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(54) **Title**: METHODS FOR PRODUCING FABS AND BI-SPECIFIC ANTIBODIES

(57) **Abstract**: The present invention provides methods for producing Fabs and bi-specific antibodies comprising designed residues in the interfaces of the heavy chain- light chain variable (V_H/V_L) domain and the heavy chain-light chain constant (C_{H1} /C_L] domain, Fabs and bi-specific antibodies produced according to said processes and host cells encoding the same.

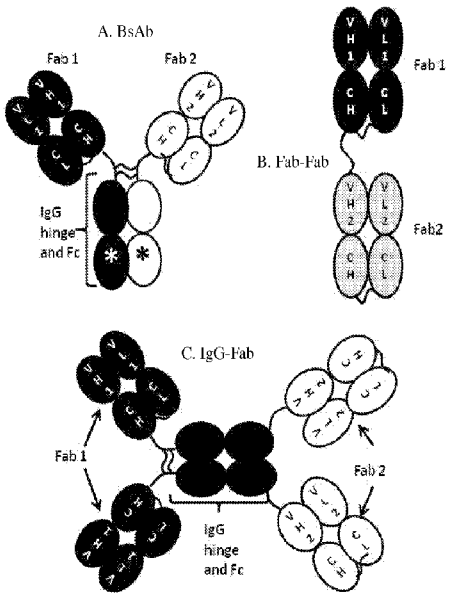


Figure 1. Schematic diagrams of IgG BsAb (*A*), Fab-Fab (*B*) and IgG-Fab(*C*) design formats. The "*" in the IgG BsAb diagram indicates a heterodimerized antibody CH3 domain

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Methods for Producing Fabs and Bi-specific Antibodies

Antibody therapies represent an ever increasing segment of the global pharmaceutical market. Approved antibody-based products include treatments for cancer, rheumatoid arthritis, infectious diseases, cardiovascular disease and autoimmune disorders. However, to improve patient outcomes, co-administration of two or more agents that perturb distinct therapeutic targets or biochemical pathways is often desired. In this context, antibody therapy has limitations.

Co-administration of two or more antibody therapies requires multiple injections or, alternatively, a single injection of a co-formulation of two different antibody compositions. While multiple injections permit flexibility in dose and timing of administration, the inconvenience and discomfort associated with multiple injections may reduce patient compliance. On the other hand, while a co-formulation of multiple antibody agents would permit fewer injections, the difficulty and/or expense associated with designing a suitable pharmaceutical formulation that provides the necessary stability and bioavailability, for each antibody ingredient, may be prohibitive. Furthermore, any treatment regime which entails administration of separate antibody agents will incur the added manufacturing and regulatory cost associated with the development of each individual agent.

The archetypical antibody is comprised of two identical antigen binding fragments (Fabs) which not only direct binding to a particular antigenic determinant, but also provide the interface for assembly between heavy chain (HC)-light chain (LC) pairs. Bispecific antibodies – single agents capable of binding to two distinct antigens - have been proposed as a means for addressing the limitations attendant with co-administration or co-formulation of separate antibody agents. Bispecific antibodies may integrate the binding activity of two separate MAb therapeutics, providing a cost and convenience benefit to the patient. Under certain circumstances, bispecific antibodies may elicit synergistic or novel activities beyond what an antibody combination can achieve. One example of novel activity provided by bispecific antibodies would be the bridging of two different cell types through the binding of distinct cell surface receptors. Alternately, bispecific antibodies could cross-link two receptors on the surface of the same cell leading to novel agonistic/antagonistic mechanisms.

The ability to generate bispecific antibodies with fully IgG architecture has been a long-standing challenge in antibody engineering. One proposal for generating fully IgG bispecific antibodies entails co-expression of nucleic acids encoding two distinct HC-LC pairs which, when expressed, assemble to form a single antibody comprising two distinct Fabs. However, challenges with this approach remain. Specifically, the expressed polypeptides of each desired Fab must assemble with good specificity to reduce generation of mis-matched byproducts, and the resulting heterotetramer must assemble with good stability. Procedures for directing assembly of particular HC-HC pairs by introducing modifications into regions of the HC-HC interface have been disclosed in the art. (See Klein et al., *mAbs*; 4(6); 1-11 (2012); Carter et al., *J. Immunol. Methods*; 248; 7-15 (2001); Gunasekaran, et al., *J. Biol. Chem.*; 285; 19637-19646 (2010); Zhu et al., *Protein Sci.*; 6: 781-788 (1997); and Igawa et al., *Protein Eng. Des. Sel.*; 23; 667-677 (2010)). However, there remains a need for alternative methods.

In accordance with the present invention, methods have been identified for achieving assembly of particular Fabs by co-expressing nucleic acids encoding particular HC-LC pairs which contain designed residues in the interface of the heavy chain-light chain variable (V_H/V_L) domains and the heavy chain-light chain constant (C_{H1}/C_L) domains. More particularly, the methods of the present invention achieve improved specificity and, or stability in assembly of particular Fabs. Even more particular, the methods of the present invention allow the binding specificities and binding activities of the variable regions of two distinct therapeutic antibodies to be combined in a single bi-specific antibody compound.

Thus, the present invention provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); and (b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid

substituted at residue 38 (38D), and said light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W), wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); and said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D).

In a separate embodiment, the present invention provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain CH1 domain comprises an arginine substituted at residue 172 (172R) and a glycine substituted at residue 174 (174G); and (b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and said light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W), wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain

variable and constant domains and said light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); and said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D).

In another embodiment, the present invention provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); and (b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and said light chain constant domain comprises a phenylalanine substituted at residue 135 (135F) and a tryptophan substituted at residue 176 (176W), wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

In another embodiment, the present invention provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said heavy chain CH1 domain comprises a WT sequence; and (b) a second nucleic acid encoding both a light chain variable domain and a

light chain constant domain wherein said light chain variable domain comprises an arginine substituted at residue 38 (38R) and said light chain constant domain comprises a WT sequence, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising the following: said first nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) and said second nucleic acid encodes a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In a separate embodiment, the present invention provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said heavy chain CH1 domain comprises an aspartic acid substituted at residue 228 (228D); and (b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain comprises an arginine substituted at residue 38 (38R) and said light chain constant domain comprises a lysine substituted at residue 122 (122K), wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising the following: said first nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) and said second nucleic acid encodes a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

The present invention also provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain CH1 domain comprises a WT sequence; and (b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and said light chain constant domain comprises a WT sequence, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

More particularly, the present invention provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said IgG heavy chain constant CH1 domain comprises an aspartic acid substituted at residue 228 (228D); and (b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D) and said light chain constant domain comprises a lysine substituted at residue 122 (122K), wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are

produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

The present invention also provides a method for producing a fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said heavy chain CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G) ; and (b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain comprises an arginine substituted at residue 38 (38R) and said light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W), wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen; (2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising the following: said first nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) and said second nucleic acid encodes a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In a more particular embodiment, the present invention provides a method for producing a first and second fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding both a first light chain variable domain and a first light

chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1(1R) and an aspartic acid substituted at residue 38 (38D), and said first light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said second IgG heavy chain constant CH1 domain comprises a WT sequence; and (d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 (38R) and said second light chain constant domain comprises a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and (3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising an leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In a further embodiment, the present invention provides a method for producing a first and second fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an arginine substituted at residue 172 (172R) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and said first light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said second IgG heavy chain constant CH1 domain comprises a WT sequence; and (d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 (38R) and said second light chain constant domain comprises a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and (3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains. More particular to this embodiment, the present invention provides a method

comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In a separate embodiment, the present invention provides A method for producing a first and second fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and said first light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said second IgG heavy chain constant CH1 domain comprises an aspartic acid substituted at residue 228 (228D); and (d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 (38R) and said second light chain constant domain comprises a lysine substituted at residue 122 (122K), wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining

regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and (3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginie substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In another embodiment, the present invention provides a method for producing a first and second fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an arginine substituted at residue 172 (172R) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at

residue 38 (38D), and said first light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said second IgG heavy chain constant CH1 domain comprises an aspartic acid substituted at residue 228 (228D); and (d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 (38R) and said second light chain constant domain comprises a lysine substituted at residue 122 (122K), wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and (3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginie substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In another particular embodiment, the present invention provides a method for producing a first and second fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1(1R) and an aspartic acid substituted at residue 38 (38D), and said first light chain constant domain comprises a phenylalanine substituted at residue 135 (135F) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y) and said second IgG heavy chain constant CH1 domain comprises a WT sequence; and (d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 (38R) and said second light chain constant domain comprises a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and (3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains.

The present invention also provides a method for producing a first and second fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y), and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain comprises an arginine substituted at residue 38 (38R), and said first light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat and said second IgG heavy chain constant CH1 domain comprises a WT sequence; and (d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D) and said second light chain constant domain comprises a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and (3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising the following: said first nucleic acid

encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) and said second nucleic acid encodes a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In another embodiment, the present invention provides a method for producing a first and second fragment, antigen binding (Fab) comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a tyrosine substituted at residue 39 (39Y), and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain comprises an arginine substituted at residue 38 (38R), and said first light chain constant domain comprises a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat and said second IgG heavy chain constant CH1 domain comprises an aspartic acid substituted at residue 228 (228D); and (d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D) and said second light chain constant domain comprises a lysine substituted at residue 122 (122K), wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and (3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and

constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains. More particular to this embodiment, the present invention provides a method comprising the following: said first nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) and said second nucleic acid encodes a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

The present invention also provides a method for producing a bispecific antibody comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant domain comprising an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1(1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y), and a CH1 constant domain comprising a WT sequence; and (d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R) and a constant domain comprising a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and (3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first

IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In a separate embodiment, the present invention provides a method for producing a bispecific antibody comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is 4 amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant domain comprising an arginine substituted at residue 172 (172R) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y), and a CH1 constant domain comprising a WT sequence; and (d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R) and a constant domain comprising a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host

cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and (3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In yet another embodiment, the present invention provides a method for producing a bispecific antibody comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant domain comprising an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y), and a CH1 constant domain comprising an aspartic acid substituted at residue 228 (228D) ; and (d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R) and a constant domain comprising a lysine substituted at residue 122 (122K), wherein each of

said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and (3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

In another embodiment, the present invention provides a method for producing a bispecific antibody comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant domain comprising an arginine substituted at residue 172 (172R) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y), and a

CH1 constant domain comprising an aspartic acid substituted at residue 228 (228D) ; and (d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R) and a constant domain comprising a lysine substituted at residue 122 (122K), wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and (3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain. More particular to this embodiment, the present invention provides a method comprising one or more of the following: said first nucleic acid encodes a heavy chain CH1 constant domain further comprising a methionine or isoleucine substituted at residue 190 (190M or 190I); said second nucleic acid encodes a light chain constant domain further comprising a leucine substituted at residue 133 (133L); said second nucleic acid encodes a light chain constant domain further comprising a glutamine or aspartic acid substituted at residue 174 (174Q or 174D), and said third nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) with said fourth nucleic acid encoding a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

The present invention also provides a method for producing a bispecific antibody comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y) , and a CH1 constant domain comprising an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding a second IgG heavy

chain, wherein said heavy chain comprises a variable domain comprising a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat and a constant domain comprising a WT sequence; and (d) a fourth nucleic acid encoding second light chain, wherein said second light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and (3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain. More particular to this embodiment, the present invention provides a method comprising the following: said first nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) and said second nucleic acid encodes a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

The present invention also provides a method for producing a bispecific antibody comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y) , and a CH1 constant domain comprising an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G); (b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding a second IgG heavy chain, wherein said heavy chain comprises a variable domain comprising a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four

amino acids upstream of the first residue of HFR3 according to Kabat and a constant domain comprising an aspartic acid substituted at residue 228 (228D); and (d) a fourth nucleic acid encoding second light chain, wherein said second light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a lysine substituted at residue 122 (122K), wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and (3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain. More particular to this embodiment, the present invention provides a method comprising the following: said first nucleic acid encodes a heavy chain variable domain further comprising an arginine substituted at residue 105 (105R) and said second nucleic acid encodes a light chain variable domain further comprising an aspartic acid substituted at residue 42 (42D).

As a further particular embodiment to the methods for producing a bispecific antibody, as provided herein, the present invention provides a method wherein one of said first and second IgG heavy chains further comprises a CH3 constant domain comprising a lysine substituted at residue 356 and a lysine substituted at residue 399, and the other of said first and second IgG heavy chains further comprises a CH3 constant domain comprising an aspartic acid substituted at residue 392 and an aspartic acid substituted at residue 409.

Even more particularly, the present invention provides a method for producing a bispecific antibody comprising: (1) co-expressing in a host cell: (a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at resiude 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3

according to Kabat, a CH1 constant domain comprising an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G) and a CH3 constant domain comprising a lysine substituted at residue 356 (356K) and a lysine substituted at residue 399 (399K); (b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y), a CH1 constant domain comprising a WT sequence and a CH3 constant domain comprising an aspartic acid substituted at residue 392 (392D) and an aspartic acid substituted at residue 409 (409D); and (d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R) and a constant domain comprising a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen; (2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and (3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain.

In other particular embodiments, the host cell for use in the methods of the present invention is a mammalian cell, more particularly a HEK293 or CHO cell, and the IgG heavy chain constant domain produced by the methods of the present invention is IgG1 or IgG4 isotype, and more particularly IgG1.

The present invention also provides Fabs, bi-specific antibodies and bi-specific antigen binding compounds, each produced according to the methods of the present invention, as well as host cells comprising nucleic acids encoding the same. In particular,

the present invention provides any of the Fabs, bispecific antibodies, nucleic acids or host cells as exemplified in any of the Examples herein.

In another particular embodiment, the present invention provides a bispecific antibody comprising a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 (39K) and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, a CH1 constant domain comprising an alanine substituted at residue 172 (172A) and a glycine substituted at residue 174 (174G) and a CH3 constant domain comprising a lysine substituted at residue 356 (356K) and a lysine substituted at residue 399 (399K); (b) a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 (1R) and an aspartic acid substituted at residue 38 (38D), and a constant domain comprising a tyrosine substituted at residue 135 (135Y) and a tryptophan substituted at residue 176 (176W); (c) a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39 (39Y), a CH1 constant domain comprising a WT sequence and a CH3 constant domain comprising an aspartic acid substituted at residue 392 (392D) and an aspartic acid substituted at residue 409 (409D); and (d) a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 (38R) and a constant domain comprising a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen.

Brief Description of Figures

Figure 1: Schematic diagrams of IgG *(A)*, Fab-Fab *(B)* and IgG-Fab *(C)* design formats. The "*" in the IgG BsAb diagram indicates a heterodimerized antibody CH3 domain. The Fab-Fab format ***(B)*** could be prepared by expressing a polypeptide linker between the C-terminus of the HC of Fab 1 and the N-terminus of the HC or LC of Fab 2. Alternately, a polypeptide could connect the C-terminus of the LC of Fab 1 and the N-terminus of the

LC or HC of Fab 2. Similarly, the additional Fab in the IgG-Fab ***(C)*** could be linked to the N-terminus of the HC or LC or the C-terminus of the LC.

Figures 2 and 3: Surface Plasmon resonance (Biacore) traces demonstrating the dual-binding behavior of the anti-HER-2/anti-EGFR ***(Fig. 2)*** and anti-cMET/anti-Axl ***(Fig. 3)*** IgG BsAbs. Bispecific binding of the Fab Redesigned BsAbs (HE Designs ***(Fig. 2)*** and MA Designs ***(Fig. 3)***) is evident by increases in signal during both injection cycles (300-540 sec and 940-1180 sec). The monospecific MAbs pertuzumab IgG1 (pG1) and matuzumab IgG1 (mG1) ***(Fig. 2)*** and METMAb IgG1 (METG1) and Anti-Axl IgG1 (AxlG1) ***(Fig. 3)*** do not demonstrate this activity. The control molecules without Fab redesigns (i.e., HE Control and MA Control), but harboring C_H3 heterodimerization mutations also demonstrate bispecific binding activity.

The general structure of an "antibody" is very well-known. For a full length antibody of the IgG type, there are four amino acid chains (two "heavy" chains and two "light" chains) that are cross-linked via intra- and inter-chain disulfide bonds. When expressed in certain biological systems, e.g. mammalian cell lines, antibodies having unmodified human Fc sequences are glycosylated in the Fc region. Antibodies may be glycosylated at other positions as well. The subunit structures and three-dimensional configurations of antibodies are well known. Each heavy chain is comprised of an N-terminal heavy chain variable region ("V_H") and a heavy chain constant region ("C_H"). The heavy chain constant region is comprised of three domains (C_H1, C_H2, and C_H3) for IgG as well as a hinge region ("hinge") between the C_{H1} and C_{H2} domains. Each light chain is comprised of a light chain variable region ("V_L") and a light chain constant region ("C_L"). The C_L and V_L regions may be of the kappa ("κ") or lambda ("λ") isotypes. IgG antibodies can be further divided into subclasses, e.g., IgG1, IgG2, IgG3, IgG4, as appreciated by one of skill in the art

The variable regions of each heavy chain - light chain pair associate to form binding sites. The heavy chain variable region (V_H) and the light chain variable region (V_L) can be subdivided into regions of hypervariability, termed complementarity determining regions ("CDRs"), interspersed with regions that are more conserved, termed framework regions ("FR"). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1,

FR2, CDR2, FR3, CDR3, FR4. CDRs of the heavy chain may be referred to as "CDRH1, CDRH2, and CDRH3" and the 3 CDRs of the light chain may be referred to as "CDRL1, CDRL2 and CDRL3." The FRs of the heavy chain may be referred to as HFR1, HFR2, HFR3 and HFR4 whereas the FRs of the light chain may be referred to as LFR1, LFR2, LFR3 and LFR4. The CDRs contain most of the residues which form specific interactions with the antigen

A wild type IgG antibody contains two identical fragments termed "fragment, antigen binding" (or Fab), each of which is composed of the V_H and C_H1 domains of one heavy chain and the V_L and C_L domains of a light chain. Each Fab directs binding of the antibody to the same antigen. As used herein, the term "bi-specific antibody" or "IgG BsAb" refers to an IgG antibody comprising two distinct Fabs, each of which direct binding to a separate antigen, and composed of two distinct heavy chains and two distinct light chains. The V_H and C_H1 domains of one heavy chain associate with the V_L and C_L domains of one light chain to form a "first" Fab, whereas the V_H and C_H1 domains of the other heavy chain associate with the V_L and C_L domains of the other light chain to form a "second" Fab. More particularly, the term "bi-specific antibody", as used herein, refers to an IgG1, IgG2 or IgG4 class of bi-specific antibody. Even more particular, the term "bi-specific antibody" refers to an IgG1 or IgG4 class of bi-specific antibody, and most particularly an IgG1 class.

The methods exemplified herein can be used to co-express two distinct Fab moieties, for example Fabs with different Fv regions, with reduced mis-matching of respective HC/LC pairs. In addition to bi-specific antibodies and individual Fabs, the methods of the present invention can be employed in the preparation of other bi-specific antigen binding compounds. As used herein, the term "bi-specific antigen binding compound" refers to Fab-Fab and IgG-Fab molecules. Figure 1, included herein, provides a schematic diagram of the structure of bi-specific antibodies (IgG BsAb) as well as the Fab-Fab and IgG-Fab formats contemplated by the methods and compounds of the present invention.

The methods and compounds of the present invention comprise designed amino acid modifications at particular residues within the variable and constant domains of heavy chain and light chain polypeptides. As one of ordinary skill in the art will appreciate, various numbering conventions may be employed for designating particular

amino acid residues within IgG variable region sequences. Commonly used numbering conventions include Kabat and EU index numbering (see, Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed, Public Health Service, National Institutes of Health, Bethesda, MD (1991)). Other conventions that include corrections or alternate numbering systems for variable domains include Chothia (Chothia C, Lesk AM (1987), *J Mol Biol* 196: 901–917; Chothia, et al. (1989), *Nature* 342: 877–883), IMGT (Lefranc, et al. (2003), *Dev Comp Immunol* 27: 55–77), and AHo (Honegger A, Pluckthun A (2001) *J Mol Biol* 309: 657–670). These references provide amino acid sequence numbering schemes for immunoglobulin variable regions that define the location of variable region amino acid residues of antibody sequences. Unless otherwise expressly stated herein, all references to immunoglobulin heavy chain variable region (i.e., V_H) amino acid residues (i.e. numbers) appearing in the Examples and Claims are based on the Kabat numbering system, as are all references to V_L and C_L residues. All references to immunoglobulin heavy chain constant region C_H1 and hinge appearing in the Examples and Claims are also based on the Kabat system, whereas all references to immunoglobulin heavy chain constant regions C_H2, and C_H3 are based on the EU Index numbering system. With knowledge of the residue number according to Kabat or EU Index numbering, one of ordinary skill can apply the teachings of the art to identify amino acid sequence modifications within the present invention, according to any commonly used numbering convention. Note, while the Examples and Claims of the present invention employ Kabat or EU Index to identify particular amino acid residues, it is understood that the SEQ IDs appearing in the Sequence Listing accompanying the present application, as generated by Patent In Version 3.5, provide sequential numbering of amino acids within a given polypeptide and, thus, do not conform to the corresponding amino acid numbers as provided by Kabat or EU index.

However, as one of skill in the art will also appreciate, CDR sequence length may vary between individual IgG molecules and, further, the numbering of individual residues within a CDR may vary depending on the numbering convention applied. Thus, to reduce ambiguity in the designation of amino acid residues within CDRs, the disclosure of the present invention first employs Kabat to identify the N-terminal (first) amino acid of the HFR3. The amino acid residue to be modified is then designated as being four (4) amino acid residues upstream (i.e. in the N-terminal direction) from the first amino acid in the

reference HFR3. For example, Design A of the present invention comprises the replacement of a WT amino acid in HCDR2 with a glutamic acid (E). This replacement is made at the residue located four amino acids upstream of the first amino acid of HFR3, according to Kabat. In the Kabat numbering system, amino acid residue X66 is the most N-terminal (first) amino acid residue of variable region heavy chain framework three (HFR3). One of ordinary skill can employ such a strategy to identify the first amino acid residue (most N-terminal) of heavy chain framework three (HFR3) from any human IgG1 or IgG4 variable region. Once this landmark is determined, one can then locate the amino acid four residues upstream (N-terminal) to this location and replace that amino acid residue (using standard insertion/deletion methods) with a glutamic acid (E) to achieve the "Design A" modification of the invention. Given any variable IgG1 or IgG4 immunoglobulin heavy chain amino acid query sequence of interest to use in the methods of the invention, one of ordinary skill in the art of antibody engineering would be able to locate the N-terminal HFR3 residue in said query sequence and then count four amino acid residues upstream therefrom to arrive at the location in HCDR2 that should be modified to glutamic acid (E).

As use herein, the phrase "a/an [amino acid name] substituted at residue . . .", in reference to a heavy chain or light chain polypeptide, refers to substitution of the parental amino acid with the indicated amino acid. For example, a heavy chain comprising "a lysine substituted at residue 39" refers to a heavy chain wherein the parental amino acid sequence has been mutated to contain a lysine at residue number 39 in place of the parental amino acid. Such mutations may also be represented by denoting a particular amino acid residue number, preceded by the parental amino acid and followed by the replacement amino acid. For example, "Q39K" refers to a replacement of a glutamine at residue 39 with a lysine. Similarly, "39K" refers to replacement of a parental amino acid with a lysine.

An antibody, Fab or other antigen binding compound of the present invention may be derived from a single copy or clone (e.g. a monoclonal antibody (mAb)), including any eukaryotic, prokaryotic, or phage clone. Preferably, a compound of the present invention exists in a homogeneous or substantially homogeneous population. In an embodiment, the antibody, Fab or other antigen binding compound, or a nucleic acid encoding the same, is provided in "isolated" form. As used herein, the term "isolated" refers to a

protein, peptide or nucleic acid which is free or substantially free from other macromolecular species found in a cellular environment.

A compound of the present invention can be produced using techniques well known in the art, e.g., recombinant technologies, phage display technologies, synthetic technologies or combinations of such technologies or other technologies readily known in the art. In particular, the methods and procedures of the Examples herein may be readily employed. An antibody, Fab or antigen binding compound of the present invention may be further engineered to comprise framework regions derived from fully human frameworks. A variety of different human framework sequences may be used in carrying out embodiments of the present invention. Preferably, the framework regions of a compound of the present invention are of human origin or are substantially human (at least 95%, 97% or 99% of human origin.) The sequences of framework regions of human origin may be obtained from *The Immunoglobulin Factsbook*, by Marie-Paule Lefranc, Gerard Lefranc, Academic Press 2001, ISBN 012441351.

Expression vectors capable of directing expression of genes to which they are operably linked are well known in the art. Expression vectors can encode a signal peptide that facilitates secretion of the desired polypeptide product(s) from a host cell. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide. Desired polypeptides, for example the components of the bi-specific antibodies or Fabs prepared according to the methods of the present invention, may be expressed independently using different promoters to which they are operably linked in a single vector or, alternatively, the desired products may be expressed independently using different promoters to which they are operably linked in separate vectors. As used herein, a "host cell" refers to a cell that is stably or transiently transfected, transformed, transduced or infected with nucleotide sequences encoding a desired polypeptide product or products. Creation and isolation of host cell lines producing a bi-specific antibody, Fab or other antigen binding compound of the present invention can be accomplished using standard techniques known in the art.

Mammalian cells are preferred host cells for expression of the Fabs, bi-specific antibodies, or antigen binding compounds according to the present invention. Particular mammalian cells are HEK 293, NS0, DG-44, and CHO cells. Preferably, expressed polypeptides are secreted into the medium in which the host cells are cultured, from

which the polypeptides can be recovered isolated. Medium, into which an expressed polypeptide has been secreted may be purified by conventional techniques. For example, the medium may be applied to and eluted from a Protein A or G column using conventional methods. Soluble aggregate and multimers may be effectively removed by common techniques, including size exclusion, hydrophobic interaction, ion exchange, or hydroxyapatite chromatography. Recovered products may be immediately frozen, for example at -70°C, or may be lyophilized.

The following Examples further illustrate the invention and provide typical procedures for carrying out various embodiments. However, it is understood that the Examples are set for the by way of illustration and not limitation, and that various modification may be made by one of ordinary skill in the art. In addition, Lewis et al., *Nature Biotech.*, 32(2); February 2014, provides additional illustration of embodiments of the present invention.

Example 1. Computational design to identify modifications of C_H1/C_L interface residues that discriminate between designed and native immunoglobulin C_H1/C_L interfaces.

Residues for initial modification at the C_H1/C_L interface are selected using a combination of computational and rational design strategies. Using a crystal structure of the IgG1/λ Fab (PDB ID 3TV3 (*see*, Lewis, S.M. and Kuhlman, B.A. (2011), *PLoS ONE* 6(6): e20872)), trimmed to heavy chain residues 112-228 and light chain residues 106A-211, the Rosetta software suite and related modeling applications are employed to evolve potential sequences for modification according to a desired fitness function (*see*, Kaufmann *et al.* (2010), *Biochemistry* 49; 2987-2998; Leaver-Fay *et al.*(2011), *Methods Enzymol.* 487; 545-574; Kuhlman *et al.* (2003), *Science* 302(5649); 1364-1368; and Leaver-Fay *et al.* (2011), *PLos ONE* 6(7): e20937). Briefly, Rosetta calculates a fitness score and binding energy based on a weighted sum of energy potentials treating phenomena such as van der Waals forces and hydrogen bonding forces. Overall, the summations of these different parameters are measured in units known as the Rosetta Energy Unit (REU). These values are interpreted as free energies, but are not directly translatable into typical units of energy.

Using a fitness function which favors the binding and stability of designed-C_H1/designed-C_L complexes and disfavors binding affinity of undesired designed-C_H1/WT-C_L and WT-C_H1/designed-C_L complexes, initial sequence modifications resulting in binding orthogonality for designed C_H1/C_L pairs are identified.

The identified mutations are subjected to computational re-docking of the C_H1/C_L complex using RosettaDock via RosettaScripts (*see*, Chaudhury *et al.*(2011), *PLoS ONE* 6(8): e2247 and Fleishman *et al.*(2011), *PLoS ONE* 6(6): e20161). This allows determination of optimal binding positions for designed complexes and allows comparison with binding energies of the similarly docked WT complexes and the undesired designed-C_H1/WT-C_L and WT-C_H1/designed-C_L complexes. A deficit in the computational total score and binding energies for these undesired complexes predicts they will bind weakly to one another compared to the WT-C_H1/WT-C_L and designed-C_H1/designed-C_L complexes. Total energies are calculated using a Rosetta standard scorefunction, "Score12 prime." (see, Leaver-Fay *et al.* (2013), *Methods Enzymol.* 523; 109-143). Binding energies are calculated as the change in free energy (ΔG) separated score as reported by Rosetta's InterfaceAnalyzer tool (*see*, Lewis, S.M. and Kuhlman, B.A. (2011), *PLoS One* 6(6): e20872). Representative design constructs and their corresponding total score and binding energies are provided in **Table 1**.

Table 1. Rosetta multi-state computational design results.

Construct (C_H1/C_λ)	Mutations[b]	HC mut/LC mut		HC mut/LC wt		HC wt/LC mut	
		total score[a]	binding energy	total score	binding energy	total score	binding energy
WT	None	-359	-29	-	-	-	-
1.0	H_F174T H_V190F L_L135F	-357	-26	-353	-23	-355	-27
2.1	H_F174G L_L135A L_S176W	-355	-26	-352	-23	-347	-22

5.0	H_D146K L_K129D	-359	-29	-355	-28	-359	-29
1.0+5.0	H_D146K H_F174T H_V190F L_L135F L_K129D	-357	-28	-352	-25	-353	-24

[a]Units for the fitness score are called Rosetta Energy Units (REUs).

[b]Mutations are designated by first identifying the heavy chain (H) or light chain (L), followed by the one letter abbreviation for the parental amino acid, the amino acid residue number and the one letter abbreviation for the replacement amino acid (For example, H_F174T indicates that residue 174 of the heavy chain is modified from a phenylalanine (F) to a threonine (T).

More than forty discrete initial designs, falling into about twenty different design paradigms (i.e., mutations with different amino acid substitution combinations and different residue positions), were identified after filtering of many more computationally-generated sequences. Select design paradigms were synthesized and further interrogated experimentally either in a full-length IgG1/λ construct or an IgG1/λ construct that lacks variable domains, each as described below (see Tables 2 and 3). Based on those experiments, three designs, Design 1.0, Design 2.1 and Design 5.0 and Design 1.0+5.0 demonstrated good biophysical properties and thermodynamic discrimination for designed-C_H1/designed-C_L association over designed-C_H1/WT-C_L or WT-C_H1/designed-C_L association as demonstrated in thermal challenge assays (described below).

Synthesis of Test Articles.

To test the designs, a pertuzumab (*see*, Nahta *et al.*(2004), *Cancer Res.* 64; 2343-2346) human IgG1 with a human chimeric kappa V_L and lambda C_L (C_λ) is created. Briefly, the pertuzumab V_H domain insert is generated using a PCR-based overlapping oligonucleotide synthesis procedure (Casimiro *et al.(1997), Structure* 5; 1407-1412) using the sequence from the published crystal structure (Franklin *et al.*(2004), *Cancer Cell* 5; 317-328). The insert contains appropriate AgeI and NheI restriction sites that enable it to be ligated directly into a linearized in-house pE vector (Lonza) containing an IgG1 constant domain sequence. The pertuzumab V_L gene is also generated using

overlapping oligonucleotide synthesis. A DNA sequence encoding the V_L domain fused to C_λ is constructed using PCR and an in-house plasmid template containing the C_λ sequence. Both 5' and 3' flanking oligonucleotides and two internal primers are designed to anneal the C-terminus of the pertuzumab V_L domain to the N-terminus of C_λ. The light chain insert is designed with HindIII and EcoRI restriction sites for direct ligation into a linearized in-house pE vector (Lonza) with a selectable GS marker system. Each pE mammalian expression vector is engineered to contain a common mouse antibody light chain signal sequence that is translated in-frame as part of the expressed protein and cleaved prior to secretion. All ligation constructs are transformed into *E. coli* strain TOP 10 competent cells (Life Technologies). Transformed bacterial colonies are picked, cultured, and the plasmids are prepped. Correct sequences are confirmed by DNA sequencing. The encoded sequences of the mature heavy chain and light chain proteins are given by SEQ ID NO:1 and SEQ ID NO:2, respectively.

To further interrogate the ability of C_H1/C_L designs to provide a new and specific interface that discriminates from WT C_H1/C_L interfaces, it is useful to remove the variable domains, which, if present, add complexity to the data interpretation. Therefore, human IgG1 and human lambda constructs *lacking* variable genes (V_H and V_L) are constructed. A recombinase-based subcloning strategy is used to remove the variable genes. Briefly, for the heavy chain plasmid, two double stranded oligonucleotides that encompass 15 base pairs 5' of an XhoI site through the common mouse antibody light chain signal sequence followed immediately by a NheI site and 15 flanking base pairs (encoding the N-terminus of the IgG1 C_H1 domain for efficient recombination) are chemically synthesized. This double stranded oligonucleotide pair has the V_H domain deleted. For the light chain plasmid, two double stranded oligonucleotides that encompass 15 base pairs 5' of a BamHI site through the common mouse antibody light chain signal sequence followed immediately by an XmaI and 15 flanking base pairs (encoding the N-terminus of the lambda C_L domain for efficient recombination) are chemically synthesized. This double stranded oligonucleotide pair has the V_L domain deleted. The variable genes are digested out of the parental pertuzumab heavy chain and ight chain plasmids using the XhoI/NheI and BamHI/XmaI enzymes, respectively. The corresponding oligonucleotide pairs are inserted into the linearized plasmids using the recombinase-based In-Fusion HD Cloning kit (Clontech) according to the manufacturer's protocol. The sequences of the

heavy chain and light chain IgG1/λ constructs lacking variable domains are given by SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

For generating small sets of mutations, the QuikChange II Site Directed Mutagenesis Kit (Agilent) may be used following the instructions provided by the manufacturer. For generating large sets of mutations (typically >3 mutations per chain), a gene synthesis strategy may be employed (G-blocks, IDT). The synthesized genes are designed to be compatible with the heavy chain and light chain construct lacking variable domains (described above). However, within the heavy chain construct, an Xho I site upstream of the common mouse light chain signal sequence is deleted using site directed mutagenesis and a new Xho I site is generated at the C-terminus (3' end) of the coding region of the C_H1 domain. Synthesized genes are ligated to the heavy chain plasmid using the NheI and new XhoI restriction sites. Synthesized genes are added to the light chain plasmid using the BamHI and EcoRI restriction sites.

Protein Expression and Characterization.

Each plasmid is scaled-up by transformation into TOP10 *E. coli*, mixed with 100 mL luria broth in a 250 mL baffled flask, and shaken O/N at 220 rpm. Large scale plasmid purifications are performed using the BenchPro 2100 (Life Technologies) according to the manufacturer's instructions.

For protein production, plasmids harboring the heavy chain and light chain DNA sequences are transfected (1:2 plasmid ratio for the heavy chain and light chain plasmids, respectively) into HEK293F cells using Freestyle transfection reagents and protocols provided by the manufacturer (Life Technologies). Transfected cells are grown at 37 °C in a 5% CO_2 incubator while shaking at 125 rpm for 5 days. Secreted protein is harvested by centrifugation at 10 K rpm for 5 min. Supernatants are passed through 2 μm filters (both large scale and small scale) for purification. Small scale (1 mL) purifications are performed by directly incubating 1 mL transfected supernatant with 100 μL resuspended, phosphate buffered saline (PBS) washed-Protein G magnetic beads (Millipore). Beads are washed 2-times with PBS and 1-time with 10-fold diluted PBS. Protein is eluted from the beads by adding 130 μL 0.01 M Acetate, pH 3.0. After harvesting, the eluants are immediately neutralized by adding 20 μL 0.1 M Tris, pH 9.0. The concentration of the

purified proteins are determined by measuring the absorbance of the solutions at 280 nm using a NanoDrop UV-Vis spectrophotometer from ThermoScientific (Grimsley, G.R. and Pace, C.N.(2004), *Curr. Protoc. Protein Sci.*, Ch. 3 (3.1)).

Identities of representative transfected plasmids and the sequences they generate are provided in **Table 2**. Methods for characterization and the resulting data for each of these heavy chain/light chain designs are described below.

Table 2. Sequence composition of the C_H1/C_L interface specificity designs based on Rosetta.

Design	Mutations	Heavy Chain SEQ ID NO:	Light Chain SEQ ID NO:
WT pertuzumab IgG1/λ	None	1	2
WT IgG1/C_λ lacking V-genes	None	3	4
Design 1.0[a]	HC_F174T, HC_V190F, LC_L135F	5	6
Design 2.1[b]	HC_F174G, LC_L135A, LC_S176W	7	8
Design 5.0[a]	HC_D146K, LC_K129D	9	10
Design 1.0+5.0[b]	HC_D146K, HC_F174T, HC_V190F, LC_K129D, LC_L135F	11	12

[a]In the pertuzumab IgG1/λ format.
[b]In the IgG1/λ format lacking variable domains

The proteins may be characterized using multiple methods, as described herein. For example, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses are used to evaluate expression and assembly of the HC and LC components. SDS-PAGE may be performed using NuPAGE® Novex 4-12% BisTris gels according to manufacturer protocols (Life Technologies). Approximately 15 µL of purified material from the magnetic bead purifications (see above) are loaded in each well. For reduced

samples, 10% 0.5 M DTT in H_2O is added. Protein bands on the gels are detected using a SimplyBlue™ Safe Stain (Life Technologies).

Enzyme-linked immunosorbent assays (ELISAs) for the detection of thermo-challenged protein samples may also be performed to compare the stability of the designed samples against the wild-type control proteins and the mis-matched designs. T_{50} values, defined as the temperature at which 50% of the ELISA signal that detects protein activity remains, after heating at elevated temperature for a specified period of time, can be determined for each sample to compare the stability of the designed HC/LC constructs relative to the stability of wild-type controls and mis-matched pairs. For the pertuzumab IgG1/λ proteins, 96-well U-bottom high protein binding 96-well plates (Greiner bio-one, cat#650061) are coated overnight at 4 °C with 100 µL/well of a polyclonal anti-human C_λ antibody (Southern Biotech, cat#2070-01) at 2 µg/mL in a 0.05 M $NaHCO_3$ buffer, pH 8.3. The plates are then washed four times with PBS with 0.02% Tween80 (PBST) and blocked for 1 hr with casein (Thermo, cat#37528) at 37 °C. The plates are washed again followed by the addition of isolated HEK293F culture supernatants containing the pertuzumab IgG1/λ protein designs (100 µL/well). Aliquots of each supernatant are pre-exposed to various temperatures for 1 hr using a PCR instrument with a 25 °C thermal gradient window. The thermal challenged pertuzumab IgG1/λ proteins (with or without mutations in the C_H1/C_L domains) are incubated on the plate for 1 hr at 37 °C. The plates are then washed and 200 ng/mL in-house biotinylated human HER-2-Fc (R&D systems, cat#1129-ER-020) is added at 100 µL/well (diluted in casein) for 1 hr at 37 °C. The plates are washed again followed by the addition of streptavidin-HRP (Jackson Immunoresearch, cat#016-030-084) diluted 1:2000 in casein. The plates are then washed and SureBlue Reserve TMB 1-component substrate (KPL, cat#53-00-01) is added at 100 µL/well. The reaction is allowed to proceed for 5-15 minutes then quenched by the addition of 1% H_3PO_4. The absorbance at 450 nm is read using a SpectraMax 190 UV plate reader (Molecular Devices). A similar procedure is followed for the detection of thermochallenged IgG1/λ minus variable gene proteins using a polyclonal anti-human C_H1 antibody (2 µg/mL in casein; Meridian Life Sciences, cat#W90075C) to capture proteins from the supernatants and a HRP-labeled polyclonal

anti-human C_λ antibody (1:2000 dilution in casein; Southern Biotech, cat#2070-05) for detection (replacing the HER-2-Fc-biotin and streptavidin-HRP).

Characterization results for each of the designs depicted in **Table 2** are provided in **Table 3** below. Three designs, Design 2.1, Design 5.0, and Design 1.0+5.0 were found where the thermal stability of the designed HC/designed LC C_H1/C_L-containing IgG protein was superior both computationally and experimentally over at least one of the mismatched Designed HC/WT LC or WT HC/Designed LC pairs (**Table 3**). In some cases (including Designs 2.1 and 1.0+5.0), the designs were tested in the IgG1/C_λ protein lacking variable domains. For both Design 1.0+5.0 and Design 2.1, a clear preference for the Designed HC/Designed LC pair could be seen by SDS-PAGE analysis as evident by a strong band at ~100 kDa resembling that of the wild-type HC/LC pairs. The Design 1.0+5.0 mismatched pairs (i.e., Designed HC/WT LC and WT HC/Designed LC) expressed too poorly to be seen on an SDS-PAGE gel. The Design 2.1 mismathed pairs demonstrated additional banding below the main band indicative of unassembled protein.

Table 3: Characterization of designed C_H1/C_L antibody proteins.

Construct	Mutation	Titer[a] (Construct vs. WT control)	Titer[a] ((HCmut/ LCwt[b]) vs WT Control)	Titer[a] ((HCwt/ LCmut[b]) vs WT Control)	SDS-PAGE Assembly[c]	T_5 (°C)	T_{50} (°C) HCmut/ LCwt	T_{50} (°C) HCwt/ LCmut
WT Pertuzumab IgG1/λ	None	1.0	n.d.[d]	n.d.	+	66	n.d.	n.d.
WT IgG1/C_λ minus V-genes	None	1.0	n.d.	n.d.	+	78	n.d.	n.d.
Design 1.0[e]	HC_F174T HC_V190F LC_L135F	1.0	3.1	-	+	67	67	n.d.
Design 2.1[f]	HC_F174G LC_S176W LC_L135A	1.1	0.2	0.5	+	>75	69	74-75
Design 5.0[e]	HC_D146K LC_K129D	0.5	0.75	0.5	+	67	60	67

Design 1.0 +5.0[f]	HC_D146K HC_F174T HC_V190F LC_K129D LC_L135F	0.6	0.0	~0.02	+	73	<45	53

[a]Titers are determined based on the protein G magnetic bead purified protein recoveries as described above. Titer values are given as a ratio of expression of the indicated designed construct or mis-matched pair (i.e. HC mut/LC wt or HC wt/LC mut) relative to expression of the appropriate WT contol.

[b]The mis-matched pairs were expressed separately by co-transfection of a designed HC or LC with a wild-type LC or HC, repectively.

[c]Assembly denoted by single band at 100 kDa by SDS-PAGE (+). Partial assembly denoted by multiple bands on SDS-PAGE including 100 kDa band (+/-). No band at 100 kDa on SDS-PAGE denoted by (-).

[d]n.d. = not determined.
[e]Determined in the pertuzumab IgG1 format.
[f]Determined in the IgG1/λ minus variable genes format.

The characterization results demonstrate that Design 1.0+5.0 maintains a high affinity and stable C_H1/C_L interface with low capability for recognizing native immunoglobulin C_H1/C_L domains. Additionally, Design 2.1 demonstrates high stability and specificity for itself while discriminating against binding to native (wt) immunoglobulin C_H1/C_L domains.

Analytical size exclusion chromatography with in-line light static scattering (SEC/LS) is another characterization tool used to confirm that the heterotetrameric HC/LC antibody complexes associate properly and continue to demonstrate monodisperse biophysical behavior. SEC./LS may be performed for each sample using 30-80 µL of purified eluant from the small scale-purification described above (concentrations ~0.1-0.4 mg/mL). For SEC/LS, the proteins are injected onto a Sepax Zenix SEC 200 analytical HPLC (7.8×300 mm) column equilibrated in 10 mM phosphate, 150 mM NaCl, 0.02% NaN_3, pH 6.8, using an Agilent 1100 HPLC system. Static light scattering data for material eluted from the SEC column are collected using a miniDAWN TREOS static light scattering detector coupled to an Optilab T-rEX in-line refractive index meter (Wyatt Technologies). UV data are analyzed using HPCHEM (Agilent). Protein molecular weights are determined by their static light scattering profiles using ASTRA V

(Wyatt Technologies). SEC/LS analysis indicates that all of the proteins described in **Table 3** were shown to be highly monodisperse with soluble aggregates <5% and indistinguishable from the wild-type IgG and IgG minus variable domain constructs.

Example 2. Optimization of Design 2.1.

Design 2.1, as described in **Example 1**, is further optimized to improve specificity of assembly of the designed C_H1/C_L interface versus a WT C_H1/C_L interface. To aid in the design process, in-house crystal structures of Design 1.0+5.0 and Design 2.1 are first solved.

To generate protein for crystallography, isolated C_H1/C_λ proteins (disulfide linked) are produced in *E. Coli*. The isolated C_H1/C_λ proteins (no variable-genes or IgG-Fc) are subcloned into the pET-DUET plasmid from Novagen. The C_H1 insert (with a pelB signal sequence for secretion into the oxidative periplasmic environment) is synthesized (with a hexahistidine C-terminal tag) using overlapping PCR and subcloned into cassette 1 of the plasmid using the NheI and BamHI sites. The C_λ insert is similarly synthesized and inserted between the NdeI and XhoI sites (no his tag). Designs 1.0+5.0 and Design 2.1 are generated from the WT plasmid using QuikChange II mutagenesis (Agilent). Each plasmid is transformed into CodonPlus BL21(DE3) chemically competent cells (Agilent) for expression. For each protein preparation, transformed and pre-cultured cells are used to inoculate 2X1.4 L luria broth supplemented with 100 μg/mL carbenicillin and 35 μg/mL chloramphenicol. The cultures are allowed to shake at 220 rpm at 37 °C until the OD_{600} reached 1-1.5. At this stage, the culture temperature is reduced to 30 °C and 1 mM Isopropyl-1-thio- β -D-galactopyranoside is added. The cultures are allowed to grow for 3-4 hrs and harvested by centrifugation for 20 min at 4000 g. The proteins are resuspended in 50 mL of a periplasmic extraction buffer (500 mM sucrose, 100 mM Tris, pH 8, 1 mM EDTA, and 100 μg/mL hen-egg white lysozyme). The extracted proteins are diluted 10-fold into a 10 mM citrate, 10 mM NaCl buffer, pH 5.5 and passed over a 5 mL SP Sepharose FF HiTrap cation exchange column (GE Healthcare) at 5 mL/min using an AKTA Explorer (GE Healthecare). The proteins are eluted from the column using a gradient up to 0.7 M NaCl. The proteins are dialyzed into PBS and captured onto a Ni-Sepharose HiTRAP affinity column. The proteins are

then eluted using a gradient up to 0.3 M imidazole. The proteins are then concentrated to ~3-10 mg/mL using VivaSpin6 centrifugal devices, dialyzed into 10 mM Tris, 100 mM NaCl, pH 8.0 and filtered.

For crystallography, the purified proteins are screened using the vapor diffusion crystallization method, whereby protein is first mixed with well solution and deposited in a small chamber with well solution, sealed and allowed to equilibrate with well solution, concentrating both protein and precipitating reagents in the protein drop. Such screens are conducted in a 96-well format (Intelli-plates, Art Robins Instrument) using the commercially available screens: PEGs, PEGs II, ComPAS, Classics, Classics II Suites (Qiagen). The initial setup utilizes a Phoenix robot (Art Robins Instrument), which deposits 0.3 μL of protein on 0.3 μL of well solution.

For the WT C_H1/C_λ protein, initial screening is performed at 6 mg/ml protein. Protein crystals grew after 2 days in the following condition: 15% Ethanol/50% MPD /10mM Sodium Acetate. These initial crystallization conditions are optimized in a set of vapor diffusion experiments where the concentration of the two components is varied, while the third is kept constant. The optimization experiments are conducted in 48-well format Intelli-plates (Art Robins Instrument). The optimization experiments result in thin needle-shaped crystals diffracting below 3 Å resolution.

Next, protein is re-screened at higher concentration of 9.2 mg/ml. Streak seeding using the needle-shaped crystals from the previous step is performed on the following day. Streak seeding is a method promoting crystal nucleation by providing a ready-made nucleus (seed crystals) to assist nucleation and facilitate the growth of ordered crystals. This technique results in a new crystallization condition: 30% PEG4K. Optimizing of this condition is performed at 21 °C by varying the PEG4K concentration, the size of the drop containing the protein and reservoir solution mixture, and the ratio of the protein and reservoir content in the drop. Crystallization drops are seeded and crystals appeared on the next day, growing to the final size within 3 days. Crystals are transferred to a cryo-protection solution of the reservoir solution with PEG4K increased by 10% and supplemented by 20% PEG400 and flash frozen by immersion in liquid nitrogen before shipping to the Advanced Photon Source for data collection.

Design 1.0+5.0 is similarly screened and streak-seeded using the WT crystal seeds. Intergrown needle-shaped crystals appeared after 3 days at two conditions: 20%

Isopropanol/20% PEG4K/100 mM tri-Sodium Citrate and 33% PEG6K/10 mM tri-Sodium Citrate. Both conditions are optimized, using the technique described above, resulting in single crystals for the optimization of the 2nd condition. Final Design 1.0+5.0 single crystals are grown at 21 °C by mixing 1.2 μL of 3.9 mg/ml protein and 1.2 μL of reservoir solution, containing 40% PEG6K and 10mM tri-Sodium Citrate dehydrate. Crystals are cryo-protected using reservoir solution increased by 10% PEG6K content and supplemented by 20% of Ethylene Glycol.

Design 2.1 crystallizes directly in conditions developed for Design 1.0+5.0. Single crystals are grown at 21 °C by mixing 1.5 μL of 5.1mg/ml protein with 1.5 μL of reservoir, containing 39% PEG6K and 10mM tri-Sodium Citrate. Crystals are cryoprotected using the same technique as for Design 1.0+5.0.

The conformations of the designed residues observed in the computational model structure and the experimental crystal structure of Design 1.0+5.0 were very similar (i.e., the designed residues adopted the same conformations in the model and the experimental in-house crystal of Design 1.0+5.0). However, the designed residues of Design 2.1 showed a substantially different orientation in the computational model than in an in-house crystal structure. Specifically, the light chain designed residue 176W and heavy chain native residue H172 of the in-house crystal structure did not adopt the conformations of the Rosetta computational model. The crystal also revealed a trans-cis isomerization between heavy chain residues G174 (mutated from F) and P175. This structural rearrangement was unanticipated by the model, but also not within the modeling degrees of freedom. Further modifications for Design 2.1 were generated following computational design methods essentially as described in Example 1, but using the in-house crystal structure of Design 2.1 as the starting point in place of the original 3TV3 crystal structure model

Following molecular biology procedures essentially as described in **Example 1**, modifications to Design 2.1 are constructed in the IgG/Cλ construct lacking variable domains. **Table 4** provides identities and corresponding mutations for representative further-optimized designs of Design 2.1.

Table 4: Optimization of Design 2.1.

Design	**C_H1/C_L Mutations**	HC SEQ ID NO:	LC SEQ ID NO:
Design 2.1.3.2	HC_H172A, HC_F174G, LC_L135F, LC_S176W	18	15
Design 2.1.3.3	HC_H172S, HC_F174G, LC_L135Y, LC_S176W	19	20
Design 2.1.3.3a	HC_H172A, HC_F174G, LC_L135Y, LC_S176W	18	20

The optimized Design 2.1 proteins are expressed using the transient HEK293F system as described in **Example 1**. Each of the designs are tested for thermal stability by thermal challenge at temperatures ranging from 70-95 °C using the methodology as described in **Example 1**. The sustained presence of each design protein after heating is determined using the IgG1/λ minus variable gene ELISA also as described in **Example 1.** Results of the thermal challenge stability test for representative optimized designs of Design 2.1 as well as WT IgG1/ C_λ (no V_H/V_L) and Design 2.1 are provided below in Table 5.

Table 5: Optimization of Design 2.1

Design	**C_H1/C_L Mutations**	**T_{50} (°C)**
WT IgG1/C_λ (no V_H/V_L)	None	78.1±0.5
Design 2.1	HC_F174G, LC_S176W, LC_L135A	>75
Design 2.1.3.2	HC_H172A, HC_F174G, LC_L135F, LC_S176W	81.4±0.5
Design 2.1.3.3	HC_H172S, HC_F174G, LC_L135Y, LC_S176W	77.7±0.5
Design 2.1.3.3a	HC_H172A, HC_F174G, LC_L135Y, LC_S176W	83±2

The thermochallenge data in Table 5 indicates Design 2.1.3.2, Design 2.1.3.3 and Design 2.1.3.3a were the most stable constructs.

To further assess specificity of optimized designs of Design 2.1, a mass spectrometry (MS) method to directly measure the specificity versus the WT C_H1/C_L domains is performed. Briefly, at the 2 mL transfection scale, a designed C_L domain is co-transfected with a WT C_L domain and either a designed heavy chain or a WT heavy chain. In this way the designed and WT C_L proteins directly *compete* with one another during protein expression for binding to the heavy chain protein and secretion into the cell media. The assembled IgG/C_L minus variable genes proteins are purified automatically using an Agilent 1100 series HPLC with autosampler and sample collector. The proteins are captured on a PG (protein G) HPLC column (Applied Biosystems, Cat#2-1002-00) at 2 mL/min, washed with phosphate buffered saline (PBS) and eluted with distilled deionized H_2O, 0.2% formic acid. The protein eluants are concentrated/dried on a Labconco CentriVap Speedvac for 3 hrs at 40 °C under vacuum. The proteins are resuspended in 100 µL distilled deionized water and neutralized with 20 mL 0.1 M Tris pH 8.0 (MediaTech, Inc., Cat#46-031-CM). The intermolecular disulfides (HC/LC and HC/HC) are reduced by the addition of 10 µL freshly solubilized 1 M dithiothreitol (DTT, Sigma, Cat#43815-1G). The samples are sequentially collected using an Agilent 1100 series HPLC with an autosampler and captured onto a reverse phase C4 analytical column for desalting using a water, 0.2% formic acid mobile phase. After separation of the protein from its buffer components, the proteins are bumped from the C4 column using a 10%/90% water/acetonitrile pulse (both with 0.2% formic acid) and injected into an Agilent 6210 time-of-flight liquid chromatography/mass spectrometry system molecular weight analyser . Theoretical mass-averaged molecular weights of the light chain and heavy chain components are determined using the GPMaw program (v. 8.20). The two separate light chains of the competition experiment can be easily discriminated from one another based on their different molecular weights. The relative counts of the ionized light chains hitting the detector are used to quantify the ratio of designed C_L and WT C_L protein that is bound to the heavy chain component and this data is shown in **Table 6**.

Table 6. Results of the competition LC specificity LC/MS assay.

LC1	LC2	HC	% Assembly[a] (LC1/HC)	% Assembly[a] (LC2/HC)	Total Expression

					(μg/mL)
2.1.3.2 Cλ	WT Cλ	WT HC	11	89	73
2.1.3.2 Cλ	WT Cλ	2.1.3.2 HC	78	22	76
2.1.3.3a Cλ	WT Cλ	WT HC	10	90	50
2.1.3.3a Cλ	WT Cλ	2.1.3.3a HC	99	1	80
2.1.3.3a Cλ	WT Cκ	WT HC	8±3	92±3	63±20
2.1.3.3a Cλ	WT Cκ	2.1.3.3a HC	100±0.2	0.2±0.2	75±20

[a] "% Assembly" is calculated as described in the legend for Table 11, below

Results of the LC/MS competition experiments indicate significant specificity versus WT sequences is obtained using Designs 2.1.3.2 and 2.1.3.3a. Both Design 2.1.3.2C_λ and Design 2.1.3.3aC_λ proteins significantly out-competed WT C_λ (and WT C_κ) for binding to their heavy chain protein counterpart containing the 2.1.3.2 or 2.1.3.3a heavy chain mutations. Conversely, Design 2.1.3.2 and Design 2.1.3.3a C_λ proteins did not compete well with WT Cλ (and WT C_κ) for binding to the WT HC protein. Design 2.1.3.3a C_λ provided slightly better specificity than Design 2.1.3.2 C_λ in the experiment.

Example 3. Designs to generate designed V_H and V_L domains that bind one another stronger than they bind WT V_L or V_H domains, respectively.

To further optimize specific heavy chain-light chain assembly when co-expressing two Fabs, variable heavy chain/variable light chain (V_H/V_L) modifications are designed. V_L position 1 and V_H position 62 are first identified for modification. V_L position 1 is modified to the positively charged residue arginine (R) and V_H position 62 is modified to the negatively charged residue glutamic acid (E). This combination of charge modifications is denoted Design A. A second design modified V_L position 38 and V_H position 39 to generate a charge pair (V_L_Q38D and V_H_Q39K) at the site of the former hydrogen bonding interaction. This charge pair introduction is denoted Design B.

Design A, Design B, and a combination of Design A and B, denoted Design AB, are introduced into the plasmids for the pertuzumab light chain and heavy chain plasmids for mammalian expression. Plasmids containing Design 1.0+5.0 (with WT petuzumab V_H and V_L) modifications are also constructed to generate heavy chains and light chains

to potentially improve specificity of the variable domain designs over pairing with fully wild type heavy and light chains . The mutations are introduced using the using the QuikChange II mutagenesis kit (Agilent) according to the manufacturer's protocols constructs. The methods followed for plasmid production and purification are essentially as described in **Example 1**. The identities and corresponding sequences of Design A, Design B, and Design AB are provided in **Table 7.**

Table 7. SEQ ID NOs of Variable Domain and Constant Domain Designs in the pertuzumab HC and LC.

Full-length HC/LC IgG Design	**Mutations**	**HC SEQ ID NO:**	**LC SEQ ID NO:**
Variable Domain Designs[a]			
Design A	HC_R62E, LC_D1R	21	22
Design B	HC_Q39K, LC_Q38D	23	24
Design AB	HC_R62E, HC_Q39K, LC_D1R, LC_Q38D	25	26
Constant Domain Designs[b]			
Design 1.0+5.0	HC_D146K, HC_F174T, HC_V190F, LC_K129D, LC_L135F	27	28

[a] The Variable Domain Designs contain the indicated mutations in the V_H and V_L domains and WT pertuzumab sequences in the C_H and C_L domains.

[b] The Constant Domain Designs contain the indicated mutations in the C_H and C_L domains and WT pertuzumab sequences in the V_H and V_L domains.

Each of the designed heavy chain and light chain plasmids are co-expressed transiently in HEK293F essentially as described in **Example 1**. To probe for specificity, the designed heavy chains and light chains are also expressed with a mismatched light chain or heavy chain as a control to probe for specificity. The mismatched heavy chain and light chain contain WT V_H and V_L domains and the Design 1.0+5.0 in the constant domains to add additional specificity (denoted in Table 8 below as HC(WT15) and

LC(WT15), respectively). The expression supernatants are subjected to a thermal challenge by incubation for 1 hr at temperatures ranging from 45-75 °C and tested for binding hHER-2-Fc using an ELISA (methods described in **Example 1**). This ELISA format is sensitive to the stability of the variable domains. Additionally, all proteins are purified from their supernatants using the protein G magnetic bead protocol, essentially as described in **Example 1,** and formed fully assembled IgG molecules. In each of the cases (Design A and Design B), the apparent thermal stability of the matched heavy chain and light chain pairs iss significantly higher than the mismatched pairs. The result indicate that the matched designed pairs are thermodynamically favored over the mismatched pairs and provides a thermodynamic basis for the specific association of the Design A heavy chain and light chain for themselves and the Design B heavy chain and light chain for themselves over the association with the heavy chain and light chains containing WT variable domains. Combining the designs into a single construct, Design AB, further improved the thermodynamic specificity and resulted in reduced expression of the mismatched pairs. Results for the thermo-challenge testing are provided in Table 8.

Table 8. Summary of the thermochallenge data with Design A, Design B, and Design AB.

Full Length HC/LC IgG Construct	**HC/LC SEQ ID NO:**	**T_{50} (°C)**
Matched pair Constructs		
HC_Design A / LC_Design A	21/22	63.6±0.6
HC_Design B / LC_Design B	23/24	59.0±1.0
HC_Design AB / LC Design AB	25/26	59.3±0.5
Mis-matched pair Constructs		
HC (WT15) / LC Design A	27/22	52.8±0.6
HC (WT15) / LC Design B	27/24	46.7±0.2
HC (WT15) / LC Design AB	27/26	<45
HC_Design A / LC (WT15)	21/28	58.1±0.2
HC_Design B / LC (WT15)	23/28	53.6±0.4
HC_Design AB / LC (WT15)	25/28	57.9±0.3

The results in **Table 8** indicate that making any of the three compensatory charge pair modifications described above maintains the thermostability of heavy chain/light chain assembly within the design constructs while reducing the thermostability of the mis-matched designs. These variable domain designs can be added to the constant domain designs described in Examples 1 and 2 above to help improve specific heavy chain/light chain assembly.

Example 4. Multi-state computational designs to create additional V_H/V_L interface modifications that discriminate from the native immunoglobulin V_H/V_L interface.

Using PDB ID 3TCL (see, McLellan *et al.* (2011), *Nature* 480(7377); 336-343) and a multi-state design protocol essentially as described in **Example 1,** additional compensatory V_H/V_L interface mutations that steer designed V_H and V_L domains from binding WT V_H and V_L domains are designed. Discrete designs are physically constructed within the pertuzumab IgG Fv region with different design paradigms (i.e., mutations with very different amino acid combinations and residue positions). The design constructs are mutated, expressed in HEK293F cells, and tested for expression level and thermal stability using similar protocols as described in **Examples 1-3** above. Three discrete designs as depicted in Table 9 (denoted H.4, H.5, and H.6) are shown to express well and are about as stable as WT pertuzumab.

Table 9. Sequence ID numbers and thermal challenge data for Designs H.4, H.5, and H.6.

Construct	Mutations	HC SEQ ID NO:	LC SEQ ID NO:	T_{50} (°C)
Pertuzumab	None	1	2	61.8±0.5
H.4	LC_Q38R, HC_Q39Y	29	30	60.3±0.5
H.5	LC_Q38R, HC_Q39F	31	30	59.2±0.5
H.6	LC_Q38R, HC_Q39W	32	30	60.6±0.5

Example 5. Combining the C_H1/C_L and V_H/V_L interface designs results in highly specific HC/LC pairing.

In this example, the variable domain Designs H.4, H.6 and AB are examined along with the constant domain Designs 2.1.3.2 and 2.1.3.3a. The specificity afforded by the V_H/V_L and C_H1/C_L designs in isolation and in combination with each other is measured using a similar LC/MS competition experiment as described in **Example 3** (with the IgG/C_L proteins lacking variable domains), but here full-length immunoglobulin heavy chains and light chains are used. In the experiment, two full-length light chains (with V_L and C_L) are co-expressed and forced to compete for binding to a single heavy chain prior to secretion. Identities of the designs and the corresponding SEQ ID numbers for the constructs used in the specificity experiments are provided in **Table 10**. Again, the pertuzumab V_H and V_L sequences were used in every construct as a vector for the designs.

Table 10. Identity and Sequence identification for constructs used in Example 5.

HC Name[a,b]	HC SEQ ID NO:	LC Name[a,b]	LC SEQ ID NO:
WT+WT HC	1	WT+WTλ LC	2
AB+WT HC	25	AB+WTλ LC	26
H.4+WT HC	29	WT+WTκ LC	33
H.6+WT HC	32	AB+WTκ LC	34
WT+2133a HC	35	H.4+WTλ LC	30
AB+2133a HC	37	H.6+WTλ LC	30
AB+2132 HC	37	WT+2133aλ LC	36
H.4+2133a HC	39	AB+2133aλ LC	38
		H.4+2133aλ LC	40
		AB+2132λ LC	42
		H.4+WTκ LC	41

[a]Nomenclature is provided by having the first two characters of each protein specifying the variable domain design, while the subsequent numbering specifies the constant

domain design (e.g., 'WT+WTκ LC' is a LC with WT pertuzumab V_L and WT kappa C_L, while 'AB+2133aλ LC' is a LC with Design AB in its V_L and Design 2.1.3.3a in its lambda C_L. Similarly, 'AB+2133a HC' is a HC with Design AB in its V_H and Design 2.1.3.3a in its C_{H1}.)

[b]Some designs only have sequence differences in their HC *or* LC (e.g., AB+2133a and AB+2132 share identical HCs).

The LC/MS specificity data for the combinations tested is provided in **Table 11**.

Table 11. LC/MS analysis of the V_H/V_L and C_H1/C_L interface designs in isolation and in combination.

LC1	LC2	HC	% Assembly[a] (LC1/HC)	% Assembly[a] (LC2/HC)	Expression (μg/mL)
WT+WTλ LC	WT+WTκ LC	WT+WT HC	18	82	69
AB+WTλ LC	WT+WTκ LC	WT+WT HC	10	90	73
AB+WTλ LC	WT+WTκ LC	AB+WT HC	61	39	108
AB+WTκ LC	WT+WTλ LC	WT+WT HC	40	60	132
AB+WTκ LC	WT+WTλ LC	AB+WT HC	39	61	112
H.4+WTλ LC	WT+WTκ LC	WT+WT HC	39	61	94
H.4+WTλ LC	WT+WTκ LC	H.4+WT HC	54	44	105
H.6+WTλ LC	WT+WTκ LC	WT+WT HC	48	52	90
H.6+WTλ LC	WT+WTκ LC	H.6+WT HC	54	46	100
H.4+WTλ	AB+WTκ	AB+WT	23	77	95

LC	LC	HC			
H.4+WTλ LC	AB+WTκ LC	H.4+WT HC	71	29	71
H.6+WTλ LC	AB+WTκ LC	AB+WT HC	25	75	67
H.6+WTλ LC	AB+WTκ LC	H.6+WT HC	69	31	94
WT+2133aλ LC	WT+WTλ LC	WT+WT HC	50	50	29
WT+2133aλ LC	WT+WTλ LC	WT+2133a HC	79	21	28
WT+2133aλ LC	WT+WTκ LC	WT+WT HC	78	22	31
WT+2133aλ LC	WT+WTκ LC	WT+2133a HC	79	21	19
AB+2133aλ LC	WT+WTλ LC	WT+WT HC	42	58	51
AB+2133aλ LC	WT+WTλ LC	AB+2133a HC	85	15	87
H.4+2133aλ LC	WT+WTλ LC	WT+WT HC	47	53	61
H.4+2133aλ LC	WT+WTλ LC	H.4+2133a HC	77	23	70
AB+2132λ LC	H.4+WTκ LC	H.4+WT HC	11	89	66
AB+2132λ LC	H.4+WTκ LC	AB+2132 HC	74	26	98
AB+2133aλ LC	H.4+WTk LC	H.4+WT HC	15	85	74
AB+2133aλ LC	H.4+WTk LC	AB+2133a HC	78	22	121
AB+2132λ LC	H.4+WTλ LC	H.4+WT HC	9	91	94

AB+2132λ LC	H.4+WTλ LC	AB+2132 HC	80	20	110
AB+2133aλ LC	H.4+WTλ LC	H.4+WT HC	9±1	91±1	76±3
AB+2133aλ LC	H.4+WTλ LC	AB+2133a HC	87±2	13±3	70±5

[a]The percent assembly is calculated based on the relative area under the deconvoluted mass spectrometry peaks (i.e., proportional to the number of counts hitting the detector) of each of the LCs co-purified bound to the HC prior to mass spectrometry analysis. Purified samples are reduced with DTT prior to analysis – as described in **Example 2**.

The data in Table 11 indicates that generating specificity at the V_H/V_L interface by combining Design AB in one Fab and Design H.4 in the other Fab resulted in significant specificity (~70-80% specificity) without any constant domain designs. Pairing the most selectively specific V_H/V_L domain designs (AB in one Fab and H.4 in the other) with the most selective constant domain designs (2.1.3.2 or 2.1.3.3a) resulted in improved specificity overall, indicating that while the variable domains may dominate, the constant domains contribute to specificity (**Table 11**). The best combination used a combination of Design AB (in V_H/V_L) and 2.1.3.3a (in C_H1/C_L) in Fab #1 and Design H.4 (in V_H/V_L) with a WT C_H1/C_L in Fab#2. This combination repeatedly generated a highly specific set of HC/LC interactions with roughly 90% specificity in both directions (**Table 11**).

Example 6. Achieving improved specific HC/LC Fab assembly within IgG bispecific antibodies (BsAbs).

Two sets of IgG bispecific antibodies (BsAbs) are constructed to test how combining the designs AB2133a and H.4WT may enable specific heavy chain/light chain assembly of specific Fabs. All subcloning and mutagenesis protocols followed are essentially as described in previous Examples. The first BsAb consists of a combination of pertuzumab (anti-HER-2) and matuzumab (anti-EGFR) (*see*, Bier *et al.* (1998), *Cancer Immunol. Immunother.* 46; 167-173) and the second consists of a combination of MetMAb (anti-cMET) *(see, Jin et al. (2008), Cancer Res.* 68; 4360-4368) and an anti-Axl antibody YW327.6S2 (see, WO2011/014457 and Ye *et al.* (2010), *Oncogene* 29; 5254-5264). All sequences for the native antibodies are publicly available. To simplify our

ability to observe specific assembly using LC/MS, all HCs are deglycosylated by mutating asparagine 297 (the site of N-linked glycosylation in the antibody C_H2 domain) to glutamine. To promote heterodimerization in the IgG-Fc, aspartic acid 399 and glutamic acid 356 are both mutated to lysine in one of the heavy chains of the anti-HER-2/anti-EGFR pair and one of the heavy chains of the anti-MET/anti-Axl pair. The remaining heavy chain in each pair had lysine 409 and lysine 392 mutated to aspartic acid (*see,* Gunasekaran *et al.* (2010), *JBC* 285; 19637-19646). It should be noted that other designs to promote heavy chain heterodimerization may be substituted to achieve the same overall affect. Sequence ID numbers of the immunoglobulin chains used to generate the IgG BsAbs are provided in **Table 12**.

Table 12. Sequence ID numbers of the HCs and LCs constructed to demonstrate the specific assembly of IgG BsAbs using the design Fab V_H/V_L and C_H1/C_L interfaces.

WT antibody sequences			
HC Name	**SEQ ID NO:**	**LC Name**	**SEQ ID NO:**
pG1 (pertuzumab)	1	pλ (pertuzumab V_L and C_λ)	2
		pκ (pertuzumab V_L and C_κ)	33
mG1 (matuzumab)	43	mλ (matuzumab V_L and C_λ)	44
		mκ (matuzumab V_L and C_κ)	45
METG1 (METMAb)	46	METλ (METMAb V_L and C_λ)	47
		METκ (METMAb V_L and C_κ)	48
AxlG1 (anti-Axl)	49	Axlλ (anti-Axl V_L and C_λ)	50
		Axlκ (anti-Axl V_L and C_κ)	51
Control IgG HCs and LCs with WT (native) V_H/V_L and C_H1/C_L interfaces[a]			
HC Name	**SEQ ID NO:**	**LC Name**	**SEQ ID NO:**
(-)pG1	52	pλ (pertuzumab V_L and C_λ)	2
(+)mG1	53	mλ (matuzumab V_L and C_λ)	44
(-)METG1	54	mκ (matuzumab V_L and C_κ)	45

(+)AxlG1	55	METλ (METMAb V_L and C_λ)	47
		Axlλ (anti-Axl V_L and C_λ)	50
		Axlκ (anti-Axl V_L and C_κ)	51
IgG HCs and LCs with design V_H/V_L and C_H1/C_L interfaces[a, b]			
HC Name	**SEQ ID NO:**	**LC Name**	**SEQ ID NO:**
AB+2133a(-)[pG1]	13	AB+2133a[pλ]	38
H.4+WT(+)[mG1]	57	AB+2133a[pκ]	56
AB+2133a(-)[METG1]	60	H.4+WT[mλ]	58
H.4+WT(+)[AxlG1]	17	H.4+WT[mκ]	59
		AB+2133a[METλ]	61
		AB+2133a[METκ]	62
		H.4+WT[Axlλ]	16
		H.4+WT[Axlk]	14

[a]The HC designs with (-) contained the K409D and K392D mutations while the HC designs with the (+) contained the D399K and E356K mutations. Both the (+) and (-) -containing HCs also have the N297Q mutation to eliminate N-linked glycosylation.

[b]Nomenclature is essentially as described in Table 10 above. The first two characters of each protein specify the variable domain design, while the subsequent numbering specifies the constant domain design (e.g., a LC designated 'AB+2133a[pλ]' contains Design AB in its V_L and Design 2.1.3.3a in its lambda C_L. Similarly, an HC designated 'AB+2133a(-)[pG1]' contains Design AB in its V_H and Design 2.1.3.3a in its C_{H1}, and the K409D and K392D substitutions in the CH3 domain.) The notation appearing within the brackets refers to the variable and constant domain of the particular parental antibody HC or LC containing the indicated design (e.g., "[pG1]" refers to a heavy chain containing a pertuzumab variable domain and IgG1 constant domain, whereas "[mG1]" refers to a heavy chain with a matuzumab variable domain and IgG1 constant domain; similarly, "[pλ] refers to a light chain with pertuzumab variable domain and λ constant domain, whereas "[METλ]" refers to a light chain with METMAb variable domain and λ constant domain)

To determine if the designed V_H/V_L (AB and H.4) and C_H1/C_L (2.1.3.3a and WT lamda or kappa) interfaces would enable specific IgG BsAb assembly over what occurs naturally with no FAb interface designs in place, transient transfections of particular designed Fab constructs followed by LC/MS analyses are performed. For each IgG BsAb, two heavy chains and two light chains are simultaneously transfected using

separate plasmids into mammalian HEK293F cells using transfection protocols essentially as described in the previous Examples. The designed IgG BsAbs include the deglycosylation mutation and Fc heterodimerization mutations as described above. Further, a control set of IgG BsAbs with the deglycosylation and Fc heterodimerization mutations and light chains with both C_κ or C_λ domains, but without the designed V_H/V_L and C_H1/C_L modifications, are also created by transfection of appropriate heavy chains and light chains. In each Designed IgG BsAb pair, one heavy chain and light chain (shown viable in both the C_λ and C_κ isotype) contained Design H.4, while the other heavy chain and light chain (shown viable in both the C_λ and C_κ isotype) contained Design AB and Design 2.1.3.3a. The exact heavy chain and light chain composition of each IgG BsAb synthesized is provided in **Table 13**.

Table 13. The HC and LC elements of each IgG BsAb and the resulting percentage of correct and incorrect IgG BsAb assembly based on the LC/MS intensities of the fully heterotetrameric species.

Anti-HER-2/Anti-EGFR IgG BsAbs							
IgG BsAb[b]	**HC1[c]**	**LC1[c]**	**HC2[c]**	**LC2[c]**	**% LC1LC2[a] (correct)**	**% $LC1_2$ (incorr.)**	**% $LC2_2$ (incorr.)**
HEControl λλ[c]	(-)pG1	pλ	(+)mG1	mλ	65	23	12
HEControl λκ	(-)pG1	pλ	(+)mG1	mκ	75	0	15
HEDesign λλ	AB+2133a (-)[pG1]	AB+2133a [pλ]	H.4+WT (+)[mG1]	H.4+WT [mλ]	90	8	2
HEDesign λκ	AB+2133a (-)[pG1]	AB+2133a [pλ]	H.4+WT (+)[mG1]	H.4+WT [mκ]	90	7	3
HEDesign κλ	AB+2133a (-)[pG1]	AB+2133a [pκ]	H.4+WT (+)[mG1]	H.4+WT [mλ]	82	18	0
HEDesign κκ	AB+2133a (-)[pG1]	AB+2133a [pκ]	H.4+WT (+)[mG1]	H.4+WT [mλ]	87	10	3
Anti-cMET/Anti-Axl IgG BsAbs							

IgG BsAb	HC1[c]	LC1[c]	HC2[c]	LC2[c]	% LC1LC[a] (correct)	% $LC1_2$[a] (incorr)	% $LC2_2$[a] (incorr)
MAControl λλ	(-)METG1	METλ	(+)AxlG1	Axlλ	70	27	3
MAControl λκ	(-)METG1	METλ	(+)AxlG1	Axlκ	61	38	1
MADesign λλ	AB+2133a (-) [METG1]	AB+2133a [METλ]	H.4+WT (+)[AxlG1]	H.4+WT [Axlλ]	95	5	0
MADesign λκ	AB+2133a (-) [METG1]	AB+2133a [METλ]	H.4+WT (+) [AxlG1]	H.4+WT [Axlκ]	100	0	0
MADesign κλ	AB+2133a (-) [METG1]	AB+2133a [METκ]	H.4+WT (+) [AxlG1]	H.4+WT [Axlλ]	97	2	1
MADesign κκ	AB+2133a (-) [METG1]	AB+2133a [METκ]	H.4+WT (+) [AxlG1]	H.4WT [Axlλ]	90	7	3

[a]The LC/MS method is sensitive for heterotetrameric IgGs containing mismatched HC pairs (HC1HC1 or HC2HC2), but none were detected. The percent values represent the relative counts detected for covalently linked (non-reduced) heterotetramers HC1HC2LC1LC2 (correctly formed) compared to incorrect HC1HC2LC1LC1 (incorr.) and incorrect HC1HC2LC2LC2 (incorr.).

[b]Each BsAb is designated λλ, λκ, κλ, or κκ based on the C_L compositions (lambda or kappa) of its LCs.

[c]Nomenclature for each heavy chain or light chain construct is essentially as described above in Tables 10 and 12

The data in **Table 13** demonstrates that incorporating the V_H/V_L and C_H1/C_L designs into the IgG BsAbs significantly improved the correct assembly of the desired heterotetrameric species (i.e., HC1/LC1+HC2/LC2). Without the Fab designs, the average correct assembly was about 70% and 65% for the anti-HER-2/anti-EGFR and anti-cMET/anti-Axl IgG BsAbs, respectively. With the Fab designs incorporated, the

average correct assembly was about 87% and 96% for the anti-HER-2/anti-EGFR and anti-cMET/anti-Axl IgG BsAbs, respectively.

Next, the IgG BsAbs may be tested for their oligomeric nature using analytical size exclusion chromatography (SEC). First, the IgG BsAbs are purified at the 1 mL scale from HEK293F supernatants using the protein G magnetic bead procedure, essentially as described in **Example 1**. For SEC analysis, between 10-50 μg of each protein was applied to a Yarra G3000 (7.8×300 mm) analytical SEC column (Phenomenex) with all other assay parameters similar to the protocol as described in **Example 1**. SEC of the pertuzumab (anti-HER-2) and matuzumab (anti-EGFR) control IgGs demonstrated slightly different SEC retention times for the two proteins due to differences in their variable domains. Further, the anti-HER-2/anti-EGFR IgG BsAbs tested demonstrate primarily monomeric behavior with SEC retention times between what was observed for non-bispecific pertuzumab and matuzumab controls, which might be expected if the proteins contain one pertuzumab Fab and one matuzumab Fab. Similar to pertuzumab and matuzumab, the METMAb and anti-Axl control IgGs demonstrate slightly different retention times by SEC. The the anti-cMet/anti-Axl IgG BsAbs also demonstrate primarily monomeric behavior with retention times approximating the average of the control antibodies, non-bispecific antibodies. In addition, the two control IgG anti-cMet/anti-Axl BsAbs that did not have the Fab specificity designs incorporated, and which demonstrated significant populations of mismatched light chain pairings, also showed multiple monomeric species by SEC. The four anti-cMet/anti-Axl BsAbs containing the Fab specificity designs did not demonstrate this behavior.

Example 7 Functional Activity of Designed IgG BSAbs

The dual-binding behavior of the synthesized BsAbs may be assessed using both sandwich ELISA and surface plasmon resonance assays as follows.

Two sandwich ELISAs are developed, one for detecting anti-HER-2/anti-EGFR BsAb activity and one for detecting anti-cMET/anti-Axl BsAb activity. For both ELISAs, clear 96-well round bottom high binding Immulon microtiter plates (Greiner bio-one, cat#650061) are coated overnight at 2-8 °C with 50 μL/well 1 μg/mL hHER-2-Fc or 1 μg/mL hHGFR(cMet)-Fc (both from R&D systems) in a 50 mM Na_2CO_3 pH 8 buffer. The plates are washed 4 times with PBST and blocked with 100 μL/well casein

buffer (Pierce) for 1 hr at 37 °C. The plates are then washed 4 times with PBST and the parental IgG controls or BsAb IgG test articles are added at 50 µL/well and 5 µg/mL and serially diluted 1:3 down the plate. The test articles are incubated on the plate for 1 hr at 37 °C. The plates are then washed 4 times with PBST and 50 µL/ well 0.2 µg/mL hEGFR-Fc-biotin or hAxl-Fc-biotin (both from R&D systems) is added for 1 hr at 37 °C. The plates are then washed 4 times with PBST followed by the addition of a 50 µL/well streptavidin-HRP (Jackson Labs) diluted 1:2000 in PBST. The streptavidin-HRP is incubated in each well for 1 hr at 37 °C. The plates are then washed 4 times with PBST and 100 µL/well 1-component TMB substrate is added (KPL laboratories). After approximately 10 minutes, 100 mL/well 1% H_3P0_4 (in H_2O) is added to each plate to quench the reaction. Absorbance (450 nm) of every well in the plates is read using a SpectraMax UV plate reader (Molecular Devices). Biotin-labeling of the hEGFR-Fc and hAxl-Fc proteins is performed using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) according to the manufacturer's protocol.

Both sandwich ELISAs were capable of demonstrating the dual-binding behavior of the BsAbs. For both the anti-HER-2/anti-EGFR and the anti-cMet/anti-Axl IgG BsAbs, the monoclonal IgG controls (pG1, mG1, METG1, or AxlG1) demonstrated no ability to generate signal in the sandwich ELISAs. The control IgG BsAbs with no Fab redesigns (HEControlλλ, HEControlλκ, MAControlλλ, MAControlλκ) generated sigmoidal binding curves in the assay. All the IgG BsAbs with the Fab redesigns also demonstrated sigmoidal binding curves. Average EC_{50} values for the control and designed IgG BsAbs were as follows: HEControl EC_{50} = 470±90 ng/mL; HEDesign EC_{50} = 280±20 ng/mL; MAControl EC_{50} = 260±80; and MADesign EC_{50} = 220±20.

Surface plasmon resonance experiments may also be performed to evaluate the dual-binding behavior of the BsAbs using, for example, on a Biacore3000 using HBS-EP as the running buffer (GE Healthcare). Briefly, hHER-2-Fc-biotin and hAxl-Fc-biotin (both at 20 µg/mL) are immobilized onto SA sensorchip surfaces by injection of 20 µL over 2 minutes at 10 µL/min. Next, 20 µL of each control IgG, control IgG BsAb, or designed IgG BsAb, diluted to 30 nM in HBS-EP buffer, is injected over these sensorchip surfaces at 5 µL/min followed by a secondary 20 µL injection of 20 nM hEGFR-Fc or hHGFR(cMet)-Fc. The hHER-2-Fc and hAxl-Fc sensorchip surfaces are

regenerated by raising the flow rate to 50 μL/min and injecting two 5 μL injections of a 0.1 M glycine solution at pH 2.5 and pH 2.0, respectively.

None of the monoclonal IgGs (pG1, mG1, METG1, or AxlG1) demonstrated dual binding activity in the assay (**Figures 2 and 3**). Both the control IgG BsAbs and the BsAbs containing Fab designs demonstrated strong dual-binding activity in the assay.

Example 8 – Optimization of Constant Domain Design 2133a Using CH1/Ckappa

In certain contexts, constant domain (C_H1/C_L) design denoted 2133a was determined to provide less correct HC/LC pairing specificity when used in C_H1/C_κ compared to its use in C_H1/C_λ (See, for example the EGFRxHER2 bispecific antibody assembly in **Table 13**). It was also found that the 2133a constant domain design destabilized when used in C_H1/C_κ compared to WT C_H1/C_κ (see **Table 15,** below).

To assess stability of the 2133a design IgG1/ C_κ proteins lacking variable domains [WT = SEQ ID NO: 3 (HC) and 63 (LC); Design 2133a = SEQ ID NO: 18 (HC) and 64 (LC)] are generated using molecular biology, expression, and purification methods as generally described in previous examples. The stability of the two purified proteins (WT and 2133a IgGs lacking variable domains or Fvs) are characterized using differential scanning calorimetry (DSC) as follows. The midpoints of the thermal unfolding transitions (denoted 'T_m') of the C_H1/C_κ domains provide a measure of their relative stability. The T_m of the 2133a-containing C_H1/C_κ domain was 67.7 °C while that of WT C_H1/C_κ was 70.8 °C. DSC is performed using an automated capillary DSC (capDSC, GE Healthcare). Protein solutions and reference (buffer) solutions are sampled automatically from a 96-well plate using the robotic attachment. Before each protein scan, at least one buffer/buffer scan is performed to define the baseline for subtraction. All 96-well plates containing protein are stored within the instrument at 6_°C. Samples are run at 1.0 mg/ml protein concentration in PBS. Scans are performed from 10 to 95_°C at 90_°C/hr using the low feedback mode. Scans are analyzed using the Origin software supplied by the manufacturer. Subsequent to the subtraction of reference baseline scans, nonzero protein scan baselines are corrected using a third-order polynomial. Based on the analyses, the IgG1/ C_κ protein harboring the 2133a design was less stable than the WT protein (see **Table 15,** below).

Utilizing an in-house crystal structure of C_H1/C_λ with the 2133a design , we identified C_H1_A172 (design 2133a already contains an H172A substitution) and C_H1_V190 as residue positions to randomize. The 2133a IgG1/ C_κ constructs lacking variable domains (SEQ ID NO: 18 and 64) are used to create the libraries for screening and analysis. The libraries are generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) using protocols provided by the manufacturer. The constructs are expressed via transient transfection in HEK293F cells as generally described in **Example 1**. Titers of the proteins are assessed using the HPLC Protein G quantitation and collection method as generally described in **Example 2**. The proteins are assessed for their stability properties using a thermal challenge assay similar to that described in **Example 1**. Unique to this assay, the plates are coated with a sheep anti-human Fd (CH1) polyclonal antibody (Meridian Life Sciences, cat#W90075C) at 1 μg/mL and 100 μL/well in a 0.05 M $NaHCO_3$ buffer, pH 8.3 for 1 hr at 37 °C or overnight at 4 °C. Thermally resistant IgG1/ $C_\kappa$protein is detected by adding a detection antibody (HRP-labeled goat anti-human kappa, Southern Biotechnology, cat#2060-5) at a 1:10,000 dilution in PBS-T into every well at 100 μL/well and incubating for 1 hr at 37 °C. Other assay parameters are generally as described previously.

From the library, three HC mutants, A172R, V190M, and V190I, are identified that stabilized the 2133a-containing C_H1/C_κ domains in the thermal challenge assay. Combinations comprising C_H1_A172R_V190M or C_H1_A172R_V190I were also generated. **Table 14** provides a listing of Sequence ID numbers for these 2133a C_H1 mutant proteins.

Larger scale (≥100 mL) transfections of the single and double mutant modifications of design 2133a C_H1/C_κconstructs are generated in HEK293F cells. The transfected cells are cultured as generally described in **Example 1** for small scale cultures. Supernatants with protein are clarified using 0.2 μm filters. The C_H1/C_κ (-Fv) proteins are purified using standard protein A affinity chromatography methods. The proteins are buffer exchanged into PBS and analyzed by DSC as described above for the WT and 2133a C_H1/C_κ (-Fv) proteins. The results of the DSC analyses showed that the single and double mutant combinations were stabilizing to the 2133a C_H1/C_κ domains (**Table 15**).

Table 14. Sequence ID numbers of the HC and LC designs improving the stability or specificity of the 2133a design in CH1/Cκ.

Modified 2133a HC for Improved Stability	**Sequence ID number**
HC (-V_H) 2133a_A172R	SEQ ID 65
HC (-V_H) 2133a_V190M	SEQ ID 66
HC (-V_H) 2133a_V190I	SEQ ID 67
HC (-V_H) 2133a_A172R_V190M	SEQ ID 68
HC (-V_H) 2133a_A172R_V190I	SEQ ID 69
Cκ_2133a_V133L	SEQ ID 70
Cκ_2133a_S174Q	SEQ ID 71
Cκ_2133a_S174D	SEQ ID 72
Cκ_2133a_V133L_S174Q	SEQ ID 73
Cκ_2133a_V133L_S174D	SEQ ID 74
(-)PG1_AB2133a[a]	SEQ ID 13
(-)PG1_AB2133a_MR[b]	SEQ ID 75
(-)PG1_AB2133a_IR[b]	SEQ ID 76
(-)MetG1_AB2133a	SEQ ID 60
(-)MetG1_AB2133a _MR[b]	SEQ ID 77
(-)MetG1_AB2133a _IR[b]	SEQ ID 78

Pκ_AB2133a	SEQ ID 56
Pκ_AB2133a_LD[b]	SEQ ID 79
Pκ_AB2133a_LQ[b]	SEQ ID 80
Metκ_AB2133a	SEQ ID 62
Metκ_AB2133a_LD[b]	SEQ ID 81
Metκ_AB2133a LQ[b]	SEQ ID 82
(+)MG1_H4[a]	SEQ ID 57
(+)TG1_H4 [a]	SEQ ID 83
Mκ_H4	SEQ ID 59
Tκ_H4	SEQ ID 87

[a] The HC designs with (-) contained the K409D and K392D mutations while the HC designs with the (+) contained the D399K and E356K mutations. Both the (+) and (-) -containing HCs also have the N297Q mutation to eliminate N-linked glycosylation.

[b] 'MR' refers to C_H1 mutations A172R and V190M. 'IR' refers to C_H1 mutations A172R and V190I. 'LD' refers to Cκ mutations V133L and S174D. 'LQ' refers to Cκ mutations V133L and S174Q.

Table 15. Expression and differential scanning calorimetry (DSC) results for mutants of the 2133a-containing IgG1/Cκ protein lacking variable domains.

Protein	**Expression Level (μg/mL)**	**DSC T_m (°C)[a]**
HC (-V_H) WT/Cκ_WT	15	70.8±0.1
HC (-V_H) 2133a/Cκ_2133a	19	67.7±0.2
HC (-V_H) 2133a_A172R/Cκ_2133a	16	70.0

HC (-V_H) 2133a_V190M/Cκ_2133a	21	69.1
HC (-V_H) 2133a_V190I/Cκ_2133a	14	68.6
HC (-V_H) 2133a_A172R_V190M/Cκ_2133a	43	70.1
HC (-V_H) 2133a_A172R_V190I/Cκ_2133a	43	69.2

[a] The DSC Tm refers to the midpoint of the cooperative unfolding transition of the C_H1/Cκ heterodimer in each of these constructs.

Using the LC competition assay as generally described in **Example 2**, the specificity afforded by the 2133a design mutations in C_H1/C_κ instead of C_H1/C_λ is assessed. As shown in **Table 16** below, the WT C_κ protein (SEQ ID 63) shows little tendency to associate with the 2133a HC (SEQ ID 18) protein with or without the stabilizing mutations depicted in **Table 15**. However, it was observed that the 2133a-containing C_κ domain (SEQ ID 64) could associate with a WT HC (SEQ ID 3) more strongly than a WT C_κ domain (SEQ ID 63, see **Table 16,** below).

Using in-house 2133a/2133a and WT/WT C_H1/C_λ crystal structures, 2133a C_κ residue positions V133 and S174 were identified as positions where potential new interactions with 2133a-containing C_H1 domains could be generated, that might be incompatible with WT C_H1. Screening for 2133a C_κ mutations that maintained binding to the 2133a design HC, while decreasing binding with WT HC, three mutations (C_κ_V133L, C_κ_S174Q, and C_κ_S174D) were found to provide beneficial specificity in the LC C_κ competition assay (**Table 16**) (Sequence ID numbers of these constructs are provided in **Table 14,** above). Each of these mutations maintained the dominance of design 2133a C_κ binding to 2133a C_H1 over WT C_κ, while decreasing the proportion of design 2133a C_κ binding to WT C_H1 compared to WT C_κ. Further, the V133L mutation could be combined with either S174Q or S174D to provide further improvements in specificity (**Table 16**).

Table 16. Competition of Ckappa proteins binding to a single IgG1 HC (no variable domains) with variants of the 2.1.3.3a design

LC1	LC2	HC	%Assembly[a] (LC1/HC)	%Assembly[a] (LC2/HC)
Cκ_2133a	Cκ_WT	HC (-V_H) WT	86.6±9.9	13.4±9.9
Cκ_2133a	Cκ_WT	HC (-V_H) 2133a	98.8±1.2	1.2±1.2
Cκ_2133a_V133L	Cκ_WT	HC (-V_H) WT	70	30
Cκ_2133a_S174Q	Cκ_WT	HC (-V_H) WT	59	41
Cκ_2133a_S174D	Cκ_WT	HC (-V_H) WT	62	38
Cκ_2133a_V133L_S174Q	Cκ_WT	HC (-V_H) WT	0	100
Cκ_2133a_V133L_S174Q	Cκ_WT	HC (-V_H) 2133a	100	0
Cκ_2133a_V133L_S174Q	Cκ_WT	HC (-V_H) 2133a A172R_V190M	100	0
Cκ_2133a_V133L_S174Q	Cκ_WT	HC (-V_H) 2133a A172R_V190I	97.8	2.2
Cκ_2133a_V133L_S174D	Cκ_WT	HC (-V_H) WT	28.5	71.5
Cκ_2133a_V133L_S174D	Cκ_WT	HC (-V_H) 2133a	100	0
Cκ_2133a_V133L_S174D	Cκ_WT	HC (-V_H) 2133a_A172R_V190M	100	0
Cκ_2133a_V133L_S174D	Cκ_WT	HC (-V_H) 2133a_A172R_V190I	100	0

[a] As described in previous examples, the % assembly was derived using the ratio of counts for each LC detected by the mass spectrometer after purifying the expressed IgG(-Fv) proteins using the anti-Fc, releasing the Cκ domains using DTT.

The 2133a C_H1 and Cκ mutants were then added to two separate full-length HCs and LCs (including variable domains) to assess their ability to impact specific bispecific antibody assembly. The 2133a C_H1 mutants A172R and V190M (denoted 'MR') and A172R and V190I (denoted 'IR') were each combined for the testing as were the compatible design 2133a Cκ mutants V133L and S174D (denoted 'LD') and V133L and

S174Q (denoted 'LQ'). The mutants were added to both a Pertuzumab (anti-HER2) and MetMAb (anti-cMet) IgG1/kappa HC/LC pair containing the 2133a designs. To promote heavy chain heterodimerization, the Pertuzumab and MetMAb HCs contained the K409D and K392D mutations, denoted '(-)', while the complementary Matuzumab and Trastuzumab HCs contained the D399K and E356K mutations, denoted '(+)' (*see*, Gunasekaran et al., (2010), *JBC* 285; 19637-19646). Both the (+) and (-) -containing HCs also have the N297Q mutation to eliminate N-linked glycosylation. The Pertuzumab HC/LC pairs were co-expressed with an H4WT-containing Matuzumab IgG1/kappa HC/LC pair to form a HER2×EGFR IgG bispecific antibody. The MetMAb HC/LC pairs were co-expressed with an H4WT-containing Trastuzumab IgG1/kappa HC/LC pair to form a cMet×HER2 IgG bispecific antibody. The Sequence ID numbers for all sequences used in the study are provided in **Table 14**, above. All LCs in this study were fully kappa (i.e., VκCκ).

The ability to co-express each of the antibody pairs consisting of 2 HCs and 2 LCs (four chains total) and have them assemble into correctly formed IgG BsAbs was determined by MS as generally described in **Example 6**, above. Results of the co-expression studies are provided in **Table 17**, below. All data points are the average of between 3 and 9 individual measurements and the error represents the standard deviation. Differences between the correct assembly in the absence and presence of the 'MR', 'IR', 'LD', and/or 'LQ' designs were tested for their significance using a standard or paired t-test.

Table 17. Specific Assembly of IgG BsAbs Using 2133a Variant CH1/Ckappa Designs.

HC1[a,b]	**LC1**[a]	**HC2**[a,b]	**LC2**[a]	**% Correct IgG BsAb w/Std.Dev.**	**P-value (One-tailed t-test)**
(-)PG1_AB2133a	Pκ_AB2133a	(+)MG1_H4	Mκ_H4	71.8±4.5 (n=9)	

(-)PG1_AB2133a_MR	Pκ_AB2133a	(+)MG1_H4	Mκ_H4	62.2±3.3 (n=5)	N/A
(-)PG1_AB2133a_IR	Pκ_AB2133a	(+)MG1_H4	Mκ_H4	56.6±2.4 (n=5)	N/A
(-)PG1_AB2133a	Pκ_AB2133a_LD	(+)MG1_H4	Mκ_H4	76.3±7.1 (n=9)	P=0.063
(-)PG1_AB2133a	Pκ_AB2133a_LQ	(+)MG1_H4	Mκ_H4	82.9±13.1 (n=8)	P=0.015
(-)PG1_AB2133a_MR	Pκ_AB2133a_LD	(+)MG1_H4	Mκ_H4	94.9±1.7 (n=5)	P<0.001
(-)PG1_AB2133a_MR	Pκ_AB2133a_LQ	(+)MG1_H4	Mκ_H4	77.3±1.7 (n=5)	P=0.01
(-)PG1_AB2133a_IR	Pκ_AB2133a_LD	(+)MG1_H4	Mκ_H4	76.2±9.6 (n=4)	P=0.14
(-)PG1_AB2133a_IR	Pκ_AB2133a_LQ	(+)MG1_H4	Mκ_H4	91.2±3.05 (n=3)	P<0.001
HC1[a,b]	**LC1[a]**	**HC2[a,b]**	**LC2[a]**	**% Correct IgG BsAb w/Std.Dev.**	**P-value (One-tailed paired t-test)**
(-)MetG1_AB2133a	Metκ_AB2133a	(+)TG1_H4	Tκ_H4	51.8±2.8 (n=3)	
(-)MetG1_AB2133a _MR	Metκ_AB2133a	(+)TG1_H4	Tκ_H4	44.9±11.6 (n=3)	N/A

(-)MetG1_AB2133a _IR	Metκ_AB2133a	(+)TG1_H4	Tκ_H4	34.8±8.4 (n=3)	N/A
(-)MetG1_AB2133a	Metκ_AB2133a_ LD	(+)TG1_H4	Tκ_H4	73.7±1.6 (n=3)	P=0.006
(-)MetG1_AB2133a	Metκ_AB2133a_ LQ	(+)TG1_H4	Tκ_H4	65.5±2.1 (n=3)	P<0.001
(-)MetG1_AB2133a _MR	Metκ_AB2133a_ LD	(+)TG1_H4	Tκ_H4	61.0±3.1 (n=3)	P=0.015
(-)MetG1_AB2133a _MR	Metκ_AB2133a_ LQ	(+)TG1_H4	Tκ_H4	50.1±1. (n=3)	Not significa nt
(-)MetG1_AB2133a _IR	Metκ_AB2133a LD	(+)TG1_H4	Tκ_H4	56.2±1.6 (n=3)	P=0.035
(-)MetG1_AB2133a _IR	Metκ_AB2133a LQ	(+)TG1_H4	Tκ_H4	50.7±2.0 (n=3)	Not significa nt

[a] All Pertuzumab and MetMAb molecules have the AB2133a HC/LC design with or without the further HC or LC mutations. All Matuzumab and Trastuzumab molecules have the H4 design mutations in their variable domains. All LCs are fully Kappa (i.e., VκCκ).

[b] The HC designs with (-) contained the K409D and K392D mutations while the HC designs with the (+) contained the D399K and E356K mutations. Both the (+) and (-) -containing HCs also have the N297Q mutation to eliminate N-linked glycosylation.

Both the 'LD' and 'LQ' paired mutations provided significant improvements in correct LC pairing within the IgG bispecific antibodies over utilization of unmodified AB2133a kappa LCs. Adding 'LD' and 'LQ' mutant combinations to the AB2133a C_κ provided an average 13% and 12%, respectively, benefit in correct LC assembly over what was obtained in their absence. While the HC 'MR' and 'IR' mutations are stabilizing to the 2133a C_H1/ C_κ interaction, their impact on specificity was less clear.

Adding the HC 'MR' and 'IR' mutations in isolation decreased the correct IgG bispecific antibody assembly in all cases. In most cases, adding the HC 'MR' or 'IR' mutants to either LC 'LQ' or 'LD' mutations led to either no increase or a decrease in correct assembly; however, the addition of 'IR' to 'LQ' within the Anti-HER2×Anti-EGFR IgG bispecific antibody led to an approximate 10% increase in correct assembly over what was observed with 'LQ' alone. Thus, while the 'MR' and 'IR' mutations improve the stability of design 2133a C_H1/ C_κ-containing bispecific antibodies, it appears to be case specific whether the 'MR' and 'IR' mutations improve specificity.

Example 9 – Additional Specificity Designs to Improve LC Specificity when expressing IgG Bispecific Antibodies

To further improve the specificity of IgG bispecific antibody assembly, additional designs within the variable domains that drive improved HC/LC pairing specificity were pursued. A charge/polar pair of amino acids at HC_Q105/LC_K42 was identified for manipulation. LC_K42 was mutated to D and HC_Q105 was mutated to R within the pertuzumab IgG1/kappa H4WT design. After analyzing expression and LC competition data (as general described in previous Examples), LC_K42D and HC_Q105R, when added to H4 variable domain designs, provided slight improvement in H4(+ K42D) pertuzumab LC pairing with its cognate HC H4(+Q105R) design when co-expressed with an AB2133a pertuzumab LC (~20% improvement). Co-expressing the H4(+K42D) pertuzumab LC with the design AB2133a pertuzumab LC and HC also did not markedly decrease the observed assembly level of the design AB2133a pertuzumab HC and LC pair. This additional design mutation (HC_Q105R/LC_K42D) is denoted as 'DR' in the **Tables 18** and **19** below.

Inspection of a Fab crystal structure (pdb id: 3HC4) reveals a lysine at HC residue 228 and an aspartic acid at LC residue 122 near the distal end of the antibody Fab located near to the HC/LC disulfide bond. To provide charge-based steering that may favor proper HC/LC pairing, these charged residues were modified (HC_K228D/LC_D122K) in Pertuzumab H4WT IgG1/kappa. (To ensure the HC/LC disulfide bond was not disrupted by the charge swap, the HC residue 230 was mutated to glycine and HC residue 127 was mutated to cysteine.) This charge swap and cysteine modification is denoted 'CS'.

To determine whether the 'CS' and 'DR' designs might improve correct HC/LC assembly when expressing IgG bispecific antibodies, these designs were tested in isolation and in combination in antibodies containing the H4WT IgG1/kappa design. The Pertuzumab IgG1/kappa molecules containing AB2133a designs were paired with other IgG1/kappa antibodies containing the H4WT designs (with and without the 'DR' and/or 'CS' mutations) including BHA10 (Jordan, JL, *et al.* (2009) *Proteins* 77:832-41), Matuzumab, and Trastuzumab (i.e., Herceptin). Additionally, Pertuzumab with H4WT IgG1/kappa (with and without the 'DR' and/or 'CS' mutations) was tested for bispecific IgG assembly with Trastuzumab-containing the AB2133a designs in IgG1/kappa. The Pertuzumab and Trastuzumab HCs containing the AB2133a Fab designs contained the K409D and K392D mutations, denoted '(-)', while the complementary H4WT designs (with and without the 'DR' and/or 'CS' mutations) contained the D399K and E356K mutations, denoted '(+)' (*see*, Gunasekaran et al., (2010), *JBC* 285; 19637-19646). Both the (+) and (-) - containing HCs also had the N297Q mutation to eliminate N-linked glycosylation. To generate IgG bispecific antibodies, the HC and LC from Pertuzumab are co-transfected with the HC and LC of a second antibody in 2 mL HEK293F cells. The resulting material is secreted from the cells, purified as described in **Example 2** and characterized by MS. The Sequence ID numbers of all the HCs and LCs used in the experiment are provided in **Table 18**.

Table 18. Sequence ID numbers of the HC and LC designs improving the specificity of HC/LC assembly.

Sequence names for constructs used to demonstrate additional improvements in the specificity of Fab Designs	**SEQ ID NO:**
(-)PG1 AB2133a	SEQ ID 13
(+)PG1 H4WT	SEQ ID 91
(+)PG1 H4WT + CS	SEQ ID 92
(+)PG1 H4WT + DR	SEQ ID 93

(+)PG1 H4WT + CS + DR	SEQ ID 94
Pκ AB2133a	SEQ ID 56
Pκ H4WT	SEQ ID 41
Pκ H4WT + CS	SEQ ID 95
Pκ H4WT + DR	SEQ ID 96
Pκ H4WT + CS + DR	SEQ ID 97
(+)BHA10G1 H4WT	SEQ ID 104
(+)BHA10G1 H4WT + CS	SEQ ID 105
(+)BHA10G1 H4WT + DR	SEQ ID 106
(+)BHA10G1 H4WT + CS+DR	SEQ ID 107
(+)MG1 H4WT	SEQ ID 57
(+)MG1 H4WT + CS	SEQ ID 98
(+)MG1 H4WT + DR	SEQ ID 99
(+)MG1 H4WT + CS+DR	SEQ ID 100
(+)TG1 H4WT	SEQ ID 83
(+)TG1 H4WT + CS	SEQ ID 84
(+)TG1 H4WT + DR	SEQ ID 85
(+)TG1 H4WT + CS+DR	SEQ ID 86
(-)TG1 AB2133a	SEQ ID 108
BHA10κ H4WT	SEQ ID 109
BHA10κ H4WT + CS	SEQ ID 110

BHA10κ H4WT + DR	SEQ ID 111
BHA10κ H4WT + CS+DR	SEQ ID 112
Mκ H4WT	SEQ ID 59
Mκ H4WT + CS	SEQ ID 101
Mκ H4WT + DR	SEQ ID 102
Mκ H4WT + CS+DR	SEQ ID 103
Tκ H4WT	SEQ ID 87
Tκ H4WT + CS	SEQ ID 88
Tκ H4WT + DR	SEQ ID 89
Tκ H4WT + CS+DR	SEQ ID 90
Tκ AB2133a	SEQ ID 113

The results of the MS measurements of percentage of correctly formed HC/LC pairs are provided in **Table 19.** All data points are the average of between 3-5 individual measurements and the error represents the standard deviation. Differences between the correct assembly in the absence and presence of the 'DR', 'CS', or 'CS + DR' designs were tested for their significance using a paired t-test.

Table 19. Specific assembly of IgG bispecific antibodies utilizing fully kappa LCs (i.e., VκCκ) with and without (i) VH_Q105R/VL_K42D (DR), (ii) CH1_S127C_K228D_C230G/ CL_D122K (CS), or the combination of (i) and (ii).

HC1[a]	LC1[b]	HC2[a]	LC2[b]	%Correct IgG BsAb w/Std.Dev.	P-value (One-tailed paired t-test)
Anti-HER2(pertuzumab)XAnti-LTbR(BHA10) IgG Coexpression					
(-)PG1 AB2133a	Pκ AB2133a	(+)BHA10G1 H4WT	BHA10κ H4WT	78.8±6.6 (n=3)	
(-)PG1 AB2133a	Pκ AB2133a	(+)BHA10G1 H4WT + CS	BHA10κ H4WT + CS	87.2±3.0 (n=3)	P=0.03
(-)PG1 AB2133a	Pκ AB2133a	(+)BHA10G1 H4WT + DR	BHA10κ H4WT + DR	84.7±3.6 (n=3)	P=0.07
(-)PG1 AB2133a	Pκ AB2133a	(+)BHA10G1 H4WT + CS+DR	BHA10κ H4WT + CS+DR	87.4±4.2 (n=3)	P=0.03
Anti-HER2(pertuzumab) X Anti-EGFR(matuzumab) IgG Coexpression					
(-)PG1 AB2133a	Pκ AB2133a	(+)MG1 H4WT	Mκ H4WT	75.1±6.1 (n=3)	
(-)PG1 AB2133a	Pκ AB2133a	(+)MG1 H4WT + CS	Mκ H4WT + CS	68.4±4.0 (n=3)	Failed
(-)PG1 AB2133a	Pκ AB2133a	(+)MG1 H4WT + DR	Mκ H4WT + DR	88.9±6.0 (n=3)	P=0.06
(-)PG1	Pκ AB2133a	(+)MG1 H4WT	Mκ H4WT +	84.4±4.1	P=0.02

AB2133a		+ CS+DR	CS+DR	(n=3)	
Anti-HER2(pertuzumab) X Anti-HER2(trastuzumab) IgG Coexpression					
(-)PG1 AB2133a	Pκ AB2133a	(+)TG1 H4WT	Tκ H4WT	69.7±5.3 (n=5)	
(-)PG1 AB2133a	Pκ AB2133a	(+)TG1 H4WT + CS	Tκ H4WT + CS	71.7±6.7 (n=5)	P=0.30
(-)PG1 AB2133a	Pκ AB2133a	(+)TG1 H4WT + DR	Tκ H4WT + DR	70.2±3.8 (n=5)	P=0.28
(-)PG1 AB2133a	Pκ AB2133a	(+)TG1 H4WT + CS+DR	Tκ H4WT + CS+DR	79.6±4.2 (n=5)	P<0.0001
Anti-HER2(pertuzumab) X Anti-HER2(trastuzumab) IgG Coexpression with H4WT and AB2133a swapped					
(+)PG1 H4WT	Pκ H4WT	(-)TG1 AB2133a	Tκ AB2133a	35.4±10.1 (n=5)	
(+)PG1 H4WT + CS	Pκ H4WT + CS	(-)TG1 AB2133a	Tκ AB2133a	41.7±16.8 (n=5)	P=0.07
(+)PG1 H4WT + DR	Pκ H4WT + DR	(-)TG1 AB2133a	Tκ AB2133a	52.0±9.0 (n=5)	P=0.003
(+)PG1 H4WT + CS+DR	Pκ H4WT + CS+DR	(-)TG1 AB2133a	Tκ AB2133a	47.6±15.1 (n=5)	P=0.01

[a]The HC designs with (-) contained the K409D and K392D mutations while the HC designs with the (+) contained the D399K and E356K mutations. Both the (+) and (-) -containing HCs also have the N297Q mutation to eliminate N-linked glycosylation. [b]All light chains in Table 19 were fully kappa (i.e., VκCκ).

As with the data in **Example 8**, it was clear from the data in **Table 19** that the AB2133a designs did not provide the same degree of specificity when using fully kappa LCs (VκCκ).

The data in **Table 19** demonstrate trends showing that both the 'DR' and 'CS' designs, when added to the H4WT-containing Fab, generally improve the specificity of correct HC/LC pairing within IgG bispecific antibodies expressed in a single cell. In a few cases, the individual 'CS' or 'DR' were found to significantly improve (i.e., $P \leq 0.05$) the correct assembly. However, when the two designs were combined ('CS + DR'), the improvements in correct HC/LC pairing specificity were statistically significant for all groups. Improvements in specificity were observed when the designs were placed on the H4WT-side of the IgG BsAb.

Overall the designs described in **Example 8** and **Example 9** can be utilized to improve the HC/LC pairing stability and specificity within IgG bispecific antibodies. This was particularly evident when both LCs of the IgG bispecific antibody were fully kappa; a scenario where the AB2133a designs provide less specificity than when the AB2133a designs are used within a VκCλ LC.

Sequences: (mutations denoted by underlined, bold-face type)

SEQ ID NO. 1: PERTUZUMAB HC (pG1)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 2: PERTUZUMAB LC (pλ, KAPPA V_L/LAMBDA C_L)

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 3: HUMAN HC (MINUS VARIABLE DOMAINS) WILD-TYPE CONSTRUCT

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 4: LAMBDA C_L DOMAIN

GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVET
TTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 5: HC DESIGN 1.0

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHT**T**PAVLQSSGLYSLSS**F**VTVPSSSLGTQTYICNVNHKPSNTKVDKKV

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 6: LC CHAIN DESIGN 1.0

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVC**F**ISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 7: HC minusV_H DESIGN 2.1, 2.1.2.1, 2.1.2.2

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT**G**PA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 8: C_L DESIGN 2.1, 2.1.1.1, AND 2.1.1.2

GQPKAAPSVTLFPPSSEELQANKATLVC**A**ISDFYPGAVTVAWKADSSPVKAGVET
TTPSKQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 9: HC DESIGN 5.0

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK**K**YFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK
VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPG

SEQ ID NO. 10: LC DESIGN 5.0

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQAN**D**ATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 11: HC minusV_H DESIGN 1.0+5.0

ASTKGPSVFPLAPSSKSTSGGTAALGCLVK**K**YFPEPVTVSWNSGALTSGVHT**T**PA
VLQSSGLYSLSS**F**VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 12: C_L DESIGN 1.0+5.0

GQPKAAPSVTLFPPSSEELQAN**D**ATLVC**F**ISDFYPGAVTVAWKADSSPVKAGVET
TTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 13: AB2133a(-)pG1 (Design AB2.1.3.3a in (-)pG1 HC)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**K**APGKGLEWVADVNP
NSGGSIYNQ**E**FKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**A**T**G**PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 14: H.4WTAx1κ (anti-Axl LC with H.4 V_L and wild-type $C_κ$)

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQ**R**KPGKAPKLLIYSASFLY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 15: C_L DESIGN 2.1.3.2

GQPKAAPSVTLFPPSSEELQANKATLVC**F**ISDFYPGAVTVAWKADSSPVKAGVET
TTPSKQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 16: H.4WTAxlλ (anti-Axl LC with H.4 V_L and wild-type C_λ)

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQ**R**KPGKAPKLLIYSASFLY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 17: H.4WT(+)AxlG1 (Design H.4 in (+)AxlG1 HC)

EVQLVESGGGLVQPGGSLRLSCAASGFSLSGSWIHWVR**Y**APGKGLEWVGWINPY
RGYAYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAREYSGWGGSS
VGYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP
VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN
TKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNG
KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPG

SEQ ID NO. 18: HC minus V_H DESIGN 2.1.3.2 AND 2.1.3.3A

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV**A**T**G**PA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 19: HC minus V_H DESIGN 2.1.3.3

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV**S**T**G**PA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 20: C_L DESIGN 2.1.3.3 AND 2.1.3.3A

GQPKAAPSVTLFPPSSEELQANKATLVC**Y**ISDFYPGAVTVAWKADSSPVKAGVET
TTPSKQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 21: HC DESIGN A

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNP
NSGGSIYNQ**E**FKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 22: LC DESIGN A

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 23: HC DESIGN B

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**K**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 24: LC DESIGN B

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 25: HC DESIGN AB

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**K**APGKGLEWVADVNP
NSGGSIYNQ**E**FKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 26: LC DESIGN AB

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 27: HC DESIGN 1.0+5.0
EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK**K**YFPEPVTVSWN
SGALTSGVHT**T**PAVLQSSGLYSLSS**F**VTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK
VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPG

SEQ ID NO. 28: LC DESIGN 1.0+5.0

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQAN**D**ATLVC**F**ISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 29: HC DESIGN H.4

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**Y**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV

SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 30: LC DESIGN H.4, H.5, H.6 LAMBDA

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**R**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 31: HC DESIGN H.5

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**F**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 32: HC DESIGN H.6

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**W**APGKGLEWVADVN
PNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYF
DYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK
KVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPG

SEQ ID NO. 33: LC PERTUZUMAB KAPPA (pκ, KAPPA V_L/KAPPA C_L)

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 34: LC DESIGN AB KAPPA

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 35: HC DESIGN 2.1.3.3a

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**A**T**G**PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 36: LC DESIGN 2.1.3.3a

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVC**Y**ISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 37: HC DESIGNS AB2.1.3.3a and AB2.1.3.2

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**K**APGKGLEWVADVNP
NSGGSIYNQ**E**FKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**A**T**G**PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 38: LC DESIGN AB2.1.3.3a (AB2133apλ)

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVC**Y**ISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 39: HC DESIGN H.42.1.3.3a

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**Y**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**A**T**G**PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 40: LC DESIGN H.42.1.3.3a

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**R**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVC**Y**ISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 41: HC DESIGN H.4 KAPPA

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**R**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 42: LC DESIGN AB2.1.3.2

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVC**F**ISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 43: Matuzumab HC (mG1)

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSHWMHWVRQAPGQGLEWIGEFN
PSNGRTNYNEKFKSKATMTVDTSTNTAYMELSSLRSEDTAVYYCASRDYDYDG
RYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKV
DKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY

KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPG

SEQ ID NO. 44: Matuzumab LC (mλ, V_L and lambda C_L [C_λ])

DIQMTQSPSSLSASVGDRVTITCSASSSVTYMYWYQQKPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSSHIFTFGQGTKVEIKGQPKA
APSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSK
QSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 45: Matuzumab LC (mκ, V_L and kappa C_L [C_κ])

DIQMTQSPSSLSASVGDRVTITCSASSSVTYMYWYQQKPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSSHIFTFGQGTKVEIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 46: METMAb HC (METG1)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYWLHWVRKAPGKGLEWVGMIDP
SNSDTRFNPNFKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCATYRSYVTPLD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 47: METMAb LC (METλ, kappa V_L and lambda C_L [C_λ])

DIQMTQSPSSLSASVGDRVTITCKSSQSLLYTSSQKNYLAWYQQKPGKAPKLLIY
WASTRESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYAYPWTFGQGTKV
EIKGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAG
VETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 48: METMAb LC (METκ, kappa V_L and kappa C_L [C_κ])

DIQMTQSPSSLSASVGDRVTITCKSSQSLLYTSSQKNYLAWYQQKPGKAPKLLIY
WASTRESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYAYPWTFGQGTKV
EIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 49: anti-Axl HC (AxlG1)

EVQLVESGGGLVQPGGSLRLSCAASGFSLSGSWIHWVRQAPGKGLEWVGWINPY
RGYAYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAREYSGWGGSS
VGYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP
VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN
TKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG
KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPG

SEQ ID NO. 50: anti-Axl LC (Axlλ, kappa V_L and lambda C_L [C_λ])

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 51: anti-Axl LC (Axlκ, kappa V_L and kappa C_L [C_κ])
DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 52: (-)pG1 (Pertuzumab IgG1 HC with CH3_K409D,K392D)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 53: (+)mG1 (Matuzumab IgG1 HC with C_H3_D399K,E356K)

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSHWMHWVRQAPGQGLEWIGEFN
PSNGRTNYNEKFKSKATMTVDTSTNTAYMELSSLRSEDTAVYYCASRDYDYDG
RYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV

SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKV
DKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPG

SEQ ID NO. 54: (-)METG1 (METMAb IgG1 HC with CH3_K409D,K392D)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYWLHWVRQAPGKGLEWVGMIDP
SNSDTRFNPNFKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCATYRSYVTPLD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 55: (+)AxlG1 (anti-Axl IgG1 HC with C_H3_D399K,E356K)

EVQLVESGGGLVQPGGSLRLSCAASGFSLSGSWIHWVRQAPGKGLEWVGWINPY
RGYAYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAREYSGWGGSS
VGYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP
VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN
TKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNG
KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPG

SEQ ID NO. 56: AB2133apκ (Pertuzumab LC with AB in V_L and 2.1.3.3a in $C_κ$)

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVC**Y**LNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDSTYSL**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 57: H.4WT(+)mG1 (Design H.4 in (+)mG1 HC)

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSHWMHWVR**Y**APGQGLEWIGEFN
PSNGRTNYNEKFKSKATMTVDTSTNTAYMELSSLRSEDTAVYYCASRDYDYDG
RYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV

SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKV
DKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPG

SEQ ID NO. 58: H.4WTmλ (Matuzumab LC with H.4 V_L and wild-type C_λ)

DIQMTQSPSSLSASVGDRVTITCSASSSVTYMYWYQ**R**KPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSSHIFTFGQGTKVEIKGQPKA
APSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSK
QSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 59: H.4WTmκ (Matuzumab LC with H.4 V_L and wild-type C_κ)

DIQMTQSPSSLSASVGDRVTITCSASSSVTYMYWYQ**R**KPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSSHIFTFGQGTKVEIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 60: AB2133a(-)METG1 (Design AB2.1.3.3a in (-)METG1 HC)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYWLHWVR**K**APGKGLEWVGMIDP
SNSDTRFNP**E**FKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCATYRSYVTPLD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**A**T**G**PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 61: AB2133aMETλ (METMAB LC with AB in V_L and 2.1.3.3a C_λ)

RIQMTQSPSSLSASVGDRVTITCKSSQSLLYTSSQKNYLAWYQ**D**KPGKAPKLLIY
WASTRESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYAYPWTFGQGTKV
EIKGQPKAAPSVTLFPPSSEELQANKATLVC**Y**ISDFYPGAVTVAWKADSSPVKAG
VETTTPSKQSNNKYAA**W**SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

SEQ ID NO. 62: AB2133aMETκ (METMAB LC with AB in V_L and 2.1.3.3a Cκ)

RIQMTQSPSSLSASVGDRVTITCKSSQSLLYTSSQKNYLAWYQ**D**KPGKAPKLLIY
WASTRESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYAYPWTFGQGTKV
EIKRTVAAPSVFIFPPSDEQLKSGTASVVC**Y**LNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTYSL**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE
C

SEQ ID NO. 63: Wild-Type Cκ

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQES
VTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 64: 2133a Cκ

RTVAAPSVFIFPPSDEQLKSGTASVVC**Y**LNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSL**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 65: HC minus V_H DESIGN 2133a + A172R

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV**R**T**G**PA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 66: HC minus V_H DESIGN 2133a + V190M

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV**A**T**G**PA
VLQSSGLYSLSS**M**VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 67: HC minus V_H DESIGN 2133a + V190I

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV**A**T**G**PA
VLQSSGLYSLSS**I**VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 68: HC minus V_H DESIGN 2133a + A172R + V190M

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV**R**T**G**PA
VLQSSGLYSLSS**M**VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 69: HC minus V_H DESIGN 2133a + A172R + V190I

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV**R**T**G**PA
VLQSSGLYSLSS**I**VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO. 70: Cκ 2133a_V133L

RTVAAPSVFIFPPSDEQLKSGTASV**L**C**Y**LNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSL**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 71: Cκ_2133a_S174Q

RTVAAPSVFIFPPSDEQLKSGTASVVC**Y**LNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTY**Q**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 72: Cκ_2133a_S174D

RTVAAPSVFIFPPSDEQLKSGTASVVC**Y**LNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTY**D**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 73: Cκ_2133a_V133L_S174Q

RTVAAPSVFIFPPSDEQLKSGTASV**L**C**Y**LNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTY**Q**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 74: Cκ_2133a_V133L_S174D

RTVAAPSVFIFPPSDEQLKSGTASV**L**C**Y**LNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTY**D**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 75: (-)PG1_AB2133a_MR (Pertuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**K**APGKGLEWVADVNP
NSGGSIYNQ**E**FKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**R**T**G**PAVLQSSGLYSLSS**M**VTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCK
VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPG

SEQ ID NO. 76: (-)PG1_AB2133a_IR (Pertuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**K**APGKGLEWVADVNP
NSGGSIYNQ**E**FKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**R**T**G**PAVLQSSGLYSLSS**I**VTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 77: (-)MetG1_AB2133a _MR (MetMAb)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYWLHWVR**K**APGKGLEWVGMIDP
SNSDTRFNP**E**FKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCATYRSYVTPLD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**R**T**G**PAVLQSSGLYSLSS**M**VTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCK
VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPG

SEQ ID NO. 78: (-)MetG1_AB2133a _IR (MetMAb)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYWLHWVR**K**APGKGLEWVGMIDP
SNSDTRFNP**E**FKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCATYRSYVTPLD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGV**R**T**G**PAVLQSSGLYSLSS**I**VTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 79: Pκ_AB2133a_LD (Pertuzumab)

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASV**L**C**Y**LNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTY**D**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 80: Pκ_AB2133a_LQ (Pertuzumab)

RIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**D**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASV**L**C**Y**LNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTY**Q**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 81: Metκ_AB2133a_LD (MetMAb)

RIQMTQSPSSLSASVGDRVTITCKSSQSLLYTSSQKNYLAWYQ**D**KPGKAPKLLIY
WASTRESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYAYPWTFGQGTKV
EIKRTVAAPSVFIFPPSDEQLKSGTASV**L**C**Y**LNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTY**D**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE
C

SEQ ID NO. 82: Metκ_AB2133a_LQ (MetMAb)

RIQMTQSPSSLSASVGDRVTITCKSSQSLLYTSSQKNYLAWYQ**D**KPGKAPKLLIY
WASTRESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYAYPWTFGQGTKV
EIKRTVAAPSVFIFPPSDEQLKSGTASV**L**C**Y**LNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTY**Q**L**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE
C

SEQ ID NO 83: (+)TG1_H4 (Trastuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR**Y**APGKGLEWVARIYPT
NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
MDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS

WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPG

SEQ ID NO 84: (+)TG1_H4 (Trastuzumab) + CS

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR**Y**APGKGLEWVARIYPT
NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
MDYWGQGTLVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KKVEP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPG

SEQ ID NO 85: (+)TG1_H4 (Trastuzumab) + DR

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR**Y**APGKGLEWVARIYPT
NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
MDYWG**R**GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPG

SEQ ID NO 86: (+)TG1_H4 (Trastuzumab) + CS+DR

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR**Y**APGKGLEWVARIYPT
NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
MDYWG**R**GTLVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KKVEP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPG

SEQ ID NO 87: Tκ_H4 (Trastuzumab)

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQ**R**KPGKAPKLLIYSASFLY
SGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO 88: Tκ_H4 (Trastuzumab) + CS

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQ**R**KPGKAPKLLIYSASFLY
SGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVA
APSVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO 89: Tκ_H4 (Trastuzumab) + DR

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQ**R**KPG**D**APKLLIYSASFLY
SGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO 90: Tκ_H4 (Trastuzumab) + CS+DR

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQ**R**KPG**D**APKLLIYSASFLY
SGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVA
APSVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO 91: (+)PG1 H4WT (Pertuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**Y**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO 92: (+)PG1 H4WT + CS (Pertuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**Y**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWGQGTLVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED

PEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCK
VSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPG

SEQ ID NO 93: (+)PG1 H4WT + DR (Pertuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**Y**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWG**R**GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO 94: (+)PG1 H4WT + CS + DR (Pertuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR**Y**APGKGLEWVADVNP
NSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFD
YWG**R**GTLVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCK
VSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPG

SEQ ID NO. 95: Pκ H4WT + CS (Pertuzumab)

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**R**KPGKAPKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 96: Pκ H4WT + DR (Pertuzumab)

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**R**KPG**D**APKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 97: Pκ H4WT + CS + DR (Pertuzumab)

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQ**R**KPG**D**APKLLIYSASYRY
TGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVA
APSVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 98: (+)MG1 H4WT + CS

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSHWMHWVR**Y**APGQGLEWIGEFN
PSNGRTNYNEKFKSKATMTVDTSTNTAYMELSSLRSEDTAVYYCASRDYDYDG
RYFDYWGQGTLVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVT
VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
VDKKVEP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPG

SEQ ID NO. 99: (+)MG1 H4WT + DR

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSHWMHWVR**Y**APGQGLEWIGEFN
PSNGRTNYNEKFKSKATMTVDTSTNTAYMELSSLRSEDTAVYYCASRDYDYDG
RYFDYWG**R**GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKV
DKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPG

SEQ ID NO. 100: (+)MG1 H4WT + CS+DR

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSHWMHWVR**Y**APGQGLEWIGEFN
PSNGRTNYNEKFKSKATMTVDTSTNTAYMELSSLRSEDTAVYYCASRDYDYDG
RYFDYWG**R**GTLVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVT
VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
VDKKVEP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPG

SEQ ID NO. 101: Mκ H4WT + CS

DIQMTQSPSSLSASVGDRVTITCSASSSVTYMYWYQ**R**KPGKAPKLLIYDTSNLAS
GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSSHIFTFGQGTKVEIKRTVAAP
SVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 102: Mκ H4WT + DR

DIQMTQSPSSLSASVGDRVTITCSASSSVTYMYWYQ**R**KPG**D**APKLLIYDTSNLAS
GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSSHIFTFGQGTKVEIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 103: Mκ H4WT + CS+DR

DIQMTQSPSSLSASVGDRVTITCSASSSVTYMYWYQ**R**KPG**D**APKLLIYDTSNLAS
GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSSHIFTFGQGTKVEIKRTVAAP
SVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 104: (+)BHA10G1 H4WT

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYYLHWVR**Y**APGQGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPG

SEQ ID NO. 105: (+)BHA10G1 H4WT + CS

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYYLHWVR**Y**APGQGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 106: (+)BHA10G1 H4WT + DR

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYYLHWVR**Y**APGQGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WG**R**GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPG

SEQ ID NO. 107: (+)BHA10G1 H4WT + CS+DR

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYYLHWVR**Y**APGQGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WG**R**GTTVTVSSASTKGPSVFPLAP**C**SKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EP**D**S**G**DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQY**Q**STYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSR**K**ELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVL**K**SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPG

SEQ ID NO. 108: (-)TG1 AB2133a (Trastuzumab)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR**K**APGKGLEWVARIYPT
NGYTRYAD**E**VKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
MDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS
WNSGALTSGV**A**T**G**PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNY**D**TTPPVLDSDGSFFLYS**D**LTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPG

SEQ ID NO. 109: BHA10k H4WT

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQ**R**KPGKAPKSLISSASYRY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTYPFTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 110: BHA10k H4WT + CS

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQ**R**KPGKAPKSLISSASYRY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTYPFTFGQGTKVEIKRTVA
APSVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 111: BHA10k H4WT + DR

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQ**R**KPG**D**APKSLISSASYRY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTYPFTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 112: BHA10k H4WT + CS+DR

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQ**R**KPG**D**APKSLISSASYRY
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTYPFTFGQGTKVEIKRTVA
APSVFIFPPS**K**EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 113: Tκ AB2133a (Trastuzumab)

RIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQ**D**KPGKAPKLLIYSASFLY
SGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVC**Y**LNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDSTYSL**W**STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

We Claim:

1. A method for producing a fragment, antigen binding (Fab) comprising:
(1) co-expressing in a host cell:
(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain CH1 domain comprises an alanine substituted at residue 172 and a glycine substituted at residue 174; and
(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and said light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen;
(2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

2. A method for producing a fragment, antigen binding (Fab) comprising:
(1) co-expressing in a host cell:
(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain CH1 domain comprises an arginine substituted at residue 172 and a glycine substituted at residue 174; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and said light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen;

(2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and (3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

3. A method according to any one of Claims 1 or 2 wherein said heavy chain CH1 domain further comprises a methionine or isoleucine substituted at residue 190.

4. A method according to any one of Claims 1 to 3 wherein said light chain constant domain is a kappa isotype and further comprises a leucine substituted at residue 133.

5. A method according to any one of Claims 1 to 4 wherein said light chain constant domain is a kappa isotype and further comprises a glutamine or aspartic acid substituted at residue 174.

6. A method for producing a fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain CH1 domain comprises an alanine substituted at residue 172 and a glycine substituted at residue 174; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and said light chain constant domain comprises a phenylalanine substituted at residue 135 and a tryptophan substituted at residue 176, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen;

(2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and

(3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

7. A method for producing a fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a tyrosine substituted at residue 39 and said heavy chain CH1 domain comprises a WT sequence; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain comprises an arginine substituted at residue 38 and said light chain constant domain comprises a WT sequence, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen;

(2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and

(3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

8. A method for producing a fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a tyrosine substituted at residue 39 and said heavy chain CH1 domain comprises an aspartic acid substituted at residue 228; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain comprises an arginine substituted at residue 38 and said light chain constant domain comprises a lysine substituted at residue 122, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen;

(2) cultivating said host cell under conditions such that said heavy chain variable and constant domains and said light chain variable and constant domains are produced; and

(3) recovering from said host cell a Fab comprising said heavy chain variable and constant domains and said light chain variable and constant domains.

9. A method according to any one of Claims 7 or 8 wherein said heavy chain variable domain further comprises an arginine substituted at residue 105 and said light chain variable domain further comprises an aspartic acid substituted at residue 42.

10. A method according to any one of Claims 1-9 wherein said host cell is a mammalian cell.

11. The method of Claim 10 wherein said host cell is a HEK293 or CHO cell.

12. A method according to any one of Claims 1-11 wherein said IgG heavy chain constant domain is IgG1 or IgG4 isotype.

13. The method according to Claim 12 wherein said IgG heavy chain constant domain is IgG1 isotype.

14. A method according to any one of Claims 1-13 wherein said light chain constant domain is kappa isotype.

15. A method for producing a first and second fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and said first light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 and said second IgG heavy chain constant CH1 domain comprises a WT sequence; and

(d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 and said second light chain constant domain comprises a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains

comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and

(3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains.

16. A method for producing a first and second fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an arginine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and said first light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 and said second IgG heavy chain constant CH1 domain comprises a WT sequence; and

(d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 and said second light chain constant domain comprises a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and

(3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains.

17. A method for producing a first and second fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and

an aspartic acid substituted at residue 38, and said first light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 and said second IgG heavy chain constant CH1 domain comprises an aspartic acid substituted at residue 228; and

(d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 and said second light chain constant domain comprises a lysine substituted at residue 122, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and

(3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains.

18. A method for producing a first and second fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain

variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an arginine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and said first light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 and said second IgG heavy chain constant CH1 domain comprises an aspartic acid substituted at residue 228; and

(d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 and said second light chain constant domain comprises a K substituted at residue 122, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and

(3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light

chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains.

19. A method according to any one of Claims 15-18, wherein said first IgG heavy chain constant CH1 domain further comprises a methionine or isoleucine substituted at residue 190.

20. A method according to any one of Claims 15-19 wherein said first light chain constant domain is is a kappa isotype and further comprises a leucine substituted at residue 133.

21. A method according to any one of Claims 15-20 wherein said first light chain constant domain is is a kappa isotype and further comprises a glutamine or aspartic acid substituted at residue 174.

22. A method according to any one of Claims 15-21 wherein said second heavy chain variable domain further comprises an arginine substituted at residue 105 and said second light chain variable domain further comprises an aspartic acid substituted at residue 42.

23. A method for producing a first and second fragment, antigen binding (Fab) comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding both a first heavy chain variable domain and a first IgG heavy chain constant CH1 domain, wherein said first heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said first IgG heavy chain constant CH1 domain comprises an alanine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding both a first light chain variable domain and a first light chain constant domain, wherein said first light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and said first light chain constant

domain comprises a phenylalanine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding both a second heavy chain variable domain and a second IgG heavy chain constant CH1 domain, wherein said second heavy chain variable domain comprises a tyrosine substituted at residue 39 and said second IgG heavy chain constant CH1 domain comprises a WT sequence; and

(d) a fourth nucleic acid encoding both a second light chain variable domain and a second light chain constant domain, wherein said second light chain variable domain comprises an arginine substituted at residue 38 and said second light chain constant domain comprises a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second heavy chain variable and IgG CH1 constant domains and said first and second light chain variable and constant domains are produced; and

(3) recovering from said host cell a first and second Fab wherein said first Fab comprises said first heavy chain variable and constant domains and said first light chain variable and constant domains, and said second Fab comprises said second heavy chain variable and constant domains and said second light chain variable and constant domains.

24. A method for producing a bispecific antibody comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant

domain comprising an alanine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and a constant domain comprising a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39, and a CH1 constant domain comprising a WT sequence; and

(d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 and a constant domain comprising a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and

(3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain.

25. A method for producing a bispecific antibody comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant

domain comprising an arginine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and a constant domain comprising a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39, and a CH1 constant domain comprising a WT sequence; and

(d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 and a constant domain comprising a WT sequence, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and

(3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain.

26. A method for producing a bispecific antibody comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant domain comprising an alanine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and a constant domain comprising a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39, and a CH1 constant domain comprising an aspartic acid substituted at residue 228 ; and

(d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 and a constant domain comprising a lysine substituted at residue 122, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and

(3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain.

27. A method for producing a bispecific antibody comprising:

(1) co-expressing in a host cell:

(a) a first nucleic acid encoding a first IgG heavy chain, wherein said first heavy chain comprises a variable domain comprising a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and a CH1 constant domain comprising an arginine substituted at residue 172 and a glycine substituted at residue 174;

(b) a second nucleic acid encoding a first light chain, wherein said first light chain comprises a kappa variable domain comprising an arginine substituted at residue 1 and an aspartic acid substituted at residue 38, and a constant domain comprising a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176;

(c) a third nucleic acid encoding a second IgG heavy chain, wherein said second heavy chain comprises a variable domain comprising a tyrosine substituted at residue 39, and a CH1 constant domain comprising an aspartic acid substituted at residue 228 ; and

(d) a fourth nucleic acid encoding a second light chain, wherein said second light chain comprises a variable domain comprising an arginine substituted at residue 38 and a constant domain comprising a lysine substituted at residue 122, wherein each of said first heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a first antigen and further wherein each of said second heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to a second antigen that differs from said first antigen;

(2) cultivating said host cell under conditions such that said first and second IgG heavy chains and said first and second light chains are produced; and

(3) recovering from said host cell a bispecific antibody comprising a first and second fragment, antigen binding (Fab) wherein said first Fab comprises said first IgG heavy chain and said first light chain and said second Fab comprises said second IgG heavy chain and said second light chain.

28. A method according to any one of Claims 24-27, wherein said first IgG heavy chain constant CH1 domain further comprises a methionine or isoleucine substituted at residue 190.

29. A method according to any one of Claims 24-28 wherein said first light chain constant domain is a kappa isotype and further comprises a leucine substituted at residue 133.

30. A method according to any one of Claims 24-29 wherein said first light chain constant domain is a kappa isotype and further comprises a glutamine or aspartic acid substituted at residue 174.

31. A method according to any one of Claims 24-30 wherein said second heavy chain variable domain further comprises an arginine substituted at residue 105 and said second light chain variable domain further comprises an aspartic acid substituted at residue 42.

32. A method according to any one of Claims 24 to 31 wherein one of said first and second IgG heavy chains further comprises a CH3 constant domain comprising a lysine substituted at residue 356 and a lysine substituted at residue 399, and the other of said first and second IgG heavy chains further comprises a CH3 constant domain comprising an aspartic acid substituted at residue 392 and an aspartic acid substituted at residue 409.

33. A method according to any one of Claims 15-32 wherein said host cell is a mammalian cell.

34. The method according to Claim 33 wherein said host cell is a HEK293 or CHO cell.

35. A method according to any one of Claims 15-34 wherein said first and second IgG heavy chain constant domains are IgG1 or IgG4 isotype.

36. The method according to Claim 35 wherein said first and second IgG heavy chain constant domains are IgG1 isotype.

37. A method according to any one of Claims 15 to 36 wherein said first and second light chain constant domains are kappa isotype.

38. A fragment, antigen binding (Fab) produced according to any one of Claims 1-23.

39. A bispecific antibody produced according to any one of Claims 24-32.

40. A host cell comprising:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain constant CH1 domain comprises an alanine substituted at residue 172 and a glycine substituted at residue 174; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38 and said light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen.

41. A host cell comprising:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain constant CH1 domain comprises an arginine substituted at residue 172 and a glycine substituted at residue 174; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38 and said light chain constant domain comprises a tyrosine substituted at residue 135 and a tryptophan substituted at residue 176, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen.

42. A host cell according to any one of Claims 40 or 41 wherein said heavy chain CH1 domain further comprises a methionine or isoleucine substituted at residue 190.

43. A host cell according to any one of Claims 40-42 wherein said light chain constant domain is a kappa isotype and further comprises a leucine substituted at residue 133.

44. A host cell according to any one of Claims 40 to 43 wherein said light chain constant domain is a kappa isotype and further comprises a glutamine or aspartic acid substituted at residue 174.

45. A host cell comprising:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a lysine substituted at residue 39 and a glutamic acid substituted at the residue which is four amino acids upstream of the first residue of HFR3 according to Kabat, and said heavy chain constant CH1 domain comprises an alanine substituted at residue 172 and a glycine substituted at residue 174; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain is a kappa isotype and comprises an arginine substituted at residue 1 and an aspartic acid substituted at residue 38 and said light chain constant domain comprises a phenylalanine substituted at residue 135 and a tryptophan substituted at residue 176, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen.

46. A host cell comprising:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a tyrosine substituted at residue 39 and said heavy chain CH1 domain comprises a WT sequence; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain comprises an arginine substituted at residue 38 and said light chain constant domain comprises a WT sequence, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen.

47. A host cell comprising:

(a) a first nucleic acid encoding both a heavy chain variable domain and an IgG heavy chain constant CH1 domain, wherein said heavy chain variable domain comprises a tyrosine substituted at residue 39 and said heavy chain CH1 domain comprises an aspartic acid substituted at residue 228; and

(b) a second nucleic acid encoding both a light chain variable domain and a light chain constant domain wherein said light chain variable domain comprises an arginine substituted at residue 38 and said light chain constant domain comprises a lysine substituted at residue 122, wherein each of said heavy chain and light chain variable domains comprise three complementarity determining regions (CDRs) which direct binding to the same antigen.

48. A host cell according to any one of Claims 46 or 47 wherein said heavy chain variable domain further comprises an arginine substituted at residue 105 and said light chain variable domain further comprises an aspartic acid substituted at residue 42.

49. A host cell comprising the first and second nucleic acids according to any one of Claims 40 to 45 and the first and second nucleic acids according to any one of Claims 46-48.

50. A host cell according to any one of Claims 40-49 wherein said host cell is a HEK293 or CHO cell.

Fig. 1/3

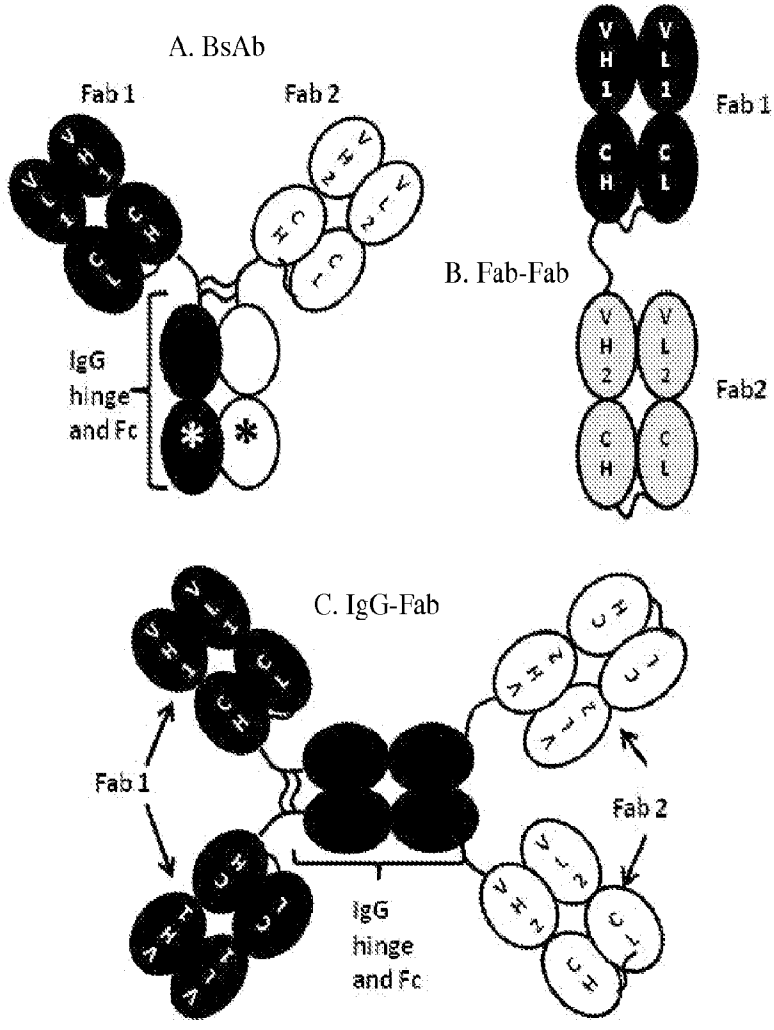


Figure 1. Schematic diagrams of IgG BsAb (*A*), Fab-Fab (*B*) and IgG-Fab(*C*) design formats. The "*" in the IgG BsAb diagram indicates a heterodimerized antibody CH3 domain

Fig. 2/3

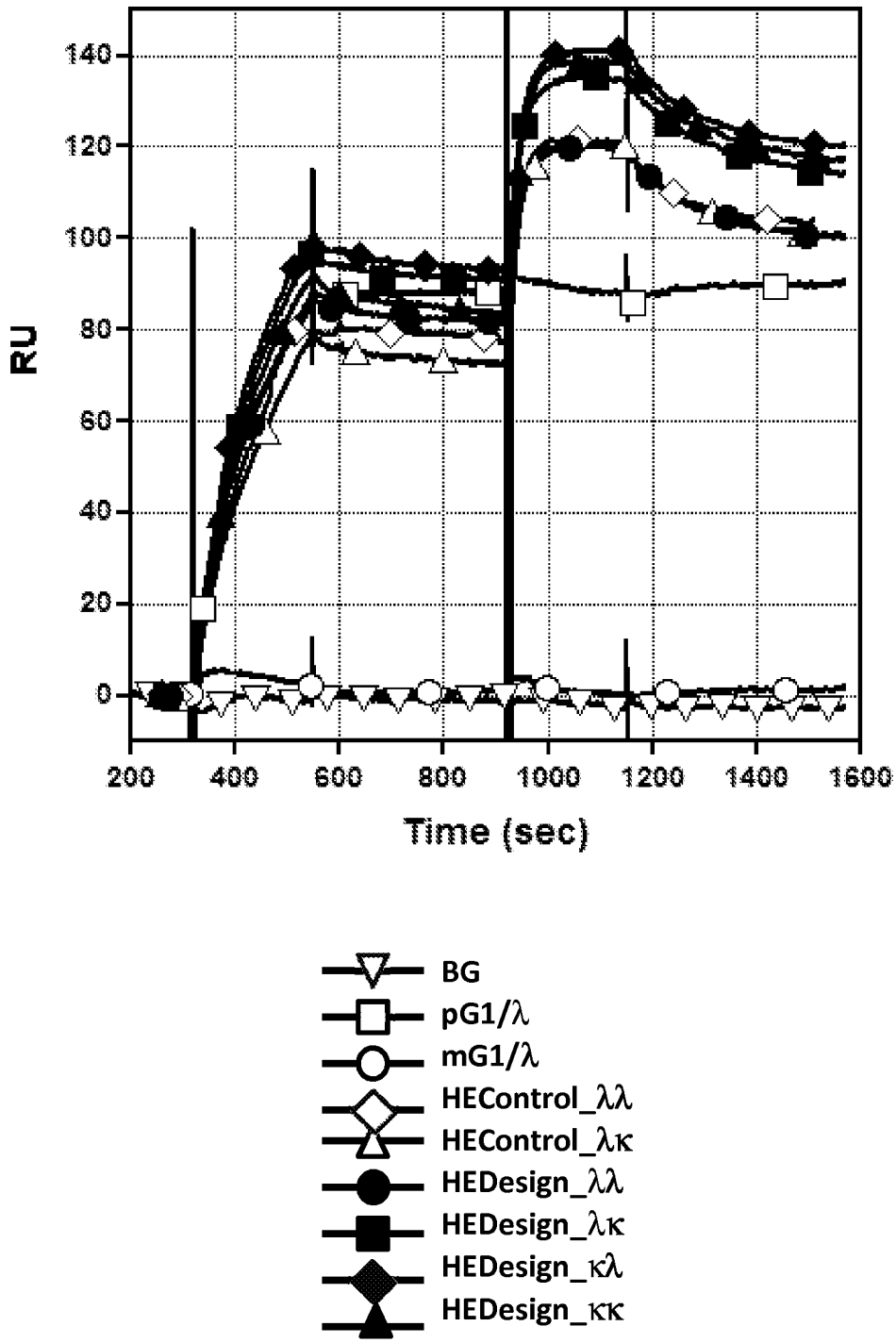
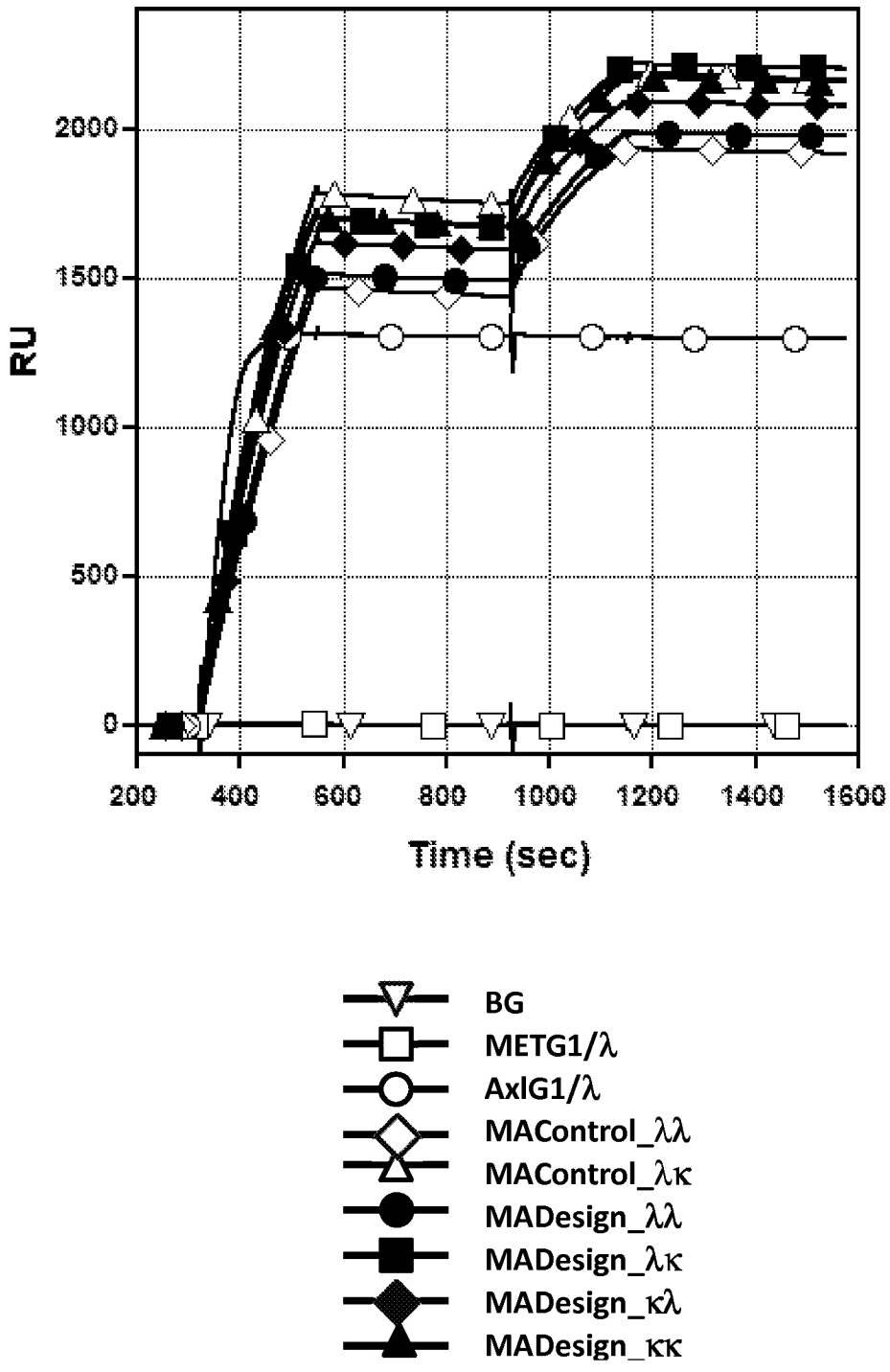

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120
100
80
60
40
20
0
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400
600
800
1000
1200
1400
1600
Time (sec)
BG
pG1/λ
mG1/λ
HEControl_λλ
HEControl_λκ
HEDesign_λλ
HEDesign_λκ
HEDesign_κλ
HEDesign_κκ

Fig. 3/3


2000
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Time (sec)
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AxlG1/λ
MAControl_λλ
MAControl_λκ
MADesign_λλ
MADesign_λκ
MADesign_κλ
MADesign_κκ

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024688

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JACOB L. JORDAN ET AL: "Structural understanding of stabilization patterns in engineered bispecific Ig-like antibody molecules", PROTEINS: STRUCTURE, FUNCTION, AND BIOINFORMATICS, vol. 77, no. 4, 1 December 2009 (2009-12-01), pages 832-841, XP055002049, ISSN: 0887-3585, DOI: 10.1002/prot.22502 The whole document, in particular Table 1 ----- -/--	1-23, 33-38, 40-50

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 June 2014	02/07/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Chapman, Rob

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/024688

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   - [ ] on paper
   - [X] in electronic form

   b. (time)

   - [X] in the international application as filed
   - [ ] together with the international application in electronic form
   - [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024688

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W. SCHAEFER ET AL: "Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 108, no. 27, 5 July 2011 (2011-07-05) , pages 11187-11192, XP055003817, ISSN: 0027-8424, DOI: 10.1073/pnas.1019002108 the whole document -----	24-50
X	WO 2007/109254 A2 (BIOGEN IDEC INC [US]; DEMAREST STEPHEN [US]; GLASER SCOTT [US]; MILLER) 27 September 2007 (2007-09-27) the whole document -----	1-23, 33-38, 40-50
A	STEVEN M. LEWIS ET AL: "Anchored Design of Protein-Protein Interfaces", PLOS ONE, vol. 6, no. 6, 17 June 2011 (2011-06-17), page e20872, XP055122659, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0020872 cited in the application the whole document -----	1-50
A	K. GUNASEKARAN ET AL: "Enhancing Antibody Fc Heterodimer Formation through Electrostatic Steering Effects: APPLICATIONS TO BISPECIFIC MOLECULES AND MONOVALENT IgG", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 285, no. 25, 18 June 2010 (2010-06-18), pages 19637-19646, XP055001947, ISSN: 0021-9258, DOI: 10.1074/jbc.M110.117382 the whole document -----	1-50
A	Ivana Spasevska: "An outlook on bispecific antibodies: Methods of production and therapeutic benefits", BioSciences Master Reviews, 21 January 2013 (2013-01-21), XP055122817, Retrieved from the Internet: URL:http://biologie.ens-lyon.fr/biologie/ressources/bibliographies/pdf/m1-12-13-biosci-reviews-spasevska-i-2c-m.pdf?lang=en [retrieved on 2014-06-11] the whole document -----	1-50

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024688

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	STEVEN M LEWIS ET AL: "Generation of bispecific IgG antibodies by structure-based design of an orthogonal Fab interface", NATURE BIOTECHNOLOGY, vol. 32, no. 2, 1 February 2014 (2014-02-01), pages 191-198, XP055122831, ISSN: 1087-0156, DOI: 10.1038/nbt.2797 the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2014/024688

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2011301331 A1	08-12-2011
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