogs fused to the N-terminal ends of both of the immunoglobulin Fc domain monomers. Figure 1E represents a monovalent heterotrimeric Fc-toxin peptide analog/Ab comprising an immunoglobulin heavy chain (HC) + immunoglobulin light chain (LC) + an immunoglobulin Fc monomer with a toxin peptide analog fused to its C-terminal end. Figure 1F represents a monovalent heterotetrameric (HT) antibody HC-toxin peptide analog fusion, with a toxin peptide analog fused to the C-terminal end of one of the HC monomers. Figure 1G represents a bivalent HT antibody Ab HC-toxin peptide analog fusion having toxin peptide analogs on the C-terminal ends of both HC monomers. Figure 1H represents a monovalent HT toxin peptide analog-LC Ab, with the toxin peptide analog fused to the N-terminal end of one of the LC monomers. Figure 1I represents a monovalent HT toxin peptide analog-HC Ab, with the toxin peptide analog fused to the N-terminal end of one of the HC monomers. Figure 1J represents a monovalent HT Ab LC-toxin peptide analog fusion (i.e., LC-toxin peptide analog

fusion + LC + 2(HC)), with the toxin peptide analog fused to the C-terminal end of one of the LC monomers. Figure 1K represents a bivalent HT Ab LC-toxin peptide analog fusion (i.e., 2(LC-toxin peptide analog fusion) + 2(HC)), with toxin peptide analogs fused to the C-terminal end of both of the LC monomers. Figure 1L represents a trivalent HT Ab LC-toxin peptide analog/HC-toxin peptide analog (i.e., 2(LC-toxin peptide analog fusion) + HC-toxin peptide analog fusion + HC), with the toxin peptide analogs fused to the C-terminal ends of both of the LC monomers and one of the HC monomers. Figure 1M represents a bivalent antibody with a toxin peptide analog moiety inserted into an internal loop of the immunoglobulin Fc domain of each HC monomer. Figure 1N represents a monovalent antibody with a toxin peptide analog moiety inserted into an internal loop of the immunoglobulin Fc domain of one of the HC monomers. Dimers or trimers will form spontaneously in certain host cells upon expression of a deoxyribonucleic acid (DNA) construct encoding a single chain. In other host cells, the cells can be placed in conditions favoring formation of dimers/trimers or the dimers/trimers can be formed in vitro. If more than one HC monomer, LC monomer, or immunoglobulin Fc domain monomer is part of a single embodiment, the individual monomers can be, if desired, identical or different from each other.

[0021] Figure 2A-B shows non-binding to IL17R by antibody embodiments of the present invention. Antibody 16429 was immobilized to a CM5 sensor chip, and 10 nM of IL-17R in the absence of antibody was used to establish the 100% binding signal of IL-17 that is free of antibody binding in solution. In Figure 2A, 10 nM, 100 nM and 1000 nM of indicated antibody samples were incubated with the 10 nM IL-17R to determine antibody binding in solution. In Figure 2B, 30,000 nM of the antibody samples were incubated with 30 nM IL-17R to determine antibody binding in solution. In Figure 2A-B, The decreased binding signal of IL-17R after the antibody incubation indicates the binding of the antibody to IL-17R in solution.

[0022] Figure 3 shows relative production of GRO-α by human foreskin fibroblasts, which were incubated with 5 ng/ml IL-17 and 0.1 μM, 1 μM, and 10 μM

of the indicated antibody samples. The conditioned cell medium was then assessed for GRO-α levels using a GRO-α sandwich ELISA.

[0023] Figure 4A shows representative elutions from two size exclusion columns in series (TSK-GEL G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) with a 100 mM sodium phosphate, 250 mM NaCl at pH 6.8 mobile phase flowed at 0.5 mL/min., of antibodies (top to bottom panels): 16435, 16436, 16439, 16440, 16441 and 16444.

[0024] Figure 4B shows a zoomed analysis of the size exclusion analysis shown in Figure 4A above, of antibodies (top to bottom panels): 16435, 16436, 16439, 16440, 16441 and 16444.

[0025] Figure 5 shows non-reducing analysis of 2 μg of antibodies 16435, 16436, 16437, 16438, 16439, 16440, 16429, 16430, 16433, 16434, 16441 and 16444 on 1.0 mm Tris-glycine 4-20% SDS-PAGEs (Novex) developed at 220V using non-reducing loading buffer and staining with QuickBlue (Boston Biologicals). Molecular weight markers are Novex SeeBlue® pre-stained standards.

[0026] Figure 6 shows reducing analysis of 2 μg of antibodies 16435, 16436, 16437, 16438, 16439, 16440, 16429, 16430, 16433, 16434, 16441 and 16444 on 1.0 mm Tris-glycine 4-20% SDS-PAGEs (Novex) developed at 220V using non-reducing loading buffer and staining with QuickBlue (Boston Biologicals). Molecular weight markers are Novex SeeBlue® pre-stained standards.

[0027] Figure 7A-B shows titers for antibodies 16435 and 16444, respectively. Expressing pools were created by transfecting CHO DHFR(-) host cells with corresponding HC and LC expression plasmid. Small scale (5-mL; Figure 7A) expression runs were conducted using a 6-day front-loaded process in CD 6-D assay media, while the large scale (3-L; Figure 7B) runs were completed using an 11-day fed-batch process with peptone medium. Titer levels were measured using a protein A HPLC based assay.

[0028] Figure 8A-B shows chomatograms of antibodies 16435 (Figure 8A) and 16444 (Figure 8B) eluted from a SP-HP sepharose column (GE Life Sciences) using a 20 column volume gradient to 50% S-Buffer B (20 mM acetic acid, 1 M NaCl, pH 5.0) at 7°C, measuring the absorbance at 300 nm.

[0029] Figure 9A-B shows analysis of the 16435 (Figure 9A) and 16444 (Figure 9B) antibodies on 1.0 mm Tris-glycine 4-20% SDS-PAGEs (Novex) developed at 220V staining with QuickBlue (Boston Biologicals). The lanes marked "NR" contained non-reducing sample buffer, while those in lanes marked "Red." contained reducing sample buffer.

[0030] Figure 10 shows zoomed size exclusion analysis, using two size exclusion columns in series (TSK-GEL G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) with a 100 mM sodium phosphate, 250 mM NaCl at pH 6.8 mobile phase flowed at 0.5 mL/min., of antibodies: 16435 and 16444.

[0031] Figure 11 shows an analysis of antibodies: an IgG2 monoclonal antibody comparator, 16444, and 16435, by DSC using a MicroCal VP-DSC where the samples were heated from 20°C to 95°C at a rate of 1°C per minute. The protein concentration was 0.5 mg/ml in 10 mM sodium acetate, 9% sucrose, pH 5.0.

[0032] Figure 12A-D shows an analysis of 16435 (Figure 12A-B) and 16444 (Figure 12C-D) antibodies by reducing (Figure 12A and Figure 12C) and non-reducing (Figure 12B and Figure 12D) CE-SDS with detection of absorbance at 220 nm. A bare-fused silica capillary 50 μm x 30.2 cm was used for the separation analysis.

[0033] Figure 13 shows size exclusion analysis of antibodies 16435 and 16444 after 3 days at room temperature covered in aluminum foil ("dark") or exposed to fluorescent light ("light"), eluted from two size exclusion columns in series (TSK-GEL G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) with a 100 mM sodium phosphate, 250 mM NaCl at pH 6.8 mobile phase flowed at 0.5 mL/min.

[0034] Figure 14A-D shows HIC analysis of the 16435 (Figure 14A and Figure 14C) and 16444 (Figure 14B and Figure 14D) antibodies, after 3 days at room temperature covered in aluminum foil ("dark", Figure 14A-B) or exposed to fluorescent light ("light", Figure 14C-D), using two Dionex ProPac HIC-10 columns in series with mobile phase A being 1 M ammonium sulfate, 20 mM sodium acetate, pH 5.0 and mobile phase B being 20 mM sodium acetate, 5% acetonitrile, pH 5.0. Samples were eluted at 0.8 ml/min with a 0 – 100% linear gradient over 50 minutes, measuring the absorbance at 220 nm.

[0035] Figure 15 shows binding of antibody to TRAIL (huTR2). Antibody 16449 was immobilized to a CM5 sensor chip, and 1 nM of TRAIL in the absence of antibody was used to establish the 100% binding signal of TRAIL that is free of antibody binding in solution. To determine antibody binding in solution, 7 pM to 10 nM of the antibody samples were incubated with the 1 nM TRAIL. The decreased binding signal of TRAIL after the antibody incubation indicates the binding of the antibody to TRAIL in solution.

[0036] Figure 16 shows non-binding to TRAIL (huTR2) by antibody embodiments of the present invention. Antibody 16449 was immobilized to a CM5 sensor chip, and 10 nM of TRAIL in the absence of antibody was used to establish the 100% binding signal of TRAIL that is free of antibody binding in solution. To determine antibody binding in solution, 50 and 1000 nM of the antibody samples were incubated with the 10 nM TRAIL. The decreased binding signal of TRAIL after the antibody incubation indicates the binding of the antibody to TRAIL in solution.

[0037] Figure 17 shows non-binding to TRAIL (huTR2) by antibody embodiments of the present invention. Antibody 16449 was immobilized to a CM5 sensor chip, and 10 nM of TRAIL in the absence of antibody was used to establish the 100% binding signal of TRAIL that is free of antibody binding in solution. To determine antibody binding in solution, 1000 nM of the antibody samples were incubated with the 10 nM TRAIL. The decreased binding signal of TRAIL after the antibody incubation indicates the binding of the antibody to TRAIL in solution.

[0038] Figure 18 shows non-binding to TRAIL (huTR2) by antibody embodiments of the present invention, listed on the y-axis. Antibody 16449 was immobilized to a CM5 sensor chip, and 10 nM of TRAIL in the absence of antibody was used to establish the 100% binding signal of TRAIL that is free of antibody binding in solution. To determine antibody binding in solution, 1000 and 10000 nM of the antibody samples were incubated with the 10 nM TRAIL. The decreased binding signal of TRAIL after the antibody incubation indicates the binding of the antibody to TRAIL in solution.

[0039] Figure 19 shows non-binding to TRAIL (huTR2) by antibody embodiments of the present invention, listed on the x-axis. Antibody 16449 was immobilized to a CM5 sensor chip, and 10 nM of TRAIL in the absence of antibody was used to establish the 100% binding signal of TRAIL that is free of antibody binding in solution. To determine antibody binding in solution, 50000 nM of the antibody samples were incubated with the 10 nM TRAIL. The decreased binding signal of TRAIL after the antibody incubation indicates the binding of the antibody to TRAIL in solution.

[0040] Figure 20A-B shows non-binding to TRAIL (huTR2) by antibody embodiments of the present invention, listed on the x-axis. Antibody 16449 was immobilized to a CM5 sensor chip, and 10 nM of TRAIL in the absence of antibody was used to establish the 100% binding signal of TRAIL that is free of antibody binding in solution. To determine antibody binding in solution, 1000, 10000 and 50000 nM of the antibody samples were incubated with the 10 nM TRAIL. The decreased binding signal of TRAIL after the antibody incubation indicates the binding of the antibody to TRAIL in solution.

[0041] Figure 20C shows results from an in vitro cell-based TRAIL activity assay. Samples of antibodies 4241 and 4341 were compared with positive control IgG1 anti-TR2 mAb 16449. The prepared antibody samples were added to TRAIL-sensitive human ascites colorectal adenocarcinoma cell line Colo205. The detection of TRAIL-mediated caspase-3 activation by measuring an increase in relative

luminescence was used as a positive marker for apoptosis. Antibodies 4241 and 4341 failed to activate caspase-3, unlike positive control antibody 16449.

[0042] Figure 21A shows non-reducing analysis of 2 μg of antibodies 1870 [aka 16451], 16449,16450, 10185, 10184, 4341, 10183 and 10182 on 1.0 mm Tris-glycine 4-20% SDS-PAGEs (Novex) developed at 220V using non-reducing loading buffer and staining with QuickBlue (Boston Biologicals). Molecular weight markers are Novex SeeBlue pre-stained standards. Molecular weight markers are Novex SeeBlue® pre-stained standards.

[0043] Figure 21B shows reducing analysis of 2 μg of antibodies 1870 [aka16451], 16449,16450, 10185, 10184, 4341, 10183 and 10182 on 1.0 mm Tris-glycine 4-20% SDS-PAGEs (Novex) developed at 220V using non-reducing loading buffer and staining with QuickBlue (Boston Biologicals). Molecular weight markers are Novex SeeBlue® pre-stained standards.

[0044] Figure 22A-F shows size exclusion chromatography on 50 μg of antibodies 4241(Figure 22A), 4341 (Figure 22B), 10182 (Figure 22C), 10183 (Figure 22D), 10184 (Figure 22E) , and 10185 (Figure 22F), injected on to a Phenomenex BioSep SEC-3000 column (7.8 x 300 mm) in 50 mM NaH_2PO_4, 250 mM NaCl, and pH 6.9 at 1 mL/min, measuring the absorbance at 280 nm.

[0045] Figure 23A-B shows titers for antibodies 4241 and 4341, respectively. Expressing pools were created by transfecting CHO DHFR(-) host cells with corresponding HC and LC expression plasmid. Small scale (5-mL; Figure 23A) expression runs were conducted using a 6-day front-loaded process in CD 6-D assay media, while the large scale (3-L; Figure 23B) runs were completed using an 11-day fed-batch process with peptone medium. Titer levels were measured using a protein A HPLC based assay.

[0046] Figure 24A-B shows reducing analysis of the in process samples for antibodies 4241 (Figure 24A) and 4341 (Figure 24B) on 1.0 mm Tris-glycine 4-20%

SDS-PAGEs (Novex) developed at 220V using non-reducing loading buffer and staining with QuickBlue (Boston Biologicals).

[0047] Figure 25 shows an overlay of the chomatograms of antibodies 4341 and 4241 on an SP-HP sepharose column (GE Life Sciences) eluted using a 20 column volume gradient to 50% S-Buffer B (20 mM acetic acid, 1 M NaCl, pH 5.0) at 7°C observing the absorbance at 300 nm.

[0048] Figure 26A-B shows analysis of the 4241 (Figure 26A) and 4341 (Figure 26B) antibodies on 1.0 mm Tris-glycine 4-20% SDS-PAGEs (Novex) developed at 220V staining with QuickBlue (Boston Biologicals). The lanes marked "NR" contained non-reducing sample buffer, while those in lanes marked "Red." contained reducing sample buffer.

[0049] Figure 27A-B shows full scale (Figure 27A) and zoomed (Figure 27B) analysis, using two size exclusion columns (TSK-GEL G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) in series with a 100 mM sodium phosphate, 250 mM NaCl at pH 6.8 mobile phase flowed at 0.5 mL/min., of antibodies: 4241 (upper panels) and 4341 (lower panels).

[0050] Figure 28 shows an analysis of antibodies 4341 and 4241 by DSC using a MicroCal VP-DSC where the samples were heated from 20°C to 95°C at a rate of 1°C per minute. The protein concentration was 0.5 mg/ml in 10 mM sodium acetate, 9% sucrose, pH 5.0.

[0051] Figure 29A-D shows an analysis of 4241 (Figure 29A-B) and 4341 (Figure 29C-D) antibodies by reducing (Figure 29A and Figure 29C) and non-reducing (Figure 29B and Figure 29D) CE-SDS with detection of absorbance at 220 nm. A bare-fused silica capillary 50 μm x 30.2 cm was used for the separation analysis.

[0052] Figure 30 shows analysis of the 4241 (upper panel) and 4341 (lower panel) antibodies using ion exchange HPLC (SP-5PW, 10 μm particle, 7.5 mm ID x 7.5 cm, TosohBioscience, 08541) using 20 mM acetic acid, pH 5.0 as buffer A and 20 mM

acetic acid, 1 M NaCl, pH 5.0 as buffer B flowed at 1 mL/min with an 80 minute linear gradient from 0 – 40% buffer B.

[0053] Figure 31A-B shows HIC analysis of the 4241 (Figure 31A) and 4341 (Figure 31B) antibodies, before and after light exposure, using two Dionex ProPac HIC-10 columns in series with mobile phase A being 1 M ammonium sulfate, 20 mM sodium acetate, pH 5.0 and mobile phase B being 20 mM sodium acetate, 5% acetonitrile, pH 5.0. Samples were eluted at 0.8 ml/min with a 0 – 100% linear gradient over 50 minutes observing the absorbance at 220 nm.

[0054] Figure 32 shows representative pharmacokinetic profiles of the 16435, 16444, 4241, and 4341 antibodies, as determined in adult Sprague-Dawley rats (8-12 weeks old) by injecting 5 mg/kg subcutaneously and collecting blood at 0, 0.25, 1, 4, 24, 48, 72, 96, 168, 336, 504, 672, 840 and 1008 hours post-dose from the lateral tail vein. Serum concentrations were then determined using an anti-human Fc based ELISA.

[0055] Figure 33 shows representative pharmacokinetic profiles of the 16435 antibody, as determined in male cynomolgus monkeys using a single IV dose at either 1 mg/kg or 10 mg/kg. Serum samples were collected pre-dose and at 0.25, 0.5, 1, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 360, 408, 456, 504, 552, 600, 648 and 672 hours after administration. Samples were assayed for 16435 antibody levels using an anti-IgG sandwich ELISA.

[0056] Figure 34 shows a schematic structural representation of one embodiment of a composition of the invention that includes one unit of a pharmacologically active toxin peptide analog (squiggle) fused, via an optional peptidyl linker moiety with one immunoglobulin.

[0057] Figure 35 shows a Coomassie brilliant blue-stained Tris-glycine 4-20% SDS-PAGE of final monovalent 16435 IgG2-L10-Shk[1-35, Q16K] products. Products were isolated from four different expression pools. Lanes 1-10 were loaded as follows: Novex Mark12 wide range protein standards (10 μl), 2 μg pool 1 product

non-reduced, 2 μg pool 2 product non-reduced, 2 μg pool 3 product non-reduced, 2 μg pool 4 product non-reduced, Novex Mark12 wide range protein standards (10 μl), 2 μg pool 1 product reduced, 2 μg pool 2 product reduced, 2 μg pool 3 product reduced, 2 μg pool 4 product reduced.

[0058] Figure 36A-D shows size exclusion chromatography on 30 μg of the final pool 1, 2, 3 & 4 of the 3742 product injected onto a Phenomenex BioSep SEC-3000 column (7.8 x 300 mm) equilibrated in 50 mM NaH2PO4, 250 mM NaCl, pH 6.9 at 1 ml/min, measuring the absorbance at 280 nm.

[0059] Figure 37A-D shows reduced light chain LC-MS analysis of the final 3742 samples. The product was chromatographed through a Waters MassPREP micro desalting column using a Waters ACQUITY UPLC system. The column was set at 80°C and the protein eluted using a linear gradient of increasing acetonitrile concentration in 0.1 % formic acid. The column effluent was directed into a Waters LCT Premier ESI-TOF mass spectrometer for mass analysis. The instrument was run in the positive V mode. The capillary voltage was set at 3,200 V and the cone voltage at 80 V. The mass spectrum was acquired from 800 to 3000 m/z and deconvoluted using the MaxEnt1 software provided by the instrument manufacturer.

[0060] Figure 38A-D shows reduced heavy chain LC-MS analysis of the final 3742 samples. The product was chromatographed through a Waters MassPREP micro desalting column using a Waters ACQUITY UPLC system. The column was set at 80°C and the protein eluted using a linear gradient of increasing acetonitrile concentration in 0.1 % formic acid. The column effluent was directed into a Waters LCT Premier ESI-TOF mass spectrometer for mass analysis. The instrument was run in the positive V mode. The capillary voltage was set at 3,200 V and the cone voltage at 80 V. The mass spectrum was acquired from 800 to 3000 m/z and deconvoluted using the MaxEnt1 software provided by the instrument manufacturer.

[0061] Figure 39A shows non-reducing analysis of the conditioned media of antibody fusions 10162, 10163 and 10164, along with the conditioned media from a mock transfection, on 1.0 mm Tris-glycine 4-20% SDS-PAGEs (Novex) developed

at 220V using non-reducing loading buffer and staining with QuickBlue (Boston Biologicals). Molecular weight markers are indicated in kDa.

[0062] Figure 39B shows a Coomassie brilliant blue stained Tris-glycine 4-20% SDS-PAGE of final 10162, 10163 & 10164 products. In lanes 1 & 5, Novex Mark 12 standards were loaded. For lanes 2-4 (non-reducing) and 6-8 (reducing), 2 μg of product was loaded.

[0063] Figure 40A-C shows size exclusion chromatography on 50 μg of fusion antibodies 10162 (Figure 40A), 10163 (Figure 40B), and 10164 (Figure 40C) injected on to a Phenomenex BioSep SEC-3000 column (7.8 x 300 mm) in 50 mM NaH_2PO_4, 250 mM NaCl, and pH 6.9 at 1 mL/min measuring the absorbance at 280 nm.

[0064] Figure 41A-C shows reduced light chain LC-MS analysis of the final 4341-ShK(1-35, Q16K) (Figure 41A), 4341-FGF21 (Figure 41B), and 16435-FGF21 (Figure 41C) samples. The product was chromatographed through a Waters MassPREP micro desalting column using a Waters ACQUITY UPLC system. The column was set at 80°C and the protein eluted using a linear gradient of increasing acetonitrile concentration in 0.1 % formic acid. The column effluent was directed into a Waters LCT Premier ESI-TOF mass spectrometer for mass analysis. The instrument was run in the positive V mode. The capillary voltage was set at 3,200 V and the cone voltage at 80 V. The mass spectrum was acquired from 800 to 3000 m/z and deconvoluted using the MaxEnt1 software provided by the instrument manufacturer.

[0065] Figure 42A-C shows reduced heavy chain LC-MS analysis of the final 4341-ShK (1-35, Q16K) (Figure 42A), 4341-FGF21 (Figure 42B), and 16435-FGF21 (Figure 42C) samples. The product was chromatographed through a Waters MassPREP micro desalting column using a Waters ACQUITY UPLC system. The column was set at 80°C and the protein eluted using a linear gradient of increasing acetonitrile concentration in 0.1 % formic acid. The column effluent was directed into a Waters LCT Premier ESI-TOF mass spectrometer for mass analysis. The

instrument was run in the positive V mode. The capillary voltage was set at 3,200 V and the cone voltage at 80 V. The mass spectrum was acquired from 800 to 3000 m/z and deconvoluted using the MaxEnt1 software provided by the instrument manufacturer.

[0066] Figure 43 shows representative PK profiles of antibodies 16435 and 4341 (both at 5 mg/kg dose) in SD rats.

[0067] Figure 44 shows representative PK profiles for sequential doses (5 mg/kg) of antibodies 16435 or 4341 in cynomolgus monkeys.

[0069] DETAILED DESCRIPTION OF EMBODIMENTS

[0070] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[0071] Definitions

[0072] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Thus, as used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the context clearly indicates otherwise. For example, reference to “a protein” includes a plurality of proteins; reference to “a cell” includes populations of a plurality of cells.

[0073] "Polypeptide” and "protein" are used interchangeably herein and include a molecular chain of two or more amino acids linked covalently through peptide bonds. The terms do not refer to a specific length of the product. Thus, "peptides," and "oligopeptides," are included within the definition of polypeptide. The terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. In addition, protein fragments, analogs, mutated or variant proteins, fusion proteins and the like are included within the meaning of polypeptide. The terms also include molecules in which one or more amino acid analogs or non-canonical or unnatural amino acids are included as can be expressed recombinantly using known protein engineering techniques. In addition, fusion proteins can be derivatized as described herein by well-known organic chemistry techniques.

[0074] The term “isolated protein” referred means that a subject protein (1) is free of at least some other proteins with which it would normally be found in nature, (2) is essentially free of other proteins from the same source, e.g., from the same species, (3) is expressed recombinantly by a cell of a heterologous species or kind, (4) has

been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature, (5) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature, and/or (6) does not occur in nature. Typically, an "isolated protein" constitutes at least about 5%, at least about 10%, at least about 25%, or at least about 50% of a given sample. Genomic DNA, cDNA, mRNA or other RNA, of synthetic origin, or any combination thereof may encode such an isolated protein. Preferably, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural environment that would interfere with its therapeutic, diagnostic, prophylactic, research or other use.

[0075] A "variant" of a polypeptide (e.g., an immunoglobulin, or an antibody) comprises an amino acid sequence wherein one or more amino acid residues are inserted into, deleted from and/or substituted into the amino acid sequence relative to another polypeptide sequence. Variants include fusion proteins.

[0076] The term "fusion protein" indicates that the protein includes polypeptide components derived from more than one parental protein or polypeptide. Typically, a fusion protein is expressed from a fusion gene in which a nucleotide sequence encoding a polypeptide sequence from one protein is appended in frame with, and optionally separated by a linker from, a nucleotide sequence encoding a polypeptide sequence from a different protein. The fusion gene can then be expressed by a recombinant host cell as a single protein.

[0077] A "secreted" protein refers to those proteins capable of being directed to the ER, secretory vesicles, or the extracellular space as a result of a secretory signal peptide sequence, as well as those proteins released into the extracellular space without necessarily containing a signal sequence. If the secreted protein is released into the extracellular space, the secreted protein can undergo extracellular processing to produce a "mature" protein. Release into the extracellular space can occur by many mechanisms, including exocytosis and proteolytic cleavage. In some other embodiments of the inventive composition, the toxin peptide analog can be

synthesized by the host cell as a secreted protein, which can then be further purified from the extracellular space and/or medium.

[0078] As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell is a protein that exists in aqueous solution; if the protein contains a twin-arginine signal amino acid sequence the soluble protein is exported to the periplasmic space in gram negative bacterial hosts, or is secreted into the culture medium by eukaryotic host cells capable of secretion, or by bacterial host possessing the appropriate genes (e.g., the kil gene). Thus, a soluble protein is a protein which is not found in an inclusion body inside the host cell. Alternatively, depending on the context, a soluble protein is a protein which is not found integrated in cellular membranes; in contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion body) in the host cell, or again depending on the context, an insoluble protein is one which is present in cell membranes, including but not limited to, cytoplasmic membranes, mitochondrial membranes, chloroplast membranes, endoplasmic reticulum membranes, etc.

[0079] "Soluble human IL-17R" is a polypeptide (huIL-17R-FpH) having the following amino acid sequence:
LRLLDHRALVCSQPGLNCTVKNSTCLDDSWIHPRNLTPSSPKDLQIQLHFAH
TQQGDLFPVAHIEWTLQTDASILYLEGAELSVLQLNTNERLCVRFEFLSKLR
HHHRRWRFTFSHFVVDPDQEYEVTVHHLPKPIPDGDPNHQSKNFLVPDCEH
ARMKVTTPCMSSGSLWDPNITVETLEAHQLRVSFTLWNESTHYQILLTSFPH
MENHSCFEHMHHIPAPRPEEFHQRSNVTLTLRNLKGCCRHQVQIQPFFSSCL
NDCLRHSATVSCPEMPDTPEPIPDYMPLWEPRSGSSDYKDDDDKGSSHHHH
HH// SEQ ID NO:89.

[0080] "Soluble human TR2" is a fusion polypeptide (huTR2 long-huFc (IgG1), in monomeric or dimeric form, having the following amino acid sequence:
MEQRGQNAPAASGARKRHGPGPREARGARPGPRVPKTLVLVVAAVLLLVS
AESALITQQDLAPQQRAAPQQKRSSPSEGLCPPGHHISEDGRDCISCKYGQDY

STHWNDLLFCLRCTRCDSGEVELSPCTTTRNTVCQCEEGTFREEDSPEMCRK
CRTGCPRGMVKVGDCTPWSDIECVHKESGTKHSGEAPAVEETVTSSPGTPAS
PCSLSGVDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCL
VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ
GNVFSCSVMHEALHNHYTQKSLSLSPGK// SEQ ID NO:82.

[0081] "Under physiological conditions" with respect to incubating buffers and immunoglobulins, or other binding assay reagents means incubation under conditions of temperature, pH, and ionic strength, that permit a biochemical reaction, such as a non-covalent binding reaction, to occur. Typically, the temperature is at room or ambient temperature up to about 37^{0}C and at pH 6.5-7.5.

[0082] The term "recombinant" indicates that the material (e.g., a nucleic acid or a polypeptide) has been artificially or synthetically (i.e., non-naturally) altered by human intervention. The alteration can be performed on the material within, or removed from, its natural environment or state. For example, a "recombinant nucleic acid" is one that is made by recombining nucleic acids, e.g., during cloning, DNA shuffling or other well known molecular biological procedures. Examples of such molecular biological procedures are found in Maniatis et al., Molecular Cloning. A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y(1982). A "recombinant DNA molecule," is comprised of segments of DNA joined together by means of such molecular biological techniques. The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule. A "recombinant host cell" is a cell that contains and/or expresses a recombinant nucleic acid.

[0083] The term "polynucleotide" or "nucleic acid" includes both single-stranded and double-stranded nucleotide polymers containing two or more nucleotide residues. The nucleotide residues comprising the polynucleotide can be

ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. Said modifications include base modifications such as bromouridine and inosine derivatives, ribose modifications such as 2',3'-dideoxyribose, and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate and phosphoroamidate.

[0084] The term "oligonucleotide" means a polynucleotide comprising 200 or fewer nucleotide residues. In some embodiments, oligonucleotides are 10 to 60 bases in length. In other embodiments, oligonucleotides are 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 nucleotides in length. Oligonucleotides may be single stranded or double stranded, e.g., for use in the construction of a mutant gene. Oligonucleotides may be sense or antisense oligonucleotides. An oligonucleotide can include a label, including a radiolabel, a fluorescent label, a hapten or an antigenic label, for detection assays. Oligonucleotides may be used, for example, as PCR primers, cloning primers or hybridization probes.

[0085] A "polynucleotide sequence" or "nucleotide sequence" or "nucleic acid sequence," as used interchangeably herein, is the primary sequence of nucleotide residues in a polynucleotide, including of an oligonucleotide, a DNA, and RNA, a nucleic acid, or a character string representing the primary sequence of nucleotide residues, depending on context. From any specified polynucleotide sequence, either the given nucleic acid or the complementary polynucleotide sequence can be determined. Included are DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Unless specified otherwise, the left-hand end of any single-stranded polynucleotide sequence discussed herein is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA transcript that are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences;" sequence regions on the DNA strand having the same sequence as the

RNA transcript that are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences."

[0086] As used herein, an "isolated nucleic acid molecule" or "isolated nucleic acid sequence" is a nucleic acid molecule that is either (1) identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid or (2) cloned, amplified, tagged, or otherwise distinguished from background nucleic acids such that the sequence of the nucleic acid of interest can be determined. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the immunoglobulin (e.g., antibody) where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

[0087] As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of ribonucleotides along the mRNA chain, and also determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the RNA sequence and for the amino acid sequence.

[0088] The term "gene" is used broadly to refer to any nucleic acid associated with a biological function. Genes typically include coding sequences and/or the regulatory sequences required for expression of such coding sequences. The term "gene" applies to a specific genomic or recombinant sequence, as well as to a cDNA or mRNA encoded by that sequence. A "fusion gene" contains a coding region that encodes a toxin peptide analog. Genes also include non-expressed nucleic acid segments that, for example, form recognition sequences for other proteins. Non-expressed regulatory sequences including transcriptional control elements to which

regulatory proteins, such as transcription factors, bind, resulting in transcription of adjacent or nearby sequences.

[0089] "Expression of a gene" or "expression of a nucleic acid" means transcription of DNA into RNA (optionally including modification of the RNA, e.g., splicing), translation of RNA into a polypeptide (possibly including subsequent post-translational modification of the polypeptide), or both transcription and translation, as indicated by the context.

[0090] As used herein the term "coding region" or "coding sequence" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of an mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (i.e., TAA, TAG, TGA).

[0091] The term "control sequence" or "control signal" refers to a polynucleotide sequence that can, in a particular host cell, affect the expression and processing of coding sequences to which it is ligated. The nature of such control sequences may depend upon the host organism. In particular embodiments, control sequences for prokaryotes may include a promoter, a ribosomal binding site, and a transcription termination sequence. Control sequences for eukaryotes may include promoters comprising one or a plurality of recognition sites for transcription factors, transcription enhancer sequences or elements, polyadenylation sites, and transcription termination sequences. Control sequences can include leader sequences and/or fusion partner sequences. Promoters and enhancers consist of short arrays of DNA that interact specifically with cellular proteins involved in transcription (Maniatis, et al., Science 236:1237 (1987)). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, i.e., promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some

eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review see Voss, et al., Trends Biochem. Sci., 11:287 (1986) and Maniatis, et al., Science 236:1237 (1987)).

[0092] The term "vector" means any molecule or entity (e.g., nucleic acid, plasmid, bacteriophage or virus) used to transfer protein coding information into a host cell.

[0093] The term "expression vector" or "expression construct" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid control sequences necessary for the expression of the operably linked coding sequence in a particular host cell. An expression vector can include, but is not limited to, sequences that affect or control transcription, translation, and, if introns are present, affect RNA splicing of a coding region operably linked thereto. Nucleic acid sequences necessary for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals. A secretory signal peptide sequence can also, optionally, be encoded by the expression vector, operably linked to the coding sequence of interest, so that the expressed polypeptide can be secreted by the recombinant host cell, for more facile isolation of the polypeptide of interest from the cell, if desired. Such techniques are well known in the art. (E.g., Goodey, Andrew R.; et al., Peptide and DNA sequences, U.S. Patent No. 5,302,697; Weiner et al., Compositions and methods for protein secretion, U.S. Patent No. 6,022,952 and U.S. Patent No. 6,335,178; Uemura et al., Protein expression vector and utilization thereof, U.S. Patent No. 7,029,909; Ruben et al., 27 human secreted proteins, US 2003/0104400 A1).

[0094] The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers

to the linkage of amino acid sequences in such a manner so that a functional protein is produced. For example, a control sequence in a vector that is "operably linked" to a protein coding sequence is ligated thereto so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences.

[0095] The term "host cell" means a cell that has been transformed, or is capable of being transformed, with a nucleic acid and thereby expresses a gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic make-up to the original parent cell, so long as the gene of interest is present. Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial host cells in culture include bacteria (such as Escherichia coli sp.), yeast (such as Saccharomyces sp.) and other fungal cells, insect cells, plant cells, mammalian (including human) cells, e.g., CHO cells and HEK-293 cells. Modifications can be made at the DNA level, as well. The peptide-encoding DNA sequence may be changed to codons more compatible with the chosen host cell. For E. coli, optimized codons are known in the art. Codons can be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected host cell. Next, the transformed host is cultured and purified. Host cells may be cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art.

[0096] The term "transfection" means the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been

introduced inside the cell membrane. A number of transfection techniques are well known in the art and are disclosed herein. See, e.g., Graham et al., 1973, Virology 52:456; Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, supra; Davis et al., 1986, Basic Methods in Molecular Biology, Elsevier; Chu et al., 1981, Gene 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

[0097] The term "transformation" refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain new DNA or RNA. For example, a cell is transformed where it is genetically modified from its native state by introducing new genetic material via transfection, transduction, or other techniques. Following transfection or transduction, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been "stably transformed" when the transforming DNA is replicated with the division of the cell.

[0098] By "physiologically acceptable salt" of a composition of matter, for example a salt of the immunoglobulin, such as an antibody, is meant any salt or salts that are known or later discovered to be pharmaceutically acceptable. Some non-limiting examples of pharmaceutically acceptable salts are: acetate; trifluoroacetate; hydrohalides, such as hydrochloride and hydrobromide; sulfate; citrate; maleate; tartrate; glycolate; gluconate; succinate; mesylate; besylate; salts of gallic acid esters (gallic acid is also known as 3,4, 5 trihydroxybenzoic acid) such as PentaGalloylGlucose (PGG) and epigallocatechin gallate (EGCG), salts of cholesteryl sulfate, pamoate, tannate and oxalate salts.

[0099] A "domain" or "region" (used interchangeably herein) of a protein is any portion of the entire protein, up to and including the complete protein, but typically comprising less than the complete protein. A domain can, but need not, fold independently of the rest of the protein chain and/or be correlated with a particular

biological, biochemical, or structural function or location (e.g., a ligand binding domain, or a cytosolic, transmembrane or extracellular domain).

[00100] "Treatment" or "treating" is an intervention performed with the intention of preventing the development or altering the pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" includes any indicia of success in the amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; diminishing of symptoms or making the injury, pathology or condition more tolerable to the patient; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating; improving a patient's physical or mental well-being. The treatment or amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination, self-reporting by a patient, neuropsychiatric exams, and/or a psychiatric evaluation.

[00101] An "effective amount" is generally an amount sufficient to reduce the severity and/or frequency of symptoms, eliminate the symptoms and/or underlying cause, prevent the occurrence of symptoms and/or their underlying cause, and/or improve or remediate the damage that results from or is associated with migraine headache. In some embodiments, the effective amount is a therapeutically effective amount or a prophylactically effective amount. A "therapeutically effective amount" is an amount sufficient to remedy a disease state (e.g., transplant rejection or GVHD, inflammation, multiple sclerosis, cancer, diabetes, neuropathy, pain) or symptom(s), particularly a state or symptom(s) associated with the disease state, or otherwise prevent, hinder, retard or reverse the progression of the disease state or any other undesirable symptom associated with the disease in any way whatsoever (i.e. that provides "therapeutic efficacy"). A "prophylactically effective amount" is an amount of a pharmaceutical composition that, when administered to a subject, will have the intended prophylactic effect, e.g., preventing or delaying the onset (or reoccurrence) of migraine headache or multiple sclerosis symptoms, or reducing the

likelihood of the onset (or reoccurrence) of migraine headache, migraine headache symptoms, or multiple sclerosis symptoms. The full therapeutic or prophylactic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, a therapeutically or prophylactically effective amount may be administered in one or more administrations.

[00102] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, rats, mice, monkeys, etc. Preferably, the mammal is human.

[00103] The term "naturally occurring" as used throughout the specification in connection with biological materials such as polypeptides, nucleic acids, host cells, and the like, refers to materials which are found in nature.

[00104] The term "antibody", or interchangeably "Ab",is used in the broadest sense and includes fully assembled antibodies, monoclonal antibodies (including human, humanized or chimeric antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments that can bind antigen (e.g., Fab, Fab', $F(ab')_2$, Fv, single chain antibodies, diabodies), comprising complementarity determining regions (CDRs) of the foregoing as long as they exhibit the desired biological activity. Multimers or aggregates of intact molecules and/or fragments, including chemically derivatized antibodies, are contemplated. Antibodies of any isotype class or subclass, including IgG, IgM, IgD, IgA, and IgE, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2, or any allotype, are contemplated. Different isotypes have different effector functions; for example, IgG1 and IgG3 isotypes have antibody-dependent cellular cytotoxicity (ADCC) activity.

[00105] The term "antigen binding protein" (ABP) includes antibodies or antibody fragments, as defined above, and recombinant peptides or other compounds that contain sequences derived from CDRs having the desired antigen-binding properties such that they specifically bind a target antigen of interest.

[00106] In general, an antigen binding protein, e.g., an antibody or antibody fragment, "specifically binds" to an antigen of interest (e.g., IL-17R or TR2) when it has a significantly higher binding affinity for, and consequently is capable of distinguishing, that antigen, compared to its affinity for other unrelated proteins, under similar binding assay conditions. Typically, an antigen binding protein is said to "specifically bind" its target antigen when the dissociation constant (K_D) is $\leq 10^{-8}$ M. The antibody specifically binds antigen with "high affinity" when the K_D is $\leq 5x\ 10^{-9}$ M, and with "very high affinity" when the K_D is $\leq 5x\ 10^{-10}$ M. In one embodiment, the antibodies will bind to the antigen of interest with a K_D of between about 10^{-8} M and 10^{-10} M, and in yet another embodiment the antibodies will bind with a $K_D \leq 5x\ 10^{-9}$.

[00107] "Antigen binding region" or "antigen binding site" means a portion of a protein, that specifically binds a specified antigen, e.g., IL-17R or TR2. For example, that portion of an antigen binding protein that contains the amino acid residues that interact with an antigen and confer on the antigen binding protein its specificity and affinity for the antigen is referred to as "antigen binding region." An antigen binding region typically includes one or more "complementary binding regions" ("CDRs"). Certain antigen binding regions also include one or more "framework" regions ("FRs"). A "CDR" is an amino acid sequence that contributes to antigen binding specificity and affinity. "Framework" regions can aid in maintaining the proper conformation of the CDRs to promote binding between the antigen binding region and an antigen. In a traditional antibody, the CDRs are embedded within a framework in the heavy and light chain variable region where they constitute the regions responsible for antigen binding and recognition. A variable region of an immunoglobulin antigen binding protein comprises at least three heavy or light chain CDRs, *see, supra* (Kabat *et al.*, 1991, *Sequences of Proteins of Immunological Interest*, Public Health Service N.I.H., Bethesda, MD; *see* also Chothia and Lesk, 1987, *J. Mol. Biol.* 196:901-917; Chothia *et al.*, 1989, *Nature* 342: 877-883), within a framework region (designated framework regions 1-4, FR1,

FR2, FR3, and FR4, by Kabat *et al.*, 1991, *supra*; *see* also Chothia and Lesk, 1987, *supra*).

[00108] An "isolated" immunoglobulin, e.g., an antibody or antibody fragment, is one that has been identified and separated from one or more components of its natural environment or of a culture medium in which it has been secreted by a producing cell. "Contaminant" components of its natural environment or medium are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody, and most preferably more than 99% by weight, or (2) to homogeneity by SDS-PAGE under reducing or nonreducing conditions, optionally using a stain, e.g., Coomassie blue or silver stain. Isolated naturally occurring antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Typically, however, isolated antibody will be prepared by at least one purification step.

[00109] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies that are antigen binding proteins are highly specific binders, being directed against an individual antigenic site or epitope, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different epitopes. Nonlimiting examples of monoclonal antibodies include murine, rabbit, rat, chicken, chimeric, humanized, or human antibodies, fully assembled antibodies, multispecific antibodies (including bispecific antibodies), antibody fragments that can bind an antigen (including, Fab, Fab', $F(ab')_2$, Fv, single chain antibodies, diabodies), maxibodies, nanobodies, and recombinant peptides comprising CDRs of the foregoing as long as they exhibit the desired biological activity, or variants or derivatives thereof.

[00110] The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., Nature, 256:495 [1975], or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature,352:624-628[1991] and Marks et al., J. Mol. Biol., 222:581-597 (1991), for example.

[00111] A "multispecific" binding agent or antigen binding protein or antibody is one that targets more than one antigen or epitope.

[00112] A "bispecific," "dual-specific" or "bifunctional" binding agent or antigen binding protein or antibody is a hybrid having two different antigen binding sites. Biantigen binding proteins, antigen binding proteins and antibodies are a species of multiantigen binding protein, antigen binding protein or multispecific antibody and may be produced by a variety of methods including, but not limited to, fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai and Lachmann, 1990, Clin. Exp. Immunol. 79:315-321; Kostelny et al., 1992, J. Immunol. 148:1547-1553. The two binding sites of a bispecific antigen binding protein or antibody will bind to two different epitopes, which may reside on the same or different protein targets.

[00113] The term "immunoglobulin" encompasses full antibodies comprising two dimerized heavy chains (HC), each covalently linked to a light chain (LC); a single undimerized immunoglobulin heavy chain and covalently linked light chain (HC + LC), or a chimeric immunoglobulin (light chain + heavy chain)-Fc heterotrimer (a so-called "hemibody"). An "immunoglobulin" is a protein, but is not necessarily an antigen binding protein.

[00114] In an "antibody", each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" chain of about 220 amino acids

(about 25 kDa) and one "heavy" chain of about 440 amino acids (about 50-70 kDa). The amino-terminal portion of each chain includes a "variable" ("V") region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. The variable region differs among different antibodies. The constant region is the same among different antibodies. Within the variable region of each heavy or light chain, there are three hypervariable subregions that help determine the antibody's specificity for antigen in the case of an antibody that is an antigen binding protein. However, within the scope of the present invention, an embodiment of the immunoglobulin, e.g., an antibody, need not be an antigen binding protein, or need not be known to specifically bind to an antigen. The variable domain residues between the hypervariable regions are called the framework residues and generally are somewhat homologous among different antibodies. Immunoglobulins can be assigned to different classes depending on the amino acid sequence of the constant domain of their heavy chains. Human light chains are classified as kappa (κ) and lambda (λ) light chains. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See generally, Fundamental Immunology, Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Within the scope of the invention, an "antibody" also encompasses a recombinantly made antibody, and antibodies that are glycosylated or lacking glycosylation.

[00115] The term "light chain" or "immunoglobulin light chain" includes a full-length light chain and fragments thereof having sufficient variable region sequence to confer binding specificity. A full-length light chain includes a variable region domain, V_L, and a constant region domain, C_L. The variable region domain of the light chain is at the amino-terminus of the polypeptide. Light chains include kappa chains and lambda chains.

[00116] The term "heavy chain" or "immunoglobulin heavy chain" includes a full-length heavy chain and fragments thereof having sufficient variable region sequence

to confer binding specificity. A full-length heavy chain includes a variable region domain, V_H, and three constant region domains, C_H1, C_H2, and C_H3. The V_H domain is at the amino-terminus of the polypeptide, and the C_H domains are at the carboxyl-terminus, with the C_H3 being closest to the carboxy-terminus of the polypeptide. Heavy chains are classified as mu (μ), delta (Δ), gamma (γ), alpha (α), and epsilon (ε), and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. In separate embodiments of the invention, heavy chains may be of any isotype, including IgG (including IgG1, IgG2, IgG3 and IgG4 subtypes), IgA (including IgA1 and IgA2 subtypes), IgM and IgE. Several of these may be further divided into subclasses or isotypes, e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. Different IgG isotypes may have different effector functions (mediated by the Fc region), such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, the Fc region of an antibody binds to Fc receptors (FcγRs) on the surface of immune effector cells such as natural killers and macrophages, leading to the phagocytosis or lysis of the targeted cells. In CDC, the antibodies kill the targeted cells by triggering the complement cascade at the cell surface.

[00117] An “Fc region”, or used interchangeably herein, “Fc domain” or “immunoglobulin Fc domain”, contains two heavy chain fragments, which in a full antibody comprise the C_H1 and C_H2 domains of the antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the C_H3 domains.

[00118] The term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

[00119] “Allotypes” are variations in antibody sequence, often in the constant region, that can be immunogenic and are encoded by specific alleles in humans. Allotypes have been identified for five of the human IGHC genes, the IGHG1, IGHG2, IGHG3, IGHA2 and IGHE genes, and are designated as G1m, G2m, G3m,

A2m, and Em allotypes, respectively. At least 18 Gm allotypes are known: nG1m(1), nG1m(2), G1m (1, 2, 3, 17) or G1m (a, x, f, z), G2m (23) or G2m (n), G3m (5, 6, 10, 11, 13, 14, 15, 16, 21, 24, 26, 27, 28) or G3m (b1, c3, b5, b0, b3, b4, s, t, g1, c5, u, v, g5). There are two A2m allotypes A2m(1) and A2m(2).

[00120] For a detailed description of the structure and generation of antibodies, see Roth, D.B., and Craig, N.L., *Cell*, 94:411-414 (1998), herein incorporated by reference in its entirety. Briefly, the process for generating DNA encoding the heavy and light chain immunoglobulin sequences occurs primarily in developing B-cells. Prior to the rearranging and joining of various immunoglobulin gene segments, the V, D, J and constant (C) gene segments are found generally in relatively close proximity on a single chromosome. During B-cell-differentiation, one of each of the appropriate family members of the V, D, J (or only V and J in the case of light chain genes) gene segments are recombined to form functionally rearranged variable regions of the heavy and light immunoglobulin genes. This gene segment rearrangement process appears to be sequential. First, heavy chain D-to-J joints are made, followed by heavy chain V-to-DJ joints and light chain V-to-J joints. In addition to the rearrangement of V, D and J segments, further diversity is generated in the primary repertoire of immunoglobulin heavy and light chains by way of variable recombination at the locations where the V and J segments in the light chain are joined and where the D and J segments of the heavy chain are joined. Such variation in the light chain typically occurs within the last codon of the V gene segment and the first codon of the J segment. Similar imprecision in joining occurs on the heavy chain chromosome between the D and J_H segments and may extend over as many as 10 nucleotides. Furthermore, several nucleotides may be inserted between the D and J_H and between the V_H and D gene segments which are not encoded by genomic DNA. The addition of these nucleotides is known as N-region diversity. The net effect of such rearrangements in the variable region gene segments and the variable recombination which may occur during such joining is the production of a primary antibody repertoire.

[00121] The term "hypervariable" region refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a complementarity determining region or CDR [i.e., residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain as described by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)]. Even a single CDR may recognize and bind antigen, although with a lower affinity than the entire antigen binding site containing all of the CDRs.

[00122] An alternative definition of residues from a hypervariable "loop" is described by Chothia et al., *J. Mol.Biol. 196*: 901-917 (1987) as residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain .

[00123] "Framework" or "FR" residues are those variable region residues other than the hypervariable region residues.

[00124] "Antibody fragments" comprise a portion of an intact full length antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng.,8(10):1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[00125] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment which contains the constant region. The Fab fragment contains all of the variable domain, as well as the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. The Fc fragment displays carbohydrates and is responsible for many antibody effector functions (such as binding complement and cell receptors), that distinguish one class of antibody from another.

[00126] Pepsin treatment yields an $F(ab')_2$ fragment that has two "Single-chain Fv" or "scFv" antibody fragments comprising the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Fab fragments differ from Fab' fragments by the inclusion of a few additional residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains that enables the Fv to form the desired structure for antigen binding. For a review of scFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. l 13, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[00127] A "Fab fragment" is comprised of one light chain and the C_H1 and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule.

[00128] A "Fab' fragment" contains one light chain and a portion of one heavy chain that contains the V_H domain and the C_H1 domain and also the region between the C_H1 and C_H2 domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form an $F(ab')_2$ molecule.

[00129] A "$F(ab')_2$ fragment" contains two light chains and two heavy chains containing a portion of the constant region between the C_H1 and C_H2 domains, such that an interchain disulfide bond is formed between the two heavy chains. A $F(ab')_2$ fragment thus is composed of two Fab' fragments that are held together by a disulfide bond between the two heavy chains.

[00130] "Fv" is the minimum antibody fragment that contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the VH VL dimer. A single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability

to recognize and bind antigen, although at a lower affinity than the entire binding site.

[00131] "Single-chain antibodies" are Fv molecules in which the heavy and light chain variable regions have been connected by a flexible linker to form a single polypeptide chain, which forms an antigen-binding region. Single chain antibodies are discussed in detail in International Patent Application Publication No. WO 88/01649 and United States Patent No. 4,946,778 and No. 5,260,203, the disclosures of which are incorporated by reference in their entireties.

[00132] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain, and optionally comprising a polypeptide linker between the V_H and V_L domains that enables the Fv to form the desired structure for antigen binding (Bird et al., *Science* 242:423-426, 1988, and Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988). An "Fd" fragment consists of the V_H and C_H1 domains.

[00133] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

[00134] A "domain antibody" is an immunologically functional immunoglobulin fragment containing only the variable region of a heavy chain or the variable region of a light chain. In some instances, two or more V_H regions are covalently joined with a peptide linker to create a bivalent domain antibody. The two V_H regions of a bivalent domain antibody may target the same or different antigens.

[00135] The term "compete" when used in the context of antigen binding proteins (e.g., neutralizing antigen binding proteins or neutralizing antibodies) that compete for the same epitope means competition between antigen binding proteins is determined by an assay in which the antigen binding protein (e.g., antibody or immunologically functional fragment thereof) under test prevents or inhibits specific binding of a reference antigen binding protein (e.g., a ligand, or a reference antibody) to a common antigen (e.g., IL-17R or a fragment thereof, or TR2 or a fragment thereof). Numerous types of competitive binding assays can be used, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see, e.g., Stahli et al., 1983, Methods in Enzymology 9:242-253); solid phase direct biotin-avidin EIA (see, e.g., Kirkland et al., 1986, J. Immunol. 137:3614-3619) solid phase direct labeled assay, solid phase direct labeled sandwich assay (see, e.g., Harlow and Lane, 1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Press); solid phase direct label RIA using I-125 label (see, e.g., Morel et al., 1988, Molec. Immunol. 25:7-15); solid phase direct biotin-avidin EIA (see, e.g., Cheung, et al., 1990, Virology 176:546-552); direct labeled RIA (Moldenhauer et al., 1990, Scand. J. Immunol. 32:77-82); and surface plasmon resonance (BIAcore®; e.g., Fischer et al., A peptide-immunoglobulin-conjugate, WO 2007/045463 A1, Example 10, which is incorporated herein by reference in its entirety), or KinExA. Typically, such an assay involves the use of purified antigen bound to a solid surface or cells bearing either of these, an unlabelled test immunoglobulin or antigen binding protein and a labeled reference antigen binding protein. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test antigen binding protein. Usually the test immunoglobulin or antigen binding protein is present in excess. Antigen binding proteins identified by competition assay (competing antigen binding proteins) include antigen binding proteins binding to the same epitope as the reference antigen binding proteins and antigen binding proteins binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference antigen binding protein for steric hindrance to occur. Additional details regarding methods for determining competitive binding are provided in the examples

herein. Usually, when a competing antigen binding protein is present in excess, it will inhibit specific binding of a reference antigen binding protein to a common antigen by at least 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75%. In some instance, binding is inhibited by at least 80%, 85%, 90%, 95%, or 97% or more.

[00136] When an immunoglobulin (e.g., an antibody or antibody fragment) "does not significantly bind" an antigen it means that the particular immunoglobulin, in excess, does not compete with a reference antigen binding protein, e.g., with a positive control antibody, to inhibit its binding to the target antigen by > 39%, or > 30%, or >20%, or > 10%. As to specific binding to soluble human IL-17R, a positive control antibody is antibody 16429, described herein. As to specific binding to soluble human TR2, a positive control antibody is antibody 16449, described herein.

[00137] Antibody-antigen interactions can be characterized by the association rate constant in $M^{-1}s^{-1}$ (k_a), or the dissociation rate constant in s^{-1} (k_d), or alternatively the dissociation equilibrium constant in M (K_D). Association rate constants, dissociation rate constants, or dissociation equilibrium constants may be readily determined using kinetic analysis techniques such as surface plasmon resonance (BIAcore®; e.g., Fischer et al., A peptide-immunoglobulin-conjugate, WO 2007/045463 A1, Example 10, which is incorporated herein by reference in its entirety), or KinExA using general procedures outlined by the manufacturer or other methods known in the art. The kinetic data obtained by BIAcore® or KinExA may be analyzed by methods described by the manufacturer.

[00138] "Measured by a surface plasmon resonance binding assay" with respect to determining whether a test immunoglobulin "does not significantly bind" means as measured in the solution equilibrium binding assay described herein to assess the binding activity of immunoglobulins based on surface plasmon resonance. A reference antigen binding protein (e.g., Antibody 16429 for human IL-17R or Antibody 16449 for human TR2) is immobilized to a BIACore® 2000, research grade sensor chip CM5 surface according to manufacturer's instructions (BIACore,

Inc., Piscataway, NJ). Carboxyl groups on the sensor chip surfaces are activated by injecting 60 μL of a mixture containing 0.2 M N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS). The reference antigen binding protein is diluted in 10 mM sodium acetate, pH 4.0 and injected over the activated chip surface at 30 μL/min for 6 minutes. Excess reactive groups on the surfaces are deactivated by injecting 60 μL of 1 M ethanolamine. The final immobilized level is typically approximately 6600 resonance units (RU). Soluble target antigen (e.g., 10 nM of soluble human IL-17R or 30 nM of soluble human TR2) in the absence of soluble antigen binding protein (e.g., antibody) is used to establish the 100% binding signal to the fixed reference antigen binding protein (e.g., the positive control anbibody). The decreased binding signal of the target antigen after incubation of the test immunoglobulin indicates its level of binding to the target antigen in solution.

[00139] The term "antigen" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antigen binding protein (including, e.g., an antibody or immunological functional fragment thereof), and additionally capable of being used in an animal to produce antibodies capable of binding to that antigen. An antigen may possess one or more epitopes that are capable of interacting with different antigen binding proteins, e.g., antibodies.

[00140] The term "epitope" is the portion of a molecule that is bound by an antigen binding protein (for example, an antibody). The term includes any determinant capable of specifically binding to an antigen binding protein, such as an antibody or to a T-cell receptor. An epitope can be contiguous or non-contiguous (e.g., in a single-chain polypeptide, amino acid residues that are not contiguous to one another in the polypeptide sequence but that within the context of the molecule are bound by the antigen binding protein). In certain embodiments, epitopes may be mimetic in that they comprise a three dimensional structure that is similar to an epitope used to generate the antigen binding protein, yet comprise none or only some of the amino acid residues found in that epitope used to generate the antigen binding protein. Most often, epitopes reside on proteins, but in some instances may reside on other

kinds of molecules, such as nucleic acids. Epitope determinants may include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl or sulfonyl groups, and may have specific three dimensional structural characteristics, and/or specific charge characteristics. Generally, antibodies specific for a particular target antigen will preferentially recognize an epitope on the target antigen in a complex mixture of proteins and/or macromolecules.

[00141] The term "identity" refers to a relationship between the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by aligning and comparing the sequences. "Percent identity" means the percent of identical residues between the amino acids or nucleotides in the compared molecules and is calculated based on the size of the smallest of the molecules being compared. For these calculations, gaps in alignments (if any) must be addressed by a particular mathematical model or computer program (i.e., an "algorithm"). Methods that can be used to calculate the identity of the aligned nucleic acids or polypeptides include those described in Computational Molecular Biology, (Lesk, A. M., ed.), 1988, New York: Oxford University Press; Biocomputing Informatics and Genome Projects, (Smith, D. W., ed.), 1993, New York: Academic Press; Computer Analysis of Sequence Data, Part I, (Griffin, A. M., and Griffin, H. G., eds.), 1994, New Jersey: Humana Press; von Heinje, G., 1987, Sequence Analysis in Molecular Biology, New York: Academic Press; Sequence Analysis Primer, (Gribskov, M. and Devereux, J., eds.), 1991, New York: M. Stockton Press; and Carillo et al., 1988, SIAM J. Applied Math. 48:1073. For example, sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. Using a computer program such as BLAST or FASTA, two polypeptide or two polynucleotide sequences are aligned for optimal matching of their respective residues (either along the full length of one or both sequences, or along a pre-determined portion of one or both sequences). The programs provide a default opening penalty and a default gap penalty, and a scoring matrix such as PAM 250 [a standard scoring matrix; see Dayhoff et al., in *Atlas of Protein Sequence and*

Structure, vol. 5, supp. 3 (1978)] can be used in conjunction with the computer program. For example, the percent identity can then be calculated as: the total number of identical matches multiplied by 100 and then divided by the sum of the length of the longer sequence within the matched span and the number of gaps introduced into the longer sequences in order to align the two sequences. In calculating percent identity, the sequences being compared are aligned in a way that gives the largest match between the sequences.

[00142] The GCG program package is a computer program that can be used to determine percent identity, which package includes GAP (Devereux et al., 1984, Nucl. Acid Res. 12:387; Genetics Computer Group, University of Wisconsin, Madison, WI). The computer algorithm GAP is used to align the two polypeptides or two polynucleotides for which the percent sequence identity is to be determined. The sequences are aligned for optimal matching of their respective amino acid or nucleotide (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3x the average diagonal, wherein the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. In certain embodiments, a standard comparison matrix (see, Dayhoff et al., 1978, Atlas of Protein Sequence and Structure 5:345-352 for the PAM 250 comparison matrix; Henikoff et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10915-10919 for the BLOSUM 62 comparison matrix) is also used by the algorithm.

[00143] Recommended parameters for determining percent identity for polypeptides or nucleotide sequences using the GAP program include the following:

[00144] Algorithm: Needleman et al., 1970, J. Mol. Biol. 48:443-453;

[00145] Comparison matrix: BLOSUM 62 from Henikoff et al., 1992, supra;

[00146] Gap Penalty: 12 (but with no penalty for end gaps)

[00147] Gap Length Penalty: 4

[00148] Threshold of Similarity: 0

[00149] Certain alignment schemes for aligning two amino acid sequences may result in matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full-length sequences. Accordingly, the selected alignment method (GAP program) can be adjusted if so desired to result in an alignment that spans at least 50 contiguous amino acids of the target polypeptide.

[00150] The term "modification" when used in connection with immunoglobulins, including antibodies and antibody fragments, of the invention, include, but are not limited to, one or more amino acid changes (including substitutions, insertions or deletions); chemical modifications; covalent modification by conjugation to therapeutic or diagnostic agents; labeling (e.g., with radionuclides or various enzymes); covalent polymer attachment such as PEGylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of non-natural amino acids. Modified immunoglobulins of the invention will retain the binding (or non-binding) properties of unmodified molecules of the invention.

[00151] The term "derivative" when used in connection with immunoglobulins (including antibodies and antibody fragments) of the invention refers to immunoglobulins that are covalently modified by conjugation to therapeutic or diagnostic agents, labeling (e.g., with radionuclides or various enzymes), covalent polymer attachment such as PEGylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of non-natural amino acids. Derivatives of the invention will retain the binding properties of underivatized molecules of the invention.

[00152] Embodiments of Immunoglobulins

[00153] In full-length immunoglobulin light and heavy chains, the variable and constant regions are joined by a "J" region of about twelve or more amino acids, with the heavy chain also including a "D" region of about ten more amino acids. *See, e.g.,* Fundamental Immunology, 2nd ed., Ch. 7 (Paul, W., ed.) 1989, New York: Raven Press (hereby incorporated by reference in its entirety for all purposes). The variable regions of each light/heavy chain pair typically form the antigen binding site.

[00154] One example of a human IgG2 heavy chain (HC) constant domain has the amino acid sequence:

[00155] ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTV ERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKC KVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK// SEQ. ID NO:86.

[00156] Constant region sequences of other IgG isotypes are known in the art for making recombinant versions of the inventive immunoglobulin having an IgG1, IgG2, IgG3, or IgG4 immunoglobulin isotype, if desired. In general, human IgG2 can be used for targets where effector functions are not desired, and human IgG1 in situations where such effector functions (e.g., antibody-dependent cytotoxicity (ADCC)) are desired. Human IgG3 has a relatively short half life and human IgG4 forms antibody "half-molecules." There are four known allotypes of human IgG1. The preferred allotype is referred to as "hIgG1z", also known as the "KEEM" allotype. Human IgG1 allotypes "hIgG1za" (KDEL), "hIgG1f" (REEM), and "hIgG1fa" are also useful; all appear to have ADCC effector function.

[00157] Human hIgG1z heavy chain (HC) constant domain has the amino acid sequence:

[00158] ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK// SEQ ID NO:87.

[00159] Human hIgG1za heavy chain (HC) constant domain has the amino acid sequence:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK// SEQ ID NO:88.

[00160] Human hIgG1f heavy chain (HC) constant domain has the amino acid sequence:

[00161] ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK// SEQ ID NO:127.

[00162] Human hIgG1fa heavy chain (HC) constant domain has the amino acid sequence:

[00163] ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK// SEQ ID NO:90.

[00164] One example of a human immunoglobulin light chain (LC) constant region sequence is the following (designated "CL-1"):

[00165] GQPKANPTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKAD GSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTV EKTVAPTECS// SEQ ID NO:91.

[00166] CL-1 is useful to increase the pI of antibodies and is convenient. There are three other human immunoglobulin light chain constant regions, designated "CL-2", "CL-3" and "CL-7", which can also be used within the scope of the present invention. CL-2 and CL-3 are more common in the human population.

[00167] CL-2 human light chain (LC) constant domain has the amino acid sequence:

Gqpkaapsvtlfppsseelqankatlvclisdfypgavtvawkadsspvkagvetttpskqsnnkyaassylsltpeq wkshrsyscqvthegstvektvaptecs// SEQ ID NO:92.

[00168] CL-3 human LC constant domain has the amino acid sequence:

gqpkaapsvtlfppsseelqankatlvclisdfypgavtvawkadsspvkagvetttpskqsnnkyaassylsltpeq wkshksyscqvthegstvektvaptecs// SEQ ID NO:93.

[00169] CL-7 human LC constant domain has the amino acid sequence:

[00170] Gqpkaapsvtlfppsseelqankatlvclvsdfypgavtvawkadgspvkvgvettkpskqsnnky aassylsltpeqwkshrsyscrvthegstvektvapaecs// SEQ ID NO:94.

[00171] Human LC kappa constant region has the amino acid sequence:

[00172] RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP VTKSFNRGEC// ESQ ID NO:129.

[00173] Variable regions of immunoglobulin chains generally exhibit the same overall structure, comprising relatively conserved framework regions (FR) joined by three hypervariable regions, more often called "complementarity determining regions" or CDRs. The CDRs from the two chains of each heavy chain/light chain pair mentioned above typically are aligned by the framework regions to form a structure that binds specifically with a specific epitope or domain on the target (*e.g.*, human IL-17R or human TR2), however within the scope of the present invention, the original CDR sequences have been deliberately modified so as not significantly to bind to human IL-17R or TR2 targets. From N-terminal to C-terminal, naturally-occurring light and heavy chain variable regions both typically conform with the following order of these elements: FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. A numbering system has been devised for assigning numbers to amino acids that occupy positions in each of these domains. This numbering system is defined in Kabat Sequences of Proteins of Immunological Interest (1987 and 1991, NIH, Bethesda, MD), or Chothia & Lesk, 1987, *J. Mol. Biol.* 196:901-917; Chothia *et al.*, 1989, *Nature* 342:878-883.

[00174] Specific examples of some of the full length light and heavy chains of the antibodies that are provided and their corresponding amino acid sequences are summarized in Table 1A and Table 1B below. Table 1A shows exemplary light chain sequences. Table 1B shows exemplary heavy chain sequences, some of which include constant region human IgG2 (SEQ ID NO:86) and some of which include constant region human IgG1f (SEQ ID NO:127). However, encompassed within the present invention are immunoglobulins with sequence changes in the constant or framework regions of those listed in Table 1A and/or Table 1B (e.g. IgG4 vs IgG2, CL2 vs CL1). Also, signal peptide (SP) sequences for all of the sequence in Table

1A and Table 1B are included, such as, the VK-1 SP signal peptide:
MDMRVPAQLLGLLLLWLRGARC (SEQ ID NO:103),
MEAPAQLLFLLLLWLPDTTG (SEQ ID NO:104),
MEWTWRVLFLVAAATGAHS (SEQ ID NO:105),
METPAQLLFLLLLWLPDTTG (SEQ ID NO:106),

[00175] MKHLWFFLLLVAAPRWVLS (SEQ ID NO:107), but any other suitable signal peptide sequence may be employed within the scope of the invention. Another example of a useful signal peptide sequence is VH21 SP MEWSWVFLFFLSVTTGVHS (SEQ ID NO:95). Other exemplary signal peptide sequences are shown in Table 1A-B.

Table 1A. Immunoglobulin Light Chain Sequences. Signal peptide sequences are indicated by underline.

SEQ ID NO:	Designation	Sequence
109	16435 (LC: P66L, D90E)	MEAPAQLLFLLLLWLPDTTGEIVMTQSPATLSV SPGERATLSCRASQSVSSNLAWFQQKPGQAPR LLIYDASTRATGVPARFSGSGSGTEFTLTISSLQ SEDFAVYYCQQYDNWPLTFGGGTKVEIKRTVA APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSS TLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
110	16435 (LC: P66L, D90E)	EIVMTQSPATLSVSPGERATLSCRASQSVSSNL AWFQQKPGQAPRLLIYDASTRATGVPARFSGS GSGTEFTLTISSLQSEDFAVYYCQQYDNWPLTF GGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESV TEQDSKDSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC
121	16444 (LC: P66L, D90E, W114A)	MEAPAQLLFLLLLWLPDTTGEIVMTQSPATLSV SPGERATLSCRASQSVSSNLAWFQQKPGQAPR LLIYDASTRATGVPARFSGSGSGTEFTLTISSLQ SEDFAVYYCQQYDNAPLTFGGGTKVEIKRTVA APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSS

		TLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
122	16444 (LC: P66L, D90E, W114A)	EIVMTQSPATLSVSPGERATLSCRASQSVSSNL AWFQQKPGQAPRLLIYDASTRATGVPARFSGS GSGTEFTLTISSLQSEDFAVYYCQQYDNAPLTF GGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESV TEQDSKDSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC
97	4241 (LC:Y53A)	METPAQLLFLLLLWLPDTTGEIVLTQSPGTLSLS PGERATLSCRASQGISRSALAWYQQKPGQAPSL LIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPE DFAVYYCQQFGSSPWTFGQGTKVEIKRTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFN RGEC
98	4241 (LC:Y53A)	EIVLTQSPGTLSLSPGERATLSCRASQGISRSAL AWYQQKPGQAPSLLIYGASSRATGIPDRFSGSG SGTDFTLTISRLEPEDFAVYYCQQFGSSPWTFG QGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQSGNSQESVT EQDSKDSTYSLSSTLTLSKADYEKHKVYACEV THQGLSSPVTKSFNRGEC
115	4341 (LC:Y53E)	METPAQLLFLLLLWLPDTTGEIVLTQSPGTLSLS PGERATLSCRASQGISRSELAWYQQKPGQAPSL LIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPE DFAVYYCQQFGSSPWTFGQGTKVEIKRTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFN RGEC
116	4341 (LC:Y53E)	EIVLTQSPGTLSLSPGERATLSCRASQGISRSELA WYQQKPGQAPSLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQFGSSPWTFGQ GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVC LLNNFYPREAKVQWKVDNALQSGNSQESVTE QDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC

Table 1B. Immunoglobulin Heavy Chain Sequences. Signal peptide sequences are indicated by underline.

SEQ ID NO:	Designation	Sequence
112	16435 (HC:R118A)	MEWTWRVLFLVAAATGAHSQVQLVQSGA EVKKPGASVKVSCKASGYTFTRYGISWVRQ APGQGLEWMGWISTYSGNTNYAQKLQGRV TMTTDTSTSTAYMELRSLRSDDTAVYYCAR AQLYFDYWGQGTLVTVSSASTKGPSVFPLA PCSRSTSESTAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVVTVPSS NFGTQTYTCNVDHKPSNTKVDKTVERKCC VECPPCPAPPVAGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVQFNWYVDGVEVH NAKTKPREEQFNSTFRVVSVLTVVHQDWL NGKEYKCKVSNKGLPAPIEKTISKTKGQPRE PQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPMLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
113	16435 (HC:R118A)	QVQLVQSGAEVKKPGASVKVSCKASGYTF TRYGISWVRQAPGQGLEWMGWISTYSGNT NYAQKLQGRVTMTTDTSTSTAYMELRSLRS DDTAVYYCARAQLYFDYWGQGTLVTVSSA STKGPSVFPLAPCSRSTSESTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSNFGTQTYTCNVDHKPSNT KVDKTVERKCCVECPPCPAPPVAGPSVFLFP PKPKDTLMISRTPEVTCVVVDVSHEDPEVQ FNWYVDGVEVHNAKTKPREEQFNSTFRVV SVLTVVHQDWLNGKEYKCKVSNKGLPAPI EKTISKTKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKT TPPMLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK
124	16444 (HC: R118A, L120Q)	MEWTWRVLFLVAAATGAHSQVQLVQSGA EVKKPGASVKVSCKASGYTFTRYGISWVRQ APGQGLEWMGWISTYSGNTNYAQKLQGRV TMTTDTSTSTAYMELRSLRSDDTAVYYCAR AQQYFDYWGQGTLVTVSSASTKGPSVFPLA PCSRSTSESTAALGCLVKDYFPEPVTVSWNS

		GALTSGVHTFPAVLQSSGLYSLSSVVTVPSS NFGTQTYTCNVDHKPSNTKVDKTVERKCC VECPPCPAPPVAGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVQFNWYVDGVEVH NAKTKPREEQFNSTFRVVSVLTVVHQDWL NGKEYKCKVSNKGLPAPIEKTISKTKGQPRE PQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPMLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
125	16444 (HC: R118A, L120Q)	QVQLVQSGAEVKKPGASVKVSCKASGYTF TRYGISWVRQAPGQGLEWMGWISTYSGNT NYAQKLQGRVTMTTDTSTSTAYMELRSLRS DDTAVYYCARAQQYFDYWGQGTLVTVSS ASTKGPSVFPLAPCSRSTSESTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSNFGTQTYTCNVDHKPSNT KVDKTVERKCCVECPPCPAPPVAGPSVFLFP PKPKDTLMISRTPEVTCVVVDVSHEDPEVQ FNWYVDGVEVHNAKTKPREEQFNSTFRVV SVLTVVHQDWLNGKEYKCKVSNKGLPAPI EKTISKTKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKT TPPMLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK
100	4241 (HC: Y125E)	MKHLWFFLLLVAAPRWVLSQVQLQESGPG LVKPSQTLSLTCTVSGGSISSGDYFWSWIRQ LPGKGLEWIGHIHNSGTTYYNPSLKSRVTIS VDTSKKQFSLRLSSVTAADTAVYYCARDRG GDYEYGMDVWGQGTTVTVSSASTKGPSVF PLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKVDKRVEP KSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSREEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK

101	4241 (HC:Y125E)	QVQLQESGPGLVKPSQTLSLTCTVSGGSISS GDYFWSWIRQLPGKGLEWIGHIHNSGTTYY NPSLKSRVTISVDTSKKQFSLRLSSVTAADT AVYYCARDRGGDYEYGMDVWGQGTTVTV SSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKRVEPKSCDKTHTCPPCPAPELLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNK ALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK
118	4341 (HC:Y125A)	MKHLWFFLLLVAAPRWVLSQVQLQESGPG LVKPSQTLSLTCTVSGGSISSGDYFWSWIRQ LPGKGLEWIGHIHNSGTTYYNPSLKSRVTIS VDTSKKQFSLRLSSVTAADTAVYYCARDRG GDYAYGMDVWGQGTTVTVSSASTKGPSVF PLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKVDKRVEP KSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSREEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK
119	4341 (HC:Y125A)	QVQLQESGPGLVKPSQTLSLTCTVSGGSISS GDYFWSWIRQLPGKGLEWIGHIHNSGTTYY NPSLKSRVTISVDTSKKQFSLRLSSVTAADT AVYYCARDRGGDYAYGMDVWGQGTTVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQ SSGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTKVDKRVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVS HEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLYSKLTVDKSRW

		QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[00176] Some useful embodiments of the isolated immunoglobulin comprising an antibody or antibody fragment, comprise:

[00177] (a) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:113, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:110, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00178] (b) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:125, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:122, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00179] (c) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:101, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:98, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00180] (d) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:119, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal,

or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:116, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both.

[00181] In some instances, such antibodies include at least one heavy chain and one light chain, whereas in other instances the variant forms contain two identical light chains and two identical heavy chains. It is within the scope of the invention that the heavy chain(s) and/or light chain(s) may have one, two, three, four or five amino acid residues lacking from the N-terminal or C-terminal, or both, in relation to any one of the heavy and light chains set forth in Tables 1A and Table 1B, e.g., due to post-translational modifications. For example, CHO cells typically cleave off a C-terminal lysine. As described herein, certain embodiments comprising conjugates with one or more pharmacologically active chemical moieties, such as a phramacologically active polypeptide can comprise heteromultimers, such as monovalent heterodimers, heterotrimers, or heterotetramers, as illustrated schematically in Figures 1F-1N (see, also Table 2D).

[00182] Variable Domains of Immunogloblins, e.g., Antibodies

[00183] The various heavy chain and light chain variable regions provided herein are depicted in Table 2A-B. Each of these variable regions may be attached to the above heavy and light chain constant regions to form a complete antibody heavy and light chain, respectively. Further, each of the so generated heavy and light chain sequences may be combined to form a complete antibody structure. It should be understood that the heavy chain and light chain variable regions provided herein can also be attached to other constant domains having different sequences than the exemplary sequences listed above.

[00184] Also provided are immunoglobulins, including antibodies or antibody fragments, that contain or include at least one immunoglobulin light chain variable region selected from V_L2, V_L3, V_L4, and V_L5, as shown in Table 2A below, and at least one immunoglobulin heavy chain variable region selected from V_H2, V_H3, V_H4,

V_H5, V_H6, V_H7, V_H8, V_H9, V_H10, and V_H11, as shown in Table 2B below, and immunologically functional fragments, derivatives, muteins and variants of these light chain and heavy chain variable regions. Examples of such embodiments are found in Table 2C and Table 2D below.

[00185] Also provided are immunoglobulins, including antibodies or antibody fragments, that contain or include at least one immunoglobulin light chain variable region selected from V_L7, V_L8, V_L9, V_L10, V_L11, V_L12, V_L13, V_L14, V_L15 and V_L16, as shown in Table 2A below, and at least one immunoglobulin heavy chain variable region selected from V_H13, V_H14, V_H15, V_H16, V_H17, V_H18, V_H19, V_H20, V_H21, V_H22, V_H23, V_H24, V_H25, V_H26, V_H27, V_H28, V_H29, V_H30, V_H31, V_H32, V_H33, V_H34, V_H35, and V_H36, as shown in Table 2B below, and immunologically functional fragments, derivatives, muteins and variants of these light chain and heavy chain variable regions. Examples of such embodiments are found in Table 2C and Table 2D below.

[00186] Exemplary embodiments of the inventive immunoglobulin include those, in which:

[00187] the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323 [VH10]; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 [VL4]; or

[00188] the light chain variable region comprises the amino acid sequence of SEQ ID NO:196 [VL8]; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:353 [VH25]; or

[00189] the light chain variable region comprises the amino acid sequence of SEQ ID NO:202 [VL11]; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349 [VH23]; or

[00190] the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325 [VH11]; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:190 [VL5].

[00191] Immunoglobulins of this type can generally be designated by the formula " V_Hx/ V_Ly," where "x" corresponds to the number of heavy chain variable regions included in the immunoglobulin and "y" corresponds to the number of the light chain variable regions included in the immunoglobulin (in general, x and y are each 1 or 2).

[00192] Table 2A. Exemplary V_L Chains. Optional N-terminal signal sequences are not shown, but may be reflected in the arbitrary "description" of the V_L.

Design ation	Description	SEQ ID NO	Amino Acid Sequence
VL1	Anti-IL-17R Wild type (WT)	182	EIVMTQSPATLSVSPGERATLSCRASQS VSSNLAWFQQKPGQAPRPLIYDASTRA TGVPARFSGSGSGTDFTLTISSLQSEDFA VYYCQQYDNWPLTFGGGTKVEIK
VL2	W114A	184	EIVMTQSPATLSVSPGERATLSCRASQS VSSNLAWFQQKPGQAPRPLIYDASTRA TGVPARFSGSGSGTDFTLTISSLQSEDFA VYYCQQYDNAPLTFGGGTKVEIK
VL3	Y111A	186	EIVMTQSPATLSVSPGERATLSCRASQS VSSNLAWFQQKPGQAPRPLIYDASTRA TGVPARFSGSGSGTDFTLTISSLQSEDFA VYYCQQADNWPLTFGGGTKVEIK
VL4	P66L, D90E	188	EIVMTQSPATLSVSPGERATLSCRASQS VSSNLAWFQQKPGQAPRLLIYDASTRA TGVPARFSGSGSGTEFTLTISSLQSEDFA VYYCQQYDNWPLTFGGGTKVEIK
VL5	P66L, D90E, W114A	190	EIVMTQSPATLSVSPGERATLSCRASQS VSSNLAWFQQKPGQAPRLLIYDASTRA TGVPARFSGSGSGTEFTLTISSLQSEDFA VYYCQQYDNAPLTFGGGTKVEIK
VL6	Anti-huTR2 Wild type (WT)	192	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSYLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQFGSSPWTFGQGTKVEIK

VL7	F112A	194	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSYLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQAGSSPWTFGQGTKVEIK
VL8	Y53A	196	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSALAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQFGSSPWTFGQGTKVEIK
VL9	W117A	198	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSYLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQFGSSPATFGQGTKVEIK
VL10	F112Y	200	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSYLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQYGSSPWTFGQGTKVEIK
VL11	Y53E	202	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSELAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQFGSSPWTFGQGTKVEIK
VL12	Y53R	204	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSRLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQFGSSPWTFGQGTKVEIK
VL13	F112E	206	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSYLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQEGSSPWTFGQGTKVEIK
VL14	F112R	208	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSYLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQRGSSPWTFGQGTKVEIK
VL15	Y53A, F112A	210	EIVLTQSPGTLSLSPGERATLSCRASQGI SRSALAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQAGSSPWTFGQGTKVEIK
VL16	G48S, I49V, R51S	212	EIVLTQSPGTLSLSPGERATLSCRASQSV SSSYLAWYQQKPGQAPSLLIYGASSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQFGSSPWTFGQGTKVEIK

[00193] Table 2B. Exemplary V_H Chains. Optional N-terminal signal sequences are not shown, but may be reflected in the arbitrary "description" of the V_H.

Design ation	Description	SEQ ID NO	Amino Acid Sequence
VH1	Anti-IL-17R Wild type (WT)	305	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQLYFD YWGQGTLVTVSS
VH2	Y124A	307	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQLYFD AWGQGTLVTVSS
VH3	F122A	309	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQLYAD YWGQGTLVTVSS
VH4	Y121A	311	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQLAFD YWGQGTLVTVSS
VH5	Y79A	313	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNAAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQLYFD YWGQGTLVTVSS
VH6	Y73A	315	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TASGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQLYFD YWGQGTLVTVSS
VH7	W69A	317	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGAIST YSGNTNYAQKLQGRVTMTTDTSTSTA YMELRSLRSDDTAVYYCARRQLYFDY WGQGTLVTVSS
VH8	Y51A	319	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRAGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQLYFD YWGQGTLVTVSS

VH9	L120Q	321	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARRQQYFD YWGQGTLVTVSS
VH10	R118A	323	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARAQLYFD YWGQGTLVTVSS
VH11	R118A, L120Q	325	QVQLVQSGAEVKKPGASVKVSCKASG YTFTRYGISWVRQAPGQGLEWMGWIS TYSGNTNYAQKLQGRVTMTTDTSTST AYMELRSLRSDDTAVYYCARAQQYFD YWGQGTLVTVSS
VH12	Anti-huTR2 Wild type (WT)	327	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH13	D123A	329	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGAYYYG MDVWGQGTTVTVSS
VH14	Y124A	331	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDAYYG MDVWGQGTTVTVSS
VH15	Y53A	333	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDAFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH16	F54A	335	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYAWSWIRQLPGKGLEWIGHIH NSGTTYYNPSLKSRVTISVDTSKKQFSL RLSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH17	F54E	337	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYEWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG

			MDVWGQGTTVTVSS
VH18	F54Y	339	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYYWSWIRQLPGKGLEWIGHIH NSGTTYYNPSLKSRVTISVDTSKKQFSL RLSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH19	F54R	341	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYRWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH20	W55A	343	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFASWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH21	Y79A	345	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTAYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH22	Y80A	347	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYANPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS
VH23	Y125A	349	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYAYG MDVWGQGTTVTVSS
VH24	Y126A	351	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYAG MDVWGQGTTVTVSS
VH25	Y125E	353	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYEYGM DVWGQGTTVTVSS
VH26	Y125R	355	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYRYGM DVWGQGTTVTVSS

VH27	F54A, Y125A	357	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYAWSWIRQLPGKGLEWIGHIH NSGTTYYNPSLKSRVTISVDTSKKQFSL RLSSVTAADTAVYYCARDRGGDYAYG MDVWGQGTTVTVSS
VH28	F54A, Y126A	359	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYAWSWIRQLPGKGLEWIGHIH NSGTTYYNPSLKSRVTISVDTSKKQFSL RLSSVTAADTAVYYCARDRGGDYYAG MDVWGQGTTVTVSS
VH29	Y126E	361	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYEGM DVWGQGTTVTVSS
VH30	Y126R	363	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYRGM DVWGQGTTVTVSS
VH31	F54A, Y125A, Y126A	365	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYAWSWIRQLPGKGLEWIGHIH NSGTTYYNPSLKSRVTISVDTSKKQFSL RLSSVTAADTAVYYCARDRGGDYAAG MDVWGQGTTVTVSS
VH32	D123A, Y124A	367	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGAAYYG MDVWGQGTTVTVSS
VH33	Y125A, Y126A	369	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYAAG MDVWGQGTTVTVSS
VH34	Y124A, Y125A, Y126A	371	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDAAAG MDVWGQGTTVTVSS
VH35	H71Y, H73Y, N74Y, T77S	373	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGYIYY SGSTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDRGGDYYYG MDVWGQGTTVTVSS

VH36	R120Y, G122D, D125Y	375	QVQLQESGPGLVKPSQTLSLTCTVSGG SISSGDYFWSWIRQLPGKGLEWIGHIHN SGTTYYNPSLKSRVTISVDTSKKQFSLR LSSVTAADTAVYYCARDYGDYYYYYY GMDVWGQGTTVTVSS

Table 2C. Embodiments of the immunoglobulins containing the indicated V_L and V_H (or multimers thereof), as disclosed in Tables 2A and 2B above. Antibodies 16429 and 16449, also listed here, are positive control antibodies for human IL-17R and TR2, respectively.

Antibody #	V_L	V_H
1869	VL6	VH13
1870	VL6	VH14
1910	VL7	VH12
1911	VL9	VH12
1912	VL6	VH15
1913	VL6	VH16
1914	VL6	VH20
1915	VL6	VH21
1916	VL6	VH22
1919	VL6	VH23
1920	VL6	VH24
1921	VL6	VH32
1922	VL6	VH34
1961	VL8	VH16
1962	VL8	VH23
1963	VL8	VH24
1964	VL7	VH24
1965	VL7	VH23

1966	VL7	VH16
2281	VL16	VH12
2301	VL10	VH12
2302	VL15	VH12
2303	VL11	VH12
2304	VL12	VH12
2305	VL13	VH12
2306	VL14	VH12
2307	VL6	VH18
2321	VL6	VH35
2322	VL6	VH36
2323	VL6	VH27
2324	VL6	VH28
2325	VL6	VH19
2326	VL6	VH25
2327	VL6	VH26
2328	VL6	VH29
2329	VL6	VH30
2330	VL6	VH33
2331	VL6	VH31
2332	VL6	VH17
4241	VL8	VH25
4341	VL11	VH23
10182	VL8	VH26
10183	VL12	VH23
10184	VL12	VH26
10185	VL11	VH25
10186	VL7	VH28
10187	VL7	VH27
10188	VL8	VH28

10189	VL8	VH27
10190	VL15	VH24
10191	VL15	VH23
10192	VL15	VH16
16429	VL1	VH1
16430	VL4	VH1
16433	VL5	VH1
16434	VL1	VH10
16435	VL4	VH10
16436	VL5	VH10
16437	VL1	VH9
16438	VL4	VH9
16439	VL5	VH9
16440	VL1	VH11
16441	VL4	VH11
16444	VL5	VH11
16449	VL6	VH12
16613	VL8	VH12
16629	VL3	VH1
16630	VL2	VH1
16631	VL1	VH8
16632	VL1	VH7
16633	VL1	VH6
16634	VL1	VH5
16635	VL1	VH4
16636	VL1	VH3
16637	VL1	VH2

Table 2D. Embodiments of the carrier antibodies containing the indicated V_L and V_H (or multimers thereof), as disclosed in Tables 2A and 2B above, and a fusion partner as described in greater detail in Examples 5-6 herein.

Antibody #	V_L	V_H	Fusion partner
3742	VL1	VH1	ShK(1-35, Q16K)
10162	VL4	VH10	FGF21
10163	VL11	VH23	FGF21
10164	VL11	VH23	ShK(1-35, Q16K)

[00194] In some embodiments, the immunoglobulin (including antibodies and antibody fragments) can be useful as a therapeutic molecule which can be used singularly or in combination with other therapeutics to achieve the desired effects. In such embodiments, the inventive immunoglobulin (including antibodies and antibody fragments) further comprises one to twenty-four, one to sixteen, one to eight, or one to four, pharmacologically active chemical moieties conjugated thereto, whether a small molecule or a polypeptide. The pharmacologically active small molecule or polypeptide chemical moieties can be conjugated at or via the N-terminal or C-terminal residue of the immunoglobulin immunoglobulin monomers (e.g., LC or HC monomers), chemical reactions known in the art and further described herein. Alternatively encompassed by the invention, is conjugation of the pharmacologically active chemical moiety, or moieties, at or via functional groups on one or more side chains of the amino acid residue(s) within the primary chain of the inventive immunoglobulin. Useful methods and internal conjugation sites (e.g., particular cysteine residues) within immunoglobulin chains are known in the art (e.g., Gegg et al., Modified Fc Molecules, published in WO 2007/022070 and US 20070269369, which are incorporated herein by reference in their entireties).

[00195] In other embodiments of the invention, in which the pharmacologically active chemical moiety is a polypeptide, a recombinant fusion protein can be produced with the pharmacologically active polypeptide being inserted in the primary amino acid sequence of the of the immunoglobulin heavy chain within an internal loop of the Fc domain of the immunoglobulin heavy chain, instead of at the N- and/or C-terminus, as further described in the Examples herein and in the art (e.g., Gegg et al., U.S. Patent No. 7,442,778; U.S. Patent No. 7,655,765; U.S. Patent No. 7,655,764; U.S. Patent No. 7,662,931; U.S. Patent No. 7,645,861; published U.S. Patent Applications US 2009/0281286; and US 2009/0286964, each of which are incorporated herein by reference in their entireties).

[00196] "Conjugated" means that at least two chemical moieties are covalently linked, or bound to each other, either directly, or optionally, via a peptidyl or non-peptidyl linker moiety that is itself covalently linked to both of the moieties. For example, covalent linkage can be via an amino acid residue of a peptide or protein, including via an alpha amino group, an alpha carboxyl group, or via a side chain. The method by which the covalent linkage is achieved is not critical, for example, whether "conjugation" is by chemical synthetic means or by recombinant expression of fused (i.e., conjugated) partners in a fusion protein.

[00197] As stated above, some embodiments of the inventive compositions involve at least one pharmacologically active polypeptide moiety conjugated to the pharmacologically inactive immunoglobulin of the invention, for example constituting a recombinant fusion protein of the pharmacologically active polypeptide moiety conjugated to the pharmacologically inactive immunoglobulin of the invention. The term "pharmacologically active" means that a substance so described is determined to have activity that affects a medical parameter (e.g., blood pressure, blood cell count, cholesterol level, pain perception) or disease state (e.g., cancer, autoimmune disorders, chronic pain), excluding mere immunogenicity, if any, of the substance. Conversely, the term "pharmacologically inactive" means that no activity affecting a medical parameter or disease state can be determined for that substance, excluding mere immunogenicity, if any, of the substance. Thus, pharmacologically active peptides or proteins comprise agonistic or mimetic and antagonistic peptides as defined below. The present invention encompasses the use of any pharmacologically active protein, which has an amino acid sequence ranging from about 5 to about 80 amino acid residues in length, and which is amenable to recombinant expression. In some useful embodiments of the invention, the pharmacologically active protein is modified in one or more ways relative to a native sequence of interest, including amino acid additions or insertions, amino acid deletions, peptide truncations, amino acid substitutions, or chemical derivatization of amino acid residues (accomplished by known chemical techniques), so long as the requisite bioactivity is maintained.

[00198] The terms "-mimetic peptide," "peptide mimetic," and "-agonist peptide" refer to a peptide or protein having biological activity comparable to a naturally occurring protein of interest, for example, but not limited to, a toxin peptide molecule, e.g., ShK or OSK1 toxin peptides, or peptide analogs thereof. These terms further include peptides that indirectly mimic the activity of a naturally occurring peptide molecule, such as by potentiating the effects of the naturally occurring molecule.

[00199] The term "-antagonist peptide," "peptide antagonist," and "inhibitor peptide" refer to a peptide that blocks or in some way interferes with the biological activity of a receptor of interest, or has biological activity comparable to a known antagonist or inhibitor of a receptor of interest (such as, but not limited to, an ion channel or a G-Protein Coupled Receptor (GPCR)).

[00200] Examples of pharmacologically active proteins that can be used within the present invention include, but are not limited to, a toxin peptide (e.g., OSK1 or an OSK1 peptide analog; ShK or an ShK peptide analog), an IL-6 binding peptide, a CGRP peptide antagonist, a bradykinin B1 receptor peptide antagonist, a parathyroid hormone (PTH) agonist peptide, a parathyroid hormone (PTH) antagonist peptide, an ang-1 binding peptide, an ang-2 binding peptide, a myostatin binding peptide, an erythropoietin-mimetic (EPO-mimetic) peptide, a FGF21 peptide, a thrombopoietin-mimetic (TPO-mimetic) peptide (e.g., AMP2 or AMP5), a nerve growth factor (NGF) binding peptide, a B cell activating factor (BAFF) binding peptide, and a glucagon-like peptide (GLP)-1 or a peptide mimetic therof or GLP-2 or a peptide mimetic thereof.

Glucagon-like peptide 1 (GLP-1) and the related peptide glucagon are produced via differential processing of proglucagon and have opposing biological activities. Proglucagon itself is produced in α-cells of the pancreas and in the enteroendocrine L-cells, which are located primarily in the distal small intestine and colon. In the pancreas, glucagon is selectively cleaved from proglucagon. In the intestine, in contrast, proglucagon is processed to form GLP-1 and glucagon-like peptide 2 (GLP-

2), which correspond to amino acid residues 78-107 and 126-158 of proglucagon, respectively (see, e.g., Irwin and Wong, 1995, *Mol. Endocrinol.* 9:267-277 and Bell *et al.*, 1983, *Nature* 304:368-371). By convention, the numbering of the amino acids of GLP-1 is based on the GLP-1 (1-37) formed from cleavage of proglucagon. The biologically active forms are generated from further processing of this peptide, which, in one numbering convention, yields GLP-1 (7-37)-OH and GLP-1 (7-36)-NH_2. Both GLP-1 (7-37)-OH (or simply GLP-1 (7-37)) and GLP-1 (7-36)-NH_2 have the same activities. For convenience, the term "GLP-1", is used to refer to both of these forms. The first amino acid of these processed peptides is His7 in this numbering convention. Another numbering convention recognized in the art, however, assumes that the numbering of the processed peptide begins with His as position 1 rather than position 7. Thus, in this numbering scheme, GLP-1 (1-31) is the same as GLP-1(7-37), and GLP-1(1-30) is the same as GLP-1 (7-36). Examples of GLP-1 mimetic polypeptide sequences include:
HGEGTFTSDQSSYLEGQAAKEFIAWLVKGRG// (SEQ ID NO:290);
HGEGTFTSDQSSYLEGQAAKEFIAWLQKGRG// (SEQ ID NO:291);
HGEGTFTSDVSSYQEGQAAKEFIAWLVKGRG// (SEQ ID NO:292);
HGEGTFTSDVSSYLEGQAAKEFIAQLVKGRG// (SEQ ID NO:293);
HGEGTFTSDVSSYLEGQAAKEFIAQLQKGRG// (SEQ ID NO:294);
HGEGTFTSDVSSYLEGQAAKEFIAWLQKGRG// (SEQ ID NO:295);
HNETTFTSDVSSYLEGQAAKEFIAWLVKGRG// (SEQ ID NO:296)
HGEGTFTSDVSSYLENQTAKEFIAWLVKGRG// (SEQ ID NO:297);
HGEGTFTSDVSSYLEGNATKEFIAWLVKGRG// (SEQ ID NO:298);
HGEGTFTSDVSSYLEGQAAKEFIAWLVNGTG// (SEQ ID NO:299);
HGEGTFTSDVSSYLEGQAAKEFIAWLVKNRT// (SEQ ID NO:300);
HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRNGT// (SEQ ID NO:301);
HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRGGTGNGT// (SEQ ID NO:302); and

[0001] HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRGGSGNGT// (SEQ ID NO:303).

[00201] Human GLP-2 and GLP-2-mimetic analogs are also known in the art. (See, e.g., Prasad et al., Glucagonlike peptide-2 analogue enhances intestinal mucosal mass after ischemia and reperfusion, J. Pediatr. Surg. 2000 Feb;35(2):357-59 (2000); Yusta et al., Glucagon-like peptide-2 receptor activation engages bad and glycogen synthase kinase-3 in a protein kinase A-dependent manner and prevents apoptosis following inhibition of phosphatidylinositol 3-kinase, J. Biol. Chem. 277(28):24896-906 (2002)).

[00202] "Toxin peptides" include peptides and polypeptides having the same amino acid sequence of a naturally occurring pharmacologically active peptide or polypeptide that can be isolated from a venom, and also include modified peptide analogs of such naturally occurring molecules. (See, e.g., Kalman et al., ShK-Dap22, a potent Kv1.3-specific immunosuppressive polypeptide, J. Biol. Chem. 273(49):32697-707 (1998); Kem et al., US Patent No. 6,077,680; Mouhat et al., OsK1 derivatives, WO 2006/002850 A2; Chandy et al., Analogs of SHK toxin and their uses in selective inhibition of Kv1.3 potassium channels, WO 2006/042151; Sullivan et al., Toxin Peptide therapeutic agents, WO 2006/116156 A2, all of which are incorporated herein by reference in their entirety). Snakes, scorpions, spiders, bees, snails and sea anemone are a few examples of organisms that produce venom that can serve as a rich source of small bioactive toxin peptides or "toxins" that potently and selectively target ion channels and receptors. An example of a toxin peptide is OSK1 (also known as OsK1), a toxin peptide isolated from Orthochirus scrobiculosus scorpion venom. (e.g., Mouhat et al., K+ channel types targeted by synthetic OSK1, a toxin from Orthochirus scrobiculosus scorpion venom, Biochem. J. 385:95-104 (2005); Mouhat et al., Pharmacological profiling of Orthochirus scrobiculosus toxin 1 analogs with a trimmed N-terminal domain, Molec. Pharmacol. 69:354- 62 (2006); Mouhat et al., OsK1 derivatives, WO 2006/002850 A2). Another example is ShK, isolated from the venom of the sea anemone Stichodactyla helianthus. (E.g., Tudor et al., Ionisation behaviour and solution properties of the potassium-channel blocker ShK toxin, Eur. J. Biochem. 251(1-2):133-41(1998); Pennington et al., Role of disulfide bonds in the structure and potassium channel

blocking activity of ShK toxin, Biochem. 38(44): 14549-58 (1999); Kem et al., ShK toxin compositions and methods of use, US Patent No. 6,077,680; Lebrun et al., Neuropeptides originating in scorpion, US Patent No. 6,689,749; Beeton et al., Targeting effector memory T cells with a selective peptide inhibitor of Kv1.3 channnels for therapy of autoimmune diseases, Molec. Pharmacol. 67(4):1369-81 (2005)).

[00203] The toxin peptides are usually between about 20 and about 80 amino acids in length, contain 2-5 disulfide linkages and form a very compact structure. Toxin peptides (e.g., from the venom of scorpions, sea anemones and cone snails) have been isolated and characterized for their impact on ion channels. Such peptides appear to have evolved from a relatively small number of structural frameworks that are particularly well suited to addressing the critical issues of potency and stability. The majority of scorpion and Conus toxin peptides, for example, contain 10-40 amino acids and up to five disulfide bonds, forming extremely compact and constrained structure (microproteins) often resistant to proteolysis. The conotoxin and scorpion toxin peptides can be divided into a number of superfamilies based on their disulfide connections and peptide folds. The solution structure of many of these has been determined by NMR spectroscopy, illustrating their compact structure and verifying conservation of their family fold. (E.g., Tudor et al., Ionisation behaviour and solution properties of the potassium-channel blocker ShK toxin, Eur. J. Biochem. 251(1-2):133-41(1998); Pennington et al., Role of disulfide bonds in the structure and potassium channel blocking activity of ShK toxin, Biochem. 38(44): 14549-58 (1999); Jaravine et al., Three-dimensional structure of toxin OSK1 from Orthochirus scrobiculosus scorpion venom, Biochem. 36(6):1223-32 (1997); del Rio-Portillo et al.; NMR solution structure of Cn12, a novel peptide from the Mexican scorpion Centruroides noxius with a typical beta-toxin sequence but with alpha-like physiological activity, Eur. J. Biochem. 271(12): 2504-16 (2004); Prochnicka-Chalufour et al., Solution structure of discrepin, a new K+-channel blocking peptide from the alpha-KTx15 subfamily, Biochem. 45(6):1795-1804 (2006)). Examples of pharmacologically active toxin peptides for which the practice

of the present invention can be useful include, but are not limited to ShK, OSK1, charybdotoxin (ChTx), kaliotoxin1 KTX1), or maurotoxin, or toxin peptide analogs of any of these, modified from the native sequences at one or more amino acid residues. Other examples are known in the art, or can be found in Sullivan et al., WO06116156 A2 or U.S. Patent Application No. 11/406,454 (titled: Toxin Peptide Therapeutic Agents, published as US 2007/0071764); Mouhat et al., OsK1 derivatives, WO 2006/002850 A2; Sullivan et al., U.S. Patent Application No. 11/978,076 (titled: Conjugated Toxin Peptide Therapeutic Agents, filed 25 October 2007, and published as US20090291885 on November 26, 2009), Sullivan et al., WO 2008/088422; Lebrun et al., U.S. Patent No. 6,689,749, and Sullivan et al., Selective and Potent Peptide Inhibitors of Kv1.3, U.S. Provisional Application No. 61/210,594, filed March 20, 2009, which are each incorporated by reference in their entireties.

[00204] The term "peptide analog" refers to a peptide having a sequence that differs from a peptide sequence existing in nature by at least one amino acid residue substitution, internal addition, or internal deletion of at least one amino acid, and/or amino- or carboxy- terminal end truncations, or additions). An "internal deletion" refers to absence of an amino acid from a sequence existing in nature at a position other than the N- or C-terminus. Likewise, an "internal addition" refers to presence of an amino acid in a sequence existing in nature at a position other than the N- or C-terminus. "Toxin peptide analogs", such as, but not limited to, an OSK1 peptide analog, ShK peptide analog, or ChTx peptide analog, contain modifications of a native toxin peptide sequence of interest (e.g., amino acid residue substitutions, internal additions or insertions, internal deletions, and/or amino- or carboxy-terminal end truncations, or additions as previously described above) relative to a native toxin peptide sequence of interest.

[00205] A "CGRP peptide antagonist" is a peptide that preferentially binds the $CGRP_1$ receptor, such as, but not limited to, a CGRP peptide analog, and that antagonizes, blocks, decreases, reduces, impedes, or inhibits $CGRP_1$ receptor activation by full length native human αCGRP or βCGRP under physiological

conditions of temperature, pH, and ionic strength. CGRP peptide antagonists include full and partial antagonists. Such antagonist activity can be detected by known *in vitro* methods or *in vivo* functional assay methods. (See, e.g., Smith et al., Modifications to the N-terminus but not the C-terminus of calcitonin gene-related peptide(8-37) produce antagonists with increased affinity, J. Med. Chem., 46:2427-2435 (2003)). Examples of useful CGRP peptide antagonists are disclosed in Gegg et al., CGRP peptide antagonists and conjugates, WO 2007/048026 A2 and U.S. Serial No. 11/584,177, filed on October 19, 2006, published as US 2008/0020978 A1, which is incorporated herein by reference in its entirety.

[00206] The terms "parathyroid hormone (PTH) agonist" and "PTH agonist"refer to a molecule that binds to PTH-1 or PTH-2 receptor and increases or decreases one or more PTH activity assay parameters as does full-length native human parathyroid hormone. Examples of useful PTH agonist peptides are disclosed in Table 1 of U.S. Patent No. 6,756,480, titled Modulators of receptors for parathyroid hormone and parathyroid hormone-related protein, which is incorporated herein by reference in its entirety. An exemplary PTH activity assay is disclosed in Example 1 of U.S. Patent No. 6,756,480.

[00207] The term "parathyroid hormone (PTH) antagonist" refers to a molecule that binds to PTH-1 or PTH-2 receptor and blocks or prevents the normal effect on those parameters by full length native human parathyroid hormone. Examples of useful PTH antagonist peptides are disclosed in Table 2 of U.S. Patent No. 6,756,480, which is incorporated herein by reference in its entirety. An exemplary PTH activity assay is disclosed in Example 2 of U.S. Patent No. 6,756,480.

[00208] The terms "bradykinin B1 receptor antagonist peptide" and "bradykinin B1 receptor peptide antagonist" mean a peptide with antagonist activity with respect to human bradykinin B1 receptor (hB1). Useful bradykinin B1 receptor antagonist peptides can be identified or derived as described in Ng et al., Antagonist of the bradykinin B1 receptor, US 2005/0215470 A1, published September 29, 2005, which issued as U.S. Patent No. 7,605,120; U.S. Patent Nos. 5,834,431 or 5,849,863. An

exemplary B1 receptor activity assays are disclosed in Examples 6-8 of US 2005/0215470 A1.

[00209] The terms "thrombopoietin (TPO)-mimetic peptide" and "TPO-mimetic peptide" refer to peptides that can be identified or derived as described in Cwirla et al. (1997), Science 276: 1696-9 , U.S. Pat. Nos. 5,869,451 and 5,932,946, which are incorporated by reference in their entireties; U.S. Pat. App. No. 2003/0176352, published Sept. 18, 2003, which is incorporated by reference in its entirety; WO 03/031589, published April 17, 2003; WO 00/24770, published May 4, 2000; and any peptides appearing in Table 5 of published application US 2006/0140934 (U.S. Serial No. 11/234,731, filed September 23, 2005, titled Modified Fc Molecules, which is incorporated herein by reference in its entirety). Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

[00210] The terms "EPO-mimetic peptide" and "erythropoietin-mimetic peptide" refers to peptides that can be identified or derived as described in Wrighton et al. (1996), Science 273: 458-63, and Naranda et al. (1999), Proc. Natl. Acad. Sci. USA 96: 7569-74, both of which are incorporated herein by reference in their entireties. Useful EPO-mimetic peptides include EPO-mimetic peptides listed in Table 5 of published U.S. patent application US 2007/0269369 A1and in U.S. Pat. No. 6,660,843, which are both hereby incorporated by reference in their entireties.

[00211] The term "ang-2-binding peptide" comprises peptides that can be identified or derived as described in U.S. Pat. App. No. 2003/0229023, published Dec. 11, 2003; WO 03/057134, published July, 17, 2003; U.S. 2003/0236193, published Dec. 25, 2003 (each of which is incorporated herein by reference in its entirety); and any peptides appearing in Table 6 of published application US 2006/0140934 (U.S. Serial No. 11/234,731, filed September 23, 2005, titled Modified Fc Molecules, which is incorporated herein by reference in its entirety). Those of ordinary skill in the art appreciate that each of these references enables one

to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

[00212] The terms "nerve growth factor (NGF) binding peptide" and "NGF-binding peptide" comprise peptides that can be identified or derived as described in WO 04/026329, published April 1, 2004 and any peptides identified in Table 7 of published application US 2006/0140934 (U.S. Serial No. 11/234,731, filed September 23, 2005, titled Modified Fc Molecules, which is incorporated herein by reference in its entirety). Those of ordinary skill in the art appreciate that this reference enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

[00213] The term "myostatin-binding peptide" comprises peptides that can be identified or derived as described in U.S. Ser. No. 10/742,379, filed December 19, 2003, which is incorporated herein by reference in its entirety, and peptides appearing in Table 8 of published application US 2006/0140934 (U.S. Serial No. 11/234,731, filed September 23, 2005, titled Modified Fc Molecules, which is incorporated herein by reference in its entirety). Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

[00214] The terms "BAFF-antagonist peptide" and "BAFF binding peptide" comprise peptides that can be identified or derived as described in U.S. Pat. Appln. No. 2003/0195156 A1, which is incorporated herein by reference in its entirety and those peptides appearing in Table 9 of published application US 2006/0140934 (U.S. Serial No. 11/234,731, filed September 23, 2005, titled Modified Fc Molecules, which is incorporated herein by reference in its entirety). Those of ordinary skill in the art appreciate that the foregoing references enable one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

[00215] The foregoing are intended merely as non-limiting examples of the pharmacologically active polypeptides that can be usefully conjugated or fused to the inventive immunoglobulins (including antibodies and antibody fragements). Any include pharmacologically active polypeptide moiety can be used within the scope of the invention, including a polypeptide having a so-called avimer structure (see, e.g., Kolkman et al., Novel Proteins with Targeted Binding, US 2005/0089932; Baker et al., IL-6 Binding Proteins, US 2008/0281076; Stemmer et al., Protein Scaffolds and Uses Thereof, US 2006/0223114 and US 2006/0234299).

[00216] Useful preclinical animal models are known in the art for use in validating a drug in a therapeutic indication of interest (e.g., an adoptive-transfer model of periodontal disease by Valverde et al., J. Bone Mineral Res. 19:155 (2004); an ultrasonic perivascular Doppler flow meter-based animal model of arterial thrombosis in Gruner et al., Blood 105:1492-99 (2005); pulmonary thromboembolism model, aorta occlusion model, and murine stroke model in Braun et al., WO 2009/115609 A1). For example, an adoptive transfer experimental autoimmune encephalomyelitis (AT-EAE) model of multiple sclerosis has been described for investigations concerning immune diseases, such as multiple sclerosis (Beeton et al., J. Immunol. 166:936 (2001); Beeton et al., PNAS 98:13942 (2001); Sullivan et al., Example 45 of WO 2008/088422 A2, incorporated herein by reference in its entirety). In the AT-EAE model, significantly reduced disease severity and increased survival are expected for animals treated with an effective amount of the inventive pharmaceutical composition, while untreated animals are expected to develop severe disease and/or mortality. For running the AT-EAE model, the encephalomyelogenic CD4+ rat T cell line, PAS, specific for myelin-basic protein (MBP) originated from Dr. Evelyne Beraud. The maintenance of these cells in vitro and their use in the AT-EAE model has been described earlier [Beeton et al. (2001) PNAS 98, 13942]. PAS T cells are maintained in vitro by alternating rounds of antigen stimulation or activation with MBP and irradiated thymocytes (2 days), and propagation with T cell growth factors (5 days). Activation of PAS T cells (3×10^5/ml) involves incubating the cells for 2 days with 10 μg/ml MBP and 15

$\times 10^6$/ml syngeneic irradiated (3500 rad) thymocytes. On day 2 after in vitro activation, 10-15 $\times 10^6$ viable PAS T cells are injected into 6-12 week old female Lewis rats (Charles River Laboratories) by tail IV. Daily subcutaneous injections of vehicle (2% Lewis rat serum in PBS) or test pharmaceutical composition are given from days –1 to 3, where day –1 represent 1 day prior to injection of PAS T cells (day 0). In vehicle treated rats, acute EAE is expected to develop 4 to 5 days after injection of PAS T cells. Typically, serum is collected by tail vein bleeding at day 4 and by cardiac puncture at day 8 (end of the study) for analysis of levels of inhibitor. Rats are typically weighed on days –1, 4, 6, and 8. Animals may be scored blinded once a day from the day of cell transfer (day 0) to day 3, and twice a day from day 4 to day 8. Clinical signs are evaluated as the total score of the degree of paresis of each limb and tail. Clinical scoring: 0 = No signs, 0.5 = distal limp tail, 1.0 = limp tail, 2.0 = mild paraparesis, ataxia, 3.0 = moderate paraparesis, 3.5 = one hind leg paralysis, 4.0 = complete hind leg paralysis, 5.0 = complete hind leg paralysis and incontinence, 5.5 = tetraplegia, 6.0 = moribund state or death. Rats reaching a score of 5.0 are typically euthanized.

[00217] Production of Antibody Embodiments of the Immunoglobulins

[00218] Polyclonal antibodies. Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. Alternatively, antigen may be injected directly into the animal's lymph node (see Kilpatrick et al., Hybridoma, 16:381-389, 1997). An improved antibody response may be obtained by conjugating the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride or other agents known in the art.

[00219] Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg of the protein or conjugate (for mice) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. At 7-14 days post-booster injection, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[00220] Monoclonal Antibodies. The inventive immunoglobulins that are provided include monoclonal antibodies. Monoclonal antibodies may be produced using any technique known in the art, e.g., by immortalizing spleen cells harvested from the transgenic animal after completion of the immunization schedule. The spleen cells can be immortalized using any technique known in the art, e.g., by fusing them with myeloma cells to produce hybridomas. For example, monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods from predetermined sequences as is useful in the present invention (e.g., Cabilly et al., Methods of producing immunoglobulins, vectors and transformed host cells for use therein, US Patent No. 6,331,415), including methods, such as the "split DHFR" method, that facilitate the generally equimolar production of light and heavy chains, optionally using mammalian cell lines (e.g., CHO cells) that can glycosylate the antibody (See, e.g., Page, Antibody production, EP0481790 A2 and US Patent No. 5,545,403).

[00221] Generally, in the hybridoma method, which is not useful in the production of the inventive immunoglobulins, but is useful to produce antigen binding proteins, a mouse or other appropriate host mammal, such as rats, hamster or macaque monkey, is immunized as herein described to elicit lymphocytes that produce or are

capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

[00222] In some instances, a hybridoma cell line is produced by immunizing a transgenic animal having human immunoglobulin sequences with an immunogen; harvesting spleen cells from the immunized animal; fusing the harvested spleen cells to a myeloma cell line, thereby generating hybridoma cells; establishing hybridoma cell lines from the hybridoma cells, and identifying a hybridoma cell line that produces an antibody that binds to an tigen of interest. Such hybridoma cell lines, and monoclonal antibodies produced by them, are aspects of the present invention.

[00223] The hybridoma cells, once prepared, are seeded and grown in a suitable culture medium that preferably 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.

[00224] Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133: 3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Myeloma cells for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas). Examples of

suitable cell lines for use in mouse fusions include Sp-20, P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XXO Bul; examples of cell lines used in rat fusions include R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210. Other cell lines useful for cell fusions are U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6.

[00225] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of the monoclonal antibody can, for example, be determined by BIAcore® or Scatchard analysis (Munson et al., Anal. Biochem., 107:220 (1980); Fischer et al., A peptide-immunoglobulin-conjugate, WO 2007/045463 A1, Example 10, which is incorporated herein by reference in its entirety).

[00226] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

[00227] Hybridomas or mAbs may be further screened to identify mAbs with particular properties, such as the ability to inhibit K^{1+} flux though Kv1.x channels. Examples of such screens are provided in the examples below. The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, affinity chromatography, or any other suitable purification technique known in the art.

[00228] Recombinant Production of Antibodies. The present invention provides isolated nucleic acids encoding any of the antibodies (polyclonal and monoclonal), including antibody fragments, of the invention described herein, optionally operably linked to control sequences recognized by a host cell, vectors and host cells comprising the nucleic acids, and recombinant techniques for the production of the antibodies, which may comprise culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture or culture medium. Similar materials and methods apply to production of polypeptide-based immunoglobulins.

[00229] Relevant amino acid sequences from an immunoglobulin or polypeptide of interest may be determined by direct protein sequencing, and suitable encoding nucleotide sequences can be designed according to a universal codon table. Alternatively, genomic or cDNA encoding the monoclonal antibodies may be isolated and sequenced from cells producing such antibodies using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies).

[00230] Cloning of DNA is carried out using standard techniques (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, which is incorporated herein by reference). For example, a cDNA library may be constructed by reverse transcription of polyA+ mRNA, preferably membrane-associated mRNA, and the library screened using probes specific for human immunoglobulin polypeptide gene sequences. In one embodiment, however, the polymerase chain reaction (PCR) is used to amplify cDNAs (or portions of full-length cDNAs) encoding an immunoglobulin gene segment of interest (e.g., a light or heavy chain variable segment). The amplified sequences can be readily cloned into any suitable vector, e.g., expression vectors, minigene vectors, or phage display vectors. It will be appreciated that the particular method of cloning used is not critical, so long as it is possible to determine the sequence of some portion of the immunoglobulin polypeptide of interest.

[00231] One source for antibody nucleic acids is a hybridoma produced by obtaining a B cell from an animal immunized with the antigen of interest and fusing it to an immortal cell. Alternatively, nucleic acid can be isolated from B cells (or whole spleen) of the immunized animal. Yet another source of nucleic acids encoding antibodies is a library of such nucleic acids generated, for example, through phage display technology. Polynucleotides encoding peptides of interest, e.g., variable region peptides with desired binding characteristics, can be identified by standard techniques such as panning.

[00232] The sequence encoding an entire variable region of the immunoglobulin polypeptide may be determined; however, it will sometimes be adequate to sequence only a portion of a variable region, for example, the CDR-encoding portion. Sequencing is carried out using standard techniques (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, and Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467, which is incorporated herein by reference). By comparing the sequence of the cloned nucleic acid with published sequences of human immunoglobulin genes and cDNAs, one of skill will readily be able to determine, depending on the region sequenced, (i) the germline segment usage of the hybridoman immunoglobulin polypeptide (including the isotype of the heavy chain) and (ii) the sequence of the heavy and light chain variable regions, including sequences resulting from N-region addition and the process of somatic mutation. One source of immunoglobulin gene sequence information is the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.

[00233] Isolated DNA can be operably linked to control sequences or placed into expression vectors, which are then transfected into host cells that do not otherwise produce immunoglobulin protein, to direct the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies is well known in the art.

[00234] Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[00235] Many vectors are known in the art. Vector components may include one or more of the following: a signal sequence (that may, for example, direct secretion of the antibody; e.g., ATGGACATGAGGGTGCCCGCTCAGCTCCTGGGGCTCCTGCTGCTGTGGCT GAGAGGTGCGCGCTGT// SEQ ID NO:102, which encodes the VK-1 signal peptide sequence MDMRVPAQLLGLLLLWLRGARC// SEQ ID NO:103), an origin of replication, one or more selective marker genes (that may, for example, confer antibiotic or other drug resistance, complement auxotrophic deficiencies, or supply critical nutrients not available in the media), an enhancer element, a promoter, and a transcription termination sequence, all of which are well known in the art.

[00236] Cell, cell line, and cell culture are often used interchangeably and all such designations herein include progeny. Transformants and transformed cells include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

[00237] Exemplary host cells include prokaryote, yeast, or higher eukaryote cells. Prokaryotic host cells include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as Escherichia, *e.g.*, E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, *e.g.*, Salmonella typhimurium, Serratia, *e.g.*, Serratia marcescans, and Shigella, as well as Bacillus such as B. subtilis and B. licheniformis, Pseudomonas, and Streptomyces. Eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for recombinant polypeptides or antibodies. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as Pichia, e.g. P. pastoris, Schizosaccharomyces pombe; Kluyveromyces, Yarrowia; Candida; Trichoderma reesia; Neurospora crassa; Schwanniomyces such as Schwanniomyces occidentalis; and filamentous fungi such as, *e.g.*, Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as A. nidulans and A. niger.

[00238] Host cells for the expression of glycosylated immunoglobulin, including antibody, can be derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mori have been identified. A variety of viral strains for transfection of such cells are publicly available, *e.g.*, the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV.

[00239] Vertebrate host cells are also suitable hosts, and recombinant production of antigen binding protein (including antibody) from such cells has become routine procedure. Examples of useful mammalian host cell lines are Chinese hamster ovary cells, including CHOK1 cells (ATCC CCL61), DXB-11, DG-44, and Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77: 4216 (1980)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in

suspension culture, [Graham et al., *J. Gen Virol. 36*: 59 (1977)]; baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human hepatoma cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y Acad. Sci. 383: 44-68 (1982)); MRC 5 cells or FS4 cells; or mammalian myeloma cells.

[00240] Host cells are transformed or transfected with the above-described nucleic acids or vectors for production immunoglobulins and are cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In addition, novel vectors and transfected cell lines with multiple copies of transcription units separated by a selective marker are particularly useful for the expression of immunoglobulins.

[00241] The host cells used to produce the immunoglobulins of the invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58: 44 (1979), Barnes et al., Anal. Biochem. 102: 255 (1980), U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO90103430; WO 87/00195; or U.S. Patent Re. No. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent

energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[00242] Upon culturing the host cells, the immunoglobulin can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the immunoglobulin is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration.

[00243] The immunoglobulin (e.g., an antibody or antibody fragment) can be purified using, for example, hydroxylapatite chromatography, cation or anion exchange chromatography, or preferably affinity chromatography, using the antigen of interest or protein A or protein G as an affinity ligand. Protein A can be used to purify proteins that include polypeptides are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., J. Immunol. Meth. *62*: 1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., EMBO J. 5: 15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the protein comprises a C_H 3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as ethanol precipitation, Reverse Phase HPLC, chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also possible depending on the antibody to be recovered.

[00244] Chimeric, Humanized and Human Engineered™ monoclonal antibodies. Chimeric monoclonal antibodies, in which the variable Ig domains of a rodent monoclonal antibody are fused to human constant Ig domains, can be generated using standard procedures known in the art (See Morrison, S. L., et al. (1984)

Chimeric Human Antibody Molecules; Mouse Antigen Binding Domains with Human Constant Region Domains, Proc. Natl. Acad. Sci. USA 81, 6841-6855; and, Boulianne, G. L., et al, Nature 312, 643-646. (1984)). A number of techniques have been described for humanizing or modifying antibody sequence to be more human-like, for example, by (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in the art as humanizing through "CDR grafting") or (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering") or (3) modifying selected non-human amino acid residues to be more human, based on each residue's likelihood of participating in antigen-binding or antibody structure and its likelihood for immunogenicity. See, e.g., Jones et al., Nature 321:522 525 (1986); Morrison et al., Proc. Natl. Acad. Sci., U.S.A., 81:6851 6855 (1984); Morrison and Oi, Adv. Immunol., 44:65 92 (1988); Verhoeyer et al., Science 239:1534 1536 (1988); Padlan, Molec. Immun. 28:489 498 (1991); Padlan, Molec. Immunol. 31(3):169 217 (1994); and Kettleborough, C.A. et al., Protein Eng. 4(7):773 83 (1991); Co, M. S., et al. (1994), J. Immunol. 152, 2968-2976); Studnicka et al. Protein Engineering 7: 805-814 (1994); each of which is incorporated herein by reference in its entirety.

[00245] A number of techniques have been described for humanizing or modifying antibody sequence to be more human-like, for example, by (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in the art as humanizing through "CDR grafting") or (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering") or (3) modifying selected non-human amino acid residues to be more human, based on each residue's likelihood of participating in antigen-binding or antibody structure and its likelihood for immunogenicity. See, e.g., Jones et al., Nature 321:522 525 (1986); Morrison et al., Proc. Natl. Acad. Sci., U.S.A., 81:6851 6855 (1984); Morrison and Oi, Adv. Immunol., 44:65 92 (1988);

Verhoeyer et al., Science 239:1534 1536 (1988); Padlan, Molec. Immun. 28:489 498 (1991); Padlan, Molec. Immunol. 31(3):169 217 (1994); and Kettleborough, C.A. et al., Protein Eng. 4(7):773 83 (1991); Co, M. S., et al. (1994), J. Immunol. 152, 2968-2976); Studnicka et al. Protein Engineering 7: 805-814 (1994); each of which is incorporated herein by reference in its entirety.

[00246] In one aspect of the invention, the light and heavy chain variable regions of the antibodies provided herein (see, Table 2A-B) are grafted to framework regions (FRs) from antibodies from the same, or a different, phylogenetic species. To create consensus human FRs, FRs from several human heavy chain or light chain amino acid sequences may be aligned to identify a consensus amino acid sequence. In other embodiments, the FRs of a heavy chain or light chain disclosed herein are replaced with the FRs from a different heavy chain or light chain. In one aspect, rare amino acids in the FRs of the heavy and light chains of the antibody are not replaced, while the rest of the FR amino acids are replaced. A "rare amino acid" is a specific amino acid that is in a position in which this particular amino acid is not usually found in an FR. Alternatively, the grafted variable regions from the one heavy or light chain may be used with a constant region that is different from the constant region of that particular heavy or light chain as disclosed herein. In other embodiments, the grafted variable regions are part of a single chain Fv antibody.

[00247] Antibodies can also be produced using transgenic animals that have no endogenous immunoglobulin production and are engineered to contain human immunoglobulin loci. For example, WO 98/24893 discloses transgenic animals having a human Ig locus wherein the animals do not produce functional endogenous immunoglobulins due to the inactivation of endogenous heavy and light chain loci. WO 91/10741 also discloses transgenic non-primate mammalian hosts capable of mounting an immune response to an immunogen, wherein the antibodies have primate constant and/or variable regions, and wherein the endogenous immunoglobulin encoding loci are substituted or inactivated. WO 96/30498 discloses the use of the Cre/Lox system to modify the immunoglobulin locus in a mammal, such as to replace all or a portion of the constant or variable region to form

a modified antibody molecule. WO 94/02602 discloses non-human mammalian hosts having inactivated endogenous Ig loci and functional human Ig loci. U.S. Patent No. 5,939,598 discloses methods of making transgenic mice in which the mice lack endogenous heavy chains, and express an exogenous immunoglobulin locus comprising one or more xenogeneic constant regions.

[00248] Using a transgenic animal described above, an immune response can be produced to a selected antigenic molecule, and antibody producing cells can be removed from the animal and used to produce hybridomas that secrete human-derived monoclonal antibodies. Immunization protocols, adjuvants, and the like are known in the art, and are used in immunization of, for example, a transgenic mouse as described in WO 96/33735. The monoclonal antibodies can be tested for the ability to inhibit or neutralize the biological activity or physiological effect of the corresponding protein. See also Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993); Mendez et al., *Nat. Genet.* 15:146-156 (1997); and U.S. Pat. No. 5,591,669, U.S. Patent No. 5,589,369, U.S. Patent No. 5,545,807; and U.S Patent Application No. 20020199213. U.S. Patent Application No. and 20030092125 describes methods for biasing the immune response of an animal to the desired epitope. Human antibodies may also be generated by *in vitro* activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

[00249] Antibody production by phage display techniques

[00250] The development of technologies for making repertoires of recombinant human antibody genes, and the display of the encoded antibody fragments on the surface of filamentous bacteriophage, has provided another means for generating human-derived antibodies. Phage display is described in e.g., Dower et al., WO 91/17271, McCafferty et al., WO 92/01047, and Caton and Koprowski, Proc. Natl. Acad. Sci. USA, 87:6450-6454 (1990), each of which is incorporated herein by reference in its entirety. The antibodies produced by phage technology are usually produced as antigen binding fragments, e.g. Fv or Fab fragments, in bacteria and thus

lack effector functions. Effector functions can be introduced by one of two strategies: The fragments can be engineered either into complete antibodies for expression in mammalian cells, or into bispecific antibody fragments with a second binding site capable of triggering an effector function.

[00251] Typically, the Fd fragment (V_H-C_H1) and light chain (V_L-C_L) of antibodies are separately cloned by PCR and recombined randomly in combinatorial phage display libraries, which can then be selected for binding to a particular antigen. The antibody fragments are expressed on the phage surface, and selection of Fv or Fab (and therefore the phage containing the DNA encoding the antibody fragment) by antigen binding is accomplished through several rounds of antigen binding and re-amplification, a procedure termed panning. Antibody fragments specific for the antigen are enriched and finally isolated.

[00252] Phage display techniques can also be used in an approach for the humanization of rodent monoclonal antibodies, called "guided selection" (see Jespers, L. S., et al., Bio/Technology 12, 899-903 (1994)). For this, the Fd fragment of the mouse monoclonal antibody can be displayed in combination with a human light chain library, and the resulting hybrid Fab library may then be selected with antigen. The mouse Fd fragment thereby provides a template to guide the selection. Subsequently, the selected human light chains are combined with a human Fd fragment library. Selection of the resulting library yields entirely human Fab.

[00253] A variety of procedures have been described for deriving human antibodies from phage-display libraries (See, for example, Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol, 222:581-597 (1991); U.S. Pat. Nos. 5,565,332 and 5,573,905; Clackson, T., and Wells, J. A., TIBTECH 12, 173-184 (1994)). In particular, in vitro selection and evolution of antibodies derived from phage display libraries has become a powerful tool (See Burton, D. R., and Barbas III, C. F., Adv. Immunol. 57, 191-280 (1994); and, Winter, G., et al., Annu. Rev. Immunol. 12, 433-455 (1994); U.S. patent application no. 20020004215 and

WO92/01047; U.S. patent application no. 20030190317 published October 9, 2003 and U.S. Patent No. 6,054,287; U.S. Patent No. 5,877,293.

[00254] Watkins, "Screening of Phage-Expressed Antibody Libraries by Capture Lift," Methods in Molecular Biology, Antibody Phage Display: Methods and Protocols 178: 187-193, and U.S. Patent Application Publication No. 20030044772 published March 6, 2003 describes methods for screening phage-expressed antibody libraries or other binding molecules by capture lift, a method involving immobilization of the candidate binding molecules on a solid support.

[00255] Other Embodiments of Immunoglobulins: Antibody Fragments

[00256] As noted above, antibody fragments comprise a portion of an intact full length antibody, preferably an antigen binding or variable region of the intact antibody, and include linear antibodies and multispecific antibodies formed from antibody fragments. Nonlimiting examples of antibody fragments include Fab, Fab', F(ab')2, Fv, Fd, domain antibody (dAb), complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single chain antibody fragments, maxibodies, diabodies, triabodies, tetrabodies, minibodies, linear antibodies, chelating recombinant antibodies, tribodies or bibodies, intrabodies, nanobodies, small modular immunopharmaceuticals (SMIPs), an antigen-binding-domain immunoglobulin fusion protein, a camelized antibody, a VHH containing antibody, or muteins or derivatives thereof, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide, such as a CDR sequence, as long as the antibody retains the desired biological activity. Such antigen fragments may be produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA technologies or peptide synthesis.

[00257] Additional antibody fragments include a domain antibody (dAb) fragment (Ward et al., *Nature* 341:544-546, 1989) which consists of a V_H domain.

[00258] "Linear antibodies" comprise a pair of tandem Fd segments (V_H -C_H1-V_H -C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific (Zapata et al. Protein Eng. 8:1057-62 (1995)).

[00259] A "minibody" consisting of scFv fused to CH3 via a peptide linker (hingeless) or via an IgG hinge has been described in Olafsen, et al., Protein Eng Des Sel. 2004 Apr;17(4):315-23.

[00260] The term "maxibody" refers to bivalent scFvs covalently attached to the Fc region of an immunoglobulin, see, for example, Fredericks et al, Protein Engineering, Design & Selection, 17:95-106 (2004) and Powers et al., Journal of Immunological Methods, 251:123-135 (2001).

[00261] Functional heavy-chain antibodies devoid of light chains are naturally occurring in certain species of animals, such as nurse sharks, wobbegong sharks and *Camelidae*, such as camels, dromedaries, alpacas and llamas. The antigen-binding site is reduced to a single domain, the VH_H domain, in these animals. These antibodies form antigen-binding regions using only heavy chain variable region, i.e., these functional antibodies are homodimers of heavy chains only having the structure H_2L_2 (referred to as "heavy-chain antibodies" or "HCAbs"). Camelized V_{HH} reportedly recombines with IgG2 and IgG3 constant regions that contain hinge, CH2, and CH3 domains and lack a CH1 domain. Classical V_H-only fragments are difficult to produce in soluble form, but improvements in solubility and specific binding can be obtained when framework residues are altered to be more VH_H-like. (See, e.g., Reichman, etal., J Immunol Methods 1999, 231:25-38.) Camelized V_{HH} domains have been found to bind to antigen with high affinity (Desmyter et al., *J. Biol. Chem.* 276:26285-90, 2001) and possess high stability in solution (Ewert et al., *Biochemistry* 41:3628-36, 2002). Methods for generating antibodies having camelized heavy chains are described in, for example, in U.S. Patent Publication Nos. 2005/0136049 and 2005/0037421. Alternative scaffolds can be made from human variable-like domains that more closely match the shark V-NAR scaffold and may provide a framework for a long penetrating loop structure.

[00262] Because the variable domain of the heavy-chain antibodies is the smallest fully functional antigen-binding fragment with a molecular mass of only 15 kDa, this entity is referred to as a nanobody (Cortez-Retamozo et al., *Cancer Research* 64:2853-57, 2004). A nanobody library may be generated from an immunized dromedary as described in Conrath et al., (*Antimicrob Agents Chemother* 45: 2807-12, 2001).

[00263] Intrabodies are single chain antibodies which demonstrate intracellular expression and can manipulate intracellular protein function (Biocca, et al., *EMBO J.* 9:101–108, 1990; Colby et al., *Proc Natl Acad Sci U S A.* 101:17616-21, 2004). Intrabodies, which comprise cell signal sequences which retain the antibody contruct in intracellular regions, may be produced as described in Mhashilkar et al (*EMBO J* 14:1542-51, 1995) and Wheeler et al. (*FASEB J.* 17:1733-5. 2003). Transbodies are cell-permeable antibodies in which a protein transduction domains (PTD) is fused with single chain variable fragment (scFv) antibodies Heng et al., (*Med Hypotheses.* 64:1105-8, 2005).

[00264] Further encompassed by the invention are antibodies that are SMIPs or binding domain immunoglobulin fusion proteins specific for target protein. These constructs are single-chain polypeptides comprising antigen binding domains fused to immunoglobulin domains necessary to carry out antibody effector functions. See e.g., WO03/041600, U.S. Patent publication 20030133939 and US Patent Publication 20030118592.

[00265] Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies, but can also be produced directly by recombinant host cells. See, for example, Better et al., Science 240: 1041-1043 (1988); Skerra et al. Science 240: 1038-1041 (1988); Carter et al., Bio/Technology 10:163-167 (1992).

[00266] Other Embodiments of Immunoglobulins: Multivalent Antibodies

[00267] In some embodiments, it may be desirable to generate multivalent or even a multispecific (e.g. bispecific, trispecific, etc.) monoclonal antibody. Such antibody may have binding specificities for at least two different epitopes of the target antigen, or alternatively it may bind to two different molecules, e.g. to the target antigen and to a cell surface protein or receptor. For example, a bispecific antibody may include an arm that binds to the target and another arm that binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g., CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the target-expressing cell. As another example, bispecific antibodies may be used to localize cytotoxic agents to cells which express target antigen. These antibodies possess a target-binding arm and an arm which binds the cytotoxic agent (e.g., saporin, anti-interferon-60, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Multispecific antibodies can be prepared as full length antibodies or antibody fragments.

[00268] Additionally, the immunoglobulins (e.g., antibodies and antibody fragments) and conjugates of the present invention can also be constructed to fold into multivalent forms, which may improve half-life in blood. Multivalent forms can be prepared by techniques known in the art.

[00269] Bispecific or multispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques. Another method is designed to make tetramers by adding a streptavidin-coding sequence at the C-terminus of the scFv. Streptavidin is composed of four subunits, so when the scFv-streptavidin is folded, four subunits associate to form a tetramer (Kipriyanov et al., Hum Antibodies Hybridomas 6(3): 93-101 (1995), the disclosure of which is incorporated herein by reference in its entirety).

[00270] According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. One interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO 96/27011 published Sept. 6, 1996.

[00271] Techniques for generating bispecific or multispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific or trispecific antibodies can be prepared using chemical linkage. Brennan et al., Science 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. Better et al., Science 240: 1041-1043 (1988) disclose secretion of functional antibody fragments from bacteria (*see, e.g.,* Better et al., Skerra et al. Science 240: 1038-1041 (1988)). For example, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form bispecific antibodies (Carter et al., Bio/Technology 10:163-167 (1992); Shalaby et al., J. Exp. Med. 175:217-225 (1992)).

[00272] Shalaby et al., J. Exp. Med. 175:217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E.coli* and subjected to directed chemical coupling *in vitro* to form the bispecfic antibody.

[00273] Various techniques for making and isolating bispecific or multispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers, e.g. GCN4. (See generally Kostelny et al., J. Immunol. 148(5):1547-1553 (1992).) The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers.

[00274] Diabodies, described above, are one example of a bispecific antibody. See, for example, Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993). Bivalent diabodies can be stabilized by disulfide linkage.

[00275] Stable monospecific or bispecific Fv tetramers can also be generated by noncovalent association in $(scFv_2)_2$ configuration or as bis-tetrabodies. Alternatively, two different scFvs can be joined in tandem to form a bis-scFv.

[00276] Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. *See* Gruber et al., J. Immunol. 152: 5368 (1994). One approach has been to link two scFv antibodies with linkers or disulfide bonds (Mallender and Voss, J. Biol. Chem. 269:199-2061994, WO 94/13806, and U.S. Patent No. 5,989,830, the disclosures of which are incorporated herein by reference in their entireties).

[00277] Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata et al. Protein Eng. 8(10):1057-1062 (1995). Briefly, these

antibodies comprise a pair of tandem Fd segments (V_H -C_H1-V_H -C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[00278] Antibodies with more than two valencies are also contemplated. For example, trispecific antibodies can be prepared. (Tutt et al., J. Immunol. 147:60 (1991)).

[00279] A "chelating recombinant antibody" is a bispecific antibody that recognizes adjacent and non-overlapping epitopes of the target antigen, and is flexible enough to bind to both epitopes simultaneously (Neri et al., *J Mol Biol.* 246:367-73, 1995).

[00280] Production of bispecific Fab-scFv ("bibody") and trispecific Fab-(scFv)(2) ("tribody") are described in Schoonjans et al. (*J Immunol.* 165:7050-57, 2000) and Willems et al. (*J Chromatogr B Analyt Technol Biomed Life Sci.* 786:161-76, 2003). For bibodies or tribodies, a scFv molecule is fused to one or both of the VL-CL (L) and VH-CH_1 (Fd) chains, e.g., to produce a tribody two scFvs are fused to C-term of Fab while in a bibody one scFv is fused to C-term of Fab.

[00281] In yet another method, dimers, trimers, and tetramers are produced after a free cysteine is introduced in the parental protein. A peptide-based cross linker with variable numbers (two to four) of maleimide groups was used to cross link the protein of interest to the free cysteines (Cochran et al., Immunity 12(3): 241-50 (2000), the disclosure of which is incorporated herein in its entirety).

[00282] Other Embodiments of Immunoglobulins

[00283] Inventive immunoglobulins also include peptibodies. The term "peptibody" refers to a molecule comprising an antibody Fc domain attached to at least one peptide. The production of peptibodies is generally described in PCT publication WO 00/24782, published May 4, 2000. Any of these peptides may be linked in tandem (i.e., sequentially), with or without linkers. Peptides containing a cysteinyl residue may be cross-linked with another Cys-containing peptide, either or

both of which may be linked to a vehicle. Any peptide having more than one Cys residue may form an intrapeptide disulfide bond, as well. Any of these peptides may be derivatized, for example the carboxyl terminus may be capped with an amino group, cysteines may be cappe, or amino acid residues may substituted by moieties other than amino acid residues (see, e.g., Bhatnagar et al., J. Med. Chem. 39: 3814-9 (1996), and Cuthbertson et al., J. Med. Chem. 40: 2876-82 (1997), which are incorporated by reference herein in their entirety). The peptide sequences may be optimized, analogous to affinity maturation for antibodies, or otherwise altered by alanine scanning or random or directed mutagenesis followed by screening to identify the best binders. Lowman, Ann. Rev. Biophys. Biomol. Struct. 26: 401-24 (1997). Various molecules can be inserted into the immunoglobulin structure, e.g., within the peptide portion itself or between the peptide and vehicle portions of the immunoglobulins, while retaining the desired activity of immunoglobulin. One can readily insert, for example, molecules such as an Fc domain or fragment thereof, polyethylene glycol or other related molecules such as dextran, a fatty acid, a lipid, a cholesterol group, a small carbohydrate, a peptide, a detectable moiety as described herein (including fluorescent agents, radiolabels such as radioisotopes), an oligosaccharide, oligonucleotide, a polynucleotide, interference (or other) RNA, enzymes, hormones, or the like. Other molecules suitable for insertion in this fashion will be appreciated by those skilled in the art, and are encompassed within the scope of the invention. This includes insertion of, for example, a desired molecule in between two consecutive amino acids, optionally joined by a suitable linker.

[00284] Linkers. A "linker" or "linker moiety", as used interchangeably herein, refers to a biologically acceptable peptidyl or non-peptidyl organic group that is covalently bound to an amino acid residue of a polypeptide chain (e.g., an immunoglobulin HC or immunoglobulin LC or immunoglobulin Fc domain) contained in the inventive composition, which linker moiety covalently joins or conjugates the polypeptide chain to another peptide or polypeptide chain in the molecule, or to a therapeutic moiety, such as a biologically active small molecule or oligopeptide, or to a half-life extending moiety, e.g., see, Sullivan et al., Toxin

Peptide Therapeutic Agents, US2007/0071764; Sullivan et al., Toxin Peptide Therapeutic Agents, PCT/US2007/022831, published as WO 2008/088422; and US Provisional Application Serial No. 61/210,594, filed March 20, 2009, which are all incorporated herein by reference in their entireties.

[00285] The presence of any linker moiety in the immunoglobulins of the present invention is optional. When present, the linker's chemical structure is not critical, since it serves primarily as a spacer to position, join, connect, or optimize presentation or position of one functional moiety in relation to one or more other functional moieties of a molecule of the inventive immunoglobulin. The presence of a linker moiety can be useful in optimizing pharamcologial activity of some embodiments of the inventive immunoglobulin (including antibodies and antibody fragments). The linker is preferably made up of amino acids linked together by peptide bonds. The linker moiety, if present, can be independently the same or different from any other linker, or linkers, that may be present in the inventive immunoglobulin.

[00286] As stated above, the linker moiety, if present (whether within the primary amino acid sequence of the immunoglobulin, or as a linker for attaching a therapeutic moiety or half-life extending moiety to the inventive immunoglobulin), can be "peptidyl" in nature (i.e., made up of amino acids linked together by peptide bonds) and made up in length, preferably, of from 1 up to about 40 amino acid residues, more preferably, of from 1 up to about 20 amino acid residues, and most preferably of from 1 to about 10 amino acid residues. Preferably, but not necessarily, the amino acid residues in the linker are from among the twenty canonical amino acids, more preferably, cysteine, glycine, alanine, proline, asparagine, glutamine, and /or serine. Even more preferably, a peptidyl linker is made up of a majority of amino acids that are sterically unhindered, such as glycine, serine, and alanine linked by a peptide bond. It is also desirable that, if present, a peptidyl linker be selected that avoids rapid proteolytic turnover in circulation *in vivo*. Some of these amino acids may be glycosylated, as is well understood by those in the art. For example, a useful linker sequence constituting a sialylation site is

$X_1X_2NX_4X_5G$ (SEQ ID NO:148), wherein X_1, X_2,X_4 and X_5 are each independently any amino acid residue.

[00287] In other embodiments, the 1 to 40 amino acids of the peptidyl linker moiety are selected from glycine, alanine, proline, asparagine, glutamine, and lysine. Preferably, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. Thus, preferred linkers include polyglycines, polyserines, and polyalanines, or combinations of any of these. Some exemplary peptidyl linkers are poly$(Gly)_{1-8}$, particularly $(Gly)_3$, $(Gly)_4$ (SEQ ID NO:149), $(Gly)_5$ (SEQ ID NO:150) and $(Gly)_7$ (SEQ ID NO:151), as well as, poly$(Gly)_4$Ser (SEQ ID NO:152), poly(Gly-Ala)$_{2-4}$ and poly$(Ala)_{1-8}$. Other specific examples of peptidyl linkers include $(Gly)_5$Lys (SEQ ID NO:154), and $(Gly)_5$LysArg (SEQ ID NO:155). Other examples of useful peptidyl linkers are: Other examples of useful peptidyl linkers are:

[00288] $(Gly)_3Lys(Gly)_4$ (SEQ ID NO:159);

[00289] $(Gly)_3AsnGlySer(Gly)_2$ (SEQ ID NO:156);

[00290] $(Gly)_3Cys(Gly)_4$ (SEQ ID NO:157); and

[00291] GlyProAsnGlyGly (SEQ ID NO:158).

[00292] To explain the above nomenclature, for example, $(Gly)_3Lys(Gly)_4$ means Gly-Gly-Gly-Lys-Gly-Gly-Gly-Gly (SEQ ID NO:159). Other combinations of Gly and Ala are also useful.

[00293] Commonly used linkers include those which may be identified herein as "L5" (GGGGS; or "G_4S"; SEQ ID NO:152), "L10" (GGGGSGGGGS; SEQ ID NO:153), "L25" (GGGGSGGGGSGGGGSGGGGSGGGGS; SEQ ID NO:146) and any linkers used in the working examples hereinafter.

[00294] In some embodiments of the compositions of this invention, which comprise a peptide linker moiety, acidic residues, for example, glutamate or

aspartate residues, are placed in the amino acid sequence of the linker moiety. Examples include the following peptide linker sequences:

[00295] GGEGGG (SEQ ID NO:160);

[00296] GGEEEGGG (SEQ ID NO:161);

[00297] GEEEG (SEQ ID NO:162);

[00298] GEEE (SEQ ID NO:163);

[00299] GGDGGG (SEQ ID NO:164);

[00300] GGDDDGG (SEQ ID NO:165);

[00301] GDDDG (SEQ ID NO:166);

[00302] GDDD (SEQ ID NO:167);

[00303] GGGGSDDSDEGSDGEDGGGGS (SEQ ID NO:168);

[00304] WEWEW (SEQ ID NO:169);

[00305] FEFEF (SEQ ID NO:170);

[00306] EEEWWW (SEQ ID NO:171);

[00307] EEEFFF (SEQ ID NO:172);

[00308] WWEEEWW (SEQ ID NO:173); or

[00309] FFEEEFF (SEQ ID NO:174).

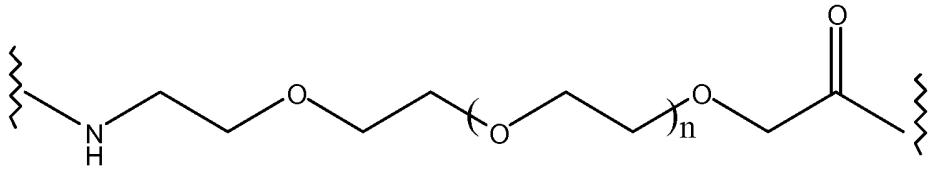
[00310] In other embodiments, the linker constitutes a phosphorylation site, e.g., $X_1X_2YX_4X_5G$ (SEQ ID NO:175), wherein X_1, X_2, X_4, and X_5 are each independently any amino acid residue; $X_1X_2SX_4X_5G$ (SEQ ID NO:176), wherein X_1, X_2,X_4 and X_5 are each independently any amino acid residue; or $X_1X_2TX_4X_5G$ (SEQ ID NO:177), wherein X_1, X_2, X_4 and X_5 are each independently any amino acid residue.

[00311] The linkers shown here are exemplary; peptidyl linkers within the scope of this invention may be much longer and may include other residues. A peptidyl linker can contain, e.g., a cysteine, another thiol, or nucleophile for conjugation with a half-life extending moiety. In another embodiment, the linker contains a cysteine or homocysteine residue, or other 2-amino-ethanethiol or 3-amino-propanethiol moiety for conjugation to maleimide, iodoacetaamide or thioester, functionalized half-life extending moiety.

[00312] Another useful peptidyl linker is a large, flexible linker comprising a random Gly/Ser/Thr sequence, for example: GSGSATGGSGSTASSGSGSATH (SEQ ID NO:178) or HGSGSATGGSGSTASSGSGSAT (SEQ ID NO:179), that is estimated to be about the size of a 1 kDa PEG molecule. Alternatively, a useful peptidyl linker may be comprised of amino acid sequences known in the art to form rigid helical structures (e.g., Rigid linker: -AEAAAKEAAAKEAAAKAGG-) (SEQ ID NO:180). Additionally, a peptidyl linker can also comprise a non-peptidyl segment such as a 6 carbon aliphatic molecule of the formula $-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$. The peptidyl linkers can be altered to form derivatives as described herein.

[00313] Optionally, a non-peptidyl linker moiety is also useful for conjugating the half-life extending moiety to the peptide portion of the half-life extending moiety-conjugated toxin peptide analog. For example, alkyl linkers such as $-NH-(CH_2)_s-C(O)-$, wherein s = 2-20 can be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, *etc*. Exemplary non-peptidyl linkers are polyethylene glycol (PEG) linkers (e.g., shown below):

[00314] (I)



[0002] wherein n is such that the linker has a molecular weight of about 100 to about 5000 Daltons (Da), preferably about 100 to about 500 Da.

[00315] In one embodiment, the non-peptidyl linker is aryl. The linkers may be altered to form derivatives in the same manner as described in the art, e.g., in Sullivan et al., Toxin Peptide Therapeutic Agents, US2007/0071764; Sullivan et al., Toxin Peptide Therapeutic Agents, PCT/US2007/022831, published as WO 2008/088422; and US Provisional Application Serial No. 61/210,594, filed March 20, 2009, which are all incorporated herein by reference in their entireties.

[00316] In addition, PEG moieties may be attached to the N-terminal amine or selected side chain amines by either reductive alkylation using PEG aldehydes or acylation using hydroxysuccinimido or carbonate esters of PEG, or by thiol conjugation.

[00317] "Aryl" is phenyl or phenyl vicinally-fused with a saturated, partially-saturated, or unsaturated 3-, 4-, or 5 membered carbon bridge, the phenyl or bridge being substituted by 0, 1, 2 or 3 substituents selected from C_{1-8} alkyl, C_{1-4} haloalkyl or halo.

[00318] "Heteroaryl" is an unsaturated 5 , 6 or 7 membered monocyclic or partially-saturated or unsaturated 6-, 7-, 8-, 9-, 10- or 11 membered bicyclic ring, wherein at least one ring is unsaturated, the monocyclic and the bicyclic rings containing 1, 2, 3 or 4 atoms selected from N, O and S, wherein the ring is substituted by 0, 1, 2 or 3 substituents selected from C_{1-8} alkyl, C_{1-4} haloalkyl and halo.

[00319] Non-peptide portions of the inventive composition of matter, such as non-peptidyl linkers or non-peptide half-life extending moieties can be synthesized by conventional organic chemistry reactions.

[00320] The above is merely illustrative and not an exhaustive treatment of the kinds of linkers that can optionally be employed in accordance with the present invention.

[00321] Production of Immunoglobulin Variants. As noted above, recombinant DNA- and/or RNA-mediated protein expression and protein engineering techniques, or any other methods of preparing peptides, are applicable to the making of the inventive compositions. For example, polypeptides can be made in transformed host cells. Briefly, a recombinant DNA molecule, or construct, coding for the peptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences encoding the peptides can be excised from DNA using suitable restriction enzymes. Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial host cells in culture include bacteria (such as Escherichia coli sp.), yeast (such as Saccharomyces sp.) and other fungal cells, insect cells, plant cells, mammalian (including human) cells, e.g., CHO cells and HEK-293 cells, and others noted herein or otherwise known in the art. Modifications can be made at the DNA level, as well. The peptide-encoding DNA sequence may be changed to codons more compatible with the chosen host cell. For E. coli, optimized codons are known in the art. Codons can be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected host cell. Next, the transformed host is cultured and purified. Host cells may be cultured under

conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art. In addition, the DNA optionally further encodes, 5' to the coding region of a fusion protein, a signal peptide sequence (e.g., a secretory signal peptide) operably linked to the expressed immunoglobulin. For further examples of appropriate recombinant methods and exemplary DNA constructs useful for recombinant expression of the inventive compositions by mammalian cells, including dimeric Fc fusion proteins ("peptibodies") or chimeric immunoglobulin (light chain + heavy chain)-Fc heterotrimers ("hemibodies"), conjugated to specific binding agents of the invention, see, e.g., Sullivan et al., Toxin Peptide Therapeutic Agents, US2007/0071764; Sullivan et al., Toxin Peptide Therapeutic Agents, PCT/US2007/022831, published as WO 2008/088422; and US Provisional Application Serial No. 61/210,594, filed March 20, 2009, which are all incorporated herein by reference in their entireties.

[00322] Amino acid sequence variants of the desired immunoglobulin may be prepared by introducing appropriate nucleotide changes into the encoding DNA, or by peptide synthesis. Such variants include, for example, deletions and/or insertions and/or substitutions of residues within the amino acid sequences of the immunoglobulins or antibodies. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the immunoglobulin, such as changing the number or position of glycosylation sites. In certain instances, immunoglobulin variants are prepared with the intent to modify those amino acid residues which are directly involved in epitope binding. In other embodiments, modification of residues which are not directly involved in epitope binding or residues not involved in epitope binding in any way, is desirable, for purposes discussed herein. Mutagenesis within any of the CDR regions and/or framework regions is contemplated. Covariance analysis techniques can be employed by the skilled artisan to design useful modifications in the amino acid sequence of the immunoglobulin, including an antibody or antibody fragment. (E.g., Choulier, et al., Covariance Analysis of

Protein Families: The Case of the Variable Domains of Antibodies, Proteins: Structure, Function, and Genetics 41:475-484 (2000); Demarest et al., Optimization of the Antibody C_H3 Domain by Residue Frequency Analysis of IgG Sequences, J. Mol. Biol. 335:41-48 (2004); Hugo et al., VL position 34 is a key determinant for the engineering of stable antibodies with fast dissociation rates, Protein Engineering 16(5):381-86 (2003); Aurora et al., Sequence covariance networks, methods and uses thereof, US 2008/0318207 A1; Glaser et al., Stabilized polypeptide compositions, US 2009/0048122 A1; Urech et al., Sequence based engineering and optimization of single chain antibodies, WO 2008/110348 A1; Borras et al., Methods of modifying antibodies, and modified antibodies with improved functional properties, WO 2009/000099 A2). Such modifications determined by covariance analysis can improve potency, pharmacokinetic, pharmacodynamic, and/or manufacturability characteristics of an immunoglobulin.

[00323] Nucleic acid molecules encoding amino acid sequence variants of the immunoglobulin or antibody are prepared by a variety of methods known in the art. Such methods include oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the immunoglobulin.

[00324] Substitutional mutagenesis within any of the hypervariable or CDR regions or framework regions is contemplated. A useful method for identification of certain residues or regions of the immunoglobulin that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells Science, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For

example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed variants are screened for the desired activity.

[00325] Some embodiments of the immunoglobulins of the present invention can also be made by synthetic methods. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides. For example, well known solid phase synthesis techniques include the use of protecting groups, linkers, and solid phase supports, as well as specific protection and deprotection reaction conditions, linker cleavage conditions, use of scavengers, and other aspects of solid phase peptide synthesis. Suitable techniques are well known in the art. (E.g., Merrifield (1973), Chem. Polypeptides, pp. 335-61 (Katsoyannis and Panayotis eds.); Merrifield (1963), J. Am. Chem. Soc. 85: 2149; Davis et al. (1985), Biochem. Intl. 10: 394-414; Stewart and Young (1969), Solid Phase Peptide Synthesis; U.S. Pat. No. 3,941,763; Finn et al. (1976), The Proteins (3rd ed.) 2: 105-253; and Erickson et al. (1976), The Proteins (3rd ed.) 2: 257-527; "Protecting Groups in Organic Synthesis," 3rd Edition, T. W. Greene and P. G. M. Wuts, Eds., John Wiley & Sons, Inc., 1999; NovaBiochem Catalog, 2000; "Synthetic Peptides, A User's Guide," G. A. Grant, Ed., W.H. Freeman & Company, New York, N.Y., 1992; "Advanced Chemtech Handbook of Combinatorial & Solid Phase Organic Chemistry," W. D. Bennet, J. W. Christensen, L. K. Hamaker, M. L. Peterson, M. R. Rhodes, and H. H. Saneii, Eds., Advanced Chemtech, 1998; "Principles of Peptide Synthesis, 2nd ed.," M. Bodanszky, Ed., Springer-Verlag, 1993; "The Practice of Peptide Synthesis, 2nd ed.," M. Bodanszky and A. Bodanszky, Eds., Springer-Verlag, 1994; "Protecting Groups," P. J. Kocienski, Ed., Georg Thieme Verlag, Stuttgart, Germany, 1994; "Fmoc Solid Phase Peptide Synthesis, A Practical Approach," W. C. Chan and P. D. White, Eds., Oxford Press, 2000, G. B. Fields et al., Synthetic Peptides: A User's Guide, 1990, 77-183). For further examples of synthetic and purification methods known in the art, which are applicable to making the inventive compositions of matter, see, e.g., Sullivan et al., Toxin Peptide Therapeutic Agents, US2007/0071764 and Sullivan et al., Toxin

Peptide Therapeutic Agents, PCT/US2007/022831, published as WO 2008/088422 A2, which are both incorporated herein by reference in their entireties.

[00326] In further describing any of the immunoglobulins herein, as well as variants, a one-letter abbreviation system is frequently applied to designate the identities of the twenty "canonical" amino acid residues generally incorporated into naturally occurring peptides and proteins (Table 3). Such one-letter abbreviations are entirely interchangeable in meaning with three-letter abbreviations, or non-abbreviated amino acid names. Within the one-letter abbreviation system used herein, an upper case letter indicates a L-amino acid, and a lower case letter indicates a D-amino acid. For example, the abbreviation "R" designates L-arginine and the abbreviation "r" designates D-arginine.

Table 3. One-letter abbreviations for the canonical amino acids. Three-letter abbreviations are in parentheses.

Alanine (Ala)	A
Glutamine (Gln)	Q
Leucine (Leu)	L
Serine (Ser)	S
Arginine (Arg)	R
Glutamic Acid (Glu)	E
Lysine (Lys)	K
Threonine (Thr)	T
Asparagine (Asn)	N
Glycine (Gly)	G
Methionine (Met)	M
Tryptophan (Trp)	W
Aspartic Acid (Asp)	D
Histidine (His)	H

Phenylalanine (Phe)	F
Tyrosine (Tyr)	Y
Cysteine (Cys)	C
Isoleucine (Ile)	I
Proline (Pro)	P
Valine (Val)	V

[00327] An amino acid substitution in an amino acid sequence is typically designated herein with a one-letter abbreviation for the amino acid residue in a particular position, followed by the numerical amino acid position relative to an original sequence of interest, which is then followed by the one-letter symbol for the amino acid residue substituted in. For example, "T30D" symbolizes a substitution of a threonine residue by an aspartate residue at amino acid position 30, relative to the original sequence of interest. Another example, "W101F" symbolizes a substitution of a tryptophan residue by a phenylalanine residue at amino acid position 101, relative to the original sequence of interest.

[00328] Non-canonical amino acid residues can be incorporated into a polypeptide within the scope of the invention by employing known techniques of protein engineering that use recombinantly expressing cells. (See, e.g., Link et al., Non-canonical amino acids in protein engineering, Current Opinion in Biotechnology, 14(6):603-609 (2003)). The term "non-canonical amino acid residue" refers to amino acid residues in D- or L-form that are not among the 20 canonical amino acids generally incorporated into naturally occurring proteins, for example, β-amino acids, homoamino acids, cyclic amino acids and amino acids with derivatized side chains. Examples include (in the L-form or D-form) β-alanine, β-aminopropionic acid, piperidinic acid, aminocaprioic acid, aminoheptanoic acid, aminopimelic acid, desmosine, diaminopimelic acid, N^{α}-ethylglycine, N^{α}-ethylaspargine, hydroxylysine, allo-hydroxylysine, isodesmosine, allo-isoleucine, ω-methylarginine, N^{α}-methylglycine, N^{α}-methylisoleucine, N^{α}-methylvaline, , γ-carboxyglutamate, ε-

N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N^{α}-acetylserine, N^{α}-formylmethionine, 3-methylhistidine, 5-hydroxylysine, and other similar amino acids, and those listed in Table 4 below, and derivatized forms of any of these as described herein. Table 4 contains some exemplary non-canonical amino acid residues that are useful in accordance with the present invention and associated abbreviations as typically used herein, although the skilled practitioner will understand that different abbreviations and nomenclatures may be applicable to the same substance and appear interchangeably herein.

Table 4. Useful non-canonical amino acids for amino acid addition, insertion, or substitution into peptide sequences in accordance with the present invention. In the event an abbreviation listed in Table 4 differs from another abbreviation for the same substance disclosed elsewhere herein, both abbreviations are understood to be applicable. The amino acids listed in Table 4 can be in the L-form or D-form.

Amino Acid	Abbreviation(s)
Acetamidomethyl	Acm
Acetylarginine	acetylarg
α-aminoadipic acid	Aad
aminobutyric acid	Abu
6-aminohexanoic acid	Ahx; εAhx
3-amino-6-hydroxy-2-piperidone	Ahp
2-aminoindane-2-carboxylic acid	Aic
α-amino-isobutyric acid	Aib
3-amino-2-naphthoic acid	Anc
2-aminotetraline-2-carboxylic acid	Atc
Aminophenylalanine	Aminophe; Amino-Phe
4-amino-phenylalanine	4AmP
4-amidino-phenylalanine	4AmPhe

2-amino-2-(1-carbamimidoylpiperidin-4-yl)acetic acid	4AmPig
Arg ψ(CH_2NH) -reduced amide bond	rArg
β-homoarginine	bhArg
β-homolysine	bhomoK
β-homo Tic	BhTic
β-homophenylalanine	BhPhe
β-homoproline	BhPro
β-homotryptophan	BhTrp
4,4'-biphenylalanine	Bip
β, β-diphenyl-alanine	BiPhA
β-phenylalanine	BPhe
p-carboxyl-phenylalanine	Cpa
Citrulline	Cit
Cyclohexylalanine	Cha
Cyclohexylglycine	Chg
Cyclopentylglycine	Cpg
2-amino-3-guanidinopropanoic acid	3G-Dpr
α, γ-diaminobutyric acid	Dab
2,4-diaminobutyric acid	Dbu
diaminopropionic acid	Dap
α, β-diaminopropionoic acid (or 2,3-diaminopropionic acid	Dpr
3,3-diphenylalanine	Dip
4-guanidino phenylalanine	Guf
4-guanidino proline	4GuaPr
Homoarginine	hArg; hR
Homocitrulline	hCit

Homoglutamine	hQ
Homolysine	hLys; hK; homoLys
Homophenylalanine	hPhe; homoPhe
4-hydroxyproline (or hydroxyproline)	Hyp
2-indanylglycine (or indanylglycine)	IgI
indoline-2-carboxylic acid	Idc
Iodotyrosine	I-Tyr
Lys ψ(CH_2NH)-reduced amide bond	rLys
methinine oxide	Met[O]
methionine sulfone	$Met[O]_2$
N^{α}-methylarginine	NMeR
Nα-[$(CH_2)_3NHCH(NH)NH_2$] substituted glycine	N-Arg
N^{α}-methylcitrulline	NMeCit
N^{α}-methylglutamine	NMeQ
N^{α}-methylhomocitrulline	N^{α}-MeHoCit
N^{α}-methylhomolysine	NMeHoK
N^{α}-methylleucine	N^{α}-MeL; NMeL; NMeLeu; NMe-Leu
N^{α}-methyllysine	NMe-Lys
$N\varepsilon$-methyl-lysine	N-eMe-K
$N\varepsilon$-ethyl-lysine	N-eEt-K
$N\varepsilon$-isopropyl-lysine	N-eIPr-K
N^{α}-methylnorleucine	NMeNle; NMe-Nle
N^{α}-methylornithine	N^{α}-MeOrn; NMeOrn
N^{α}-methylphenylalanine	NMe-Phe
4-methyl-phenylalanine	MePhe
α-methylphenyalanine	AMeF
N^{α}-methylthreonine	NMe-Thr; NMeThr

N^{α}-methylvaline	NMeVal; NMe-Val
*N*ε-(O-(aminoethyl)-O'-(2-propanoyl)-undecaethyleneglycol)-Lysine	K(NPeg11)
*N*ε-(O-(aminoethyl)-O'-(2-propanoyl)-(ethyleneglycol)27-Lysine	K(NPeg27)
3-(1-naphthyl)alanine	1-Nal; 1Nal
3-(2-naphthyl)alanine	2-Nal; 2Nal
nipecotic acid	Nip
Nitrophenylalanine	nitrophe
norleucine	Nle
norvaline	Nva or Nvl
O-methyltyrosine	Ome-Tyr
octahydroindole-2-carboxylic acid	Oic
Ornithine	Orn
Orn ψ(CH2NH)-reduced amide bond	rOrn
4-piperidinylalanine	4PipA
4-pyridinylalanine	4Pal
3-pyridinylalanine	3Pal
2-pyridinylalanine	2Pal
para-aminophenylalanine	4AmP; 4-Amino-Phe
para-iodophenylalanine (or 4-iodophenylalanine)	pI-Phe
Phenylglycine	Phg
4-phenyl-phenylalanine (or biphenylalanine)	4Bip
4,4'-biphenyl alanine	Bip
pipecolic acid	Pip
4-amino-1-piperidine-4-carboxylic acid	4Pip
Sarcosine	Sar

1,2,3,4-tetrahydroisoquinoline	Tic
1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid	Tiq
1,2,3,4-tetrahydroisoquinoline-7-hydroxy-3-carboxylic acid	Hydroxyl-Tic
1,2,3,4-tetrahydronorharman-3-carboxylic acid	Tpi
thiazolidine-4-carboxylic acid	Thz
3-thienylalanine	Thi

[00329] Nomenclature and Symbolism for Amino Acids and Peptides by the UPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) have been published in the following documents: Biochem. J., 1984, 219, 345-373; Eur. J. Biochem., 1984, 138, 9-37; 1985, 152, 1; 1993, 213, 2; Internat. J. Pept. Prot. Res., 1984, 24, following p 84; J. Biol. Chem., 1985, 260, 14-42; Pure Appl. Chem., 1984, 56, 595-624; Amino Acids and Peptides, 1985, 16, 387-410; Biochemical Nomenclature and Related Documents, 2nd edition, Portland Press, 1992, pages 39-69.

[00330] The one or more useful modifications to peptide domains of the inventive immunoglobulin can include amino acid additions or insertions, amino acid deletions, peptide truncations, amino acid substitutions, and/or chemical derivatization of amino acid residues, accomplished by known chemical techniques. For example, the thusly modified amino acid sequence includes at least one amino acid residue inserted or substituted therein, relative to the amino acid sequence of the native sequence of interest, in which the inserted or substituted amino acid residue has a side chain comprising a nucleophilic or electrophilic reactive functional group by which the peptide is conjugated to a linker and/or half-life extending moiety. In accordance with the invention, useful examples of such a nucleophilic or electrophilic reactive functional group include, but are not limited to, a thiol, a primary amine, a seleno, a hydrazide, an aldehyde, a carboxylic acid, a ketone, an

aminooxy, a masked (protected) aldehyde, or a masked (protected) keto functional group. Examples of amino acid residues having a side chain comprising a nucleophilic reactive functional group include, but are not limited to, a lysine residue, a homolysine, an α,β-diaminopropionic acid residue, an α,γ-diaminobutyric acid residue, an ornithine residue, a cysteine, a homocysteine, a glutamic acid residue, an aspartic acid residue, or a selenocysteine residue.

[00331] Amino acid residues are commonly categorized according to different chemical and/or physical characteristics. The term "acidic amino acid residue" refers to amino acid residues in D- or L-form having side chains comprising acidic groups. Exemplary acidic residues include aspartatic acid and glutamatic acid residues. The term "alkyl amino acid residue" refers to amino acid residues in D- or L-form having C_{1-6}alkyl side chains which may be linear, branched, or cyclized, including to the amino acid amine as in proline, wherein the C_{1-6}alkyl is substituted by 0, 1, 2 or 3 substituents selected from C_{1-4}haloalkyl, halo, cyano, nitro, $-C(=O)R^b$, $-C(=O)OR^a$, $-C(=O)NR^aR^a$, $-C(=NR^a)NR^aR^a$, $-NR^aC(=NR^a)NR^aR^a$, $-OR^a$, $-OC(=O)R^b$, $-OC(=O)NR^aR^a$, $-OC_{2-6}$alkylNR^aR^a, $-OC_{2-6}$alkylOR^a, $-SR^a$, $-S(=O)R^b$, $-S(=O)_2R^b$, $-S(=O)_2NR^aR^a$, $-NR^aR^a$, $-N(R^a)C(=O)R^b$, $-N(R^a)C(=O)OR^b$, $-N(R^a)C(=O)NR^aR^a$, $-N(R^a)C(=NR^a)NR^aR^a$, $-N(R^a)S(=O)_2R^b$, $-N(R^a)S(=O)_2NR^aR^a$, $-NR^aC_{2-6}$alkylNR^aR^a and $-NR^aC_{2-6}$alkylOR^a; wherein R^a is independently, at each instance, H or R^b; and R^b is independently, at each instance C_{1-6}alkyl substituted by 0, 1, 2 or 3 substituents selected from halo, C_{1-4}alk, C_{1-3}haloalk, $-OC_{1-4}$alk, $-NH_2$, $-NHC_{1-4}$alk, and $-N(C_{1-4}alk)C_{1-4}$alk; or any protonated form thereof, including alanine, valine, leucine, isoleucine, proline, serine, threonine, lysine, arginine, histidine, aspartate, glutamate, asparagine, glutamine, cysteine, methionine, hydroxyproline, but which residues do not contain an aryl or aromatic group. The term "aromatic amino acid residue" refers to amino acid residues in D- or L-form having side chains comprising aromatic groups. Exemplary aromatic residues include tryptophan, tyrosine, 3-(1-naphthyl)alanine, or phenylalanine residues. The term "basic amino acid residue" refers to amino acid residues in D- or L-form having side chains comprising basic groups. Exemplary basic amino acid residues include histidine, lysine, homolysine,

ornithine, arginine, N-methyl-arginine, ω-aminoarginine, ω-methyl-arginine, 1-methyl-histidine, 3-methyl-histidine, and homoarginine (hR) residues. The term "hydrophilic amino acid residue" refers to amino acid residues in D- or L-form having side chains comprising polar groups. Exemplary hydrophilic residues include cysteine, serine, threonine, histidine, lysine, asparagine, aspartate, glutamate, glutamine, and citrulline (Cit) residues. The terms "lipophilic amino acid residue" refers to amino acid residues in D- or L-form having sidechains comprising uncharged, aliphatic or aromatic groups. Exemplary lipophilic sidechains include phenylalanine, isoleucine, leucine, methionine, valine, tryptophan, and tyrosine. Alanine (A) is amphiphilic—it is capable of acting as a hydrophilic or lipophilic residue. Alanine, therefore, is included within the definition of both "lipophilic residue" and "hydrophilic residue." The term "nonfunctional amino acid residue" refers to amino acid residues in D- or L-form having side chains that lack acidic, basic, or aromatic groups. Exemplary neutral amino acid residues include methionine, glycine, alanine, valine, isoleucine, leucine, and norleucine (Nle) residues.

[00332] Additional useful embodiments of can result from conservative modifications of the amino acid sequences of the polypeptides disclosed herein. Conservative modifications will produce half-life extending moiety-conjugated peptides having functional, physical, and chemical characteristics similar to those of the conjugated (e.g., PEG-conjugated) peptide from which such modifications are made. Such conservatively modified forms of the conjugated polypeptides disclosed herein are also contemplated as being an embodiment of the present invention.

[00333] In contrast, substantial modifications in the functional and/or chemical characteristics of peptides may be accomplished by selecting substitutions in the amino acid sequence that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the region of the substitution, for example, as an α-helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the size of the molecule.

[00334] For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a nonnative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis" (see, for example, MacLennan et al., Acta Physiol. Scand. Suppl., 643:55-67 (1998); Sasaki et al., 1998, Adv. Biophys. 35:1-24 (1998), which discuss alanine scanning mutagenesis).

[00335] Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important residues of the peptide sequence, or to increase or decrease the affinity of the peptide or vehicle-conjugated peptide molecules described herein.

[00336] Naturally occurring residues may be divided into classes based on common side chain properties:

1) hydrophobic: norleucine (Nor or Nle), Met, Ala, Val, Leu, Ile;
2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
3) acidic: Asp, Glu;
4) basic: His, Lys, Arg;
5) residues that influence chain orientation: Gly, Pro; and
6) aromatic: Trp, Tyr, Phe.

[00337] Conservative amino acid substitutions may involve exchange of a member of one of these classes with another member of the same class. Conservative amino acid substitutions may encompass non-naturally occurring amino acid residues, which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics and other reversed or inverted forms of amino acid moieties.

[00338] Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of the toxin peptide analog.

[00339] In making such changes, according to certain embodiments, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. They are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

[00340] The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is understood in the art (see, *for example*, Kyte *et al.*, 1982, *J. Mol. Biol.* 157:105-131). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, in certain embodiments, the substitution of amino acids whose hydropathic indices are within ±2 is included. In certain embodiments, those that are within ±1 are included, and in certain embodiments, those within ±0.5 are included.

[00341] It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biologically functional protein or peptide thereby created is intended for use in immunological embodiments, as disclosed herein. In certain embodiments, the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.*, with a biological property of the protein.

[00342] The following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4);

proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5) and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, in certain embodiments, the substitution of amino acids whose hydrophilicity values are within ±2 is included, in certain embodiments, those that are within ±1 are included, and in certain embodiments, those within ±0.5 are included. One may also identify epitopes from primary amino acid sequences on the basis of hydrophilicity. These regions are also referred to as "epitopic core regions."

[00343] Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine norleucine, alanine, or methionine for another, the substitution of one polar (hydrophilic) amino acid residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic amino acid residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another. The phrase “conservative amino acid substitution” also includes the use of a chemically derivatized residue in place of a non-derivatized residue, provided that such polypeptide displays the requisite bioactivity. Other exemplary amino acid substitutions that can be useful in accordance with the present invention are set forth in Table 5 below.

Table 5. Some Useful Amino Acid Substitutions.

Original Residues	Exemplary Substitutions
Ala	Val, Leu, Ile
Arg	Lys, Gln, Asn

Asn	Gln
Asp	Glu
Cys	Ser, Ala
Gln	Asn
Glu	Asp
Gly	Pro, Ala
His	Asn, Gln, Lys, Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine
Leu	Norleucine, Ile, Val, Met, Ala, Phe
Lys	Arg, 1,4-Diamino-butyric Acid, Gln, Asn
Met	Leu, Phe, Ile
Phe	Leu, Val, Ile, Ala, Tyr

Pro	Ala
Ser	Thr, Ala, Cys
Thr	Ser
Trp	Tyr, Phe
Tyr	Trp, Phe, Thr, Ser
Val	Ile, Met, Leu, Phe, Ala, Norleucine

[00344]

[00345] Ordinarily, amino acid sequence variants of the immunoglobulin will have an amino acid sequence having at least 60% amino acid sequence identity with the original immunoglobulin or antibody amino acid sequences of either the heavy or the light chain variable region, or at least 65%, or at least 70%, or at least 75% or at least 80% identity, more preferably at least 85% identity, even more preferably at least 90% identity, and most preferably at least 95% identity, including for example, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the original sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the immunoglobulin or antibody sequence shall be construed as affecting sequence identity or homology.

[00346] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or

more residues, as well as intra-sequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an immunoglobulin with an N-terminal methionyl residue or the immunoglobulin (including antibody or antibody fragment) fused to an epitope tag or a salvage receptor binding epitope. Other insertional variants of the immunoglobulin or antibody molecule include the fusion to a polypeptide which increases the serum half-life of the immunoglobulin, e.g. at the N-terminus or C-terminus.

[00347] Examples of epitope tags include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol. 8: 2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Mol. Cell. Biol. 5(12): 3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering 3(6): 547-553 (1990)]. Other exemplary tags are a poly-histidine sequence, generally around six histidine residues, that permits isolation of a compound so labeled using nickel chelation. Other labels and tags, such as the FLAG® tag (Eastman Kodak, Rochester, NY) are well known and routinely used in the art.

[00348] Some particular, non-limiting, embodiments of amino acid substitution variants of the inventive immunoglobulins, including antibodies and antibody fragments are exemplified below.

[00349] Any cysteine residue not involved in maintaining the proper conformation of the immunoglobulin also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the immunoglobulin to improve its stability (particularly where the immunoglobulin is an antibody fragment such as an Fv fragment).

[00350] In certain instances, immunoglobulin variants are prepared with the intent to modify those amino acid residues which are directly involved in epitope binding in a starting sequence. In other embodiments, modification of residues which are not directly involved in epitope binding or residues not involved in epitope binding in

any way, is desirable, for purposes discussed herein. Mutagenesis within any of the CDR regions and/or framework regions is contemplated.

[00351] In order to determine which antigen binding protein amino acid residues are important for epitope recognition and binding, alanine scanning mutagenesis can be performed to produce substitution variants. See, for example, Cunningham et al., Science, 244:1081-1085 (1989), the disclosure of which is incorporated herein by reference in its entirety. In this method, individual amino acid residues are replaced one-at-a-time with an alanine residue and the resulting antibody is screened for its ability to bind its specific epitope relative to the unmodified polypeptide. Modified antigen binding proteins with reduced binding capacity are sequenced to determine which residue was changed, indicating its significance in binding or biological properties.

[00352] Substitution variants of antigen binding proteins can be prepared by affinity maturation wherein random amino acid changes are introduced into the parent polypeptide sequence. See, for example, Ouwehand et al., Vox Sang 74 (Suppl 2):223-232, 1998; Rader et al., Proc. Natl. Acad. Sci. USA 95:8910-8915, 1998; Dall'Acqua et al., Curr. Opin. Struct. Biol. 8:443-450, 1998, the disclosures of which are incorporated herein by reference in their entireties. Affinity maturation involves preparing and screening the antigen binding proteins, or variants thereof and selecting from the resulting variants those that have modified biological properties, such as increased binding affinity relative to the parent antigen binding protein. A convenient way for generating substitutional variants is affinity maturation using phage display. Briefly, several hypervariable region sites are mutated to generate all possible amino substitutions at each site. The variants thus generated are expressed in a monovalent fashion on the surface of filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.*, binding affinity). See e.g., WO 92/01047, WO 93/112366, WO 95/15388 and WO 93/19172.

[00353] Current antibody affinity maturation methods belong to two mutagenesis categories: stochastic and nonstochastic. Error prone PCR, mutator bacterial strains (Low et al., *J. Mol. Biol.* 260, 359-68, 1996), and saturation mutagenesis (Nishimiya et al., *J. Biol. Chem.* 275:12813-20, 2000; Chowdhury, P. S. *Methods Mol. Biol.* 178, 269-85, 2002) are typical examples of stochastic mutagenesis methods (Rajpal et al., *Proc Natl Acad Sci U S A.* 102:8466-71, 2005). Nonstochastic techniques often use alanine-scanning or site-directed mutagenesis to generate limited collections of specific muteins. Some methods are described in further detail below.

[00354] *Affinity maturation via panning methods*—Affinity maturation of recombinant antibodies is commonly performed through several rounds of panning of candidate antibodies in the presence of decreasing amounts of antigen. Decreasing the amount of antigen per round selects the antibodies with the highest affinity to the antigen thereby yielding antibodies of high affinity from a large pool of starting material. Affinity maturation via panning is well known in the art and is described, for example, in Huls et al. (*Cancer Immunol Immunother.* 50:163-71, 2001). Methods of affinity maturation using phage display technologies are described elsewhere herein and known in the art (see e.g., Daugherty et al., *Proc Natl Acad Sci U S A.* 97:2029-34, 2000).

[00355] Look-through mutagenesis—Look-through mutagenesis (LTM) (Rajpal et al., Proc Natl Acad Sci U S A. 102:8466-71, 2005) provides a method for rapidly mapping the antibody-binding site. For LTM, nine amino acids, representative of the major side-chain chemistries provided by the 20 natural amino acids, are selected to dissect the functional side-chain contributions to binding at every position in all six CDRs of an antibody. LTM generates a positional series of single mutations within a CDR where each "wild type" residue is systematically substituted by one of nine selected amino acids. Mutated CDRs are combined to generate combinatorial single-chain variable fragment (scFv) libraries of increasing complexity and size without becoming prohibitive to the quantitative display of all muteins. After positive selection, clones with improved binding are sequenced, and beneficial mutations are mapped.

[00356] Error-prone PCR—Error-prone PCR involves the randomization of nucleic acids between different selection rounds. The randomization occurs at a low rate by the intrinsic error rate of the polymerase used but can be enhanced by error-prone PCR (Zaccolo et al., J. Mol. Biol. 285:775-783, 1999) using a polymerase having a high intrinsic error rate during transcription (Hawkins et al., J Mol Biol. 226:889-96, 1992). After the mutation cycles, clones with improved affinity for the antigen are selected using routine methods in the art.

[00357] Techniques utilizing gene shuffling and directed evolution may also be used to prepare and screen antigen binding proteins, or variants thereof, for desired activity. For example, Jermutus et al., Proc Natl Acad Sci U S A., 98(1):75-80 (2001) showed that tailored *in vitro* selection strategies based on ribosome display were combined with *in vitro* diversification by DNA shuffling to evolve either the off-rate or thermodynamic stability of scFvs; Fermer et al., Tumour Biol. 2004 Jan-Apr;25(1-2):7-13 reported that use of phage display in combination with DNA shuffling raised affinity by almost three orders of magnitude. Dougherty et al., Proc Natl Acad Sci U S A. 2000 Feb. 29; 97(5):2029-2034 reported that (i) functional clones occur at an unexpectedly high frequency in hypermutated libraries, (ii) gain-of-function mutants are well represented in such libraries, and (iii) the majority of the scFv mutations leading to higher affinity correspond to residues distant from the binding site.

[00358] Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen, or to use computer software to model such contact points. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, they are subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

[00359] Immunoglobulins with modified carbohydrate

[00360] Immunoglobulin variants can also be produced that have a modified glycosylation pattern relative to the parent polypeptide, for example, adding or deleting one or more of the carbohydrate moieties bound to the immunoglobulin, and/or adding or deleting one or more glycosylation sites in the immunoglobulin.

[00361] Glycosylation of polypeptides, including antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. The presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Thus, N-linked glycosylation sites may be added to an immunoglobulin by altering the amino acid sequence such that it contains one or more of these tripeptide sequences. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. O-linked glycosylation sites may be added to an immunoglobulin by inserting or substituting one or more serine or threonine residues to the sequence of the original immunoglobulin or antibody.

[00362] Altered Effector Function

[00363] Cysteine residue(s) may be removed or introduced in the Fc region of an antibody or Fc-containing polypeptide, thereby eliminating or increasing interchain disulfide bond formation in this region. A homodimeric immunoglobulin thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., J. Exp Med. 176: 1191-1195 (1992) and Shopes, B. J. Immunol. 148: 2918-2922 (1992). Homodimeric immunoglobulins or antibodies may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., Cancer Research 53: 2560-2565 (1993). Alternatively, an immunoglobulin can be engineered which has dual Fc regions and may thereby have enhanced

complement lysis and ADCC capabilities. See Stevenson et al., Anti-CancerDrug Design 3: 219-230 (1989).

[00364] It is also contemplated that one or more of the N-terminal 20 amino acid residues (e.g., a signal sequence) of the heavy or light chain are removed.

[00365] Modifications to increase serum half-life also may desirable, for example, by incorporation of or addition of a salvage receptor binding epitope (e.g., by mutation of the appropriate region or by incorporating the epitope into a peptide tag that is then fused to the immunoglobulin at either end or in the middle, e.g., by DNA or peptide synthesis) (see, e.g., WO96/32478) or adding molecules such as PEG or other water soluble polymers, including polysaccharide polymers.

[00366] The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the immunoglobulin or fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or VH region, or more than one such region, of the immunoglobulin or antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the immunoglobulin fragment. See also International applications WO 97/34631 and WO 96/32478 which describe Fc variants and their interaction with the salvage receptor.

[00367] Other sites and amino acid residue(s) of the constant region have been identified that are responsible for complement dependent cytotoxicity (CDC), such as the C1q binding site, and/or the antibody-dependent cellular cytotoxicity (ADCC) [see, e.g., Molec. Immunol. 29 (5): 633-9 (1992); Shields et al., J. Biol. Chem., 276(9):6591-6604 (2001); Lazar et al., Proc. Nat'l. Acad. Sci. 103(11): 4005 (2006) which describe the effect of mutations at specific positions, each of which is incorporated by reference herein in its entirety]. Mutation of residues within Fc receptor binding sites can result in altered (i.e. increased or decreased) effector

function, such as altered affinity for Fc receptors, altered ADCC or CDC activity, or altered half-life. As described above, potential mutations include insertion, deletion or substitution of one or more residues, including substitution with alanine, a conservative substitution, a non-conservative substitution, or replacement with a corresponding amino acid residue at the same position from a different subclass (e.g. replacing an IgG1 residue with a corresponding IgG2 residue at that position).

[00368] The invention also encompasses production of immunoglobulin molecules, including antibodies and antibody fragments, with altered carbohydrate structure resulting in altered effector activity, including antibody molecules with absent or reduced fucosylation that exhibit improved ADCC activity. A variety of ways are known in the art to accomplish this. For example, ADCC effector activity is mediated by binding of the antibody molecule to the FcγRIII receptor, which has been shown to be dependent on the carbohydrate structure of the N-linked glycosylation at the Asn-297 of the CH2 domain. Non-fucosylated antibodies bind this receptor with increased affinity and trigger FcγRIII-mediated effector functions more efficiently than native, fucosylated antibodies. For example, recombinant production of non-fucosylated antibody in CHO cells in which the alpha-1,6-fucosyl transferase enzyme has been knocked out results in antibody with 100-fold increased ADCC activity (Yamane-Ohnuki et al., Biotechnol Bioeng. 2004 Sep 5;87(5):614-22). Similar effects can be accomplished through decreasing the activity of this or other enzymes in the fucosylation pathway, e.g., through siRNA or antisense RNA treatment, engineering cell lines to knockout the enzyme(s), or culturing with selective glycosylation inhibitors (Rothman et al., Mol Immunol. 1989 Dec;26(12):1113-23). Some host cell strains, e.g. Lec13 or rat hybridoma YB2/0 cell line naturally produce antibodies with lower fucosylation levels. Shields et al., J Biol Chem. 2002 Jul 26;277(30):26733-40; Shinkawa et al., J Biol Chem. 2003 Jan 31;278(5):3466-73. An increase in the level of bisected carbohydrate, e.g. through recombinantly producing antibody in cells that overexpress GnTIII enzyme, has also been determined to increase ADCC activity. Umana et al., Nat Biotechnol. 1999 Feb;17(2):176-80. It has been predicted that the absence of only one of the two

fucose residues may be sufficient to increase ADCC activity. (Ferrara et al., J Biol Chem. 2005 Dec 5).

[00369] Other Covalent Modifications of Immunoglobulins

[00370] Other particular covalent modifications of the immunoglobulin, are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the immunoglobulin or antibody, if applicable. Other types of covalent modifications can be introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

[00371] Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, .alpha.-bromo-β-(5-imidozoyl) propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

[00372] Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

[00373] Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing .alpha.-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

[00374] Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

[00375] The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

[00376] Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N.dbd.C.dbd.N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[00377] Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

[00378] Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the .alpha.-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[00379] Another type of covalent modification involves chemically or enzymatically coupling glycosides to the immunoglobulin (e.g., antibody or antibody fragment). These procedures are advantageous in that they do not require production of the immunoglobulin in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO87/05330 published 11 Sep. 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

[00380] Removal of any carbohydrate moieties present on the immunoglobulin may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the immunoglobulin to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the immunoglobulin intact. Chemical deglycosylation is described by Hakimuddin, et al. Arch. Biochem. Biophys. 259: 52 (1987) and by Edge et al. Anal. Biochem., 118: 131 (1981). Enzymatic cleavage of carbohydrate moieties on an immunoglobulin can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. Meth. Enzymol. 138: 350 (1987).

[00381] Another type of covalent modification of the immunoglobulins of the invention (including antibodies and antibody fragments) comprises linking the immunoglobulin to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyethylated polyols, polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol, polyoxyalkylenes, or polysaccharide polymers such as dextran. Such methods are known in the art, see, e.g. U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192, 4,179,337, 4,766,106, 4,179,337, 4,495,285, 4,609,546 or EP 315 456.

[00382] Isolated nucleic acids

[00383] Another aspect of the present invention is an isolated nucleic acid that encodes an immunoglobulin of the invention, such as, but not limited to, an isolated nucleic acid that encodes an antibody or antibody fragment of the invention. Such nucleic acids are made by recombinant techniques known in the art and/or disclosed herein.

[00384] In other embodiments the isolated nucleic acid encodes an immunoglobulin comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

[00385] (a) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 or SEQ ID NO:190; or

[00386] (b) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:321 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 or SEQ ID NO:190; or

[00387] (c) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:182, SEQ ID NO:188, or SEQ ID NO:190.

[00388] And in some embodiments the isolated nucleic acid encodes an immunoglobulin comprising comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

[00389] (a) the light chain variable region comprises the amino acid sequence of SEQ ID NO:196 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335, SEQ ID NO:349, SEQ ID NO:351, SEQ ID NO:353, SEQ ID NO:355, or SEQ ID NO:359; or

[00390] (b) the light chain variable region comprises the amino acid sequence of SEQ ID NO:204 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349 or SEQ ID NO:355; or

[00391] (c) the light chain variable region comprises the amino acid sequence of SEQ ID NO:202 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349; or

[00392] (d) the light chain variable region comprises the amino acid sequence of SEQ ID NO:192 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:357, SEQ ID NO:359, or SEQ ID NO:369; or

[00393] (e) the light chain variable region comprises the amino acid sequence of SEQ ID NO:194 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335, SEQ ID NO:349, or SEQ ID NO:351.

[00394] In other examples, the isolated nucleic acid encodes an immunoglobulin comprising an immunoglobulin heavy chain and an immunoglobulin light chain wherein:

[00395] (a) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188; or

[00396] (b) the light chain variable region comprises the amino acid sequence of SEQ ID NO:196; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:353; or

[00397] (c) the light chain variable region comprises the amino acid sequence of SEQ ID NO:202; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349; or

[00398] (d) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:190.

[00399] Or in some embodiments, the isolated nucleic acid encodes an immunoglobulin comprising:

[00400] an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:113, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:110, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00401] an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:125, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:122, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00402] an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:101, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:98, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00403] an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:119, or comprising the foregoing sequence from which one, two, three,

four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:116, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both.

[00404] The present invention is also directed to vectors, including expression vectors that comprise any of the inventive isolated nucleic acids. An isolated host cell that comprises the expression vector is also encompassed by the present invention, which is made by molecular biological techniques known in the art and/or disclosed herein.

[00405] The invention is also directed to a method involving:

[00406] culturing the host cell in a culture medium under conditions permitting expression of the immunoglobulin encoded by the expression vector; and

[00407] recovering the immunoglobulin from the culture medium. Recovering the immunoglobulin is accomplished by known methods of antbody purification, such as but not limited to, antibody purification techniques disclosed in Example 1 and elsewhere herein.

[00408] Gene Therapy

[00409] Delivery of a therapeutic immunoglobulin to appropriate cells can be effected via gene therapy ex vivo, in situ, or in vivo by use of any suitable approach known in the art. For example, for in vivo therapy, a nucleic acid encoding the desired immunoglobulin or antibody, either alone or in conjunction with a vector, liposome, or precipitate may be injected directly into the subject, and in some embodiments, may be injected at the site where the expression of the immunoglobulin compound is desired. For ex vivo treatment, the subject's cells are removed, the nucleic acid is introduced into these cells, and the modified cells are returned to the subject either directly or, for example, encapsulated within porous

membranes which are implanted into the patient. See, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187.

[00410] There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, chemical treatments, DEAE-dextran, and calcium phosphate precipitation. Other in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, adeno-associated virus or retrovirus) and lipid-based systems. The nucleic acid and transfection agent are optionally associated with a microparticle. Exemplary transfection agents include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, quaternary ammonium amphiphile DOTMA ((dioleoyloxypropyl) trimethylammonium bromide, commercialized as Lipofectin by GIBCO-BRL))(Felgner et al, (1987) Proc. Natl. Acad. Sci. USA 84, 7413-7417; Malone et al. (1989) Proc. Natl Acad. Sci. USA 86 6077-6081); lipophilic glutamate diesters with pendent trimethylammonium heads (Ito et al. (1990) Biochem. Biophys. Acta 1023, 124-132); the metabolizable parent lipids such as the cationic lipid dioctadecylamido glycylspermine (DOGS, Transfectam, Promega) and dipalmitoylphosphatidyl ethanolamylspermine (DPPES)(J. P. Behr (1986) Tetrahedron Lett. 27, 5861-5864; J. P. Behr et al. (1989) Proc. Natl. Acad. Sci. USA 86, 6982-6986); metabolizable quaternary ammonium salts (DOTB, N-(1-[2,3-dioleoyloxy]propyl)-N,N,N-trimethylammonium methylsulfate (DOTAP)(Boehringer Mannheim), polyethyleneimine (PEI), dioleoyl esters, ChoTB, ChoSC, DOSC)(Leventis et al. (1990) Biochim. Inter. 22, 235-241); 3beta[N-(N', N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), dioleoylphosphatidyl ethanolamine (DOPE)/3beta[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterolDC-Chol in one to one mixtures (Gao et al., (1991) Biochim. Biophys. Acta 1065, 8-14), spermine, spermidine, lipopolyamines (Behr et al., Bioconjugate Chem, 1994, 5: 382-389), lipophilic polylysines (LPLL) (Zhou et al.,

(1991) Biochim. Biophys. Acta 939, 8-18), [[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide (DEBDA hydroxide) with excess phosphatidylcholine/cholesterol (Ballas et al., (1988) Biochim. Biophys. Acta 939, 8-18), cetyltrimethylammonium bromide (CTAB)/DOPE mixtures (Pinnaduwage et al, (1989) Biochim. Biophys. Acta 985, 33-37), lipophilic diester of glutamic acid (TMAG) with DOPE, CTAB, DEBDA, didodecylammonium bromide (DDAB), and stearylamine in admixture with phosphatidylethanolamine (Rose et al., (1991) Biotechnique 10, 520-525), DDAB/DOPE (TransfectACE, GIBCO BRL), and oligogalactose bearing lipids. Exemplary transfection enhancer agents that increase the efficiency of transfer include, for example, DEAE-dextran, polybrene, lysosome-disruptive peptide (Ohmori N I et al, Biochem Biophys Res Commun Jun. 27, 1997;235(3):726-9), chondroitan-based proteoglycans, sulfated proteoglycans, polyethylenimine, polylysine (Pollard H et al. J Biol Chem, 1998 273 (13):7507-11), integrin-binding peptide CYGGRGDTP (SEQ ID NO:235), linear dextran nonasaccharide, glycerol, cholesteryl groups tethered at the 3'-terminal internucleoside link of an oligonucleotide (Letsinger, R. L. 1989 Proc Natl Acad Sci USA 86: (17):6553-6), lysophosphatide, lysophosphatidylcholine, lysophosphatidylethanolamine, and 1-oleoyl lysophosphatidylcholine.

[00411] In some situations it may be desirable to deliver the nucleic acid with an agent that directs the nucleic acid-containing vector to target cells. Such "targeting" molecules include antigen binding proteins specific for a cell-surface membrane protein on the target cell, or a ligand for a receptor on the target cell. Where liposomes are employed, proteins which bind to a cell-surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake. Examples of such proteins include capsid proteins and fragments thereof tropic for a particular cell type, antigen binding proteins for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. In other embodiments, receptor-mediated endocytosis can be used. Such methods are described, for example, in Wu et al., 1987 or Wagner et al., 1990. For review of the currently known gene marking and gene therapy

protocols, see Anderson 1992. See also WO 93/25673 and the references cited therein. For additional reviews of gene therapy technology, see Friedmann, Science, 244: 1275-1281 (1989); Anderson, Nature, supplement to vol. 392, no 6679, pp. 25-30 (1998); Verma, Scientific American: 68-84 (1990); and Miller, Nature, 357: 455460 (1992).

[00412] Administration and Preparation of Pharmaceutical Formulations

[00413] The immunoglobulins or antibodies used in the practice of a method of the invention may be formulated into pharmaceutical compositions and medicaments comprising a carrier suitable for the desired delivery method. Suitable carriers include any material which, when combined with the immunoglobulin or antibody, and is nonreactive with the subject's immune systems. Examples include, but are not limited to, any of a number of standard pharmaceutical carriers such as sterile phosphate buffered saline solutions, bacteriostatic water, and the like. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like, and may include other proteins for enhanced stability, such as albumin, lipoprotein, globulin, etc., subjected to mild chemical modifications or the like.

[00414] Exemplary immunoglobulin concentrations in the formulation may range from about 0.1 mg/ml to about 180 mg/ml or from about 0.1 mg/mL to about 50 mg/mL, or from about 0.5 mg/mL to about 25 mg/mL, or alternatively from about 2 mg/mL to about 10 mg/mL. An aqueous formulation of the immunoglobulin may be prepared in a pH-buffered solution, for example, at pH ranging from about 4.5 to about 6.5, or from about 4.8 to about 5.5, or alternatively about 5.0. Examples of buffers that are suitable for a pH within this range include acetate (e.g. sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. The buffer concentration can be from about 1 mM to about 200 mM, or from about 10 mM to about 60 mM, depending, for example, on the buffer and the desired isotonicity of the formulation.

[00415] A tonicity agent, which may also stabilize the immunoglobulin, may be included in the formulation. Exemplary tonicity agents include polyols, such as

mannitol, sucrose or trehalose. Preferably the aqueous formulation is isotonic, although hypertonic or hypotonic solutions may be suitable. Exemplary concentrations of the polyol in the formulation may range from about 1% to about 15% w/v.

[00416] A surfactant may also be added to the immunoglobulin formulation to reduce aggregation of the formulated immunoglobulin and/or minimize the formation of particulates in the formulation and/or reduce adsorption. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbate 20, or polysorbate 80) or poloxamers (e.g. poloxamer 188). Exemplary concentrations of surfactant may range from about 0.001% to about 0.5%, or from about 0.005% to about 0.2%, or alternatively from about 0.004% to about 0.01% w/v.

[00417] In one embodiment, the formulation contains the above-identified agents (i.e. immunoglobulin, buffer, polyol and surfactant) and is essentially free of one or more preservatives, such as benzyl alcohol, phenol, m-cresol, chlorobutanol and benzethonium Cl. In another embodiment, a preservative may be included in the formulation, e.g., at concentrations ranging from about 0.1% to about 2%, or alternatively from about 0.5% to about 1%. One or more other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may be included in the formulation provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; co-solvents; antoxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

[00418] Therapeutic formulations of the immunoglobulin are prepared for storage by mixing the immunoglobulin having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's

Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, maltose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

[00419] In one embodiment, a suitable formulation of the claimed invention contains an isotonic buffer such as a phosphate, acetate, or Tris buffer in combination with a tonicity agent such as a polyol, Sorbitol, sucrose or sodium chloride which tonicifies and stabilizes. One example of such a tonicity agent is 5% Sorbitol or sucrose. In addition, the formulation could optionally include a surfactant such as to prevent aggregation and for stabilization at 0.01 to 0.02% wt/vol. The pH of the formulation may range from 4.5-6.5 or 4.5 to 5.5. Other exemplary descriptions of pharmaceutical formulations for antibodies may be found in US 2003/0113316 and US patent no. 6,171,586, each incorporated herein by reference in its entirety.

[00420] The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may

be desirable to further provide an immunosuppressive agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[00421] The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

[00422] Suspensions and crystal forms of immunoglobulins are also contemplated. Methods to make suspensions and crystal forms are known to one of skill in the art.

[00423] The formulations to be used for in vivo administration must be sterile. The compositions of the invention may be sterilized by conventional, well known sterilization techniques. For example, sterilization is readily accomplished by filtration through sterile filtration membranes. The resulting solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration.

[00424] The process of freeze-drying is often employed to stabilize polypeptides for long-term storage, particularly when the polypeptide is relatively unstable in liquid compositions. A lyophilization cycle is usually composed of three steps: freezing, primary drying, and secondary drying; Williams and Polli, Journal of Parenteral Science and Technology, Volume 38, Number 2, pages 48-59 (1984). In the freezing step, the solution is cooled until it is adequately frozen. Bulk water in the solution forms ice at this stage. The ice sublimes in the primary drying stage, which is conducted by reducing chamber pressure below the vapor pressure of the ice, using a vacuum. Finally, sorbed or bound water is removed at the secondary drying stage under reduced chamber pressure and an elevated shelf temperature. The

process produces a material known as a lyophilized cake. Thereafter the cake can be reconstituted prior to use.

[00425] The standard reconstitution practice for lyophilized material is to add back a volume of pure water (typically equivalent to the volume removed during lyophilization), although dilute solutions of antibacterial agents are sometimes used in the production of pharmaceuticals for parenteral administration; Chen, Drug Development and Industrial Pharmacy, Volume 18, Numbers 11 and 12, pages 1311-1354 (1992).

[00426] Excipients have been noted in some cases to act as stabilizers for freeze-dried products; Carpenter et al., Developments in Biological Standardization, Volume 74, pages 225-239 (1991). For example, known excipients include polyols (including mannitol, sorbitol and glycerol); sugars (including glucose and sucrose); and amino acids (including alanine, glycine and glutamic acid).

[00427] In addition, polyols and sugars are also often used to protect polypeptides from freezing and drying-induced damage and to enhance the stability during storage in the dried state. In general, sugars, in particular disaccharides, are effective in both the freeze-drying process and during storage. Other classes of molecules, including mono- and di-saccharides and polymers such as PVP, have also been reported as stabilizers of lyophilized products.

[00428] For injection, the pharmaceutical formulation and/or medicament may be a powder suitable for reconstitution with an appropriate solution as described above. Examples of these include, but are not limited to, freeze dried, rotary dried or spray dried powders, amorphous powders, granules, precipitates, or particulates. For injection, the formulations may optionally contain stabilizers, pH modifiers, surfactants, bioavailability modifiers and combinations of these.

[00429] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the immunoglobulin, which matrices are in the form of shaped

articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919), copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S--S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[00430] The formulations of the invention may be designed to be short-acting, fast-releasing, long-acting, or sustained-releasing as described herein. Thus, the pharmaceutical formulations may also be formulated for controlled release or for slow release.

[00431] Specific dosages may be adjusted depending on conditions of disease, the age, body weight, general health conditions, sex, and diet of the subject, dose intervals, administration routes, excretion rate, and combinations of drugs. Any of the above dosage forms containing effective amounts are well within the bounds of routine experimentation and therefore, well within the scope of the instant invention.

[00432] The immunoglobulin is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include

intravenous, intraarterial, intraperitoneal, intramuscular, intradermal or subcutaneous administration. In addition, the immunoglobulin is suitably administered by pulse infusion, particularly with declining doses of the immunoglobulin or antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Other administration methods are contemplated, including topical, particularly transdermal, transmucosal, rectal, oral or local administration e.g. through a catheter placed close to the desired site. Most preferably, the immunoglobulin of the invention is administered intravenously in a physiological solution at a dose ranging between 0.01 mg/kg to 100 mg/kg at a frequency ranging from daily to weekly to monthly (e.g. every day, every other day, every third day, or 2, 3, 4, 5, or 6 times per week), preferably a dose ranging from 0.1 to 45 mg/kg, 0.1 to 15 mg/kg or 0.1 to 10 mg/kg at a frequency of 2 or 3 times per week, or up to 45mg/kg once a month.

[00433] Embodiments or aspects of the invention can include but are not limited to the following:

[00434] 1. An isolated immunoglobulin, comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

[00435] (a) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 or SEQ ID NO:190; or

[00436] (b) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:321 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 or SEQ ID NO:190; or

[00437] (c) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:182, SEQ ID NO:188, or SEQ ID NO:190.

[00438] 2. An isolated immunoglobulin, comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

[00439] (a) the light chain variable region comprises the amino acid sequence of SEQ ID NO:196 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335, SEQ ID NO:349, SEQ ID NO:351, SEQ ID NO:353, SEQ ID NO:355, or SEQ ID NO:359; or

[00440] (b) the light chain variable region comprises the amino acid sequence of SEQ ID NO:204 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349 or SEQ ID NO:355; or

[00441] (c) the light chain variable region comprises the amino acid sequence of SEQ ID NO:202 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349; or

[00442] (d) the light chain variable region comprises the amino acid sequence of SEQ ID NO:192 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:357, SEQ ID NO:359, or SEQ ID NO:369; or

[00443] (e) the light chain variable region comprises the amino acid sequence of SEQ ID NO:194 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335, SEQ ID NO:349, or SEQ ID NO:351.

[00444] 3. The isolated immunoglobulin of Claim 1, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188.

[00445] 4. The isolated immunoglobulin of Claim 2, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO:196; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:353.

[00446] 5. The isolated immunoglobulin of Claim 2, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO:202; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349.

[00447] 6. The isolated immunoglobulin of Claim 1, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:190.

[00448] 7. The isolated immunoglobulin of Claim 1 or Claim 2, wherein the isolated immunoglobulin comprises an antibody or antibody fragment.

[00449] 8. The isolated immunoglobulin of any of Claims 1-7, comprising an IgG1, IgG2, IgG3 or IgG4.

[00450] 9. The isolated immunoglobulin of any of Claims 1-8, comprising a monoclonal antibody.

[00451] 10. The isolated immunoglobulin of any of Claims 1-9, comprising a human antibody.

[00452] 11. The isolated immunoglobulin of Claim 10, comprising:

[00453] (a) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:113, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:110, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00454] (b) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:125, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of

SEQ ID NO:122, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00455] (c) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:101, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:98, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

[00456] (d) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:119, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:116, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both.

[00457] 12. The isolated immunoglobulin of any of Claims 1-11, further comprising one to twenty-four pharmacologically active chemical moieties conjugated thereto.

[00458] 13. The isolated immunoglobulin of any of Claims 1-12, wherein the pharmacologically active chemical moiety is a pharmacologically active polypeptide.

[00459] 14. The isolated immunoglobulin of any of Claims 1-13, wherein the immunoglobulin is recombinantly produced.

[00460] 15. The isolated immunoglobulin of Claim 14, wherein the immunoglobulin comprises at least one immunoglobulin heavy chain and at least one immunoglobulin light chain, and wherein the pharmacologically active polypeptide

is inserted in the primary amino acid sequence of the of the immunoglobulin heavy chain within an internal loop of the Fc domain of the immunoglobulin heavy chain.

[00461] 16. The isolated immunoglobulin of Claim 13 or 14, wherein the immunoglobulin comprises at least one immunoglobulin heavy chain and at least one immunoglobulin light chain, and wherein the pharmacologically active polypeptide is conjugated at the N-terminal or C-terminal of the immunoglobulin heavy chain.

[00462] 17. The isolated immunoglobulin of Claim 13 or 14, wherein the immunoglobulin comprises at least one immunoglobulin heavy chain and at least one immunoglobulin light chain, and wherein the pharmacologically active polypeptide is conjugated at the N-terminal or C-terminal of the immunoglobulin light chain.

[00463] 18. The isolated immunoglobulin of Claim 13 or 14, wherein the pharmacologically active polypeptide is a toxin peptide, an IL-6 binding peptide, a CGRP peptide antagonist, a bradykinin B1 receptor peptide antagonist, a PTH agonist peptide, a PTH antagonist peptide, an ang-1 binding peptide, an ang-2 binding peptide, a myostatin binding peptide, an EPO-mimetic peptide, a FGF21 peptide, a TPO-mimetic peptide, a NGF binding peptide, a BAFF antagonist peptide, a GLP-1 or peptide mimetic thereof, or a GLP-2 or peptide mimetic thereof.

[00464] 19. The isolated immunoglobulin of Claim 18, wherein the toxin peptide is ShK or a ShK peptide analog.

[00465] 20. A pharmaceutical composition comprising the immunoglobulin of any of Claims 1-19; and a pharmaceutically acceptable diluent, excipient or carrier.

[00466] 21. An isolated nucleic acid that encodes the immunoglobulin of any of Claims 1-11.

[00467] 22. An isolated nucleic acid that encodes the immunoglobulin of Claim 3.

[00468] 23. An isolated nucleic acid that encodes the immunoglobulin of Claim 4.

[00469] 24. An isolated nucleic acid that encodes the immunoglobulin of Claim 5.

[00470] 25. An isolated nucleic acid that encodes the immunoglobulin of Claim 6.

[00471] 26. An isolated nucleic acid that encodes the immunoglobulin of Claim 11.

[00472] 27. An isolated nucleic acid that encodes the immunoglobulin of any of Claims 13-19.

[00473] 28. A vector comprising the isolated nucleic acid of Claims 21.

[00474] 29. A vector comprising the isolated nucleic acid of any of Claims 22-26.

[00475] 30. A vector comprising the isolated nucleic acid of Claim 27.

[00476] 31. The vector of Claim 28, comprising an expression vector.

[00477] 32. The vector of Claim 29, comprising an expression vector.

[00478] 33. The vector of Claim 30, comprising an expression vector.

[00479] 34. An isolated host cell, comprising the expression vector of any of Claima 31-33.

[00480] 35. A method, comprising:

[00481] (a) culturing the host cell of claim 34 in a culture medium under conditions permitting expression of the immunoglobulin encoded by the expression vector; and

[00482] (b) recovering the immunoglobulin from the culture medium.

[00483] 36. The immunoglobulin of Claim 1, wherein the immunoglobulin at 30 micromolar concentration does not significantly bind soluble human IL-17R (SEQ ID NO:89) at 30 nanomolar concentration in an aqueous solution incubated under physiological conditions, as measured by a surface plasmon resonance binding assay.

[00484] 37. The immunoglobulin of Claim 2, wherein the immunoglobulin at 10 micromolar concentration does not significantly bind soluble human TR2 (SEQ ID NO:82) at 10 nanomolar concentration in an aqueous solution incubated under physiological conditions, as measured by a surface plasmon resonance binding assay.

[00485] The invention is illustrated by the following further examples, which are not intended to be limiting in any way.

[00486] **Examples**

[00487] **Example 1**

[00488] **Generation of antibodies to human IL-17R and screening**

[00489] Cloning and engineering. The Antibody 16429 DNA sequences encoding immunoglobulin heavy chain (comprising VH1) and light chain (comprising VL1) subunits for anti-huIL-17R antibodies were obtained from Tocker et al. (WO 2008/054603 A2) and were cloned using standard recombinant technology. In order to eliminate the binding ability of these antibodies a series of site directed mutagenesis clones were generated using polymerase chain reaction (PCR) amplification. The amino acids to be changed were selected on the basis of location in the complementarity determining regions (CDRs), change from germline sequence, estimated solvent exposure, and aromatic and charge nature. The initial set of mutants was germlining and alanine scanning mutants. Subsequently, mutations were combined and in several cases the alanine scanning mutants were mutated to introduce negative charge, by replacing the alanine with glutamic acid, or positive charge, by replacing the alanine with arginine.

[00490] A representative example of the PCR site direct mutation procedure is the introduction of an alanine in place of a tryptophan the CDR3 of the anti-IL17 light chain.

[00491] PCR amplification was done as a three step process with a 5' and 3' PCR used to introduce the mutation and a final overlap PCR to join the two ends of the mutated anti-IL17R light chain. The 5' PCR use the forward primer, 5'- AAG CTC GAG GTC GAC TAG ACC ACC ATG GAA GCC CCA GCG CAG -3' (SEQ ID NO:31) and the reverse primer, 5'- GAA AGT GAG CGG AGC GTT ATC ATA CTG CTG ACA -3' (SEQ ID NO:32). The 3' PCR use the forward primer, 5'- TGT CAG CAG TAT GAT AAC GCT CCG CTC ACT TTC -3' (SEQ ID NO:33) and the reverse primer, 5'- AAC CGT TTA AAC GCG GCC GCT CAA CAC TCT CCC CTG TTG AA -3' (SEQ ID NO:34). The overlap PCR use the forward primer, 5'- AAG CTC GAG GTC GAC TAG ACC ACC ATG GAA GCC CCA GCG CAG -3' (SEQ ID NO:31) and the reverse primer, 5'- AAC CGT TTA AAC GCG GCC GCT CAA CAC TCT CCC CTG TTG AA -3' (SEQ ID NO:34).

[00492] The PCRs were performed with Phusion HF DNA polymerase (Finnzyme). The PCR reaction cycles for the 5' and 3' PCRs consisted of a 20 second denaturation of the anti-IL-17R light chain DNA at 94°C, followed by three cycles of amplification with each cycle consisting of 20 seconds at 94°C; 30 seconds at 55°C; and 30 seconds at 72°C plus an additional 27 cycles consisting of 20 seconds at 94°C; 30 seconds at 60°C; and 30 seconds at 72°C. The reactions were then incubated for 7 minutes at 72°C following the last PCR cycle to insure complete elongation. The PCR reaction cycles for the overlap PCR consisted of a 20 second denaturation of the 5' and 3' PCR DNAs at 94°C, followed by three cycles of amplification with each cycles consisting of 20 seconds at 94°C; 60 seconds at 55°C; and 40 seconds at 72°C plus an additional 27 cycles consisting of 20 seconds at 94°C; 30 seconds at 60°C; and 40 seconds at 72°C. The reaction was then incubated for 7 minutes at 72°C following the last PCR cycle to insure complete elongation. The overlap PCR product was cloned into pTT5 expression vector (NRCC) and its sequences determined using ABI DNA sequencing instrument (Perkin Elmer). Further detail about construct development is found in Example 5 and Example 6 herein. Table 6 (below) contains details about the primers and templates used in cloning the component subunits of various embodiments of the inventive

immunoglobulins and conjugates, based on the same PCR cycling conditions described in this paragraph.

[00493] Transient expression to generate recombinant monoclonal antibodies.

[00494] Transient transfections were carried out in HEK 293-6E cells as follows. The human embryonic kidney 293 cell line stably expressing Epstein Barr virus Nuclear Antigen-1 (293-6E cells) was obtained from the National Research Council (Montreal, Canada). Cells were maintained as serum-free suspension cultures using F17 medium (Invitrogen, Carlsbad, CA) supplemented with 6 mM L-glutamine (Invitrogen, Carlsbad, CA), 1.1% F-68 Pluronic (Invitrogen, Carlsbad, CA) and 250 μg/ul Geneticin (Invitrigen, Carlsbad, CA). The suspension cell cultures were maintained in Erlenmeyer shake flask cultures. The culture flasks were shaken at 65 rpm at 37 °C in a humidified, 5% CO_2 atmosphere. A stock solution (1mg/ml) of 25-kDa linear PEI (Polysciences, Warrington, PA) was prepared in water, acidified with HCl to pH 2.0 until dissolved, then neutralized with NaOH, sterilized by filtration (0.2 μm), aliquoted, and stored at -20°C until used. Tryptone N1 was obtained from OrganoTechni S.A. (TekniScience, QC, Canada). A stock solution (20%, w/v) was prepared in Freestyle medium (Invitrogem, Carlsbad, CA), sterilized by filtration through 0.2 μm filters, and stored at 4°C until use. Typically, transfections were performed at the 1L scale. Cells (293-6E) were grown too a viable cell density of 1.1 X 10^6 cells/ml then transfection complexes were prepared in 1/10th volume of the final culture volume. For a 1-L transfection culture, transfection complexes were prepared in 100 ml F17 basal medium, and 500 μg plasmid DNA (heavy chain and light chain DNA, 1:1 ratio) was first diluted in 100 ml F17 medium. After a 5-minute incubation at room temperature, 1.5 ml of PEI solution was added. The complexes were vortexed mildly, then incubated for 15 minutes at room temperature. The cells were transfected by adding the transfection complex mix to the cells in the shale flask culture. 24 hours post-transfection, Tryptone N1 was added to the transfected culture to a final concentration of 0.5%, and the transfected cultures were maintained on a shaker at 65 rpm at 37°C in a humidified, 5% CO_2 atmosphere for another 5 days after which they were harvested. The conditioned medium was

harvested by centrifugation at 4000 rpm, and then sterile filtered through 0.2 μm filter (Corning Inc.).

[00495] Purification of Antibodies. The transiently expressed antibodies were purified using recombinant protein A sepharose (GE Healthcare) directly loading the conditioned media on the column at 5 ml/min at 7 °C. The column was then washed with 10 column volumes of Dulbecco's PBS without divalent cations and then eluted with 100 mM acetic acid, pH 3.5. The eluted antibody was pooled based on the chromatographic profile and the pH was adjusted to 5.0 using 2 M Tris base. The pool was then filtered through a 0.8/0.22 μm syringe filter and then dialyzed against 10 mM acetic acid, 9% sucrose, pH 5.0. The buffer exchanged antibody was then concentrated using a Vivaspin 30 kDa centrifugal concentration (Sartorius), and the concentrated product was filtered through a 0.22 μm cellulose acetate filter.

[00496] BIAcore® binding assays. The lead candidates were then selected based on lack of binding to the IL-17R extracellular domain as determined by BIAcore analysis. Antibody 16429 is a human antibody that specifically binds to huIL-17R. A solution equilibrium binding assay was developed to assess the binding activity of a set of antibodies to huIL-17R. Antibody 16429 was immobilized to a BIACore® 2000, research grade sensor chip CM5 surface according to manufacturer's instructions (BIACore, Inc., Piscataway, NJ). Briefly, carboxyl groups on the sensor chip surfaces were activated by injecting 60 μL of a mixture containing 0.2 M N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS). Antibody 16429 was diluted in 10 mM sodium acetate, pH 4.0 and injected over the activated chip surface at 30 μL/min for 6 minutes. Excess reactive groups on the surfaces were deactivated by injecting 60 μL of 1 M ethanolamine. The final immobilized level was approximately 6600 resonance units (RU). As represented in Figure 2A, 10 nM of IL-17R in the absence of soluble antibody was used to establish the 100% binding signal of IL-17R to the fixed 16429 anbibody. To determine antibody binding in solution, 10 nM, 100 nM and 1000 nM of the antibody samples were incubated with the 10 nM IL-17R. The decreased binding signal of IL-17R after the antibody incubation indicates the binding of the

antibody to IL-17R in solution. Based on this assay, the 16435, 16438, 16439, 16440, 16441, and 16444 antibodies demonstrated substantial reduction in IL-17R binding capability. As represented in Figure 2B, 30 nM IL-17R and 30 μM antibody samples were used to further demonstrate that the selected antibodies lost their IL-17R binding activity. Based on this assay, all six antibodies examined (16435, 16438, 16439, 16440, 16441, and 16444) showed no signficant IL-17R binding activity at up to 30 μM antibody.

[00497] Cell Based Activity Assay. Interaction of IL-17 with the IL-17R on cells induces the production of various factors, including growth-related oncogene alpha (GRO-α), from these cells. A cell-based characterization assay was developed to measure GRO-α released using sandwich ELISA. In this ELISA, a GRO-α capture antibody is utilized to bind GRO-α, and then a biotinylated GRO-α detection antibody is used to detect the captured protein. Streptavidin conjugated to horseradish peroxidise (HRP) is then added to detect the amount of biotinylated GRO-α detection antibody bound. The amount of HRP bound is measured by evaluation of absorbance at 450 nm. An increase in absorbance at 450 nm is indicative of an increase in the amount of GRO-α produced. In this assay, human foreskin fibroblasts (HFF) are incubated with 5 ng/ml IL-17 and 0.1 μM, 1 μM and 10 μM of antibody samples. The conditioned cell medium is then harvested and processed for assessment of GRO-α levels using a GRO-α sandwich ELISA. All six experimental carrier antibodies (16435, 16438, 16439, 16440, 16441, and 16444) showed no significant blocking activity in this assay at up to 10 μM antibody (Figure 3).

[00498] Analysis of homogeneity. Antibodies produced by transient expression were analyzed for homogeneity using two size exclusion columns (TSK-GEL G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) in series with a 100 mM sodium phosphate, 250 mM NaCl, pH 6.8, mobile phase flowed at 0.5 mL/min (Figure 4A-B). While all the antibodies showed relatively low levels of high molecular weight species, 16439 and 16435 had the least, while 16440 had the most. The lead antibodies were further analyzed for product quality on a 1.0-mm

Tris-glycine 4-20% SDS-PAGE (Novex) using reducing (Figure 6) and non-reducing loading buffer (Figure 5). All candidates appeared quite similar by both non-reducing and reducing SDS-PAGE; however, 16433 did show some additional high molecular mass material on the reducing SDS-PAGE. Lead candidates were further selected based on SEC behavior, SDS-PAGE uniformity, BIAcore binding analysis, cell based assay results and expression levels. Based on these criteria, 16435 and 16444 were chosen for further evaluation.

[00499] Stable expression of antibodies. Antibody 16435 and 16444 expressing pools were created by transfecting CHO DHFR(-) host cells with corresponding HC and LC expression plasmid set using a standard electroporation procedure. Per each antibody molecule, 3-4 different transfections were performed to generate multiple pools. After transfection the cells were grown as a pool in a serum free, (-) GHT (selective growth media to allow for selection and recovery of the plasmid containing cells. Cell pools grown in (–) GHT selective media were cultured until they reached > 85% viability. The selected cell pools were amplified with 150 nm MTX. When the viability of the MTX amplified pools reached >85% viability, the pools were screened using an abbreviated six day batch production assay with an enriched production media to assess expression. The best pool was chosen based on the six day assay titer and correct mass confirmation. Subsequently, scale-up production using 11-day fed-batch process was performed for the antibody generation, followed by harvest and purification.

[00500] Titers were determined by HPLC assay (Figure 7A-B) using a Poros A column, 20 μm, 2.1 x 30 mm (Applied Biosystems, part #1-5024-12). Briefly, Antibodies in conditioned media were filtered using Spin-X columns (Corning, part #8160) prior to analysis by HPLC, and a blank injection of 1X PBS (Invitrogen, part #14190-144) was performed prior to injection of test antibodies and after each analysis run. In addition, conditioned media without antibody was injected prior to analysis to condition the column, and new columns were conditioned by triplicate injection of 100μg of control antibody. After a 9-minute wash with PBS at 0.6 ml/min, the antibody was eluted with ImmunoPure IgG Elution Buffer (Pierce, part

#21009) and the absorbance at 280 nm was observed. Antibody titers were quantified against a standard plot of control antibody concentration versus peak area. A control antibody stock was prepared at a concentration of 4 mg/ml, and five standard antibody concentrations were prepared by dilution of the antibody control stock in a volume of PBS (0.1 μg/μl to 1.6 μg/μl). By extending the standard curve, the lower limit of detection is 0.02 μg/μl of antibody, and the higher limit of quantification is 4 μg/μl. An assumption was made that test antibodies have similar absorbance characteristics as the control; however titers can be adjusted by multiplying titer an extinction coefficient ratio of the control antibody over the extinction coefficient of the test antibody. The titer assay results show that after scale up to the fed batch process, the 16435 antibody demonstrated marginally better expression than the 16444 carrier antibody.

[00501] Purification of stably expressed antibodies. Stably expressed antibodies were purified by Mab Select Sure chromatography (GE Life Sciences) using 8 column volumes of Dulbecco's PBS without divalent cations as the wash buffer and 100 mM acetic acid, pH 3.5, as the elution buffer at 7°C. The elution peak was pooled based on the chromatogram, and the pH was raised to about 5.0 using 2 M Tris base. The pool was then diluted with at least 3 volumes of water, filtered through a 0.22-μm cellulose acetate filter and then loaded on to an SP-HP sepharose column (GE Life Sciences) and washed with 10 column volumes of S-Buffer A (20 mM acetic acid, pH 5.0) followed by elution using a 20 column volume gradient to 50% S-Buffer B (20 mM acetic acid, 1 M NaCl, pH 5.0) at 7 °C. A pool was made based on the chromatogram and SDS-PAGE analysis, then the material was concentrated about 6-fold and diafiltered against about 5 volumes of 10 mM acetic acid, 9% sucrose, pH 5.0 using a VivaFlow TFF cassette with a 30 kDa membrane. The dialyzed material was then filtered through a 0.8/0.2-μm cellulose acetate filter and the concentration was determined by the absorbance at 280 nm. Comparison of the ion exchange chromatographic profiles of the 16435 and 16444 variants showed no significant differences (Figure 8A-B).

[00502] Analysis of stably expressed antibodies. Analysis of the variants using 1.0-mm Tris-glycine 4-20% SDS-PAGE (Novex) with reducing and non-reducing loading buffer also showed no significant difference between the variants (Figure 9A-B). However analysis using two size exclusion columns (TSK-GEL G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) in series with a 100 mM sodium phosphate, 250 mM NaCl, pH 6.8, mobile phase flowed at 0.5 mL/min showed that 16444 possessed more high molecular weight species, and 16435 had a more prominent pre-peak (Figure 10).

[00503] Antibodies were also analyzed for thermoresistance by DSC using a MicroCal VP-DSC where the samples were heated from 20°C to 95°C at a rate of 1°C per minute. DSC directly measures heat changes that occur in biomolecules during controlled increase or decrease in temperature, making it possible to study materials in their native state.

[00504] DSC measures the enthalpy (ΔH) of unfolding due to heat denaturation. A biomolecule in solution is in equilibrium between the native (folded) conformation and its denatured (unfolded) state. The higher the thermal transition midpoint (Tm), when 50% of the biomolecules are unfolded, the more stable the molecule. DSC is also used to determine the change in heat capacity (ΔCp) of denaturation (see, Figure 11). The proteins were incubated at 0.5 mg/ml in 10 mM sodium acetate, 9% sucrose, pH 5.0 (Figure 11). The 16435 antibody produced the most desirable melting profile, with a higher temperature for the secondary transition.

[00505] The antibodies were analyzed by reducing and non-reducing CE-SDS (Figure 12A-D). All CE SDS experiments were performed using Beckman PA800 CE system (Fullerton, CA) equipped with UV diode detector employing 221 nm and 220 nm wavelength. A bare-fused silica capillary 50 μm x 30.2 cm was used for the separation analysis. Buffer vial preparation and loading as well as capillary cartridge installation were conducted as described in the Beckman Coulter manual for IgG Purity/Heterogeneity. The running conditions for reduced and non-reduced CE-SDS were similar to those described in Beckman Coulter manual for IgG

Purity/Heterogeneity with some modifications which are briefly described below. For non-reducing conditions, the antibody sample (150 μg) was added to 20 μl of SDS reaction buffer and 5 μl of 70 mM N-ethylmaleimide. Water was then added to make final volume 35 μl and the protein concentration was brought to 4.3 mg/ml. The SDS reaction buffer was made of 4% SDS, 0.01 M citrate phosphate buffer (Sigma) and 0.036 M sodium phosphate dibasic. The preparation was vortexed thoroughly, and heated at 45°C for 5 min. The preparation was then combined with an additional 115 μl of 4% SDS. After being vortexed and centrifuged, the preparation was placed in a 200 μL PCR vial and then loaded onto the PA800 instrument. The sample was injected at the anode with reverse polarity using -10 kV for 30 sec, and was then separated at -15 kV with 20 psi pressure at both ends of capillary during the 35 min separation. For reducing conditions, the antibody sample was diluted to 2.1 mg/ml by adding purified H_2O, and 95 μl of the antibody was added to 105 μL of SDS sample buffer (Beckman) with 5.6% beta mercaptoethanol. The preparation was then vortexed thoroughly and then heated at 70 °C for 10 min. After being centrifuged, the supernatant was placed in a 200 μl PCR vial and then loaded onto the PA800 instrument. The sample was injected at the anode with reverse polarity using -5 kV for 20 sec, and was then separated at -15 kV with 20 psi pressure at both ends of capillary during 30 min separation. Both 16435 and 16444 antibodies produced very similar profiles with both reducing and non-reducing CE-SDS (Figure 12A-D).

[00506] To measure the light sensitivity of the antibodies, they were incubated in ambient lab fluorescent lighting or covered in aluminum foil for 3 days at room temperature. Light exposed and dark control antibodies were then analyzed using two size exclusion columns (TSK-GEL G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) in series with a 100 mM sodium phosphate, 250 mM NaCl, pH 6.8, mobile phase flowed at 0.5 mL/min. Based on the SEC chromatograms, 16444 showed significantly more light sensitivity than 16435 (Figure 13). The antibodies were then analyzed by hydrophobic interaction chromatography (HIC) using two Dionex ProPac HIC-10 columns in series with

mobile phase A being 1 M ammonium sulfate, 20 mM sodium acetate, pH 5.0 and mobile phase B being 20 mM sodium acetate, 5% acetonitrile, pH 5.0. Samples were eluted at 0.8 ml/min with a 0 – 100% linear gradient over 50 minutes observing the absorbance at 220 nm. Based on the HIC chromatograms, 16435 had a narrower main peak, indicating more product uniformity (Figure 14). Based on the lower light sensitivity, better purification yield (1219 mg/L vs. 1008 mg/L), better DSC profile, better SEC profile and fewer mutations from the parental antibody, 16435 was chosen as the primary lead for this family of antibodies.

Table 6. PCR primer sets and templates used to clone the indicated products.

Primer Sets SEQ ID NOS:	With Template SEQ ID NO:	Product + Primer Set SEQ ID NOS:	**Final Product SEQ ID NO:**
Round One Cloning			
(31, 32)(33, 34)	187	(31, 34)	189
(35, 37)(38, 36)	304	(35, 36)	322
(35, 39)(40, 36)	304	(35, 36)	320
(35, 41)(42, 36)	304	(35, 36)	324
(278, 43)(44, 36)	326	(278, 36)	328
(278, 45)(46, 36)	326	(278, 36)	330
Round Two Cloning			
(31, 213)(214, 34)	181	(31, 34)	185
(31, 215)(216, 34)	181	(31, 34)	183
(35, 217)(218, 36)	304	(35, 36)	318
(35, 219)(220, 36)	304	(35, 36)	316

(35, 221)(222, 36)	304	(35, 36)	314
(35, 223)(224, 36)	304	(35, 36)	312
(35, 225)(226, 36)	304	(35, 36)	310
(35, 227)(228, 36)	304	(35, 36)	308
(35, 229)(230, 36)	304	(35, 36)	306
(231, 232)(233, 34)	191	(231, 34)	195
(231, 234)(235, 34)	191	(231, 34)	193
(231, 236)(237, 34)	191	(231, 34)	197
(278, 238)(239, 36)	326	(278, 36)	332
(278, 240)(241, 36)	326	(278, 36)	334
(278, 242)(243, 36)	326	(278, 36)	342
(278, 244)(245, 36)	326	(278, 36)	344
(278, 246)(247, 36)	326	(278, 36)	346
(278, 248)(249, 36)	326	(278, 36)	328
(278, 250)(251, 36)	326	(278, 36)	330
(278, 252)(253, 36)	326	(278, 36)	348
(278, 254)(255, 36)	326	(278, 36)	350
(278, 256)(257, 36)	326	(278, 36)	366
(278, 258)(259, 36)	326	(278, 36)	370
Round Three Cloning (double mutants & germlining)			
(231, 132)(133, 34)	191	(231, 34)	211
(231, 134)(135, 34)	191	(231, 34)	199
(278, 136)(137, 36)	326	(278, 36)	338
(278, 138)(139, 36)	326	(278, 36)	372
(278, 140)(141, 36)	326	(278, 36)	374
(231, 234)(235, 34)	195	(231, 34)	209

(278, 240)(241, 36)	348	(278, 36)	356
(278, 240)(241, 36)	350	(278, 36)	358
Round Four Cloning (charge mutants [A to E or R] and triple mutants)			
(231, 142)(143, 34)	191	(231, 34)	201
(231, 144)(145, 34)	191	(231, 34)	203
(231, 260)(261, 34)	191	(231, 34)	205
(231, 262)(263, 34)	191	(231, 34)	207
(278, 264)(265, 36)	326	(278, 36)	336
(278, 266)(267, 36)	326	(278, 36)	340
(278, 268)(269, 36)	326	(278, 36)	352
(278, 270)(271, 36)	326	(278, 36)	354
(278, 272)(273, 36)	326	(278, 36)	360
(278, 274)(275, 36)	326	(278, 36)	362
(278, 276)(277, 36)	326	(278, 36)	368
(278, 276)(277, 36)	334	(278, 36)	364

[00507] **Example 2**

[00508] **Generation of antibodies to human TRAIL R2 and screening**

[00509] Cloning and engineering. The Antibody 16449 DNA sequences encoding immunoglobulin heavy chain (comprising VH12) and light chain (comprising VL6) subunits for anti-huTR2 antibodies were obtained from Gliniak et al. (US Patent No. 7,521,048) and were cloned using standard recombinant technology. In order to eliminate the binding ability of these antibodies a series of site directed mutagenesis clones were generated using polymerase chain reaction (PCR) amplification. The amino acids to be changed were selected on the basis of location in the complementarity determining regions (CDRs), change from germline sequence, estimated solvent exposure, and aromatic and charge nature. The initial set of mutants was germlining and alanine scanning mutants. Subsequently, mutations were combined and in several cases the alanine scanning mutants were mutated to introduce negative charge, by replacing the alanine with glutamic acid, or positive charge, by replacing the alanine with arginine. Further detail about construct development is found in Example 5 and Table 6 herein.

[00510] Transient expression to generate recombinant monoclonal antibodies. Transient transfections were carried out in HEK 293-6E cells as follows. The human embryonic kidney 293 cell line stably expressing Epstein Barr virus Nuclear Antigen-1 (293-6E cells) was obtained from the National Research Council (Montreal, Canada). Cells were maintained as serum-free suspension cultures using F17 medium (Invitrogen, Carlsbad, CA) supplemented with 6 mM L-glutamine (Invitrogen, Carlsbad, CA), 1.1% F-68 Pluronic (Invitrogen, Carlsbad, CA) and 250 μg/ul Geneticin (Invitrigen, Carlsbad, CA). The suspension cell cultures were maintained in Erlenmeyer shake flask cultures. The culture flasks were shaken at 65 rpm at 37 °C in a humidified, 5% CO_2 atmosphere. A stock solution (1 mg/ml) of 25-kDa linear PEI (Polysciences, Warrington, PA) was prepared in water, acidified with HCl to pH 2.0 until dissolved, then neutralized with NaOH, sterilized by

filtration (0.2 μm), aliquoted, and stored at -20°C until used. Tryptone N1 was obtained from OrganoTechni S.A. (TekniScience, QC, Canada). A stock solution (20%, w/v) was prepared in Freestyle medium (Invitrogem, Carlsbad, CA), sterilized by filtration through 0.2 μm filters, and stored at 4°C until use. Typically, transfections were performed at the 1L scale. Cells (293-6E) were grown too a viable cell density of 1.1 X 10^6 cells/ml then transfection complexes were prepared in 1/10th volume of the final culture volume. For a 1-L transfection culture, transfection complexes were prepared in 100 ml F17 basal medium, and 500 μg plasmid DNA (heavy chain and light chain DNA, 1:1 ratio) was first diluted in 100 ml F17 medium. After a 5-minute incubation at room temperature, 1.5 ml of PEI solution was added. The complexes were vortexed mildly, then incubated for 15 minutes at room temperature. The cells were transfected by adding the transfection complex mix to the cells in the shale flask culture. 24 hours post-transfection, Tryptone N1 was added to the transfected culture to a final concentration of 0.5%, and the transfected cultures were maintained on a shaker at 65 rpm at 37°C in a humidified, 5% CO_2 atmosphere for another 5 days after which they were harvested. The conditioned medium was harvested by centrifugation at 4000 rpm, and then sterile filtered through 0.2 μm filter (Corning Inc.).

[00511] Purification of Antibodies. The transiently expressed antibodies were purified using recombinant protein A sepharose (GE Healthcare) directly loading the conditioned media on the column at 5 ml/min at 7 °C. The column was then washed with 10 column volumes of Dulbecco's PBS without divalent cations and then eluted with 100 mM acetic acid, pH 3.5. The eluted antibody was pooled based on the chromatographic profile and the pH was adjusted to 5.0 using 2 M tris base. The pool was then filtered through a 0.8/0.22 μm syringe filter and then dialyzed against 10 mM acetic acid, 9% sucrose, pH 5.0. The buffer exchanged antibody was then concentrated using a Vivaspin 30 kDa centrifugal concentrator (Sartorius), and the concentrated product was filtered through a 0.22 μm cellulose acetate filter.

[00512] BIAcore binding assays. Antibody 16449 is a human antibody that specifically binds to Trail Receptor 2 (TR2). A solution equilibrium binding assay

was developed to assess the binding activity of a set of antibodies to TR2. Antibody 16449 was immobilized to a CM5 sensor chip surface as described in Example 1 above. Final immobilized level was approximately 8000 resonance units (RU). TR2 (1 nM) in the absence of antibody was used to establish the 100% binding signal of TR2 that is free of antibody binding in solution. To determine antibody binding in solution, serial diluted antibody samples in a range of 7 pM to 10 nM were incubated with the 1 nM TR2. The decreased binding signal of TR2 after the antibody incubation indicates the binding of the antibody to TR2 in solution. The results in Figure 15 indicate that all three new antibody constructs (16449, 1869, and 1870) retained TR2 binding activity similar to that of the original construct.

[00513] In other experiments, 10 nM TR2 was incubated with 50 nM and 1 μM antibody samples in the assay as described above. 10 nM TR2 was used to define the 100% binding signal. Although several of the antibodies showed significant lack of binding at 50 nM (16613, 1919, 1913, 1910, 1920 and 1922), none showed complete lack of binding at 1000 nM (results shown in Figure 16). Additional point mutagenesis yielded antibodies with lower affinity for TR2 (Figure 17). Two sites (heavy chain Y125 and light chain Y53) showed exceptional sensitivity to mutagenesis, particularly with charged substitutions at position Y125. Double alanine substitutions produced variants with even further decreased binding affinity for TR2 (Figure 18). Combining the alanine mutations with charged mutations in a pairwise, or greater order, fashion produced several molecules that did not show significant binding to TR2 even at 10 μM antibody (Figure 19). From these data, five of the best variants (10186, 10184, 4341, 10183, and 4241) were advanced for binding studies at 50 μM antibody (Figure 20A-B). All but 10186 showed no significant binding to TR2 even at 50 μM.

[00514] Cell Based Activity Assay. Colo205 is a human colon carcinoma cell line that is sensitive to the presence of TRAIL. Binding of positive control IgG1 anti-TR2 mAb molecules (antibody 16449) to TR2 on the surface of Colo205 results in cell apoptosis. A Colo205 based cell assay was developed to verify the cell killing efficacy of antibodies. The in vitro biological activity of the Antibody 16449 (anti-

TRAIL-R2 antibody) is analyzed by its ability to induce apoptosis in human ascites colorectal adenocarcinoma cell line Colo205. The detection of caspase-3 activation is used as a positive marker for apoptosis, using the Apo-One™ Homogeneous caspase™-3/7 assay kit (PromegaCorporation, Madison, WI), according to the manufacturer's instruction. (see, Niles et al., The Apo-One™.Homogeneous Caspase™-3/7 assay: a simplified "solution" for apoptosis detection, Cell Notes 2:2-3 (2001)). In this method, luminescent caspase-3/7 reagent provides a sensitive and robust monitoring of anti-TRAIL-R2 induced caspase activation in Colo205 cells. Luminescence produced is proportional to the amount of caspase activity present. The luminescence of each sample is measured in a plate-reading luminometer. Biological activity of the test sample is determined by comparing test sample response to Reference Standard response. To compare the samples with standard control antibody, 200 nM and 10 μM of antibody samples were pre-incubated with 1 or 100 μg/ml of protein G. The mixtures were then added to Colo205 cultures. Figure 20C indicates that, unlike the control anti-TR2 mAb molecules, the antibody samples do not have the ability to kill the cells even at very high concentrations (e.g., 30 μg/mL of antibody).

[00515] Analysis of homogeneity. The lead antibodies were analyzed for product quality on a 1.0-mm Tris-glycine 4-20% SDS-PAGE (Novex) using reducing (Figure 21B) and non-reducing loading buffer (Figure 21A). All candidates appeared quite similar by both non-reducing and reducing SDS-PAGE. Antibodies were further analyzed for homogeneity using one size exclusion column (Phenomenex SEC3000, 7.8 x 300 mm) with a 50 mM sodium phosphate, 250 mM NaCl, pH 6.8, mobile phase flowed at 1.0 mL/min (representative results are shown in Figure 22). While all the antibodies showed relatively low levels of high molecular weight species, 10185 and 10184 showed slightly more high molecular mass material. Lead candidates were selected based on SEC behavior, BIAcore binding analysis, cell based assay results, estimated proteolytic vulnerability and lower shift in the calculated isoelectric point. Based on these criteria, 4241 and 4341 were chosen for further evaluation.

[00516] Construct development for stable expression. Pools of stably expressed antibodies 4241 and 4341 were created by transfecting CHO DHFR(-) host cells with corresponding HC and LC expression plasmid set using a standard electroporation procedure. Per each antibody molecule, 3-4 different transfections were performed to generate multiple pools. After transfection, the cells were grown as a pool in a serum free (-)GHT selective growth media to allow for selection and recovery of the plasmid containing cells. Cell pools grown in (–)GHT selective media were cultured until they reached > 85% viability. The selected cell pools were amplified with 150 nm methotrexate (MTX). When the viability of the MTX-amplified pools reached >85% viability, the pools were screened using an abbreviated six-day batch production assay with an enriched production media to assess expression. The best pool was chosen based on the six-day assay titer and correct mass confirmation. Subsequently, scale-up production using 11-day fed-batch process was performed for the antibody generation, followed by harvest and purification.

[00517] Titers were determined by HPLC assay using a Poros A column, 20μm, 2.1 x 30mm (Applied Biosystems, part #1-5024-12). Briefly, Antibodies in conditioned media were filtered using Spin-X columns (Corning, part #8160) prior to analysis by HPLC, and a blank injection of 1X PBS (Invitrogen, part #14190-144) was performed prior to injection of test antibodies and after each analysis run. In addition, conditioned media without antibody was injected prior to analysis to condition the column, and new columns were conditioned by triplicate injection of 100 μg of control antibody. After a 9-minute wash with PBS at 0.6 ml/min, the antibody was eluted with ImmunoPure IgG Elution Buffer (Pierce, part #21009) and the absorbance at 280 nm was measured.

[00518] Antibody titers were quantified against a standard plot of control antibody concentration versus peak area. A control antibody stock was prepared at a concentration of 4 mg/ml, and five standard antibody concentrations were prepared by dilution of the antibody control stock in a volume of PBS (0.1 μg/μl to 1.6 μg/μl). By extending the standard curve, the lower limit of detection is 0.02 μg/μl of antibody, and the higher limit of quantification is 4 μg/μl. An assumption is made

that test antibodies have similar absorbance characteristics as the control; however Titers can be adjusted by multiplying titer an extinction coefficient ratio of the control antibody over the extinction coefficient of the test antibody. The titer assay results show that after scale up to the fed batch process, the 4241 antibody demonstrated marginally better expression than the 4341 antibody (Figure 23A-B).

[00519] Purification of stably expressed antibodies. Stably expressed antibodies were purified by Mab Select Sure chromatography (GE Life Sciences) using 8 column volumes of Dulbecco's PBS without divalent cations as the wash buffer and 100 mM acetic acid, pH 3.5, as the elution buffer at 7°C. The elution peak was pooled based on the chromatogram, and the pH was raised to about 5.0 using 2 M Tris base. The pool was then diluted with at least 3 volumes of water, filtered through a 0.22-μm cellulose acetate filter and then loaded on to an SP-HP sepharose column (GE Life Sciences) and washed with 10 column volumes of S-Buffer A (20 mM acetic acid, pH 5.0) followed by elution using a 20 column volume gradient to 50% S-Buffer B (20 mM acetic acid, 1 M NaCl, pH 5.0) at 7 °C. A pool was made based on the chromatogram and SDS-PAGE analysis, then the material was concentrated about 6-fold and diafiltered against about 5 volumes of 10 mM acetic acid, 9% sucrose, pH 5.0 using a VivaFlow TFF cassette with a 30 kDa membrane. The dialyzed material was then filtered through a 0.8/0.2-μm cellulose acetate filter and the concentration was determined by the absorbance at 280 nm. The purification processed samples were analyzed using a 1.0-mm Tris-glycine 4-20% SDS-PAGE (Novex) reducing loading buffer (Figure 24A-B). These data showed that both 4241 and 4341 antibodies had similar purification characteristics, with no steps producing unexpected sample losses.

[00520] Analysis of stably expressed antibodies. Comparison of the ion exchange chromatographic profiles of the 4241 and 4341 variants (Figure 25) showed that 4341 has a narrower main peak indicating less heterogeneity than 4241. Analysis of the variants using 1.0-mm Tris-glycine 4-20% SDS-PAGE (Novex) with reducing and non-reducing loading buffer showed no significant difference between the variants (Figure 26A-B). Analysis using two size exclusion columns (TSK-GEL

G3000SWXL, 5 mm particle size, 7.8 x 300 mm, TosohBioscience, 08541) in series with a 100 mM sodium phosphate, 250 mM NaCl, pH 6.8, mobile phase flowed at 0.5 mL/min also showed no significant difference between the 4241 and 4341 variants (Figure 27A-B). The antibodies were analyzed for thermoresistance by DSC using a MicroCal VP-DSC where the samples were heated from 20°C to 95°C at a rate of 1°C per minute. The proteins were at 0.5 mg/ml in 10 mM sodium acetate, 9% sucrose, pH 5.0 (Figure 28). The 4241 antibody produced the most desirable melting profile, with a higher temperature for the secondary transition, compared to antibody 4341.

[00521] The 4241 and 4341 antibodies were analyzed by reducing and non-reducing CE-SDS (Figure 29A-D). All CE SDS experiments were performed using Beckman PA800 CE system (Fullerton, CA) equipped with UV diode detector employing 221 nm and 220 nm wavelength. A bare-fused silica capillary 50 μm x 30.2 cm was used for the separation analysis. Buffer vial preparation and loading as well as capillary cartridge installation were conducted as described in the Beckman Coulter manual for IgG Purity/Heterogeneity. The running conditions for reduced and non-reduced CE-SDS were similar to those described in Beckman Coulter manual for IgG Purity/Heterogeneity with some modifications which are briefly described below. For non-reducing conditions, the antibody sample (150 μg) was added to 20 μl of SDS reaction buffer and 5 μl of 70 mM N-ethylmaleimide. Water was then added to make final volume 35 μl and the protein concentration was brought to 4.3 mg/ml. The SDS reaction buffer was made of 4% SDS, 0.01 M citrate phosphate buffer (Sigma) and 0.036 M sodium phosphate dibasic. The preparation was vortexed thoroughly, and heated at 45°C for 5 min. The preparation was then combined with an additional 115 μl of 4% SDS. After being vortexed and centrifuged, the preparation was placed in a 200 μl PCR vial and then loaded onto the PA800 instrument. The sample was injected at the anode with reverse polarity using -10 kV for 30 sec, and was then separated at -15 kV with 20 psi pressure at both ends of capillary during the 35-min separation. For reducing conditions, the antibody sample was diluted to 2.1 mg/ml by adding purified H_2O, and 95 μl of the

antibody was added to 105 µl of SDS sample buffer (Beckman) with 5.6% beta mercaptoethanol. The preparation was then vortexed thoroughly and then heated at 70 °C for 10 min. After being centrifuged, the supernatant was placed in a 200 µl PCR vial and then loaded onto the PA800 instrument. The sample was injected at the anode with reverse polarity using -5 kV for 20 sec, and was then separated at -15 kV with 20 psi pressure at both ends of capillary during 30 min separation. Neither of the antibodies showed significant differenced by CE-SDS analysis (Figure 29A-D).

[00522] Antibodies were also analyzed for homogeneity using high performance ion exchange chromotography (SP-5PW, 10 µm particle, 7.5 mm ID x 7.5 cm, TosohBioscience, 08541) using 20 mM acetic acid, pH 5.0 as buffer A and 20 mM acetic acid, 1 M NaCl, pH 5.0 as buffer B flowed at 1 mL/min with an 80 minute linear gradient from 0 – 40% buffer B. Neither purified 4241 or 4341 antibody showed significant difference in the high performance ion exchange profiles with this method (Figure 30). To measure the light sensitivity of the antibodies, they were incubated in ambient lab fluorescent lighting or covered in aluminum foil for 3 days at room temperature. The antibodies were then analyzed by hydrophobic interaction chromatography using two Dionex ProPac HIC-10 columns in series with mobile phase A being 1 M ammonium sulfate, 20 mM sodium acetate, pH 5.0 and mobile phase B being 20 mM sodium acetate, 5% acetonitrile, pH 5.0. Samples were eluted at 0.8 ml/min with a 0 – 100% linear gradient over 50 minutes observing the absorbance at 220 nm. Based on the HIC chromatograms both with and without light exposure, neither antibody displayed significant differences (Figure 31A-B). Based primarily on the more uniform ion exchange chromatography peak during purification 4341 was chosen as the primary lead for this family of antibodies.

[00523] **Example 3**

[00524] **Human tissue cross-reactivity assessment**

[00525] In general accordance with the guidance laid out in Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (U.S. Department of Health and Human Services, Food and Drug Administraton, Center for Biologics Evaluation and Research (1997)), a preliminary non-GLP study was carried out to determine cross-reactivity of inventive antibodies with a variety of human tissues. If an antibody is intended for drug development, a more extensive testing under GLP conditions is required. The tissue cross-reactivity of antibodies 16435 and 4341 was evaluated (Charles River Laboratories, Preclinical Services, Reno, NV) with cryosections of selected human tissues using Alexa Fluor 488 labeled forms of the test articles. Normal human tissues from two unique individuals (unless otherwise indicated) were obtained from the Special Pathology Services Human Tissue Bank collected by the National Disease Research Interchange (NDRI, Philadelphia, PA), Cureline, Inc. (Burlingame, CA), Cybrdi (Rockville, MD), or Rocky Mountain Lions Eye Bank (Aurora, CO). Tissues tested included human cerebellum, lung, cerebral cortex, ovary (from mature female), eye, placenta, gastrointestinal tract (small intestine), skin (1 individual), heart, spleen, kidney (1 individual), thyroid, liver, testis. Sections of fresh-frozen human tissues and control bead blocks (human serum albumin [HSA] beads) were cut on the cryostat and thaw mounted onto capillary gap slides. The tissue and control bead slides were fixed in cold acetone for approximately 10 minutes at -10°C to -25°C. The fixed slides were allowed to dry for at least one hour (to overnight). If stored frozen, fixed slides were removed from the freezer on the day prior to an experiment and allowed to thaw overnight prior to use. All the following steps were performed at room temperature unless otherwise specified. The slides were incubated with 1X Morphosave™ for approximately 15 minutes to preserve tissue morphology then washed two times for approximately 5 minutes each in1X phosphate-buffered saline (PBS). To block endogenous peroxidase, the slides were incubated in a glucose oxidase solution for approximately 1 hour at approximately 37°C. The slides were washed two times in

1X PBS for approximately 5 minutes each. Endogenous biotin was blocked by sequential incubation (approximately 15 minutes each) in avidin and biotin solutions. Following the incubation in biotin, the tissue sections were blocked with a blocking antibody solution for approximately 25 minutes. Alexa Fluor 488-Ab 16435, and Alexa Fluor 488 anti-Ab 4341 were applied to sections at the optimal concentration (2.0 μg/mL) or 5 times the optimal concentration (10.0 μg/mL) for approximately 25 minutes. Slides were washed 3 times with wash buffer and then incubated with the secondary antibody (rabbit anti-Alexa Fluor 488) for approximately 25 minutes. Following incubation with the secondary antibody, slides were washed 4 times with wash buffer then incubated with the tertiary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG antibody) for approximately 25 minutes and binding visualized with a diaminobenzidine (DAB) chromogen substrate. HSA beads were used as a negative control. Tissues were qualified as adequate for immunohistochemistry via staining with an antibody against CD31 (anti-CD31) i.e., platelet endothelial cell adhesion molecule (PECAM-1). There was no specific staining in any human tissue examined at either 2.0 or 10.0 μg/mL concentration for any of the tested antibodies.

[00526] **Example 4**

[00527] **Pharmacokinetic (PK) Studies of Antibody Embodiments of the Invention in Rats and Cynomolgus Monkeys**

[00528] The pharmacokinetic profile of the 16435, 16444, 4241, and 4341 carrier antibodies was determined in adult Sprague-Dawley rats (8-12 weeks old) by injecting 5 mg/kg subcutaneously and collecting approximately 250 μL of blood in Microtainer® serum separator tubes at 0, 0.25, 1, 4, 24, 48, 72, 96, 168, 336, 504, 672, 840 and 1008 hours post-dose from the lateral tail vein. Each sample was maintained at room temperature following collection, and following a 30-40 minute clotting period, samples were centrifuged at 2-8°C at 11,500 rpm for about 10 minutes using a calibrated Eppendorf 5417R Centrifuge System (Brinkmann

Instruments, Inc., Westbury, NY). The collected serum was then transferred into a pre-labeled (for each rat), cryogenic storage tube and stored at -60°C to -80°C for future analysis. To measure the serum sample concentrations from the PK study samples, the following method was used: ½ area black plate (Corning 3694) was coated with 2 µg/ml of anti-hu Fc, antibody 1.35.1 in PBS and then incubated overnight at 4°C. The plate was then washed and blocked with I-Block™ (Applied Biosystems) overnight at 4°C. If samples needed to be diluted, then they were diluted in Rat SD control serum. The standards and samples were then diluted 1: 20 in I-Block™ + 5% BSA into 380 µl of diluting buffer. The plate was washed and 50-µl samples of pretreated standards and samples were transferred into an antibody 1.35.1 coated plate and incubated for 1.5 h at room temperature. The plate was washed, then 50 µl of 100 ng/ml of anti-hu Fc antibody 21.1-HRP conjugate in I-Block™ +5% BSA was added and incubated for 1.5 h. The plate was washed, then 50 µl of Pico substrate were added, after which the plate was immediately analyzed with a luminometer. The pharmacokentic profile was not significantly different for any of the four antibodies (Figure 32) with AUC_{0-t} ±SD of 18,492 ±2,104; 21,021 ±2,832; 24,045 ±2,480 and 24,513 ±972 µg/h/mL for antibodies 16435, 16444, 4241 and 4341, respectively.

[00529] The pharmacokinetic profile of the 16435 antibody was determined in cynomolgus monkeys (Macaca fascicularis) to assess the in vivo parameters. Briefly, a single IV bolus dose of 16435, either 1 mg/kg or 10 mg/kg, was administered to mature male cynomolgus monkeys (n=2 per group). Serum samples were collected pre-dose and at timepoints 0.25, 0.5, 1, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 360, 408, 456, 504, 552, 600, 648 and 672 hours after antibody administration. Samples were assayed for 16435 antibody levels by using an anti-IgG sandwich ELISA. Time concentration data were analyzed using non-compartmental methods with WinNonLin® (Enterprise version 5.1.1, 2006, Pharsight® Corp. Mountain View, CA). The resulting pharmacokinetic profile did not show any significant abnormalities (Figure 33).

[00530] **Example 5**

[00531] **Antibody 16435-ShK[1-35, Q16K] Fusion Cloning, Purification & Analysis**

[00532] Cloning and expression. The components of the monovalent 16435-ShK fusion (Antibody 3742) include:

[00533] (a) 16435 kappa LC (SEQ ID NO:109);

[00534] (b) 16435 IgG2 HC (SEQ ID NO:112); and

[00535] (c) 16435 IgG2-ShK[1-35, Q16K] (SEQ ID NO:377):

[00536] QVQLVQSGAEVKKPGASVKVSCKASGYTFTRYGISWVRQAPGQG
LEWMGWISTYSGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVY
YCARAQLYFDYWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYT
CNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVL
TVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEM
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKL
TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGGGGGSGGGGSRSCID
TIPKSRCTAFKCKHSMKYRLSFCRKTCGTC// SEQ ID NO:377.

[00537] The desired product antibody fusion (3742) was a full antibody with the ShK[1-35, Q16K] peptide (SEQ ID NO:76) fused to the C-terminus of one heavy chain (see, schematic representation in Figure 34). With two different heavy chains sharing one variety of light chain, the ratio of heavy chain:light chain:heavy chain-ShK[1-35, Q16K] was 1:2:1. The expected expression products are 16435 IgG2, monovalent 16435 IgG2-ShK[1-35, Q16K], and divalent 16435 IgG2-ShK[1-35, Q16K]. The monovalent 16435 IgG2-Shk fusion protein was isolated from the mix using cation exchange chromatography, as described herein.

[00538] The ShK[1-35, Q16K] fragment was generated using construct pTT5-aKLH120.6-IgG2-HC-L10-ShK[1-35, Q16K], encoding (SEQ ID NO:389), as a template, which was digested with StuI and NotI and purified with the PCR Purification Kit (Qiagen). At the same time, pDC324 (SEQ ID NO:111) was digested with StuI and NotI, treated with Calf Intestine Phosphatase (CIP) and run out on a 1% agarose gel. The larger fragment was cut out and gel purified by Qiagen's Gel Purification Kit. The purified Shk[1-35, Q16K] fragment was ligated to the large vector fragment and transformed into OneShot Top10 bacteria. DNAs from transformed bacterial colonies were isolated and submitted for sequencing. Although analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final pDC324-16435-IgG2-HC-L10-ShK[1-35, Q16K] construct encoded an IgG2-HC-L10-ShK[1-35, Q16K] fusion polypeptide (SEQ ID:377).

[00539] Purification. Initial purification of the 3742 conditioned media was done by affinity FPLC capture of the Fc region using Protein A Sepharose (GE Healthcare) followed by a column wash with Dulbecco's PBS without divalent cations (Invitrogen) and step elution with 100 mM acetic acid, pH 3.5. Protein containing fractions were pooled and neutralized to pH 5.0 with 10 N NaOH and diluted 5-times volume with water. The material was filtered through a 0.45 μm cellulose acetate filter (Corning) and further purified by cation exchange FPLC (SP Sepharose High Performance; GE Healthcare). Samples were loaded onto a column equilibrated with 100% buffer A (50mM acetic acid, pH 5.0) and eluted with a gradient of 0 to 800 mM NaCl over 30 column volumes. Peaks containing monovalent species were pooled and formulated into 10 mM sodium acetate, 9% sucrose, pH 5.0.

[00540] Analysis. Reducing and non-reducing SDS-PAGE analysis was done on 3742 pools using 4-12% tris-glycine gels (Invitrogen) with 2 μg of protein, stained with QuickBlue (Boston Biologicals). Based on the SDS-PAGE there were no significant differences between the pools (Figure 35). Analytical SEC was done using a Biosep SEC-S3000 column (Phenomenex) with an isocratic elution using 50

mM sodium phosphate, 250 mM NaCl, pH 6.9 as the mobile phase at 1 ml/min (Figure 36A-D). All four pools showed relatively low levels of aggregate based on the SEC data; however, pool 1 showed somewhat higher levels than the other pools.

[00541] The final 3742 samples were characterized by LC-MS analysis of reduced heavy chain (Figure 38A-D) and light chain (Figure 37A-D). The product was chromatographed through a Waters MassPREP micro desalting column using a Waters ACQUITY UPLC system. The column was set at 80°C and the protein eluted using a linear gradient of increasing acetonitrile concentration in 0.1 % formic acid. The column effluent was directed into a Waters LCT Premier ESI-TOF mass spectrometer for mass analysis. The instrument was run in the positive V mode. The capillary voltage was set at 3,200 V and the cone voltage at 80 V. The mass spectrum was acquired from 800 to 3000 m/z and deconvoluted using the MaxEnt1 software provided by the instrument manufacturer. All four pools yielded the expected mass within the error of the instrument, indicating all pools were producing the expected product (Figure 37A-D and Figure 38A-D).

[00542] Whole Blood Assay. An *ex vivo* assay was employed to examine impact of toxin peptide analog Kv1.3 inhibitors on secretion of IL-2 and IFN-γ. The potency of ShK analogs and conjugates in blocking T cell inflammation in human whole blood was examined using an *ex vivo* assay that has been described earlier (see Example 46 of WO 2008/088422 A2, incorporated herein by reference in its entirety). In brief, 50% human whole blood was stimulated with thapsigargin to induce store depletion, calcium mobilization and cytokine secretion. To assess the potency of molecules in blocking T cell cytokine secretion, various concentrations of Kv1.3 blocking peptides and peptide-conjugates were pre-incubated with the human whole blood sample for 30-60 min prior to addition of the thapsigargin stimulus. After 48 hours at 37°C and 5% CO_2, conditioned medium was collected and the level of cytokine secretion was determined using a 4-spot electrochemiluminescent immunoassay from MesoScale Discovery. Using thapsigargin stimulus, the cytokines IL-2 and IFN-g were secreted robustly from blood isolated from multiple donors. The IL-2 and IFN-g produced in human whole blood following thapsigargin

stimulation were produced from T cells, as revealed by intracellular cytokine staining and fluorescence-activated cell sorting (FACS) analysis. Kv1.3 is the major voltage-gated potassium channel present on T cells. Allowing for K^+ efflux, Kv1.3 provides the driving force for continued Ca^{2+} influx which is necessary for the sustained elevation in intracellular calcium needed for efficient T cell activation and cytokine secretion. Kv1.3 inhibitors have been shown earlier to suppress this calcium flux induced by TCR ligation (G.C. Koo et al., 1999, Cell. Immunol. 197, 99-107). Thapsigargin-induced store-depletion and TCR ligation elicits similar patterns of Ca^{2+} mobilization in isolated T cells (E. Donnadieu et al., 1991, J. Biol. Chem. 267, 25864-25872), but we have found thapsigargin gives a more robust response in whole blood. Therefore, we employed a bioassay whereby the bioactivity of Kv1.3 inhibitors is assessed by examining their ability to block thapsigargin-induced cytokine secretion from T cells in human whole blood. Since whole blood is a complex fluid containing high protein levels, the activity of peptides and peptide conjugates in this whole blood assay has an additional advantage in assessing the molecules stability over 48 hours in a biologically relevant fluid. The whole blood assay provides important confirmation of the Kv1.3 potency of molecules determined by electrophysiology (ePhys), since ePhys assays are generally of short duration (<1-2 hours) and use physiological saline containing no protein. The longer duration of the whole blood assay may allow for more effective determination of equilibrium binding kinetics relative to ePhys studies which are of short duration. As seen in Table 7A (below), all four pools of 3742-ShK(1-35, Q16K) showed good potency in the human whole blood assay, indicating the isolated molecules have obtained the proper tertiary structure and are reasonably stable in serum for 48 hours. Table 7B (below) shows that the potency was comparable to other ShK-conjugated molecules.

Table 7A. Human whole blood ("WB") assays of four pools of 3742 (SEQ ID NOS: 377; 109, 112; 109) of IL-2 and interferon-gamma ("IFNγ") were conducted as described in Example 5 herein.

Pool	IC_{50} IFNγ (pM)	IC_{50} IL-2 (pM)
1	708	2220
2	599	2461
3	598	1649
4	412	909

Table 7B. Data demonstrating potency of various conjugates of [Lys16]ShK in the Whole Blood Assay. Toxin peptides and toxin peptide analogs were PEGylated as described in Example 9 herein. Immunoglobulin-containing compounds were recombinantly expressed and purified as described in Example 8. Human whole blood ("WB") assays of IL-2 and interferon-gamma ("IFNg") were conducted as described in Example 5 herein).

SEQ ID NO or citation	Conjugate Type	Designation	WB (IL-2) IC50 (nM)	WB (IFNg) IC50 (nM)	Potency Relative to ShK (WB, IL2)
378	none	ShK(1-35)	0.067	0.078	1.00
76	none	[Lys16]ShK	0.110	0.158	1.64
379	none	[Lys16]ShK-Ala	0.138	0.266	2.06
380	PEG	20kDa-PEG-ShK	0.380	0.840	5.67
381	PEG	20kDa-PEG-[Lys16]ShK	0.092	0.160	1.37
382	PEG	20kDa-PEG-[Lys16]ShK-Ala	0.754	1.187	11.25
377;109;112;109	IgG2	Monovalent antibody # 3742-ShK(1-35, Q16K), Pool 4	0.412	0.909	6.15
Example 1, WO2008/088422 A2	IgG1	Bivalent Fc-L10-ShK[1-35] homodimer	0.386	0.320	5.76
Example 2, WO2008/088422	IgG1	Bivalent Fc-L10-ShK[2-35]	0.585	2.285	8.73

A2		homodimer			
Example 2, WO2008/088422 A2	IgG1	Monovalent Fc/Fc-L10-ShK[2-35] heterodimer	2.149	5.199	32.07
1; 26	IgG2	Monovalent Fc/Fc-ShK(1-35 Q16K) heterodimer	0.160	0.499	2.39
26; 26	IgG2	Bivalent Fc-ShK(1-35, Q16K) homodimer	1.850	3.140	27.61

[00543] **Example 6**

[00544] **Ab 4341-ShK(1-35, Q16K), 4341-FGF21 and 16435-FGF21 Fusion Construct Generation**

[00545] Antibody 16435-huFGF21 Fusion (Ab 10162). The components of the 16435-huFGF21 fusion include:

[00546] (a) 16435 kappa LC (SEQ ID NO:109);

[00547] (b) 16435 HC (R118A; SEQ ID NO:112); and

[00548] (c) 16435 IgG2-HC-huFGF21 [1-181] (SEQ ID NO:384):

[00549] QVQLVQSGAEVKKPGASVKVSCKASGYTFTRYGISWVRQAPGQG
LEWMGWISTYSGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVY
YCARAQLYFDYWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYT
CNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVL
TVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEM
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKL
TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGGGGGGSGGGSGGGG
SHPIPDSSPLLQFGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPES
LLQLKALKPGVIQILGVKTSRFLCQRPDGALYGSLHFDPEACSFRELLLEDGY
NVYQSEAHGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPAPPEPPGILAPQPP
DVGSSDPLSMVGPSQGRSPSYAS// SEQ ID NO:384.

[00550] The 16435 huIgG2-HC-L15-huFGF21 [1-181] fragment was generated using construct pTT5-aKLH120.6-IgG2-HC-L15-huFGF21 [1-181] (SEQ ID NO:130) as a template, which was digested with BsmBI and NotI and purified with the Qiagen Gel Purification Kit. At the same time, pTT5-16435 IgG2 HC was digested with BsmBI and NotI, and run out on a 1% agarose gel. The vector fragments, which contained the 16435 heavy chain variable region, were cut out and

gel purified by Qiagen Gel Purification Kit. The purified huIgG2-HC-L15-huFGF21 [1-181] fragment was ligated to the vector fragments containing the 16435 heavy chain variable region and transformed into DH10b bacteria. DNAs from transformed bacterial colonies were isolated and submitted for sequencing. Although analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final pTT5-16435-IgG2-HC-L15-huFGF21 [1-181] construct encoded an IgG2-HC-L15-huFGF21 [1-181] fusion polypeptide (SEQ ID:384)

[00551] Antibody 4341-huFGF21 Fusion (Ab 10163). The components of the 4341-ShK[1-35, Q16K] fusion (Ab 10163) include:

[00552] (a) 4341 kappa LC (SEQ ID NO:115);

[00553] (b) 4341 HC (Y125A; SEQ ID NO:118); and

[00554] (c) 4341 IgG2-HC-huFGF21 [1-181] (SEQ ID NO:386):

[00555] QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYFWSWIRQLPGKG
LEWIGHIHNSGTTYYNPSLKSRVTISVDTSKKQFSLRLSSVTAADTAVYYCA
RDRGGDYAYGMDVWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGC
LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQ
TYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLM
ISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVV
SVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSR
EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGGGGGGSGGGS
GGGGSHPIPDSSPLLQFGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAAD
QSPESLLQLKALKPGVIQILGVKTSRFLCQRPDGALYGSLHFDPEACSFRELL
LEDGYNVYQSEAHGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPAPPEPPGI
LAPQPPDVGSSDPLSMVGPSQGRSPSYAS// SEQ ID NO:386.

[00556] The huIgG2-HC-L15-huFGF21 [1-181] fragment was generated using construct pTT5-aKLH120.6-IgG2-HC-L15-huFGF21 [1-181] as a template, which was digested with BsmBI and NotI and purified with the Qiagen Gel Purification Kit. At the same time, pTT5-4341 IgG2 HC was digested with BsmBI and NotI, and run out on a 1% agarose gel. The larger fragment, which contained the 4341 heavy chain variable region, was cut out and gel purified by Qiagen Gel Purification Kit. The purified huIgG2-HC-L15-huFGF21 [1-181] fragment was ligated to the large vector fragment containing the 4341 heavy chain variable region and transformed into DH10b bacteria. DNAs from transformed bacterial colonies were isolated and submitted for sequencing. Although analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final pTT5-4341-IgG2-HC-L15-huFGF21 [1-181] construct encoded an IgG2-HC-L15-huFGF21 [1-181] fusion polypeptide (SEQ ID:386).

[00557] 4341-ShK[1-35, Q16K] Fusion (antibody 10164). The components of the 4341-ShK[1-35, Q16K] fusion (Ab 10164) include:

[00558] (a) 4341 kappa LC (SEQ ID NO:115);

[00559] (b) 4341 HC (Y125A; SEQ ID NO:118); and

[00560] (c) 4341 IgG2-HC-ShK [1-35, Q16K] (SEQ ID NO:388):

[00561] QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYFWSWIRQLPGKG
LEWIGHIHNSGTTYYNPSLKSRVTISVDTSKKQFSLRLSSVTAADTAVYYCA
RDRGGDYAYGMDVWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGC
LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQ
TYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLM
ISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVV
SVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSR
EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFL

YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGGGGGSGGGGS RSCIDTIPKSRCTAFKCKHSMKYRLSFCRKTCGTC// SEQ ID NO:388.

[00562] The huIgG2-HC-L10-ShK [1-35, Q16K] fragment was generated using construct pDC324-16435-HC-L10-IgG2-ShK [1-35, Q16K] (SEQ ID NO:376) as a template, which was digested with BsmBI and NotI and purified with the Qiagen Gel Purification Kit. At the same time, pTT5-4341 IgG2 HC was digested with BsmBI and NotI, and run out on a 1% agarose gel. The larger fragment, which contained the 4341 heavy chain variable region, was cut out and gel purified by Qiagen Gel Purification Kit. The purified huIgG2-HC-L10-ShK [1-35, Q16K] fragment was ligated to the large vector fragment containing the 4341 heavy chain variable region and transformed into DH10b bacteria. DNAs from transformed bacterial colonies were isolated and submitted for sequencing. Although analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final pTT5-4341-IgG2-HC-L10-ShK [1-35, Q16K] construct encoded an IgG2-HC-L10-ShK [1-35, Q16K] fusion polypeptide (SEQ ID NO:388).

[00563] **Example 7**

[00564] **Ab 4341-ShK, 4341-FGF21 and 16435-FGF21 Fusion Expression, Purification & Analysis**

[00565] Transient transfections were carried out in HEK 293-6E cells as follows. The human embryonic kidney 293 cell line stably expressing Epstein Barr virus Nuclear Antigen-1 (293-6E cells) was obtained from the National Research Council (Montreal, Canada). Cells were maintained as serum-free suspension cultures using F17 medium (Invitrogen, Carlsbad, CA) supplemented with 6 mM L-glutamine (Invitrogen, Carlsbad, CA), 1.1% F-68 Pluronic (Invitrogen, Carlsbad, CA) and 250

μg/ul Geneticin (Invitrogen, Carlsbad, CA). The suspension cell cultures were maintained in Erlenmeyer shake flask cultures. The culture flasks were shaken at 65 rpm at 37 °C in a humidified, 5% CO_2 atmosphere. A stock solution (1mg/ml) of 25-kDa linear PEI (Polysciences, Warrington, PA) was prepared in water, acidified with HCl to pH 2.0 until dissolved, then neutralized with NaOH, sterilized by filtration (0.2 μm), aliquoted, and stored at -20°C until used. Tryptone N1 was obtained from OrganoTechni S.A. (TekniScience, QC, Canada). A stock solution (20%, w/v) was prepared in F17 medium, sterilized by filtration through 0.2 μm filters, and stored at 4°C until use. Typically, transfections were performed at the 1L scale. Cells (293-6E) were grown too a viable cell density of 1.1 X 10^6 cells/ml then transfection complexes were prepared in 1/10th volume of the final culture volume. For a 1-L transfection culture, transfection complexes were prepared in 100 ml F17 basal medium, and 500 μg plasmid DNA (heavy chain and light chain DNA, 1:1 ratio) was first diluted in 100 ml F17 medium. After a 5-minute incubation at room temperature, 1.5 ml of PEI solution was added. The complexes were vortexed mildly, then incubated for 15 minutes at room temperature. The cells were transfected by adding the transfection complex mix to the cells in the shake flask culture. 24 hours post-transfection, Tryptone N1 was added to the transfected culture to a final concentration of 0.5%, and the transfected cultures were maintained on a shaker at 65 rpm at 37°C in a humidified, 5% CO_2 atmosphere for another 5 days after which they were harvested. The conditioned medium was harvested by centrifugation at 4000 rpm, and then sterile filtered through 0.2 μm filter (Corning Inc.).

[00566] The transiently expressed antibodies were purified using recombinant protein A sepharose (GE Healthcare) directly loading the conditioned media on the column at 5 ml/min at 7 °C. The column was then washed with 10 column volumes of Dulbecco's PBS without divalent cations and then eluted with 100 mM acetic acid, pH 3.5. The eluted antibodies were pooled based on the chromatographic profile and the pH was adjusted to 5.0 using 2 M tris base. The pools were then filtered through a 0.8/0.22 um syringe filter and then dialyzed against 10 mM acetic

acid, 9% sucrose, pH 5.0. The buffer exchanged antibodies were then concentrated using a Vivaspin 30 kDa centrifugal concentration (Sartorius), and the concentrated products were filtered through a 0.22 um cellulose acetate filter. All conditioned media, including a mock transfection, were analyzed using a 1.0 mm Tris-glycine 4-20% SDS-PAGE run at 35 mA/1000V/250W for 55 min (Figure 39A) loading 10 µl conditioned media. The band above the 250 molecular weight marker not observed in the mock transfection sample is likely the expressed product. All three experimental transfections showed a signficant quantity of the expected product on the SDS-PAGE.

[00567] Antibody fusions were analyzed for product quality on a 1.0-mm Tris-glycine 4-20% SDS-PAGE (Novex) using reducing and non-reducing loading buffer (Figure 39B). All candidates electrophoresed as expected by both non-reducing and reducing SDS-PAGE; however, 10162 and 10163 show some slower migrating than expected bands, possibly indicating partial glycosylation. Antibodies were further analyzed for homogeneity using one size exclusion column (Phenomenex SEC3000, 7.8 x 300 mm) with a 50 mM sodium phosphate, 250 mM NaCl, pH 6.8, mobile phase flowed at 1.0 mL/min (Figure 40). The 10162 and 10163 fusions eluted as expected and showed relatively low levels of high molecular weight species; however, the 10164 fusion eluted earlier than expected, possibly indicating aggregation.

[00568] LC-MS analysis was conducted of reduced light chain (Figure 41A-C) and heavy chain (Figure 42A-C), respectively, of the final 4341-ShK, 4341-FGF21, and 16435-FGF21 samples. The FGF21 fusion samples were deglycosylated prior to reduction using the PNGase F technique as described by the manufacturer (QA Bio, LLC, Palm Desert, CA), except that the substrate to enzyme ratio was 10 µg substrate to 1 µL enzyme. The product was chromatographed through a Zorbax SB300 C8 50x1 mm 3 micron column using an Agilent 1100 capillary HPLC system. The column was set at 75°C and the protein eluted using a gradient of increasing n-propanol concentration in 0.1 % trifluoroacetic acid. The column effluent was directed into an Agilent-TOF mass spectrometer for mass analysis. The

capillary voltage was set at 3,200 V and the fragmentor voltage at 225 V. The mass spectrum was acquired from 800 to 3000 m/z and deconvoluted using the MassHunter software provided by the instrument manufacturer. All samples possessed the expected mass within the error of the instrument, indicating all pools contained the expected product.

[00569] **Example 8**

[00570] **Expression and Purification of Monovalent or Multivalent Immunoglobulin- and/or Fc domain-Toxin Peptide Analog Fusions**

[00571] An assortment of monovalent, bivalent and trivalent structures were expressed and purified for comparison, including exemplary embodiments of the invention, as illustrated in Table 7B in Example 5. Those included antibody IgG2- or IgG1-ShK fusion variants (see Figure 1F-L). For example, bivalent Fc-L10-ShK[1-35], monovalent immunoglobulin heavy chain-[Lys16]ShK fusion antibody; see Figure 1F). IgG2 Fc/Fc-ShK variants (see Figure 1A), bivalent Fc-L10-ShK[2-35], monovalent Fc/Fc-L10-ShK[2-35] were also made for comparison, by recombinant methods as described in Sullivan et al., WO 2008/088422 A2, and in particular Examples 1, 2, and 56 therein, incorporated by reference in its entirety, or as modified herein.

[00572] Transient expression system used to generate toxin peptide analog-Fc fusions ("peptibodies") or other immunoglobulin fusion embodiments. HEK 293-6E cells were maintained in 3L Fernbach Erlenmeyer Flasks between 2e5 and 1.2e6 cells/ml in F17 medium supplemented with L-Glutamine (6 mM) and Geneticin (25 μg/ml) at 37°C, 5% CO_2, and shaken at 65 RPM. At the time of transfection, cells were diluted to 1.1 x 10^6 cells/mL in the F17 medium mentioned above at 90% of the final culture volume. DNA complex was prepared in Freestyle293 medium at 10% of the final culture volume. DNA complex includes 500ug total DNA per liter of culture and 1.5ml PEImax per liter of culture. DNA complex is briefly shaken once

ingredients are added and incubated at room temperature for 10 to 20 minutes before being added to the cell culture and placed back in the incubator. The day after transfection, Tryptone N1 (5g/L) was added to the culture from liquid 20% stock. Six days after transfection, culture was centrifuged at 4,000 RPM for 40 minutes to pellet the cells and the cultured medium was harvested through a 0.45um filter.

[00573] In preparing the DNA complex, the ratio of plasmids was proportional to the desired molar ratio of the peptides needed to generate the intended product. The components of the IgG2 Fc/Fc-ShK include IgG2 Fc and IgG2 Fc-ShK at a 1:1 ratio. During expression these assemble into IgG2 Fc homodimers, IgG2 Fc/Fc-ShK heterodimers, and IgG2 Fc-ShK homodimers. The IgG2 Fc/Fc-ShK heterodimer (monovalent form) was isolated during purification using cation exchange chromatography.

[00574] IgG2 Fc-ShK[2-35]; IgG2 Fc Shk[2-35, Q16K];IgG2 Fc-Shk[1-35]; IgG2 Fc-ShK[1-35, Q16K] mammalian expression. DNA sequences coding for the immunoglobulin Fc domain of human IgG2:

[00575] MEWSWVFLFFLSVTTGVHSERKVECPPCPAPPVAGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNST FRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK// SEQ ID NO:1,

fused in-frame to a monomer of the Kv1.3 inhibitor peptide ShK[2-35] or a mutated ShK[2-35, Q16K] were constructed using standard PCR technology. The ShK[2-35] or ShK[2-35, Q16K] and the 10 amino acid linker portion of the molecule were generated in a PCR reaction using the original Fc-2xL-ShK[2-35] in pcDNA3.1(+)CMVi as a template (see Sullivan et al., WO 2008/088422 A2, Example 2, Figure 15A-B therein). The ShK[1-35] was generated in a PCR reaction using the original Fc-2xL-ShK[1-35] in pcDNA3.1(+)CMVi as a template (Sullivan et al., WO 2008/088422 A2, Example 1, Figure 14A-B therein). These ShK

constructs have the following modified VH21 Signal peptide amino acid sequence of MEWSWVFLFFLSVTTGVHS// SEQ ID NO:2 generated from a pSelexis-Vh21-hIgG2-Fc template with the following oligos:

[00576] 5'- CAT GAA TTC CCC ACC ATG GAA TGG AGC TGG -3' (SEQ ID NO:3); and

[00577] 5'- CA CGG TGG GCA CTC GAC TTT GCG CTC GGA GTG GAC ACC -3' (SEQ ID NO:4).

[00578] Wild Type ShK[2-35] with N-terminal linker extension (amino acid sequence GGGGSGGGGSSCIDTIPKSRCTAFQCKHSMKYRLSFCRKTCGTC// SEQ ID NO:6) was encoded by the DNA sequence below: GGAGGAGGAGGATCCGGAGGAGGAGGAAGCAGCTGCATCGACACCATC CCCAAGAGCCGCTGCACCGCCTTCCAGTGCAAGCACAGCATGAAGTACC GCCTGAGCTTCTGCCGCAAGACCTGCGGCACCTGC// SEQ ID NO:5. A fragment containing this coding sequence (SEQ ID NO:5) was generated using the oligos below (SEQ ID NO:7 and SEQ ID NO:8)-and the original Fc-L10-ShK[2-35] in pcDNA3.1(+)CMVi as a template (Sullivan et al., WO 2008/088422 A2, Example 2, Figure 15A-B therein, incorporated by reference):

[00579] 5'-GTC CAC TCC GAG CGC AAA GTC GAG TGC CCA CCG TGC C-3' (SEQ ID NO:7); and

[00580] 5'- TCC TCC TCC TTT ACC CGG AGA CAG GGA GAG -3'// (SEQ ID NO:8).

[00581] Mutant ShK[2-35, Q16K] was generated using site directed mutagenesis with Stratagene's QuikChange Multi site-Directed Mutagenesis kit cat# 200531 per the manufacterer's instruction. Oligos used to generate the mutagenesis were:

[00582] 5'-GCT GCA CCG CCT TCA AGT GCA AGC ACA GC 3' (SEQ ID NO:9); and

[00583] 5'- GCT GTG CTT GCA CTT GAA GGC GGT GCA GC -3' (SEQ ID NO:10); and using the original Fc-L10-ShK[2-35] in pcDNA3.1(+)CMVi as a template (Sullivan et al., WO 2008/088422 A2, Example 2, Figure 15A-B therein) resulting in the DNA coding sequence

[00584] Ggaggaggaggatccggaggaggaggaagcagctgcatcgacaccatccccaagagccgctgcacc gccttcaagtgcaagcacagcatgaagtaccgcctgagcttctgccgcaagacctgcggcacctgc// (SEQ ID NO:11), which encodes the amino acid sequence Shk(2-35, K16) with a N-terminal linker extension:

[00585] ggggsggggsscidtipksrctafkckhsmkyrlsfcrktcgtc// SEQ ID NO:12).

[00586] ShK[1-35]WT fragment was generated using the original Fc-2xL-ShK[1-35] in pcDNA3.1(+)CMVi as a template (Sullivan et al., WO 2008/088422 A2, Example 1, Figure 14A-B therein) and oligos:

[00587] 5'-GTC CAC TCC GAG CGC AAA GTC GAG TGC CCA CCG TGC C-3' (SEQ ID NO:7); and

[00588] 5'- TCC TCC TCC TTT ACC CGG AGA CAG GGA GAG -3' (SEQ ID NO:8).

[00589] The IgG2Fc region was generated using oligos:

[00590] 5'-CCG GGT AAA GGA GGA GGA GGA TCC GGA G-3' (SEQ ID NO:13); and

[00591] 5'- CAT GCG GCC GCT CAT TAG CAG GTG -3' (SEQ ID NO:14), and the pSelexis Vh21-hIgG2-Fc template resulting in a fragment containing the following DNA coding sequence:

[00592] gcaccacctgtggcaggaccgtcagtcttcctcttccccccaaaacccaaggacaccctcatgatctcccg gacccctgaggtcacgtgcgtggtggtggacgtgagccacgaagaccccgaggtccagttcaactggtacgtggacgg cgtggaggtgcataatgccaagacaaagccacgggaggagcagttcaacagcacgttccgtgtggtcagcgtcctcac cgttgtgcaccaggactggctgaacggcaaggagtacaagtgcaaggtctccaacaaaggcctcccagcccccatcga

gaaaaccatctccaaaaccaaagggcagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatga
ccaagaaccaggtcagcctgacctgcctggtcaaaggcttctaccccagcgacatcgccgtggagtgggagagcaatg
ggcagccggagaacaactacaagaccacacctcccatgctggactccgacggctccttcttcctctacagcaagctcacc
gtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgc
agaagagcctctccctgtctccgggtaaa // SEQ ID NO:15, which encodes the amino acid sequence
appvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvlt
vvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlppsreemtknqvsltclvkgfypsdiavewes
ngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk SEQ ID NO:16).

[00593] The PCR fragments were generated and the products were run out on a gel. After gel purification, the DNA fragments were put together in a PCR tube and sewn together with outside primers:

[00594] 5'- CAT GAA TTC CCC ACC ATG GAA TGG AGC TGG -3' (SEQ ID NO:3); and

[00595] 5'- CAT GCG GCC GCT CAT TAG CAG GTG -3' (SEQ ID NO:14).

[00596] The PCR products were digested with EcoRI and NotI (Roche) restriction enzymes and agarose gel purified by Gel Purification Kit. At the same time, the pTT14 vector (an Amgen vector containing a CMV promoter, Poly A tail and a Puromycin resistance gene) was digested with EcoRI and NotI restriction enzymes and the large fragment was purified by Gel Purification Kit. Each purified PCR product was ligated to the large fragment and transformed into OneShot Top10 bacteria. DNAs from transformed bacterial colonies were isolated and subjected to EcoRI and NotI restriction enzyme digestions and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. Although, analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone of each construct was selected for large scaled plasmid purification. The final pTT14-VH1SP-IgG2-Fc construct encoded IgG2-Fc-L10-ShK(2-35) fusion polypeptide having the following sequence:

[00597] Mewswvflfflsvttgvhserkvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshe dpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqpr epqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqg nvfscsvmhealhnhytqkslslspgkggggsggggsscidtipksrctafqckhsmkyrlsfcrktcgtc// (SEQ ID NO:17).

[00598] The pTT14-VH21SP-IgG2-Fc-L10-ShK(2-35,Q16K) construct encoded a IgG2-Fc L10-ShK(2-35, Q16K) fusion polypeptide sequence:

[00599] Mewswvflfflsvttgvhserkvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshe dpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqpr epqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqg nvfscsvmhealhnhytqkslslspgkggggsggggsscidtipksrctafKckhsmkyrlsfcrktcgtc// SEQ ID NO:18;

[00600] and pTT14-VH21SP-IgG2-Fc ShK1-35 construct contained a coding sequence for IgG2 Fc-L10-ShK(1-35) fusion polypeptide having the following sequence:

[00601] mewswvflfflsvttgvhserkvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshe dpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqpr epqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqg nvfscsvmhealhnhytqkslslspgkggggsggggsrscidtipksrctafqckhsmkyrlsfcrktcgtc// (SEQ ID NO:19).

[00602] Generating the VH21SP-IgG2-Fc-only construct in pYD16 (an Amgen vector containing a CMV promoter, Poly A tail and a Hygromycin resistance gene) occurred as follows: The VH21 signal peptide was generated using the following oligos:

[00603] 5'-CAT AAG CTT CCC ACC ATG GAA TGG AGC TGG-3' (SEQ ID NO:20); and

[00604] 5'- CA CGG TGG GCA CTC GAC TTT GCG CTC GGA GTG GAC ACC -3' (SEQ ID NO:4), and using the pSelexis template as noted above.

[00605] The Fc region was generated using the pSelexis template described above and following oligos:

[00606] 5'-GTC CAC TCC GAG CGC AAA GTC GAG TGC CCA CCG TGC C-3' (SEQ ID NO:7); and

[00607] 5'- CAT GGA TCC TCA TTT ACC CGG AGA CAG GGA G -3' (SEQ ID NO:21).

[00608] The PCR fragments were gel purified and sewn together in single PCR reaction using outside primers GGT TGA GAG GTG CCA GAT GTC AGG GCT GCA GCA GCG GC// SEQ ID NO:391 and CAG CTG CAC CTG ACC ACC ACC TCC ACC GCT ATG CTG AGC GCG// SEQ ID NO:392. The resulting PCR fragment was gel purified, and digested by HindIII and BamHI. Concurrently, pYD16 vector (an Amgen vector containing a CMV promoter, Poly A tail and a Hygromycin resistance gene) was also cut by HindIII and BamHI and the large vector fragment was purified by Qiagen's Gel Purification Kit. The purified PCR product was ligated to the large fragment and transformed into OneShot Top10 bacteria. DNA from transformed bacterial colonies were isolated and subjected to HindIII and BamHI restriction enzyme digestions and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. Although, analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final pYD16-VH21SP-IgG2-Fc construct encoded human IgG2-Fc (SEQ ID NO:1 above).

[00609] IgG2-Fc ShK[1-35, Q16K] mammalian expression. Using the DNA pTT5-aKLH120.6-VK1SP-IgG2-HC-L10-ShK[1-35, Q16K] construct, the fragment containing the DNA coding sequence

[00610] ggatccggaggaggaggaagccgcagctgcatcgacaccatccccaagagccgctgcaccgccttca agtgcaagcacagcatgaagtaccgcctgagcttctgccgcaagacctgcggcacctgctaatgagcggccgctcgag gccggcaaggccggatcc// (SEQ ID NO:22)

[00611] was cut out using BamHI/BamHI. This coding sequence (SEQ ID NO:23) encodes ShK(1-35, Q16K) with an N-terminal linker sequence: GSGGGGSRSCIDTIPKSRCTAFKCKHSMKYRLSFCRKTCGTC// (SEQ ID NO:23).

[00612] At the same time, pTT14-hIgG2-Fc-ShK[1-35]WT construct, was also digested by BamHI/BamHI, thereby removing the Shk[1-35] coding region to yield the coding sequence

[00613] Atggaatggagctgggtctttctcttcttcctgtcagtaacgactggtgtccactccgagcgcaaagtcga gtgcccaccgtgcccagcaccacctgtggcaggaccgtcagtcttcctcttccccccaaaacccaaggacaccctcatga tctcccggacccctgaggtcacgtgcgtggtggtggacgtgagccacgaagaccccgaggtccagttcaactggtacgt ggacggcgtggaggtgcataatgccaagacaaagccacgggaggagcagttcaacagcacgttccgtgtggtcagcg tcctcaccgttgtgcaccaggactggctgaacggcaaggagtacaagtgcaaggtctccaacaaaggcctcccagcccc catcgagaaaaccatctccaaaaccaaagggcagccccgagaaccacaggtgtacaccctgcccccatcccgggagg agatgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctaccccagcgacatcgccgtggagtgggaga gcaatgggcagccggagaacaactacaagaccacacctcccatgctggactccgacggctccttcttcctctacagcaa gctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccac tacacgcagaagagcctctccctgtctccgggtaaaggaggagga // (SEQ ID NO:24), encoding the amino acid sequence mewswvflfflsvttgvhserkvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnw yvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlpps reemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmh ealhnhytqkslslspgkggg// (SEQ ID NO:25).

[00614] The pTT14-hIgG2-Fc vector with the ShK removed was treated with Calf Intestine Phosphatase (CIP) to remove the 5' Phosphate group and Phenol/Chloroform extracted to prevent religation of the vector upon itself. The insert ShK[1-35, Q16K] fragment was gel purified away from its vector and cleaned

up with Qiagen Gel Purification Kit. . The purified insert was ligated to the large vector fragment and transformed into OneShot Top10 bacteria. DNAs from transformed bacterial colonies were isolated and subjected to BamHI restriction enzyme digestion and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. Although, analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final pTT14-IgG2-Fc-ShK[1-35, Q16K] construct encoded the following IgG2 Fc-L10-ShK(1-35, Q16K) fusion protein sequence:

[00615] mewswvflfflsvttgvhserkvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshe dpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqpr epqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqg nvfscsvmhealhnhytqkslslspgkggggsggggsrscidtipksrctafkckhsmkyrlsfcrktcgtc// (SEQ ID NO:26).

[00616] The amino acid sequence for IgG2 Fc-L10-ShK(1-35) is:

[00617] mewswvflfflsvttgvhserkvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshe dpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqpr epqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqg nvfscsvmhealhnhytqkslslspgkggggsggggsrscidtipksrctafqckhsmkyrlsfcrktcgtC// (SEQ ID NO:30).

[00618] The desired aKLH IgG2/Fc-ShK product contained one copy of each of components (a)-(c), immediately above, configured as in Figure 1E. Because of this, the ratio was 1:1:1. This product can be described as half antibody and half Fc fusion ("hemibody"), coupled together at the Fc domain. Additional peptide assemblies that had to be removed from the culture were the aKLH Ab and the Fc-ShK homodimer.

[00619] The ShK[1-35]WT fragment was generated using the original Fc-L10-ShK[1-35] in pcDNA3.1(+)CMVi as a template (described in Example 1, Figure

14A-14B in Sullivan et al., Toxin Peptide Therapeutic Agents, PCT/US2007/022831, published as WO 2008/088422, which is incorporated herein by reference in its entirety) and the oligos:

[00620] 5'-TCC CTG TCT CCG GGT GGA GGA GGA GGA TCC GGA G-3' (SEQ ID NO:47); and 5'- CAT GCG GCC GCT CAT TAG CAG GTG -3' (SEQ ID NO:14).

[00621] The PCR products were run on a 1% agarose gel. The bands were punched for an agarose plug and the plugs were placed in a fresh PCR reaction tube. The agarose plugs were then amplified by PCR using the outside primers SEQ ID NO:357 and SEQ ID NO:330. The PCR product was then digested by XbaI and NotI and PCR clean up kit (Qiagen) purified. At the same time, pTT5 Vector (an Amgen vector containing a CMV promoter and Poly A tail) was cut by XbaI and NotI. The pTT5 vector was run out on a 1% agarose gel and the larger fragment was cut out and gel purified by Qiagen's Gel Purification Kit. The purified PCR product was ligated to the large vector fragment and transformed into OneShot Top10 bacteria. DNAs from transformed bacterial colonies were isolated and subjected to XbaI and NotI restriction enzyme digestions and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. Although, analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final pTT5-aKLH 120.6-VK1SP-IgG2-HC-L10-ShK[1-35] construct encoded an IgG2-HC-L10-ShK[1-35] fusion polypeptide with the amino acid sequence:

[00622] Mdmrvpaqllgllllwlrgarcqvqlvqsgaevkkpgasvkvsckasgytftgyhmhwvrqapgq
glewmgwinpnsggtnyaqkfqgrvtmtrdtsistaymelsrlrsddtavyycardrgsyywfdpwgqgtlvtvss
astkgpsvfplapcsrstsestaalgclvkdyfpepvtvswnsgaltsgvhtfpavlqssglyslssvvtvpssnfgtqty
tcnvdhkpsntkvdktverkccvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnw
yvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlpps
reemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmh

ealhnhytqkslslspgggggsggggsrscidtipksrctafqckhsmkyrlsfcrktcgtc// (SEQ ID NO:48).

[00623] To generate the ShK[1-35, Q16K] mutant version of this construct, site-directed mutagenesis was performed using the Stratagene Quikchange Multi site Directed Mutagenesis Kit (Cat#200531), per manufacturer's instructions, and oligos:

[00624] 5'-GCT GCA CCG CCT TCA AGT GCA AGC ACA GC 3' (SEQ ID NO:9); and

[00625] 5'- GCT GTG CTT GCA CTT GAA GGC GGT GCA GC -3' (SEQ ID NO:10). The final construct pTT5-aKLH120.6-VK1SP-IgG2-HC-L10-ShK[1-35, Q16K] encoded IgG2-HC-L10-ShK[1-35, Q16K] fusion polypeptide with the following amino acid sequence:

[00626] Mdmrvpaqllgllllwlrgarcqvqlvqsgaevkkpgasvkvsckasgytftgyhmhwvrqapgq glewmgwinpnsggtnyaqkfqgrvtmtrdtsistaymelsrlrsddtavyycardrgsyywfdpwgqgtlvtvss astkgpsvfplapcsrstsestaalgclvkdyfpepvtvswnsgaltsgvhtfpavlqssglyslssvvtvpssnfgtqty tcnvdhkpsntkvdktverkccvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnw yvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlpps reemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmh ealhnhytqkslslspgggggsggggsrscidtipksrctafkckhsmkyrlsfcrktcgtc// (SEQ ID NO:49).

[00627] aKLH-IgG2 Heavy Chain-L10-ShK[2-35, Q16K] mammalian expression. Using DNA construct pTT5-aKLH 120.6-VK1SP-IgG2-HC-L10-ShK[1-35] as the vector, the ShK[1-35] was cut out using BamHI/BamHI. The vector fragment from pTT5-aKLH 120.6-VK1SP-IgG2-HC without ShK[1-35] contained the coding sequence:

[00628] atggacatgagggtgcccgctcagctcctggggctcctgctgctgtggctgagaggtgccagatgtcag gtgcagctggtgcagtctggggctgaggtgaagaagcctggggcctcagtgaaggtctcctgcaaggcttctggataca ccttcaccggctaccacatgcactgggtgcgacaggcccctggacaagggcttgagtggatgggatggatcaaccctaa

cagtggtggcacaaactatgcacagaagtttcagggcagggtcaccatgaccagggacacgtccatcagcacagccta
catggagctgagcaggctgagatctgacgacacggccgtgtattactgtgcgagagatcgtgggagctactactggttcg
acccctggggccagggaaccctggtcaccgtctcctcagcctccaccaagggcccatcggtcttcccctggcgccctg
ctccaggagcacctccgagagcacagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtc
gtggaactcaggcgctctgaccagcggcgtgcacaccttcccagctgtcctacagtcctcaggactctactccctcagca
gcgtggtgaccgtgccctccagcaacttcggcacccagacctacacctgcaacgtagatcacaagcccagcaacacca
aggtggacaagacagttgagcgcaaatgttgtgtcgagtgcccaccgtgcccagcaccacctgtggcaggaccgtcagt
cttcctcttcccccaaaacccaaggacaccctcatgatctcccggaccctgaggtcacgtgcgtggtggtggacgtga
gccacgaagaccccgaggtccagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccacgg
gaggagcagttcaacagcacgttccgtgtggtcagcgtcctcaccgttgtgcaccaggactggctgaacggcaaggagt
acaagtgcaaggtctccaacaaaggcctcccagcccccatcgagaaaaccatctccaaaaccaaagggcagccccga
gaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaaccaggtcagcctgacctgcctggtcaaa
ggcttctaccccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacacctcc
catgctggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtc
ttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtggaggagg
a // (SEQ ID NO:50),

[00629] encoding the amino acid sequence

[00630] mdmrvpaqllgllllwlrgarcqvqlvqsgaevkkpgasvkvsckasgytftgyhmhwvrqapgq
glewmgwinpnsggtnyaqkfqgrvtmtrdtsistaymelsrlrsddtavyycardrgsyywfdpwgqgtlvtvss
astkgpsvfplapcsrstsestaalgclvkdyfpepvtvswnsgaltsgvhtfpavlqssglyslssvvtvpssnfgtqty
tcnvdhkpsntkvdktverkccvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnw
yvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlpps
reemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmh
ealhnhytqkslslspgggg// (SEQ ID NO:51).

[00631] The vector fragment was then treated with Calf Intestine Phosphatase (CIP) to remove the 5' Phosphate group and Phenol/Chloroform extracted to prevent religation of the vector upon itself. The insert came from pTT14-VH21SP-IgG2-Fc-ShK[2-35, Q16K] encoding IgG2 Fc-L10-ShK(2-35, Q16K):

[00632] mewswvflfflsvttgvhserkvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgkggggsggggsscidtipksrctafkckhsmkyrlsfcrktcgtc// (SEQ ID NO:18),

[00633] and the insert was also digested out using BamHI/BamHI. The insert ShK[2-35, Q16K] fragment was gel purified away from its vector and cleaned up with Qiagen Gel Purification Kit. A purified DNA insert containing the coding sequence

[00634] gga tcc gga gga gga gga agc agc tgc atc gac acc atc ccc aag agc cgc tgc acc gcc ttc aag tgc aag cac agc atg aag tac cgc ctg agc ttc tgc cgc aag acc tgc ggc acc tgc taa tga // (SEQ ID NO:52),

[00635] encoding the amino acid sequence GSGGGGSSCIDTIPKSRCTAFKCKHSMKYRLSFCRKTCGTC (SEQ ID NO:53), was ligated to the large vector fragment and transformed into OneShot Top10 bacteria. DNAs from transformed bacterial colonies were isolated and subjected to BamHI restriction enzyme digestion and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. Although, analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final construct pTT5-aKLH-IgG2 HC-L10-ShK[2-35,Q16K] encoded an IgG2 HC-L10-ShK[2-35,Q16K] fusion polypeptide:

[00636] Mdmrvpaqllgllllwlrgarcqvqlvqsgaevkkpgasvkvsckasgytftgyhmhwvrqapgqglewmgwinpnsggtnyaqkfqgrvtmtrdtsistaymelsrlrsddtavyycardrgsyywfdpwgqgtlvtvssastkgpsvfplapcsrstsestaalgclvkdyfpepvtvswnsgaltsgvhtfpavlqssglyslssvvtvpssnfgtqtytcnvdhkpsntkvdktverkccvecppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgggggsggggsscidtipksrctafkckhsmkyrlsfcrktcgtc// (SEQ ID NO:54).

[00637] VH21SP-N-terminus ShK[1-35] Wild Type-IgG1-Fc mammalian expression. A DNA sequence coding for a monomer of the Kv1.3 inhibitor peptide ShK[1-35] fused in-frame to the N-terminal Fc region of human IgG1 was constructed as described below.

[00638] For construction of VH21 SP-ShK(1-35)-L10-IgG1 Fc expression vector, a PCR strategy was employed to generate the VH21 signal peptide ShK(1-35) gene linked to a four glycine and one serine amino acid flanked by HindIII and BamHI restriction sites and a four glycine and one serine amino acid linked to IgG1 Fc fragment flanked by BamHI and NotI restriction sites was generated in a PCR reaction using the Fc-L10-OSK1in pcDNA3.1(+)CMVi as a template (described in Example 41 and Figure 42A-B of Sullivan et al., WO 2008/088422A2, incorporated by reference).

[00639] To generate VH21 SP-ShK(1-35)-G_4S, two oligos with the sequence as depicted below were used in a PCR reaction with PfuTurbo HotStart DNA polymerase (Stratagene) at 95°C-30sec, 55°C-30sec, 75°C-45sec for 35 cycles; HindIII (aagctt) and BamHI (ggatcc) restriction sites are underlined:

[00640] Forward primer:
tgcagaagcttctagaccaccatggaatggagctgggtctttctcttcttcctgtcagtaacgactggtgtccactcccgcag ctgcatcgacaccatccccaagagccgctgcaccgccttccagt// (SEQ ID NO:55); and

[00641] Reverse primer:

[00642] Ctccggatcctcctcctccgcaggtgccgcaggtcttgcggcagaagctcaggcggtacttcatgctgtg cttgcactggaaggcggtgcagcggctcttggggatggtgtcgat// (SEQ ID NO:56).

[00643] The resulting PCR products were resolved as the 202bp bands on a two percent agarose gel. The 202bp PCR product was purified using PCR Purification Kit (Qiagen), then digested with HindIII and BamHI (Roche) restriction enzymes, and agarose gel was purified by Gel Extraction Kit (Qiagen).

[00644] To generate G_4S-IgG1 Fc, two oligos with the sequence as depicted below were used in a PCR reaction with PfuTurbo HotStart DNA polymerase (Stratagene) at 95°C-30sec, 55°C-30sec, 75°C-1min for 30 cycles; BamHI (ggatcc) and NotI (gcggccgc) restriction sites are underlined:

[00645] Forward primer:

[00646] gtaggatccggaggaggaggaagcgacaaaactcacac// (SEQ ID NO:57); and

[00647] Reverse primer:

[00648] Cgagcggccgcttactatttacccggagacaggga// (SEQ ID NO:58).

[00649] The resulting PCR products were resolved as the 721-bp bands on a one percent agarose gel. The 721-bp PCR product was purified using PCR Purification Kit (Qiagen), then digested with BamHI and NotI (Roche) restriction enzymes, and agarose gel was purified by Gel Extraction Kit (Qiagen).

[00650] The pcDNA3.1(+)CMVi-Fc-L10-OSK1 vector was digested with BamHI and NotI restriction enzymes and the large fragment was purified by Gel Extraction Kit. The gel purified 4GS-IgG1 Fc fragment was ligated to the purified large fragment and transformed into One Shot® Top10 (Invitrogen) to create a pCMVi-Fc-L10-IgG1 Fc vector. Subsequently, pCMVi-Fc-L10-IgG1 Fc vector was digested with HindIII and BamHI restriction enzymes and the large fragment was purified by Gel Extraction Kit. The gel purified VH21 SP-ShK(1-35)-4GS fragment was ligated to the purified large fragment and transformed into One Shot® Top10 (Invitrogen) resulting in a pCMVi-VH21 SP-ShK(1-35)-L10-IgG1 Fc construct. DNAs from transformed bacterial colonies were isolated and digested with BamHI and NotI restriction enzymes and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. Although, analysis of several sequences of clones yielded a 100% percent match with the above sequences, only one clone from each gene was selected for large scaled plasmid purification. The DNA from VH21 SP-ShK(1-35)-L10-IgG1 Fc in pCMVi vector was resequenced to

confirm the Fc and linker regions and the sequence was 100% identical to the above sequence. Fragment VH21 SP-ShK(1-35)-L10-IgG1 Fc contained the coding sequence

[00651] atggaatggagctgggtctttctcttcttcctgtcagtaacgactggtgtccactcccgcagctgcatcgaca ccatccccaagagccgctgcaccgccttccagtgcaagcacagcatgaagtaccgcctgagcttctgccgcaagacctg cggcacctgcggaggaggaggatccggaggaggaggaagcgacaaaactcacacatgcccaccgtgcccagcacct gaactcctgggggggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctcccggacccctgaggt cacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggaggtgca taatgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcacca ggactggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctc caaagccaaagggcagccccgagaaccacaggtgtacaccctgcccccatcccgggatgagctgaccaagaaccag gtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggag aacaactacaagaccacgcctcccgtgctggactccgacggctccttcttcctctacagcaagctcaccgtggacaagag caggtggcagcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctc tccctgtctccgggtaaatagtaa// (SEQ ID NO:59),

[00652] encoding VH21 SP-ShK(1-35)-L10-IgG1 Fc amino acid sequence mewswvflfflsvttgvhsrscidtipksrctafqckhsmkyrlsfcrktcgtcggggsggggsdkthtcppcpapell ggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkfnwyvdgvevhnaktkpreeqynstyrvvsvltvlhq dwlngkeykckvsnkalpapiektiskakgqprepqvytlppsrdeltknqvsltclvkgfypsdiavewesngqpe nnykttppvldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk// (SEQ ID NO:60).

[00653] Mammalian expression of N-terminus ShK[1-35, Q16K]-aKLH HC; and N-terminus ShK[1-35Q16K]-aKLH LC. Using a construct encoding N-terminus ShK[1-35]Wild Type-L10-IgG1-Fc, site directed mutagenesis was performed using the following oligos to produce a Q16K mutation in the ShK region:

[00654] 5'-GCT GCA CCG CCT TCA AGT GCA AGC ACA GC-3'// (SEQ ID NO:9); and

[00655] 5'- GCT GTG CTT GCA CTT GAA GGC GGT GCA GC -3' (SEQ ID NO:10).

[00656] The Stratagene QuikChange Multi Site Directed Mutagenesis Kit was used according to the manufacturer's instructions. The final construct for pCMVi-N-terminus-ShK[1-35Q16K]-L10-IgG1-Fc encoded the following Signal peptide (VH21 SP)-ShK[1-35, Q16K]-L10-IgG1-Fc fusion polypeptide:

[00657] Mewswvflfflsvttgvhsrscidtipksrctafkckhsmkyrlsfcrktcgtcggggsggggsdktht cppcpapellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkfnwyvdgvevhnaktkpreeqynstyr vvsvltvlhqdwlngkeykckvsnkalpapiektiskakgqprepqvytlppsrdeltknqvsltclvkgfypsdiav ewesngqpennykttppvldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk// (SEQ ID NO:61).

[00658] To generate the N-terminus ShK[1-35, Q16K]-aKLH HC construct, a PCR product containing the Signal peptide-ShK[1-35Q16K]-L10 linker was produced using the following oligos:

[00659] 5'-CAT TCT AGA CCA CCA TGG AAT GG-3' (SEQ ID NO:62);

[00660] 5'- CAG CTG CAC CTG GCT TCC TCC TCC TCC GG -3' (SEQ ID NO:63);

[00661] and template pCMVi-N-terminus-ShK[1-35, Q16K]-L10-IgG1-Fc, resulted in a fragment containing the coding sequence atggaatggagctgggtctttctcttcttcctgtcagtaacgactggtgtccactcccgcagctgcatcgacaccatccccaa gagccgctgcaccgccttcaagtgcaagcacagcatgaagtaccgcctgagcttctgccgcaagacctgcggcacctg cggaggaggaggatccggaggaggaggaagc// (SEQ ID NO:64),

[00662] encoding the VH21 SP-ShK(1-35, Q16K)-L10 amino acid sequence MEWSWVFLFFLSVTTGVHSRSCIDTIPKSRCTAFKCKHSMKYRLSFCRKTCG TCGGGGSGGGGS// (SEQ ID NO:65).

[00663] To generate the aKLH-HC fragment, a PCR product was created using oligos:

[00664] 5'-GGA GGA GGA AGC CAG GTG CAG CTG GTG CAG-3' (SEQ ID NO:66);

[00665] 5'- CAT GCG GCC GCT CAT TTA CCC -3' (SEQ ID NO:67);

[00666] and template pTT5-aKLH 120.6-HC, resulting in a DNA fragment containing the coding sequence

[00667] caggtgcagctggtgcagtctggggctgaggtgaagaagcctggggcctcagtgaaggtctcctgcaa ggcttctggatacaccttcaccggctaccacatgcactgggtgcgacaggcccctggacaagggcttgagtggatggga tggatcaaccctaacagtggtggcacaaactatgcacagaagtttcagggcagggtcaccatgaccagggacacgtcca tcagcacagcctacatggagctgagcaggctgagatctgacgacacggccgtgtattactgtgcgagagatcgtgggag ctactactggttcgacccctggggccagggaaccctggtcaccgtctcctcagcctccaccaagggcccatcggtcttcc ccctggcgccctgctccaggagcacctccgagagcacagcggccctgggctgcctggtcaaggactacttccccgaac cggtgacggtgtcgtggaactcaggcgctctgaccagcggcgtgcacaccttcccagctgtcctacagtcctcaggactc tactccctcagcagcgtggtgaccgtgccctccagcaacttcggcacccagacctacacctgcaacgtagatcacaagc ccagcaacaccaaggtggacaagacagttgagcgcaaatgttgtgtcgagtgcccaccgtgcccagcaccacctgtgg caggaccgtcagtcttcctcttccccccaaaacccaaggacaccctcatgatctcccggacccctgaggtcacgtgcgtg gtggtggacgtgagccacgaagaccccgaggtccagttcaactggtacgtggacggcgtggaggtgcataatgccaag acaaagccacgggaggagcagttcaacagcacgttccgtgtggtcagcgtcctcaccgttgtgcaccaggactggctga acggcaaggagtacaagtgcaaggtctccaacaaaggcctcccagcccccatcgagaaaaccatctccaaaaccaaag ggcagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaaccaggtcagcctgacc tgcctggtcaaaggcttctaccccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaag accacacctcccatgctggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagca ggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgg gtaaatga// (SEQ ID NO:68),

[00668] encoding amino acid sequence qvqlvqsgaevkkpgasvkvsckasgytftgyhmhwvrqapgqglewmgwinpnsggtnyaqkfqgrvtmtr dtsistaymelsrlrsddtavyycardrgsyywfdpwgqgtlvtvssastkgpsvfplapcsrstsestaalgclvkdyf pepvtvswnsgaltsgvhtfpavlqssglyslssvvtvpssnfgtqtytcnvdhkpsntkvdktverkccvecppcpa ppvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltv vhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlppsreemtknqvsltclvkgfypsdiavewesn

gqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk// (SEQ ID NO:69).

[00669] The two PCR products were run out on a gel and the appropriate sized band was punched for an agarose plug. The agarose plugs were placed in a single new PCR reaction, and the fragments were sewn together using outer most primers (SEQ ID NO:62) and (SEQ ID NO:67). The PCR fragment was cut using XbaI and NotI and cleaned with Qiagen PCR Cleanup Kit. At the same time, pTT5 vector was also cut by XbaI and NotI and gel purified. The purified insert was ligated to the large vector fragment and transformed into OneShot Top10 bacteria. DNAs from transformed bacterial colonies were isolated and subjected to XbaI and NotI restriction enzyme digestions and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. Although, analysis of several sequences of clones yielded a 100% percent match with the above sequence, only one clone was selected for large scaled plasmid purification. The final construct pTT5-N-terminus ShK[1-35Q16K]-L10-aKLH120.6-HC encoded a VH21 SP-ShK[1-35, Q16K]-L10-aKLH120.6-HC fusion polypeptide:

[00670] Mewswvflfflsvttgvhsrscidtipksrctafkckhsmkyrlsfcrktcgtcggggsggggsqvqlv qsgaevkkpgasvkvsckasgytftgyhmhwvrqapgqglewmgwinpnsggtnyaqkfqgrvtmtrdtsista ymelsrlrsddtavyycardrgsyywfdpwgqgtlvtvssastkgpsvfplapcsrstsestaalgclvkdyfpepvtv swnsgaltsgvhtfpavlqssglyslssvvtvpssnfgtqtytcnvdhkpsntkvdktverkccvecppcpappvag psvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnwyvdgvevhnaktkpreeqfnstfrvvsvltvvhqdw lngkeykckvsnkglpapiektisktkgqprepqvytlppsreemtknqvsltclvkgfypsdiavewesngqpen nykttppmldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk// (SEQ ID NO:70).

[00671] Lastly, the N-terminus-ShK[1-35, Q16K]-L10-aKLH120.6 Light Chain (LC) was generated in the same manner as above. A PCR product containing the signal peptide-ShK[1-35, Q16K]-L10 was created using oligos:

[00672] 5'-CAT TCT AGA CCA CCA TGG AAT GG-3' (SEQ ID NO:62); and

[00673] 5'- CAT CTG GAT GTC GCT TCC TCC TCC TCC GG -3' (SEQ ID NO:71);

[00674] and template pCMVi-N-terminus-ShK[1-35Q16K]-L10-IgG1-Fc, resulting in a DNA fragment containing the coding sequence atggaatggagctgggtctttctcttcttcctgtcagtaacgactggtgtccactcccgcagctgcatcgacaccatccccaa gagccgctgcaccgccttcaagtgcaagcacagcatgaagtaccgcctgagcttctgccgcaagacctgcggcacctg cggaggaggaggatccggaggaggaggaagc// (SEQ ID NO:64),

[00675] encoding the amino acid sequence for a signal peptide (VH21 SP)-ShK(1-35, Q16K)-L10 linker:

[00676] mewswvflfflsvttgvhsrscidtipksrctafkckhsmkyrlsfcrktcgtcggggsggggs// (SEQ ID NO:65).

[00677] Using template and oligos:

[00678] 5'-GGA GGA GGA AGC GAC ATC CAG ATG ACC CAG TC-3' (SEQ ID NO:72); and

[00679] 5'- CAT CTC GAG CGG CCG CTC AAC -3' (SEQ ID NO:73).

[00680] The resulting cloned PCR fragment contained the coding sequence atggaatggagctgggtctttctcttcttcctgtcagtaacgactggtgtccactcccgcagctgcatcgacaccatccccaa gagccgctgcaccgccttcaagtgcaagcacagcatgaagtaccgcctgagcttctgccgcaagacctgcggcacctg cggaggaggaggatccggaggaggaggaagcgacatccagatgacccagtctccatcctccctgtctgcatctgtagg agacagagtcaccatcacttgccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaaccagggaaa gcccctaaacgcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcagcggcagtggatctgggac agaattcactctcacaatcagcagcctgcagcctgaagattttgcaacttattactgtctacagcataatagttacccgctcac tttcggcggagggaccaaggtggagatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagca gttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaagtacagtggaaggtggat aacgccctccaatcgggtaactcccaggagagtgtcacagagcaggacagcaaggacagcacctacagcctcagcag caccctgacgctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaagtcacccatcagggcctgagctc gcccgtcacaaagagcttcaacaggggagagtgttga// (SEQ ID NO:74) was generated,

[00681] encoding the amino acid sequence for N-terminus VH21 SP-ShK[1-35, Q16K]-L10-aKLH120.6 Light Chain (LC) with an N-terminal signal peptide:

[00682] mewswvflfflsvttgvhsrscidtipksrctafkckhsmkyrlsfcrktcgtcggggsggggsdiqmt qspsslsasvgdrvtitcrasqgirndlgwyqqkpgkapkrliyaasslqsgvpsrfsgsgsgteftltisslqpedfatyy clqhnsypltfgggtkveikrtvaapsvfifppsdeqlksgtasvvcllnnfypreakvqwkvdnalqsgnsqesvte qdskdstyslsstltlskadyekhkvyacevthqglsspvtksfnrgec// (SEQ ID NO:75).

[00683] Both PCR fragments (DNA fragment containing the coding sequence (SEQ ID NO:64) and aKLH 120.6 Light Chain LC fragment containing the coding sequence (SEQ ID NO:74) were run out on a gel, and the appropriate sized band was punched for an agarose plug. The agarose plugs were placed in a single new PCR reaction, and the fragments were sewn together using outer most primers (SEQ ID NO:62) and (SEQ ID NO:73). The resulting PCR fragment was cut using XbaI and NotI and cleaned with Qiagen PCR Cleanup Kit.

[00684] At the same time, pTT14 vector (an Amgen vector containing a CMV promoter, Poly A tail and a Puromycin resistance gene) was also cut by XbaI and NotI and gel purified. The purified insert was ligated to the large vector fragment and transformed into OneShot Top10 bacteria. DNAs from transformed bacterial colonies were isolated and subjected to XbaI and NotI restriction enzyme digestions and resolved on a one percent agarose gel. DNAs resulting in an expected pattern were submitted for sequencing. The final construct pTT14-N-terminus ShK[1-35Q16K]-L10-aKLH120.6-LC encoding a Signal Peptide-ShK[1-35, Q16K]-L10-aKLH120.6-LC fusion polypeptide sequence (i.e., SEQ ID NO:75).

[00685] **Example 9**

[00686] **Purifications and Evaluation of Comparator Molecules: Monovalent Fc/Fc-L10-ShK[2-35] Heterodimers and Monovalent or Bivalent Fc/Fc-ShK(1-35 Q16K)(IgG2) Heterodimers and Other Polypeptide Molecules**

[00687] Monovalent or bivalent Fc-L10-ShK[2-35], monovalent or bivalent Fc-L10-ShK[1-35], monovalent or bivalent Fc-L10-ShK(1-35, Q16K), and other ShK-related polypeptide molecules molecules listed in Table 7B (in Example 5 herein), were expressed, isolated and purified by methods described herein. PEGylated and un-PEGylated toxin peptide comparators in Table 7B were prepared synthetically as follows:

[00688] Peptide Synthesis. N^{α}-Fmoc, side-chain protected amino acids and H-Cys(Trt)-2Cl-Trt resin were purchased from Novabiochem, Bachem, or Sigma Aldrich. The following side-chain protection strategy was employed: Asp(OtBu), Arg(Pbf), Cys(Trt), Glu(OtBu), His(Trt), Lys(N^{ε}-Boc), Ser(OtBu), Thr(OtBu) and Tyr(OtBu). ShK (RSCIDTIPKSRCTAFQCKHSMKYRLSFCRKTCGTC// SEQ ID NO:378), [Lys16]ShK (RSCIDTIPKSRCTAFKCKHSMKYRLSFCRKTCGTC// SEQ ID NO:76), or other toxin peptide analog amino acid sequences, were synthesized in a stepwise manner on an CS Bio peptide synthesizer by SPPS using DIC/HOBt coupling chemistry at 0.2 mmol equivalent scale using H-Cys(Trt)-2Cl-Trt resin (0.2 mmol, 0.32 mmol/g loading). For each coupling cycle, 1 mmol N^{α}-Fmoc-amino acid was dissolved in 2.5 mL of 0.4 M 1-hydroxybenzotriazole (HOBt) in N,N-dimethylformamide (DMF). To the solution was added 1.0 mL of 1.0 M N,N'-diisopropylcarbodiimide (DIC) in DMF. The solution was agitated with nitrogen bubbling for 15 min to accomplish pre-activation and then added to the resin. The mixture was shaken for 2 h. The resin was filtered and washed three times with DMF, twice with dichloromethane (DCM), and three times with DMF.

Fmoc deprotections were carried out by treatment with 20% piperdine in DMF (5 mL, 2 x 15 min). The first 23 residues were single coupled through repetition of the Fmoc-amino acid coupling and Fmoc removal steps described above. The remaining residues were double coupled by performing the coupling step twice before proceeding with Fmoc-removal.

[00689] Following synthesis, the resin was then drained, and washed sequentially with DCM, DMF, DCM, and then dried in vacuo. The peptide-resin was transferred to a 250-mL plastic round bottom flask. The peptide was deprotected and released from the resin by treatment with triisopropylsilane (1.5 mL), 3,6-dioxa-1,8-octane-dithiol (DODT, 1.5 mL), water (1.5 mL), trifluoroacetic acid (TFA, 20 mL), and a stir bar, and the mixture was stirred for 3 h. The mixture was filtered through a 150-mL sintered glass funnel into a 250-mL plastic round bottom flask. The mixture was filtered through a 150-mL sintered glass funnel into a 250-mL plastic round bottom flask, and the filtrate was concentrated in vacuo. The crude peptide was precipitated with the addition of cold diethyl ether, collected by centrifugation, and dried under vacuum.

[00690] Peptide Folding. The dry crude linear peptide (about 600 mg), for example [Lys16]ShK peptide (SEQ ID NO:76) or [Lys16]ShK-Ala (also known as [Lys16, Ala36]-ShK; SEQ ID NO:379) peptide, was dissolved in 16 mL acetic acid, 64 mL water, and 40 mL acetonitrile. The mixture was stirred rapidly for 15 min to complete dissolution. The peptide solution was added to a 2-L plastic bottle that contained 1700 mL of water and a large stir bar. To the thus diluted solution was added 20 mL of concentrated ammonium hydroxide to raise the pH of the solution to 9.5. The pH was adjusted with small amounts of acetic acid or NH_4OH as necessary. The solution was stirred at 80 rpm overnight and monitored by LC-MS. Folding was usually judged to be complete in 24 to 48 h, and the solution was quenched by the addition of acetic acid and TFA (pH = 2.5). The aqueous solution was filtered (0.45 μm cellulose membrane).

[00691] Reversed-Phase HPLC Purification. Reversed-phase high-performance liquid chromatography was performed on an analytical (C18, 5 μm, 0.46 cm × 25 cm) or a preparative (C18, 10 μm, 2.2 cm × 25 cm) column. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 90% aq. ACN containing 0.09% TFA) typically 5-95% over 35 min at a flow rate of 1 mL/min for analytical analysis and 5-65% over 90 min at 20 mL/min for preparative separations. Analytical and preparative HPLC fractions were characterized by ESMS and photodiode array (PDA) HPLC, combined and lyophilized.

[00692] Mass Spectrometry. Mass spectra were acquired on a single quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (25 μL) were injected into a moving solvent (10 μL/min; 30:50:20 ACN/MeOH containing 0.05% TFA) coupled directly to the ionization source via a fused silica capillary interface (50 μm i.d.). Sample droplets were ionized at a positive potential of 5 kV and entered the analyzer through an interface plate and subsequently through an orifice (100-120 μm diameter) at a potential of 60 V. Full scan mass spectra were acquired over the mass range 400-2200 Da with a scan step size of 0.1 Da. Molecular masses were derived from the observed m/z values.

[00693] PEGylation, Purification and Analysis. Peptide, e.g., [Lys16]ShK (SEQ ID NO:76) or [Lys16]ShK-Ala (SEQ ID NO:379), was selectively PEGylated by reductive alkylation at its N-terminus, using activated linear or branched PEG. Conjugation was performed at 2 mg/ml in 50 mM NaH_2PO_4, pH 4.5 reaction buffer containing 20mM sodium cyanoborohydride and a 2 molar excess of 20 kDa monomethoxy-PEG-aldehyde (NOF, Japan). Conjugation reactions were stirred for approximately 5 hrs at room temperature, and their progress was monitored by RP-HPLC. Completed reactions were quenched by 4-fold dilution with 20 mM NaOAc, pH 4 and chilled to 4°C. The PEG-peptides were then purified chromatographically at 40C; using SP Sepharose HP columns (GE Healthcare, Piscataway, NJ) eluted with linear 0-1M NaCl gradients in 20mM NaOAc, pH 4.0. Eluted peak fractions were analyzed by SDS-PAGE and RP-HPLC and pooling determined by purity

>97%. Principle contaminants observed were di-PEGylated toxin peptide analog. Selected pools were concentrated to 2-5 mg/ml by centrifugal filtration against 3 kDa MWCO membranes and dialyzed into 10 mM NaOAc, pH 4 with 5% sorbitol. Dialyzed pools were then sterile filtered through 0.2 micron filters and purity determined to be >97% by SDS-PAGE (data not shown). Reverse-phase HPLC was performed on an Agilent 1100 model HPLC running a Zorbax® 5μm 300SB-C8 4.6 x 50 mm column (Agilent) in 0.1% TFA/H_2O at 1 ml/min and column temperature maintained at 40°C. Samples of PEG-peptide (20 μg) were injected and eluted in a linear 6-60% gradient while monitoring wavelength 215 nm.

[00694] Fusion Proteins. Generally, Figure 1A and Figure 1B show a schematic representation of monovalent and bivalent Fc-toxin peptide (or toxin peptide analog) fusion proteins (or "peptibodies"), respectively. The bivalent Fc-ShK molecule is a homodimer containing two Fc-ShK chains. The monovalent Fc-ShK toxin peptide (or toxin peptide analog) molecule is a heterodimer containing one Fc chain and one Fc-ShK (or analog) chain. Since the monovalent Fc-ShK molecule contains just a single ShK peptide per dimer, it is considered monovalent. Constructs or chains referred to as Fc-(toxin peptide analog), contain an N-terminal Fc region and an optional flexible linker sequence (e.g., L10 peptidyl linker GGGGSGGGGS; SEQ ID NO:153) covalently attached to the toxin peptide or toxin peptide analog, such that the orientation from N- to C-terminus would be: Fc-linker-toxin peptide or toxin peptide analog.

[00695] In Examples 1 and 2 of Sullivan et al., WO 2008/088422A2, were described the activity of bivalent Fc-ShK peptibodies, Fc-L10-ShK(1-35) and Fc-L10-ShK(2-35) expressed from mammalian cells. In Example 1 of WO 2008/088422A2, was also described isolation of a monovalent Fc-L10-ShK(1-35) molecule, formed as a small by-product during expression. The monovalent antibody #3742-ShK(1-35, Q16K) conjugate provided potent blockade of T cell cytokine secretion in human whole blood (see, Table 7A-B, in Example 5 herein).

[00696] **Example 10**

[00697] **Pharmacokinetic (PK) studies in rats and cynomolgus monkeys.**

Rat PK. The pharmacokinetic profiles of the 16435 and 4341 antibodies were determined in adult Sprague-Dawley (SD) rats (n=3 per group) by injecting 5 mg/kg subcutaneously and collecting approximately 250 µL of blood in Microtainer® serum separator tubes at 0, 0.25, 1, 4, 24, 48, 72, 168, 336, 504, 672, 840 and 1008 hours post-dose from the lateral tail vein. Each sample was maintained at room temperature following collection, and following a 30-40 minute clotting period, samples were centrifuged at 2-8°C at 11,500 rpm for about 10 minutes using a calibrated Eppendorf 5417R Centrifuge System (Brinkmann Instruments, Inc., Westbury, NY). The collected serum was then transferred into a pre-labeled (for each rat), cryogenic storage tube and stored at -60°C to -80°C for future analysis. To measure the serum sample concentrations from the PK study samples, the following method was used: ½ area black plate (Corning 3694) was coated with 2 µg/ml of anti-hu Fc, antibody 1.35.1 in PBS and then incubated overnight at 4°C. The plate was then washed and blocked with I-Block™ (Applied Biosystems) overnight at 4°C. If samples needed to be diluted, then they were diluted in Rat SD serum. The standards and samples were then diluted 1: 20 in 1X PBS +1M NaCl+0.5% Tween 20 and 1% BSA buffer (5% serum). The plate was washed and 50-µl samples of diluted standards and samples were transferred into an antibody 1.35.1 coated plate and incubated for 1.5 h at room temperature. The plate was washed, then 50 µl of 100 ng/ml of anti-hu Fc antibody 21.1-HRP conjugate in I-Block™ +5% BSA was added and incubated for 1.5 h. The plate was washed, then 50 µl of Pico substrate were added, after which the plate was immediately analyzed with a luminometer. Time concentration data were analyzed using non-compartmental methods with WinNonLin® (Enterprise version 5.1.1, 2006, Pharsight® Corp. Mountain View, CA) (figure 34.0). The pharmacokentic profiles of these two antibodies in Sprague-Dawley rat are shown in Figure 43. The PK parameters of 16435 and 4341 antibodies in SD Rats are summarized in the Table 8 (below). Both molecules have good PK profile in rats with half life of over 10 days.

Table 8. PK parameters of antibodies 16435 and 4341 in SD Rats.

Compound	SC Dose (mg/kg)	$T_{1/2}$ (h)	Tmax (h)	Cmax (ng/mL)	MRT (h)	CL/F (mL/h/kg)	AUC_{0-t} (ng*h/mL)	AUC_{0-inf} (ng*h/mL)
16435	5	226	104	44,080	395	0.368	16,038,601	20,048,353
4341	5	365	136	38,963	580	0.190	22,280,335	26,661,802

Cynomolgus PK. The pharmacokinetic profiles of the 16435 and 4341 antibodies were also determined in cynomolgus monkeys (n=2 per group) by injecting of two subsequent subcutaneous doses of 1 mg/kg at day 0 and 5 mg/kg at day 57. Serum samples were collected at pre-dose, 0.5, 2, 4, 8, 12, 24, 48, 96, 168, 336, 504, 672, 840, 1008, 1176, 1344 (prior to second dose) hours post 1st dose at 1 mg/kg and 0.5, 2, 4, 8, 12, 24, 48, 96, 168, 336, 360, 384, 432, 504, 672, 840, 1008, 1176, 1344 following post 2nd dose at 5 mg/kg. The samples were assayed for the 16435 and 4341 antibody levels by using an anti-IgG sandwich ELISA as described above. Time concentration data were analyzed using non-compartmental methods with WinNonLin® . The pharmacokentic profiles of these two antibodies in cynomolgus monkey are shown in Figure 44. The PK parameters of 16435 and 4341 antibodies in cynomolgus monkeys are summarized in the Table 9 (below). Both molecules exhibited a good PK profile in cynos, with half life of about 12 and 21 days for 16435 and 4341, respectively. The 4341 antibody has better PK attributes than 16435 and has shown normal hu IgG clearance in monkey based on FcRn binding and in the absence of any target mediated drug disposition (TMDD) clearance mechanism. In addition, the results in Figure 44 show that even with multiple dosing in the cynos, both antibodies 16435 and 4341 had no indication of a signficant change in the clearance mediated by an immune response in the cynos. If there had been a significant immune response causing abnormal antibody clearance, it would have been expected after the second dose, due to immune system priming by the first dose.

Table 9. PK parameters of antibodies 16435 and 4341 in cynomolgus monkeys.

Compound	SC Dose (mg/kg)	$T_{1/2}$ (h)	Tmax (h)	Cmax (ng/mL)	MRT (h)	CL/F (mL/h/kg)	AUC_{0-t} (ng*h/mL)	AUC_{0-inf} (ng*h/mL)
16435	5	285	96	58,682	450	0.161	29,924,604	31,230,285
4341	5	502	96	68,166	740	0.096	43,578,088	51,909,826

Abbreviations

Abbreviations used throughout this specification are as defined below, unless otherwise defined in specific circumstances.

Ac	acetyl (used to refer to acetylated residues)
AcBpa	acetylated p-benzoyl-L-phenylalanine
ACN	acetonitrile
AcOH	acetic acid
ADCC	antibody-dependent cellular cytotoxicity
Aib	aminoisobutyric acid
bA	beta-alanine
Bpa	p-benzoyl-L-phenylalanine
BrAc	bromoacetyl ($BrCH_2C(O)$
BSA	Bovine serum albumin
Bzl	Benzyl
Cap	Caproic acid
CBC	complete blood count
COPD	Chronic obstructive pulmonary disease
CTL	Cytotoxic T lymphocytes
DCC	Dicylcohexylcarbodiimide
Dde	1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)ethyl
DNP	2,4-dinitrophenol

DOPC	1,2-Dioleoyl-sn-Glycero-3-phosphocholine
DOPE	1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine
DPPC	1,2-Dipalmitoyl-sn-Glycero-3-phosphocholine
DSPC	1,2-Distearoyl-sn-Glycero-3-phosphocholine
DTT	Dithiothreitol
EAE	experimental autoimmune encephalomyelitis
ECL	enhanced chemiluminescence
ESI-MS	Electron spray ionization mass spectrometry
FACS	fluorescence-activated cell sorting
Fmoc	fluorenylmethoxycarbonyl
GHT	glycine, hypoxanthine, thymidine
HOBt	1-Hydroxybenzotriazole
HPLC	high performance liquid chromatography
HSL	homoserine lactone
IB	inclusion bodies
KCa	calcium-activated potassium channel (including IKCa, BKCa, SKCa)
KLH	Keyhole Limpet Hemocyanin
Kv	voltage-gated potassium channel
Lau	Lauric acid
LPS	lipopolysaccharide
LYMPH	lymphocytes
MALDI-MS	Matrix-assisted laser desorption ionization mass spectrometry
Me	methyl
MeO	methoxy
MeOH	methanol
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
1-Nap	1-napthylalanine
NEUT	neutrophils

Nle	norleucine
NMP	N-methyl-2-pyrrolidinone
OAc	acetate
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
Pbf	2,2,4,6,7-pendamethyldihydrobenzofuran-5-sulfonyl
PCR	polymerase chain reaction
PD	pharmacodynamic
Pec	pipecolic acid
PEG	Poly(ethylene glycol)
pGlu	pyroglutamic acid
Pic	picolinic acid
PK	pharmacokinetic
pY	phosphotyrosine
RBS	ribosome binding site
RT	room temperature (about 25°C)
Sar	sarcosine
SDS	sodium dodecyl sulfate
STK	serine-threonine kinases
t-Boc	tert-Butoxycarbonyl
tBu	tert-Butyl
TCR	T cell receptor
TFA	trifluoroacetic acid
THF	thymic humoral factor
Trt	trityl

CLAIMS

What is claimed is:

1. An isolated immunoglobulin, comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

(a) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 or SEQ ID NO:190; or

(b) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:321 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188 or SEQ ID NO:190; or

(c) the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325 and the light chain variable region comprises the amino acid sequence of SEQ ID NO:182, SEQ ID NO:188, or SEQ ID NO:190.

2. An isolated immunoglobulin, comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, wherein:

(a) the light chain variable region comprises the amino acid sequence of SEQ ID NO:196 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335, SEQ ID NO:349, SEQ ID NO:351, SEQ ID NO:353, SEQ ID NO:355, or SEQ ID NO:359; or

(b) the light chain variable region comprises the amino acid sequence of SEQ ID NO:204 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349 or SEQ ID NO:355; or

(c) the light chain variable region comprises the amino acid sequence of SEQ ID NO:202 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349; or

(d) the light chain variable region comprises the amino acid sequence of SEQ ID NO:192 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:357, SEQ ID NO:359, or SEQ ID NO:369; or

(e) the light chain variable region comprises the amino acid sequence of SEQ ID NO:194 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:335, SEQ ID NO:349, or SEQ ID NO:351.

3. The isolated immunoglobulin of Claim 1, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:323; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:188.

4. The isolated immunoglobulin of Claim 2, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO:196; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:353.

5. The isolated immunoglobulin of Claim 2, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO:202; and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:349.

6. The isolated immunoglobulin of Claim 1, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO:325; and the light chain variable region comprises the amino acid sequence of SEQ ID NO:190.

7. The isolated immunoglobulin of Claim 1 or Claim 2, wherein the isolated immunoglobulin comprises an antibody or antibody fragment.

8. The isolated immunoglobulin of Claim 7, comprising an IgG1, IgG2,

IgG3 or IgG4.

9. The isolated immunoglobulin of Claim 7, comprising a monoclonal antibody.

10. The isolated immunoglobulin of Claim 9, comprising a human antibody.

11. The isolated immunoglobulin of Claim 10, comprising:

(a) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:113, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:110, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

(b) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:125, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:122, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

(c) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:101, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:98, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; or

(d) an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:119, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both; and an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO:116, or comprising the foregoing sequence from which one, two, three, four or five amino acid residues are lacking from the N-terminal or C-terminal, or both.

12. The isolated immunoglobulin of any of Claims 1-6 or 8-11, further comprising one to twenty-four pharmacologically active chemical moieties conjugated thereto.

13. The isolated immunoglobulin of Claim 12, wherein the pharmacologically active chemical moiety is a pharmacologically active polypeptide.

14. The isolated immunoglobulin of Claim 13, wherein the immunoglobulin is recombinantly produced.

15. The isolated immunoglobulin of Claim 14, wherein the immunoglobulin comprises at least one immunoglobulin heavy chain and at least one immunoglobulin light chain, and wherein the pharmacologically active polypeptide is inserted in the primary amino acid sequence of the of the immunoglobulin heavy chain within an internal loop of the Fc domain of the immunoglobulin heavy chain.

16. The isolated immunoglobulin of Claim 13, wherein the immunoglobulin comprises at least one immunoglobulin heavy chain and at least one immunoglobulin light chain, and wherein the pharmacologically active polypeptide is conjugated at the N-terminal or C-terminal of the immunoglobulin heavy chain.

17. The isolated immunoglobulin of Claim 13, wherein the immunoglobulin comprises at least one immunoglobulin heavy chain and at least one

immunoglobulin light chain, and wherein the pharmacologically active polypeptide is conjugated at the N-terminal or C-terminal of the immunoglobulin light chain.

18. The isolated immunoglobulin of Claim 13, wherein the pharmacologically active polypeptide is a toxin peptide, an IL-6 binding peptide, a CGRP peptide antagonist, a bradykinin B1 receptor peptide antagonist, a PTH agonist peptide, a PTH antagonist peptide, an ang-1 binding peptide, an ang-2 binding peptide, a myostatin binding peptide, an EPO-mimetic peptide, a FGF21 peptide, a TPO-mimetic peptide, a NGF binding peptide, a BAFF antagonist peptide, a GLP-1 or peptide mimetic thereof, or a GLP-2 or peptide mimetic thereof.

19. The isolated immunoglobulin of Claim 18, wherein the toxin peptide is ShK or a ShK peptide analog.

20. A pharmaceutical composition comprising the immunoglobulin of any of Claims 1-19; and a pharmaceutically acceptable diluent, excipient or carrier.

21. An isolated nucleic acid that encodes the immunoglobulin of any of Claims 1-11.

22. An isolated nucleic acid that encodes the immunoglobulin of Claim 3.

23. An isolated nucleic acid that encodes the immunoglobulin of Claim 4.

24. An isolated nucleic acid that encodes the immunoglobulin of Claim 5.

25. An isolated nucleic acid that encodes the immunoglobulin of Claim 6.

26. An isolated nucleic acid that encodes the immunoglobulin of Claim 11.

27. An isolated nucleic acid that encodes the immunoglobulin of any of Claims 13-19.

28. A vector comprising the isolated nucleic acid of Claims 21.

29. A vector comprising the isolated nucleic acid of any of Claims 22-26.

30. A vector comprising the isolated nucleic acid of Claim 27.

31. The vector of Claim 28, comprising an expression vector.

32. The vector of Claim 29, comprising an expression vector.

33. The vector of Claim 30, comprising an expression vector.

34. An isolated host cell, comprising the expression vector of any of Claims 31-33.

35. A method, comprising:

(a) culturing the host cell of claim 34 in a culture medium under conditions permitting expression of the immunoglobulin encoded by the expression vector; and

(b) recovering the immunoglobulin from the culture medium.

36. The immunoglobulin of Claim 1, wherein the immunoglobulin at 30 micromolar concentration does not significantly bind soluble human IL-17R (SEQ ID NO:89) at 30 nanomolar concentration in an aqueous solution incubated under physiological conditions, as measured by a surface plasmon resonance binding assay.

37. The immunoglobulin of Claim 2, wherein the immunoglobulin at 10 micromolar concentration does not significantly bind soluble human TR2 (SEQ ID

NO:82) at 10 nanomolar concentration in an aqueous solution incubated under physiological conditions, as measured by a surface plasmon resonance binding assay.

FIG. 1A

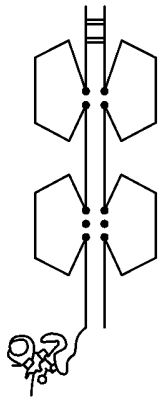


FIG. 1B

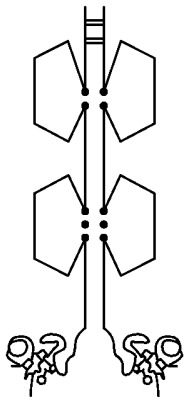


FIG. 1C

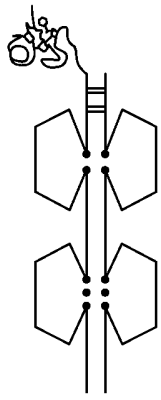


FIG. 1D

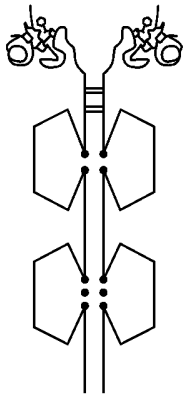


FIG. 1E

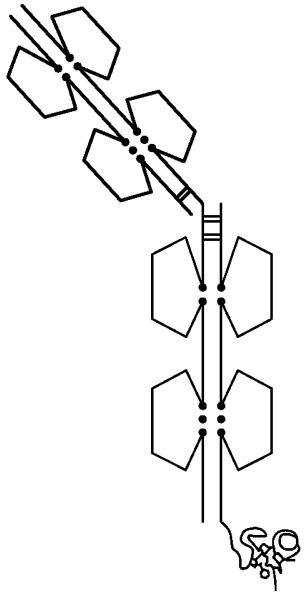


FIG. 1F

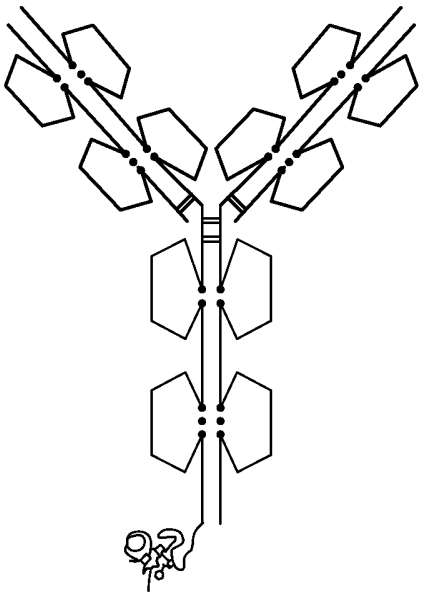


FIG. 1G

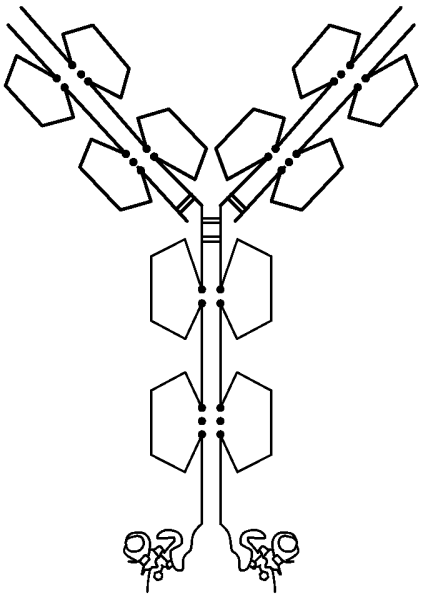


FIG. 1H

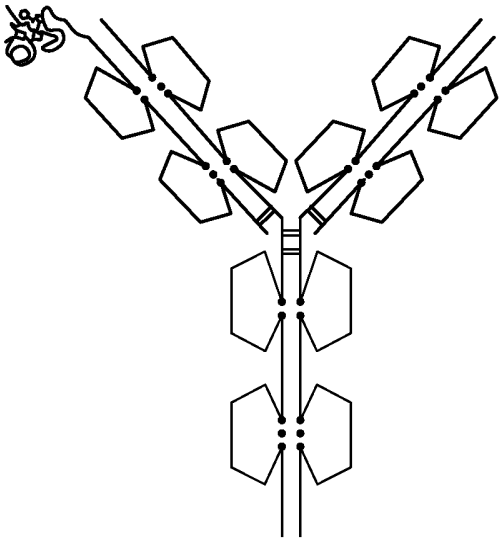


FIG. 1I

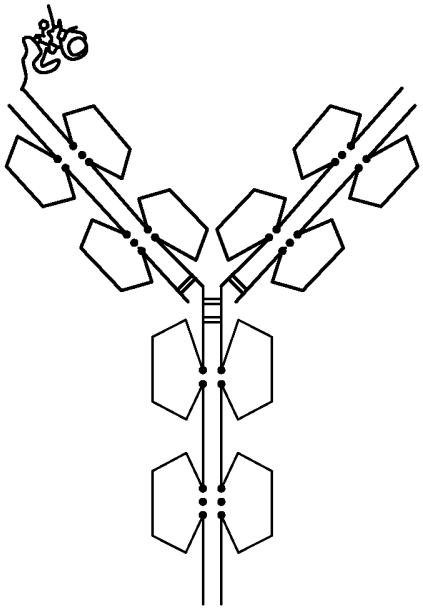


FIG. 1J

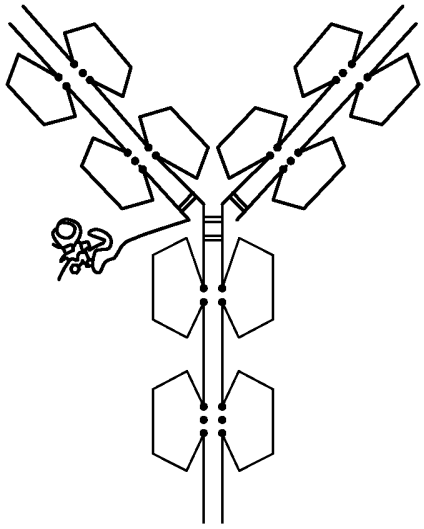


FIG. 1K

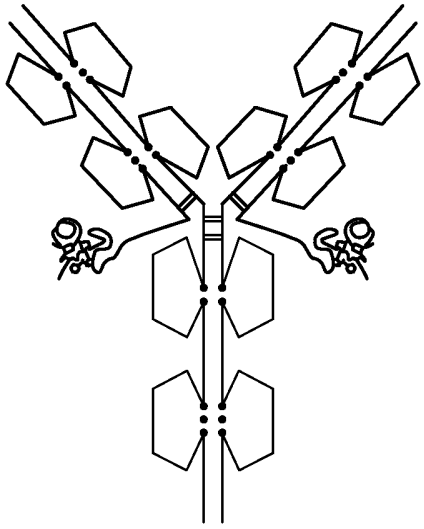


FIG. 1L

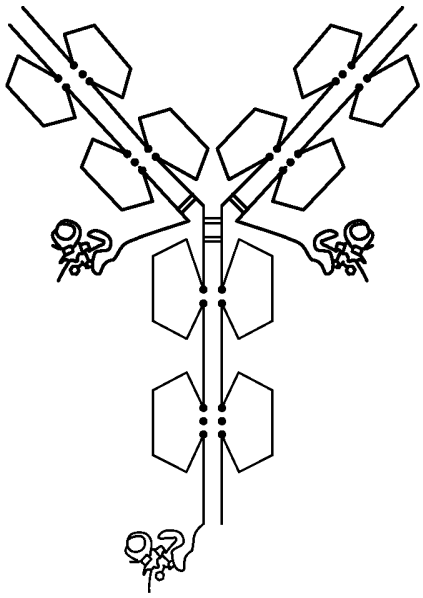


FIG. 1M

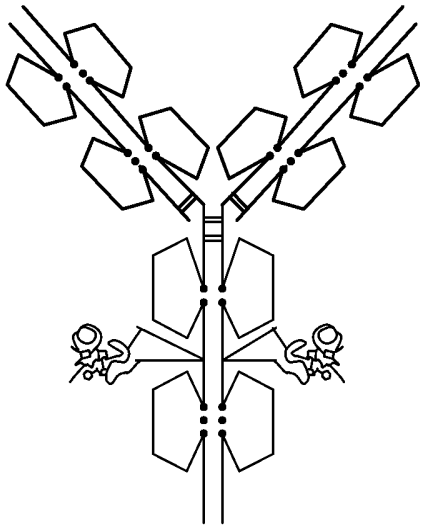


FIG. 1N

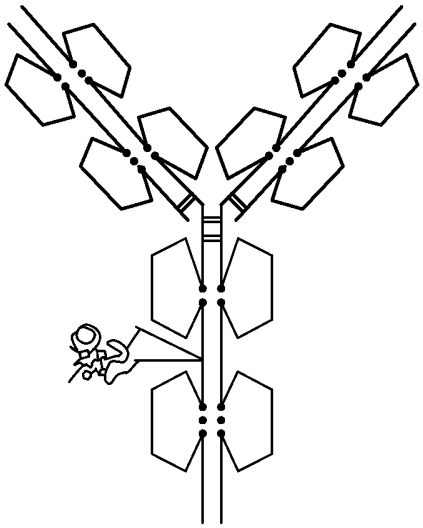


FIG. 2A

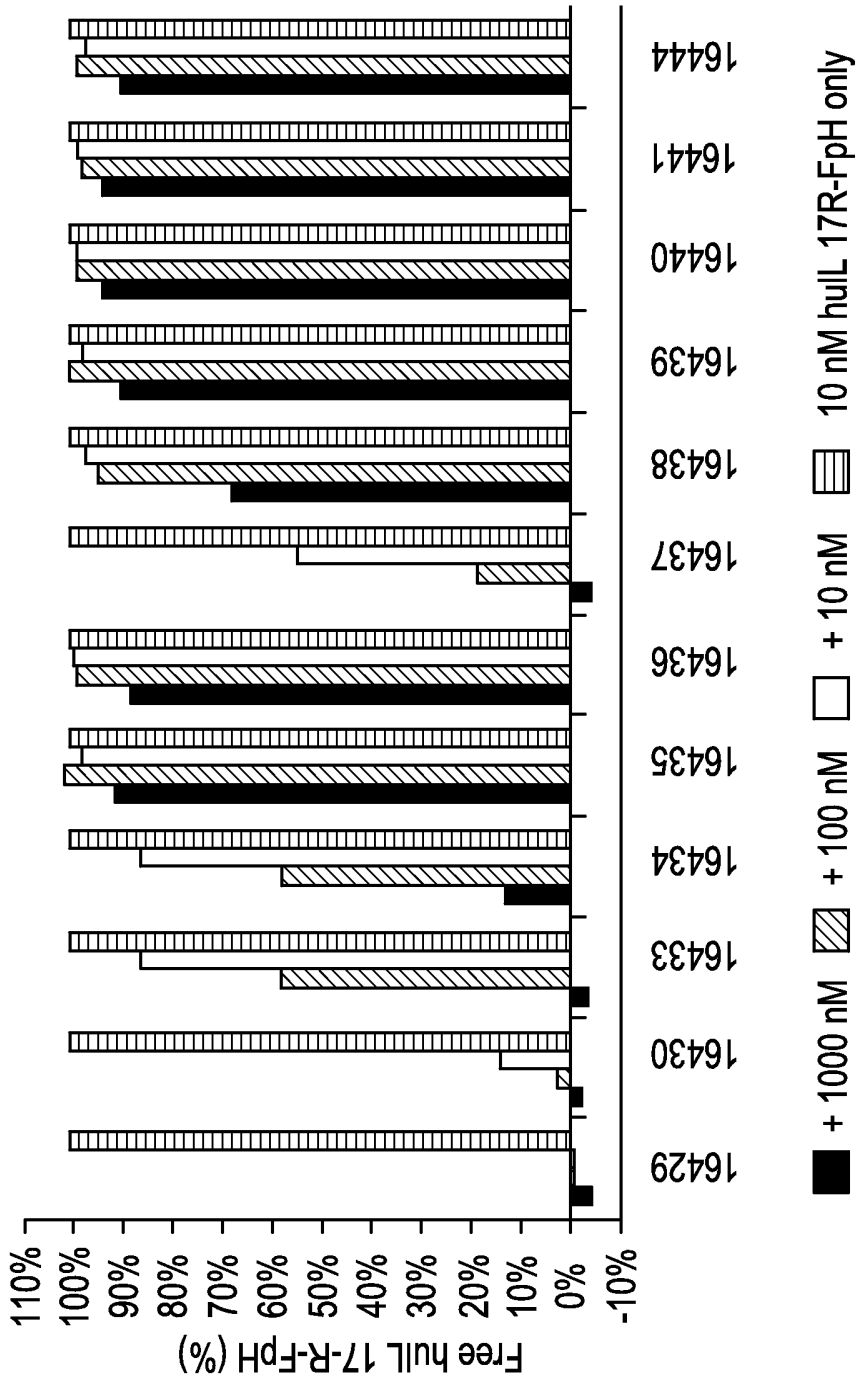

Free huIL 17-R-FpH (%)
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
16429
16430
16433
16434
16435
16436
16437
16438
16439
16440
16441
16444
+ 1000 nM
+ 100 nM
+ 10 nM
10 nM huIL 17R-FpH only

FIG. 2B

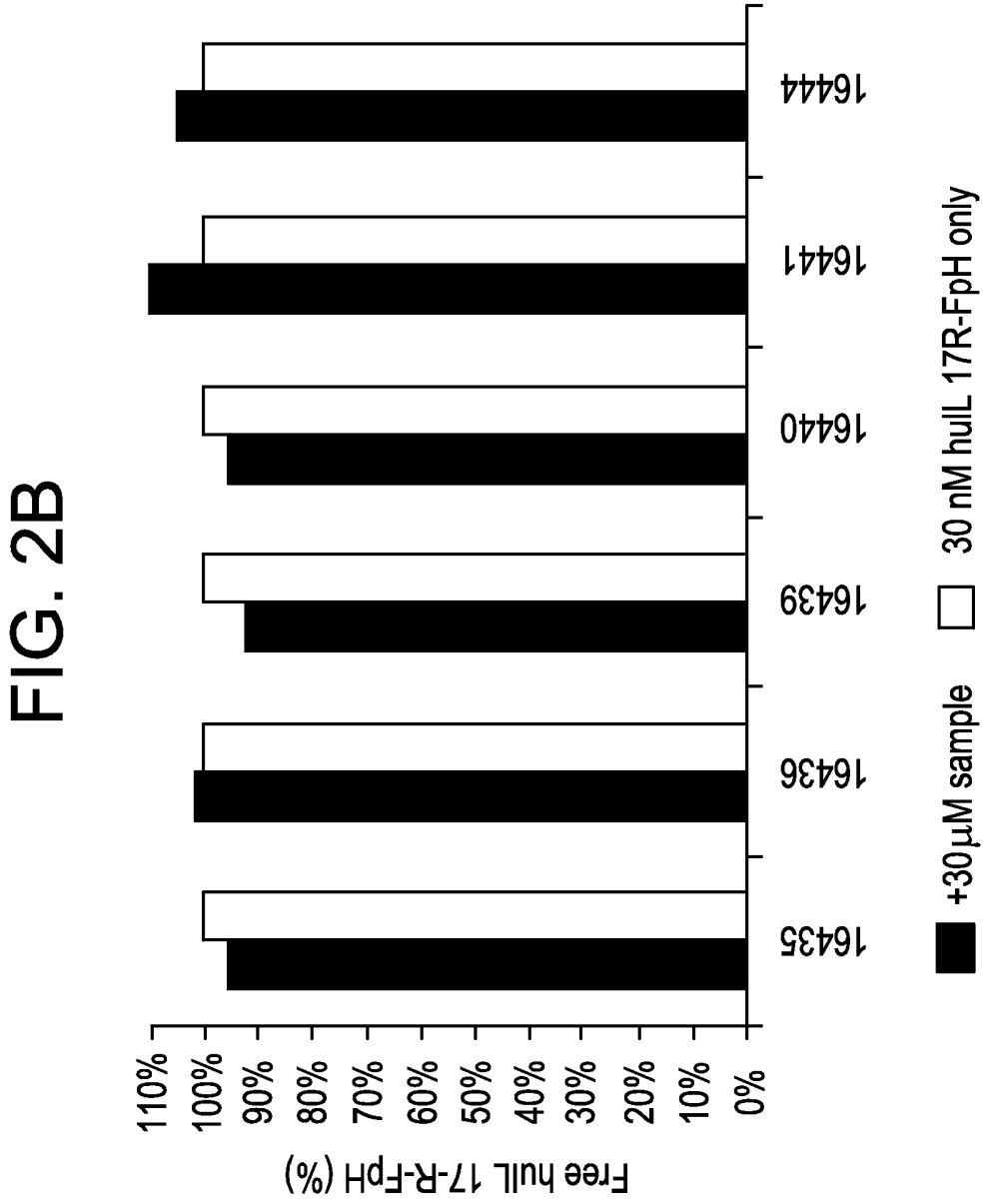

Free huIL 17-R-FpH (%)
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
16435
16436
16439
16440
16441
16444
+30µM sample
30 nM huIL 17R-FpH only

FIG. 3

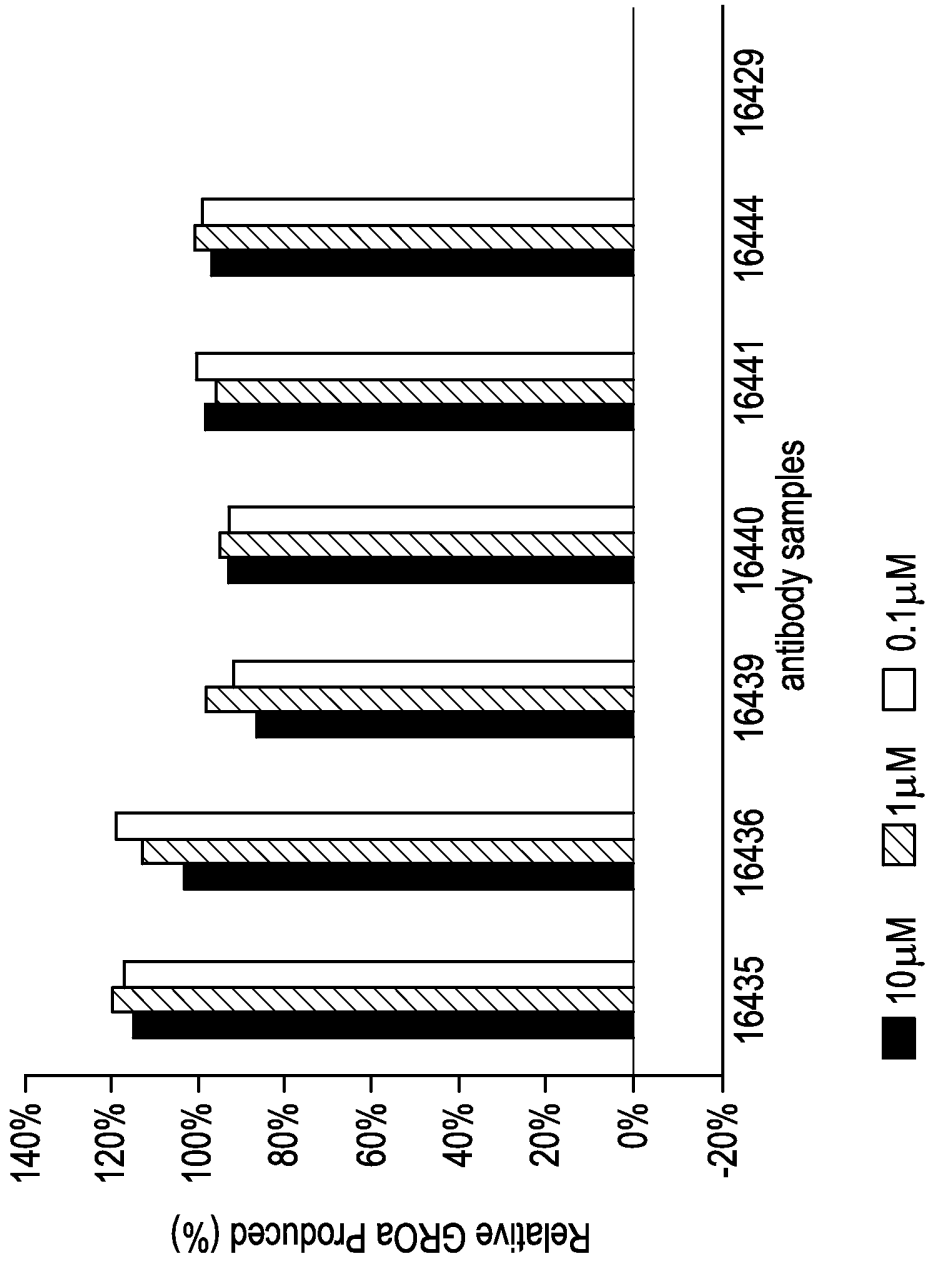

Relative GROa Produced (%)
140%
120%
100%
80%
60%
40%
20%
0%
-20%
16435
16436
16439
16440
16441
16444
16429
antibody samples
10μM
1μM
0.1μM

FIG. 4A

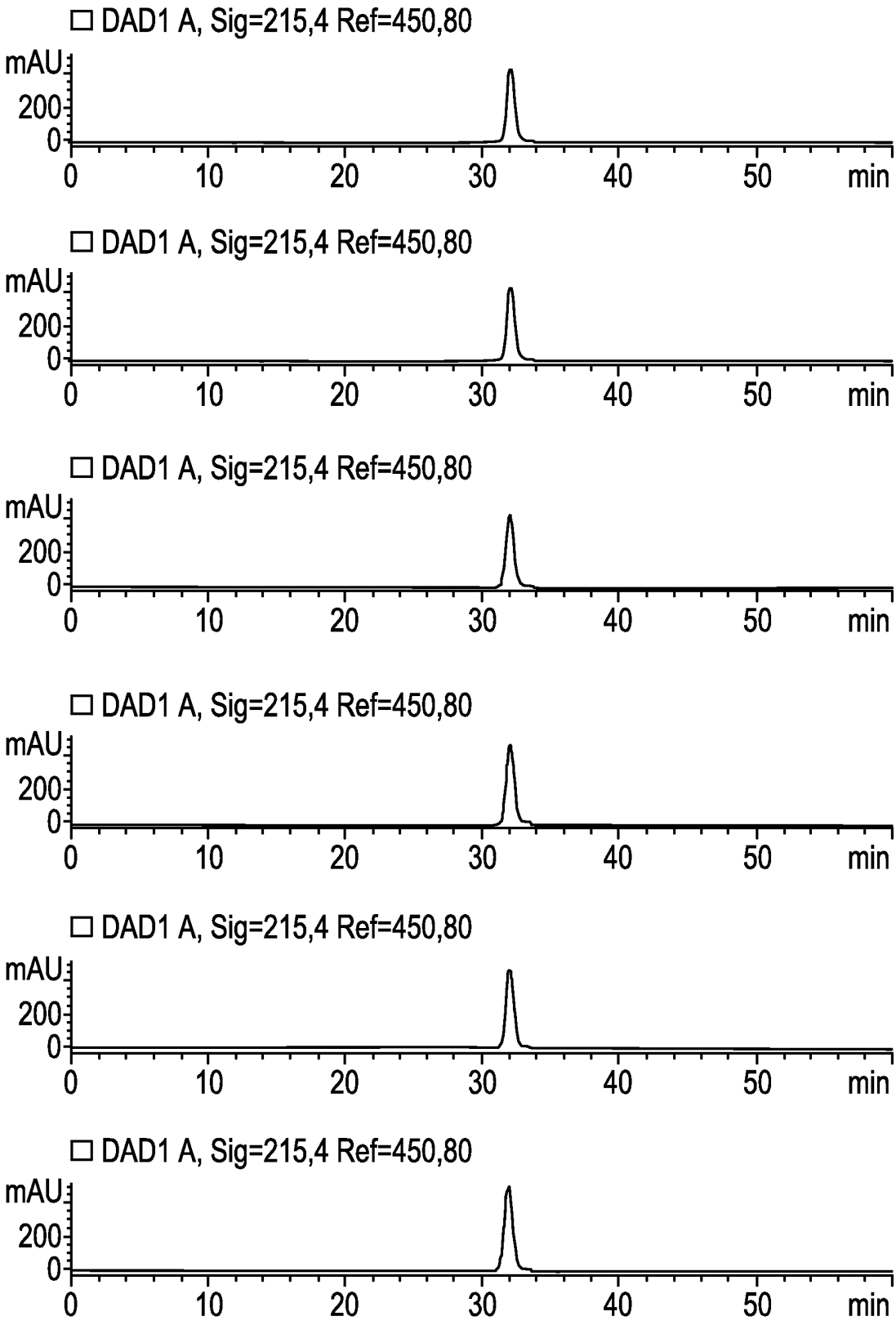


FIG. 4B

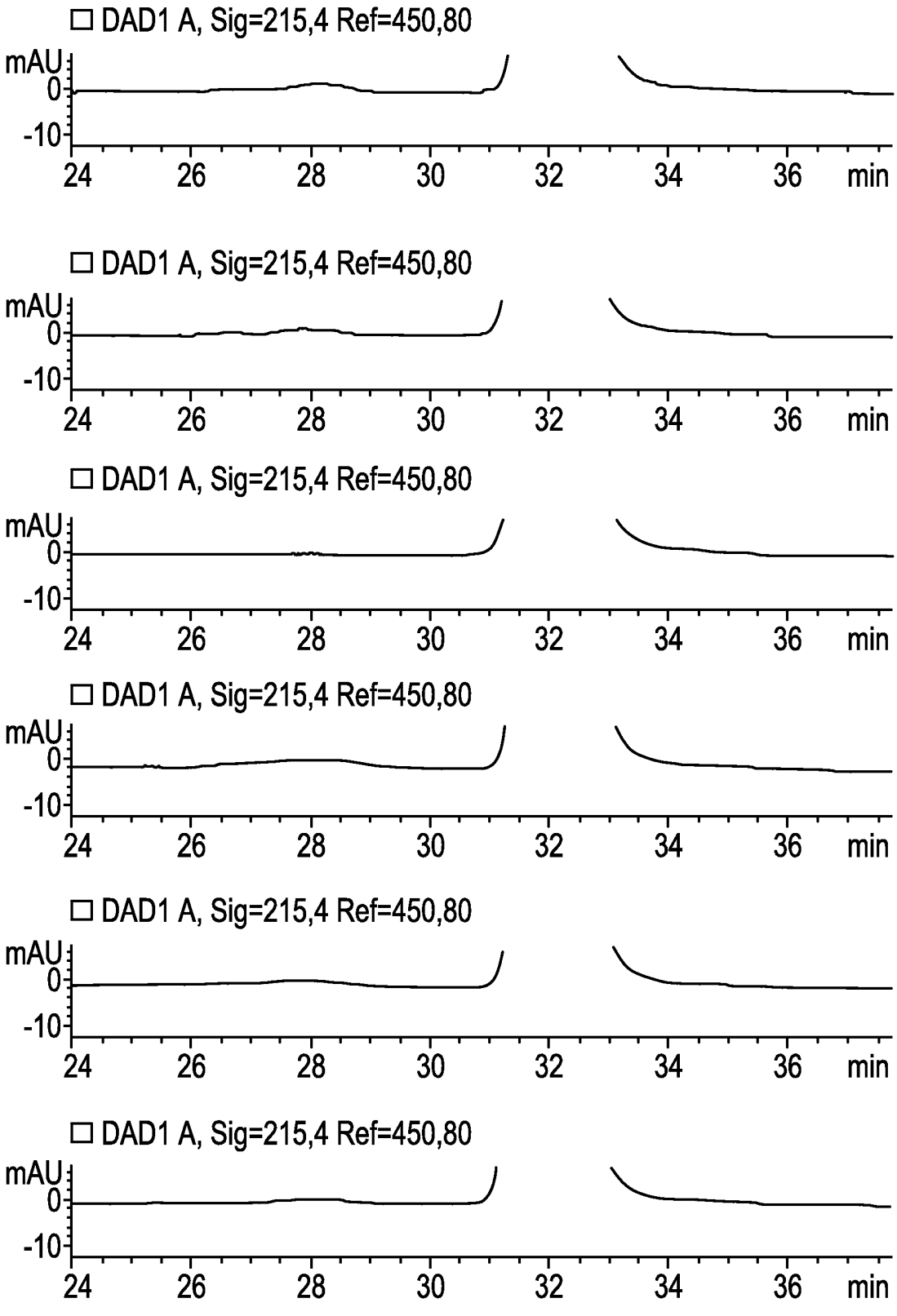


FIG. 5

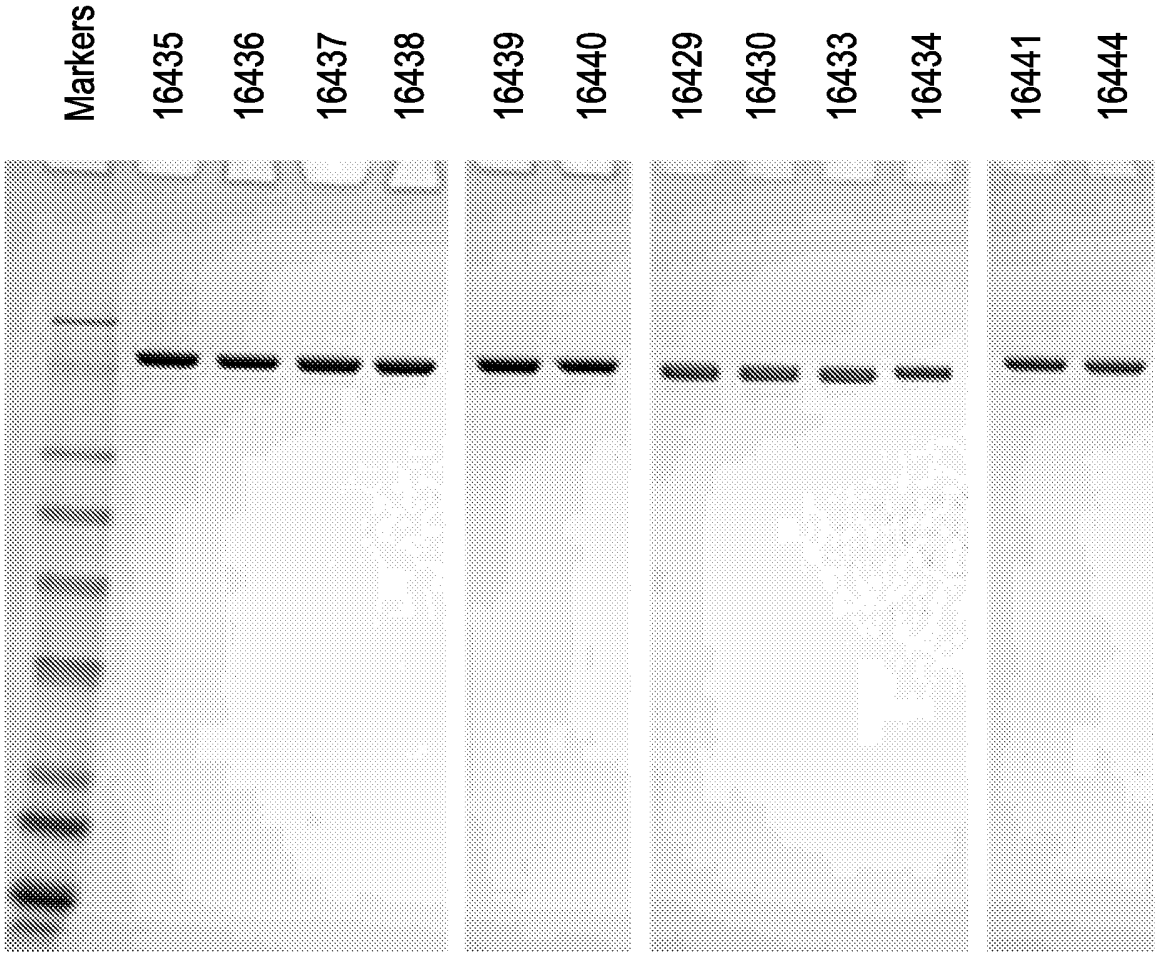

Markers
16435
16436
16437
16438
16439
16440
16429
16430
16433
16434
16441
16444

FIG. 6

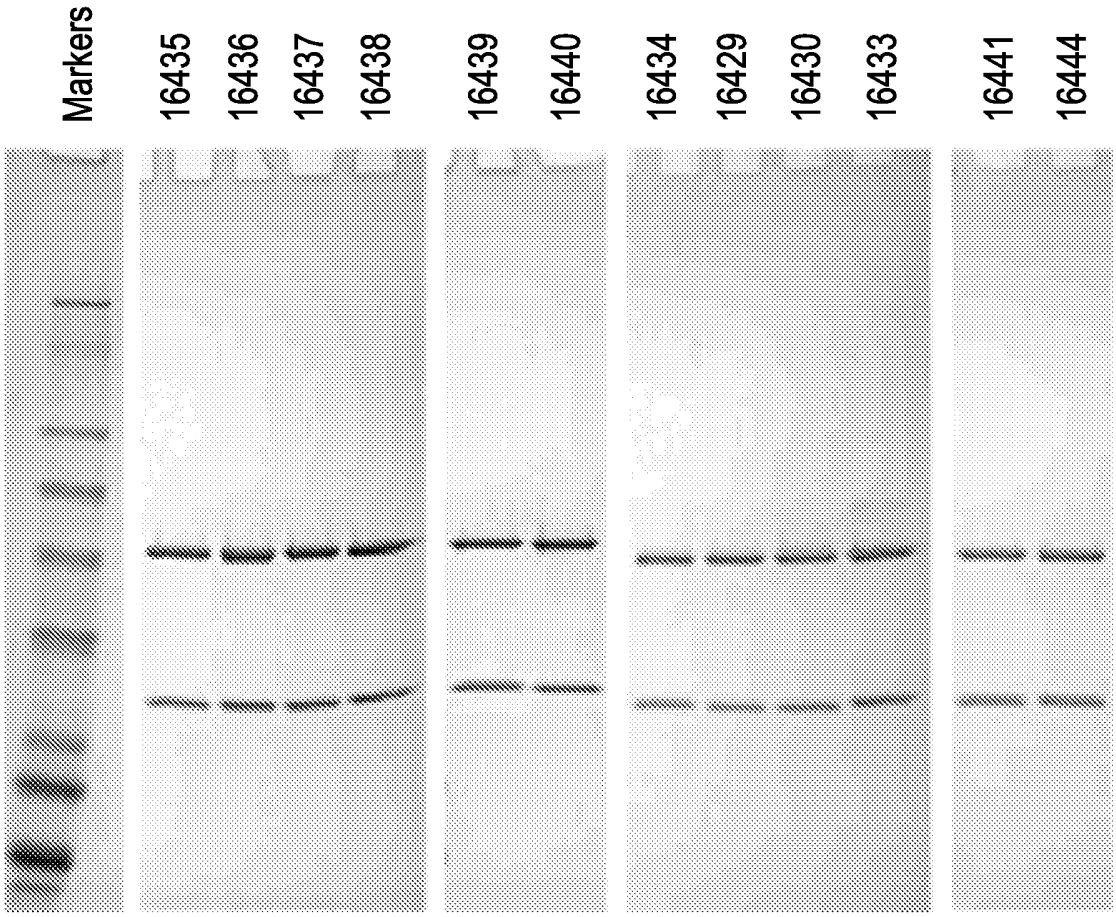

Markers
16435
16436
16437
16438
16439
16440
16434
16429
16430
16433
16441
16444

FIG. 7A

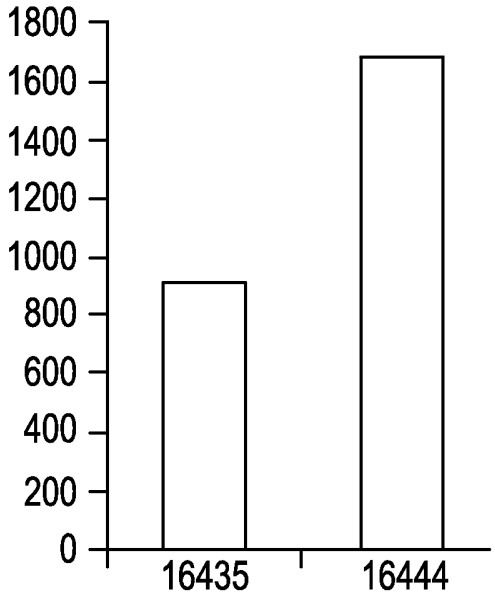

1800
1600
1400
1200
1000
800
600
400
200
0
16435
16444

FIG. 7B

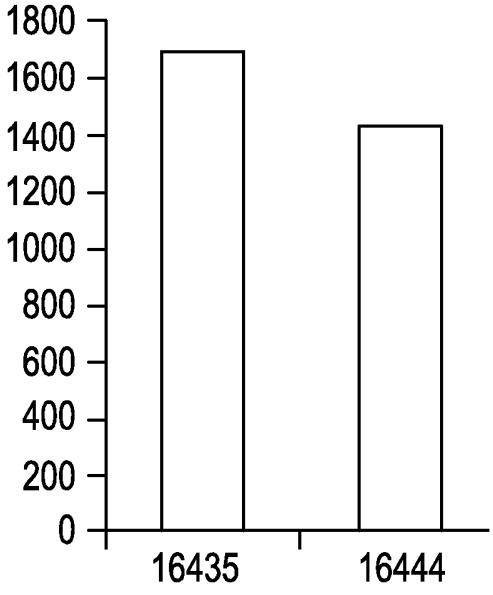

1800
1600
1400
1200
1000
800
600
400
200
0
16435
16444

FIG. 8A

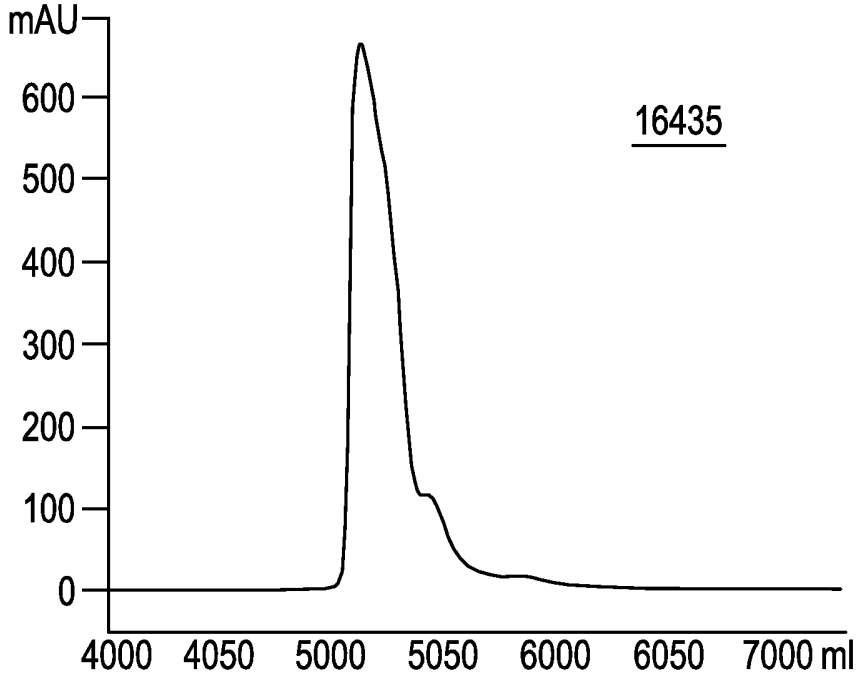

mAU
600
500
400
300
200
100
0
16435
4000
4050
5000
5050
6000
6050
7000 ml

FIG. 8B

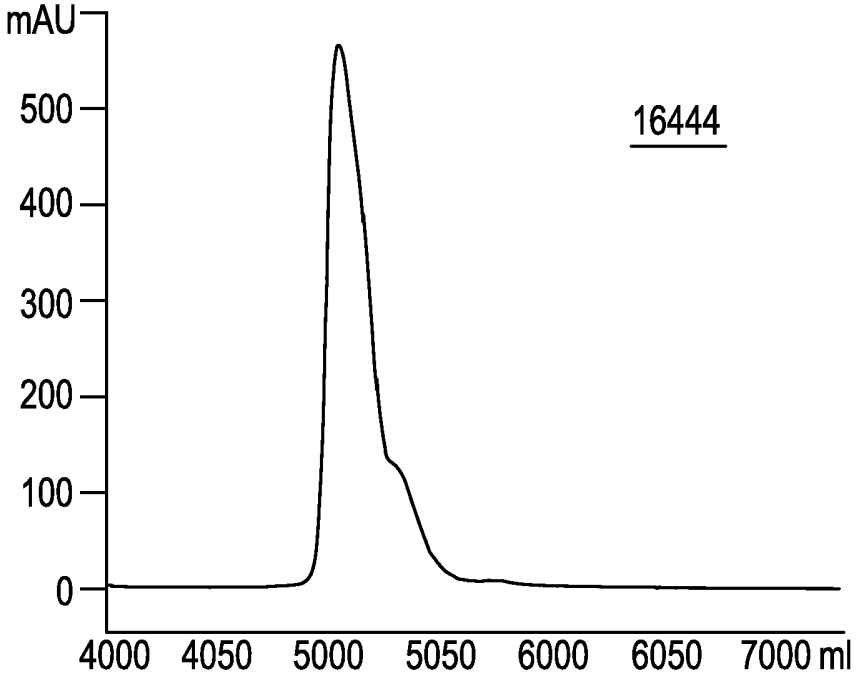

mAU
500
400
300
200
100
0
16444
4000
4050
5000
5050
6000
6050
7000 ml

FIG. 9A

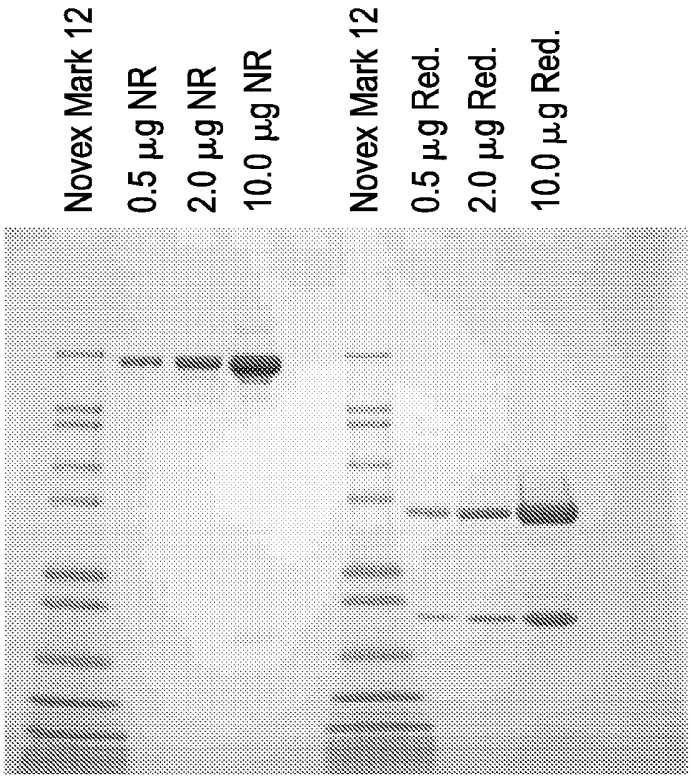

Novex Mark 12
0.5 µg NR
2.0 µg NR
10.0 µg NR
Novex Mark 12
0.5 µg Red.
2.0 µg Red.
10.0 µg Red.

FIG. 9B

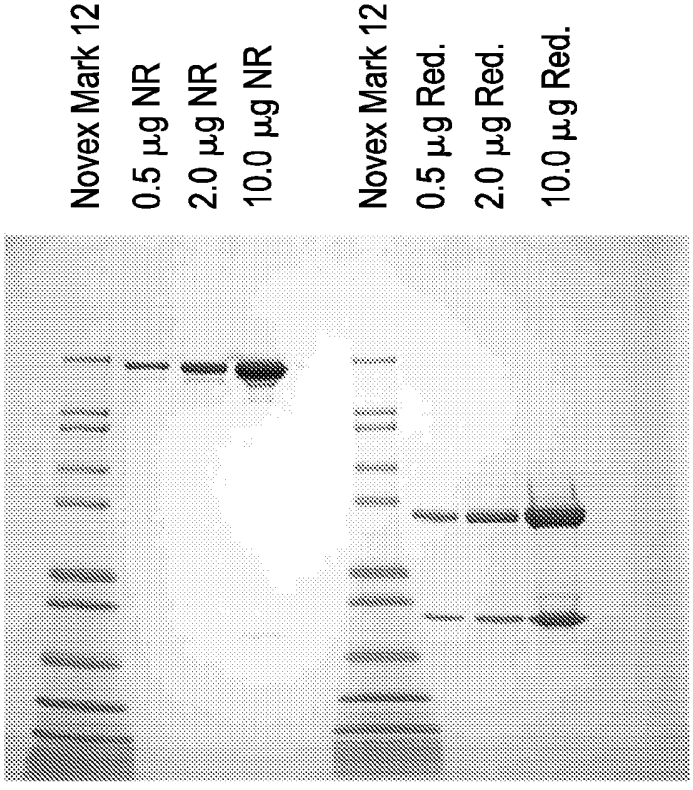

Novex Mark 12
0.5 µg NR
2.0 µg NR
10.0 µg NR
Novex Mark 12
0.5 µg Red.
2.0 µg Red.
10.0 µg Red.

FIG. 10

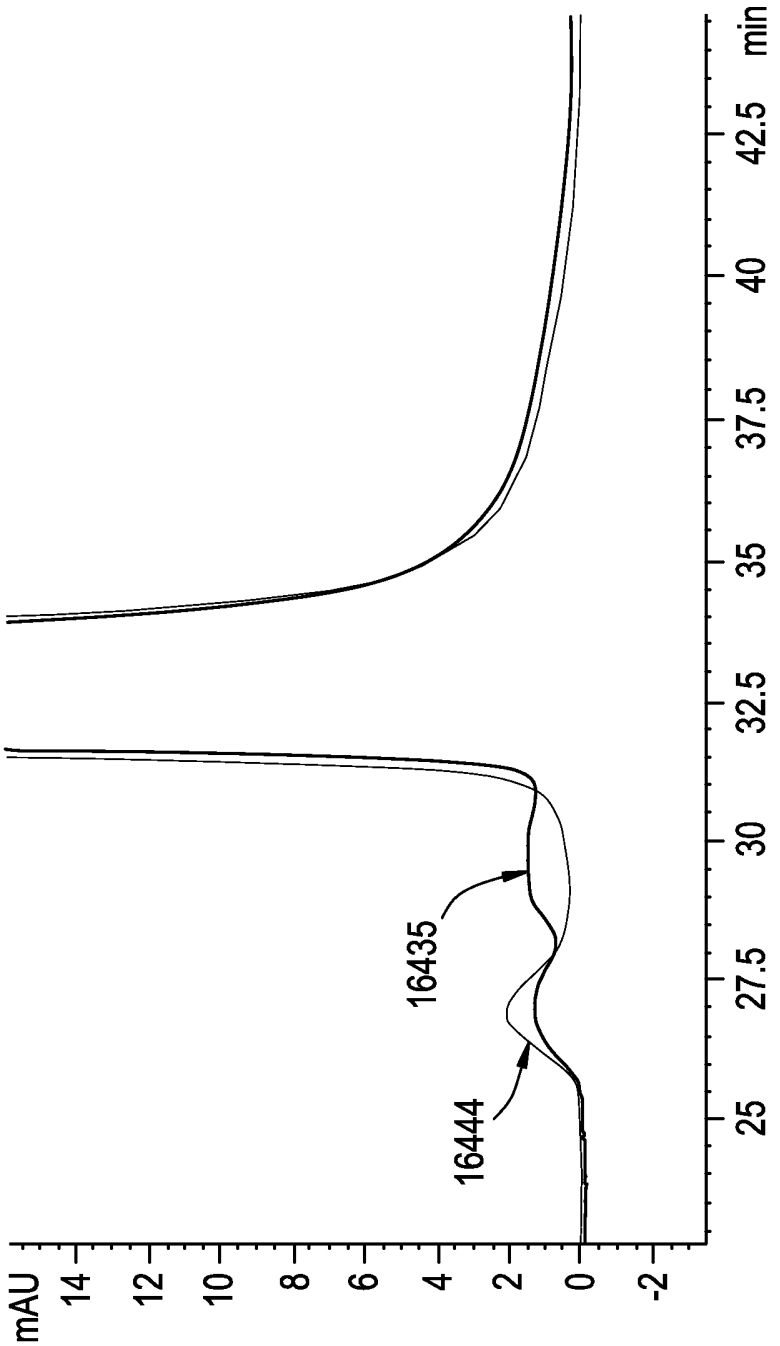


FIG. 11

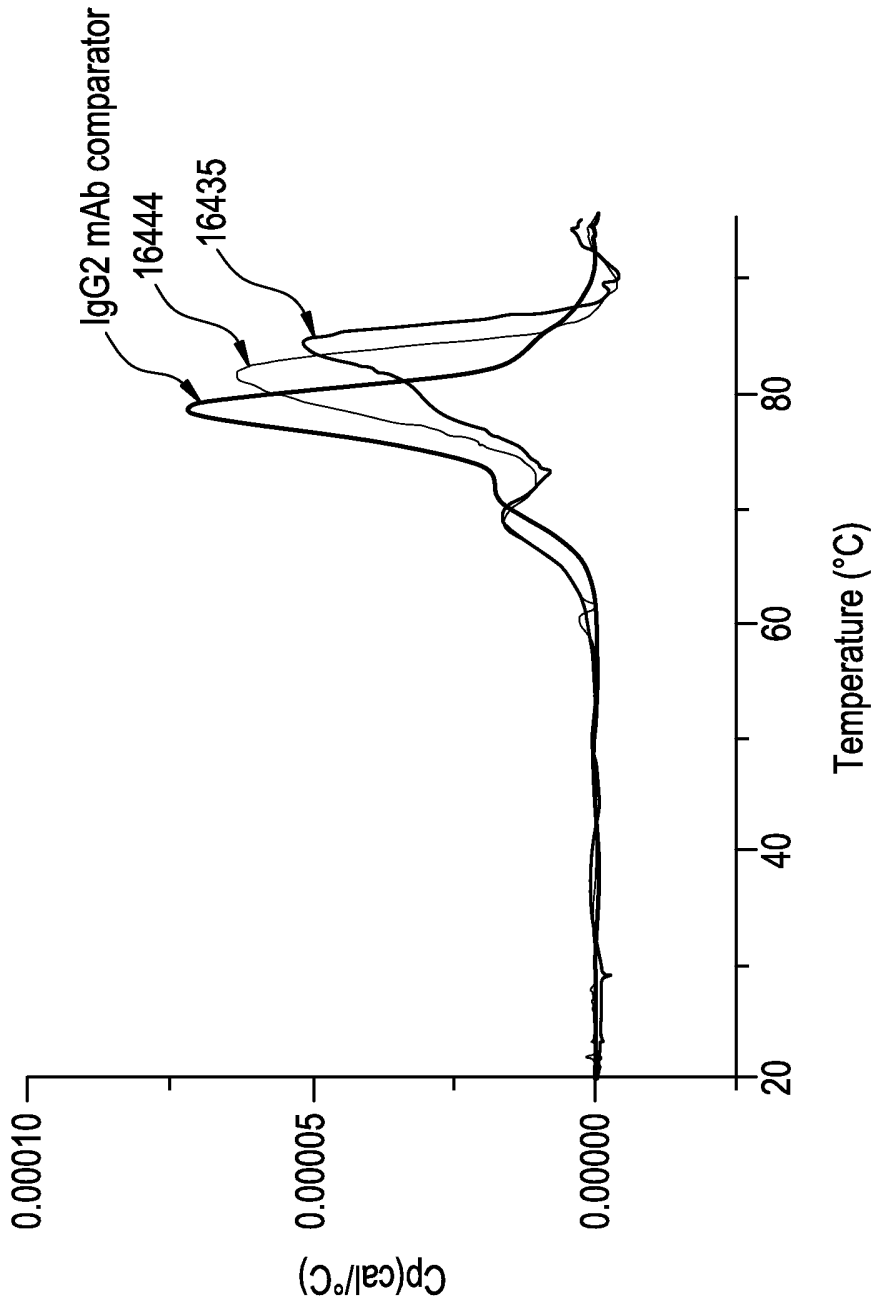

IgG2 mAb comparator
16444
16435
0.00010
0.00005
0.00000
Cp(cal/°C)
20
40
60
80
Temperature (°C)

FIG. 12A

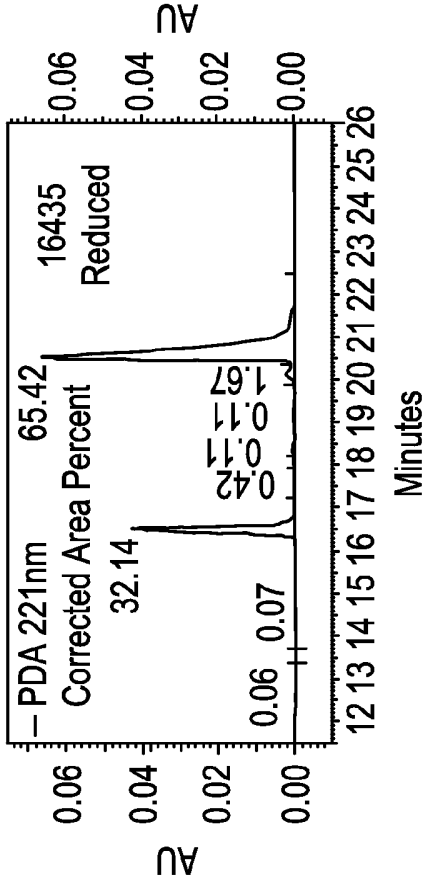


FIG. 12B

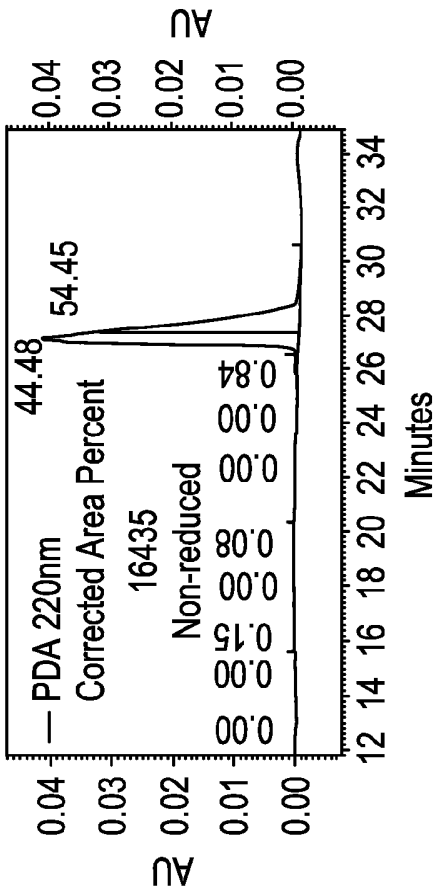


FIG. 12C

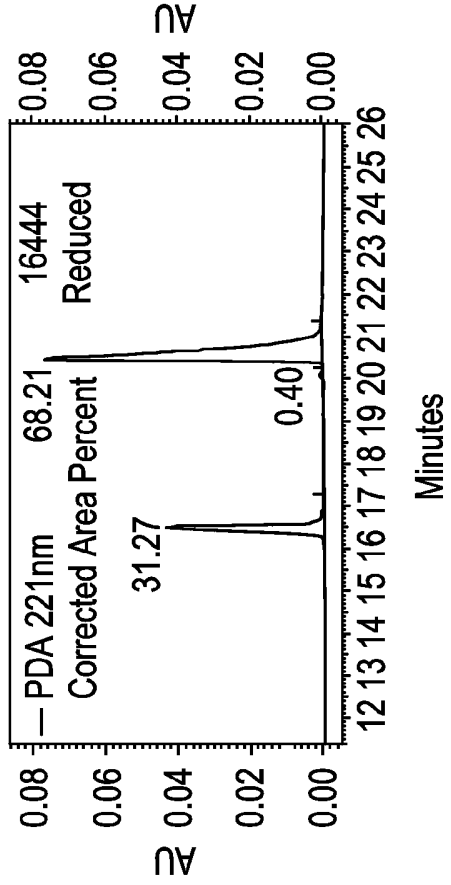


FIG. 12D

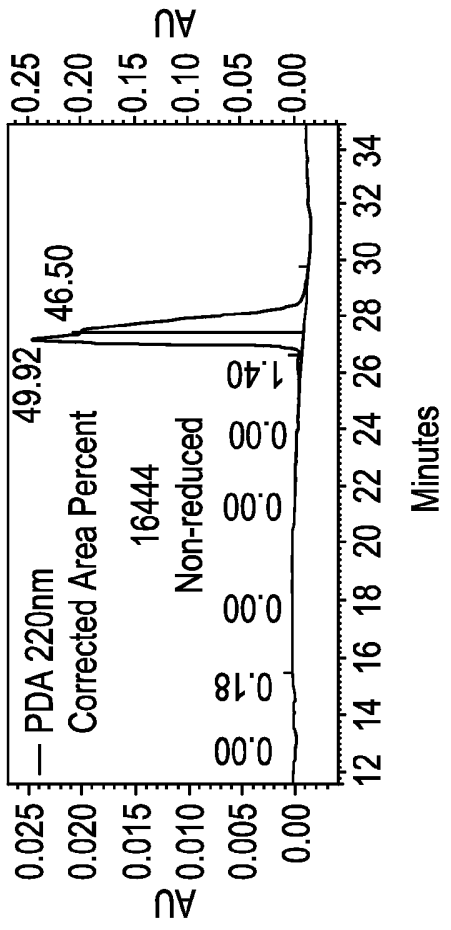


FIG. 13

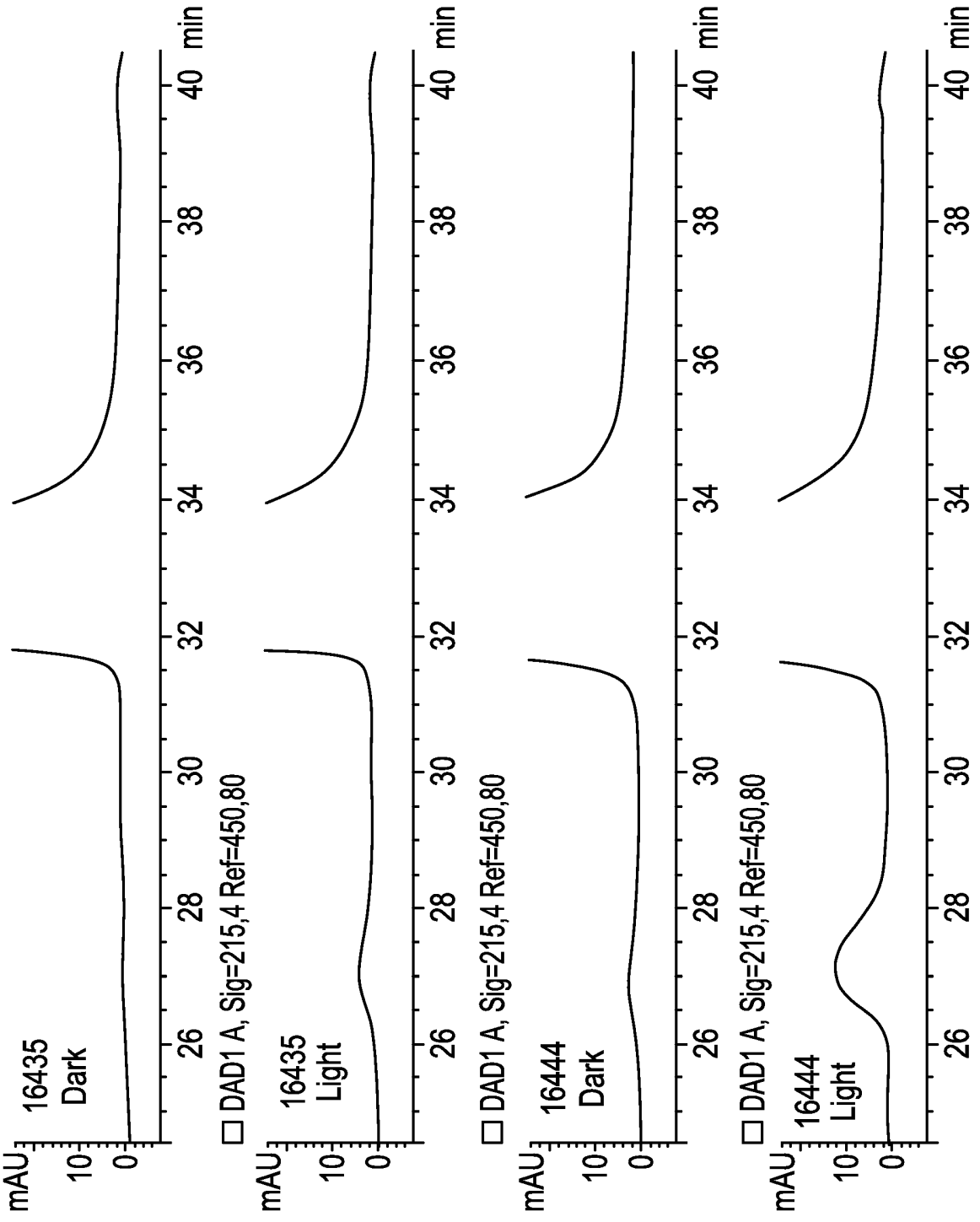

DAD1 A, Sig=215,4 Ref=450,80
16435
Dark
mAU
10
0
26
28
30
32
34
36
38
40 min
DAD1 A, Sig=215,4 Ref=450,80
16435
Light
mAU
10
0
26
28
30
32
34
36
38
40 min
DAD1 A, Sig=215,4 Ref=450,80
16444
Dark
mAU
10
0
26
28
30
32
34
36
38
40 min
DAD1 A, Sig=215,4 Ref=450,80
16444
Light
mAU
10
0
26
28
30
32
34
36
38
40 min

FIG. 14A

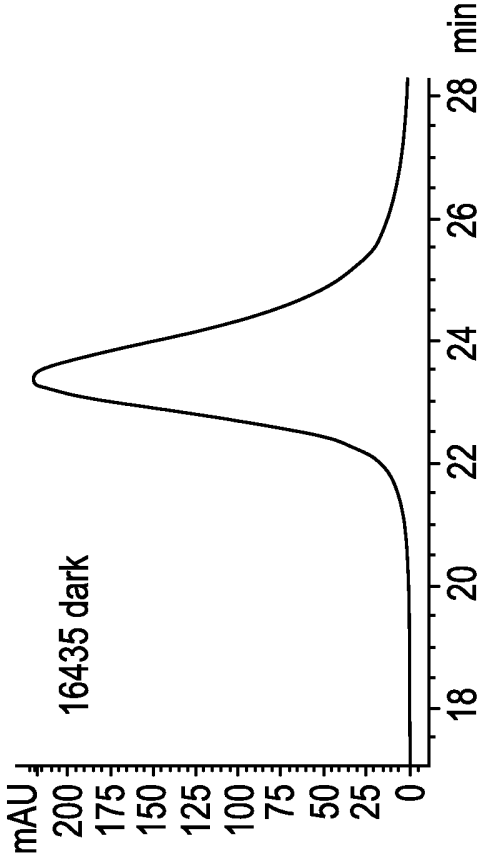


FIG. 14B

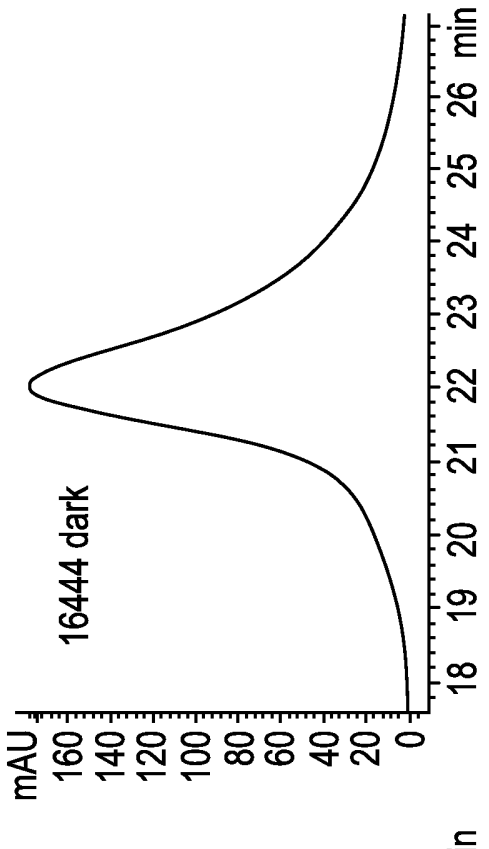


FIG. 14C

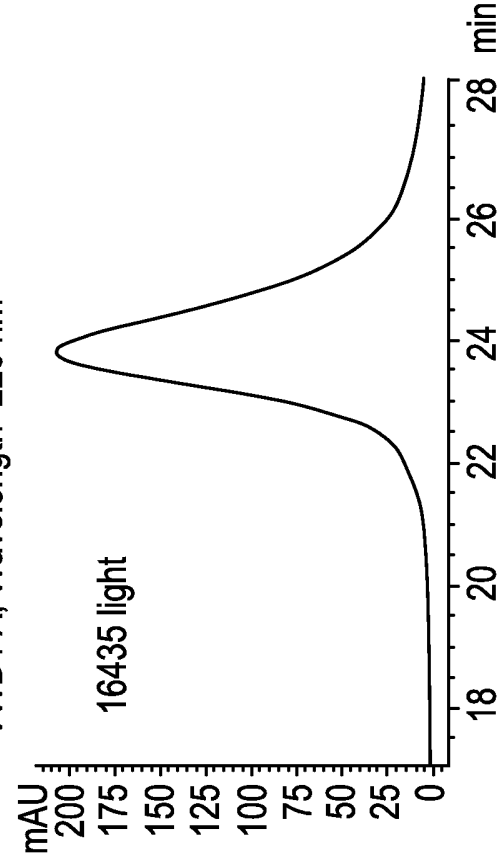


FIG. 14D

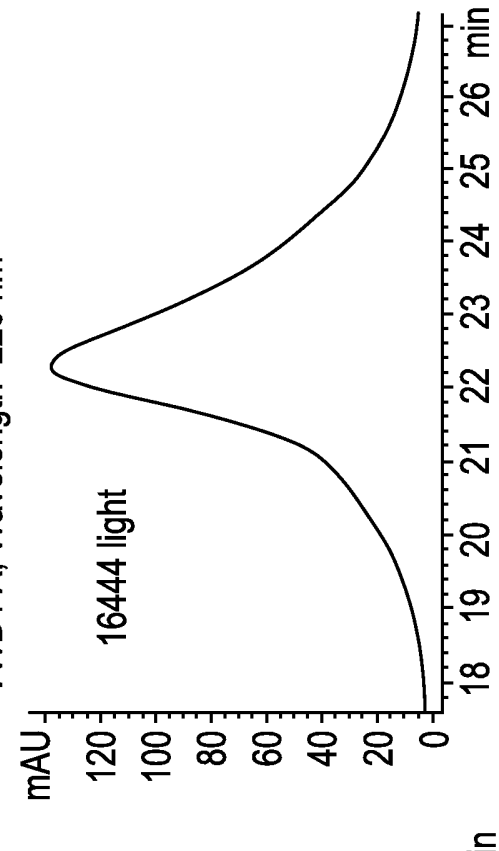


FIG. 15

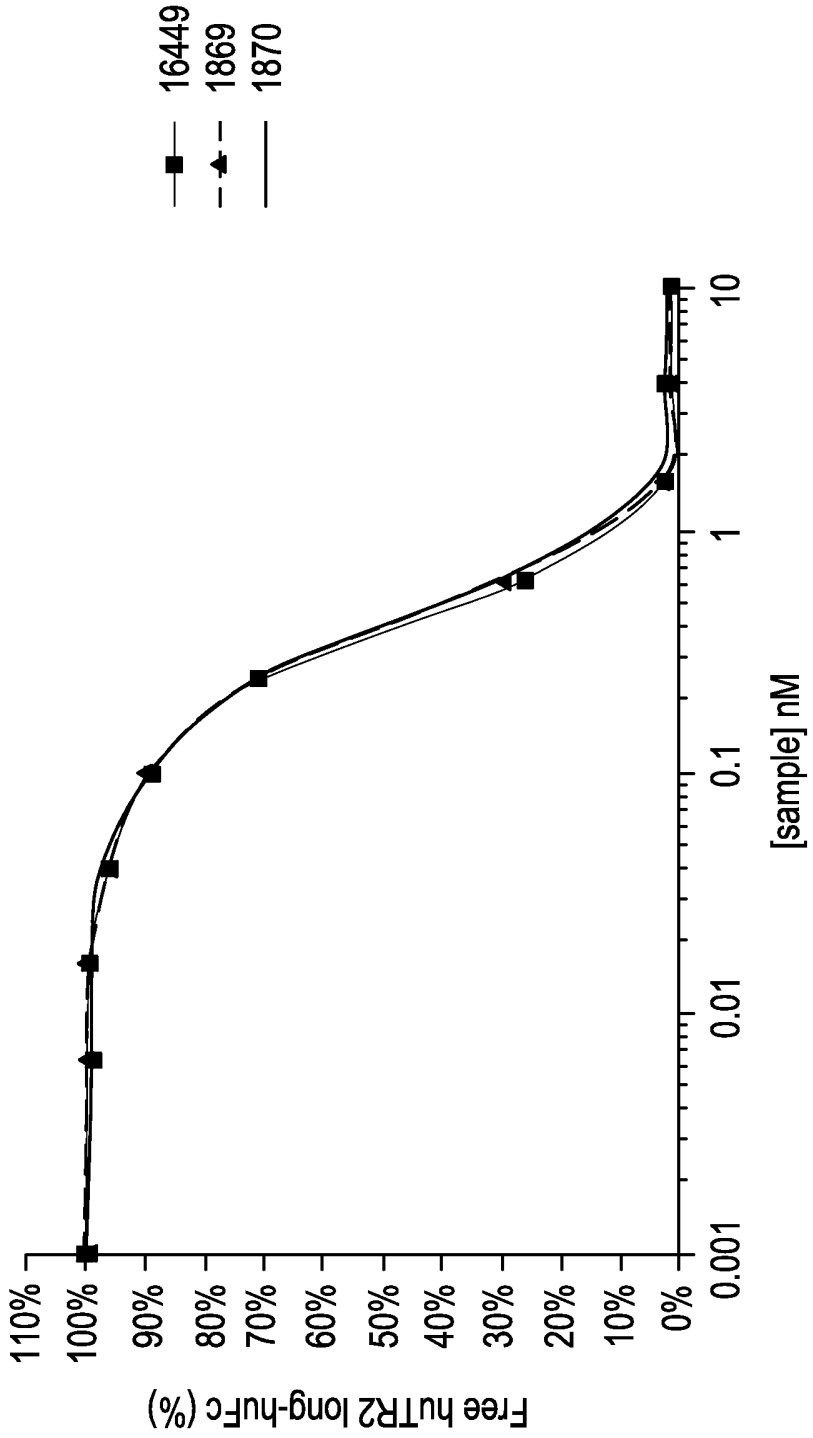

16449
1869
1870
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Free huTR2 long-huFc (%)
0.001
0.01
0.1
1
10
[sample] nM

FIG. 16

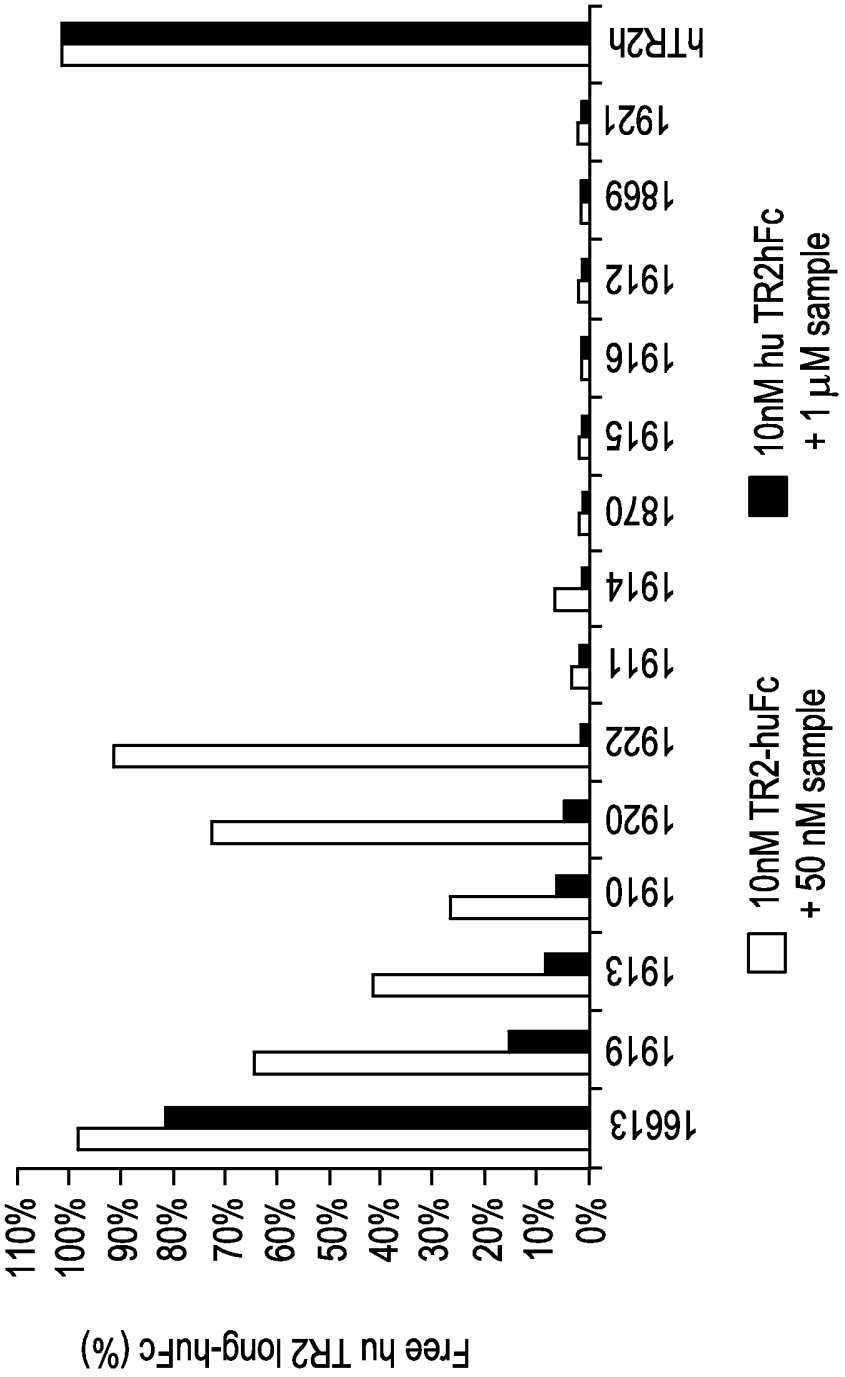

Free hu TR2 long-huFc (%)
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
16613
1919
1913
1910
1920
1922
1911
1914
1870
1915
1916
1912
1869
1921
hTR2h
10nM TR2-huFc
+ 50 nM sample
10nM hu TR2hFc
+ 1 µM sample

FIG. 17

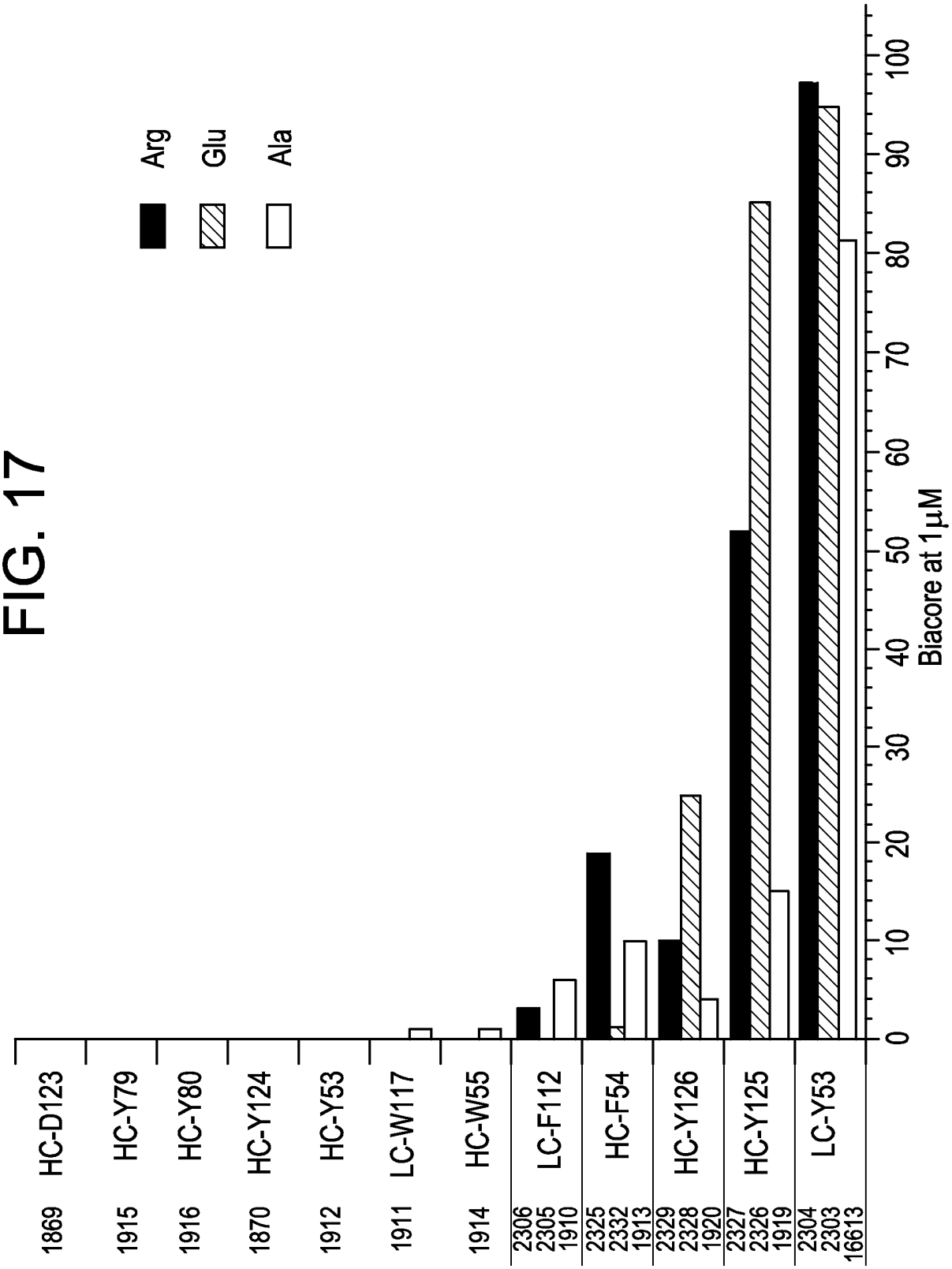

Arg
Glu
Ala
Biacore at 1µM
0
10
20
30
40
50
60
70
80
90
100
1869 HC-D123
1915 HC-Y79
1916 HC-Y80
1870 HC-Y124
1912 HC-Y53
1911 LC-W117
1914 HC-W55
2306 2305 1910 LC-F112
2325 2332 1913 HC-F54
2329 2328 1920 HC-Y126
2327 2326 1919 HC-Y125
2304 2303 16613 LC-Y53

FIG. 18

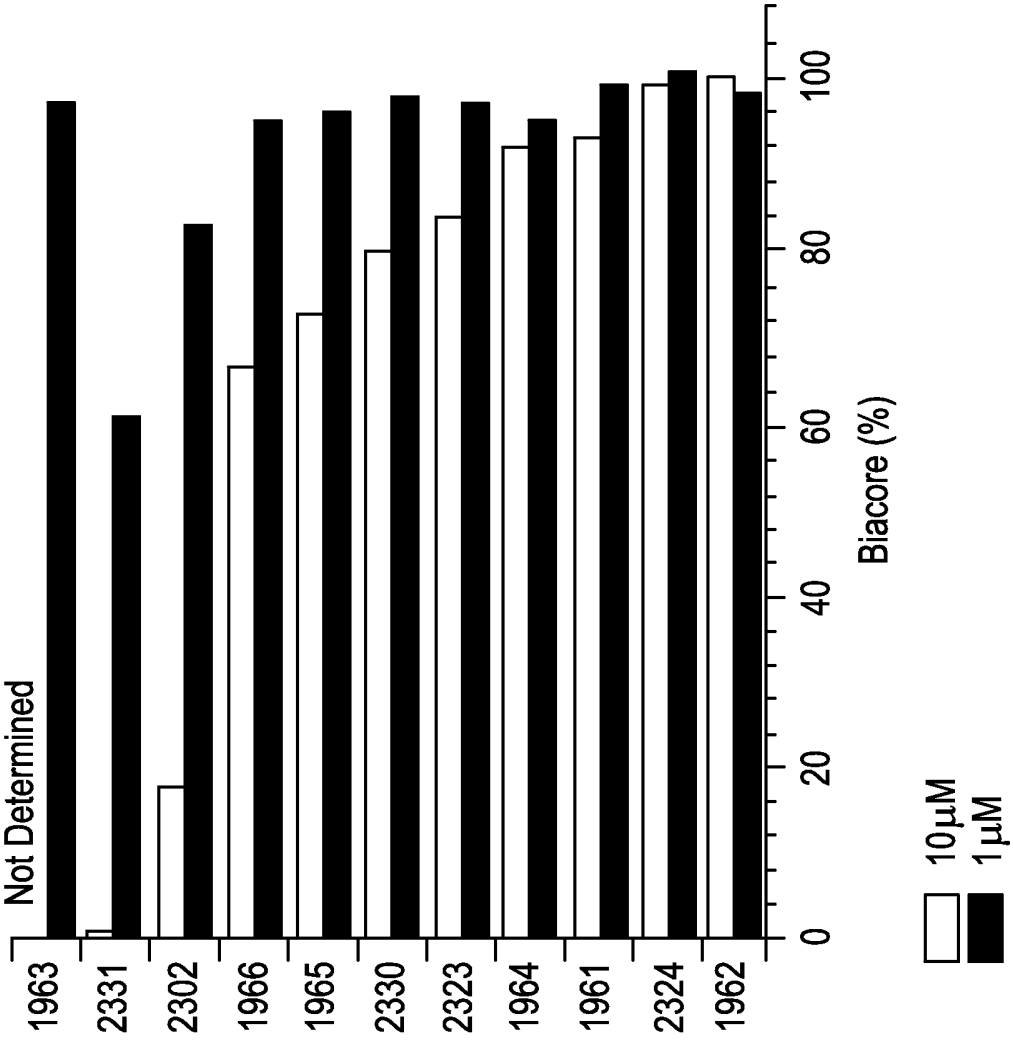

Not Determined
1963
2331
2302
1966
1965
2330
2323
1964
1961
2324
1962
0
20
40
60
80
100
Biacore (%)
10μM
1μM

FIG. 19

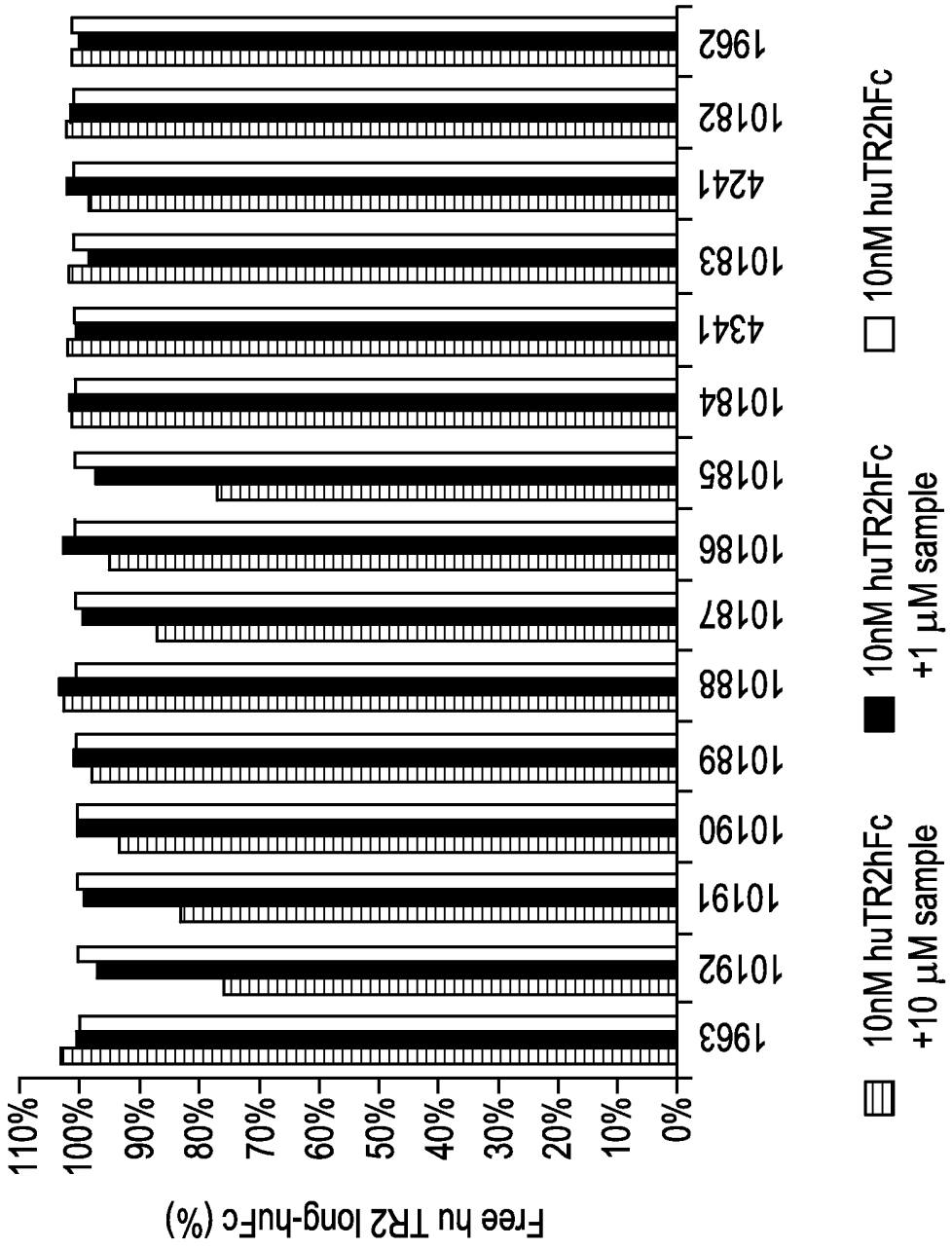

Free hu TR2 long-huFc (%)
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1963
10192
10191
10190
10189
10188
10187
10186
10185
10184
4341
10183
4241
10182
1962
10nM huTR2hFc +10 µM sample
10nM huTR2hFc +1 µM sample
10nM huTR2hFc

FIG. 20A

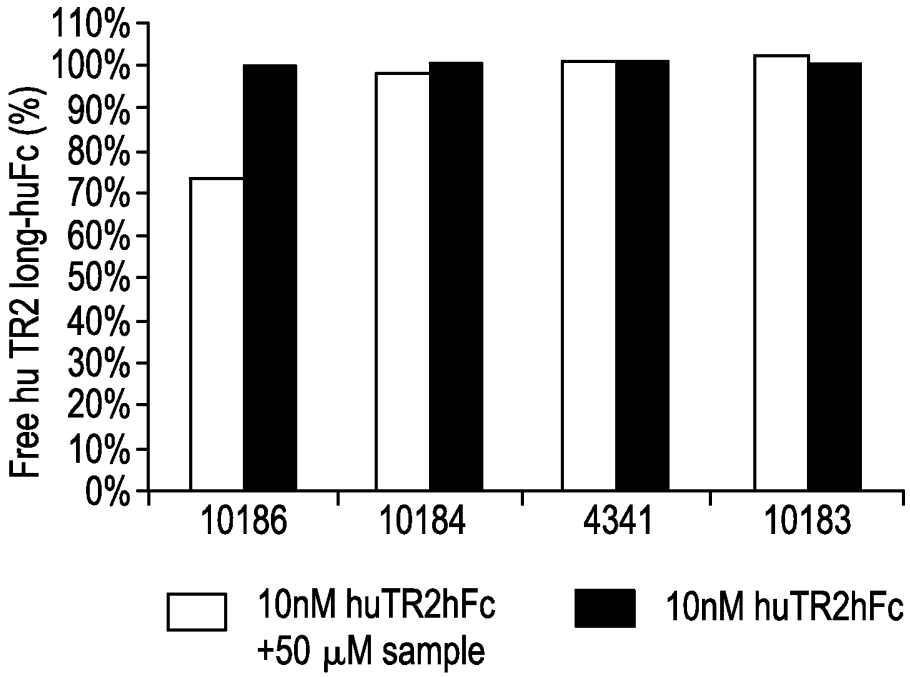


FIG. 20B

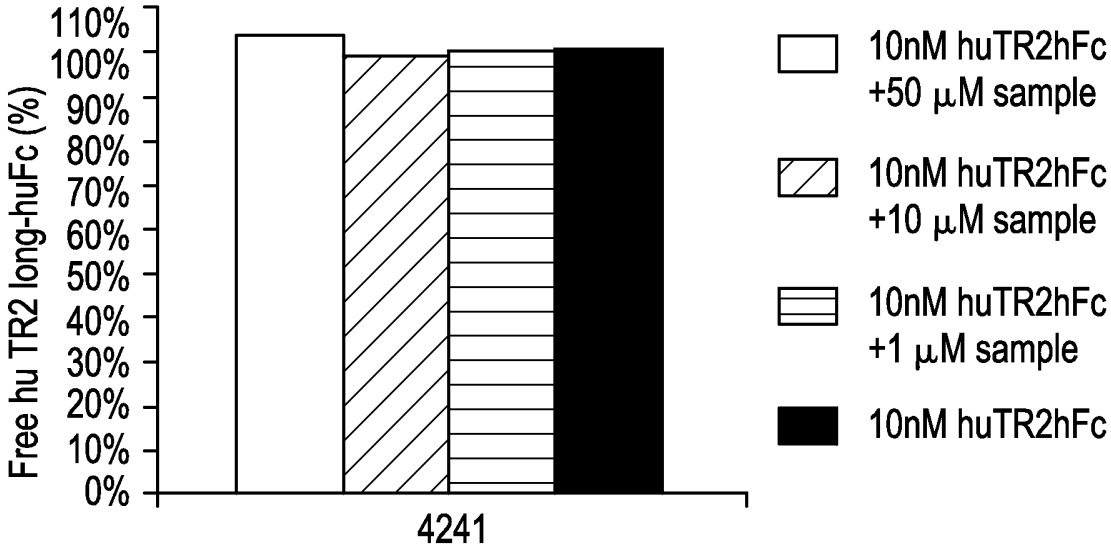


FIG. 20C

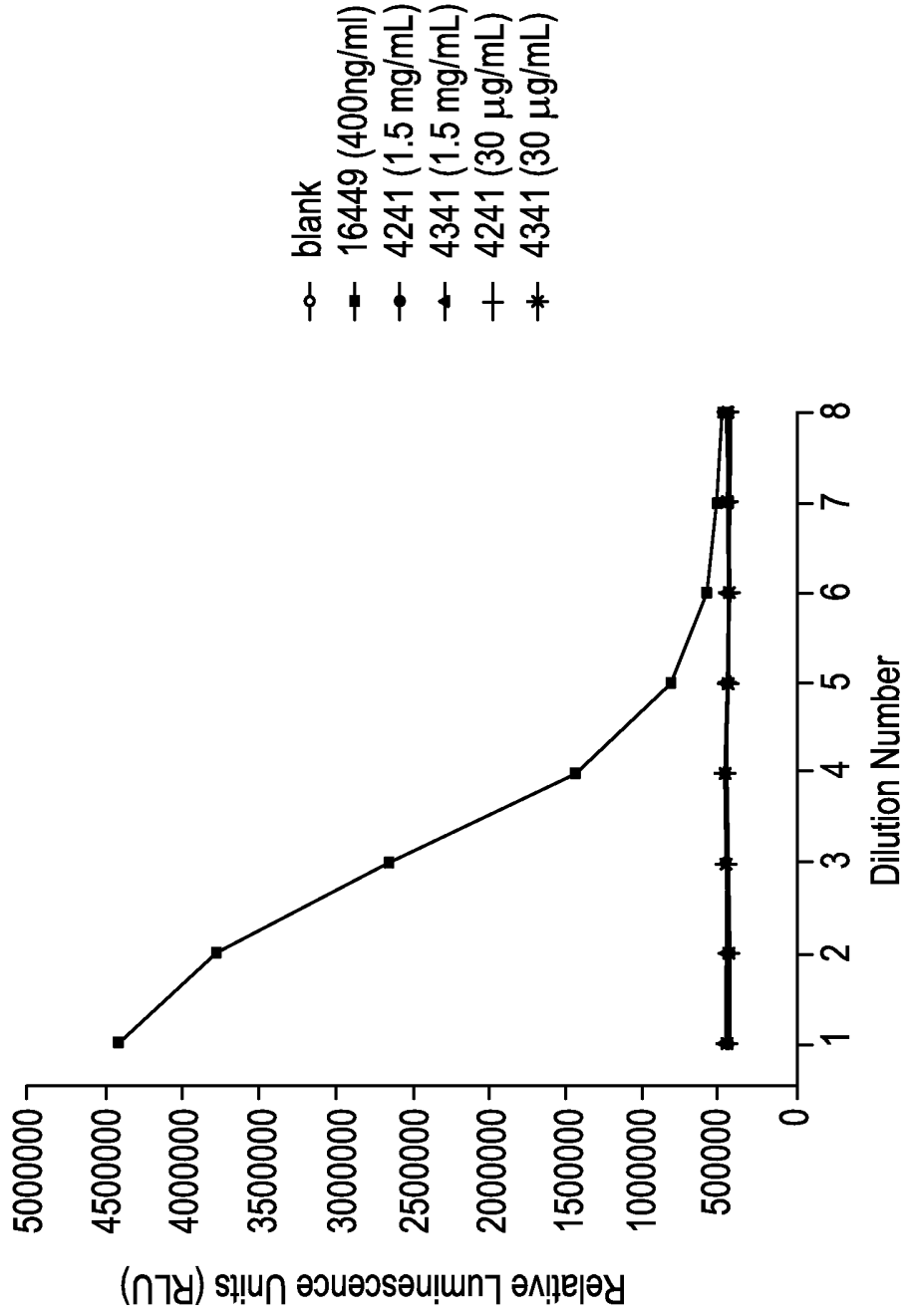

blank
16449 (400ng/ml)
4241 (1.5 mg/mL)
4341 (1.5 mg/mL)
4241 (30 µg/mL)
4341 (30 µg/mL)
Relative Luminescence Units (RLU)
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
1
2
3
4
5
6
7
8
Dilution Number

FIG. 21A

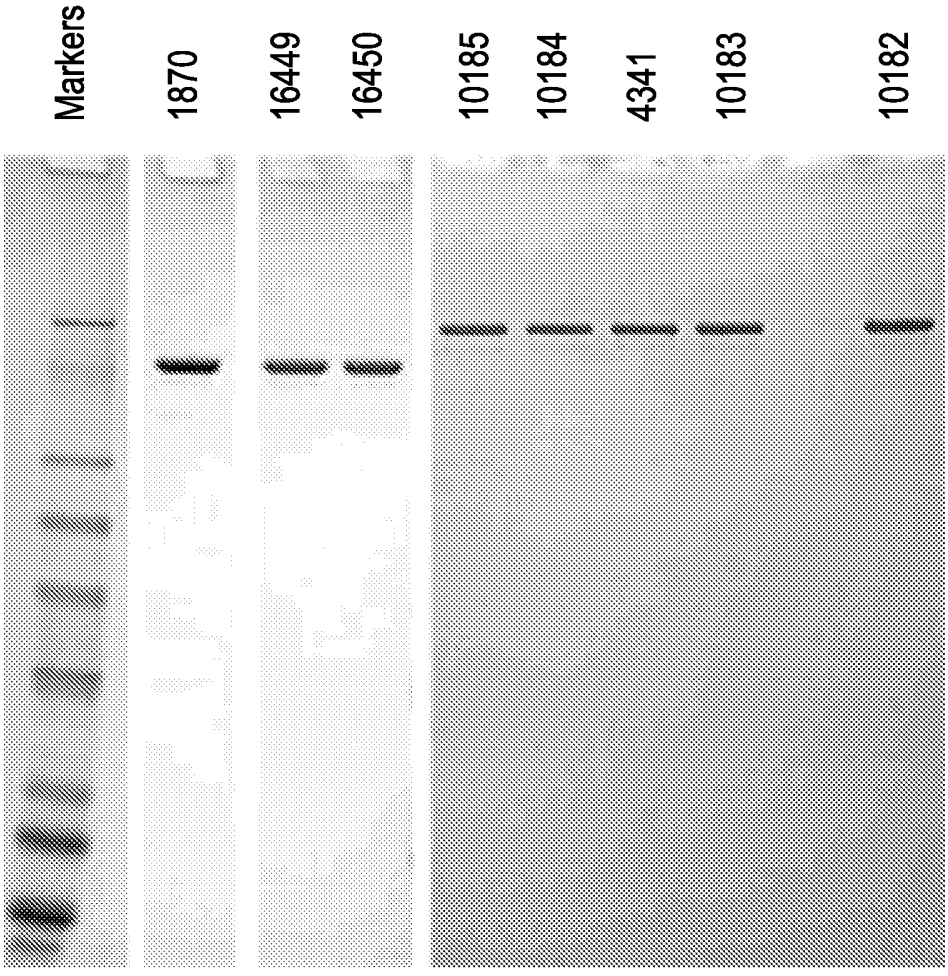


FIG. 21B

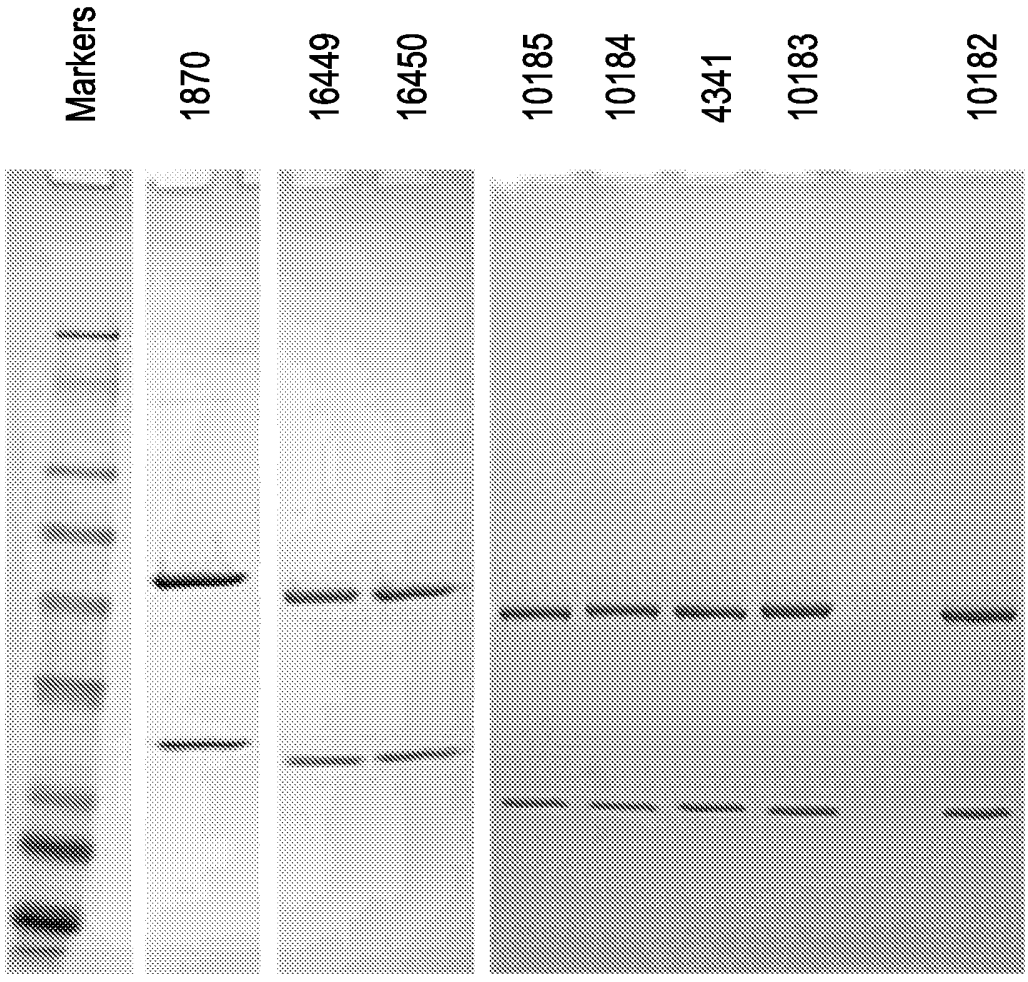


FIG. 22A

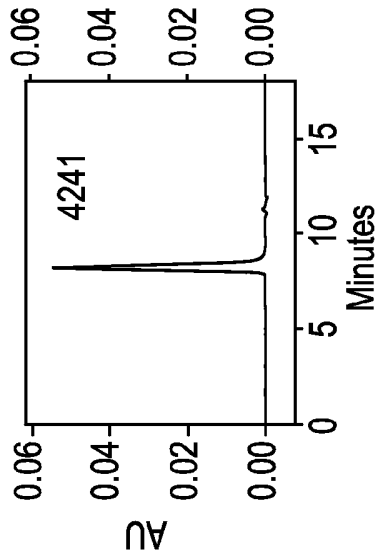


FIG. 22B

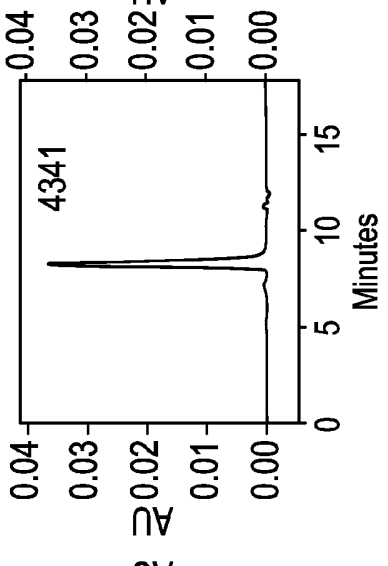


FIG. 22C

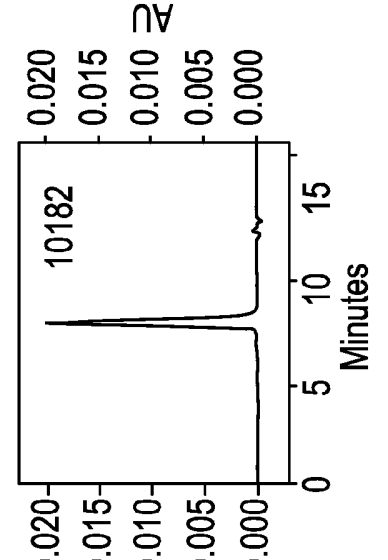


FIG. 22D

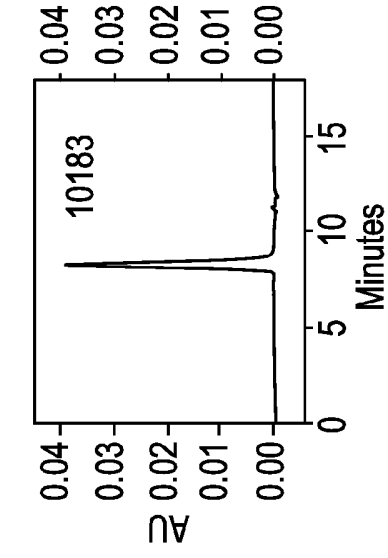


FIG. 22E

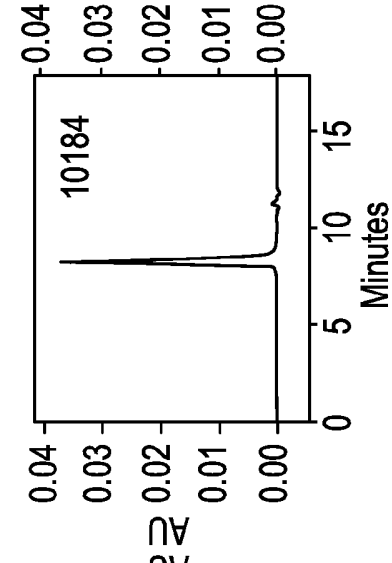


FIG. 22F

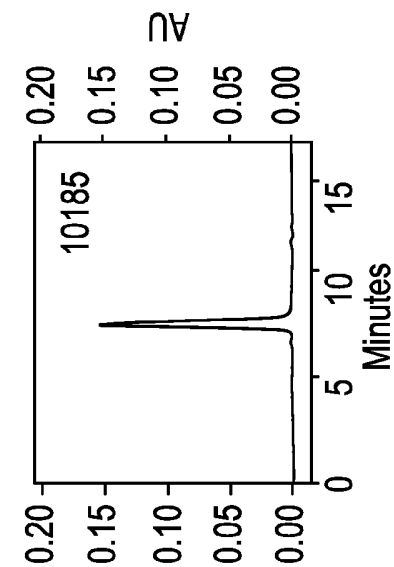


FIG. 23A

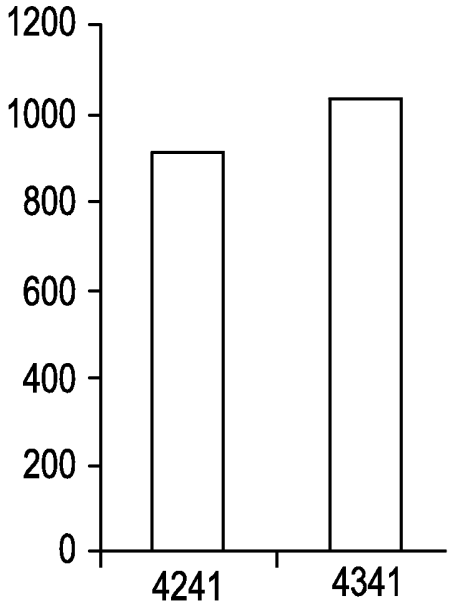


FIG. 23B

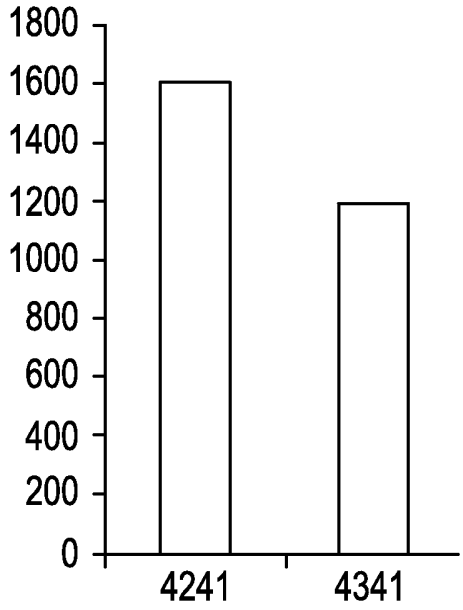


FIG. 24A

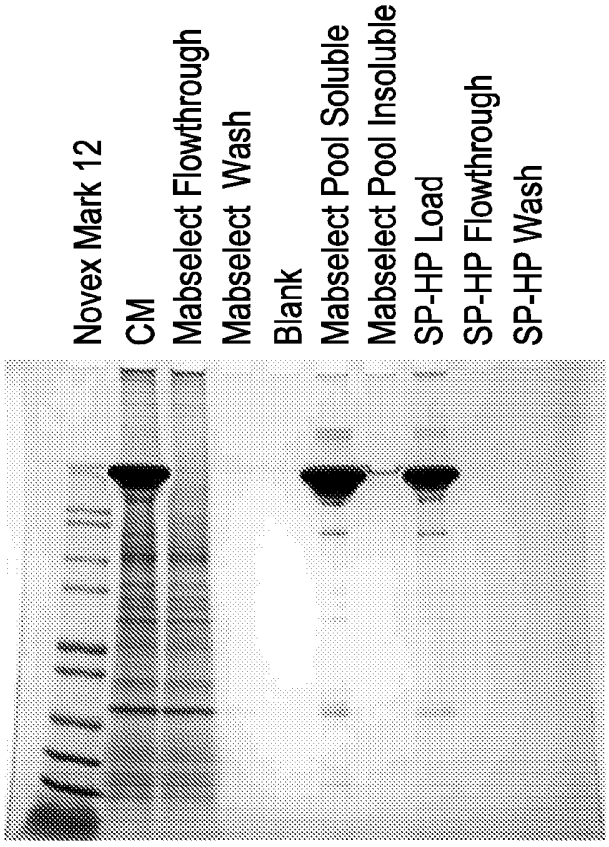


FIG. 24B

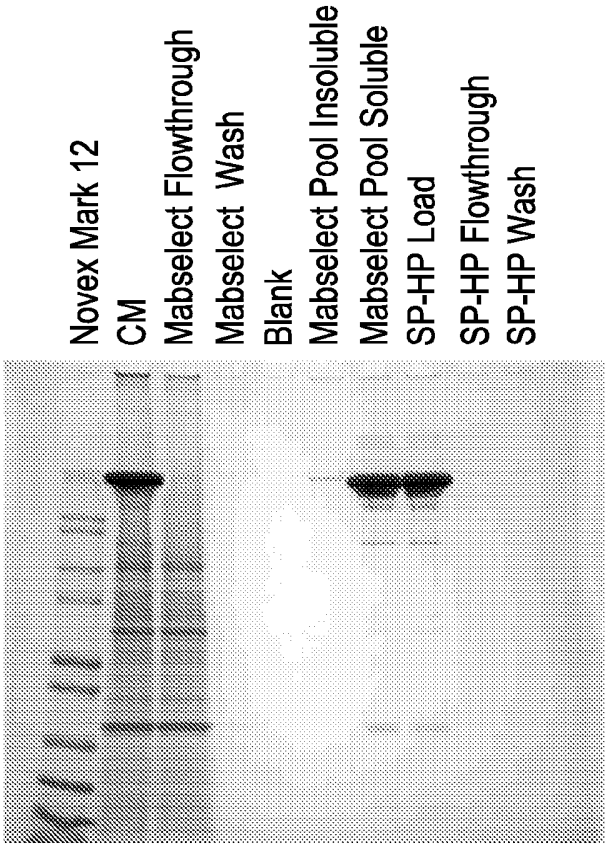


FIG. 25

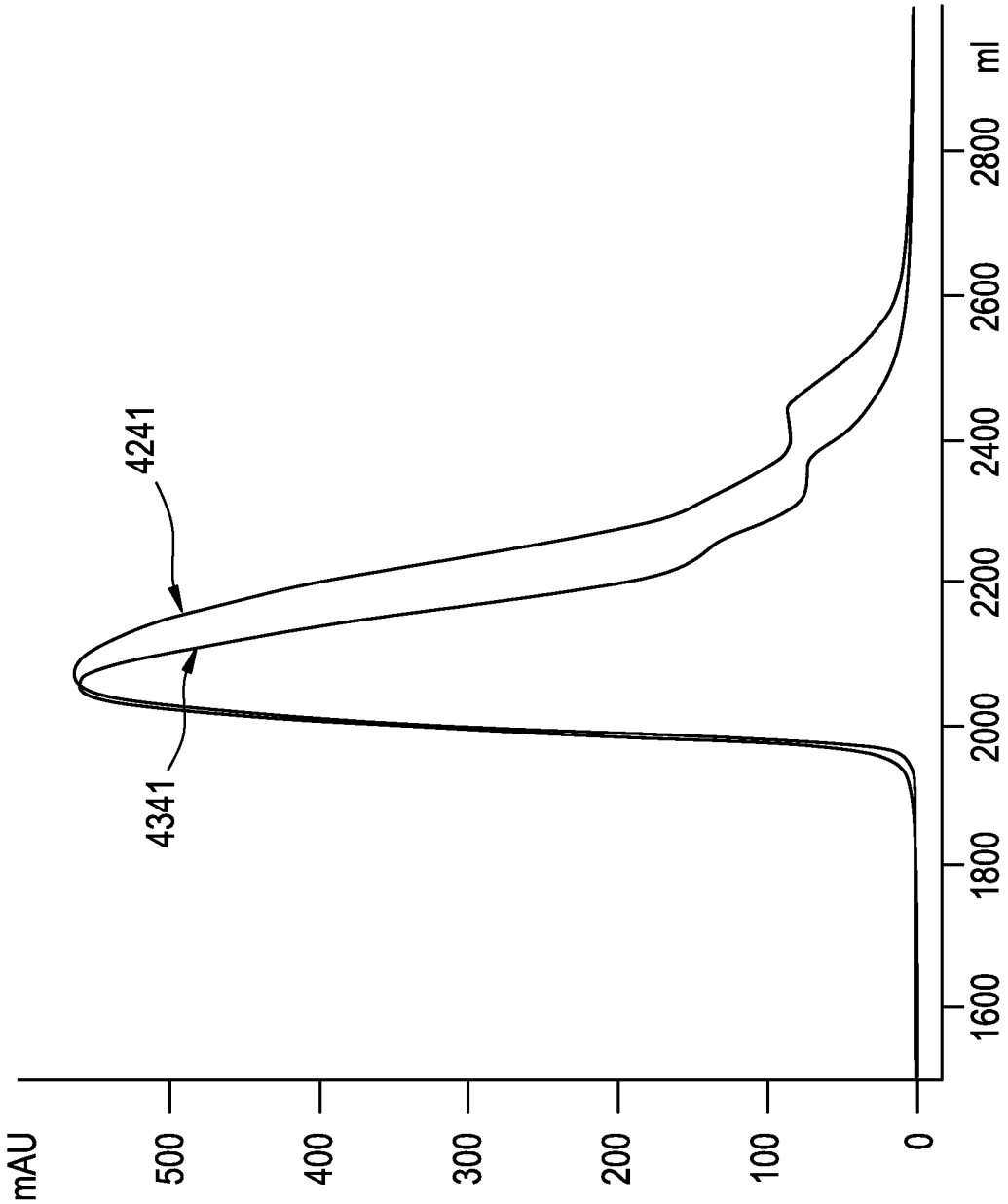

4241
4341
mAU
500
400
300
200
100
0
1600
1800
2000
2200
2400
2600
2800
ml

FIG. 26A

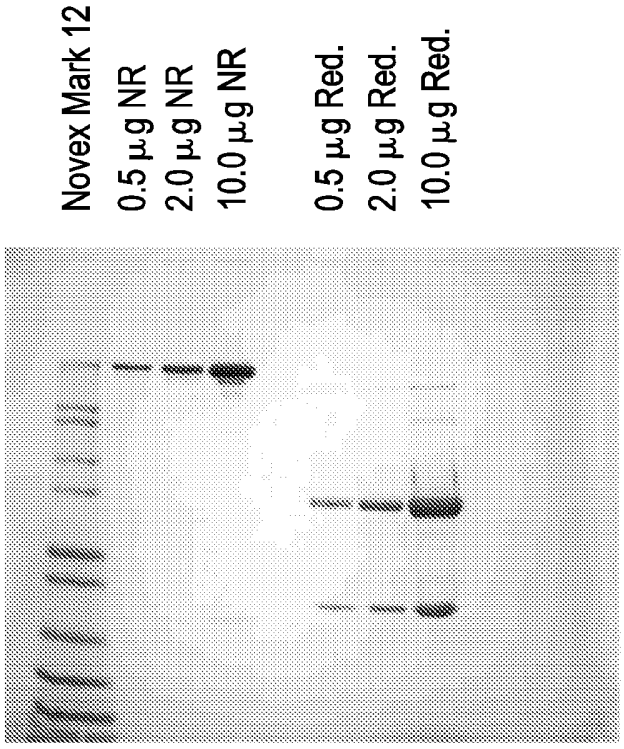


FIG. 26B

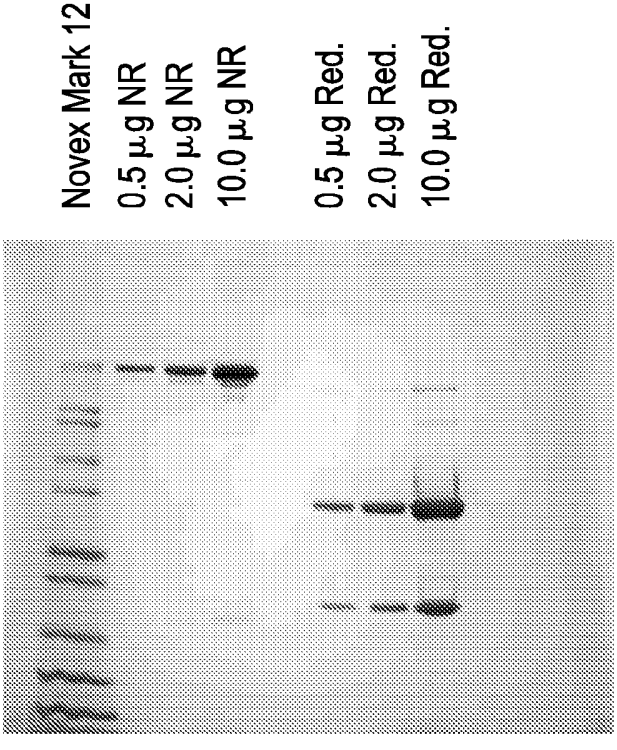


FIG. 27A

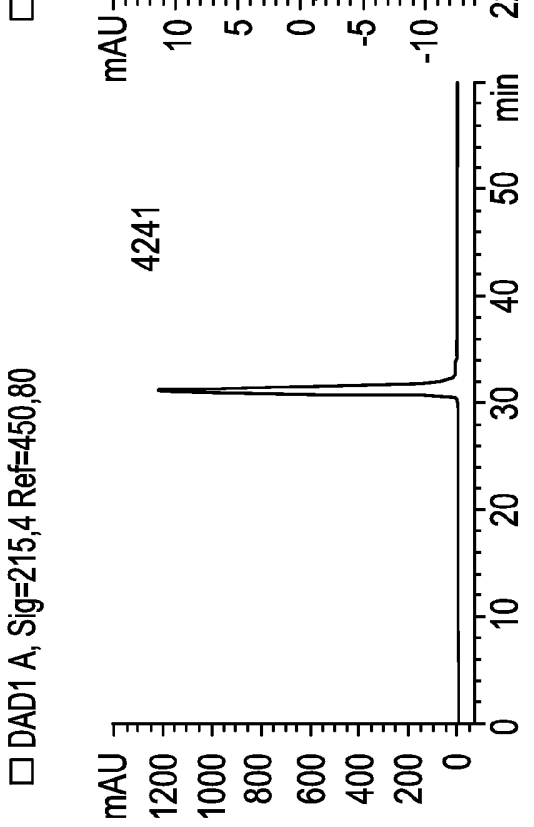


FIG. 27B

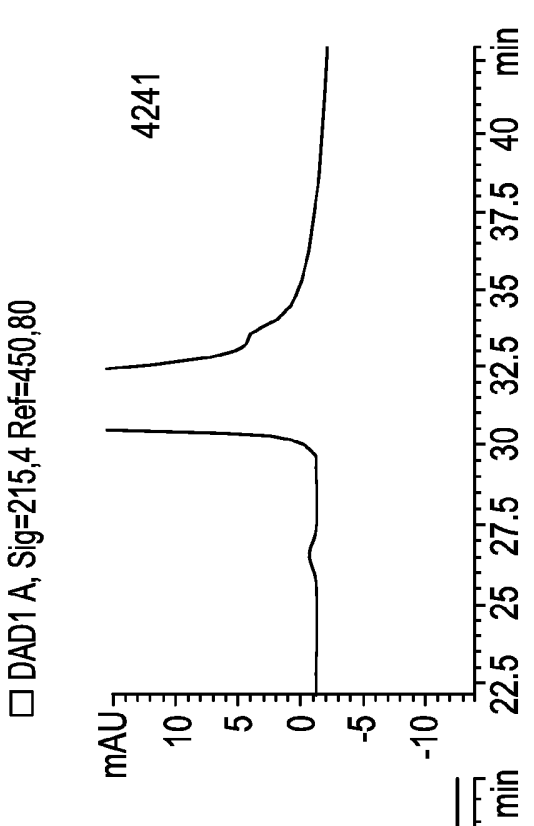


FIG. 28

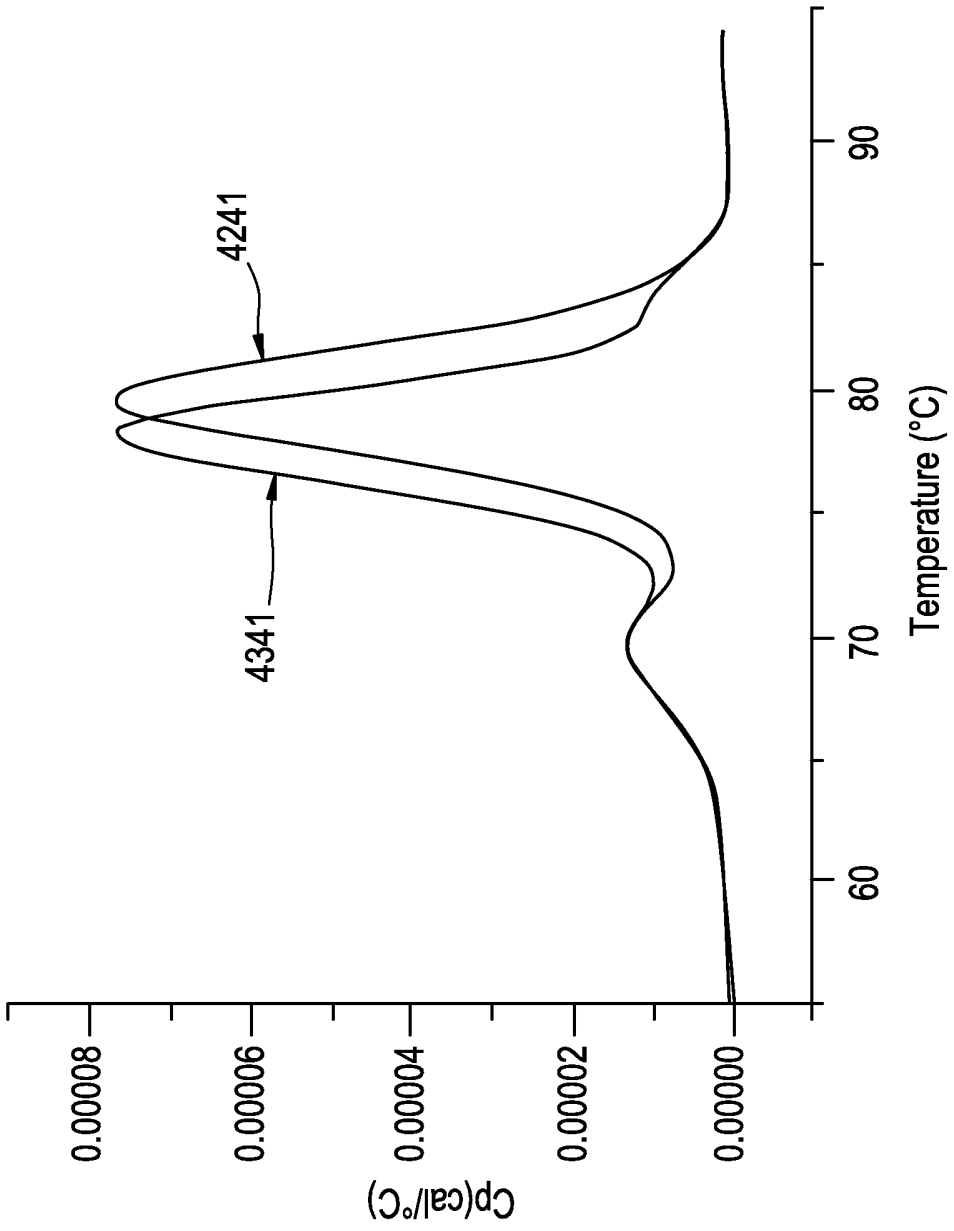

4241
4341
0.00008
0.00006
0.00004
0.00002
0.00000
Cp(cal/°C)
60
70
80
90
Temperature (°C)

FIG. 29A

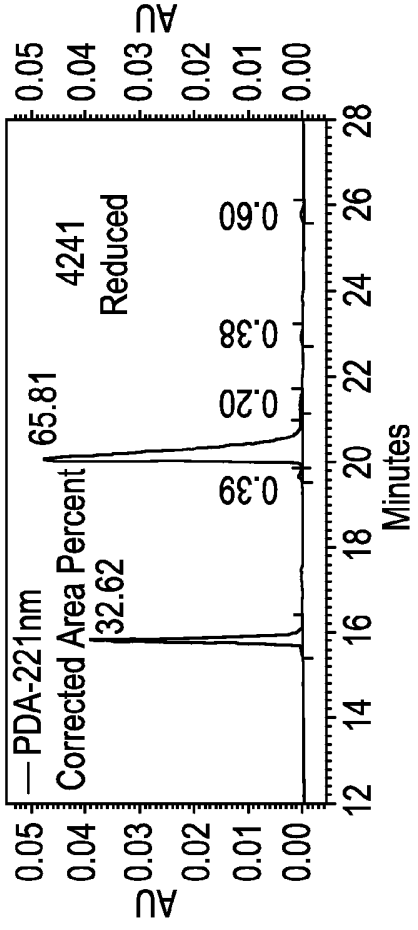


FIG. 29B

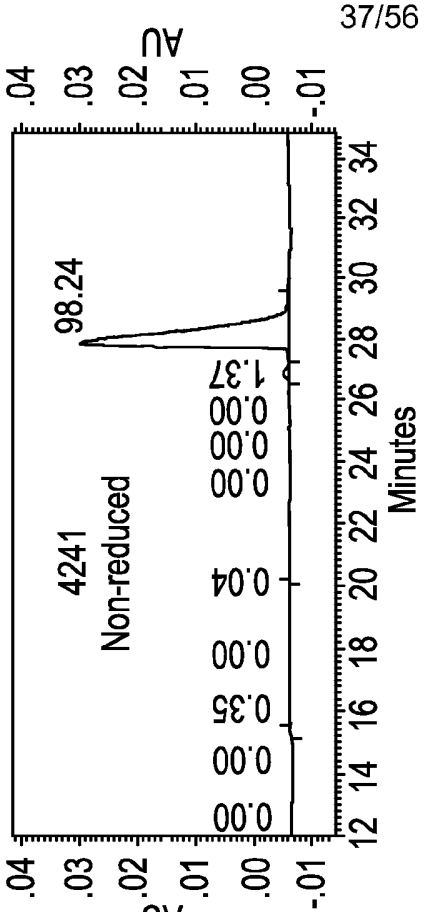


FIG. 29C

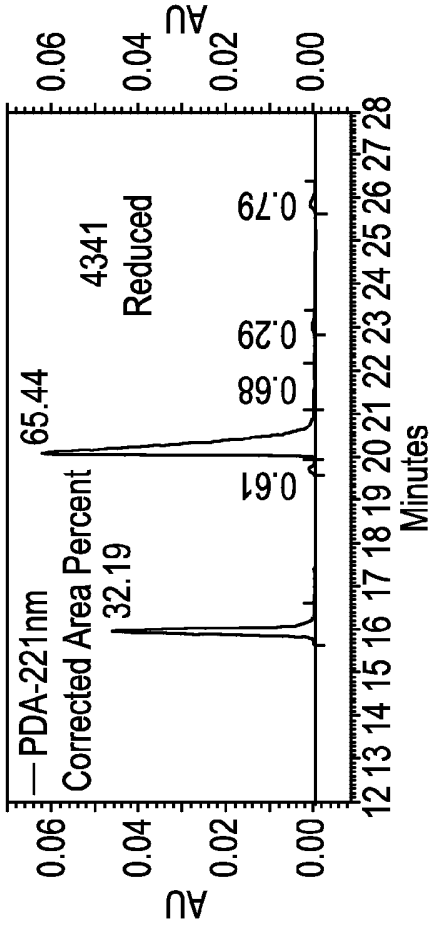


FIG. 29D

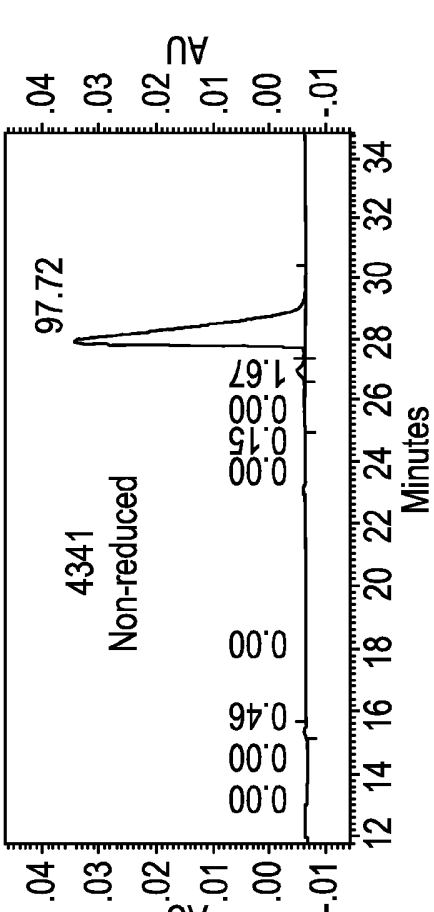


FIG. 30

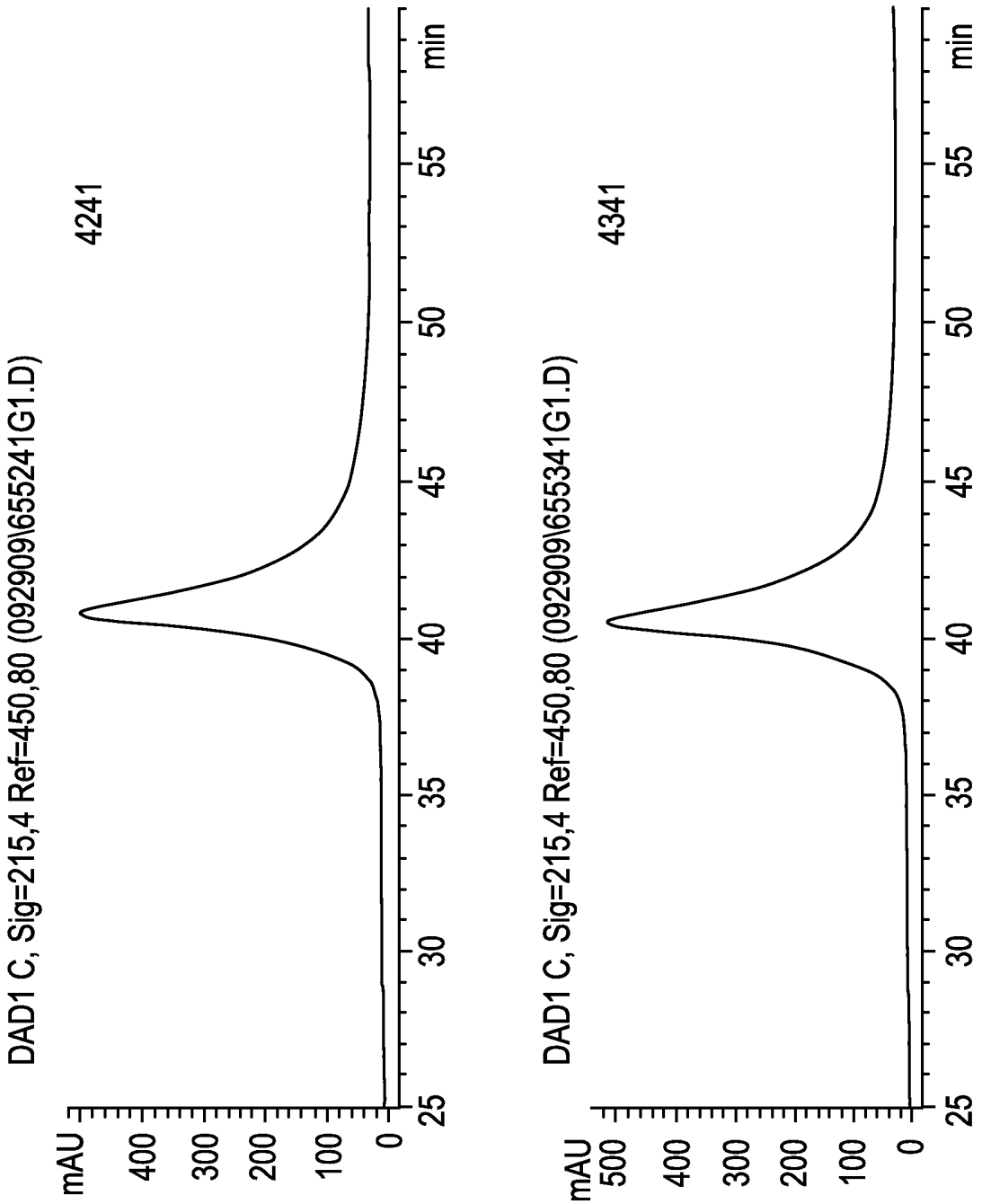
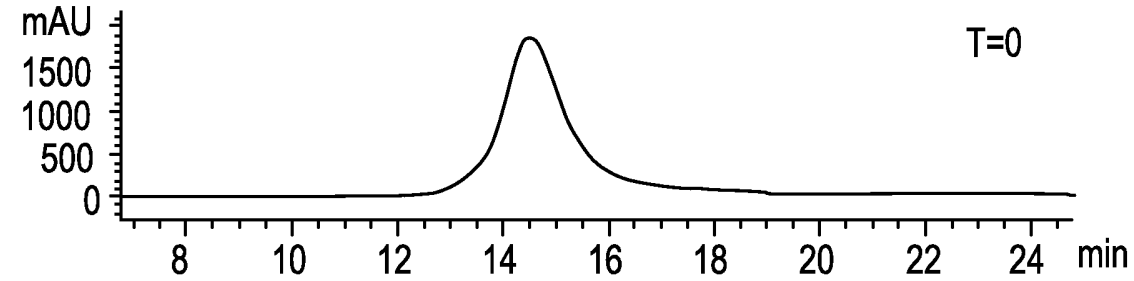
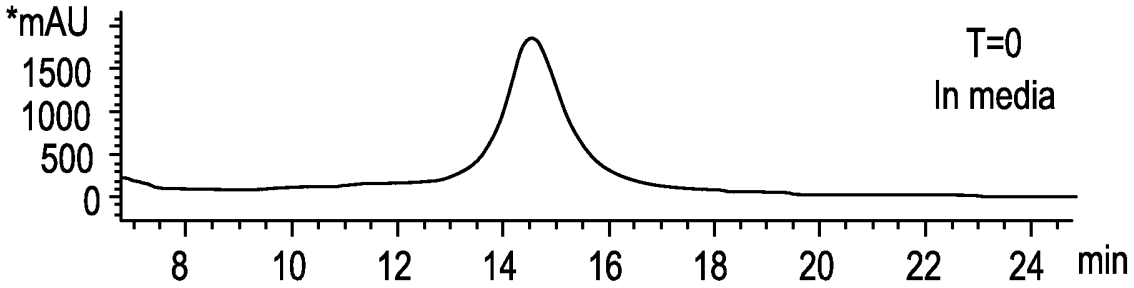
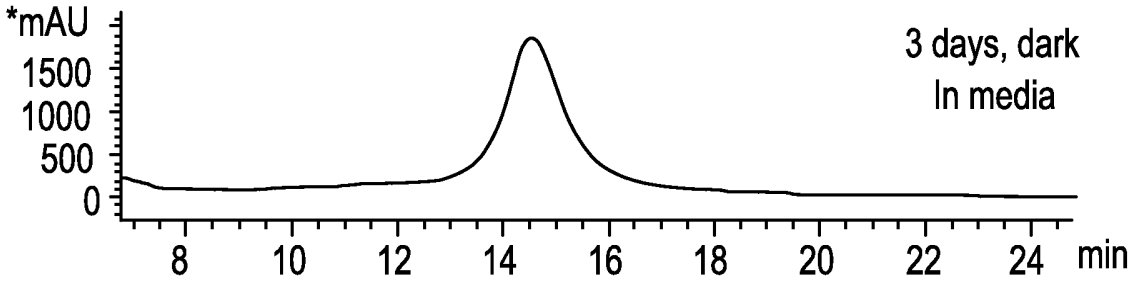

DAD1 C, Sig=215,4 Ref=450,80 (092909\65241G1.D)
4241
mAU
400
300
200
100
0
25
30
35
40
45
50
55
min
DAD1 C, Sig=215,4 Ref=450,80 (092909\65341G1.D)
4341
mAU
500
400
300
200
100
0
25
30
35
40
45
50
55
min

FIG. 31A


mAU
1500
1000
500
0
8
10
12
14
16
18
20
22
24
min
T=0


*mAU
1500
1000
500
0
8
10
12
14
16
18
20
22
24
min
T=0
In media


*mAU
1500
1000
500
0
8
10
12
14
16
18
20
22
24
min
3 days, dark
In media

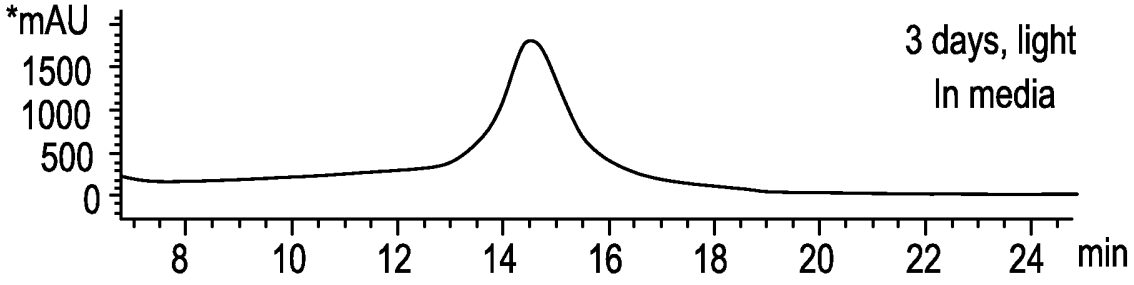

*mAU
1500
1000
500
0
8
10
12
14
16
18
20
22
24
min
3 days, light
In media

FIG. 31B

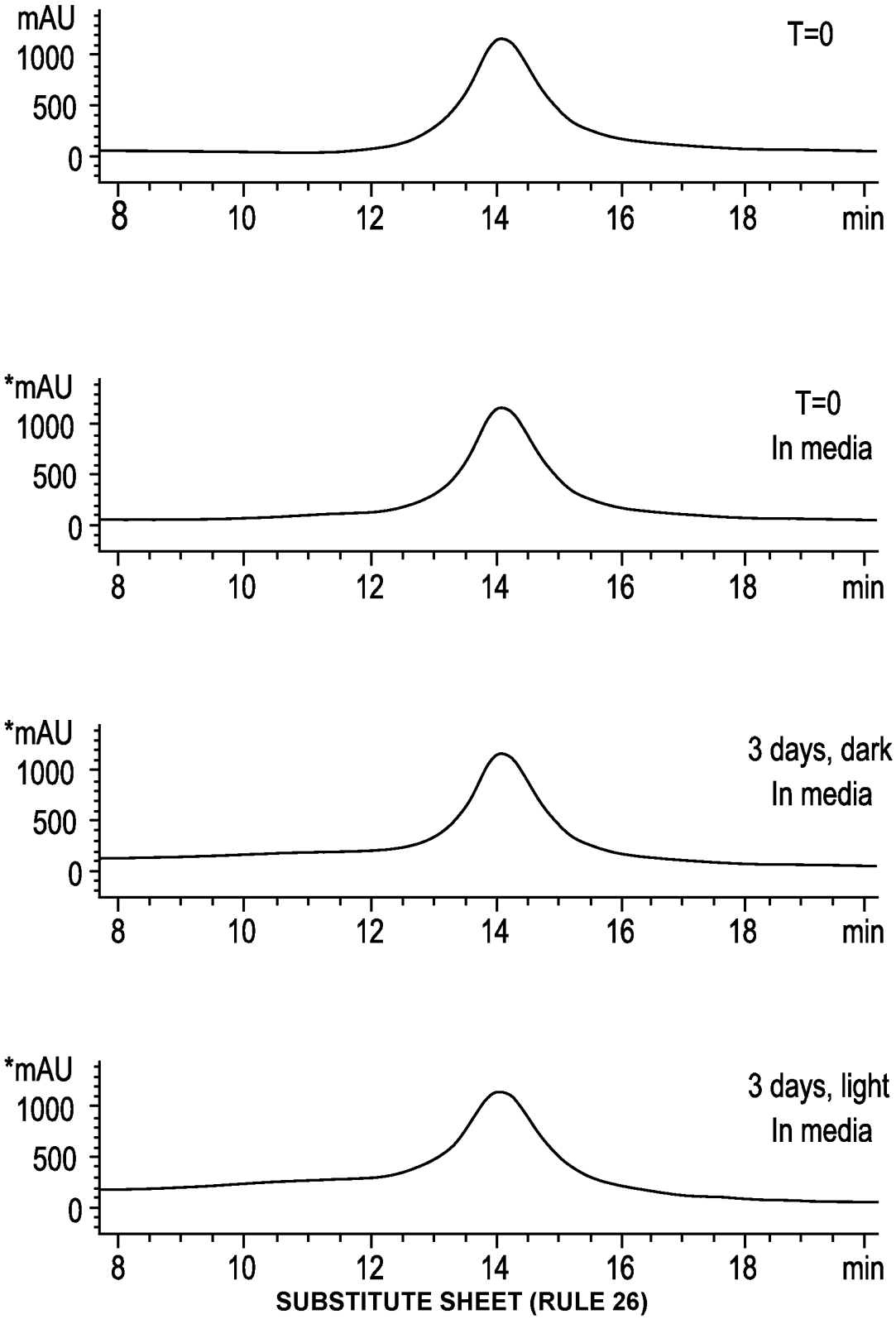

mAU
1000
500
0
8
10
12
14
16
18
min
T=0
*mAU
1000
500
0
8
10
12
14
16
18
min
T=0
In media
*mAU
1000
500
0
8
10
12
14
16
18
min
3 days, dark
In media
*mAU
1000
500
0
8
10
12
14
16
18
min
3 days, light
In media

FIG. 32

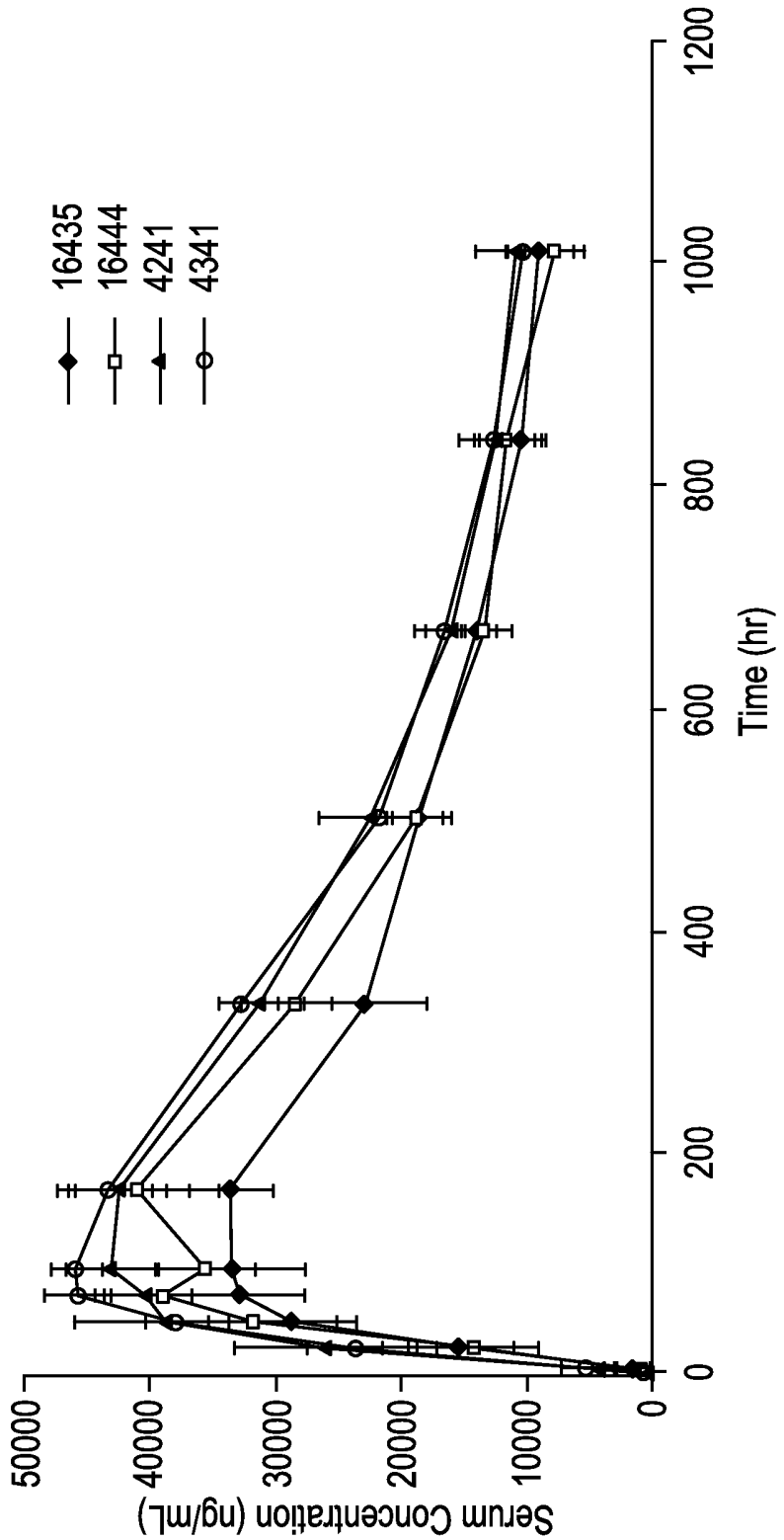

16435
16444
4241
4341
Serum Concentration (ng/mL)
50000
40000
30000
20000
10000
0
0
200
400
600
800
1000
1200
Time (hr)

FIG. 33

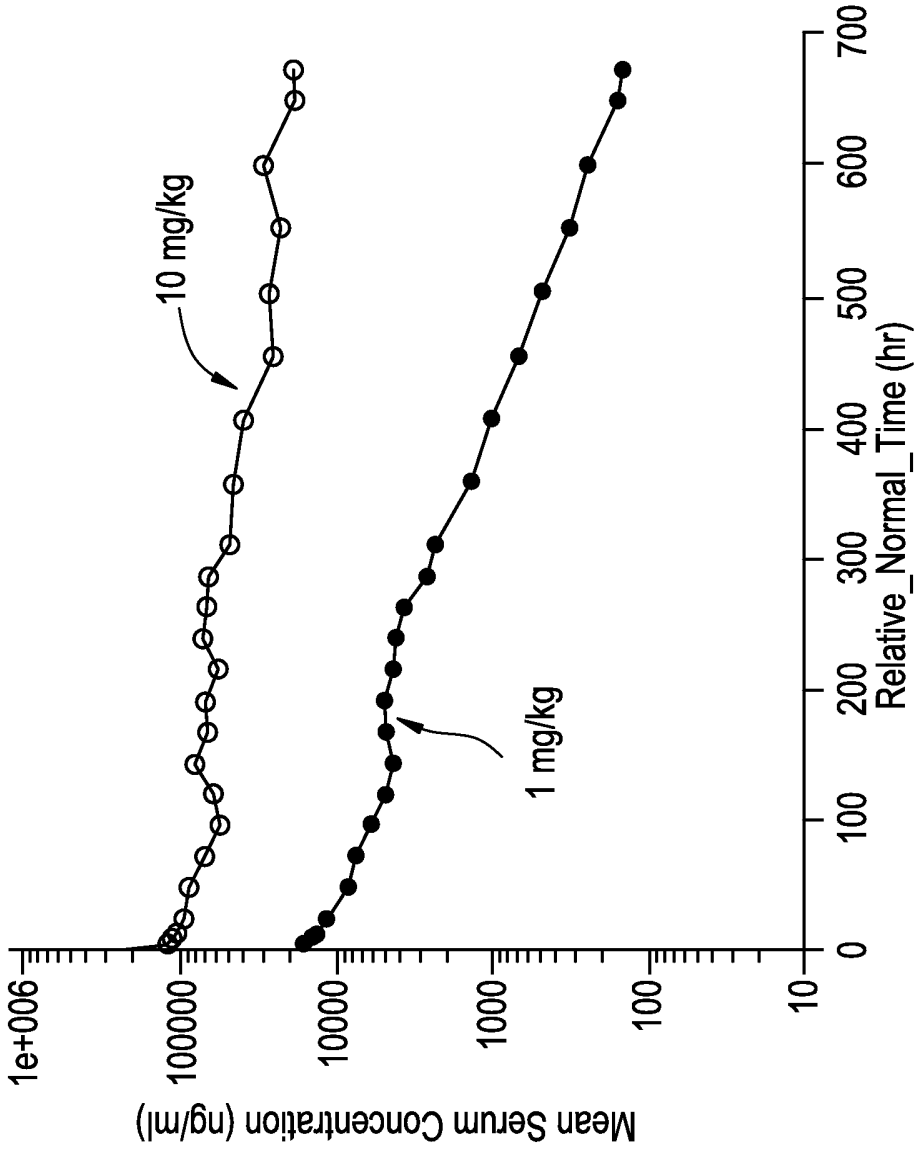

Mean Serum Concentration (ng/ml)
1e+006
100000
10000
1000
100
10
0
100
200
300
400
500
600
700
Relative_Normal_Time (hr)
10 mg/kg
1 mg/kg

FIG. 34

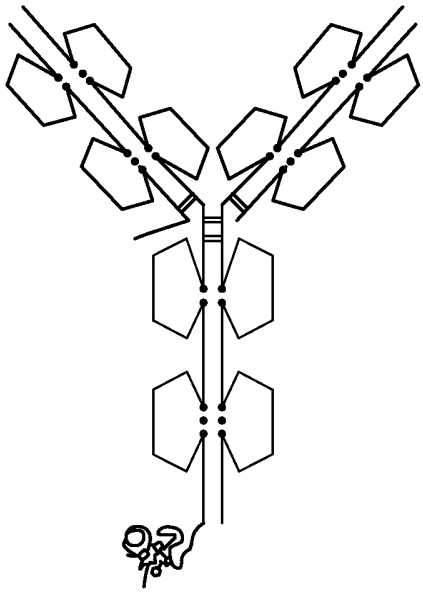


FIG. 35

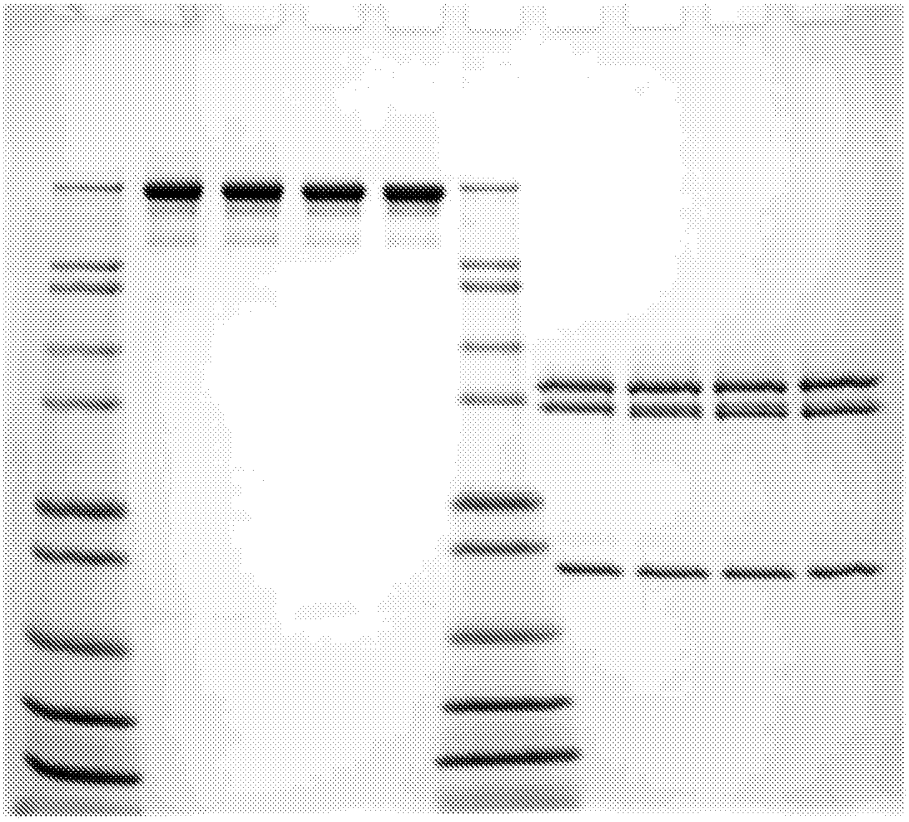


FIG. 36A

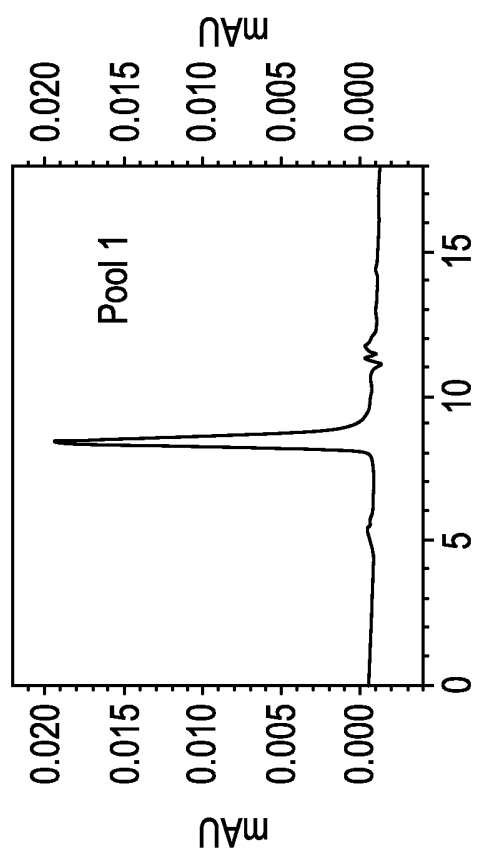


FIG. 36B

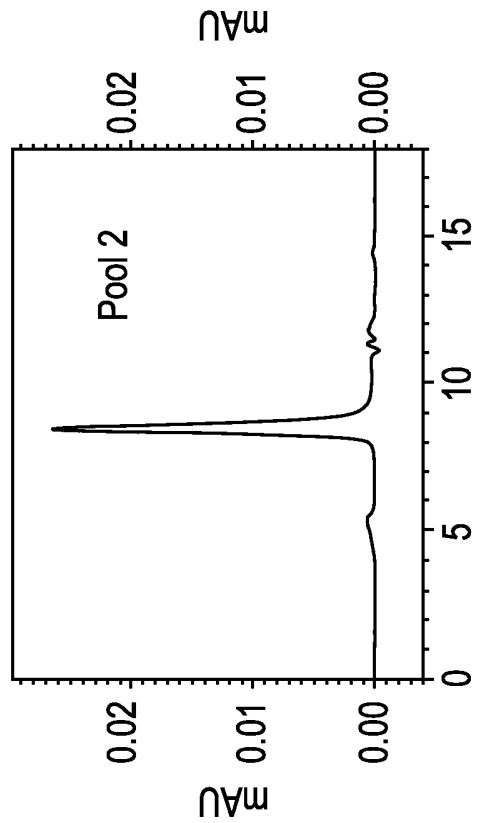


FIG. 36C

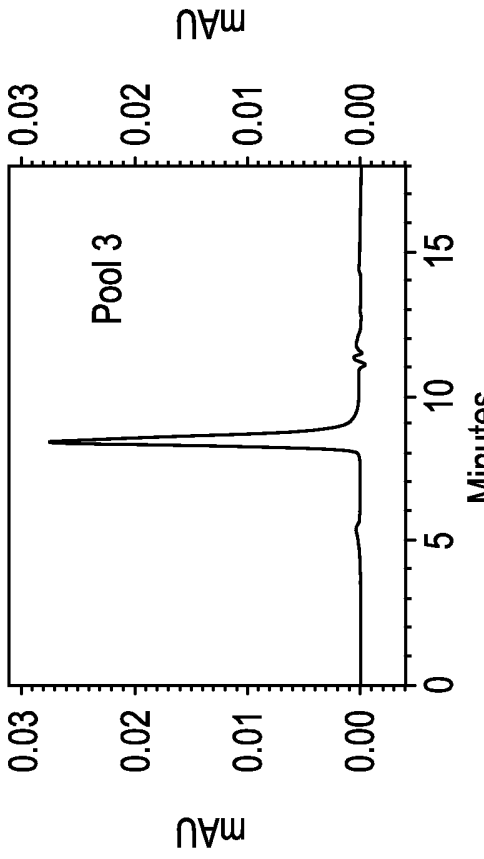


FIG. 36D

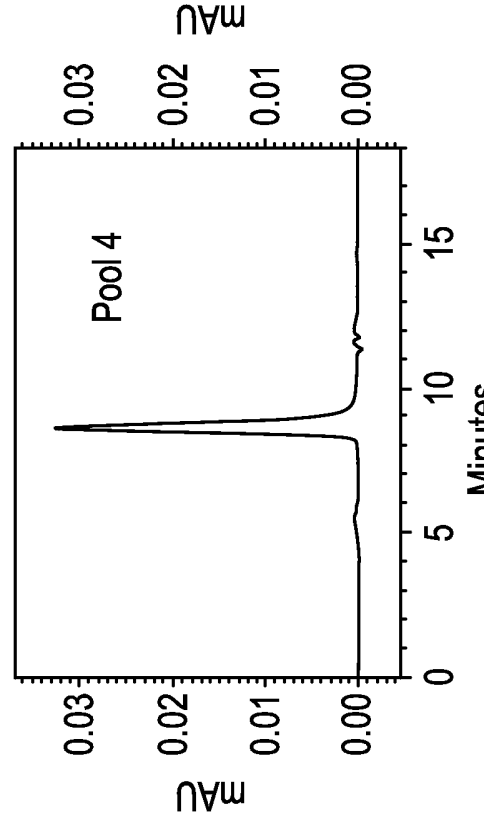


FIG. 37A

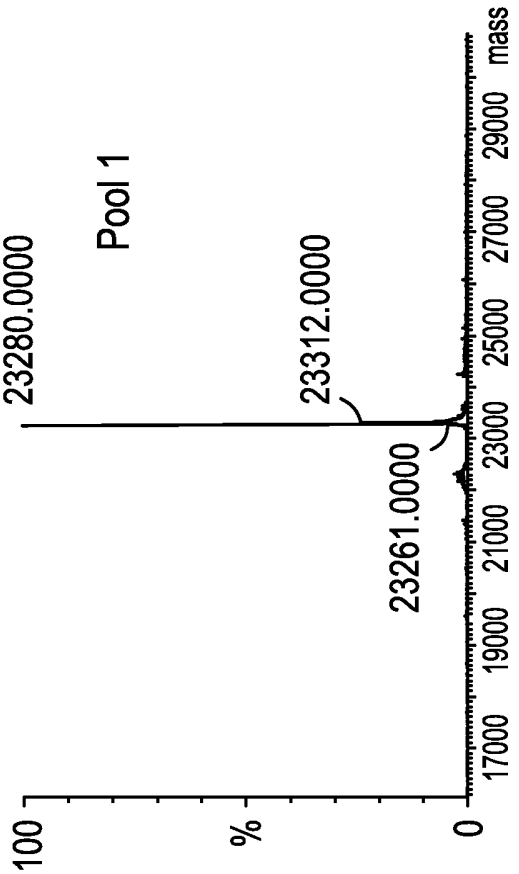


FIG. 37B

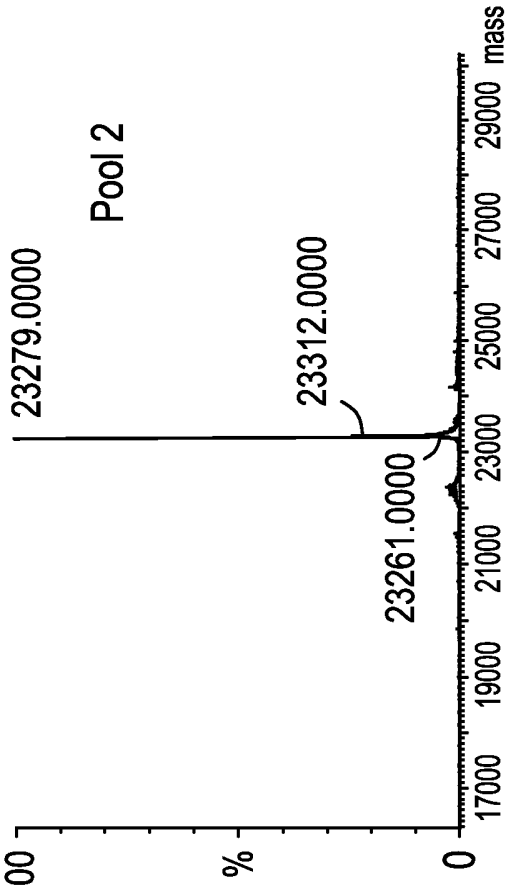


FIG. 37C

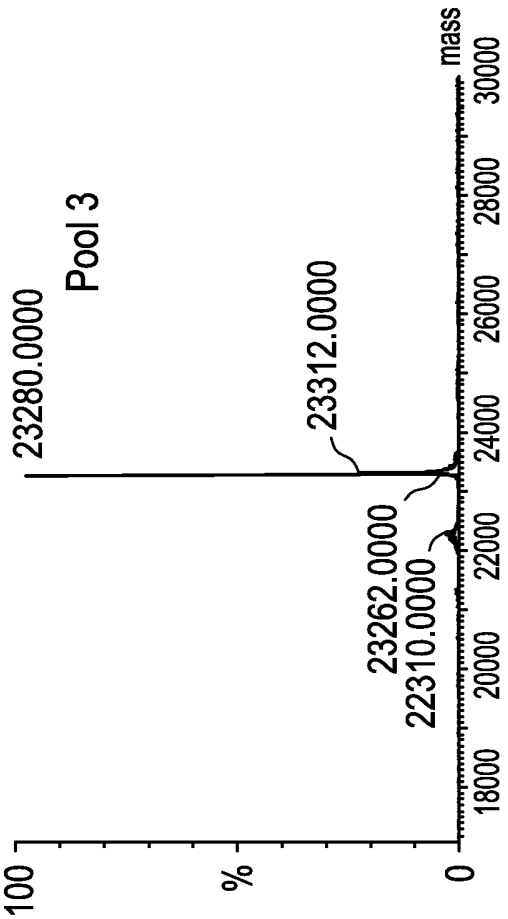


FIG. 37D

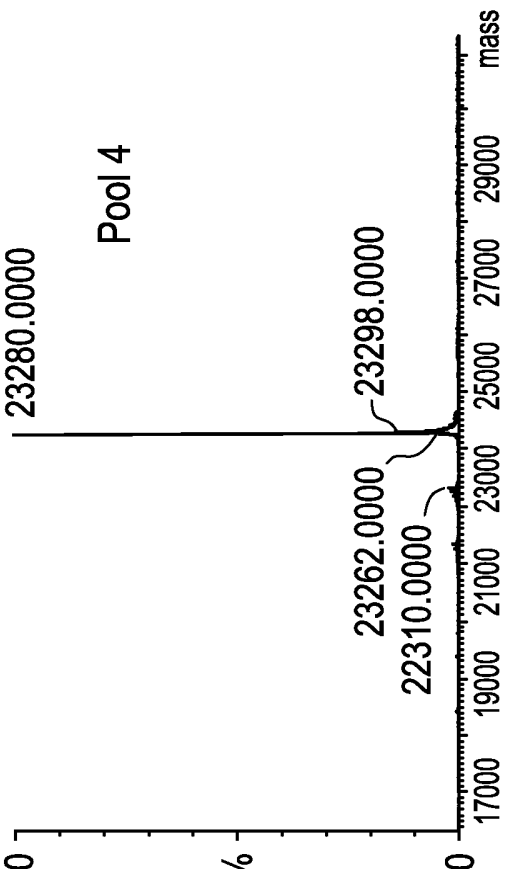


FIG. 38A

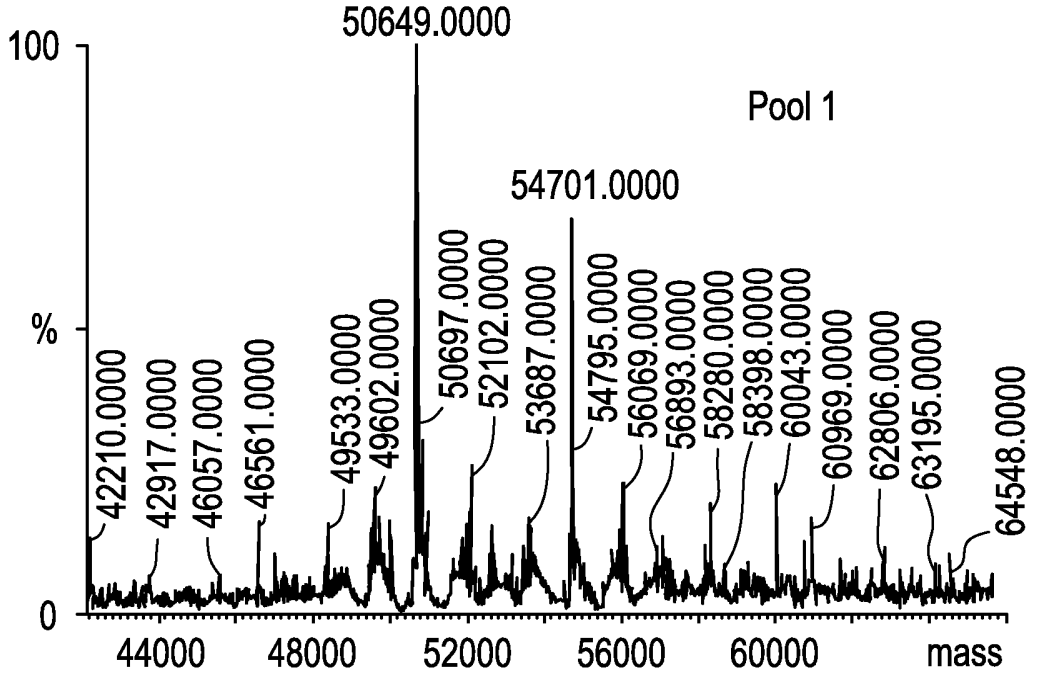


FIG. 38B

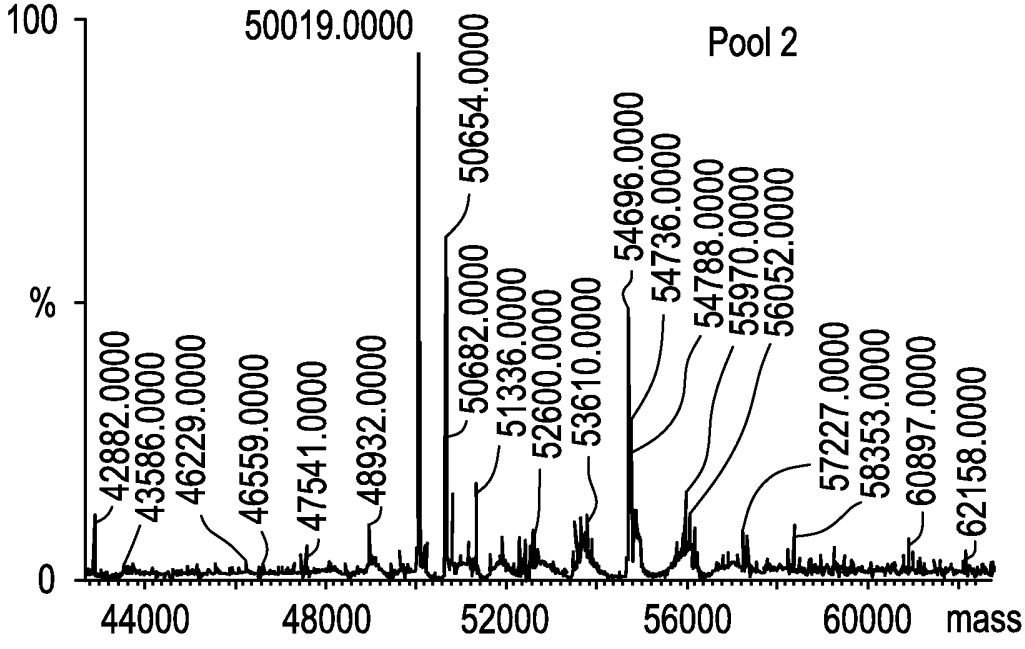


FIG. 38C

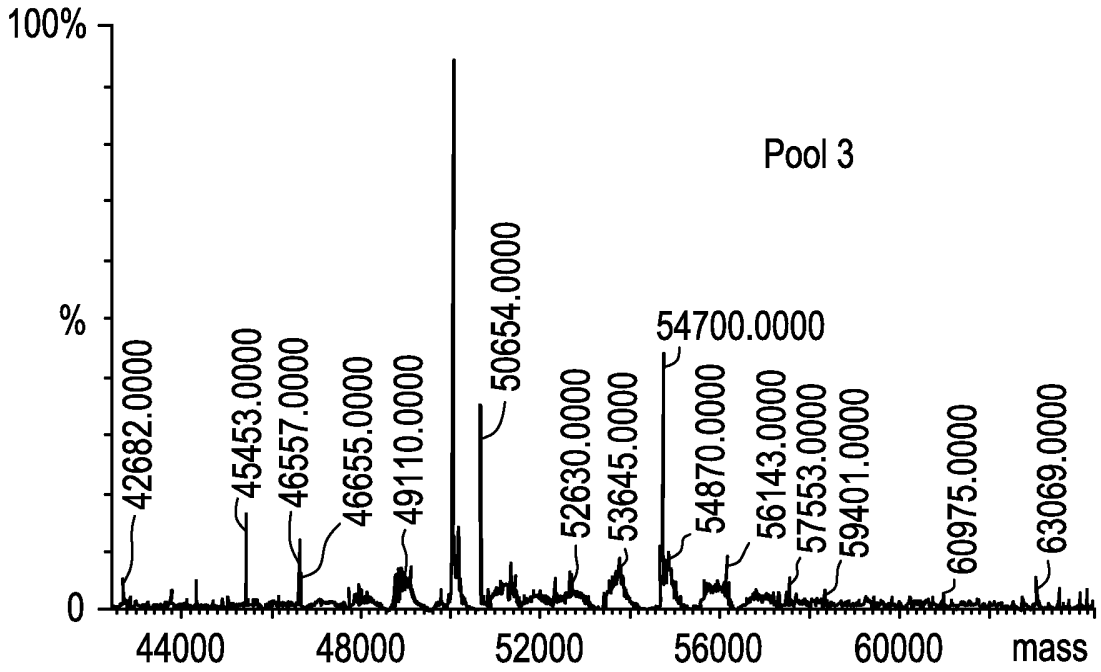


FIG. 38D

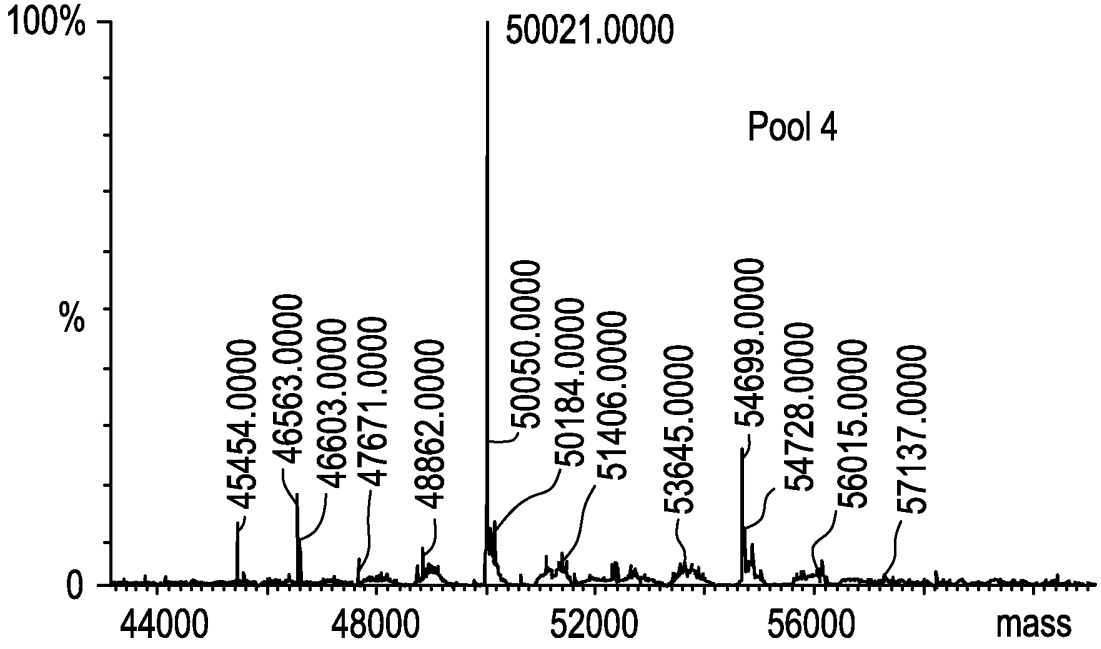


FIG. 39A

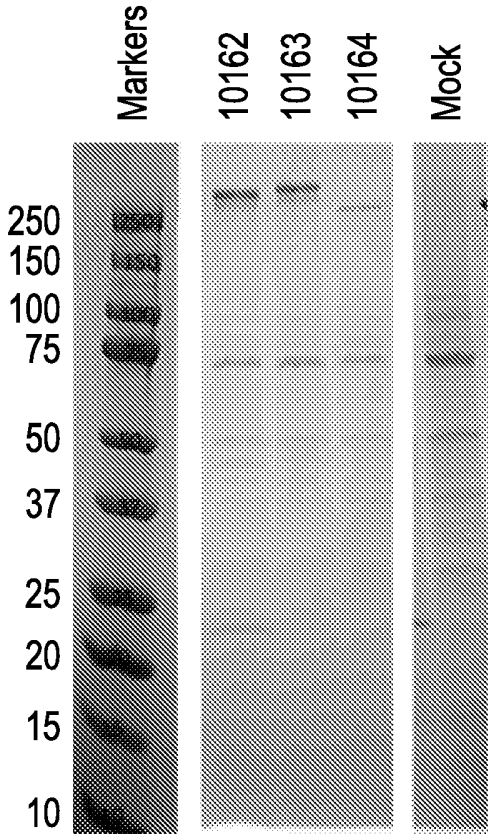


FIG. 39B

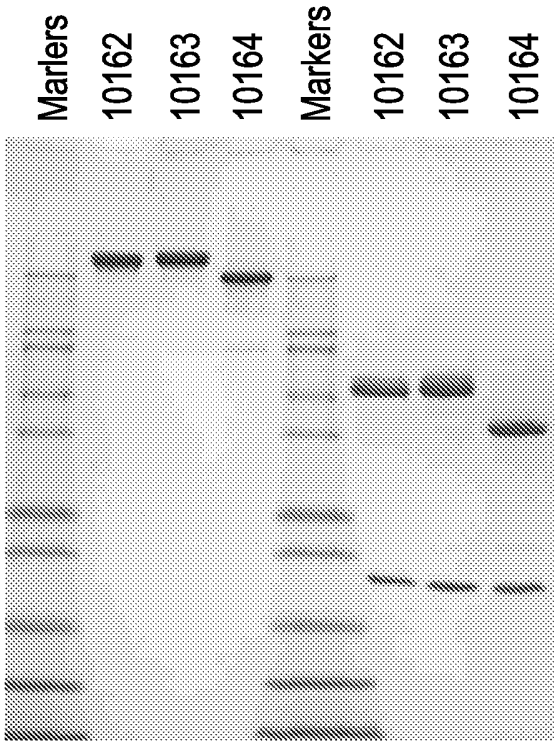


FIG. 40A

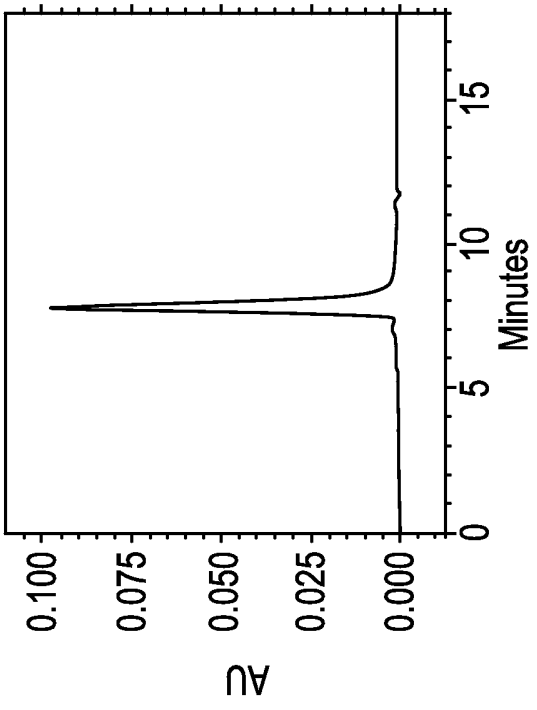


FIG. 40B

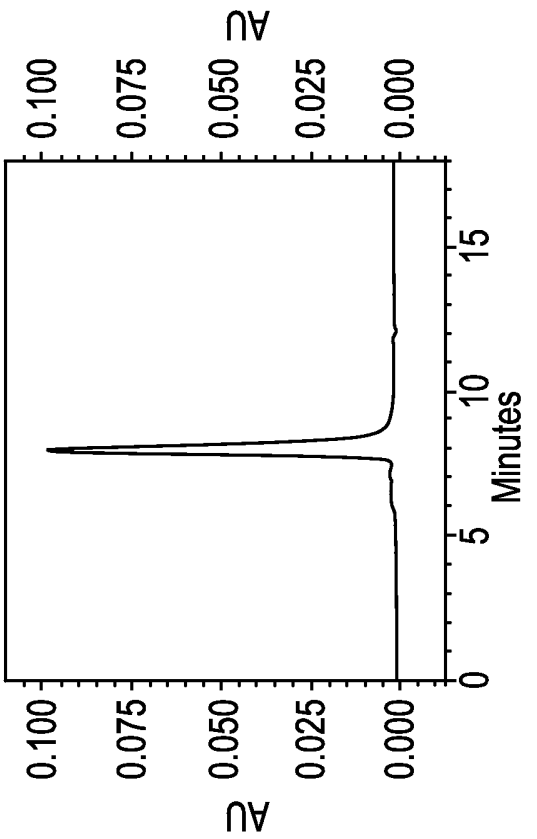


FIG. 40C

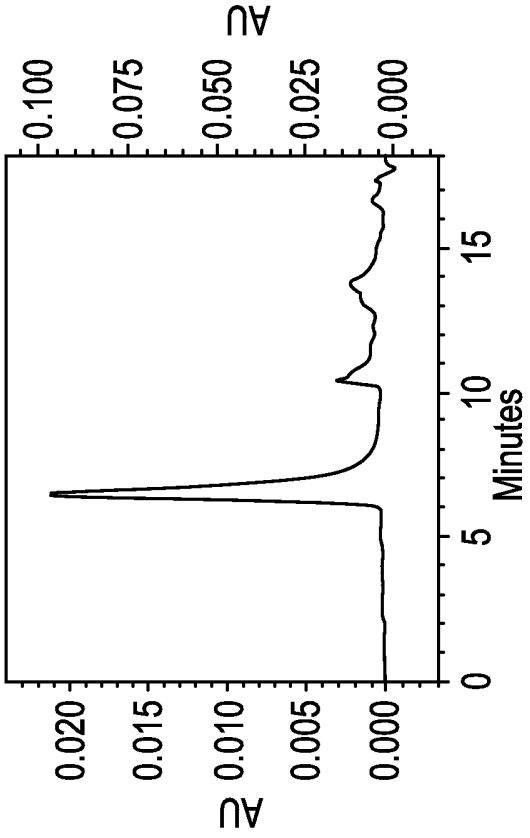


FIG. 41A

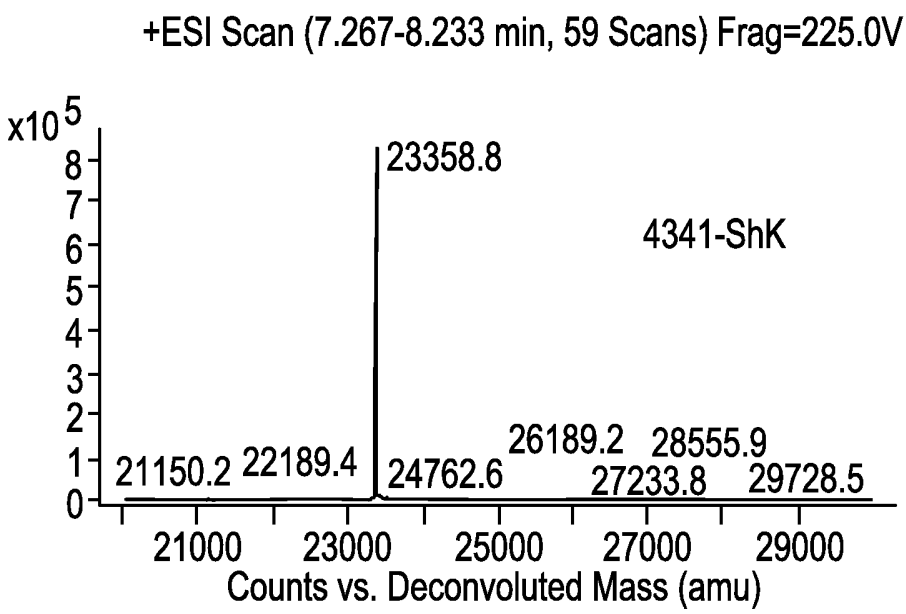


FIG. 41B

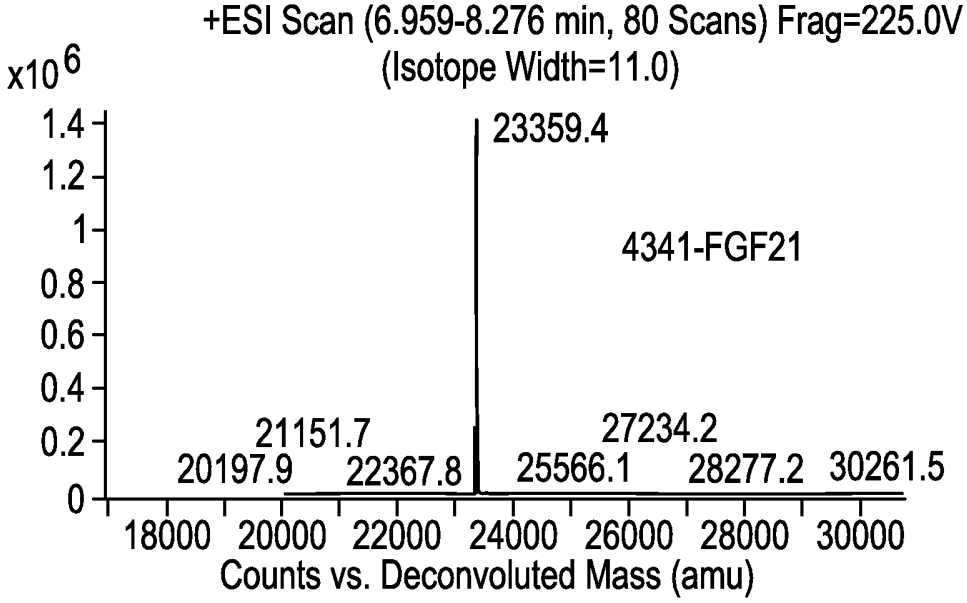


FIG. 41C

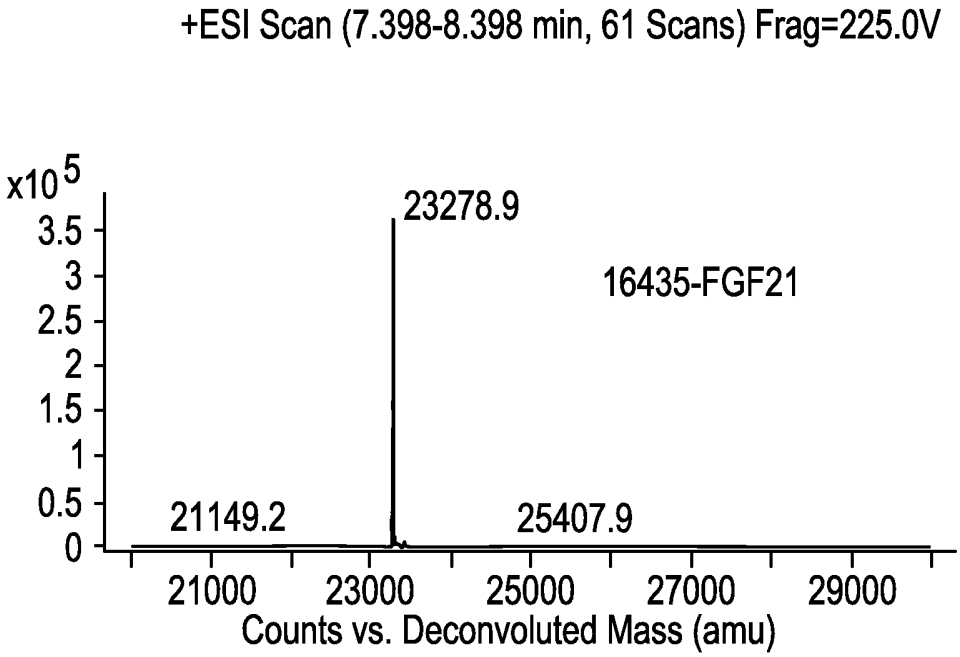

+ESI Scan (7.398-8.398 min, 61 Scans) Frag=225.0V
x10 5
3.5
3
2.5
2
1.5
1
0.5
0
23278.9
16435-FGF21
21149.2
25407.9
21000
23000
25000
27000
29000
Counts vs. Deconvoluted Mass (amu)

FIG. 42A

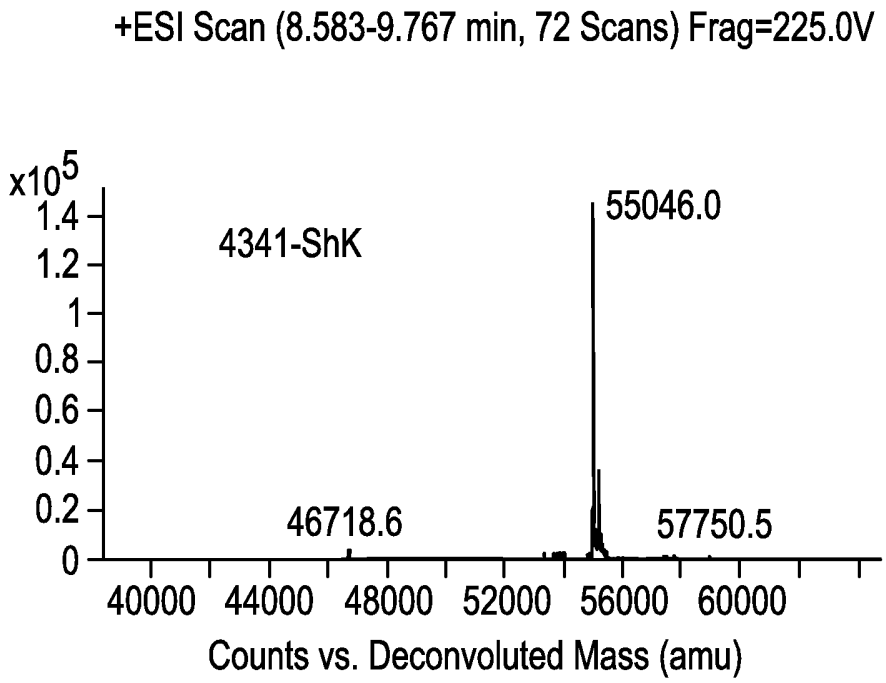


FIG. 42B

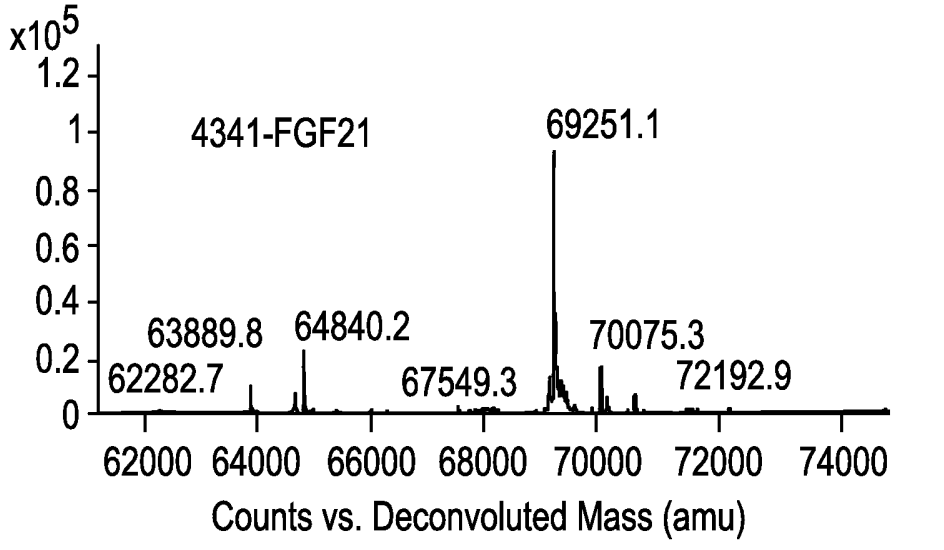


FIG. 42C

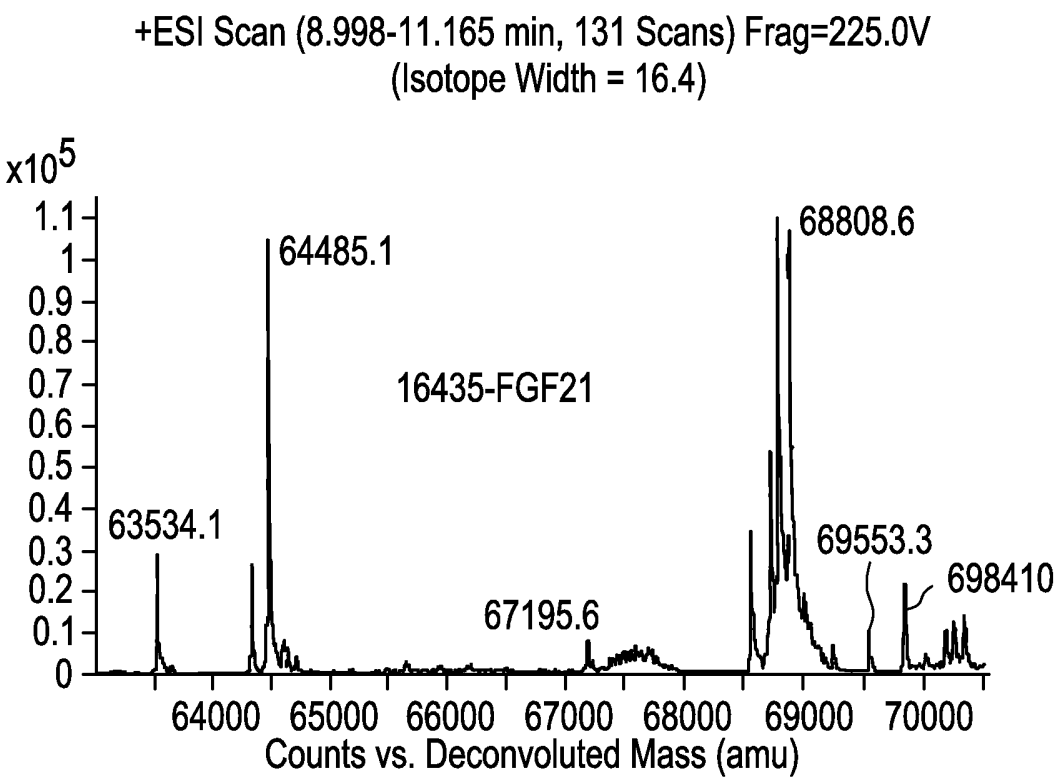

+ESI Scan (8.998-11.165 min, 131 Scans) Frag=225.0V
(Isotope Width = 16.4)
x10^5
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
64485.1
68808.6
16435-FGF21
63534.1
69553.3
698410
67195.6
64000 65000 66000 67000 68000 69000 70000
Counts vs. Deconvoluted Mass (amu)

FIG. 43

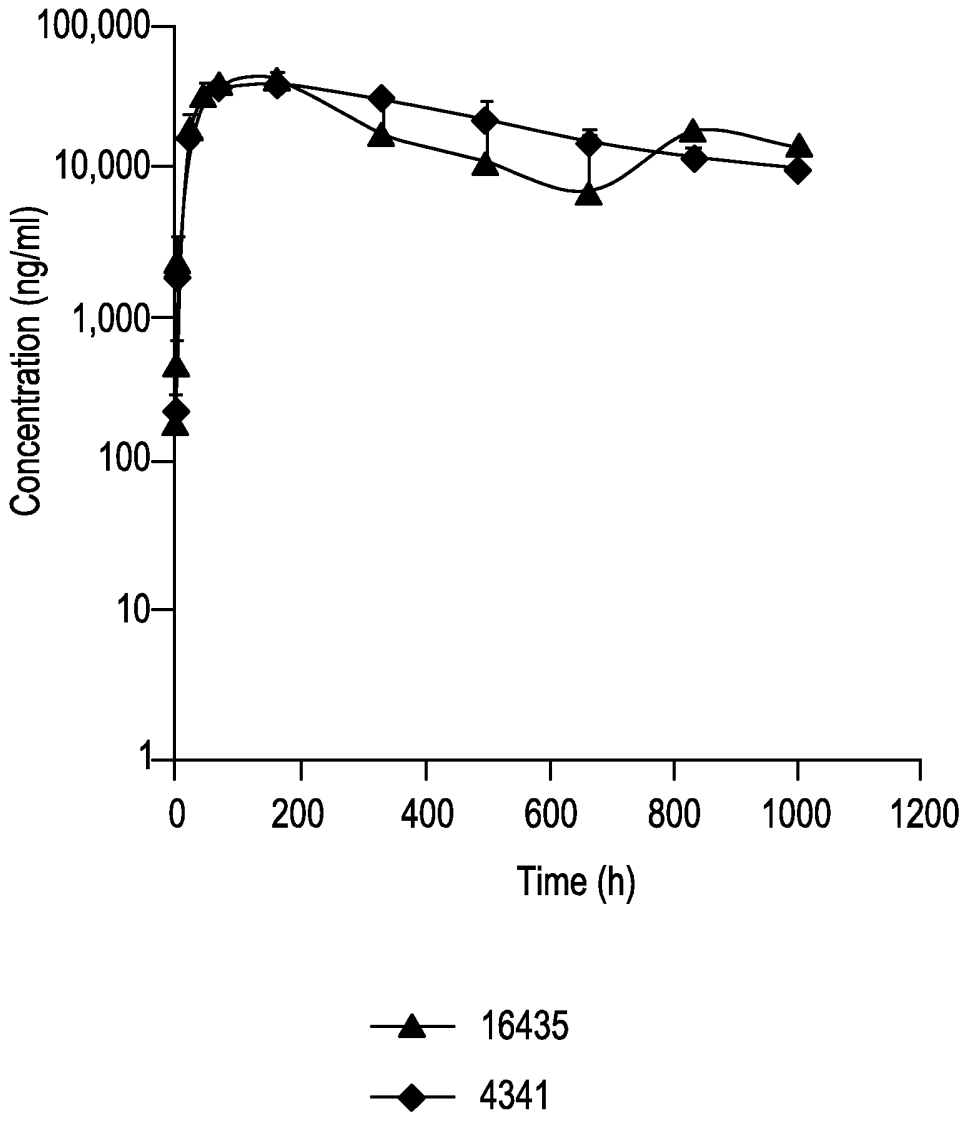
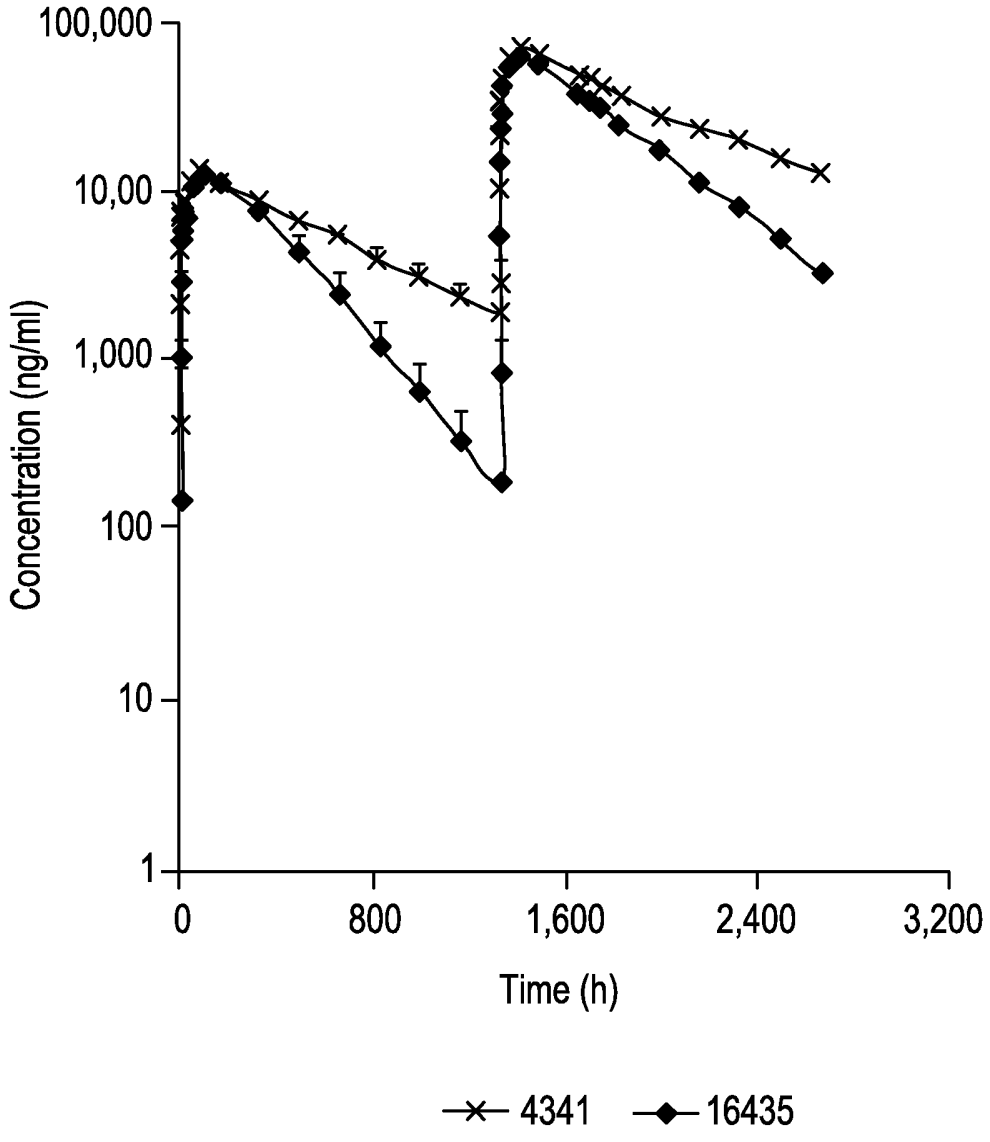

100,000
10,000
1,000
100
10
1
Concentration (ng/ml)
0
200
400
600
800
1000
1200
Time (h)
16435
4341

FIG. 44


100,000
10,00
1,000
100
10
1
Concentration (ng/ml)
0
800
1,600
2,400
3,200
Time (h)
4341
16435