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DESCRIPTION

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/829,973, filed May 31, 2013.

INTRODUCTION

2.1. Field of the Invention

[0002] The present invention relates to the field of therapeutic antibody design and specifically to polypeptides comprising a heterodimeric Fc region which has been modified in order to silence effector functions mediated by the Fc region.

2.2 Background of the Invention

[0003] Therapeutic antibodies have been developed for the treatment of many disease indications. In some of these cases the efficacy of the therapeutic antibody results, at least in part, from the ability of the Fc region of the antibody to mediate one or more effector functions. These effector functions result from the interaction of antibodies and antibody-antigen complexes with cells of the immune system to stimulate a variety of responses, including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) (reviewed in Daeron, Annu. Rev. Immunol. 15:203-234 (1997); Ward and Ghetie, Therapeutic Immunol. 2:77-94 (1995); and Ravetch and Kinet, Annu. Rev. Immunol. 9:457-492 (1991)). Several antibody effector functions are mediated by Fc receptors (FcRs), which bind the Fc region of an antibody. FcRs are defined by their specificity for immunoglobulin isotypes; Fc receptors for IgG antibodies are referred to as FcγR, for IgE antibodies as FcεR, for IgA antibodies as FcαR and so on. The Fc region of antibodies also mediates functions, such as binding to FcRn, that operate independently of antigen binding and that confer persistence in the circulation and the ability to be transferred across cellular barriers by transcytosis (Ward and Ghetie, Therapeutic Immunology 2:77-94 (1995)).

[0004] For certain disease indications, however, effector functions mediated by the Fc region of the antibody can cause undesirable adverse effects and thus efforts have been made to engineer antibodies with reduced or silenced effector functions.

[0005] Recent publications describe strategies that have been used to engineer antibodies with reduced or silenced effector activity (see Strohl, WR (2009), Curr **O**pin Biotech 20:685-691, and Strohl, WR and Strohl LM, "Antibody Fc engineering for optimal antibody performance" In Therapeutic Antibody Engineering, Cambridge: Woodhead Publishing (2012), pp 225-249). These strategies include reduction of effector function through modification of glycosylation, use of IgG2/IgG4 scaffolds, or the introduction of mutations in the hinge or CH2 regions of the Fc region of the antibody.

[0006] In addition, US Patent Publication No. 2011/0212087 (Strohl) describe antibodies and other Fc-containing molecules with variations in the Fc region that reduce binding to FcγRs (Fc gamma receptors) and resulting activity and can be used in the treatment of various diseases and disorders.

[0007] International Patent Publication No. W**O** 2006/105338 (Xencor) describes Fc variants with optimized properties, methods for their generation, Fc polypeptides comprising Fc variants with optimized properties, and methods for using Fc variants with optimized properties.

[0008] US Patent Publication No. 2012/0225058 (Xencor) describes an Fc variant of a parent IgG Fc construct, wherein said Fc variant exhibits altered binding to one or more FcγRs, wherein said Fc variant comprises at least one amino acid insertion in the Fc region of said parent IgG Fc construct.

[0009] US Patent Publication No. 2012/0251531 (Genentech) describes engineered polypeptides comprising Fc variants and their uses, and more specifically, Fc variants exhibiting reduced effector function. These variants are also described as causing a benefit for a patient suffering from a disease which could be treated with an antibody for which it is desirable to reduce the effector function elicited by antibodies.

[0010] Strop et al ((2012) J. Mol. Biol. 420: 204-219) describe the introduction of charged mutations in the core hinge region and CH3 region of human IgG1 and IgG2 to improve bi-specific antibody formation.

[0011] International Patent Publication No. WO 2012/058768 (Zymeworks Inc.) describes scaffolds that have heavy chains that are asymmetric in the various domains (e.g.CH2 and CH3) to accomplish selectivity between the various Fc receptors involved in modulating effector function, beyond those achievable with a natural homodimeric

[0012] (symmetric) Fc molecule, and increased stability and purity of the resulting variant Fc heterodimers. These molecules comprise complexes of heterogeneous components designed to alter the natural way antibodies behave and that find use in therapeutics.

[0013] International Patent Publication No. WO 2012/133782 (Chugai Pharmaceutical) describes the discovery that by modifying the Fc region of an antigen-binding molecule to an Fc region in which a heterocomplex containing a bimolecular FcRn and four activating Fcγ receptors does not form in the neutral pH range, pharmacokinetics improve due to the antigen-binding molecule, and immune response decreases due to the antigen-binding molecule. In addition, the publication describes the discovery of a method for manufacturing antigen-binding molecules having the abovementioned characteristics, and also resulted in the discovery that when a drug composition, which contains such antigen-binding molecules or antigen-binding molecules manufactured according to the manufacturing method disclosed as an active ingredient, is administered, the antigen-binding molecules have excellent characteristics, such as improving pharmacokinetics and decreasing immune response by a living organism that has been administered the drug, compared to antigen-binding molecules of the prior art.

[0014] European Patent Application EP2698431 (Chugai Pharmaceutical) describes the discovery that the modification of the Fc region of an antigen-binding molecule into an Fc region that does not form in a neutral pH range a heterotetramer complex containing two molecules of FcRn and an active Fcγ receptor improved the pharmacokinetics of the antigen-binding molecule and reduced the immune response to the antigen-binding molecule. The application also revealed methods for producing antigen-binding molecules having the properties described above, and successfully demonstrated that pharmaceutical compositions containing as an active ingredient such an antigen-binding molecule or an antigen-binding molecule produced by a production method disclosed have excellent features over conventional antigen-binding molecules in that when administered, they exhibit improved pharmacokinetics and reduced in vivo immune response.

[0015] International Patent Publication No. WO 2012/125850 (Amgen Inc.) describes Fc-containing proteins comprising a binding region and a variant Fc region that can elicit one or more immune effector function and/or bind to an Fc receptor more effectively than a similar Fc-containing protein comprising a wild type Fc region. Also described are nucleic acids encoding such Fc-containing proteins, methods for making such proteins, and methods of treatment utilizing such proteins.

[0016] Shields et al ((2001) J. of Biological Chemistry 276: 6591-6604) describe IgG Fc receptors that play a critical role in linking IgG antibody-mediated immune responses with cellular effector functions. A high resolution map of the binding site on human IgG1 for human FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA, and FcRn receptor was determined. A common set of IgG1 residues involved in binding to all FcγR: FcγRII and FcγRIII also utilize residues outside this common set. In addition to residues which, when altered, abrogated binding to one or more

of the receptors, several residues were found that improved binding only to specific receptors or simultaneously improved binding to one type of receptor and reduced binding to another type. Select IgG1 variants with improved binding to FcγRIIIA were also described to exhibit up to 100% enhancement in antibody-dependent cell cytotoxicity using human effector cells; these variants included changes at residues not found at the binding interface in the IgG/FcγRIIIA co-crystal structure (Sondermann, P., Huber, R., **O**osthuizen, V., and Jacob, U. (2000) Nature 406, 267-273.

[0017] Hezareh et al ((2001) J. of Virology 75: 12161-12168) describe that the human IgG1 b12 neutralizes a broad range of human immunodeficiency virus-type 1 (HIV-1) isolates in vitro and is able to protect against viral challenge in animal models. Neutralization of free virus, which is an antiviral activity of antibody that generally does not require the antibody Fc fragment, is described as likely playing an important role in the protection observed. The role of Fc-mediated effector function, which may reduce infection by inducing phagocytosis and lysis of virions and infected cells, however, is less clear. Hezareh et al constructed a panel of IgG1 b12 mutants with point mutations in the CH2 domain, and this did not affect gp120 binding or HIV-1 neutralization. IgG1 b12 mediated strong antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) of HIV-1-infected cells, but these activities were reduced or abrogated for the antibody mutants. Two mutants were of particular interest. K322A showed a twofold reduction in FcγR binding affinity and ADCC, while C1q binding and CDC were abolished. In this study, Hezareh et al confirm that K322 forms part of the C1q binding site in human IgG1 and plays an important role in the molecular interactions leading to complement activation. Hezareh et al also describe that the lower hinge region in human IgG1 has a strong modulating effect on C1q binding and CDC. The b12 mutants K322A and L234A, L235A are described as providing useful tools for dissecting the in vivo roles of ADCC and CDC in the anti-HIV-1 activity of neutralizing antibodies.

[0018] Mimoto et al ((2013) J. mAbs 5: 229-236) describe Fc engineering as a promising approach to enhance the antitumor efficacy of monoclonal antibodies (mAbs) through antibody-dependent cell-mediated cytotoxicity (ADCC). Glyco- and protein-Fc engineering have been employed to enhance FcγR binding and ADCC activity of mAbs; the drawbacks of previous approaches lie in their binding affinity to both FcγRIIIa allotypes, the ratio of activation FcγR binding to inhibitory FcγR binding (A/I ratio) or the melting temperature (T_m) of the CH2 domain. Mimoto et al describe that, to date, no engineered Fc variant has been reported that satisfies all these points. Mimoto et al report a novel Fc engineering approach that introduces different substitutions in each Fc domain asymmetrically, conferring optimal binding affinity to FcγR and specificity to the activating FcγR without impairing the stability.Mimoto et al further report designing an asymmetric Fc variant with the highest binding affinity for both FcγRIIIa allotypes and the highest A/I ratio compared with previously reported symmetrically engineered Fc variants, and superior or at least comparable in vitro ADCC activity compared afucosylated Fc variants. The asymmetric Fc engineering approach is described as offering a higher stability by minimizing the use of substitutions that reduce the T_m of the CH2 domain compared with the symmetric approach.

SUMMARY OF THE INVENTION

[0019] The invention is directed to heteromultimers comprising an IgG Fc construct having a first and a second Fc polypeptide, the IgG Fc construct displaying reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding wild-type IgG Fc construct, wherein: A) each Fc polypeptide comprises a modified lower hinge region as compared to the corresponding wild-type IgG Fc polypeptide, the modified lower hinge region of the first Fc polypeptide comprising amino acid modifications at L234 and/or L235 that increase the net positive charge in the modified lower hinge region of the first Fc polypeptide at about physiological pH conditions, and the modified lower hinge region of the second Fc polypeptide comprising at least one amino acid modification which is different from at least one amino acid modification of the first Fc polypeptide, and wherein the modified lower hinge region of at least one of the first or second Fc polypeptides comprises two or more amino acid modifications, or B) the first Fc polypeptide comprises the amino acid modifications E269Q/D270N and the second Fc polypeptide comprises the amino acid modifications E269K/D270R, or C) the first Fc polypeptide comprises the amino acid modifications L235K/A327K and the second Fc polypeptide does not comprise an amino acid modification at the hinge or lower hinge region as compared to the corresponding wild-type IgG Fc

polypeptide, wherein the IgG Fc construct is a human IgG1, IgG3 or IgG4 construct, and wherein the numbering of amino acids is according to the EU index as in Kabat.

[0020] In certain disclosures is a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified lower hinge region wherein: the modified lower hinge region of said first Fc polypeptide comprises at least one amino acid modification, the modified lower hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, and the IgG Fc construct displays reduced binding to all Fcγ receptors and to C1q protein as compared to a corresponding parent IgG Fc construct.

[0021] In certain disclosures is a heteromultimer described herein, wherein the at least one amino acid modification in the modified lower hinge region of the first Fc polypeptide increases the net positive charge in the modified lower hinge region, and the at least one amino acid modification in the second Fc polypeptide increases the total number of negative charges or is charge neutral relative to the wild-type hinge region; or the at least one amino acid modification in the modified lower hinge region of the first Fc polypeptide increases the net negative charge in the modified lower hinge region, and the at least one amino acid modification in the second Fc polypeptide increases the total number of positive charges.

[0022] In some embodiments is a heteromultimer described herein, wherein the modified lower hinge region of at least one of said first and second Fc polypeptides comprises two or more amino acid modifications.

[0023] Provided herein is a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified hinge region wherein: the modified lower hinge region of said first Fc polypeptide comprising amino acid modifications at L234 and/or L235 that increase the net charge in the modified hinge region of the first Fc polypeptide at about physiological pH conditions, the modified hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide.

[0024] In some disclosures is a heteromultimer described herein wherein the increase in net charge is an increase in net positive charge on the first Fc polypeptide. In some disclosures, said increase in net positive charge is an increase in the total number of positively charged amino acids on the first Fc polypeptide, or a decrease in the total number of negatively charged amino acids on the first Fc polypeptide. In certain disclosures is a heteromultimer described herein, wherein the at least one amino acid modification on the modified hinge region of said first Fc polypeptide increases the total number of positively charged amino acids on said first Fc polypeptide, and the at least one amino acid modification on the modified hinge region of said second Fc polypeptide increases the total number of negative charges on said second Fc polypeptide or is charge neutral.

[0025] In a particular disclosure, the increase in net charge is an increase in the net negative charge on the first Fc polypeptide. In certain disclosures, the increase in net negative charge is an increase in the total number of negatively charged amino acids or a decrease in the total number of positively charged amino acids on the first Fc polypeptide. In certain disclosures when the net negative charge is an increase in the total number of negatively charged amino acids, the at least one amino acid modification on the second Fc polypeptide increases the total number of positive charges.

[0026] In some embodiments is provided a heteromultimer described herein, wherein the at least one amino acid modification in the modified lower hinge region of the first Fc polypeptide combined with the at least one amino acid modification in the second Fc polypeptide increases the overall positive charge of the IgG Fc construct compared to a corresponding parent IgG Fc construct not comprising the hinge region modifications.

[0027] In certain embodiments is provided a heteromultimer described herein, wherein the modified lower hinge region of at least one of said first and second Fc polypeptides comprises two or more amino acid modifications.

[0028] In certain embodiments is provided a heteromultimer described herein, wherein the modified lower hinge

region of said first and second Fc polypeptides comprises two or more amino acid modifications.

[0029] In certain embodiments is provided a heteromultimer described herein, wherein the at least one amino acid modification in the modified hinge region of the first and second Fc polypeptides is in the lower hinge region.

[0030] Provided is a heteromultimer described herein, wherein the IgG Fc construct has a K_D of greater than 10 μM for FcγRIIaH, a K_D of greater than 10 μM for FcγRIIaR, a K_D of greater than 10 μM for FcγRIIb, a K_D of greater than 6 μM for FcγRIIIaF, a K_D of greater than 6 μM for FcγRIIIaV, and a K_D of greater than 6.5 nM for FcγRIa.

[0031] In certain disclosures is provided a heteromultimer described herein, wherein the IgG Fc construct mediates reduced effector function compared to a corresponding IgG Fc construct not comprising the amino acid modifications. In some disclosures, the IgG Fc construct mediates less than 70%, less than 50%, less than 30%, or less than 10% of effector function as measured by EC_{50}. In some disclosures, the IgG Fc construct mediates less than 10%, less than 5%, less than 2%, or less than 1% of the effector function as measured by maximum lysis of cells. In an embodiment, the effector function is selected from ADCC, ADCP, CDC or any combination thereof.

[0032] In certain embodiments is provided a heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications at L234 and/or L235. In some embodiments, the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications selected from L234K, L234R, L234A, L235K, L235R, and L235A. In a further embodiment one of said first and second Fc polypeptides further comprises an amino acid modification at E233. In some embodiments, said modification at E233 is selected from E233A, E233K, and E233R.

[0033] In certain embodiments is provided a heteromultimer described herein, wherein the modified hinge region of both said first and second Fc polypeptides comprises amino acid modifications at L234 and/or L235. In some embodiments, the modification at L234 and/or L235 is selected from L234A, L234K, L234R, L234D, L234E, L235K, L235R, L235E, L235A, and L235D. In a further embodiment, the modified hinge region of the first and/or the second Fc polypeptide further comprises an amino acid modification at E233. In some embodiments, either or both amino acid modifications are independently E233A or E233D.

[0034] In certain embodiments is provided a heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises the amino acid modifications L234K/L235K, E233A/L234R/L235R, E233K/L234R/L235R, or E233K/L234A/L235K.

[0035] In certain disclosures is provided a heteromultimer described herein, wherein the modified hinge region of the first or second Fc polypeptide comprises the amino acid modifications L234A/L235A, L234D/L235E, E233A/L234D/L235E, or E233A/L234K/L235A.

[0036] In certain embodiments is provided a heteromultimer described herein, wherein: the modified hinge region of the first Fc polypeptide comprises the amino acid modifications L234K/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234A/L235A; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications L234K/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234D/L235E; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233A/L234R/L235R and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications E233A/L234D/L235E; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234D/L235E; or the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233K/L234A/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications E233A/L234K/L235A.

[0037] In certain disclosures is provided a heteromultimer described herein, wherein: the first Fc polypeptide comprises the amino acid modifications L234D/L235E and the second Fc polypeptide comprises the amino acid modifications L234R/L235R/E233K; the first Fc polypeptide comprises the amino acid modifications

L234D/L235E/D265S and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/D265S; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/K322A; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/P329W and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/P329W; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322A; or the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322E/E333K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322E/E333K.

[0038] Provided herein is a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide, wherein: said first Fc polypeptide comprises the amino acid modifications E269Q/D270N and the second Fc polypeptide comprises the amino acid modifications E269K/D270R; or said first Fc polypeptide comprises the amino acid modifications L235K/A327K and the second Fc polypeptide does not comprise a modification at the hinge or lower hinge region.

[0039] In some embodiments is a heteromultimer described herein, wherein the IgG Fc construct is aglycosylated. In some embodiments is a heteromultimer described herein, wherein the IgG Fc construct is deglycosylated.

[0040] In some disclosures is a heteromultimer described herein, wherein the onset of melting of the IgG Fc construct in a thermogram is greater than or equal to 68°C.

[0041] In some disclosures is a heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is greater than or equal to the melting temperature of a corresponding parent CH2 region not comprising the hinge region modifications.

[0042] In a particular disclosure is the heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is about 1 to 2°C greater than the melting temperature of the parent CH2 region.

[0043] In a particular disclosure is the heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is about 2 to 3°C greater than the melting temperature of the parent CH2 region.

[0044] In an embodiment is the heteromultimer described herein, wherein the IgG Fc construct comprises a variant CH3 region comprising amino acid modifications that promote the formation of a heterodimeric Fc region in comparison to a homodimeric Fc region, when said heteromultimer is expressed. In some embodiments, one of said first and second Fc polypeptides comprises the CH3 amino acid modifications T366L/N390R/K392M/T394W and the other Fc polypeptide comprises the CH3 amino acid modifications L351Y/S400E/F405A/Y407V. In some embodiments, the first and/or second Fc polypeptides comprise the amino acid modification T350V. In a further embodiment, heteromultimers comprising a heterodimeric Fc region are resolved from expression products comprising a homodimeric Fc region using charge-based purification methods.

[0045] In an embodiment is the heteromultimer described herein, wherein the heteromultimer further comprises at least one antigen-binding construct fused to the IgG Fc construct.

[0046] In an embodiment is the heteromultimer described herein, wherein the at least one antigen-binding construct is selected from a Fab fragment, an scFv, an sdAb, an antigen binding peptide, an Fc fusion protein, or a protein or fragment thereof capable of binding the antigen. In some embodiments is a heteromultimer described herein, comprising one antigen-binding construct. In an embodiment is a heteromultimer described herein, comprising two antigen-binding constructs.

[0047] In an embodiment is a heteromultimer described herein, wherein the IgG Fc construct is linked to one or more toxic drug molecules.

[0048] In an embodiment is a heteromultimer described herein, wherein the IgG Fc construct is linked to one or more heterologous polypeptides. In some embodiments, the one or more heterologous polypeptides are selected from enzymes and toxins.

[0049] In an embodiment is a heteromultimer described herein, wherein the IgG is IgG1.

[0050] Provided herein is a nucleic acid encoding the first or second Fc polypeptide of the heteromultimer described herein. Provided is a host cell comprising the nucleic acid described herein. Provided is a method of preparing the heteromultimer described herein, the method comprising the steps of: (a) culturing the host cell described herein; and (b) recovering the heteromultimer from the host cell culture. In certain embodiments is the method of preparing the heteromultimer, further comprising the step of isolating the heteromultimer using charge-based purification methods. In some embodiments is method of preparing the heteromultimer described herein, wherein the charge-based purification method is ion-exhange chromatography.

[0051] Provided is a pharmaceutical composition comprising the heteromultimer described herein and a pharmaceutically acceptable carrier. Disclosed is a method of treating a disease comprising providing to a patient in need thereof an effective amount of the pharmaceutical composition described herein. In some disclosures is use of the heteromultimer described herein in the preparation of a medicament for the treatment of a disease.

[0052] In some embodiments is use of a heteromultimer described herein for use in the treatment of a disease in a patient in need thereof.

[0053] Provided herein is a method of reducing the effector function of an IgG construct comprising: modifying the lower hinge region of a first and a second Fc polypeptide, wherein; the modified lower hinge region of said first Fc polypeptide comprises at least one amino acid modification, the modified lower hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, the IgG Fc construct displays reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding wild-type IgG Fc construct and wherein the numbering of the amino acid is according to the EU index as in Kabat.

[0054] The invention encompasses a method of reducing the effector function of an IgG Fc construct comprising a first and second Fc polypeptide, the method comprising: modifying the lower hinge region of at least one of the first and second Fc polypeptides, wherein: the modified lower hinge region of the first Fc polypeptide comprises amino acid modifications at L234 and/or L235 that increase the net positive charge in the modified lower hinge region of the first Fc polypeptide at about physiological pH conditions, and the modified lower hinge region of the second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of the first Fc polypeptide, wherein the modified lower hinge region of at least one of the first or second Fc polypeptides comprises two or more amino acid modifications, wherein the IgG construct is a human IgG1, IgG3 or IgG4 Fc construct, wherein the IgG Fc construct displays reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding wild-type IgG Fc construct, and wherein the numbering of the amino acids is according to the EU index as in Kabat.

BRIEF DESCRIPTION OF THE FIGURES

[0055] These and other features of the invention will become more apparent in the following detailed description in which reference is made to the appended drawings.

Figure 1 depicts regions of the Fc region that are involved in FcγR binding. (A) 3b/Fc crystal structure (PDB:1E4K)

denoting the loops and lower hinge of the CH2 domain of the Fc (shown in gray) that are involved in FcγR binding (shown in black). (B) Topology of the CH2 domain. Strands are denoted as S1, S2, S3, S4, S5, S6, and S7; Loops are denoted L1, L2, and L3.

Figure 2 depicts thermograms of exemplary asymmetric antibody constructs compared to wild-type antibody (WT). Figure 2A depicts the thermograms for WT, and AAC2 - AAC6. Figure 2B depicts the identity of the two transitions in the WT. The first transition corresponds to the unfolding of the CH2 domain, the second transition corresponds to the unfolding of the CH3+Fab. Figure 2C depicts the overlay of a number of samples showing how the CH2 transition of the variants has shifted to a higher Tm value, indicating higher stability.

Figure 3A depicts exemplary results showing the resolution by ion exchange chromatography of components produced when exemplary asymmetric antibody constructs are purified. Figure 3B depicts separation of components using a pH gradient (upper panel), or a salt gradient (lower panel) using exemplary asymmetric variant AAC4.

Figure 4 depicts the ability of a control variant (v1051) and an exemplary heteromultimer of the invention (AAC6) to mediate ADCC.

Figure 5 depicts the amino acid sequence of the human IgG1 Fc region (SEQ ID NO:1); the amino acid sequence of the heavy chain of trastuzumab (SEQ ID NO:2), the amino acid sequence of the light chain of trastuzumab (SEQ ID NO:3), the amino acid sequence of the heavy chain of rituximab (SEQ ID NO:4), the amino acid sequence of the light chain of rituximab (SEQ ID NO:5).

Figure 6 depicts the ability of variants AAC9 - AAC12, AAC14, and AAC15 to mediate ADCC in Daudi cells. Figure 6A depicts the results for AAC10 and AAC11 compared to controls including commercially available rituximab; Figure 6B depicts the results for AAC12 and AAC14 compared to controls including commercially available rituximab; Figure 6C depicts the results for AAC9 and AAC15 compared to controls including commercially available rituximab.

Figure 7 depicts the ability of variants AAC9 - AAC12, AAC14, and AAC15 to mediate CDC in Daudi cells.

Figure 8 depicts sequences for SEQ ID NOs: 6-69 filed herewith.

DETAILED DESCRIPTION

[0056] The present invention provides a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide, the IgG Fc construct displaying reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding wild-type IgG Fc construct, wherein: A) each Fc polypeptide comprises a modified lower hinge region as compared to the corresponding wild-type IgG Fc polypeptide, the modified lower hinge region of the first Fc polypeptide comprising amino acid modifications at L234 and/or L235 that increase the net positive charge in the modified lower hinge region of the first Fc polypeptide at about physiological pH conditions, and the modified lower hinge region of the second Fc polypeptide comprising at least one amino acid modification which is different from at least one amino acid modification of the first Fc polypeptide, and wherein the modified lower hinge region of at least one of the first or second Fc polypeptides comprises two or more amino acid modifications, or B) the first Fc polypeptide comprises the amino acid modifications E269Q/D270N and the second Fc polypeptide comprises the amino acid modifications E269K/D270R, or C) the first Fc polypeptide comprises the amino acid modifications L235K/A327K and the second Fc polypeptide does not comprise an amino acid modification at the hinge or lower hinge region as compared to the corresponding wild-type IgG Fc polypeptide, wherein the IgG Fc construct is a human IgG1, IgG3 or IgG4 construct, and wherein the numbering of amino acids is according to the EU index as in Kabat.

[0057] In certain embodiments, a heteromultimer comprising an IgG Fc construct. The IgG Fc construct comprises two Fc polypeptides, each having a modified hinge region, wherein the modified hinge region comprises

asymmetric amino acid modifications that reduce or eliminate binding of the IgG Fc construct to FcγRIIaH, FcγRIIaR, FcγRIIb FcγRIIIaF, FcγRIIIaV, and FcγRIa receptors and to complement factor C1q protein. Such reduction or elimination of this binding results in reduction or silencing of effector functions typically mediated by the wild-type IgG Fc region. As noted, the modified hinge region comprises asymmetric amino acid modifications and as such, the amino acid modifications in the hinge region of one polypeptide of the IgG Fc construct are different from those on the hinge region of the other polypeptide. In some disclosures, the isolated antibody constructs are stable and capable of binding to FcRn.

[0058] In certain embodiments, the modified hinge region of each polypeptide of the IgG Fc construct comprises one or more amino acid modifications, selected in order to increase the positive charge on one polypeptide of the IgG Fc construct compared to the other polypeptide of the IgG Fc construct. In certain embodiments, the modified hinge region of each polypeptide of the IgG Fc construct comprises one or more amino acid modifications, selected in order to increase the negative charge on one polypeptide of the IgG Fc construct compared to the other polypeptide of the IgG Fc construct.

[0059] The heteromultimer according to the invention can be useful in the development of therapeutic antibodies where effector functions are undesirable due to resulting side-effects such as cytotoxicity. In some embodiments, the heteromultimers also exhibit properties that facilitate their purification using charge-based methods.

[0060] Disclosed herein are heteromultimer constructs with reduced or silenced effector function. In an embodiment is provided a heteromultimer construct comprising an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified lower hinge region wherein: the modified lower hinge region of said first Fc polypeptide comprises at least one amino acid modification, the modified lower hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, and the IgG Fc construct displays reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding parent IgG Fc construct. In certain embodiments, the heteromultimer construct displays negligible binding to Fcγ receptors as compared to a corresponding parent IgG Fc construct that does not have the modifications described herein. In some embodiments, the heteromultimer construct displays reduced binding to all Fcγ receptors and negligible binding to at least one of the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors. In certain embodiments, the heteromultimer construct described herein displays reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors and negligible binding to C1q protein. In certain embodiments, at least one of said first and second Fc polypeptides comprises a further modification of at least one amino acid which is not in the lower hinge region. In certain embodiments, at least one of said first and second Fc polypeptides further comprises modification of at least one amino acid which is in the hinge region. In some embodiments, at least one of said first and second Fc polypeptides further comprises modification of at least one amino acid which is in the CH3 region.

[0061] Disclosed herein is a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified hinge region wherein: the modified hinge region of said first Fc polypeptide comprises at least one amino acid modification that increases the net charge in the modified hinge region of the first Fc polypeptide at about physiological pH conditions, the modified hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, and the IgG Fc construct displays reduced binding to all Fcγ receptors and to C1q protein as compared to a corresponding parent IgG Fc construct. In some embodiments, the increase in net charge is an increase in the net positive charge on the first Fc polypeptide. In a particular disclosure, the increase in the net positive charge is an increase in the total number of positively charged amino acids on the first Fc polypeptide. In certain disclosures, said increase in net charge is an increase in the net negative charges on the first Fc polypeptide. In a particular disclosure, the increase in the net negative charge is an increase in the total number of negatively charged amino acids on the first Fc polypeptide. In some embodiments, the heteromultimer construct displays negligible binding to FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding parent IgG Fc construct that does not have the modifications described herein. In particular embodiments, the heteromultimer construct displays reduced binding to FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors and negligible binding to at least one Fcγ receptor. In an embodiment, the

heteromultimer construct described herein displays reduced binding to FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors and negligible binding to C1q protein. In certain embodiments, at least one of said first and second Fc polypeptides further comprises modification of at least one amino acid which is in the lower hinge region. In some embodiments, at least one of said first and second Fc polypeptides further comprises modification of at least one amino acid which is in the CH3 region.

[0062] In some embodiments is the heteromultimer provided herein, wherein the one or more amino acid modifications in the modified hinge region of the second Fc polypeptide modifies the number of negative or positive charges in the hinge region, or is charge neutral relative to the wild-type hinge region. In certain embodiments is the heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises two or more amino acid modifications. In an embodiment is provided the heteromultimer described herein, wherein the modified hinge region of said second Fc polypeptide comprises two or more amino acid modifications. In certain disclosures is a heteromultimer construct described herein, wherein the one or more amino acid modifications in the modified hinge region of the first and second Fc polypeptides are in the lower hinge region (amino acids 16-23 of SEQ ID NO:1, see Figure 5).

[0063] In an embodiment is a heteromultimer described herein, wherein the IgG Fc construct has a KD of at least about 10 μM for FcγRIIaH, a KD of at least about 10 μM for FcγRIIaR, a KD of at least about 10 μM for FcγRIIb, a KD of at least about 6 μM for FcγRIIIaF, a KD of at least about 6 μM for FcγRIIIaV, and a KD of at least about 6.5 nM for FcγRIa. In some embodiments is a heteromultimer described herein, wherein the IgG Fc construct has a KD of greater than 10 μM for FcγRIIaH, a KD of greater than 10 μM for FcγRIIaR, a KD of greater than 10 μM for FcγRIIb, a KD of greater than 6 μM for FcγRIIIaF, a KD of greater than 6 μM for FcγRIIIaV, and a KD of greater than 6.5 nM for FcγRIa.

[0064] In some embodiments is a heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications of at least one of L234 and L235. In an embodiment is the heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications selected from L234K, L234R, L234A, L235K, L235R, and L235A. In an embodiment is the heteromultimer described herein, said one of first and second Fc polypeptides comprises an amino acid modification at E233. In an embodiment is the heteromultimer provided herein, wherein said modification at E233 is selected from E233A, E233K, and E233R. In some embodiments is the heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications of at least one of L234 and L235. In an embodiment is the heteromultimer described herein, wherein said modification of at least one of L234 and L235 is selected from L234A, L234K, L234R, L234D, L234E, L235K, L235R, L235E, L235A, and L235D. In one embodiment is the heteromultimer described herein, wherein the modified hinge region of the second Fc polypeptide further comprises amino acid modifications at E233. In an embodiment, the first or second Fc polypeptide comprises amino acid modifications selected from E233A or E233D. In certain embodiments, at least one of said first or second Fc polypeptides further comprises at least one amino acid modification selected from D265S, E269K, K322A, P329W, and E333K. In some embodiments, said first and second Fc polypeptides further comprise at least one amino acid modification selected from D265S, E269K, K322A, P329W, and E333K.

[0065] Provided herein is a heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises the amino acid modifications L234A/L235K, E233A/L234R/L235R, E233K/L234R/L235R, or E233K/L234A/L235K. In some embodiments is a heteromultimer described herein, wherein the modified hinge region of the first or second Fc polypeptide comprises the amino acid modifications L234A/L235A, L234D/L235E, E233A/L234D/L235E, or E233A/L234K/L235A.

[0066] Provided herein is a heteromultimer as described herein, wherein the modified hinge region of the first Fc polypeptide comprises the amino acid modifications L234K/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234A/L235K; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications L234K/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234D/L235E; the modified hinge region of the first Fc

polypeptide comprises the amino acid modifications E233A/L234R/L235R and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications E233A/L234D/L235E; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234D/L235E; or the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233K/L234A/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications E233A/L234K/L235A.

[0067] In some embodiments is the heteromultimer provided herein, wherein at least one of said first or second Fc polypeptides further comprises at least one amino acid modification selected from D265S, E269K, K322A, P329W, and E333K. For instance, in a particular disclosure is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E and the second Fc polypeptide comprises the amino acid modifications L234R / L235R/E233K. In another disclosure is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/D265S and the second Fc polypeptide comprises the amino acid modifications L234R/L235R/D265S. In a further disclosure is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K. In another disclosure is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/K322A. In yet another disclosure is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/P329W and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/P329W. In an additional disclosure is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322A. In further disclosures is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322E/E333K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322E/E333K.

[0068] Disclosed is a heteromultimer as described herein, wherein: the first Fc polypeptide comprises the amino acid modifications L234D/L235E and the second Fc polypeptide comprises the amino acid modifications L234R/L235R/E233K; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/D265S and the second Fc polypeptide comprises the amino acid modifications L234R/L235R/D265S; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/K322A; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/P329W and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/P329W; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322A; or the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322E/E333K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322E/E333K.

[0069] Provided is a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide wherein: said first Fc polypeptide comprises the amino acid modifications E269Q/D270N and the second Fc polypeptide comprises the amino acid modifications E269K/D270R; or said first Fc polypeptide comprises the amino acid modifications L235K/A327K and the second Fc polypeptide does not comprise a modification at the hinge or lower hinge region; and wherein the IgG Fc construct displays reduced binding to all Fcγ receptors and to C1q protein as compared to a corresponding parent IgG Fc construct.

[0070] In some embodiments is the heteromultimer provided herein, wherein the IgG Fc construct is aglycosylated. In an embodiment is the heteromultimer described herein, wherein the IgG Fc construct is deglycosylated.

[0071] In some disclosures is the heteromultimer described herein, wherein the onset of melting of the IgG Fc construct in a thermogram is greater than or equal to 68°C. In some disclosures is the heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is comparable to the melting temperature of the parent CH2 region. In some disclosures is the heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is greater than or about the same as the melting temperature of the parent CH2 region. In some disclosures is the heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is about 1 to 2°C greater than the melting temperature of the parent CH2 region. In a particular disclosure is the heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is about 2 to 3°C greater than the melting temperature of the parent CH2 region. In a particular disclosure is the heteromultimer described herein, wherein the IgG Fc construct has a CH2 region with a melting temperature that is about 5°C greater than the melting temperature of the parent CH2 region.

[0072] Provided herein is a heteromultimer construct, wherein the IgG Fc construct comprises a variant CH3 region comprising amino acid modifications that promote the formation of a heterodimer Fc region.

[0073] In some embodiments is a heteromultimer construct described herein, wherein one of said first and second Fc polypeptides comprises the CH3 amino acid modifications T366L/N390R/K392M/T394W and the other Fc polypeptide comprises the CH3 amino acid modifications L351Y/S400E/F405A/Y407V.

[0074] In a particular disclosure is a heteromultimer construct described herein, wherein the IgG Fc construct comprises amino acid modifications that increase the stability of the CH3 region. In one embodiment the first and second Fc polypeptides comprise the amino acid modification T350V. In certain embodiments, any antibody constructs with homodimeric Fc constructs are clearly resolved from said heteromultimer using charge-based purification methods.

[0075] Provided are heteromultimers as described herein, wherein the heteromultimer further comprises at least one antigen-binding construct fused to the IgG Fc construct. In certain embodiments, the at least one antigen-binding construct is selected from a Fab fragment, an scFv, an sdAb, an antigen binding peptide, an Fc fusion protein, or a protein or fragment thereof capable of binding the antigen. In some embodiments is a heteromultimer comprising one antigen-binding construct. In some embodiments is a heteromultimer comprising two antigen-binding constructs. In an embodiment is a heteromultimer wherein the IgG Fc construct is linked to one or more toxic drug molecules. In certain embodiments, the IgG Fc construct is linked to one or more heterologous polypeptides. In some embodiments the one or more heterologous polypeptides is selected from enzymes and toxins.

[0076] Provided is a heteromultimer described herein, wherein the IgG is IgG1.

[0077] Provided herein is a nucleic acid encoding the first or second Fc polypeptide of the heteromultimer described herein. In some embodiments is a host cell comprising the nucleic acid described herein. In certain embodiments is a method of preparing the heteromultimer described herein, the method comprising the steps of: (a) culturing the host cell described herein; and (b) recovering the heteromultimer from the host cell culture.

[0078] Provided herein are pharmaceutical compositions comprising the heteromultimer described herein and a pharmaceutically acceptable carrier. In certain disclosures is a method of treating a disease comprising providing to a patient in need thereof an effective amount of the pharmaceutical composition described herein. In some disclosures is the use of the heteromultimer described herein in the preparation of a medicament for the treatment of a disease. In some embodiments is the use of a therapeutic amount of the heteromultimer described herein for use in the treatment of a disease in a patient in need thereof.

[0079] Disclosed herein is a method of reducing the ADCC of an antibody construct comprising: modifying the lower hinge region of a first and a second Fc polypeptide, wherein the modified lower hinge region of said first Fc polypeptide comprises at least one amino acid modification, the modified lower hinge region of said second Fc

polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, and the IgG Fc construct displays reduced binding to all Fcγ receptors or to C1q protein as compared to a corresponding parent IgG Fc construct. In certain embodiments is the method of reducing ADCC, wherein said modifications result in negligible binding to Fc receptors.

Definitions

[0080] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the claimed subject matter belongs. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

[0081] It is to be understood that the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of any subject matter claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise.

[0082] "Parent CH2 domain" refers to a CH2 domain polypeptide of an Fc region comprising an amino acid sequence which lacks the one or more amino acid modifications in the hinge region of the first and second Fc polypeptides disclosed herein, but which may include one or more of disulfide hinge modifications, added CH2 disulfides, glycosylation modifications or different CH3 domain stabilities, and which differs in effector function compared to the CH2 domain of the heteromultimers of the disclosure. The parent CH2 domain may comprise a native sequence Fc region or an Fc region with pre-existing amino acid sequence modifications (such as additions, deletions and/or substitutions).

[0083] "Aglycosylated antibodies" refers to antibodies or heteromultimers that are not glycosylated during expression. Aglycosylated antibodies can be prepared by expression in systems lacking a mammalian glycosylation pathway, such as E. coli, or mutating one or more glycosylation sites such as N297.

[0084] "Deglycosylated antibodies" refers to antibodies or heteromultimers that are initially glycosylated during expression, but that subsequently undergo a biochemical reaction such as, for example, PNGase F treatment, that removed the glycan.

[0085] "Amino acid with neutral side chain" refers to an amino acid that contains a side chain that lacks a charge at neutral pH. All amino acids except lysine, arginine, aspartate, glutamate, and histidine are considered neutral. In some embodiments, depending on the structural environment it is found in, lysine, arginine, aspartate, glutamate and histidine are also considered neutral. In one embodiment, an "amino acid with neutral side chain" refers to an amino acid that contains a side chain that lacks overall charge at physiological pH.

[0086] "Amino acid with positively charged side chain" refers to a polar amino acid that contains a side chain that is protonated and hence positively charged at neutral pH. These amino acids are often referred as basic. Examples of amino acids with positively charged side chains include lysine, arginine. In some embodiments, depending on the structural environment it is found in, histidine can also be protonated and have a positively charged side chain. In one embodiment, an "amino acid with a positively charged side chain" refers to an amino acid that contains a side chain that is positively charged at physiological pH.

[0087] "Amino acid with a negatively charged side chain" is a polar amino acid whose side chain is deprotonated and hence negatively charged at neutral pH. These amino acids are often referred as acidic. Examples of amino acids with negatively charged side chains include aspartic acid and glutamic acid. In one embodiment, an "amino acid with a negatively charged side chain" refers to an amino acid that contains a side chain that is negatively charged at physiological pH.

[0088] "Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen or FcγR). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd or K_D). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies bind antigen (or FcγR) weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigen (or FcγR) more tightly and remain bound longer.

[0089] The term "Fc region" (or fragment crystallizable region) is used to define the C-terminal region of an antibody. The Fc region is composed of two identical protein fragments, derived from the second and third constant domains of the antibody's two heavy chains: Chain A and Chain B. The second and third constant domains are known as the CH2 domain and the CH3 domain, respectively. The CH2 domain comprises a CH2 domain sequence of Chain A and a CH2 domain sequence of Chain B. The CH3 domain comprises a CH3 domain sequence of Chain A and a CH3 domain sequence of Chain B. As used herein, the Fc region includes the hinge region as defined below.

[0090] The term "Fc region sequence" is used to define a C-terminal region of an immunoglobulin heavy chain. The "Fc region sequence" may be a native Fc region sequence or a variant Fc region sequence. Although the boundaries of the Fc region sequence of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region sequence is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof.

[0091] The "CH2 domain sequence" of a human IgG Fc region sequence (also referred to as "Cγ2" domain sequence) usually extends from about amino acid 231 to about amino acid 340. The CH2 domain sequence is unique in that it is not closely paired with another domain sequence. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domain sequences of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, Molec. Immunol.22: 161-206 (1985).

[0092] The "CH3 domain sequence" comprises the stretch of residues C-terminal to a CH2 domain sequence in an Fc region sequence (i.e. from about amino acid residue 341 to about amino acid residue 447 of an IgG).

[0093] A "functional Fc region" possesses the "effector functions" of a native Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays as herein disclosed, for example.

[0094] A "native Fc region sequence" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

[0095] A "variant Fc region sequence" comprises an amino acid sequence which differs from that of a native Fc region sequence by virtue of "one or more amino acid modifications" as herein defined. The variant Fc region sequence has at least one amino acid substitution compared to a native Fc region sequence or to the Fc region sequence of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native Fc region sequence or in the Fc region sequence of the parent polypeptide. In certain embodiments, the variant Fc region sequence herein possesses at least about 80% identity with a native Fc region sequence and/or with an Fc region sequence of a parent polypeptide, and most preferably at least about 90% identity therewith, more preferably at least about 95% identity therewith.

[0096] The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody.

The preferred FcR is a native human FcR. Moreover, in certain embodiments, the FcR is one which binds an IgG antibody (a gamma receptor, FcγR) and includes receptors of the FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain, (see review M. in Daeron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991); Capel et al., Immunomethods 4:25-34 (1994); and de Haas et al., J. Lab. Clin. Med. 126:330-41 (1995). Other FcRs, including those to be identified in the future are encompassed by the term "FcR" herein. The term "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 1 17:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)). The term "FcγR" does not include the FcRn.

[0097] "Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express FcRs (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII and low levels of FcγRIIc, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991).

[0098] "Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

[0099] A heteromultimer with "greatly reduced," "silenced," "negligible" or "ablated" FcγR binding affinity and C1q binding affinity is one which has diminished FcγR binding activity and C1q binding activity compared to a parent polypeptide or to a polypeptide comprising a native Fc region sequence. In some disclosures, an heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" FcγR binding affinity and C1q binding affinity is also a heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" ADCC, ADCP and CDC activity compared to a parent polypeptide or to a polypeptide comprising a native Fc region sequence. A heteromultimer which "displays decreased or undetectable binding" to FcγR binds all FcγRs with lower affinity than the parent polypeptide. Such variants which display decreased binding to an FcγR may possess little or no appreciable binding to an FcγR. In one disclosure, the variant displays 0-20% binding to the FcγR compared to a native IgG Fc region, e.g. as determined in the Examples herein or as measured by change in equilibrium constant. In one disclosure, the variant displays 0-10% binding to the FcγR compared to a native IgG Fc region. In one disclosure, the variant displays 0-5% binding to the FcγR compared to a native IgG Fc region. In one disclosure, the variant displays 0-1% binding to the FcγR compared to a native IgG Fc region.

[0100] In another embodiment, a heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" binding to FcγRIIaH has a Kd for FcγRIIaH that is greater than 5 μM as measured by SPR (surface plasmon resonance). In another embodiment, a heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" binding to FcγRIIaR has a Kd for FcγRIIaR that is greater than 10 μM as measured by SPR (surface plasmon resonance). In another embodiment, a heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" binding to FcγRIIb has a K_D for FcγRIIb that is greater than 30 μM as measured by SPR (surface plasmon resonance). In another embodiment, a heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" binding to FcγRIIIaF has a K_D for FcγRIIIaF that is greater than 20 μM as measured by SPR (surface plasmon resonance). In another embodiment, a heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" binding to FcγRIIIaV has a K_D for FcγRIIIaV that is greater than 6 μM as measured by SPR (surface plasmon resonance). In another embodiment, a heteromultimer with "greatly reduced," "silenced," negligible" or "ablated" binding to FcγRIa has a K_D for FcγRIa that is greater than 6.5 nM as measured by SPR (surface plasmon resonance).

[0101] The heteromultimer which "mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells more effectively" than a parent antibody is one which in vitro or in vivo is substantially more effective at mediating ADCC, when the amounts of heteromultimer and parent antibody used in the assay are essentially the same. Generally, such polypeptides will be identified using the in vitro ADCC assay as herein disclosed, but other assays or methods for determining ADCC activity, e.g. in an animal model etc, are contemplated. The preferred polypeptide is from about 1.5 fold to about 100 fold, e.g. from about two fold to about fifty fold, more effective at mediating ADCC than the parent, e.g. in the *in vitro* assay disclosed herein.

[0102] A "parent antibody," "parent polypeptide," or "polypeptide comprising a native Fc region" refers to a construct that does not comprise amino acid modifications to the hinge region. In one embodiment, the parent antibody is one that does not comprise amino acid modifications to the hinge region and comprises modifications to the CH3 domain that promote the formation of a heterodimeric Fc region.

[0103] An "amino acid modification" refers to a change in the amino acid sequence of a predetermined amino acid sequence. Exemplary modifications include an amino acid substitution, insertion and/or deletion. In certain embodiments the amino acid modification herein is a substitution. An "amino acid modification at" a specified position, e.g. of the Fc region, refers to the substitution or deletion of the specified residue, or the insertion of at least one amino acid residue adjacent the specified residue. By insertion "adjacent" a specified residue is meant insertion within one to two residues thereof. In certain embodiments the insertion is N-terminal or C-terminal to the specified residue.

[0104] An "amino acid substitution" refers to the replacement of at least one existing amino acid residue in a predetermined amino acid sequence with another different "replacement" amino acid residue. The replacement residue or residues may be "naturally occurring amino acid residues" (i.e. encoded by the genetic code) and selected from the group consisting of: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); histidine (His); Isoleucine (Ile): leucine (Leu); lysine (Lys); methionine (Met); phenylalanine (Phe); proline (Pro): serine (Ser); threonine (Thr); tryptophan (Trp); tyrosine (Tyr); and valine (Val). Preferably, the replacement residue is not cysteine. Substitution with one or more non-naturally occurring amino acid residues is also encompassed by the definition of an amino acid substitution herein. A "non-naturally occurring amino acid residue" refers to a residue, other than those naturally occurring amino acid residues listed above, which is able to covalently bind adjacent amino acid residues(s) in a polypeptide chain. Examples of non-naturally occurring amino acid residues include norleucine, ornithine, norvaline, homoserine and other amino acid residue analogues such as those described in Ellman et al. Meth. Enzym. 202:301-336 (1991). To generate such non-naturally occurring amino acid residues, the procedures of Noren et al. Science 244: 182 (1989) and Ellman et al., supra, can be used. Briefly, these procedures involve chemically activating a suppressor tRNA with a non-naturally occurring amino acid residue followed by in vitro transcription and translation of the RNA.

[0105] An "amino acid insertion" refers to the incorporation of at least one amino acid into a predetermined amino acid sequence. In certain embodiments, the insertion consists of the insertion of one or two amino acid residues. In certain other embodiments, are larger "peptide insertions", e.g. insertion of about three to about five or even up to about ten amino acid residues. In these embodiments the inserted residue(s) are naturally occurring or non-naturally occurring as disclosed above.

[0106] An "amino acid deletion" refers to the removal of at least one amino acid residue from a predetermined amino acid sequence.

[0107] "Hinge region" is generally defined from Glu216 to Pro238 of human IgG1, of which Cys226 to Pro230 form the 'core' hinge region (Burton, Molec. Immunol.22: 161-206 (1985)), while Ala231 to Pro238 form the 'lower' hinge region. Glu216 to Thr225 form the "upper" hinge region. Hinge regions of other IgG isotypes may be aligned with the IgG 1 sequence by placing the first and last cysteine residues forming inter-heavy chain S--S bonds in the same positions.

[0108] "C1q" is a mulitmer of polypeptides that includes a binding site for the Fc region of an immunoglobulin. C1q together with two serine proteases, C1r and C1s, forms the complex C1, the first component of the complement dependent cytotoxicity (CDC) pathway. Human C1q can be purchased commercially from, e.g. Quidel, San Diego, Calif.

[0109] The term "binding domain" refers to the region of a polypeptide that binds to another molecule. In the case of an FcR, the binding domain can comprise a portion of a polypeptide chain thereof (e.g. the α chain thereof) which is responsible for binding an Fc region. One useful binding domain is the extracellular domain of an FcRα chain.

[0110] The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multi-specific antibodies (e.g., bi-specific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

[0111] "Antibody fragments", as defined herein, comprise a portion of an intact antibody, generally including at least one antigen binding or variable region of the intact antibody or the Fc region of an antibody. Examples of antibody fragments include linear antibodies; single-chain antibody molecules; and multi-specific antibodies formed from antibody fragments. In certain embodiments, the antibody fragments retain at least part of the hinge and optionally the CH1 region of an IgG heavy chain. In some embodiments the antibody fragments retain the entire constant region of an IgG heavy chain, and include an IgG light chain.

[0112] 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 are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. 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. In certain embodiments the monoclonal antibodies to be used in accordance with the present disclosure are 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. Pat. No. 4,816,567). In some embodiments "monoclonal antibodies" are 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.

[0113] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA 81 :6851-6855 (1984)).

[0114] A "disorder" is any condition that would benefit from treatment with the polypeptide variant. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. In one embodiment, the disorder is cancer.

[0115] The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. In certain embodiments the label is itself detectable (e.g., radioisotope labels or fluorescent labels). In some other embodiments, the label catalyzes chemical alteration of a substrate compound or composition which is detectable. An exemplary embodiment comprises an enzymatic label that catalyzes a chemical alteration of a substrate compound or composition which is detectable.

[0116] An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the polypeptide nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the polypeptide where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

[0117] The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

[0118] 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.

[0119] As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "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. Where distinct designations are intended, it will be clear from the context.

[0120] The phrase "low affinity receptor" denotes a receptor that has a weak binding affinity for a ligand of interest, e.g. having a dissociation constant of about 50 nM or worse affinity. Exemplary low affinity receptors include FcγRII and FcγRIII.

[0121] By "Fc-fusion" as used herein is meant a protein wherein one or more polypeptides is operably linked to an Fc region or a derivative thereof. Fc fusion is herein meant to be synonymous with the terms "immunoadhesin", "Ig fusion", "Ig chimera", and "receptor globulin" (sometimes with dashes) as used in the art (Chamow et al., 1996, Trends Biotechnol 14:52-60; Ashkenazi et al., 1997, Curr Opin Immunol 9:195-200). An Fc fusion combines the Fc region of an immunoglobulin with a fusion partner, which in general can be any protein or small molecule. The role of the non-Fc part of an Fc fusion, i.e. the fusion partner, is to mediate target binding, and thus it is functionally analogous to the variable regions of an antibody. Virtually any protein or small molecule may be linked to Fc to generate an Fc fusion. Protein fusion partners may include, but are not limited to, the target-binding region of a receptor, an adhesion molecule, a ligand, an enzyme, a cytokine, a chemokine, or some other protein or protein domain. Small molecule fusion partners may include any therapeutic agent that directs the Fc fusion to a therapeutic target. Such targets may be any molecule, preferably an extracellular receptor, that is implicated in disease. Two families of surface receptors that are targets of a number of approved small molecule drugs are G-Protein Coupled Receptors (GPCRs), and ion channels, including .+, Na+, Ca+ channels. Nearly 70% of all drugs currently marketed worldwide target GPCRs. Thus the Fc proteins described herein may be fused to a small molecule that targets, for example, one or more GABA receptors, purinergic receptors, adrenergic receptors, histaminergic receptors, opioid receptors, chemokine receptors, glutamate receptors, nicotinic receptors, the 5HT (serotonin) receptor, and estrogen receptors. A fusion partner may be a small-molecule mimetic of a protein that targets a therapeutically useful target. Specific examples of particular drugs that may serve as Fc fusion partners can be found in L. S. Goodman et al., Eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics

(McGraw-Hill, New York, ed. 9, 1996). Fusion partners include not only small molecules and proteins that bind known targets for existing drugs, but orphan receptors that do not yet exist as drug targets. The completion of the genome and proteome projects are proving to be a driving force in drug discovery, and these projects have yielded a trove of orphan receptors. There is enormous potential to validate these new molecules as drug targets, and develop protein and small molecule therapeutics that target them. Such protein and small molecule therapeutics are contemplated as Fc fusion partners that employ the IgG Fc constructs described herein. A variety of linkers, defined and described below, may be used to covalently link Fc to a fusion partner to generate an Fc fusion.

[0122] By "target antigen" as used herein is meant the molecule that is bound specifically by the variable region of a given antibody. A target antigen may be a protein, carbohydrate, lipid, or other chemical compound.

[0123] By "target cell" as used herein is meant a cell that expresses a target antigen.

[0124] Throughout the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (19 1), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

1. Heteromultimers comprising an IgG Fc construct

[0125] Disclosed herein are heteromultimer constructs with reduced or silenced effector function. In a particular disclosure is provided a heteromultimer construct comprising an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified hinge region wherein: the modified hinge region of said first Fc polypeptide comprises at least one amino acid modification, the modified hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, and the IgG Fc construct displays reduced binding to all Fcγ receptors and to C1q protein as compared to a corresponding parent IgG Fc construct.

[0126] Disclosed herein are heteromultimer constructs with reduced or silenced effector function. In an embodiment is provided a heteromultimer construct comprising an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified lower hinge region wherein: the modified lower hinge region of said first Fc polypeptide comprising amino acid modifications at L234 and/or L235, the modified lower hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, and the IgG Fc construct displays reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors and to C1q protein as compared to a corresponding parent IgG Fc construct. In certain embodiments, the heteromultimer construct displays negligible binding the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding parent IgG Fc construct that does not have the modifications described herein. In some disclosures, the heteromultimer construct displays reduced binding to all Fcγ receptors and negligible binding to at least one Fcγ receptor. In certain disclosures, the heteromultimer construct described herein displays reduced binding to Fcγ receptors and negligible binding to C1q protein.

[0127] Disclosed herein is a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified hinge region wherein: the modified hinge region of said first Fc polypeptide comprises at least one amino acid modification that increases the net charge in the modified hinge region of the first Fc polypeptide at about physiological pH conditions, the modified hinge region of said second Fc polypeptide comprises at least one amino acid modification which is different from at least one amino acid modification of said first Fc polypeptide, and the IgG Fc construct displays reduced binding to all Fcγ receptors and to C1q protein as compared to a corresponding parent IgG Fc construct. In some embodiments, the increase in net charge is an increase in the net positive charge on the first Fc polypeptide. In an embodiment, the increase in the net positive charge is an increase in the total number of positively charged amino acids on the first Fc polypeptide. In certain disclosures, said increase in net charge is an increase in the net negative charge on the

first Fc polypeptide. In a particular disclosure, the increase in the net negative charge is an increase in the total number of negatively charged amino acids on the first Fc polypeptide. In some embodiments, the heteromultimer construct displays negligible binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors as compared to a corresponding parent IgG Fc construct that does not have the modifications described herein. In particular disclosures, the heteromultimer construct displays reduced binding to all Fcγ receptors and negligible binding to at least one Fcγ receptor. In an embodiment, the heteromultimer construct described herein displays reduced binding to the FcγRIa, FcγRIIa, FcγRIIb and FcγRIIIa receptors and negligible binding to C1q protein.

[0128] Disclosed are heteromultimers comprising an IgG Fc construct, said IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified hinge region wherein the modified hinge region of said first Fc polypeptide comprises one or more amino acid modifications that increase the number of positive charges in the modified hinge region of the first Fc polypeptide, wherein the modified hinge region of said second Fc polypeptide comprises one or more amino acid modifications different from the one or more amino acid modifications of said first Fc polypeptide, and wherein the IgG Fc construct does not bind to Fcγ receptors or to C1q protein.

1.1 Fc region and hinge region

[0129] The heteromultimers comprise an IgG Fc construct (Fc region) which includes the hinge region. The Fc region of an antibody typically comprises two polypeptide chains, each of which comprises a C-terminal fragment of an IgG heavy chain polypeptide. Accordingly, the IgG Fc construct has two Fc polypeptides, each derived from an IgG heavy chain polypeptide and including the regions that mediate binding to FcγRs, complement, and FcRn, as well as the hinge region.

[0130] In one embodiment the heteromultimer comprises an IgG Fc construct that is derived from a human IgG heavy chain polypeptide. Several human IgG heavy chain polypeptides sub-types are known in the art and include IgG1, IgG2, IgG3, and IgG4. Of these human IgG sub-types, IgG1, IgG2, and IgG3 are known to activate complement, and IgG1 and IgG3 mediate the effector function ADCC (antibody-dependent cell-mediated cytotoxicity) more effectively than IgG2 and IgG4. In one embodiment, the heteromultimer comprises an IgG Fc construct that is derived from a human IgG1 heavy chain polypeptide. The amino acid sequence of the human IgG1 heavy chain is known in the art (see for example IMGT Accession No. J00228). In one embodiment, the heteromultimer comprises an IgG Fc construct that is derived from a human IgG3 heavy chain polypeptide. The sequence of the human IgG3 heavy chain is known in the art (see for example IMGT Accession No. X03604). In one embodiment, the heteromultimer comprises an IgG Fc construct that is derived from a human IgG4 heavy chain polypeptide. The sequence of the human IgG4 heavy chain is known in the art (see for example IMGT Accession No. K01316). The amino acid sequence of a human IgG1 Fc region, including the hinge region is shown in Figure 5 (SEQ ID NO:1).

[0131] In a further embodiment, the heteromultimer can comprise an IgG Fc region that is derived from an allotype of IgG. IgG allotypes are known in the art (see, for example, Jefferies et al. (2009) Mabs 1(4):332-338).

[0132] In one embodiment, the IgG Fc region is derived from a humanized monoclonal antibody with therapeutic potential, selected from: alemtuzumab, apolizumab, aselizumab, atlizumab, bapineuzumab, bevacizumab, bivatuzumab, cantuzumab, cedelizumab, certolizumab pegol, cidfusituzumab, cidtuzumab, daclizumab, eculizumab, efalizumab, epratuzumab, erlizumab, felvizumab, fontolizumab, gemtuzumab ozogamicin, inotuzumab ozogamicin, ipilimumab, labetuzumab, lintuzumab, matuzumab, mepolizumab, motavizumab, motovizumab, natalizumab, nimotuzumab, nolovizumab, numavizumab, ocrelizumab, omalizumab, palivizumab, pascolizumab, pecfusituzumab, pectuzumab, pertuzumab, pexelizumab, ralivizumab, ranibizumab, reslivizumab, reslizumab, resyvizumab, rovelizumab, ruplizumab, sibrotuzumab, siplizumab, sontuzumab, tacatuzumab tetraxetan, tadocizumab, talizumab, tefibazumab, tocilizumab, toralizumab, trastuzumab, tucotuzumab celmoleukin, tucusituzumab, umavizumab, urtoxazumab, and visilizumab.

[0133] In another embodiment, the IgG Fc region is derived from a therapeutic antibody such as, for example, rituximab.

[0134] Each Fc polypeptide of the IgG Fc construct comprises at least a portion of the Fc region of the IgG heavy chain polypeptide including the hinge region. The Fc region of IgG polypeptides includes binding sites for multiple receptors that mediate the effector functions of the Fc region. Examples of such receptors include FcγRIa, FcγRIIa, FcγRIIb, FcγRIIIa. The Fc region further comprises a region that binds to complement factor C1q protein.

[0135] The Fc region of the human IgG1 heavy chain polypeptide comprises amino acids 216-447 of the IgG1 heavy chain (see SEQ ID NO:2, Figure 5). The length and sequence of the human IgG hinge region varies with the IgG isotype as shown in Table 1 below:

Table 1: Comparison of human IgG hinge region amino acid sequences

IgG	length	CH1	'upper' hinge	'core' hinge	'lower' hinge (CH2)
IgG1	15	VDKRV	EPKSCDKTHT	CPPCP	APELLGGP
IgG2	12	VDKTV	ELK CCVE	CPPCP	APPVAGP
IgG3	62	VDKRV	ELKTPLGDTTHT	CPRCP (EPKSCDTPPPCPRCP)x3	APELLGGP
IgG4	12	VDKRV	ESKYGPP	CPSCP	APELLGGP

[0136] The hinge region of the IgG1 polypeptide comprises amino acid residues from 216 to 238, while the lower hinge region of the human IgG1 polypeptide comprises amino acid residues from 231 to 238.

[0137] Thus, in one embodiment, each Fc polypeptide of the IgG Fc construct comprises amino acids 216 to 447 of the human IgG1 heavy chain, wherein said first Fc polypeptide includes at least one modification in the hinge region comprising amino acid residues 216 to 238, and said second Fc polypeptide comprises at least one amino acid modification in the hinge region which is different from the at least one amino acid modification in the first Fc polypeptide. In another embodiment, each Fc polypeptide of the IgG Fc construct comprises amino acids 231 to 447 of the human IgG1 heavy chain, wherein said first Fc polypeptide includes at least one modification in the lower hinge region comprising amino acid residues 231 to 238, and said second Fc polypeptide comprises at least one amino acid modification in the lower hinge region that is different from the at least one amino acid modification in the first Fc polypeptide.

1.1.2 Amino acid modifications in the modified hinge region

[0138] The first and second Fc polypeptides of the IgG Fc constructs comprise a modified hinge region that is asymmetrically modified to generate heteromultimers with greatly decreased or ablated effector function. The terms "first" and "second" with reference to Fc polypeptide can be used interchangeably provided that each IgG Fc construct comprises one first Fc polypeptide and one second Fc polypeptide. The amino acid modifications are introduced into the hinge region of the first and second Fc polypeptides in an asymmetric fashion as described in more detail below.

[0139] The heteromultimers comprise first and second Fc polypeptide comprising core amino acid modifications described in the following paragraphs.

[0140] In some embodiments is a heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications at least one of L234 and L235. In an embodiment is the heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications selected from L234K, L234R, L234A, L235K, L235R, and L235A. In an embodiment is the heteromultimer described herein, said one of first and second Fc

polypeptides further comprises an amino acid modification at E233. In an embodiment is the heteromultimer provided herein, wherein said modification at E233 is selected from E233A, E233K, and E233R. In some embodiments is the heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises amino acid modifications of at least one of L234 and L235. In an embodiment is the heteromultimer described herein, wherein said modification at least one of L234 and L235 is selected from L234A, L234K, L234R, L234D, L234E, L235K, L235R, L235E, L235A, and L235D. In one embodiment is the heteromultimer described herein, wherein the modified hinge region of the second Fc polypeptide further comprises amino acid modifications at E233. In an embodiment, the first or second Fc polypeptide comprises amino acid modifications selected from E233A or E233D.

[0141] Provided herein is a heteromultimer described herein, wherein the modified hinge region of at least one of said first and second Fc polypeptides comprises the amino acid modifications L234A/L235K, L234K/L235K, E233A/L234R/L235R, E233K/L234R/L235R, or E233K/L234A/L235K. In some embodiments is a heteromultimer described herein, wherein the modified hinge region of the first or second Fc polypeptide comprises the amino acid modifications L234A/L235A, L234D/L235E, E233A/L234D/L235E, or E233A/L234K/L235A.

[0142] Provided herein is a heteromultimer as described herein, wherein the modified hinge region of the first Fc polypeptide comprises the amino acid modifications L234K/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234A/L235K; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications L234K/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234A/L235A; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications L234K/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234D/L235E; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233A/L234R/L235R and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications E233A/L234D/L235E; the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications L234D/L235E; or the modified hinge region of the first Fc polypeptide comprises the amino acid modifications E233K/L234A/L235K and the modified hinge region of the second Fc polypeptide comprises the amino acid modifications E233A/L234K/L235A.

[0143] In some embodiments, the heteromultimer comprise first and second polypeptides comprising the core amino acid modifications, and comprise the following additional amino acid modification. Thus in some embodiments is the heteromultimer provided herein, wherein at least one of said first or second Fc polypeptides further comprises at least one amino acid modification selected from D265S, E269K, K322A, P329W, and E333K. For instance, in an embodiment is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E and the second Fc polypeptide comprises the amino acid modifications L234R / L235R/E233K. In another embodiment is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/D265S and the second Fc polypeptide comprises the amino acid modifications L234R/L235R/D265S. In a further embodiment is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K. In another embodiment is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/K322A. In yet another embodiment is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/P329W and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/P329W. In an additional embodiment is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322A. In further embodiments is a heteromultimer as described herein, wherein the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322E/E333K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322E/E333K.

[0144] Disclosed is a heteromultimer as described herein, wherein: the first Fc polypeptide comprises the amino acid modifications L234D/L235E and the second Fc polypeptide comprises the amino acid modifications L234R/L235R/E233K; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/D265S and the second Fc polypeptide comprises the amino acid modifications L234R/L235R/D265S; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/K322A; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/P329W and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/P329W; the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322A and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322A; or the first Fc polypeptide comprises the amino acid modifications L234D/L235E/E269K/D265S/K322E/E333K and the second Fc polypeptide comprises the amino acid modifications E233K/L234R/L235R/E269K/D265S/K322E/E333K.

First Fc polypeptide

[0145] Each of the two Fc polypeptides of the IgG Fc construct comprises a modified hinge region. The modified hinge region of the first Fc polypeptide comprises one or more amino acid modifications that increase the positive charge of the modified hinge region of that Fc polypeptide. By "increase the positive charge of the modified hinge region" is meant that the modified hinge region with one or more amino acid modifications has an overall positive charge that is greater than that of the wild-type, unmodified hinge region. The modified hinge region of the second Fc polypeptide comprises one or more amino acid modifications that are different from the one or more amino acid modifications of the other Fc polypeptide. As used herein, asymmetric amino acid modifications are any modification wherein an amino acid at a specific position on one polypeptide (e.g., "first polypeptide") is different from the amino acid on the second polypeptide (e.g., "second polypeptide") at the same position. This can be a result of modification of only one of the two amino acids or modification of both amino acids to two different amino acids from the first or second polypeptide of the IgG Fc construct.

[0146] For example, if the first Fc polypeptide comprises the amino acid modification L235K, amino acid modifications that are different would include modification of other amino acids in the hinge region, such as L234 or E233, or modification of L235 other than L235K, such as L235A or L235D. Thus, in one embodiment, the modified hinge region of a first Fc polypeptide comprises one or more amino acid modifications that increase the positive charge of the modified hinge region of the first Fc polypeptide, and the modified hinge region of a second Fc polypeptide comprises one or more amino acid modifications different from the two or more amino acid modifications of said first Fc polypeptide. In another embodiment, the modified hinge region of said first Fc polypeptide comprises two or more amino acid modifications that increase the positive charge of the modified hinge region of the first Fc polypeptide, and the modified hinge region of said second Fc polypeptide comprises one or more amino acid modifications different from the one or more amino acid modifications of said first Fc polypeptide. In an alternate embodiment, the modified hinge region of said first Fc polypeptide comprises two or more amino acid modifications that increase the positive charge of the modified hinge region of the first Fc polypeptide, and the modified hinge region of said second Fc polypeptide comprises two or more amino acid modifications different from the two or more amino acid modifications of said first Fc polypeptide.

[0147] In one embodiment, when the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises one amino acid modification, the amino acid modification is made to substitute an amino acid having a neutral or negatively charged side chain with an amino acid having a positively charged side chain. For example, L234 or L235 of the hinge region can be substituted with Lys (K), Orn (O) or Arg (R). Thus, in embodiments where the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises one amino acid modification, the following amino acid residues of the lower hinge region of human IgG1 can be substituted with Lys, Orn, or Arg: A231, P232, E233, L234, L235, G236, G237, or P238.

[0148] In embodiments where the modified hinge region of the first Fc polypeptide comprises two or more amino acid modifications, the overall result of the combination of modifications is an increase in positive charge of the modified hinge region. This overall increase in positive charge can result from combinations of amino acid modifications substituting amino acids having negatively charged or neutral side chains with amino acids having positively charged side chains or neutral side chains. For example, in one embodiment, the two or more amino acid modifications that increase the positive charge of the lower hinge region of the first Fc polypeptide of an IgG1 IgG Fc construct can be selected from E233K, E233R, E233A, L234K, L234R, L234A, L235K, L235R, and L235A provided that the combination of the two or more amino acid modifications increases the positive charge of the modified hinge region. Similarly, other amino acid modifications within the hinge region may be made as long as they increase the positive charge of the hinge region.

[0149] In one embodiment, the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L234 or L235 that increase the positive charge of the modified hinge region. In another embodiment, the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L234 and L235 that increase the positive charge of the modified hinge region. In another embodiment, the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L234, L235 or E233 that increase the positive charge of the modified hinge region. In another embodiment, the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L234 and E233 that increase the positive charge of the modified hinge region. In another embodiment, the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L235 and E233 that increase the positive charge of the modified hinge region. In another embodiment, the modified hinge region of the first Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L234, L235 and E233 that increase the positive charge of the modified hinge region.

[0150] In one embodiment, the first Fc polypeptide of the IgG Fc construct comprises a modified hinge region comprising the amino acid modifications L234A/L235K, E233A/ L234R/L235R, E233K/L234R/L235R, or E233K/L234A/L235K.

Second Fc polypeptide

[0151] In one disclosure, the modified hinge region of the second Fc polypeptide of the IgG Fc construct comprises one or more amino acid modifications that are different from the amino acid modifications of the first Fc polypeptide, and that increase the negative charge of the modified hinge region or that are charge neutral relative to the wild-type hinge region. By "increase the negative charge of the modified hinge region" is meant that the modified hinge region of the second Fc polypeptide of the IgG Fc construct with one or more amino acid modifications has an overall negative charge that is greater than that of the wild-type, unmodified hinge region. By "charge neutral" is meant that the one or more amino acid modifications do not result in a change in the overall charge of the modified hinge region of the second Fc polypeptide of the IgG Fc construct compared to that of the wild-type hinge region. Thus, amino acid modifications of the second polypeptide of the IgG Fc construct include combinations of amino acid modifications that replace amino acids having a neutral side chain with amino acids having a negatively charged side chain, a positively charged sided chain or a different neutral side chain, and/or amino acid modifications that replace amino acids having a negatively charged side chain with amino acids having a neutral side chain. Combinations of these amino acid modifications are suitable as long as they result in an increase in the negative charge of the modified hinge region or are charge neutral with respect to the wild-type hinge region.

[0152] In one embodiment, the second Fc polypeptide of the IgG Fc construct comprises an amino acid modification at L234 or L235. In another embodiment, the second Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L234 and L235. In one embodiment, the second Fc polypeptide of the IgG Fc construct comprises amino acid modifications selected from L234A, L234K, L234R, L234D, L234E, L235K, L235R, L235E, L235A, and L235D.

[0153] In one embodiment, the second Fc polypeptide of the IgG Fc construct comprises amino acid modifications at L234 and/or L235, and E233. In one embodiment, the second Fc polypeptide of the IgG Fc construct comprises amino acid modifications selected from L234A, L234K, L234R, L234D, L234E, L235K, L235R, L235E, L235A, L235D, E233A and E233D.

[0154] In one embodiment, the modified hinge region of the second Fc polypeptide of the IgG Fc construct comprises the amino acid modifications L234A/L235A, L234D/L235E, E233A/L234D/L235E, or E233A/L234K/L235A.

2. Other modifications to the IgG Fc construct

[0155] In some embodiments, the heteromultimers according to the invention comprise additional modifications as described below.

[0156] In one embodiment of the invention, the heteromultimer comprises an IgG Fc construct comprising modified Fc polypeptides that have been further modified promote the formation of a heterodimeric Fc region. Such further modified Fc polypeptides are useful in the production of heteromultimers in the context of bi-specific antibodies. In one embodiment, the Fc polypeptides comprise variant CH3 domains having amino acid modifications that promote the formation of heterodimeric Fc regions. Suitable variant CH3 domains are known in the art and include, for example, those described in International Patent Publication No. WO 2012/058768, and U.S. Patent Nos. 5,821,333, 7,695,936. In one embodiment, the heteromultimer according to the invention comprises an IgG Fc construct wherein one of said first and second Fc polypeptides comprises the CH3 amino acid modifications T366L/N390R/K392M/T394W and the other Fc polypeptide comprises the CH3 amino acid modifications L351Y/S400E/F405A/Y407V.

[0157] Additional methods for modifying Fc polypeptides to promote heterodimeric Fc formation are described in International Patent Publication No. WO 96/027011 (knobs into holes), in Gunasekaran *et al.* (Gunasekaran K. et al. (2010) J Biol Chem. 285, 19637-46, electrostatic design to achieve selective heterodimerization), in Davis *et al.* (Davis, JH. et al. (2010) Prot Eng Des Sel ;23(4): 195-202, strand exchange engineered domain (SEED) technology), and in Moore et al (2011) Mabs 3:6, 546-557.

[0158] In one embodiment, the heteromultimer comprises an IgG Fc construct further comprising amino acid modifications that promote the formation of a heterodimeric Fc region. In one embodiment, the heteromultimer comprises an IgG Fc construct further comprising amino acid modifications in the CH3 region of each Fc polypeptide that promote the formation of a heterodimeric Fc region.

[0159] In some disclosures, the heteromultimer comprises an IgG Fc construct further comprising amino acid modifications that increase the stability of the IgG Fc construct, as determined by the melting temperature of the CH2 domain. Suitable amino acid modifications are known in the art and include, for example, those described in International Patent Application No. PCT/CA2012/050780. Specifically, in one embodiment, the heteromultimer comprises an IgG Fc construct comprising the amino acid modification T350V in both the first Fc polypeptide and the second Fc polypeptide.

[0160] Provided is a heteromultimer comprising an IgG Fc construct having a first and a second Fc polypeptide wherein: said first Fc polypeptide comprises the amino acid modifications E269Q/D270N and the second Fc polypeptide comprises the amino acid modifications E269K/D270R; or said first Fc polypeptide comprises the amino acid modifications L235K/A327K and the second Fc polypeptide does not comprise a modification at the hinge or lower hinge region; and wherein the IgG Fc construct displays reduced binding to all Fcγ receptors and to C1q protein as compared to a corresponding parent IgG Fc construct.

[0161] In one embodiment, the Fc is an IgG1 Fc construct, an IgG3 Fc construct, or an IgG4 Fc construct.

[0162] In some embodiments, an IgG Fc construct comprises at least one CH3 domain that has at least one amino acid modification that promotes the formation of a heterodimeric Fc with stability comparable to a wild-type homodimeric Fc. Exemplary modifications are described below. In some disclosures, the dimerized CH3 domains of the heterodimeric Fc have a melting temperature (Tm) as measured by differential scanning calorimetry (DSC) of about 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 77.5, 78, 79, 80, 81, 82, 83, 84, or 85°C or higher. In some disclosures, the dimeric Fc is a heterodimer formed with a purity greater than about 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% when produced; or wherein the Fc is a heterodimer formed with a purity greater than about 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% when expressed or when expressed via a single cell.

[0163] In some aspects, the Fc comprises one or more modifications in at least one of the CH3 sequences. In some disclosures, the Fc comprises one or more modifications in at least one of the CH2 sequences.

[0164] In some aspects, Fc is a Fc described in patent applications PCT/CA2011/001238, filed November 4, 2011 or PCT/CA2012/050780, filed November 2, 2012.

[0165] In some aspects, a Fc construct described herein comprises a heterodimeric Fc comprising a modified CH3 domain that has been asymmetrically modified. The heterodimeric Fc can comprise two heavy chain constant domain polypeptides: a first heavy chain polypeptide and a second heavy chain polypeptide, which can be used interchangeably provided that Fc comprises one first heavy chain polypeptide and one second heavy chain polypeptide. Generally, the first heavy chain polypeptide comprises a first CH3 sequence and the second heavy chain polypeptide comprises a second CH3 sequence.

[0166] Two CH3 sequences that comprise one or more amino acid modifications introduced in an asymmetric fashion generally results in a heterodimeric Fc, rather than a homodimer, when the two CH3 sequences dimerize. As used herein, "asymmetric amino acid modifications" refers to any modification where an amino acid at a specific position on a first CH3 sequence is different from the amino acid on a second CH3 sequence at the same position, and the first and second CH3 sequence preferentially pair to form a heterodimer, rather than a homodimer. This heterodimerization can be a result of modification of only one of the two amino acids at the same respective amino acid position on each sequence; or modification of both amino acids on each sequence at the same respective position on each of the first and second CH3 sequences. The first and second CH3 sequence of a heterodimeric Fc can comprise one or more than one asymmetric amino acid modification.

[0167] *Table X provides the amino acid sequence of a human IgG1 Fc sequence, corresponding to amino acids 231 to 447 of a full-length human IgG1 heavy chain. The CH3 sequence comprises amino acid 341-447 of the full-length human IgG1 heavy chain. Typically an Fc can include two contiguous heavy chain sequences (A and B) that are capable of dimerizing. In some aspects, one or both sequences of an Fc include one or more mutations or modifications at the following locations: L351, F405, Y407, T366, K392, T394, T350, S400, and/or N390, using EU numbering. In some aspects, a Fc includes a mutant sequence shown in Table X. In some aspects, a Fc includes the mutations of Variant 1 A-B. In some aspects, a Fc includes the mutations of Variant 2 A-B. In some aspects, a Fc includes the mutations of Variant 3 A-B. In some aspects, a Fc includes the mutations of Variant 4 A-B. In some aspects, a Fc includes the mutations of Variant 5 A-B.*

Table X: Exemplary Fc sequence and CH3 modifications

Human IgG1 Fc sequence 231-447 (EU-numbering)	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGK (SEQ ID NO:70)	
Variant IgG1 Fc sequence (231-447)	Chain	Mutations
1	A	L351Y_F405A_Y407V
1	B	T366L_K392M_T394W

Variant IgG1 Fc sequence (231-447)	Chain	Mutations
2	A	L351Y_F405A_Y407V
2	B	T366L_K392L_T394W
3	A	T350V_L351Y_F405A_Y407V
3	B	T350V_T366L_K392L_T394W
4	A	T350V_L351Y_F405A_Y407V
4	B	T350V_T366L_K392M_T394W
5	A	T350V_L351Y_ S400E_ F405A_Y407V
5	B	T350V_T366L_N390R_K392M_T394W

[0168] The first and second CH3 sequences can comprise amino acid mutations as described herein, with reference to amino acids 231 to 447 of the full-length human IgG1 heavy chain. In one embodiment, the heterodimeric Fc comprises a modified CH3 domain with a first CH3 sequence having amino acid modifications at positions F405 and Y407, and a second CH3 sequence having amino acid modifications at position T394. In one embodiment, the heterodimeric Fc comprises a modified CH3 domain with a first CH3 sequence having one or more amino acid modifications selected from L351Y, F405A, and Y407V, and the second CH3 sequence having one or more amino acid modifications selected from T366L, T366I, K392L, K392M, and T394W.

[0169] In one embodiment, an Fc construct comprises a heterodimeric Fc which comprises a modified CH3 domain with a first CH3 sequence having amino acid modifications at positions L351, F405 and Y407, and a second CH3 sequence having amino acid modifications at positions T366, K392, and T394, and one of the first or second CH3 sequences further comprising amino acid modifications at position Q347, and the other CH3 sequence further comprising amino acid modification at position K360. In another embodiment, a heterodimeric Fc comprises a modified CH3 domain with a first CH3 sequence having amino acid modifications at positions L351, F405 and Y407, and a second CH3 sequence having amino acid modifications at position T366, K392, and T394, one of the first or second CH3 sequences further comprising amino acid modifications at position Q347, and the other CH3 sequence further comprising amino acid modification at position K360, and one or both of said CH3 sequences further comprise the amino acid modification T350V.

[0170] In one embodiment, an Fc construct provided herein comprises a heterodimeric Fc comprises a modified CH3 domain with a first CH3 sequence having amino acid modifications at positions L351, F405 and Y407, and a second CH3 sequence having amino acid modifications at positions T366, K392, and T394 and one of said first and second CH3 sequences further comprising amino acid modification of D399R or D399K and the other CH3 sequence comprising one or more of T411E, T411D, K409E, K409D, K392E and K392D. In another embodiment, a heterodimeric Fc comprises a modified CH3 domain with a first CH3 sequence having amino acid modifications at positions L351, F405 and Y407, and a second CH3 sequence having amino acid modifications at positions T366, K392, and T394, one of said first and second CH3 sequences further comprises amino acid modification of D399R or D399K and the other CH3 sequence comprising one or more of T411E, T411D, K409E, K409D, K392E and K392D, and one or both of said CH3 sequences further comprise the amino acid modification T350V.

[0171] In one embodiment, a heterodimeric Fc comprises a modified CH3 domain with a first CH3 sequence having amino acid modifications at positions L351, F405 and Y407, and a second CH3 sequence having amino acid modifications at positions T366, K392, and T394, wherein one or both of said CH3 sequences further comprise the amino acid modification of T350V.

[0172] In one embodiment, a heterodimeric Fc comprises a modified CH3 domain comprising the following amino acid modifications, where "A" represents the amino acid modifications to the first CH3 sequence, and "B" represents the amino acid modifications to the second CH3 sequence: **A**:L351Y_F405A_Y407V,

B:T366L_K392M_T394W, **A**:L351Y_F405A_Y407V, **B**:T366L_K392L_T394W, **A**:T350V_L351Y_F405A_Y407V, **B**:T350V_T366L_K392L_T394W, **A**:T350V_L351Y_F405A_Y407V, **B**:T350V_T366L_K392M_T394W, **A**:T350V_L351Y_S400E_F405A_Y407V, and/or **B**:T350V_T366L_N390R_K392M_T394W.

[0173] The one or more asymmetric amino acid modifications can promote the formation of a heterodimeric Fc in which the heterodimeric CH3 domain has a stability that is comparable to a wild-type homodimeric CH3 domain. In an embodiment, the one or more asymmetric amino acid modifications promote the formation of a heterodimeric Fc domain in which the heterodimeric Fc domain has a stability that is comparable to a wild-type homodimeric Fc domain. In a particular disclosure, the one or more asymmetric amino acid modifications promote the formation of a heterodimeric Fc domain in which the heterodimeric Fc domain has a stability observed via the melting temperature (Tm) in a differential scanning calorimetry study, and where the melting temperature is within 4°C of that observed for the corresponding symmetric wild-type homodimeric Fc domain. In some aspects, the Fc comprises one or more modifications in at least one of the CH3 sequences that promote the formation of a heterodimeric Fc with stability comparable to a wild-type homodimeric Fc.

[0174] In one disclosure, the stability of the CH3 domain can be assessed by measuring the melting temperature of the CH3 domain, for example by differential scanning calorimetry (DSC). Thus, in a further disclosure, the CH3 domain has a melting temperature of about 68°C or higher. In another disclosure, the CH3 domain has a melting temperature of about 70°C or higher. In another disclosure, the CH3 domain has a melting temperature of about 72°C or higher. In another disclosure, the CH3 domain has a melting temperature of about 73°C or higher. In another disclosure, the CH3 domain has a melting temperature of about 75°C or higher. In another disclosure, the CH3 domain has a melting temperature of about 78°C or higher. In some disclosures, the dimerized CH3 sequences have a melting temperature (Tm) of about 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 77.5, 78, 79, 80, 81, 82, 83, 84, or 85°C or higher.

[0175] In some disclosures is a Fc construct comprising a heterodimeric Fc further comprising modified CH3 sequences can be formed with a purity of at least about 75% as compared to homodimeric Fc in the expressed product. In another disclosure, the heterodimeric Fc is formed with a purity greater than about 80%. In another disclosure, the heterodimeric Fc is formed with a purity greater than about 85%. In another disclosure, the heterodimeric Fc is formed with a purity greater than about 90%. In another disclosure, the heterodimeric Fc is formed with a purity greater than about 95%. In another disclosure, the heterodimeric Fc is formed with a purity greater than about 97%. In some disclosures, the Fc is a heterodimer formed with a purity greater than about 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% when expressed. In some disclosures, the Fc is a heterodimer formed with a purity greater than about 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% when expressed via a single cell.

[0176] Additional methods for modifying monomeric Fc polypeptides to promote heterodimeric Fc formation are described in International Patent Publication No. WO 96/027011 (knobs into holes), in Gunasekaran et al. (Gunasekaran K. et al. (2010) J Biol Chem. 285, 19637-46, electrostatic design to achieve selective heterodimerization), in Davis et al. (Davis, JH. et al. (2010) Prot Eng Des Sel; 23(4): 195-202, strand exchange engineered domain (SEED) technology), and in Labrijn et al [Efficient generation of stable bi-specific IgG1 by controlled Fab-arm exchange. Labrijn AF, Meesters JI, de Goeij BE, van den Bremer ET, Neijssen J, van Kampen MD, Strumane K, Verploegen S, Kundu A, Gramer MJ, van Berkel PH, van de Winkel JG, Schuurman J, Parren PW. Proc Natl Acad Sci U S A. 2013 Mar 26;110(13):5145-50.

3. Functional characteristics of heteromultimers

[0177] The heteromultimers according to the disclosure exhibit greatly reduced/ablated binding to FcγRs and to C1q. In addition, in certain disclosures, the heteromultimers also exhibit additional desirable properties such as stability, the ability to bind FcRn, and properties that facilitate purification of desired expression products from undesired products or impurities.

[0178] In one disclosure, the heteromultimer comprises an IgG Fc construct that does not measurably bind to FcγR receptors, but does bind to FcRn, which makes it a desired candidate for applications in which the half life of the antibody *in vivo* is important yet effector functions (such as CDC, ADCP and ADCC) are unnecessary or deleterious.

[0179] Methods for determining the ability of the heteromultimers comprising IgG Fc constructs to either bind to FcγRs or C1q are known in the art and described elsewhere, herein.

3a. Reduced/ablated binding to FcγR and complement

[0180] The heteromultimers according to the disclosure exhibit reduced/ablated binding to FcγR and C1q as compared to the parent polypeptide. In one embodiment, the heteromultimer according to the invention exhibits K_Ds for FcγRs and C1q that are at least 5 times higher than the K_D of the parent polypeptide. In another embodiment, the heteromultimer according to the invention exhibits K_Ds for FcγRs and C1q that are at least 10 times greater than that of the parent polypeptide, as measured by binding assays known in the art. Binding assays known in the art, include but are not limited to FRET (Fluorescence Resonance Energy Transfer) and BRET (Bioluminescence Resonance Energy Transfer) -based assays, AlphaScreen™ (Amplified Luminescent Proximity Homogeneous Assay), Scintillation Proximity Assay, ELISA (Enzyme-Linked Immunosorbent Assay), SPR (Surface Plasmon Resonance, also known as Biacore™), isothermal titration calorimetry, differential scanning calorimetry, gel electrophoresis, and chromatography including gel filtration. These and other methods may take advantage of some fusion partner or label of the antibody. Assays may employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels.

[0181] In one embodiment, the heteromultimer comprising an IgG Fc construct has a K_D of greater than 5 μM for FcγRIIaH, a K_D of greater than 10 μM for FcγRIIaR, a K_D of greater than 30 μM for FcγRIIb, a K_D of greater than 20 μM for FcγRIIIaF, a K_D of greater than 6 μM for FcγRIIIaV, and a K_D of greater than 6.5 nM for FcγRIa, as measured by surface plasmon resonance (SPR). In another embodiment, the heteromultimer comprising an IgG Fc construct has a K_D of greater than 10 μM for FcγRIIaH, a K_D of greater than 10 μM for FcγRIIaR, a K_D of greater than 10 μM for FcγRIIb, a K_D of greater than 6 μM for FcγRIIIaF, a K_D of greater than 6 μM for FcγRIIIaV, a K_D of greater than 30 nM for FcγRIa, and does not bind to C1q, as measured by surface plasmon resonance (SPR).

[0182] In one embodiment, the heteromultimers comprising an IgG Fc construct do not exhibit detectable levels of ADCC, ADCP, and CDC as measured by standard assays. Non-limiting examples of standard assays to test effector function include those describe in the Examples provided here.

3b. Stability

[0183] The biophysical properties of the heteromultimers including for example stability, is assessed using a variety of methods known in the art. Protein stability may be determined by measuring the thermodynamic equilibrium between folded and unfolded states. For example, heteromultimers of the present invention may be unfolded using chemical denaturant, heat, or pH, and this transition may be monitored using methods including but not limited to circular dichroism spectroscopy, fluorescence spectroscopy, absorbance spectroscopy, NMR spectroscopy, calorimetry, and proteolysis. As will be appreciated by those skilled in the art, the kinetic parameters of the folding and unfolding transitions may also be monitored using these and other techniques. The solubility and overall structural integrity of heteromultimer may be quantitatively or qualitatively determined using a wide range of methods that are known in the art.

[0184] Methods which can be used to characterize the biophysical properties of heteromultimers include gel

electrophoresis, isoelectric focusing, capillary electrophoresis, chromatography such as size exclusion chromatography, ionexchange chromatography, and reversed-phase high performance liquid chromatography, peptide mapping, oligosaccharide mapping, mass spectrometry, ultraviolet absorbance spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, differential scanning calorimetry, analytical ultra-centrifugation, dynamic light scattering, proteolysis, and cross-linking, turbidity measurement, filter retardation assays, immunological assays, fluorescent dye binding assays, protein-staining assays, microscopy, and detection of aggregates via ELISA or other binding assay. Structural analysis employing X-ray crystallographic techniques and NMR spectroscopy may also find use. In one embodiment, stability and/or solubility may be measured by determining the amount of protein solution after some defined period of time. In this assay, the protein may or may not be exposed to some extreme condition, for example elevated temperature, low pH, or the presence of denaturant. Because function typically requires a stable, soluble, and/or well-folded/structured protein, the aforementioned functional and binding assays also provide ways to perform such a measurement. For example, a solution comprising an heteromultimer could be assayed for its ability to bind target antigen, then exposed to elevated temperature for one or more defined periods of time, then assayed for antigen binding again. Because unfolded and aggregated protein is not expected to be capable of binding antigen, the amount of activity remaining provides a measure of the antibody's stability and solubility.

[0185] In one disclosure, the heteromultimers comprising an IgG Fc construct are stable as measured by the melting temperature of one or more domains of the heteromultimer comprising an IgG Fc construct. The melting temperature of the heteromultimers can be determined according to methods known in the art and described in more detail elsewhere herein. For example, the melting temperature of the heteromultimers can be determined by differential scanning calorimetery (DSC), and a thermogram of the heteromultimer generated. In one disclosure, the heteromultimer comprises an IgG Fc construct wherein the onset of melting of the IgG Fc construct in a thermogram is greater than or equal to 65°C. In one disclosure, the heteromultimer comprises an IgG Fc construct wherein the onset of melting of the IgG Fc construct in a thermogram is greater than or equal to 66°C. In one disclosure, the heteromultimer comprises an IgG Fc construct wherein the onset of melting of the IgG Fc construct in a thermogram is greater than or equal to 68°C. In one disclosure, the heteromultimer comprises an IgG Fc construct wherein the onset of melting of the IgG Fc construct in a thermogram is greater than or equal to 70°C.

[0186] In another disclosure, the heteromultimer comprises an IgG Fc construct wherein the IgG Fc construct has a CH2 domain with a melting temperature that is greater than or equal to the melting temperature of the CH2 domain of the parent polypeptide or antibody. In one disclosure the heteromultimer comprises an IgG Fc construct, wherein the IgG Fc construct has a CH2 domain with a melting temperature that is about 1 to 2°C greater than the melting temperature of the parent CH2 domain. In one disclosure the heteromultimer comprises an IgG Fc construct, wherein the IgG Fc construct has a CH2 domain with a melting temperature that is about 2 to 3°C greater than the melting temperature of the parent CH2 domain. In one disclosure, the heteromultimer comprises an IgG Fc construct, wherein the IgG Fc construct has a CH2 domain with a melting temperature that is about 1 to 2°C greater than the melting temperature of the parent CH2 domain, wherein the heteromultimer comprises the amino acid modifications exemplified by AAC4 and AAC5. In one disclosure, the heteromultimer comprises an IgG Fc construct, wherein the IgG Fc construct has a CH2 domain with a melting temperature that is about 2 to 3°C greater than the melting temperature of the parent CH2 domain, wherein the heteromultimer comprises the amino acid modifications exemplified by AAC2, AAC9, AAC10, AAC11, AAC12, AAC13, AAC14, and AAC15. In another disclosure, the heteromultimer comprises an IgG Fc construct, wherein the IgG Fc construct has a CH2 domain with a melting temperature that is about 4°C greater than the melting temperature of the parent CH2 domain, wherein the heteromultimer comprises the amino acid modifications exemplified by AAC6.

3c. FcRn binding

[0187] As is known in the art, binding to FcRn recycles endocytosed antibody from the endosome back to the bloodstream (Raghavan et al., 1996, Annu Rev Cell Dev Biol 12:181-220; Ghetie et al., 2000, Annu Rev Immunol 18:739-766). This process, coupled with preclusion of kidney filtration due to the large size of the full-length molecule, results in favorable antibody serum half-lives ranging from one to three weeks. Binding of Fc to FcRn

also plays a key role in antibody transport. Thus, in one disclosure, the heteromultimers are able to bind FcRn.

4. Format of heteromultimer

4a. Antigen-binding domains

[0188] In one embodiment, the heteromultimer according to the invention consists only of an IgG Fc construct. In other embodiments, the heteromultimer according to the invention comprises an IgG Fc construct and one or more antigen binding domains. In one embodiment, the heteromultimer according to the invention comprises an IgG Fc construct and one antigen-binding domain. In another embodiment, the heteromultimer according to the invention comprises an IgG Fc construct and two antigen binding domains. In another embodiment, the heteromultimer according to the invention comprises an IgG Fc construct and three antigen binding domains. In another embodiment, the heteromultimer according to the invention comprises an IgG Fc construct and four antigen binding domains. In another embodiment, the heteromultimer according to the invention comprises an IgG Fc construct and up to six antigen binding domains.

[0189] The antigen-binding domains can be fused to the IgG Fc construct according to methods known in the art.

[0190] In one embodiment, the heteromultimer according to the invention comprises an IgG Fc construct comprising at least one antigen-binding domain, wherein the at least one antigen-binding domain is selected from a Fab fragment, an scFv, an sdAb, an antigen binding peptide, an Fc fusion protein, or a protein domain capable of binding an antigen.

[0191] In one embodiment, the heteromultimer comprises an IgG Fc construct comprising at least one antigen-binding domain, wherein the at least one antigen-binding domain binds a target antigen selected from α-chain (CD25) of IL-2R, Amyloid beta, EpCAM, CD3, BLyS (or BAFF), CD11a, CD20, CD22, CD23, CD3, CD4, CD52, CD80, CTLA-4, EGFR, F protein of RSV, G250, glycoprotein IIb/IIIa R, HER2, HER2/neu receptor, Hsp90, IgE antibody, IL-12 / IL-23, IL-Iβ, IL-5, IL-6 receptor, Integrin alpha-4/beta-1, Mucin 16/CA- 125, RAN L, TNF alpha, VEGF-A, and other therapeutically advantageous targets.

[0192] In one embodiment, the heteromultimer comprises an IgG Fc construct having a first and a second Fc polypeptide, each Fc polypeptide comprising a modified hinge region, wherein the heteromultimer is derived from a humanized monoclonal antibody with therapeutic potential, selected from: alemtuzumab, apolizumab, aselizumab, atlizumab, bapineuzumab, bevacizumab, bivatuzumab mertansine, cantuzumab mertansine, cedelizumab, certolizumab pegol, cidfusituzumab, cidtuzumab, daclizumab, eculizumab, efalizumab, epratuzumab, erlizumab, felvizumab, fontolizumab, gemtuzumab ozogamicin, inotuzumab ozogamicin, ipilimumab, labetuzumab, lintuzumab, matuzumab, mepolizumab, motavizumab, motovizumab, natalizumab, nimotuzumab, nolovizumab, numavizumab, ocrelizumab, omalizumab, palivizumab, pascolizumab, pecfusituzumab, pectuzumab, pertuzumab, pexelizumab, ralivizumab, ranibizumab, reslivizumab, reslizumab, resyvizumab, rovelizumab, ruplizumab, sibrotuzumab, siplizumab, sontuzumab, tacatuzumab tetraxetan, tadocizumab, talizumab, tefibazumab, tocilizumab, toralizumab, trastuzumab, tucotuzumab celmoleukin, tucusituzumab, umavizumab, urtoxazumab, and visilizumab.

[0193] In another embodiment, the heteromultimer comprises an IgG Fc construct derived from a therapeutic antibody such as, for example, rituximab or trastuzumab.

4b. Antibody-drug conjugates

[0194] It is further contemplated that the heteromultimer according to the invention can comprise one or more

toxic drug molecules linked to the IgG Fc construct and/or to other domains of the heteromultimer. Toxic drug molecules include substances that inhibit or prevent the function of cells and/or cause destruction of cells. In one embodiment, the one or more toxic drug molecules can be linked to an heteromultimer comprising an IgG Fc construct. In another embodiment, the one or more toxic drug molecules can be linked to a heteromultimer comprising an IgG Fc construct and at least one antigen-binding domain. Suitable toxic drug molecules that can be linked to the IgG Fc construct are selected from radioactive isotopes (e.g. At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

[0195] Suitable chemotherapeutic agents that can be linked to the IgG Fc construct are selected from alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®) and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, e.g., paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANE™), and doxetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; pharmaceutically acceptable salts, acids or derivatives of any of the

above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovovin.

4c. Heterologous peptides or polypeptides

[0196] It is further contemplated that the heteromultimers according to the invention comprises an IgG Fc construct that is linked to one or more heterologous peptides or polypeptides. The one or more heterologous peptide or polypeptides is selected from, for example, a detectable marker, a member of a ligand-receptor pair, a member of an enzyme-substrate pair and a member of a fluorescence resonance energy transfer pair.

5. Methods of making and purifying heteromultimers

[0197] As described above, the heteromultimer according to the invention comprises an IgG Fc construct comprising a first and a second Fc polypeptide. Both Fc polypeptides can readily be prepared using recombinant DNA technology known in the art, whether in embodiments where the heteromultimer comprises an IgG Fc region alone, or in embodiments where the heteromultimers further comprise one or more antigen-binding domains or heterologous proteins. The design of nucleic acid that encode such molecules is well within the common knowledge of a worker skilled in the art. Standard techniques such as, for example, those described in Sambrook and Russell, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 3rd ed., 2001); Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2nd ed., 1989); Short Protocols in Molecular Biology (Ausubel et al., John Wiley and Sons, New York, 4th ed., 1999); and Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA (ASM Press, Washington, D.C., 2nd ed., 1998) can be used for recombinant nucleic acid methods, nucleic acid synthesis, cell culture, transgene incorporation, and recombinant protein expression.

[0198] As indicated elsewhere herein, the nucleic acid and amino acid sequences of the Fc polypeptides derived from the IgG heavy chain are known in the art or can be readily determined using nucleic acid and/or protein sequencing methods. Methods of genetically fusing the heterologous proteins or toxic drug molecules described herein to the Fc polypeptides are known in the art, and some are described below and in the Examples.

[0199] Expression vectors and host cells suitable for expression of the Fc polypeptides and, where required polypeptides encoding antigen-binding domains are also well known in the art as described below.

5.1 Vectors and Host Cells

[0200] Recombinant expression of the polypeptides of the heteromultimer requires construction of an expression vector containing a polynucleotide that encodes the necessary polypeptides. Once a polynucleotide encoding the polypeptide has been obtained, the vector for the production of the polypeptide may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing the polypeptide-encoding nucleotide sequence are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing polypeptide coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding polypeptides of the heteromultimer, operably linked to a promoter.

[0201] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are

then cultured by conventional techniques to produce the polypeptide for use in the method of the invention. In specific embodiments the polypeptide for use in the method are co-expressed in the host cell for expression of an entire immunoglobulin molecule, as detailed below.

[0202] A variety of host-expression vector systems may be utilized to express the polypeptides. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the polypeptides in situ. These include but are not limited to microorganisms such as bacteria (e.g., E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the polypeptide coding sequences; yeast (e.g., Saccharomyces Pichia) transformed with recombinant yeast expression vectors containing modified heavy and light chain coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing polypeptide coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing polypeptide coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, HEK-293, NSO, and 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). In certain embodiments, bacterial cells such as Escherichia coli, or eukaryotic cells, are used for the expression of polypeptide, which is a recombinant antibody or fusion protein molecules. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., 1986, Gene 45:101; and Cockett et al., 1990, Bio/Technology 8:2). In a specific embodiment, the expression of nucleotide sequences encoding the immunoglobulin heavy and light chains of each heterodimer is regulated by a constitutive promoter, inducible promoter or tissue specific promoter.

[0203] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the modified heavy and light chain coding sequences of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the polypeptide in infected hosts (e.g., see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g., Bittner et al., 1987, Methods in Enzymol. 153:516-544).

[0204] The expression of the polypeptides of the heteromultimers may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control the expression of the gene encoding polypeptide include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981 , Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981 , Proc. Natl. Acad. Sci. U.S.A. 78.1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), the tetracycline (Tet) promoter (Gossen et al., 1995, Proc. Nat. Acad. Sci. USA 89:5547-5551); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff et al, 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25; see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981 , Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or

other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1 :268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1 :161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286); neuronal-specific enolase (NSE) which is active in neuronal cells (Morelli et al., 1999, Gen. Virol. 80:571-83); brain-derived neurotrophic factor (BDNF) gene control region which is active in neuronal cells (Tabuchi et al., 1998, Biochem. Biophysic. Res. Com. 253:818-823); glial fibrillary acidic protein (GFAP) promoter which is active in astrocytes (Gomes et al., 1999, Braz J Med Biol Res 32(5): 619-631 ; Morelli et al., 1999, Gen. Virol. 80:571-83) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

[0205] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered fusion protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system will produce an unglycosylated product and expression in yeast will produce a glycosylated product. Eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript (e.g., glycosylation, and phosphorylation) of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERY, BHK, Hela, COS, MDCK, HEK-293, 3T3, WI38, NSO, and in particular, neuronal cell lines such as, for example, SK-N-AS, SK-N-FI, SK-N-DZ human neuroblastomas (Sugimoto et al., 1984, J. Natl. Cancer Inst. 73: 51-57), SK-N-SH human neuroblastoma (Biochim. Biophys. Acta, 1982, 704: 450-460), Daoy human cerebellar medulloblastoma (He et al., 1992, Cancer Res. 52: 1144-1148) DBTRG-05MG glioblastoma cells (Kruse et al., 1992, In Vitro Cell. Dev. Biol. 28A: 609-614), IMR-32 human neuroblastoma (Cancer Res., 1970, 30: 2110-2118), 1321 N1 human astrocytoma (Proc. Natl. Acad. Sci. USA, 1977, 74: 4816), MOG-G-CCM human astrocytoma (Br. J. Cancer, 1984, 49: 269), U87MG human glioblastoma-astrocytoma (Acta Pathol. Microbiol. Scand., 1968, 74: 465-486), A172 human glioblastoma (Olopade et al., 1992, Cancer Res. 52: 2523-2529), C6 rat glioma cells (Benda et al., 1968, Science 161 : 370-371), Neuro-2a mouse neuroblastoma (Proc. Natl. Acad. Sci. USA, 1970, 65: 129-136), NB41A3 mouse neuroblastoma (Proc. Natl. Acad. Sci. USA, 1962, 48: 1184-1190), SCP sheep choroid plexus (Bolin et al., 1994, J. Virol. Methods 48: 211-221), G355-5, PG-4 Cat normal astrocyte (Haapala et al., 1985, J. Virol. 53: 827-833), Mpf ferret brain (Trowbridge et al., 1982, In Vitro 18: 952-960), and normal cell lines such as, for example, CTX TNA2 rat normal cortex brain (Radany et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6467-6471) such as, for example, CRL7030 and Hs578Bst. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

[0206] For long-term, high-yield production of recombinant proteins, stable expression is often preferred. For example, cell lines that stably express the polypeptide of the invention (e.g., antibody or fusion protein) may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the

introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn can be cloned and expanded into cell lines.

[0207] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt-cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147) genes.

[0208] Methods of preparing antibody-drug conjugates are known in the art and a description of same is found in US Patent Publication No. 2011/0200596.

5.2 Purification of heteromultimers

[0209] When using recombinant techniques, the heteromultimers can be produced intracellularly, or directly secreted into the medium. If the heteromultimer is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Where the heteromultimer is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0210] The heteromultimers composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc region that is present in the heteromultimer. Protein A can be used to purify antibodies that 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 heteromultimers comprises a CH3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the heteromultimer to be recovered.

[0211] Following any preliminary purification step(s), the mixture comprising the heteromultimer of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

[0212] The heteromultimers of the invention comprise asymmetric amino acid modifications in the first and second Fc polypeptides of the IgG Fc construct. Accordingly, due to the inherent properties of the Fc polypeptides, when the Fc polypeptides are expressed together, the products that result will include homodimers of the first Fc polypeptide, homodimers of the second Fc polypeptide, and heterodimers of the first and second polypeptide.

[0213] In one embodiment, heteromultimers are purified or isolated after expression. Methods of expression are described elsewhere herein. Proteins may be isolated or purified in a variety of ways known to those skilled in the art. Standard purification methods include chromatographic techniques, including ion exchange, hydrophobic interaction, affinity, sizing or gel filtration, and reversed-phase, carried out at atmospheric pressure or at high pressure using systems such as FPLC and HPLC. Purification methods also include electrophoretic, immunological, precipitation, dialysis, and chromatofocusing techniques. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. As is well known in the art, a variety of natural proteins bind Fc and antibodies, and these proteins can find use in the present invention for purification of heteromultimers. For example, the bacterial proteins A and G bind to the Fc region. Likewise, the bacterial protein L binds to the Fab region of some antibodies, as of course does the antibody's target antigen. Purification can often be enabled by a particular fusion partner. For example, antibodies may be purified using glutathione resin if a GST fusion is employed, Ni+2affinity chromatography if a His-tag is employed, or immobilized anti-flag antibody if a flag-tag is used. For general guidance in suitable purification techniques, see, e.g. Protein Purification: Principles and Practice, 3rd Ed., Scopes, Springer-Verlag, NY, 1994. The degree of purification necessary will vary depending on the screen or use of the antibodies. In some instances no purification is necessary. For example in one embodiment, if the antibodies are secreted, screening may take place directly from the media. As is well known in the art, some methods of selection do not involve purification of proteins. Thus, for example, if a library of antibodies is made into a phage display library, protein purification may not be performed.

[0214] Thus, in one embodiment, the heteromultimers comprising an IgG Fc construct, when expression of said IgG Fc construct results in a mixture of IgG Fc constructs with homodimeric Fc regions and IgG Fc constructs with heterodimeric Fc regions, the IgG Fc constructs with homodimeric Fc regions are clearly resolved from the IgG Fc constructs with heterodimeric Fc regions using charge-based purification methods, such as, for example ion exchange chromatography.

[0215] In an additional embodiment, heteromultimers comprising an IgG Fc construct described herein can also comprise a variant CH3 region comprising amino acid modifications that promote the formation of a heterodimeric Fc region rather than formation of a homodimeric Fc region. Expression of these heteromultimers may result in a mixture of heteromultimers having homodimeric Fc regions and heterodimeric Fc regions. Such mixtures can also be resolved using charge-based purification methods as indicated above. Exemplary variants that can be purified in this manner include AAC3, AAC4, and AAC5.

6. Testing of heteromultimers

6.1 FcγR, FcRn and C1q binding

[0216] In certain embodiments, the Fc activities of the produced immunoglobulin are measured to ensure that only the desired properties are maintained. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. Methods of assessing effector function are described in Jiang et al. (2011) Nature Reviews Drug Discovery 10:101-111. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the heteromultimer lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). An example of an in vitro assay to assess ADCC activity of a molecule of interest is described in U.S. Pat. No. 5,500,362 or 5,821,337. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the heteromultimer is unable to bind C1q and hence lacks CDC activity. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), may

be performed. FcRn binding and in vivo clearance/half life determinations can also be performed using methods known in the art.

[0217] FcγR and C1q binding can also be measured by Surface Plasmon Resonance (SPR), or ELISA-based methods. FcγR binding can also be measured by FACS (fluorescence activated cell sorting). Commercially available may also be used to measure the ability of the heteromultimers to bind to FcγR or C1q.

6.2 Stability

[0218] The thermal stability of the heteromultimers can be determined according to methods known in the art. The melting temperature of the IgG Fc construct is indicative of its thermal stability. The melting point of the IgG Fc construct may be measured using techniques such as differential scanning calorimetry (Chen et al (2003) Pharm Res 20:1952-60; Ghirlando et al (1999) Immunol Lett 68:47-52). Alternatively, the thermal stability of the IgG Fc construct may be measured using circular dichroism (Murray et al. (2002) J. Chromatogr Sci 40:343-9).

[0219] The methodology for determining the Tm of the parent CH2 domain is well described in the art (see for example Ionescu et al (2008) J Pharm Sci 97(4):1414-26). In short, melting of the Fc region of IGG1 produces two transitions: one for the melting of the CH2 domain and one for that of the CH3 domain. These transitions are independent of the Fab present, but can be masked by the Fab transition. Typically, melting of IGG1 Fc gives a transition with a Tm of 71°C for the CH2 domain and one with a Tm of 82°C for the CH3 domain. The Tm of the CH2 domain is affected by its glycosylation state, the nature of the hinge region, and the intrinsic stability of the CH3 domain. Aglycosylation and deglycosylation are known to decrease the Tm of the CH2 domain by 10°C. Removal of hinge disulfides are known to decrease the Tm of the CH2 domain by more than 10°C. Changes to the CH3 domain that decrease its stability below that of the CH2 domain are likely to produce changes in the Tm of the CH2 domain, but the effect is harder to predict.

7. Pharmaceutical Compositions

[0220] The present invention also provides pharmaceutical compositions comprising the heteromultimers according to the invention. Such compositions comprise a therapeutically effective amount of the heteromultimer and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[0221] In certain embodiments, the composition comprising the heteromultimer is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anaesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachet indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0222] In certain embodiments, the compositions described herein are formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxide isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0223] The amount of the composition described herein which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a therapeutic protein can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses are extrapolated from dose-response curves derived from in vitro or animal model test systems.

8. Methods of treatment/Uses

[0224] The heteromultimers generated by any of the above described methods may be used to diagnose, treat, detect, or modulate human disease or specific pathologies in cells, tissues, organs, fluid, or, generally, a host. As taught herein, modification of the Fc region of an antibody, Fc-fusion protein, or Fc fragment to reduce or ablate Fc gamma receptor binding and specified effector functions, but where the heteromultimer retains the original targeting properties, provides antibodies and IgG Fc constructs with a superior spectrum of activities, biophysical properties, stability and ability to persist in the body of a host.

[0225] The diseases or pathologies that may be amenable to treatment using a composition provided by the invention include, but are not limited to: neurological disorders, such as but not limited to Alzheimer's disease and including neuropathic pain; dermatological disease; metabolic diseases; osteoarthritis; and conditions resulting from burns or injury; cardiovascular disorders including but not limited to myocardial infarction, congestive heart failure, stroke, ischemic stroke, and hemorrhage; as well as general immune mediated disorders, including the rheumatic diseases, psoriasis, and scleroderma.

[0226] In one embodiment, the heteromultimers according to the present invention are used in the treatment of diseases where antibodies are used to target cell surface molecules where depletion of these molecules resulting from FcγR mediated effector function has adverse effects.

[0227] In one embodiment, the heteromultimers according to the present invention are used to improve the safety index for antibodies that form immune complexes with their targets.

[0228] Strohl, WR and Strohl LM, "Antibody Fc engineering for optimal antibody performance" In Therapeutic Antibody Engineering, Cambridge: Woodhead Publishing (2012), pp 225-249, provides a description of the advantages of using antibodies that lack FcγR- and complement-mediated effector functions for the treatment of

disease. It is contemplated that heteromultimers comprising the IgG Fc constructs according the present invention are useful in preparing antibodies that lack FcγR- and complement-mediated effector functions for the treatment of disease.

9. Kits

[0229] The present disclosure additionally provides for kits comprising one or more heteromultimers. Individual components of the kit would be packaged in separate containers and, associated with such containers, can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale. The kit may optionally contain instructions or directions outlining the method of use or administration regimen for the heterodimer pairs.

[0230] When one or more components of the kit are provided as solutions, for example an aqueous solution, or a sterile aqueous solution, the container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the solution may be administered to a subject or applied to and mixed with the other components of the kit.

[0231] The components of the kit may also be provided in dried or lyophilised form and the kit can additionally contain a suitable solvent for reconstitution of the lyophilised components. Irrespective of the number or type of containers, the kits may comprise an instrument for assisting with the administration of the composition to a patient. Such an instrument may be an inhalant, nasal spray device, syringe, pipette, forceps, measured spoon, eye dropper or similar medically approved delivery vehicle.

EXAMPLES

[0232] The examples below are given so as to illustrate the practice of this invention. They are not intended to limit or define the entire scope of this invention.

Example 1: Preparation and expression of antibody constructs (heteromultimers)

[0233] The following antibody constructs were prepared. All antibody constructs were based on the sequence of the wild-type anti-Her2 antibody trastuzumab (see Figure 5, SEQ ID NO:2 for wild-type trastuzumab Heavy chain amino acid sequence, SEQ ID NO:3 for wild-type trastuzumab light chain sequence) with the following added modifications in the heavy chain CH3 domain introduced in order to promote the formation of a heterodimer Fc domain with increased stability as compared to a CH3 domain that does not comprise amino acid mutations.

Chain A: T350V/L351Y/S400E/F405A/Y407V, and

Chain B: T350V/T366L/N390R/K392M/T394W

[0234] This construct, with the above modifications is referred to as v791. All sequences described herein are numbered using the EU numbering system.

[0235] Additional variants were constructed based on v791, with amino acid modifications in the hinge region and or CH2 domain of the heavy chain as shown in Table A1. All variants included the trastuzumab light chain sequence as set forth in SEQ ID NO: 67 (amino acid) and/or SEQ ID NO:34 (DNA).

Table A1: Asymmetric antibody constructs based on trastuzumab

Variant	Heavy Chain A	SEQ ID No.:(amino acid/DNA)	Heavy Chain B	SEQ ID No.: (amino acid/DNA)
1051/ control	L234A/L235A	6/7	L234A/L235A	8/9
AAC1	L234A/L235A	6/7	--	20/21
AAC2	L234A/L235A	6/7	L234K/L235K	22/23
AAC3	L234D/L235E	10/11	L234K/L235K	22/23
AAC4	E233A/L234D/L235E	12/13	E233A/L234R/L235R	24/25
AAC5	L234D/L235E	10/11	E233K/L234R/L235R	26/27
AAC6	E233A/L234K/L235A	14/15	E233K/L234A/L235K	28/29
AAC7	E269Q/D270N	16/17	E269K/D270R	30/31
AAC8	--	18/19	L235K/A327K	32/33
1051 is a control variant described in Strohl (2009) Current Opinion in Biotechnology 20:685-691. AAC1 is another control variant which is an asymmetric version of 1051, in which only one of the heavy chains has the L234/L235 double mutation. AAC2-AAC8 are asymmetric designs.				

[0236] The antibodies and controls were cloned and expressed as follows. v791 was prepared by site-directed mutagenesis using standard methods. The final DNA was sub-cloned into the vector pTT5 (see International Patent Publication No. WO 2009/137911). Expression was carried out in either 2mL or 50mL or 500mL CHO 3E7 cells. CHO cells were transfected in exponential growth phase (1.5 to 2 million cells/mL) with aqueous 1mg/mL 25kDa polyethylenimine (PEI, Polysciences) at a PEI:DNA ratio of 2.5:1.(Raymond C. et al. A simplified polyethylenimine-mediated transfection process for large-scale and high-throughput applications. Methods. 55(1):44-51 (2011)). In order to determine the optimal concentration range for forming heterodimers, the DNA was transfected in optimal DNA ratios of the heavy chain A (HC-A), light chain (LC), and heavy chain B that allow for heterodimer formation (e.g. HC-A/HC-B/LC ratios = 25:25:50%). Transfected cells were harvested after 5-6 days with the culture medium collected after centrifugation at 4000rpm and clarified using a 0.45µm filter.

[0237] Purification protocols: The clarified culture medium was loaded onto a MabSelect SuRe (GE Healthcare) Protein-A column and washed with 10 column volumes of PBS buffer at pH 7.2. The antibody was eluted with 10 column volumes of citrate buffer at pH 3.6 with the pooled fractions containing the antibody neutralized with TRIS at pH 11. The Protein-A purified antibody was further purified by gel filtration (SEC). For gel filtration, 3.5mg of the antibody mixture was concentrated to 1.5mL and loaded onto a Sephadex 200 HiLoad 16/600 200 pg column (GE Healthcare) via an ÄKTA Express FPLC at a flow-rate of 1mL/min. PBS buffer at pH 7.4 was used at a flow-rate of 1mL/min. Fractions corresponding to the purified antibody were collected, concentrated to ~1mg/mL and stored at -80°C.

Table A2 summarizes the expression yields for the various samples.

Variant	50 mL Expression Protein-A yield [mg/L]	50 mL Expression SEC yield [mg/L]	500 mL Expression Protein-A yield [mg/L]	500 mL Expression SEC yield [mg/L]
WT	30	n/d*	n/d	n/d
1051/control	48	20	48	23
AAC1	n/d	n/d	n/d	n/d
AAC2	63	24	n/d	n/d
AAC3	39	20	n/d	n/d
AAC4	42	26	n/d	n/d
AAC5	44	16	n/d	n/d
AAC6	31	13	15	10

Variant	50 mL Expression Protein-A yield [mg/L]	50 mL Expression SEC yield [mg/L]	500 mL Expression Protein-A yield [mg/L]	500 mL Expression SEC yield [mg/L]
AAC7	n/d	n/d	n/d	n/d
AAC8	n/d	n/d	n/d	n/d
*n/d = not determined				

[0238] The majority of the samples showed levels of expression similar to the WT or control.

Example 2: Asymmetric antibody constructs based on trastuzumab do not bind to FcγR

[0239] The ability of the asymmetric antibody constructs to bind to FcγRIIaH, FcγRIIaR, FcγRIIb FcγRIIIaF, FcγRIIIaV, and FcγRIa was assessed by surface plasmon resonance (SPR).

[0240] Affinity of FcγRs to antibody Fc was measured by SPR using a ProteOn XPR36 at 25°C with 10mM HEPES, 150mM NaCl, 3.4mM EDTA, and 0.05% Tween 20 at pH 7.4. Recombinant HER-2 was captured on the activated GLM sensorchip by injecting 4.0µg/mL in 10mM NaOAc (pH 4.5) at 25µL/min until approx. 3000 resonance units (RUs) were immobilized with the remaining active groups quenched. 40µg/mL of purified HER-2/neu-based antibodies were indirectly captured when injected at 25µL/min for 240s (resulting in approx. 500RUs) following a buffer injection to establish a stable baseline. FcγRs were injected at 60µL/min for 120s with a 180s dissociation phase to obtain a set of binding sensograms. Resultant kD values were determined from binding isotherms using the Equilibrium Fit model with reported values as the mean of two or three independent runs.

[0241] The in vitro binding Ka ratio with respect to the WT for each variant, as determined by SPR, is shown in Table B.

Table B: SPR Ka ratio for binding to Fcγ receptors with respect to wild-type trastuzumab

Variant	2aH[1]	2aR[2]	2b[3]	3aF[4]	3aV[5]	1a[6]
WT	1.00	1.00	1.00	1.00	1.00	1.00
control /1051	0.06	0.18	0.52	0.29	0.10	0.01
AAC1	n/d*	n/d	n/d	0.87	0.71	0.48
AAC2	NB	NB	NB	LOW	LOW	LOW
AAC3	NB	NB	NB	LOW	LOW	LOW
AAC4	NB	NB	NB	LOW	LOW	LOW
AAC5	NB	NB	NB	LOW	LOW	LOW
AAC6	NB	NB	NB	NB	LOW	LOW
AAC7	n/d	n/d	n/d	LOW	LOW	0.15
AAC8	n/d	n/d	n/d	0.19	0.10	0.13

*n/d = not determined
1. The Kd of 2ah was 0.48 µM. Receptor was run at 10 µM. LOW means that Kd >>10 µM, NB means Kd>>100 µM where >> indicates "much greater than".
2. The Kd of 2ar was 0.87 µM. Receptor was run at 10 µM. LOW means that Kd >>10 µM, NB means Kd>>100 µM.
3. The Kd of 2b was 3.4 µM. Receptor was run at 10 µM. LOW means that Kd >>10 µM, NB means Kd>>100 µM.
4. The Kd of 3af was 1.9 µM. Receptor was run at 6 µM. LOW means that Kd >>6 µM, NB means Kd>>60 µM.
5. The Kd of 3av was 0.60 µM. Receptor was run at 6 µM. LOW means that Kd >>6 µM, NB means Kd>>60 µM.
6. The Kd of 1a was 0.65 nM. Receptor was run at 30 nM. LOW means that Kd >>80 nM, NB means

Kd>>800 nM.

[0242] All of the variants showed significantly decreased binding to all of the receptors. In most cases the binding was undetectable or unquantifiable due to the low affinity.

Example 3: Asymmetric antibody constructs based on trastuzumab do not bind to C1q

[0243] The ability of the asymmetric antibody constructs to bind to C1q was tested as follows. Human C1q was purchased from GenWay Biotech (San Diego, CA). SPR chip immobilization of antibodies was as described in Example 2. 30nM C1q injected over mAb variants captured onto a HER2 SPR surface using standard protocols as also described in Example 2. The results are shown in Table C below.

Table C: Results of C1q binding assay

Variant	C1q[1]
WT	yes
Control/1051	NB
AAC1	partial
AAC2	NB
AAC3	NB
AAC4	NB
AAC5	NB
AAC6	NB
AAC7	NB
AAC8	NB

1. C1q is a hexamer of heterotrimers with a potential stoichiomentry mAb:C1q of 6:1. The binding kinetics were very complex, and a proper Kd could not be determined. Receptor was tested at 30nM. 'partial' means diminished binding, 'NB' means no detectable binding

[0244] All of the variants showed undetectable binding to C1q, except for AAC1 which showed decreased, but detectable binding to C1q.

Example 4: Asymmetric antibody constructs based on trastuzumab bind to FcRn

[0245] The ability of the asymmetric antibodies to bind to FcRn was tested by SPR as follows.

[0246] SPR chip capture surface was prepared with goat anti-hIgG polyclonal. Variants were captured from supernatants on the vertical line. A flow of FcRn at 1μM maximum with a 3x dilution series was run on the horizontal line. Duplicate runs at pH 6 produced similar results. One run at pH 7.4 was performed to check for lack of binding. The results are shown in Table D below.

Table D: FcRn binding

Variant	FcRn[1]
WT	Yes
Control/1051	Yes
AAC1	n/d*
AAC2	Yes

Variant	FcRn[1]
AAC3	Yes
AAC4	Yes
AAC5	Yes
AAC6	Yes
AAC7	n/d
AAC8	n/d

*n/d = not determined
1. FcRn binding was measured at pH 6.5 and 7.4. Variants with WT binding at pH 6.5 and no detectable binding at pH 7.4 are denoted as 'Yes'

Example 5: Asymmetric antibody constructs are thermally stable

[0247] The thermal stability of the CH2 domains of the asymmetric antibody constructs was determined using differential scanning calorimetry as follows. Each antibody construct was purified as described in Example 1 and diluted to 0.2 mg/mL in PBS, and a total of 400 µL was used for DSC analysis with a VP-Capillary DSC (GE Healthcare). At the start of each DSC run, five buffer blank injections were performed to stabilize the baseline, and a buffer injection was placed before each antibody construct injection for referencing. Each sample was scanned from 20 to 100°C at a 60°C/hr rate, with low feedback, 8 sec filter, 5 min preTstat, and 70 psi nitrogen pressure. The resulting thermograms were referenced and analyzed using Origin 7 software.

[0248] Thermal unfolding curves for the heterodimers tested are shown in Figure 2. The melting temperatures of the heteromultimers tested are shown in Table E below.

Table E: Thermal stability of heteromultimers

Variant	Tm onset (WT ~66.5C) [1]	Tm (WT ~71.0C)[2]
Control/1051	66.5	71.8
AAC1	n/d	n/d*
AAC2	70.5	74
AAC3	65.8	71.5
AAC4	66.7	72.8
AAC5	67.0	72.9
AAC6	68.7	75.0
AAC7	n/d	n/d
AAC8	n/d	n/d

*n/d = not determined
1. Tm onset was visually taken as the first point where the thermogram in Figure 2 significantly goes above baseline.
2. The Tm was measured by deconvolution using a non-2 state model of the first transition in the thermograms shown in Figure 2.

[0249] These results indicate that a number of designs have higher Tm onset and Tm of the CH2 domain when compared to the control WT.

Example 6: Purification of asymmetric antibody constructs based on trastuzumab

[0250] Selected asymmetric antibody constructs were expressed and purified by UPLC IEX (Ultra Performance Liquid Chromatography - Ion exchange chromatography) as follows.

[0251] Chain A and Chain B of variants 791 (WT heterodimer), AAC3 (L234D/L235E[Chain A]|L234K/L235K[Chain B]) and AAC5 (L234D/L235E[Chain A]|E233K/L234K/L235K[Chain B]) were expressed in ratios 1:0 (A), 1:1 (C) and 0:1 (E). in 50 mL CHO cultures. Ratios A and E produced homodimers of Chain A and Chain B, respectively. All of the samples were purified by Protein A, and then by Size Exclusion Chromatography (SEC) using a Superdex 200 16/600 column in PBS buffer prior to loading them into the UPLC IEX. UPLC IEX was carried out under the following conditions (pH gradient): Solvents: A, 0.1 M NaH_2PO_4, pH 4.44; B, 0.1 M Na_2HPO_4, pH 9.20; C, MilliQ water; D, 0.5 M Na Acetate, pH 9.13 (lot# 03-Dec-12). Initial Buffer: 18% A, 2% B, 68% C, 12% D = 20 mM $NaPO_4$, 60 mM NaAcetate, pH ~5.9; Gradient: to 2% A, 18% B, 68% C, 12% D = 20 mM $NaPO_4$, 60 mM NaAcetate, pH ~7.9 in 7.2 column volumes. Flow rate: 0.3 ml/min. Temperature: 30°C. Pressure: ~4200 psi. Column: Agilent BioMAb, 4.6 x 50 mm, 1.7 µm particles, SN USDJA01061.

[0252] The results are shown in Figure 3A. Traces for ratios A, C, and E corresponding to the homodimers or heterodimers are labelled. Repeat runs, when carried out, are also shown.

[0253] Figure 3A shows that the introduction of asymmetric charges on the lower hinge region result in a design that not only has lower receptor binding (Example 3) and higher thermal stability (Example 5), but it also can be purified from homodimer impurities by Ion Exchange Chromatography.

[0254] The separation of one variant, AAC4, was tested under two conditions. It was eluted under a pH gradient as described above, or under a salt gradient as follows: Solvents: A 0.1 M NaH2PO4, pH 4.44; B 0.1 M Na2HPO4, pH 9.20; C MilliQ water; D 0.5 M NaCl. Initial Buffer: 18% A, 2% B, 68% C, 12% D = 20 mM $NaPO_4$, 60 mM NaCl, pH ~5.9. Salt gradient to 18% A, 2% B, 0% C, 80% D (= 20 mM $NaPO_4$, 400 mM NaCl, pH ~5.9) in 7.2 column volumes.

[0255] The results are shown in Figure 3B. The figure shows that the separation of the homodimers and heterodimers with a salt gradient was similar to that with a pH gradient.

Example 7: Asymmetric antibody constructs based on trastuzumab do not stimulate ADCC (antibody-dependent cell-mediated cytotoxicity) in SK-BR-3 cells

[0256] An exemplary variant was tested for its ability to stimulate ADCC in SK-BR-3 cells in order to assess whether the lack of measured binding to FcγR translated into an inability to mediate effector function as measured by ADCC. SK-BR-3 cells express HER2 on their surface and thus bind to trastuzumab, allowing for NK cell mediated ADCC in the presence of trastuzumab. The activity of AA6 in this assay was compared to that of the control variant described in Table A, and to the positive control trastuzumab.

[0257] Cell lines used: SK-BR-3 cell line (ATCC#HTB-30), NK92/ CD16a(158V/V) Detection device: FlexStation3, Molecular Devices.
Positive control antibody: Herceptin™ (Trastuzumab).

[0258] Cell Culture. Frozen cells were thawed by gently swirling the vial in the 37°C water bath. After 1-2min, the medium in the vial was completely thawed. The outside of the vial was wiped with 70% ethanol. The cell suspension was then transferred to a 15ml centrifuge tube, followed by addition of 5ml of pre-warmed complete medium. After centrifugation for 3-5min at 500 g, the supernatant was aspirated. 10ml of complete medium was added and the cells were resuspended by pipetting up and down for a few times. Cell viability was determined by Trypan blue staining method. The cell suspension was then seeded in flasks. The cells were incubated at 37°C, 5% CO2 overnight.

[0259] Cells were maintained at 37 °C / 5% CO2 and regularly sub-cultured with suitable medium supplemented with 10% FBS according to protocol from ATCC.

[0260] The antibody sample and the Standard were delivered in dry shipper and stored at -20 °C before testing. The sample and the Standard were stored at 4 °C after they were thawed on ice. The sample and the Standard were diluted with Phenol red free MEM medium (supplemented with 1% FBS and 1% Pen/Strep) and applied to the tests.

[0261] ADCC assay buffer was composed of 98% Phenol red free MEM medium, 1% Pen/Strep and 1% FBS.

[0262] NK92/ FcRγ3a(158V/V) cells were conventionally maintained.

[0263] Target cells were harvested by centrifugation at 800 rpm for 3 min, washed with assay medium once and centrifuged; the medium above the pellet was removed completely. Cells were gently suspended with assay medium to make single cell solution. The target cell number was adjusted to 4x cell stock (10,000 cells in 50 µl assay medium). Test articles were prepared at interested concentrations. 50µl 4x target cell stock were seeded to 96-well assay plates and 50 µl 4x sample diluents added. The plates were incubated at room temperature for 30min in cell culture incubator. 100 µl effector cells (E/T=5:1, i.e, 50,000 effector cells per well) were added to initiate the reaction and mixed gently by cross shaking. Triton X-100 was added to cell controls without effector cells and antibody in a final concentration of 1% to lyze the target cells and it served as the maximum lysis control; assay buffers were added in to cell controls without effector cells and antibody and it served as the minimum LDH release control. Target cells incubated with effector cells without the presence of antibodies were set as background control of non-specific LDH release when both cells were incubated together. Plate was incubated at 37°C/5%CO2 incubator for 4-6 hours. The cell viability was assayed with an LDH kit. The absorbance data at OD492nm and OD650nm were measured on Flexstation 3.

[0264] The background (OD650nm) subtracted OD492nm data was analyzed to study the LDH release.The percentages of cell lysis were calculated according to the formula:
Cell lysis %=100*(1-(ODSample data- ODtumor cells plus effector cells) / (ODMaximum release - ODMinimum release))

[0265] The results are shown in Figure 4 and indicate that exemplary heteromultimer AA6 is able to silence ADCC activity in this assay.

Example 8: Preparation and expression of antibody constructs based on the anti-CD20 antibody, Rituximab (heteromultimers)

[0266] The following antibody constructs were prepared. All antibody constructs were based on the sequence of the wild-type anti-CD20 antibody rituximab (see Figure 5, SEQ ID NO:4 for wild-type rituximab Heavy chain amino acid sequence, SEQ ID NO:5 for wild-type rituximab light chain sequence) with the following added modifications in the heavy chain CH3 domain introduced in order to promote the formation of a heterodimer Fc domain with increased stability as compared to a CH3 domain that does not comprise amino acid mutations:

Chain A: T350V/L351Y/F405A/Y407V, and

Chain B: T350V/T366L/K392L/T394W

[0267] This construct with the above mutations is referred to as v1261.

[0268] Additional variants were constructed based on v1261, with amino acid modifications in the hinge region and

or CH2 domain of the heavy chain as shown in Table F. All variants additionally comprise the light chain sequence as set forth in SEQ ID NO: 68 (amino acid) and or SEQ ID NO:69 (DNA).

Table F: Asymmetric antibody constructs based on rituximab

Variant	Chain A	SEQ ID No.: (amino acid/DNA)	Chain B	SEQ ID No.:(amino acid/DNA)
Control WT Rituximab 1261	--	35/36	--	37/38
AAC9	L234D / L235E	39/40	E233K / L234R / L235R	41/42
AAC10	L234D / L235E+D265S	43/44	E233K / L234R / L235R+D265S	45/46
AAC11	L234D / L235E+E269K	47/48	E233K / L234R / L235R+E269K	49/50
AAC12	L234D / L235E+K322A	51/52	E233K / L234R / L235R+K322A	53/54
AAC13	L234D / L235E+P329W	55/56	E233K / L234R / L235R +P329W	57/58
AAC14	L234D / L235E+E269K+D265S+K322A	59/60	E233K / L234R / L235R +E269K+D265S+K322A	61/62
AAC15	L234D / L235E +E269K+D265S+K322E+E333K	63/64	E233K / L234R / L235R +E269K+D265S+K322E+E333K	65/66

[0269] The antibodies and controls were cloned and expressed as follows. V1261 was prepared by site-directed mutagenesis using standard methods. The final DNA was sub-cloned into the vector pTT5 (see International Patent Publication No. WO 2009/137911). Expression was carried out in either 50mL or 250mL CHO 3E7 cells. CHO cells were transfected in exponential growth phase (1.5 to 2 million cells/mL) with aqueous 1mg/mL 25kDa polyethylenimine (PEI, Polysciences) at a PEI:DNA ratio of 2.5:1.(Raymond C. et al. A simplified polyethylenimine-mediated transfection process for large-scale and high-throughput applications. Methods. 55(1):44-51 (2011)). The DNA was transfected in a DNA ratios of the heavy chain A (HC-A), light chain (LC), and heavy chain B of HC-A/HC-B/LC ratios = 30:30:40%). Transfected cells were harvested after 5-6 days with the culture medium collected after centrifugation at 4000rpm and clarified using a 0.45µm filter.

[0270] Purification protocols: The clarified culture medium was loaded onto a MabSelect SuRe (GE Healthcare) Protein-A column and washed with 10 column volumes of PBS buffer at pH 7.2. The antibody was eluted with 10 column volumes of citrate buffer at pH 3.6 with the pooled fractions containing the antibody neutralized with TRIS at pH 11. The Protein-A purified antibody was further purified by gel filtration (SEC). For gel filtration, 3.5mg of the antibody mixture was concentrated to 1.5mL and loaded onto a Sephadex 200 HiLoad 16/600 200 pg column (GE Healthcare) via an AKTA Express FPLC at a flow-rate of 1mL/min. PBS buffer at pH 7.4 was used at a flow-rate of 1mL/min. Fractions corresponding to the purified antibody were collected, concentrated to ~1mg/mL and stored at -80°C.

[0271] The expression yields are the following:

Table G - Expression yields

Variant	50 mL Expression Protein-A yield [mg/L]	50 mL Expression SEC yield [mg/L]	250 mL Expression Protein-A yield [mg/L]	250 mL Expression SEC yield [mg/L]
Control WT Rituximab 1261			28	15
AAC9	11	6	8	3

Variant	50 mL Expression Protein-A yield [mg/L]	50 mL Expression SEC yield [mg/L]	250 mL Expression Protein-A yield [mg/L]	250 mL Expression SEC yield [mg/L]
AAC10	12	5	24	11
AAC11	12	3	24	9
AAC12	11	4	11	9
AAC13	15	5	7	3
AAC14	10	3	13	11
AAC15	18	5	8	3

[0272] Considering the batch to batch variablity in yield, all samples expressed well to levels comparable to the control WT Rituximab.

Example 9: Asymmetric antibody constructs based on rituximab do not bind to FcγR

[0273] The ability of the asymmetric antibody constructs based on rituximab to bind to FcγRIIaH, FcγRIIaR, FcγRIIb FcγRIIIaF, and FcγRIIIaV was assessed by surface plasmon resonance (SPR).

[0274] Affinity of FcgR to antibody Fc was measured by SPR using a ProteOn XPR36 at 25°C with PBS containing 3.4mM EDTA, and 0.05% Tween 20 at pH 7.4 as the running buffer. Goat polyclonal anti-IgG antibodies were immobilized on a NHS/EDC activated GLC sensorchip by injecting 4.0µg/mL in 10mM NaOAc (pH 4.5) at 25µL/min until approximately 3000 resonance units (RUs) was reached, which was followed by quenching the remaining active groups with ethanolamine. 40 µg/mL of purified rituximab-based antibodies were indirectly captured by injecting at 25µL/min for 240s (resulting in approx. 500RUs capture) in the ligand direction, following a buffer injection to establish a stable baseline in the analyte direction. FcγRs were subsequently injected at 50µL/min for 120s with a 180s dissociation phase to obtain a set of binding sensorgrams. Resultant Kd (affinity) values were determined from double-referenced sensorgrams using the Equilibrium Fit model in the Proteon Manager software. Reported values as the mean of two or three independent runs.

[0275] The *in vitro* binding Ka ratio with respect to the WT for each variant, as determined by SPR, is shown in Table H.

Table H: SPR Ka ratio for binding to Fcγ receptors with respect to wild-type trastuzumab

Variant	CD16aF	CD16aV	CD32b	CD32aH	CD32aR
Trastuzumab	1.00	1.00	1.00	1.00	1.00
Control WT Rituximab 1261	1.36	1.34	1.85	1.87	1.47
AAC9	NB	0.08	NB	NB	NB
AAC10	NB	LOW	NB	NB	NB
AAC11	NB	LOW	NB	NB	NB
AAC12	NB	0.08	NB	NB	NB
AAC13	NB	LOW	NB	NB	NB
AAC14	NB	LOW	NB	NB	NB
AAC15	NB	LOW	NB	NB	NB

[0276] The heterodimer driving mutations on the control WT Rituximab 1261 marginally brought up affinity towards the receptors when compared to homodimeric WT trastuzumab. The mutants, which contained the heterodimer driving mutations, showed significantly reduced or undetectable binding to the Fcγ receptors.

Example 10: Asymmetric antibody constructs based on rituximab are thermally stable

[0277] The thermal stability of the CH2 domains of the asymmetric antibody constructs based on rituximab was determined using differential scanning calorimetry as follows. Each antibody construct was purified as described in Example 8 and diluted to 0.2 mg/mL in PBS, and a total of 400 µL was used for DSC analysis with a VP-Capillary DSC (GE Healthcare). At the start of each DSC run, five buffer blank injections were performed to stabilize the baseline, and a buffer injection was placed before each antibody construct injection for referencing. Each sample was scanned from 20 to 100°C at a 60°C/hr rate, with low feedback, 8 sec filter, 5 min preTstat, and 70 psi nitrogen pressure. The resulting thermograms were referenced and analyzed using Origin 7 software.

[0278] The melting temperatures of the heteromultimers tested are shown in Table I below.
Table I: Thermal stability of heteromultimers

Variant	Tm [°C][1]
Control WT Rituximab 1261	73.0
AAC9	75.3
AAC10	75.3
AAC11	75.4
AAC12	75.4
AAC13	75.4
AAC14	75.2 (noisy)
AAC15	67.5

1. The first transition included the unfolding of both the Rituximab FAB and CH2 domain. The Tm was measured by deconvolution using a non-2 state model of the first transition.

[0279] These results indicate that a number of designs have higher Tm onset and Tm of the CH2 domain when compared to the control WT.

Example 11: Asymmetric antibody constructs based on rituximab do not stimulate ADCC in Daudi cells

[0280] Selected variants were tested for their ability to stimulate ADCC in Daudi cells in order to assess whether the lack of measured binding to FcγR translated into an inability to mediate effector function as measured by ADCC. Daudi cells express CD20 on their surface and thus bind to rituximab, allowing for NK cell mediated ADCC in the presence of rituximab. The activity of the selected variants in this assay was compared to that of the control rituximab variant described in Table F, and to commerically obtained rituximab.

[0281] Cell lines used: Daudi cell line (ATCC, Cat# CCL-213), NK92/ CD16a (158V/V) Detection device: FlexStation3, Molecular Devices.
Positive control antibody: Rituximab.

[0282] Cell Culture. Frozen cells were thawed by gently swirling the vial in the 37°C water bath. After 1-2min, the medium in the vial was completely thawed. The outside of the vial was wiped with 70% ethanol. The cell suspension was then transferred to a 15ml centrifuge tube, followed by addition of 5ml of pre-warmed complete medium. After centrifugation for 3-5min at 500 g, the supernatant was aspirated. 10ml of complete medium was added and the cells were resuspended by pipetting up and down for a few times. Cell viability was determined by Trypan blue staining method. The cell suspension was then seeded in flasks. The cells were incubated at 37°C, 5% CO2 overnight.

[0283] Cells were maintained at 37 °C / 5% CO2 and regularly sub-cultured with suitable medium supplemented with 10% FBS according to protocol from ATCC.

[0284] The antibody sample and the Standard (Rituximab) were delivered in dry shipper and stored at -20 °C before testing. The sample and the Standard were stored at 4 °C after they were thawed on ice. The sample and the Standard were diluted with Phenol red free MEM medium (supplemented with 1% FBS and 1% Pen/Strep) and applied to the tests.

[0285] ADCC assay buffer was composed of 98% Phenol red free MEM medium, 1% Pen/Strep and 1% FBS.

[0286] NK92/ FcRγ3a(158V/V) cells were conventionally maintained.

[0287] Target cells were harvested by centrifugation at 800 rpm for 3 min, washed with assay medium once and centrifuged; the medium above the pellet was removed completely. Cells were gently suspended with assay medium to make single cell solution. The target cell number was adjusted to 4x cell stock (10,000 cells in 50 µl assay medium). Test articles were prepared at interested concentrations. 50µl 4x target cell stock were seeded to 96-well assay plates and 50 µl 4x sample diluents added. The plates were incubated at room temperature for 30min in cell culture incubator. 100 µl effector cells (E/T=5:1, i.e, 50,000 effector cells per well) were added to initiate the reaction and mixed gently by cross shaking. Triton X-100 was added to cell controls without effector cells and antibody in a final concentration of 1% to lyze the target cells and it served as the maximum lysis control; assay buffers were added in to cell controls without effector cells and antibody and it served as the minimum LDH release control. Target cells incubated with effector cells without the presence of antibodies were set as background control of non-specific LDH release when both cells were incubated together. Plate was incubated at 37°C/5%CO2 incubator for 4-6 hours. The cell viability was assayed with an LDH kit. The absorbance data at OD492nm and OD650nm were measured on Flexstation 3.

[0288] The background (OD650nm) subtracted OD492nm data was analyzed to study the LDH release.The percentages of cell lysis were calculated according to the formula:
Cell lysis %=100*(1-(ODSample data- ODtumor cells plus effector cells) / (ODMaximum release - ODMinimum release))

[0289] The results are shown in Figure 6 and indicate that all variants showed significantly decreased or undetectable ADCC activity.
- The following table summarizes the results:
Table J: ADCC activity of the variant

Variant	EC50 [nM]	Maximum Lysis
Control WT Rituximab 1261	0.1	66
AAC9	Non-lytic	Non-lytic
AAC10	7.4	45
AAC11	>100	Low
AAC12	23.1	20
AAC13	n/d*	n/d*
AAC14	Non-lytic	Non-lytic
AAC15	Non-lytic	Non-lytic
*n/d = not determined		

[0290] The KO variants showed significantly decreased ADCC lytic activity, and for many variants, no activity was detected at all.

Example 12: Asymmetric antibody constructs based on rituximab reduce CDC (complement-dependent cytotoxicity) in Daudi cells

[0291] Although not tested for their ability to bind to C1q, selected variants were tested to determine whether they were able to mediate CDC in Daudi cells. The activity of the selected variants in this assay was compared to that of the control rituximab variant described in Table F, and to commerically obtained rituximab.

[0292] Cell lines used: Daudi cell line (ATCC, Cat# CCL-213), NK92/ CD16a (158V/V). Detection device: F PHERAstarPlus, BMG Labtech. Positive control antibody: Rituximab.

[0293] Cell Culture. Daudi cells were harvested by centrifugation and the pallets were washed with assay buffer once. Viable cells were counted by Trypan Blue dye. Cell population was only allowed of >99% viability for the assay. The cell concentration was adjusted and 5,000 cells were seeded in 20 µl CDC buffer. 10 µl diluted samples were added (8 concentrations with a dilution factor of 1:10 descending from 600nM, in triplicates). Samples and Rituxan control were incubated at room temperature for 30 min. 10 µl NHS (10% final concentration in 40 µl reaction volume) were added to each well to initiate the CDC assay. Plate was incubated at 37°C/ 5% CO2 incubator for 2 hours. Cell viability test was performed with CellTiter-Glo® Luminescent Cell Viability Assay Kit. Luminescence was read on PHERAStar Plus and record the relative light unit data.

Data analysis

[0294] The percentage of cell lysis was calculated with the formula:
%Cell lysis =100 ×(1- (RLUsample) / (RLUcell+NHS),
in which NHS stands for normal human serum.

[0295] The results are shown in Figure 7 and indicate that all samples showed significantly lower CDC activity.
Table K: CDC activity of the variants

Variant	EC50 [nM]	Maximum Lysis
Control WT Rituximab 1261	2.9	96
AAC9	47.9	68
AAC10	82.8	79
AAC11	51.6	63
AAC12	54.6	67
AAC13	n/d*	n/d*
AAC14	69.7	72
AAC15	~ 55.87	43
*n/d = not determined		

[0296] The KO variants showed significantly decreased CDC lytic activity.

[0297] The reagents employed in the examples are commercially available or can be prepared using commercially available instrumentation, methods, or reagents known in the art. The foregoing examples illustrate various aspects of the invention and practice of the methods of the invention. The examples are not intended to provide an exhaustive description of the different embodiments of the invention.

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Patentkrav

1. Heteromultimer, der omfatter et IgG-Fc-konstrukt med et første og et andet Fc-polypeptid, hvor IgG-Fc-konstruktet viser reduceret binding til FcγRIa-, FcγRIIa-, FcγRIIb- og FcγRIIIa-receptorerne i sammenligning med et tilsvarende vildtype-IgG-Fc-konstrukt, hvor:

A) hvert Fc-polypeptid omfatter en modificeret nedre hængselsregion i sammenligning med det tilsvarende vildtype-IgG-Fc-polypeptid, hvor den modificerede nedre hængselsregion i det første Fc-polypeptid omfatter aminosyremodifikationer ved L234 og/eller L235, som forøger den positive nettoladning i den modificerede nedre hængselsregion i det første Fc-polypeptid ved omkring fysiologiske pH-betingelser, og den modificerede nedre hængselsregion i det andet Fc-polypeptid omfatter mindst én aminosyremodifikation, der er forskellig fra mindst én aminosyremodifikation af det første Fc-polypeptid, og hvor den modificerede nedre hængselsregion i mindst ét af det første eller det andet Fc-polypeptid omfatter to eller flere aminosyremodifikationer, eller

B) det første Fc-polypeptid omfatter aminosyremodifikationerne E269Q/D270N, og det andet Fc-polypeptid omfatter aminosyremodifikationerne E269K/D270R, eller

C) det første Fc-polypeptid omfatter aminosyremodifikationerne L235K/A327K, og det andet Fc-polypeptid ikke omfatter en aminosyremodifikation i hænglet eller den nedre hængselsregion i sammenligning med det tilsvarende vildtype-IgG-Fc-polypeptid,

hvor IgG-Fc-konstruktet er et human IgG1-, IgG3- eller IgG4-konstrukt, og hvor nummereringen af aminosyrer er ifølge EU-indekset som i Kabat.

2. Heteromultimer ifølge krav 1A), hvor:

i) den mindst ene aminosyremodifikation på det andet Fc-polypeptid forøger det samlede antal negative ladninger på det andet Fc-polypeptid eller er ladningsneutral, eller

ii) den mindst ene aminosyremodifikation i den modificerede nedre hængselsregion i det første Fc-polypeptid kombineret med den mindst ene aminosyremodifikation i det andet Fc-polypeptid forøger den samlede

positive ladning af IgG-Fc-konstruktet sammenlignet med udgangs-IgG-Fc-konstruktet.

3. Heteromultimer ifølge krav 1 eller 2, hvor den modificerede hængselsregion i hver af det første og det andet Fc-polypeptid omfatter to eller flere aminosyremodifikationer.

4. Heteromultimer ifølge et hvilket som helst af kravene 1 til 3, hvor IgG-Fc-konstruktet har en K_D på mere end 10 µM for FcγRIIaH, en K_D på mere end 10 µM for FcγRIIaR, en K_D på mere end 10 µM for FcγRIIb, en K_D på mere end 6 µM for FcγRIIIaF, en K_D på mere end 6 µM for FcγRIIIaV og en K_D på mere end 6,5 nM for FcγRIa.

5. Heteromultimer ifølge et hvilket som helst af kravene 1 til 4, hvor:

A) IgG-Fc-konstruktet medierer reduceret effektorfunktion sammenlignet med det tilsvarende vildtype-IgG-Fc-konstrukt, valgfrit hvor effektorfunktionen er ADCC, ADCP, CDC eller en hvilken som helst kombination deraf, og/eller

B) IgG-Fc-konstruktet yderligere viser reduceret binding til C1q-protein i sammenligning med det tilsvarende vildtype-IgG-Fc-konstrukt.

6. Heteromultimer ifølge et hvilket som helst af kravene 1 til 5, hvor modifikationen ved L234 er valgt blandt L234A, L234K, L234R, L234D og L234E, og modifikationen ved L235 er valgt blandt L235K, L235R, L235E, L235A og L235D.

7. Heteromultimer ifølge krav 6, hvor den modificerede hængselsregion i det første og/eller det andet Fc-polypeptid yderligere omfatter en aminosyremodifikation ved E233, valgfrit hvor den ene eller den anden aminosyremodifikation eller begge to ved E233 uafhængigt af hinanden er E233A, E233D, E233K eller E233R.

8. Heteromultimer ifølge et hvilket som helst af kravene 1 til 7, hvor den modificerede nedre hængselsregion i mindst ét af det første og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234A/L235A, L234D/L235E,

L234K/L235K, E233A/L234D/L235E, E233A/L234K/L235A, E233A/L234R/L235R, E233K/L234R/L235R eller E233K/L234A/L235K, valgfrit hvor det første og/eller det andet Fc-polypeptid yderligere omfatter én eller flere aminosyremodifikationer, der er valgt blandt D265S, E269K, K322A, K322E, P329W og E333K.

9. Heteromultimer ifølge krav 1A), hvor:

a. den modificerede nedre hængselsregion i det første Fc-polypeptid omfatter aminosyremodifikationerne L234K/L235K, og den modificerede nedre hængselsregion i det andet Fc-polypeptid omfatter aminosyremodifikationerne L234A/L235A; eller

b. den modificerede nedre hængselsregion i det første Fc-polypeptid omfatter aminosyremodifikationerne L234K/L235K, og den modificerede nedre hængselsregion i det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E; eller

c. den modificerede nedre hængselsregion i det første Fc-polypeptid omfatter aminosyremodifikationerne E233A/L234R/L235R, og den modificerede nedre hængselsregion i det andet Fc-polypeptid omfatter aminosyremodifikationerne E233A/L234D/L235E; eller

d. den modificerede nedre hængselsregion i det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R, og den modificerede nedre hængselsregion i det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E; eller

e. den modificerede nedre hængselsregion i det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234A/L235K, og den modificerede nedre hængselsregion i det andet Fc-polypeptid omfatter aminosyremodifikationerne E233A/L234K/L235A; eller

f. det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R, og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E; eller

g. det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R/D265S, og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E/D265S; eller

h. det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R/E269K, og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E/E269K; eller

i. det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R/K322A, og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E/K322A; eller

j. det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R/P329W, og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E/P329W; eller

k. det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R/E269K/D265S/K322A, og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E/E269K/D265S/K322A; eller

l. det første Fc-polypeptid omfatter aminosyremodifikationerne E233K/L234R/L235R/E269K/D265S/K322E/E333K, og det andet Fc-polypeptid omfatter aminosyremodifikationerne L234D/L235E/E269K/D265S/K322E/E333K.

10. Heteromultimer ifølge et hvilket som helst af kravene 1 til 9, hvor IgG-Fc-konstruktet er aglycosyleret eller deglycosyleret.

11. Heteromultimer ifølge et hvilket som helst af kravene 1 til 10, hvor IgG-Fc-konstruktet omfatter en variant-CH3-region, der omfatter aminosyremodifikationer, som fremmer dannelsen af en heterodimer Fc-region i sammenligning med en homodimer Fc-region, når heteromultimeren udtrykkes, valgfrit hvor:

a. ét af det første og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T366L/N390R/K392M/T394W, og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne L351Y/S400E/F405A/Y407V, eller

b. ét af det første og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne L351Y/F405A/Y407V, og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T366L/K392M/T394W, eller

c. ét af det første og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne L351Y/F405A/Y407V, og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T366L/K392L/T394W, eller

d. ét af det første og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T350V/L351Y/F405A/Y407V, og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T350V/T366L/K392M/T394W, eller

e. ét af det første og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T350V/L351Y/F405A/Y407V, og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T350V/T366L/K392L/T394W, eller

f. ét af det første og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T350V/L351Y/S400E/F405A/Y407V, og det andet Fc-polypeptid omfatter CH3-aminosyremodifikationerne T350V/T366L/N390R/K392M/T394W.

12. Heteromultimer ifølge et hvilket som helst af kravene 1 til 11, hvor heteromultimeren yderligere omfatter mindst ét antigenbindende konstrukt, der er fusioneret til IgG-Fc-konstruktet, valgfrit hvor det mindst ene antigenbindende konstrukt er et Fab-fragment, et scFv, et sdAb, et antigenbindende peptid, et Fc-fusionsprotein eller et protein eller fragment deraf, som er i stand til at binde antigenet.

13. Heteromultimer ifølge et hvilket som helst af kravene 1 til 12, hvor IgG'et er IgG1.

14. Heteromultimer ifølge et hvilket som helst af kravene 1 til 13, hvor heteromultimeren er et antistof eller antistoffragment, valgfrit et bispecifikt antistof eller antistoffragment.

15. Heteromultimer ifølge et hvilket som helst af kravene 1 til 14, hvor IgG-Fc-konstruktet er forbundet med ét eller flere toksiske lægemiddelmolekyler eller heterologe polypeptider, valgfrit hvor det ene eller flere heterologe polypeptider er

enzymer eller toksiner.

16. Nukleinsyre, der koder for det første eller det andet Fc-polypeptid i heteromultimeren ifølge et hvilket som helst af kravene 1 til 14.

17. Værtscelle, som omfatter én eller flere nukleinsyrer, der koder for heteromultimeren ifølge et hvilket som helst af kravene 1 til 14.

18. Fremgangsmåde til fremstilling af heteromultimeren ifølge et hvilket som helst af kravene 1 til 14, hvilken fremgangsmåde omfatter følgende trin: (a) dyrkning af værtscellen ifølge krav 17; og (b) isolering af heteromultimeren fra værtscellekulturen, idet fremgangsmåden valgfrit endvidere omfatter (c) isolering af heteromultimeren under anvendelse af ladningsbaserede oprensningsmetoder, såsom ionbytningskromatografi.

19. Farmaceutisk sammensætning, der omfatter heteromultimeren ifølge et hvilket som helst af kravene 1 til 15 og en farmaceutisk acceptabel bærer.

20. Heteromultimer ifølge et hvilket som helst af kravene 1 til 15 til anvendelse ved behandling af en sygdom hos en patient, som har behov derfor.

21. Fremgangsmåde til reduktion af effektorfunktionen af et IgG-Fc-konstrukt, der omfatter et første og et andet Fc-polypeptid, hvilken fremgangsmåde omfatter:
modifikation af den nedre hængselsregion i mindst ét af det første og det andet Fc-polypeptid, hvor:
den modificerede nedre hængselsregion i det første Fc-polypeptid omfatter aminosyremodifikationer ved L234 og/eller L235, der forøger den positive nettoladning i den modificerede nedre hængselsregion i det første Fc-polypeptid ved omkring fysiologiske pH-betingelser, og den modificerede nedre hængselsregion i det andet Fc-polypeptid omfatter mindst én aminosyremodifikation, som er forskellig fra mindst én aminosyremodifikation af det første Fc-polypeptid, hvor den modificerede nedre hængselsregion i mindst ét af det første eller det andet Fc-polypeptid omfatter to eller flere aminosyremodifikationer,

hvor IgG-konstruktet er et human IgG1-, IgG3- eller IgG4-Fc-konstrukt, hvor IgG-Fc-konstruktet viser reduceret binding til FcγRIa-, FcγRIIa-, FcγRIIb- og FcγRIIIa-receptorer i sammenligning med et tilsvarende vildtype-IgG-Fc-konstrukt, og hvor nummereringen af aminosyrerne er ifølge EU-indekset som i Kabat.

DRAWINGS

FIGURE 1

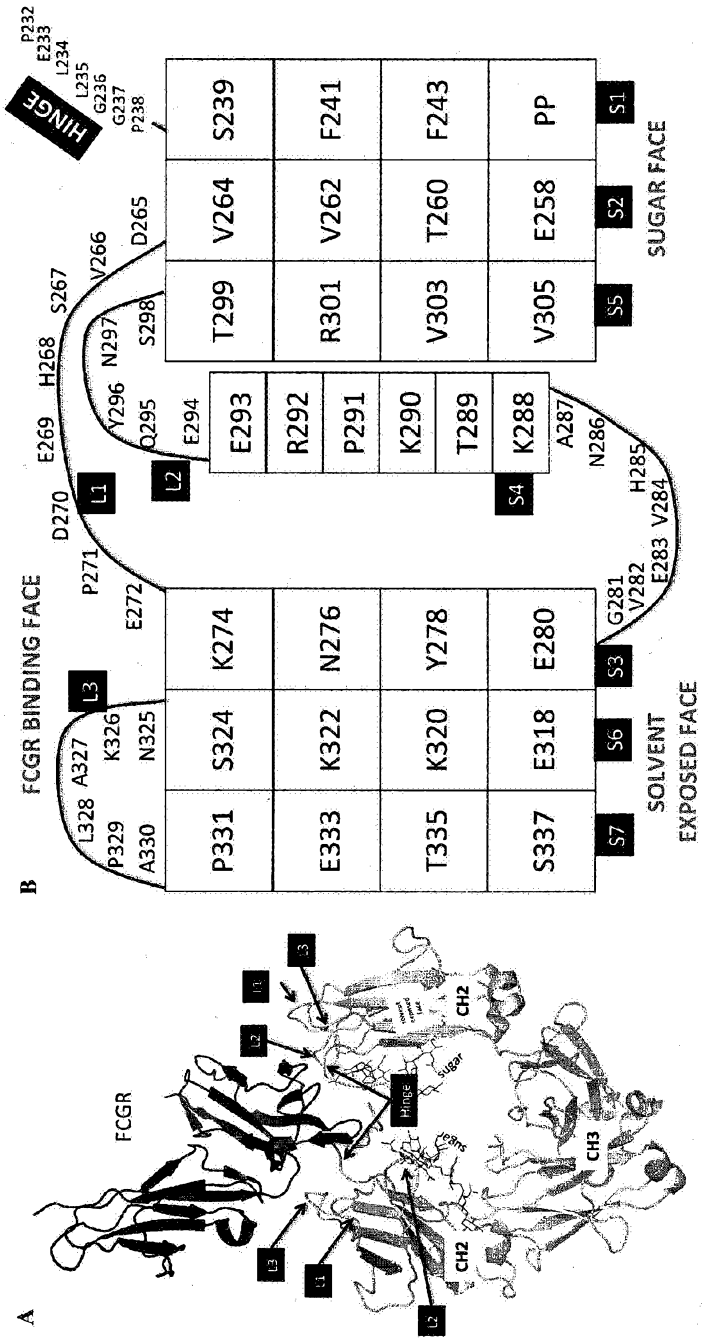

FIGURE 2

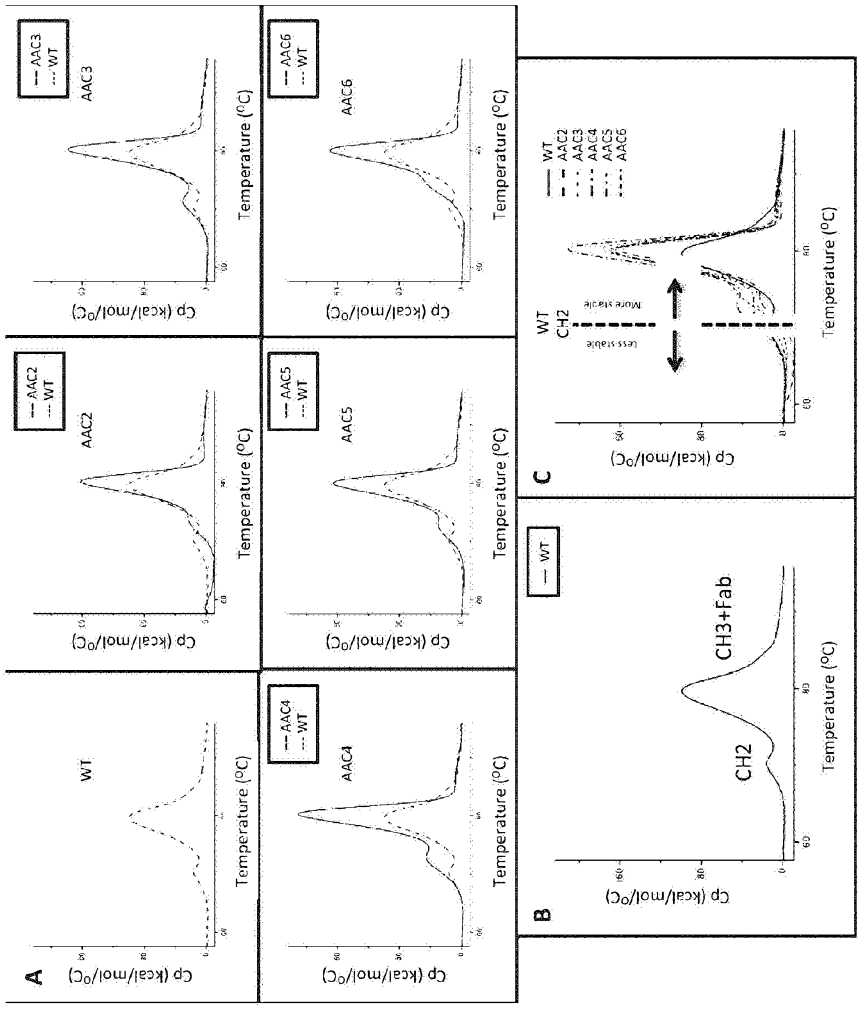
A
WT
AAC2
AAC3
AAC4
AAC5
AAC6
Cp (kcal/mol/°C)
Temperature (°C)
B
CH3+Fab
CH2
C
WT
CH2
Less stable
More stable

FIGURE 3A

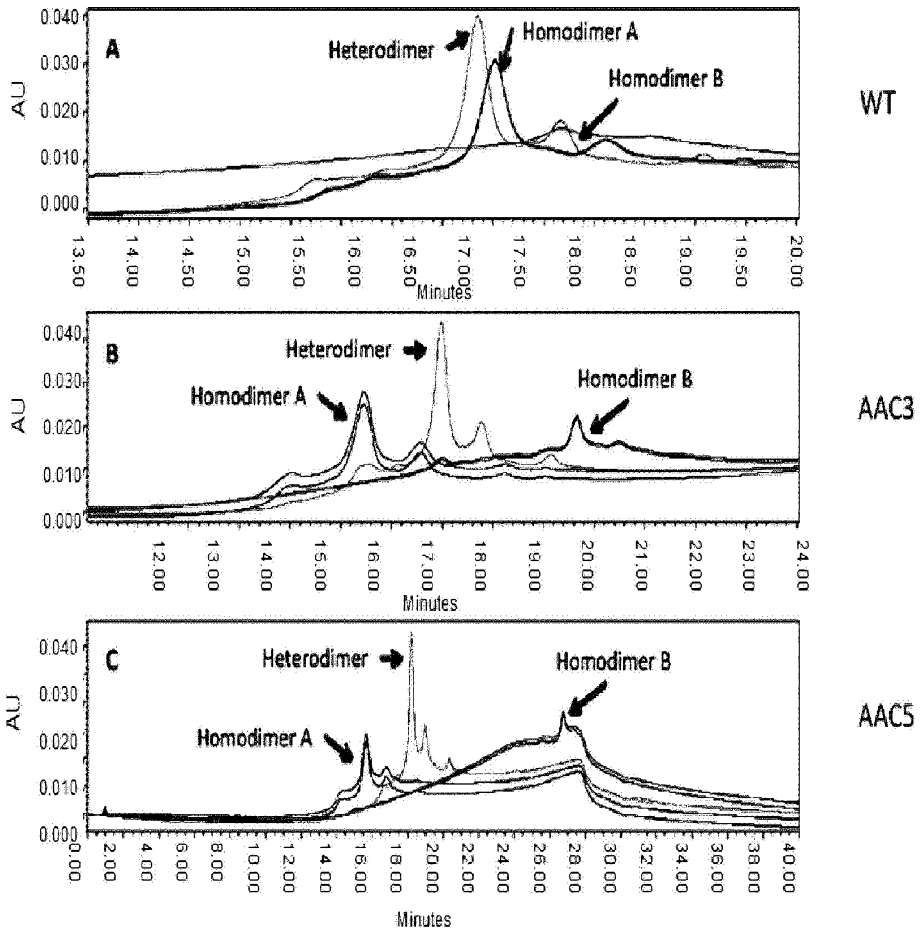
A
Heterodimer
Homodimer A
Homodimer B
WT
AU
Minutes
B
Heterodimer
Homodimer A
Homodimer B
AAC3
AU
Minutes
C
Heterodimer
Homodimer B
Homodimer A
AAC5
AU
Minutes

FIGURE 3B

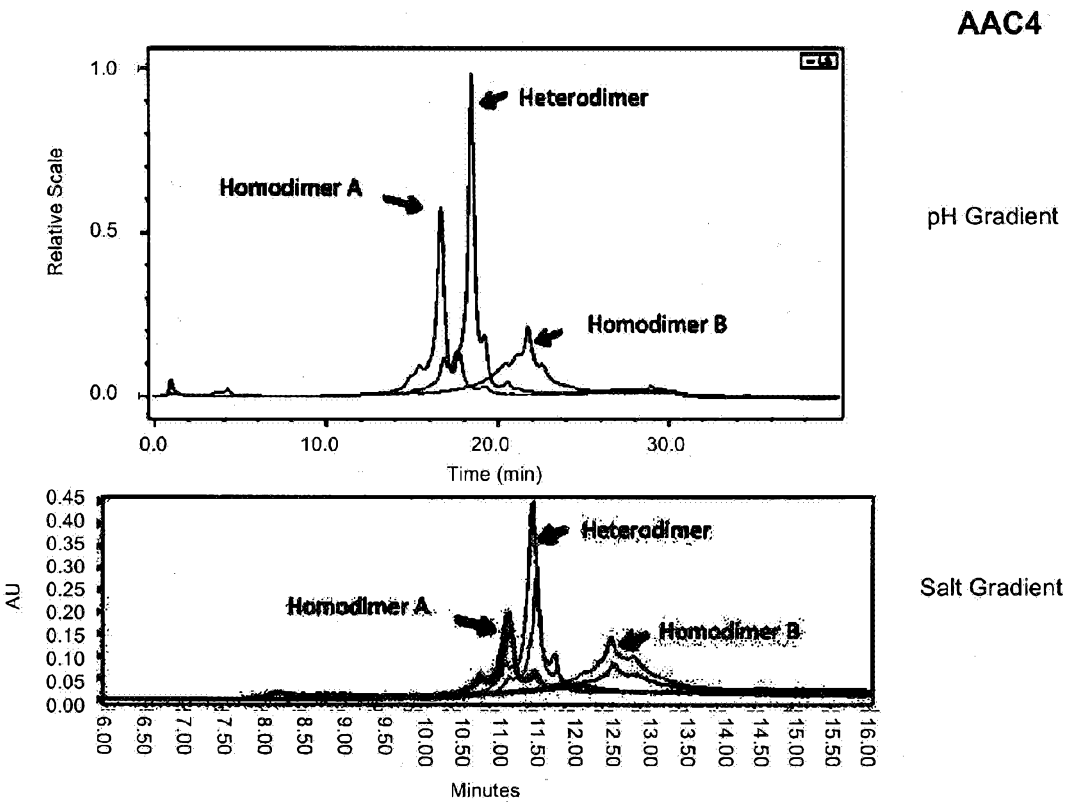
AAC4
Heterodimer
Homodimer A
Homodimer B
Relative Scale
1.0
0.5
0.0
0.0
10.0
20.0
30.0
Time (min)
pH Gradient
AU
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Heterodimer
Homodimer A
Homodimer B
6.00
6.50
7.00
7.50
8.00
8.50
9.00
9.50
10.00
10.50
11.00
11.50
12.00
12.50
13.00
13.50
14.00
14.50
15.00
15.50
16.00
Minutes
Salt Gradient

FIGURE 4

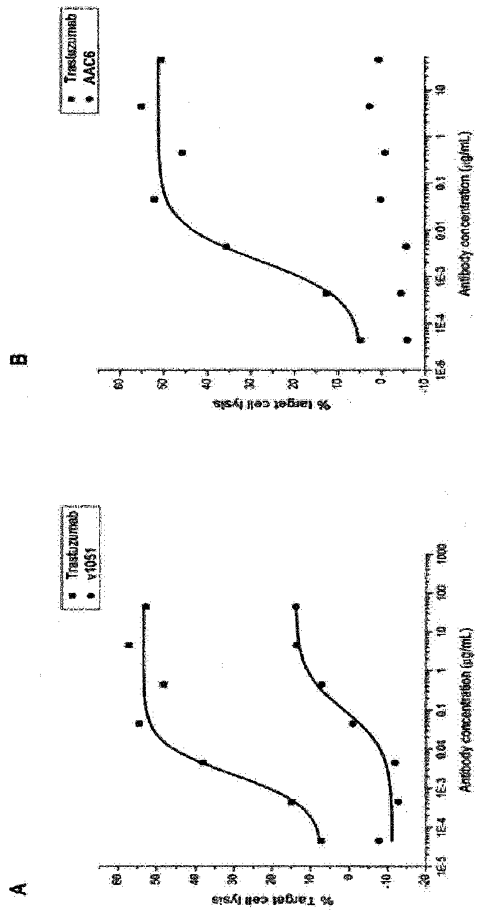
A
Trastuzumab
v1051
% Target cell lysis
Antibody concentration (µg/mL)
B
Trastuzumab
AAC6
% Target cell lysis
Antibody concentration (µg/mL)

FIGURE 5

SEQ ID NO:1:

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:2:

>Trastuzumab_Heavy chain

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTR
YADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLV
TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS
KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:3:

>Trastuzumab_Light chain

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVP
SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO:4:

>Rituximab+Heavy chain

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGD
TSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGT
TVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF
PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC
PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ
VYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:5:

>Rituximab+Light chain

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVR
FSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPS
DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTL
TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIGURE 6

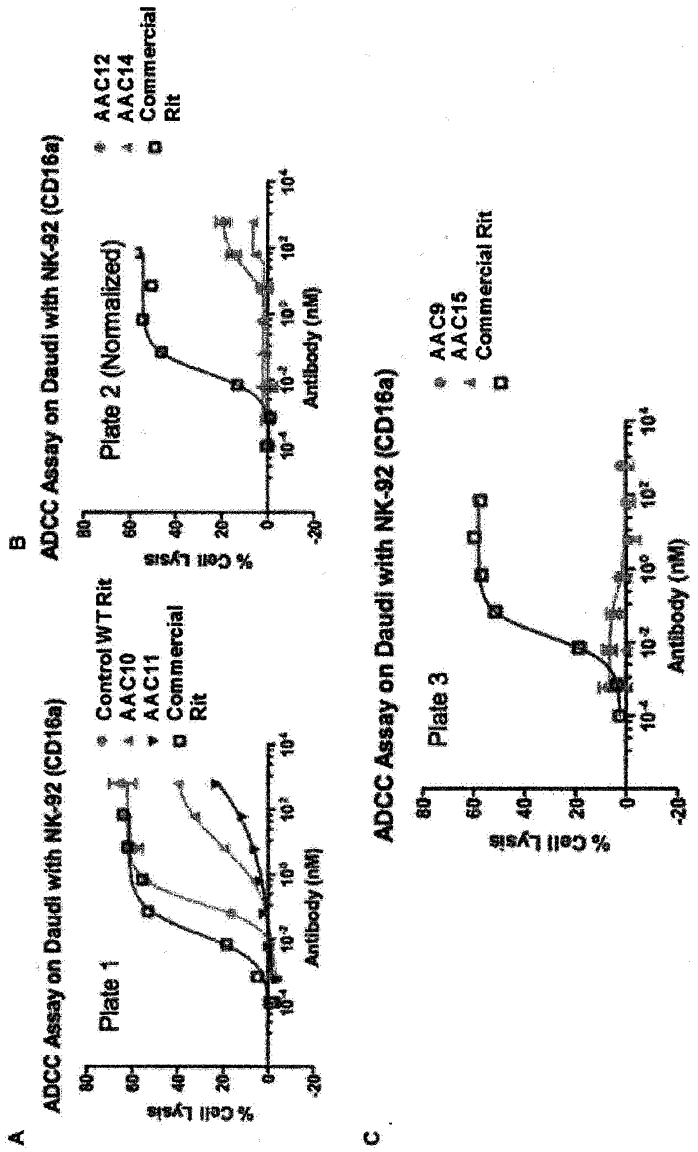
A
ADCC Assay on Daudi with NK-92 (CD16a)
Plate 1
% Cell Lysis
Antibody (nM)
Control WT Rit
AAC10
AAC11
Commercial Rit
B
ADCC Assay on Daudi with NK-92 (CD16a)
Plate 2 (Normalized)
% Cell Lysis
Antibody (nM)
AAC12
AAC14
Commercial Rit
C
ADCC Assay on Daudi with NK-92 (CD16a)
Plate 3
% Cell Lysis
Antibody (nM)
AAC9
AAC15
Commercial Rit

FIGURE 7

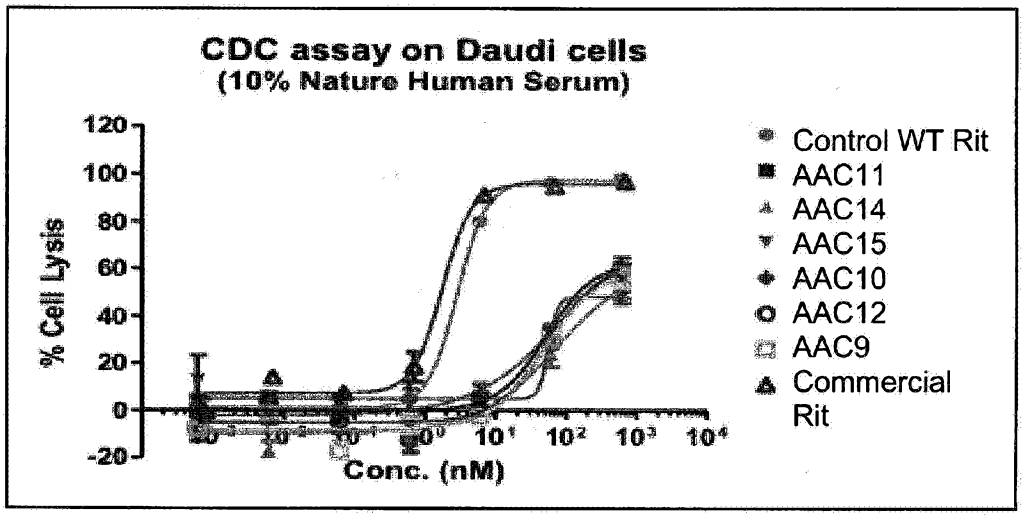
CDC assay on Daudi cells
(10% Nature Human Serum)
% Cell Lysis
120
100
80
60
40
20
0
-20
Conc. (nM)
Control WT Rit
AAC11
AAC14
AAC15
AAC10
AAC12
AAC9
Commercial Rit

FIGURE 8

SEQ ID NO:	Type	
6	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDEDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
8	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTW PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
10	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDEDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
12	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPADEGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDEDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK

14	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPAKAGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDEDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
16	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHQNPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDEDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
18	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDEDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
20	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTWP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
22	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEKKGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP

		QVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTWP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
24	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPARRGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTW PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
26	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTW PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
28	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKAKGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTW PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
30	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHKRPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTWP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
32	polypeptide	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ

		TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELKGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKKLPAPIEKTISKAKGQPREP QVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENRYMTWP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
35	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK TTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL SLSPGK
37	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNYL TWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPGK
39	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
41	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNY LTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK

43	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFL FPPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
45	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFL FPPKPKDTLMISRTPEVTCVVVSVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNY LTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK
47	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHKDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
49	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHKDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNY LTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK
51	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKG

		QPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
53	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKG QPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNY LTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK
55	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALWAPIEKTISKAKG QPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
57	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALWAPIEKTISKAKG QPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNY LTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK
59	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFL FPPKPKDTLMISRTPEVTCVVVSVSHKDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKG QPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
61	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS

		LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFL FPPKPKDTLMISRTPEVTCVVVSVSHKDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKG QPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNY LTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK
63	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEDEGGPSVFL FPPKPKDTLMISRTPEVTCVVVSVSHKDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCEVSNKALPAPIKKTISKAKG QPREPQVYVYPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFALVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
65	polypeptide	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPKRRGGPSVFL FPPKPKDTLMISRTPEVTCVVVSVSHKDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCEVSNKALPAPIKKTISKAKG QPREPQVYVLPPSRDELTKNQVSLLCLVKGFYPSDIAVEWESNGQPENNY LTWPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK
67	polypeptide	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGT KVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSS PVTKSFNRGEC
68	polypeptide	QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATS NLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGT KLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA LQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP VTKSFNRGEC
7	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC

		CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAAGCC GCCGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGTACCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGACATGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAATTACAA GACCACACCTCCAGTGCTGGACGAGGATGGCAGCTTCGCCCTGGTGT CCAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTT AGTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAG AGCCTGTCCCTGTCTCCCGGCAAA
9	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAAGCC GCCGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA

		GCCTGTCCCTGTCTCCCGGCAAA
11	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAAGAC GAGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGTACCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGACATGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAATTACAA GACCACACCTCCAGTGCTGGACGAGGATGGCAGCTTCGCCCTGGTGT CCAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTT AGTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAG AGCCTGTCCCTGTCTCCCGGCAAA
13	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC

		CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGCCGAC GAGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGTACCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGACATGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAATTACAA GACCACACCTCCAGTGCTGGACGAGGATGGCAGCTTCGCCCTGGTGT CCAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTT AGTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAG AGCCTGTCCCTGTCTCCCGGCAAA
15	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGCCAAG GCCGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGTACCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGACATGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAATTACAA GACCACACCTCCAGTGCTGGACGAGGATGGCAGCTTCGCCCTGGTGT CCAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTT AGTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAG AGCCTGTCCCTGTCTCCCGGCAAA

17	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAACTG CTGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACCAGAACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGTACCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGACATGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAATTACAA GACCACACCTCCAGTGCTGGACGAGGATGGCAGCTTCGCCCTGGTGT CCAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTT AGTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAG AGCCTGTCCCTGTCTCCCGGCAAA
19	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAACTG CTGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG

		TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGTACCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGACATGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAATTACAA GACCACACCTCCAGTGCTGGACGAGGATGGCAGCTTCGCCCTGGTGT CCAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTT AGTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAG AGCCTGTCCCTGTCTCCCGGCAAA
21	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAACTG CTGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA GCCTGTCCCTGTCTCCCGGCAAA
23	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG

		GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAAAAG AAGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA GCCTGTCCCTGTCTCCCGGCAAA
25	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGCCAGA AGAGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT

		GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA GCCTGTCCCTGTCTCCCGGCAAA
27	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAAAGAGA AGAGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA GCCTGTCCCTGTCTCCCGGCAAA
29	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC

		CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAAAGGCC AAGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA GCCTGTCCCTGTCTCCCGGCAAA
31	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAACTG CTGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACAAGAGACCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGGCCCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA GCCTGTCCCTGTCTCCCGGCAAA

33	DNA	GAGGTGCAGCTGGTGGAAAGCGGAGGAGGACTGGTGCAGCCAGGA GGATCTCTGCGACTGAGTTGCGCCGCTTCAGGATTCAACATCAAGGAC ACCTACATTCACTGGGTGCGACAGGCTCCAGGAAAAGGACTGGAGTG GGTGGCTCGAATCTATCCCACTAATGGATACACCCGGTATGCCGACTC CGTGAAGGGGAGGTTTACTATTAGCGCCGATACATCCAAAAACACTG CTTACCTGCAGATGAACAGCCTGCGAGCCGAAGATACCGCTGTGTACT ATTGCAGTCGATGGGGAGGAGACGGATTCTACGCTATGGATTATTGG GGACAGGGGACCCTGGTGACAGTGAGCTCCGCCTCTACCAAGGGCCC CAGTGTGTTTCCCCTGGCTCCTTCTAGTAAATCCACCTCTGGAGGGAC AGCCGCTCTGGGATGTCTGGTGAAGGACTATTTCCCCGAGCCTGTGAC CGTGAGTTGGAACTCAGGCGCCCTGACAAGCGGAGTGCACACTTTTC CTGCTGTGCTGCAGTCAAGCGGGCTGTACTCCCTGTCCTCTGTGGTGA CAGTGCCAAGTTCAAGCCTGGGCACACAGACTTATATCTGCAACGTGA ATCATAAGCCCTCAAATACAAAAGTGGACAAGAAAGTGGAGCCCAAG AGCTGTGATAAGACCCACACCTGCCCTCCCTGTCCAGCTCCAGAACTG AAGGGAGGACCTAGCGTGTTCCTGTTTCCCCCTAAGCCAAAAGACACT CTGATGATTTCCAGGACTCCCGAGGTGACCTGCGTGGTGGTGGACGT GTCTCACGAGGACCCCGAAGTGAAGTTCAACTGGTACGTGGATGGCG TGGAAGTGCATAATGCTAAGACAAAACCAAGAGAGGAACAGTACAAC TCCACTTATCGCGTCGTGAGCGTGCTGACCGTGCTGCACCAGGACTGG CTGAACGGGAAGGAGTATAAGTGCAAAGTCAGTAATAAGAAGCTGCC TGCTCCAATCGAAAAAACCATCTCTAAGGCCAAAGGCCAGCCAAGGG AGCCCCAGGTGTACGTGCTGCCACCCAGCAGAGACGAACTGACCAAG AACCAGGTGTCCCTGCTGTGTCTGGTGAAAGGCTTCTATCCTAGTGAT ATTGCTGTGGAGTGGGAATCAAATGGACAGCCAGAGAACAGATACAT GACCTGGCCTCCAGTGCTGGACAGCGATGGCAGCTTCTTCCTGTATTC CAAGCTGACAGTGGATAAATCTCGATGGCAGCAGGGGAACGTGTTTA GTTGTTCAGTGATGCATGAAGCCCTGCACAATCATTACACTCAGAAGA GCCTGTCCCTGTCTCCCGGCAAA
34	DNA	GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGA GACAGAGTCACCATCACTTGCCGGGCAAGTCAGGACGTTAACACCGC TGTAGCTTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGAT CTATTCTGCATCCTTTTTGTACAGTGGGGTCCCATCAAGGTTCAGTGGC AGTCGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT GAAGATTTTGCAACTTACTACTGTCAACAGCATTACACTACCCCACCCA CTTTCGGCCAAGGGACCAAAGTGGAGATCAAACGAACTGTGGCTGCA CCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCA AAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAA GAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCA GCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTC TACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG AGCTTCAACAGGGGAGAGTGT
36	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT

		TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGCT GCTGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA
38	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGCT GCTGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG

		GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
40	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGGA CGAGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA
42	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT

		CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAAAGAG AAGAGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
44	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGGA CGAGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGAGCGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA

46	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAAAGAG AAGAGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGAGCGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
48	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGGA CGAGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACAAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG

		TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA
50	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAAAGAG AAGAGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACAAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
52	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG

		GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGGA CGAGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCGCCGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA
54	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAAAGAG AAGAGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCGCCGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC

		TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
56	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGGA CGAGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGT GGGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA
58	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT

		CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAAAGAG AAGAGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGGACGT GAGCCACGAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCAAGGTGTCCAACAAGGCCCTGT GGGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
60	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGGA CGAGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGAGCGT GAGCCACAAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCGCCGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA

62	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAAAGAG AAGAGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGAGCGT GAGCCACAAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCGCCGTGTCCAACAAGGCCCTGC CCGCACCTATCGAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
64	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAGAGGA CGAGGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGAGCGT GAGCCACAAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG

		TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCGAGGTGTCCAACAAGGCCCTGC CCGCACCTATCAAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGTACCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGACCTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACA AGACTACCCCCCCTGTGCTGGACTCAGATGGGAGCTTCGCCCTGGTGT CCAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTT TCCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAG TCCCTGAGCCTGTCACCCGGCAAA
66	DNA	CAGGTCCAGCTGCAGCAGCCCGGAGCTGAACTGGTCAAACCTGGCGC ATCCGTGAAAATGTCTTGCAAGGCTAGTGGCTACACATTCACTTCCTAT AACATGCACTGGGTGAAGCAGACACCAGGACGAGGACTGGAGTGGA TCGGAGCAATCTACCCTGGAAACGGCGACACTTCTTATAATCAGAAGT TTAAAGGCAAGGCCACCCTGACAGCTGATAAGAGCTCCTCTACCGCCT ACATGCAGCTGAGTTCACTGACAAGTGAAGACTCAGCAGTGTACTATT GCGCCAGAAGCACCTACTATGGCGGGGATTGGTACTTCAACGTGTGG GGGGCAGGAACCACAGTCACCGTGAGCGCCGCTTCCACAAAAGGACC AAGCGTGTTTCCACTGGCACCAAGCTCCAAGTCAACCAGCGGAGGAA CAGCAGCCCTGGGATGTCTGGTGAAGGACTACTTCCCAGAGCCCGTC ACCGTGTCTTGGAACAGTGGCGCCCTGACAAGCGGGGTCCATACTTTT CCCGCTGTGCTGCAGTCTAGTGGCCTGTACAGCCTGTCAAGCGTGGTC ACCGTCCCTTCCTCTAGTCTGGGGACTCAGACCTATATCTGCAACGTG AATCACAAACCTTCTAATACAAAGGTCGACAAGAAAGTGGAACCAAA AAGTTGTGATAAGACACATACTTGCCCACCTTGTCCTGCACCAAAGAG AAGAGGAGGACCATCCGTGTTCCTGTTTCCACCCAAACCCAAGGACAC TCTGATGATTAGCCGGACTCCTGAAGTCACCTGCGTGGTCGTGAGCGT GAGCCACAAGGACCCCGAAGTCAAATTCAACTGGTACGTGGATGGCG TCGAGGTGCATAATGCCAAAACAAAGCCCCGGGAGGAACAGTACAAC TCAACATATAGAGTCGTGAGCGTCCTGACTGTGCTGCACCAGGACTG GCTGAACGGCAAGGAGTATAAATGCGAGGTGTCCAACAAGGCCCTGC CCGCACCTATCAAGAAGACTATTTCTAAAGCCAAGGGCCAGCCTAGG GAACCACAGGTGTACGTGCTGCCTCCAAGCCGCGACGAGCTGACTAA AAACCAGGTCTCCCTGCTGTGTCTGGTGAAGGGGTTCTATCCAAGTGA TATCGCTGTGGAGTGGGAATCAAATGGACAGCCCGAGAACAATTACC TGACTTGGCCCCCTGTGCTGGACTCAGATGGGAGCTTCTTTCTGTATTC CAAACTGACCGTGGATAAGTCTCGGTGGCAGCAGGGAAATGTCTTTT CCTGTTCTGTGATGCACGAAGCACTGCACAATCACTACACCCAGAAGT CCCTGAGCCTGTCACCCGGCAAA
69	DNA	CAGATTGTCCTGTCTCAGAGTCCCGCTATCCTGTCAGCAAGCCCTGGG GAGAAGGTGACCATGACATGCCGAGCCAGCTCCTCTGTCAGCTACATC CACTGGTTCCAGCAGAAGCCAGGCAGTTCACCTAAACCATGGATCTAC GCCACATCTAACCTGGCTAGTGGAGTGCCCGTCCGGTTTTCCGGCTCT GGGAGTGGAACATCATACAGCCTGACTATTTCCAGAGTGGAGGCCGA AGACGCCGCTACCTACTATTGCCAGCAGTGGACCTCTAATCCCCCTAC ATTCGGCGGGGGAACTAAGCTGGAGATCAAAAGGACTGTGGCAGCC

		CCTTCTGTCTTCATTTTTCCACCCAGTGACGAACAGCTGAAATCAGGAA CCGCTTCCGTGGTCTGTCTGCTGAACAACTTCTACCCCCGCGAGGCAA AGGTGCAGTGGAAAGTCGATAACGCCCTGCAGTCCGGCAATTCTCAG GAGAGTGTGACCGAACAGGACTCAAAGGATAGCACATATTCCCTGAG CTCCACTCTGACCCTGTCCAAAGCTGATTACGAAAAGCATAAAGTGTA TGCATGTGAGGTCACCCACCAGGGGCTGAGTAGTCCCGTCACAAAGA GTTTCAATAGAGGAGAGTGT