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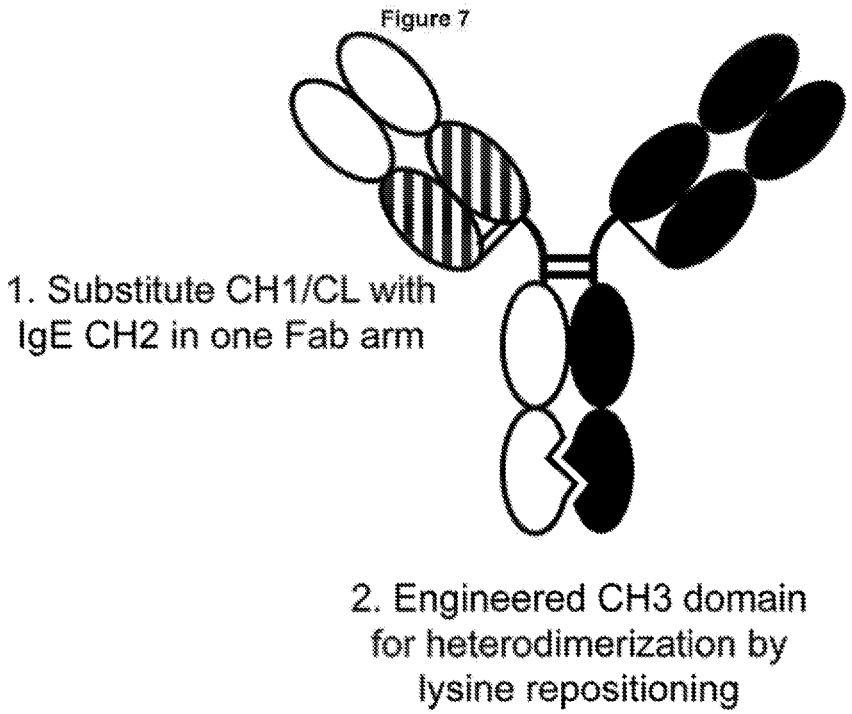
(54) Title: BISPECIFIC ANTIBODY PLATFORM

Figure 7



(57) Abstract: Provided herein are solutions to the heavy and light chain mispairing problems in bispecific antibodies. One part of the solution involves Fc regions that are engineered in their CH3 domains by lysine repositioning to drive heterodimerization of the two heavy chains of the bispecific antibody. The second part of the solution involves a modification of one of the two Fab arms of the bispecific antibody so as to prevent mispairing of light chains in the bispecific antibody. Specifically, the CH1 and CL domains of one of the Fab arms of the bispecific antibody are substituted with an IgE CH2 domain or an IgM CH2 domain.

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BISPECIFIC ANTIBODY PLATFORM

Cross-Reference to Related Application

This application claims the benefit of priority to U.S. Provisional Appl. No. 62/269,664, filed December 18, 2015, the contents of which are incorporated by reference in their entirety herein.

Background

There is increasing interest in the use of bispecific antibodies as biologics drugs due, in large part, to the potential of achieving novel mechanisms of action that cannot be achieved with a combination of two conventional monospecific antibodies. Efficient methods for generating bispecific antibodies are therefore sought after. Initial attempts to produce bispecific antibodies as biologics drugs involved chemical conjugation of monospecific antibodies and fusion of mAb-expressing cells, but low efficiency and the necessity of purification from abundant side products are disadvantages of these strategies. Advanced methods in protein engineering and molecular biology have enabled the generation of a variety of new bispecific antibody formats. However, altered biochemical/biophysical properties, serum half-life, or stability of these engineered bispecific antibody formats can be unfavorable. Thus, an efficient platform for the generation of bispecific antibodies that might overcome some of these problems would be useful.

Summary

This application relates to an antibody platform technology that can convert any two antibodies that bind to different epitopes into a bispecific antibody. This platform technology involves, in part, Fc regions that are engineered in their CH3 domains by lysine repositioning to drive heterodimerization of the two heavy chains of the bispecific antibody. In addition, this technology involves a modification of one of the two Fab arms of the bispecific antibody so as to prevent mispairing of light chains in the bispecific antibody. Specifically, the CH1 and CL domains of one of the Fab arms of the bispecific antibody are substituted with an IgE CH2 domain or an IgM CH2 domain. In some instances, the CH1 and CL domains of one of the Fab arms of the bispecific antibody are substituted with a fragment of an IgE CH2 domain (or an IgM

CH2 domain), wherein the fragment can still dimerize with the IgE CH2 domain (or the IgM CH2 domain).

In one aspect, this disclosure provides an antibody or antigen-binding fragment thereof that comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen. The first VH is either directly linked or linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (SEQ ID NO:1). The first VL is either directly linked or linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The first polypeptide and the second polypeptide pair to form a dimer.

In certain embodiments of this aspect, the first polypeptide and/or the second polypeptide is directly linked to an Fc domain. In certain instances, the Fc domain comprises a CH2 and CH3 domain of an IgG1 antibody. In certain instances, the Fc domain comprises a CH2 and CH3 domain of an IgG4 antibody. In certain instances, the Fc domain comprises a CH2 of an IgG4 antibody and a CH3 domain of an IgG1 antibody. In some of all of these embodiments, the Fc domain comprises a hinge region of an IgG4 antibody (e.g., IgG4P – i.e., IgG4 hinge region with the S228P mutation). In some embodiments, the first polypeptide and/or the second polypeptide comprise the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In certain embodiments, the first polypeptide and/or the second polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:5. In a specific embodiment, the first polypeptide comprises the amino acid sequence set forth in SEQ ID NO:5. In some embodiments, the second polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:6. In a specific embodiment, the second polypeptide comprises an amino acid sequence that is identical to the amino acid sequence set forth in SEQ ID NO:6. In some embodiments, the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in

amino acids 9-107 of SEQ ID NO:1 at twelve or fewer amino acid residues. In some embodiments, the first polypeptide and/or the second polypeptide contain an amino acid other than cysteine at at least one of the two cysteine residues of SEQ ID NO:1 that do not form an intrachain disulfide bond. In certain embodiments, the first polypeptide and the second polypeptide each contain a mutation of the N-linked glycosylation site in the IgE CH2 domain or fragment thereof such that the N-linked glycosylation site is not glycosylated (*e.g.*, the asparagine and/or threonine residue is substituted with another amino acid). In certain embodiments, the first polypeptide or the second polypeptide contain a mutation of the N-linked glycosylation site in the IgE CH2 domain or fragment thereof such that the N-linked glycosylation site is not glycosylated. In certain embodiments, the antibody or antigen-binding fragment thereof comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen. In certain embodiments, the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the antibody or antigen-binding fragment described above. In some embodiments, expression vectors are provided comprising the polynucleotide or polynucleotides described above. In other embodiments, host cells comprising the polynucleotide or polynucleotides or the expression vectors are provided. In yet other embodiments, provided are methods of making the antibody or antigen-binding fragment described above comprising culturing the host cells described above under conditions that result in the expression of the antibody or antigen-binding fragment described above and isolating the antibody or antigen-binding fragment from the cell culture. In certain embodiments, the isolated antibody or antigen-binding fragment is formulated as a sterile composition for administration to a human subject in need thereof.

In a second aspect, the disclosure provides an antibody or antigen-binding fragment thereof comprising a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the

CH2 domain of human immunoglobulin M (IgM) (SEQ ID NO:2). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The first polypeptide and the second polypeptide pair to form a dimer.

In certain embodiments of this aspect, the first polypeptide and/or the second polypeptide is directly linked to an Fc domain. In certain instances, the Fc domain comprises a CH2 and CH3 domain of an IgG1 antibody. In certain instances, the Fc domain comprises a CH2 and CH3 domain of an IgG4 antibody. In certain instances, the Fc domain comprises a CH2 of an IgG4 antibody and a CH3 domain of an IgG1 antibody. In some of all of these embodiments, the Fc domain comprises a hinge region of an IgG4 antibody (e.g., IgG4P – i.e., IgG4 hinge region with the S228P mutation). In some embodiments, the first polypeptide and/or the second polypeptide comprise the amino acid sequence set forth in SEQ ID NO:2. In some embodiments, the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide and/or the second polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In some embodiments, the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in amino acids 7-112 of SEQ ID NO:2 at twelve or fewer amino acid residues. In some embodiments, the first polypeptide and/or the second polypeptide contain an amino acid other than cysteine at the cysteine residue of SEQ ID NO:2 that does not form an intrachain disulfide bond. In certain embodiments, the first polypeptide and the second polypeptide each contain a mutation of the N-linked glycosylation site in the IgM CH2 domain or fragment thereof such that the N-linked glycosylation site is not glycosylated (*e.g.,* the asparagine and/or serine residue is substituted with another amino acid). In certain embodiments, the first polypeptide or the second polypeptide contain a mutation of the N-linked glycosylation site in the IgM CH2 domain or fragment thereof such that the N-linked glycosylation site is not glycosylated. In certain embodiments, the antibody or antigen-binding fragment thereof comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen. In certain embodiments, the second VH

is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the antibody or antigen-binding fragment described above. In some embodiments, expression vectors are provided comprising the polynucleotide or polynucleotides described above. In other embodiments, host cells comprising the polynucleotide or polynucleotides or the expression vectors are provided. In yet other embodiments, provided are methods of making the antibody or antigen-binding fragment described above comprising culturing the host cells described above under conditions that result in the expression of the antibody or antigen-binding fragment described above and isolating the antibody or antigen-binding fragment from the cell culture. In certain embodiments, the isolated antibody or antigen-binding fragment is formulated as a sterile composition for administration to a human subject in need thereof.

In another aspect, the disclosure provides a bispecific antibody that comprises a first fragment antigen-binding (first Fab) comprising a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (SEQ ID NO:1). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The bispecific antibody further comprises a second Fab comprising a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region, and wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain. The first Fab and the second Fab specifically bind to different antigens or to different epitopes of the same antigen and the first Fab is connected to the second Fab.

In some embodiments of this aspect, the first Fab is connected to the second Fab by a linker. In certain embodiments, the first Fab is connected to the second Fab by a heterologous polypeptide. In some embodiments, the heterologous polypeptide is human serum albumin. In some embodiments, the heterologous polypeptide is an XTEN (*e.g.*, AE144, AE288). In some embodiments, the first Fab is connected to the second Fab by polyethylene glycol (PEG). In

certain embodiments, the first polypeptide and/or the second polypeptide each comprise the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In certain embodiments, the first polypeptide and/or the second polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:5. In some embodiments, the first polypeptide comprises the amino acid sequence set forth in SEQ ID NO:5. In certain embodiments, the second polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:6. In some embodiments, the second polypeptide comprises the amino acid sequence set forth in SEQ ID NO:6. In certain embodiments, the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in amino acids 9-107 of SEQ ID NO:1 at twelve amino acid residues or fewer. In other embodiments, the first polypeptide and/or the second polypeptide contain an amino acid other than cysteine at at least one of the two cysteine residues of SEQ ID NO:1 that can form an interchain disulfide bond. In certain embodiments, the first polypeptide and/or the second polypeptide contain a mutation of the N-linked glycosylation site in the IgE CH2 domain or fragment thereof such that the N-linked glycosylation site is not glycosylated. For example, the asparagine and/or threonine of the IgE CH2 domain N-linked glycosylation site may be substituted with another amino acid to prevent glycosylation of this motif. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the bispecific antibody described above. In some embodiments, expression vectors are provided comprising the bispecific antibody described above. In other embodiments, host cells comprising the bispecific antibody or the expression vectors are provided. In yet other embodiments, provided are methods of making the bispecific antibody comprising culturing the host cells described above under conditions that result in the expression of the bispecific antibody and isolating the bispecific antibody from the cell culture. In certain embodiments, the isolated bispecific antibody is formulated as a sterile composition for administration to a human subject in need thereof.

In another aspect, this disclosure provides a bispecific antibody comprising a first fragment antigen-binding (first Fab) comprising a first VH and a first VL, wherein the first VH

and the first VL pair to form a first variable region. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (SEQ ID NO:2). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The bispecific antibody further comprises a second Fab comprising a second VH and a second VL. The second VH and the second VL pair to form a second variable region, and wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain. The first Fab and the second Fab specifically bind to different antigens or to different epitopes of the same antigen. The first Fab is connected to the second Fab.

In some embodiments of this aspect, the first Fab is connected to the second Fab by a linker. In certain embodiments, the first Fab is connected to the second Fab by a heterologous polypeptide. In some embodiments, the heterologous polypeptide is human serum albumin. In some embodiments, the heterologous polypeptide is an XTEN (*e.g.,* AE144, AE288). In some embodiments, the first Fab is connected to the second Fab by polyethylene glycol (PEG). In certain embodiments, the first polypeptide and/or the second polypeptide each comprise the amino acid sequence set forth in SEQ ID NO:2. In some embodiments, the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide and/or the second polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in amino acids 7-112 of SEQ ID NO:2 at twelve amino acid residues or fewer. In other embodiments, the first polypeptide and/or the second polypeptide contain an amino acid other than cysteine at the cysteine residue of SEQ ID NO:2 that can form an interchain disulfide bond. In certain embodiments, the first polypeptide and/or the second polypeptide contain a mutation of the N-linked glycosylation site in the IgM CH2 domain or fragment thereof such that the N-linked glycosylation site is not glycosylated. For example, the asparagine and/or serine of the IgM CH2 domain N-linked glycosylation site may be substituted with another amino acid to

prevent glycosylation of this motif. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the bispecific antibody described above. In some embodiments, expression vectors are provided comprising the bispecific antibody described above. In other embodiments, host cells comprising the bispecific antibody or the expression vectors are provided. In yet other embodiments, provided are methods of making the bispecific antibody comprising culturing the host cells described above under conditions that result in the expression of the bispecific antibody and isolating the bispecific antibody from the cell culture. In certain embodiments, the isolated bispecific antibody is formulated as a sterile composition for administration to a human subject in need thereof.

In another aspect, this disclosure provides a tetravalent bispecific antibody that comprises a whole IgG antibody that specifically binds to a first epitope of a first antigen, the whole IgG antibody comprising a first CH3 domain and a second CH3 domain; and a first Fab and a second Fab. The first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a second epitope of the first antigen or to a second antigen. The second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the same epitope as the first Fab. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (SEQ ID NO:1). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The second VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The second VL is either (i) directly linked or (ii) linked via a linker to a fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The first Fab is connected to the C-terminus of the first CH3 domain of the whole IgG antibody and

the second Fab is connected to the C-terminus of the second CH3 domain of the whole IgG antibody.

In some embodiments of this aspect, the first Fab is connected to the C-terminus of the first CH3 domain of the whole antibody through a first linker and the second Fab is connected to the C-terminus of the second CH3 domain of the whole antibody through a second linker. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the first VH. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the first VL. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the first polypeptide. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the second polypeptide. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the second VH. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the second VL. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the third polypeptide. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the fourth polypeptide. In certain embodiments, the first and second linkers are peptide linkers. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise the amino acid sequence set forth in SEQ ID NO:1. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide and the third polypeptide each comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:5. In certain embodiments, the first polypeptide and the third polypeptide each comprise the amino acid sequence set forth in SEQ ID NO:5. In certain embodiments, the second polypeptide and the fourth polypeptide each comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set

forth in SEQ ID NO:6. In some embodiments, the second polypeptide and the fourth polypeptide each comprise the amino acid sequence set forth in SEQ ID NO:6. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in amino acids 9-107 of SEQ ID NO:1 at at least twelve amino acid residues. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the bispecific antibody described above. In some embodiments, expression vectors are provided comprising the bispecific antibody described above. In other embodiments, host cells comprising the bispecific antibody or the expression vectors are provided. In yet other embodiments, provided are methods of making the bispecific antibody comprising culturing the host cells described above under conditions that result in the expression of the bispecific antibody and isolating the bispecific antibody from the cell culture. In certain embodiments, the isolated bispecific antibody is formulated as a sterile composition for administration to a human subject in need thereof.

In another aspect, this disclosure provides a tetravalent bispecific antibody comprising a whole IgG antibody that specifically binds to a first epitope of a first antigen, the whole IgG antibody comprising a first CH3 domain and a second CH3 domain; and a first Fab and a second Fab. The first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a second epitope of the first antigen or to a second antigen. The second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the same epitope as the first Fab. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (SEQ ID NO:2). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The second VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The second VL is either (i) directly linked or (ii) linked via a linker to a

fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The first Fab is connected to the C-terminus of the first CH3 domain of the whole IgG antibody and the second Fab is connected to the C-terminus of the second CH3 domain of the whole IgG antibody.

In some embodiments of this aspect, the first Fab is connected to the C-terminus of the first CH3 domain of the whole antibody through a first linker and the second Fab is connected to the C-terminus of the second CH3 domain of the whole antibody through a second linker. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the first VH. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the first VL. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the first polypeptide. In certain embodiments, the first linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the second polypeptide. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the second VH. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the second VL. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the third polypeptide. In certain embodiments, the second linker links the C-terminus of the first CH3 domain of the whole antibody and the N-terminus of the fourth polypeptide. In certain embodiments, the first and second linkers are peptide linkers. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in amino acids 7-112 of SEQ ID NO:2 at at least twelve

amino acid residues. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the bispecific antibody described above. In some embodiments, expression vectors are provided comprising the bispecific antibody described above. In other embodiments, host cells comprising the bispecific antibody or the expression vectors are provided. In yet other embodiments, provided are methods of making the bispecific antibody comprising culturing the host cells described above under conditions that result in the expression of the bispecific antibody and isolating the bispecific antibody from the cell culture. In certain embodiments, the isolated bispecific antibody is formulated as a sterile composition for administration to a human subject in need thereof.

In another aspect, the disclosure relates to a tetravalent bispecific antibody comprising a first Fab and a second Fab, wherein the first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, and wherein the second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the first epitope of the first antigen. The tetravalent bispecific antibody also comprises a whole antibody comprising a first heavy chain comprising a first IgG CH2 domain and a first IgG CH3 domain, a second heavy chain comprising a second IgG CH2 domain and a second IgG CH3 domain, a first light chain, and a second light chain, wherein the antibody comprises a third VH and third VL and a fourth VH and a fourth VL, wherein the third VH and the third VL pair to form a third variable region that binds specifically to an epitope of a second antigen, and wherein the fourth VH and the fourth VL pair to form a fourth variable region that binds specifically to the same epitope of the second antigen. The third VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (SEQ ID NO:1). The third VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The fourth VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the

CH2 domain of human IgE (SEQ ID NO:1). The fourth VL is either (i) directly linked or (ii) linked via a linker to a fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The first polypeptide is connected to the N-terminus of the first IgG CH2 domain and the third polypeptide is connected to the N-terminus of the second IgG CH2 domain. The first Fab is connected to the C-terminus of the first IgG CH3 domain and the second Fab is connected to the C-terminus of the second IgG CH3 domain.

In some embodiments of this aspect, the first Fab is connected to the C-terminus of the first CH3 domain of the whole antibody through a first linker and the second Fab is connected to the C-terminus of the second CH3 domain of the whole antibody through a second linker. In certain embodiments, the first and second linkers are peptide linkers. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise the amino acid sequence set forth in SEQ ID NO:1. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the first polypeptide and the third polypeptide each comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:5. In certain embodiments, the first polypeptide and the third polypeptide each comprise the amino acid sequence set forth in SEQ ID NO:5. In certain embodiments, the second polypeptide and the fourth polypeptide each comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:6. In some embodiments, the second polypeptide and the fourth polypeptide each comprise the amino acid sequence set forth in SEQ ID NO:6. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in amino acids 9-107 of SEQ ID NO:1 at at least twelve amino acid residues. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the bispecific antibody described above. In some embodiments, expression vectors are provided comprising the bispecific antibody described above. In other embodiments, host cells comprising the bispecific antibody or the expression

vectors are provided. In yet other embodiments, provided are methods of making the bispecific antibody comprising culturing the host cells described above under conditions that result in the expression of the bispecific antibody and isolating the bispecific antibody from the cell culture. In certain embodiments, the isolated bispecific antibody is formulated as a sterile composition for administration to a human subject in need thereof.

In another aspect, the disclosure relates to a tetravalent bispecific antibody comprising a first Fab and a second Fab, wherein the first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, and wherein the second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the first epitope of the first antigen. The tetravalent bispecific antibody also comprises a whole antibody comprising a first heavy chain comprising a first IgG CH2 domain and a first IgG CH3 domain, a second heavy chain comprising a second IgG CH2 domain and a second IgG CH3 domain, a first light chain, and a second light chain, wherein the antibody comprises a third VH and third VL and a fourth VH and a fourth VL, wherein the third VH and the third VL pair to form a third variable region that binds specifically to an epitope of a second antigen, and wherein the fourth VH and the fourth VL pair to form a fourth variable region that binds specifically to the same epitope of the second antigen. The third VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (SEQ ID NO:2). The third VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The fourth VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The fourth VL is either (i) directly linked or (ii) linked via a linker to a fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The first polypeptide is connected to the N-terminus of the first IgG CH2

domain and the third polypeptide is connected to the N-terminus of the second IgG CH2 domain. The first Fab is connected to the C-terminus of the first IgG CH3 domain and the second Fab is connected to the C-terminus of the second IgG CH3 domain.

In some embodiments of this aspect, the first Fab is connected to the C-terminus of the first CH3 domain of the whole antibody through a first linker and the second Fab is connected to the C-terminus of the second CH3 domain of the whole antibody through a second linker. In certain embodiments, the first and second linkers are peptide linkers. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In some embodiments, the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in amino acids 7-112 of SEQ ID NO:2 at at least twelve amino acid residues. In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the bispecific antibody described above. In some embodiments, expression vectors are provided comprising the bispecific antibody described above. In other embodiments, host cells comprising the bispecific antibody or the expression vectors are provided. In yet other embodiments, provided are methods of making the bispecific antibody comprising culturing the host cells described above under conditions that result in the expression of the bispecific antibody and isolating the bispecific antibody from the cell culture. In certain embodiments, the isolated bispecific antibody is formulated as a sterile composition for administration to a human subject in need thereof.

In yet another aspect, the disclosure provides a heterodimerization module comprising a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a serine; and a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acid at position 370 is a serine and the amino acids at positions 405 and 409

are lysines. The amino acid positions are based on the EU numbering system. The first IgG1 CH3 domain and the second IgG1 CH3 pair to form a heterodimer.

In certain embodiments of this aspect, the heterodimerization module includes a first IgG1 CH2 domain and a second IgG1 CH2 domain, wherein the first IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain.

In some embodiments, the heterodimerization module includes a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is linked to the N-terminus of a first hinge region, and wherein the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain.

In other embodiments, the heterodimerization module comprises a linker that links the C-terminus of the first IgG1 CH3 domain to the N-terminus of a second hinge region that is linked to the N-terminus of the second IgG1 CH2 domain. In certain embodiments, the heterodimerization module includes a second Fab linked via a second hinge region to the N-terminus of the second IgG1 CH2 domain.

In certain embodiments, the heterodimerization module comprises a VH domain, a CH1 domain, a VL domain, and a CL domain. The C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first hinge region, and the C-terminus of the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain that is directly linked to the first IgG1 CH3 domain. The C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second hinge region, and the C-terminus of the second hinge region is linked to the N-terminus of the second IgG1 CH2 domain that is directly linked to the second IgG1 CH3 domain. The VH domain and the VL domain pair to form a variable region that binds specifically to an antigen.

In certain embodiments, the heterodimerization module comprises a first VH and a first VL and a second VH and a second VL. The first VH and the first VL pair to form a first variable region that binds specifically to a first antigen. The second VH and the second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a

second antigen. In some instances, the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

In certain embodiments, the heterodimerization module comprises a first IgG4 CH2 domain and a second IgG4 CH2 domain, wherein the first IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain. In some instances, the heterodimerization module includes a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is linked to the N-terminus of a first IgG4 hinge region, and wherein the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain. In some instances, the first IgG4 hinge region comprises the S228P mutation (EU numbering). In some instances, the heterodimerization module includes a linker that links the C-terminus of the first IgG1 CH3 domain to the N-terminus of a second IgG4 hinge region that is linked to the N-terminus of the second IgG4 CH2 domain. In certain instances, the second IgG4 hinge region comprises the S228P mutation (EU numbering). In some instances, the heterodimerization module includes a second Fab linked via a second IgG4 hinge region to the N-terminus of the second IgG4 CH2 domain. In certain instances, the second IgG4 hinge region comprises the S228P mutation (EU numbering).

In some embodiments, the heterodimerization module comprises a VH domain, a CH1 domain, a VL domain, and a CL domain. The C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first IgG4 hinge region, and the C-terminus of the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain that is directly linked to the first IgG1 CH3 domain. The C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second IgG4 hinge region, and the C-terminus of the second IgG4 hinge region is linked to the N-terminus of the second IgG4 CH2 domain that is directly linked to the second IgG1 CH3 domain. The VH domain and the VL domain pair to form a variable region that binds specifically to an antigen. In some instances, the first IgG4 hinge region and the second IgG4 hinge region each comprise the S228P mutation (EU numbering).

In some embodiments, the heterodimerization module comprises a first VH and a first VL and a second VH and a second VL. The first VH and the first VL pair to form a first variable region that binds specifically to a first antigen. The second VH and the second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a second antigen. In some instances, the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

In certain embodiments, the disclosure features a polynucleotide or polynucleotides that encode the above-described heterodimerization module. In some instances, an expression vector or expression vectors comprising the polynucleotide or polynucleotides are featured. In other instances, host cells are provided that comprise the polynucleotide or polynucleotides or expression vector or vectors. In some instances, methods of producing the heterodimerization module are encompassed. The methods involve culturing the host cells under conditions that result in expression of the heterodimerization module and isolation thereof.

In another aspect, the disclosure relates to a heterodimerization module comprising a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a leucine; and a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acid at position 370 is a serine, the amino acid at position 397 is an isoleucine, and the amino acids at positions 405 and 409 are lysines. The amino acid positions are based on the EU numbering system. The first IgG1 CH3 domain and the second IgG1 CH3 pair to form a heterodimer.

In certain embodiments of this aspect, the heterodimerization module includes a first IgG1 CH2 domain and a second IgG1 CH2 domain, wherein the first IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain.

In some embodiments, the heterodimerization module includes a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is linked to the N-terminus of a first hinge region, and wherein the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain.

In other embodiments, the heterodimerization module comprises a linker that links the C-terminus of the first IgG1 CH3 domain to the N-terminus of a second hinge region that is linked to the N-terminus of the second IgG1 CH2 domain. In certain embodiments, the heterodimerization module includes a second Fab linked via a second hinge region to the N-terminus of the second IgG1 CH2 domain.

In certain embodiments, the heterodimerization module comprises a VH domain, a CH1 domain, a VL domain, and a CL domain. The C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first hinge region, and the C-terminus of the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain that is directly linked to the first IgG1 CH3 domain. The C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second hinge region, and the C-terminus of the second hinge region is linked to the N-terminus of the second IgG1 CH2 domain that is directly linked to the second IgG1 CH3 domain. The VH domain and the VL domain pair to form a variable region that binds specifically to an antigen.

In certain embodiments, the heterodimerization module comprises a first VH and a first VL and a second VH and a second VL. The first VH and the first VL pair to form a first variable region that binds specifically to a first antigen. The second VH and the second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a second antigen. In some instances, the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

In certain embodiments, the heterodimerization module comprises a first IgG4 CH2 domain and a second IgG4 CH2 domain, wherein the first IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain. In some instances, the heterodimerization module includes a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is linked to the N-terminus of a first IgG4 hinge region, and wherein the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain. In some instances, the first IgG4 hinge region comprises the S228P mutation (EU numbering). In some instances, the heterodimerization module includes a linker that links the C-terminus of the first IgG1 CH3

domain to the N-terminus of a second IgG4 hinge region that is linked to the N-terminus of the second IgG4 CH2 domain. In certain instances, the second IgG4 hinge region comprises the S228P mutation (EU numbering). In some instances, the heterodimerization module includes a second Fab linked via a second IgG4 hinge region to the N-terminus of the second IgG4 CH2 domain. In certain instances, the second IgG4 hinge region comprises the S228P mutation (EU numbering).

In some embodiments, the heterodimerization module comprises a VH domain, a CH1 domain, a VL domain, and a CL domain. The C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first IgG4 hinge region, and the C-terminus of the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain that is directly linked to the first IgG1 CH3 domain. The C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second IgG4 hinge region, and the C-terminus of the second IgG4 hinge region is linked to the N-terminus of the second IgG4 CH2 domain that is directly linked to the second IgG1 CH3 domain. The VH domain and the VL domain pair to form a variable region that binds specifically to an antigen. In some instances, the first IgG4 hinge region and the second IgG4 hinge region each comprise the S228P mutation (EU numbering).

In some embodiments, the heterodimerization module comprises a first VH and a first VL and a second VH and a second VL. The first VH and the first VL pair to form a first variable region that binds specifically to a first antigen. The second VH and the second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a second antigen. In some instances, the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

In certain embodiments, the disclosure features a polynucleotide or polynucleotides that encode the above-described heterodimerization module. In some instances, an expression vector or expression vectors comprising the polynucleotide or polynucleotides are featured. In other instances, host cells are provided that comprise the polynucleotide or polynucleotides or expression vector or vectors. In some instances, methods of producing the heterodimerization module are encompassed. The methods involve culturing the host cells under conditions that result in expression of the heterodimerization module and isolation thereof.

In another aspect, the disclosure provides a bispecific antibody that includes a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (SEQ ID NO:1). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (SEQ ID NO:1). The first polypeptide and the second polypeptide pair to form a dimer. The bispecific antibody also includes a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen. The second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain. The second VL is either (i) directly linked or (ii) linked via a linker to a CL domain. The CH1 domain and the CL domain pair to form a dimer. The bispecific antibody also includes a heterodimerization module comprising a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a serine. The heterodimerization module also includes a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acid at position 370 is a serine and the amino acids at positions 405 and 409 are lysines. The amino acid positions above are all based on the EU numbering system. (It is important to note that the IgE CH2 domain can be part of the polypeptide comprising the first IgG1 CH3 domain or the second IgG1 CH3 domain.)

In another aspect, the disclosure provides a bispecific antibody that includes a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (SEQ ID NO:1). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE

(SEQ ID NO:1). The first polypeptide and the second polypeptide pair to form a dimer. The bispecific antibody also includes a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen. The second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain. The second VL is either (i) directly linked or (ii) linked via a linker to a CL domain. The CH1 domain and the CL domain pair to form a dimer. The bispecific antibody also includes a heterodimerization module comprising a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a leucine. The heterodimerization module also includes a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acid at position 370 is a serine, the amino acid at position 397 is an isoleucine, and the amino acids at positions 405 and 409 are lysines. The amino acid positions above are all based on the EU numbering system. (It is important to note that the IgE CH2 domain can be part of the polypeptide comprising the first IgG1 CH3 domain or the second IgG1 CH3 domain.)

In some embodiments of the above two aspects, the bispecific antibody includes two IgG1 CH2 domains. In some embodiments of the above two aspects, the bispecific antibody includes two IgG4 CH2 domains. In some embodiments, a single polypeptide chain comprises the first VH, the first polypeptide, and the first IgG1 CH3 domain. In some embodiments, a single polypeptide chain comprises the first VH, the first polypeptide, and the second IgG1 CH3 domain. In other embodiments, a single polypeptide chain comprises the first VL, the second polypeptide, and the first IgG1 CH3 domain. In yet other embodiments, a single polypeptide chain comprises the first VL, the second polypeptide, and the second IgG1 CH3 domain. In some embodiments, a second single polypeptide chain comprises the second VH, the CH1 domain, and the second IgG1 CH3 domain. In some embodiments, a second single polypeptide chain comprises the second VH, the CH1 domain, and the first IgG1 CH3 domain. In some embodiments, a second single polypeptide chain comprises the second VL, the CL domain, and the second IgG1 CH3 domain. In some embodiments, a second single polypeptide chain comprises the second VL, the CL domain, and the first IgG1 CH3 domain. In some embodiments, the first polypeptide and/or the second polypeptide comprise the amino acid

sequence set forth in SEQ ID NO:1. In certain embodiments, the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In other embodiments, the first polypeptide and/or the second polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in SEQ ID NO:1. In other embodiments, the first polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:5. In other embodiments, the first polypeptide comprises the amino acid sequence set forth in SEQ ID NO:5. In certain embodiments, the second polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in SEQ ID NO:6. In some embodiments, the second polypeptide comprises the amino acid sequence set forth in SEQ ID NO:6. In certain embodiments, the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in SEQ ID NO:1 at at least twelve amino acid residues. In some embodiments, the first polypeptide and the second polypeptide each contain an amino acid other than cysteine at at least one of the two cysteine residues of SEQ ID NO:1 that do not form intrachain disulfide bond. In certain embodiments, the first polypeptide and the second polypeptide have a mutation or mutations at the N-linked glycosylation site such that the first polypeptide and the second polypeptide are not glycosylated at the N-linked glycosylation site. In other embodiments, the first polypeptide or the second polypeptide have a mutation or mutations at the N-linked glycosylation site such that the first polypeptide and the second polypeptide are not glycosylated at the N-linked glycosylation site. These mutations can be of the asparagine or the threonine or serine of the N-linked glycosylation site to other amino acid(s).

In another aspect, the disclosure provides a bispecific antibody comprising a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (SEQ ID NO:2). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The first polypeptide and the second polypeptide pair to form a dimer. The bispecific antibody also comprises a second VH and a second VL, wherein the second VH and

the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen. The second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain. The second VL is either (i) directly linked or (ii) linked via a linker to a CL domain, and wherein the CH1 domain and the CL domain pair to form a dimer. The bispecific antibody also comprises a heterodimerization module that includes a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a serine; and a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acid at position 370 is a serine and the amino acids at positions 405 and 409 are lysines. The amino acid positions above are based on the EU numbering system.

In another aspect, the disclosure relates to a bispecific antibody comprising a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen. The first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (SEQ ID NO:2). The first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (SEQ ID NO:2). The first polypeptide and the second polypeptide pair to form a dimer. The bispecific antibody also comprises a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen. The second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain. The second VL is either (i) directly linked or (ii) linked via a linker to a CL domain, and wherein the CH1 domain and the CL domain pair to form a dimer. The bispecific antibody also comprises a heterodimerization module that includes a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a leucine; and a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:11, wherein the amino acid at position 370 is a serine, the amino acid at position 397 is an isoleucine, and the

amino acids at positions 405 and 409 are lysines. The amino acid positions above are based on the EU numbering system.

In some embodiments of the above two aspects, the bispecific antibody includes two IgG1 CH2 domains. In some embodiments of the above two aspects, the bispecific antibody includes two IgG4 CH2 domains. In some embodiments, a single polypeptide chain comprises the first VH and the first IgG1 CH3 domain. In some embodiments, a single polypeptide chain comprises the first VH and the second IgG1 CH3 domain. In other embodiments, a single polypeptide chain comprises the first VL and the first IgG1 CH3 domain. In yet other embodiments, a single polypeptide chain comprises the first VL and the second IgG1 CH3 domain. In some embodiments, single polypeptide chain comprises the second VH and the second IgG1 CH3 domain. In some embodiments, single polypeptide chain comprises the second VH and the first IgG1 CH3 domain. In some embodiments, single polypeptide chain comprises the second VH and the second IgG1 CH3 domain. In some embodiments, the first polypeptide and/or the second polypeptide comprise the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In other embodiments, the first polypeptide and/or the second polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in SEQ ID NO:2. In certain embodiments, the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in SEQ ID NO:2 at at least twelve amino acid residues. In some embodiments, the first polypeptide and the second polypeptide each contain an amino acid other than cysteine at the cysteine residue of SEQ ID NO:2 that does not form an intrachain disulfide bond. In certain embodiments, the first polypeptide and the second polypeptide have a mutation or mutations at the N-linked glycosylation site such that the first polypeptide and the second polypeptide are not glycosylated at the N-linked glycosylation site. In other embodiments, the first polypeptide or the second polypeptide have a mutation or mutations at the N-linked glycosylation site such that the first polypeptide and the second polypeptide are not glycosylated at the N-linked glycosylation site. These mutations can be of the asparagine or the threonine or serine of the N-linked glycosylation site to other amino acid(s). In certain embodiments, the disclosure provides a polynucleotide or polynucleotides encoding the bispecific antibody described above. In some embodiments, expression vectors are provided comprising the

bispecific antibody described above. In other embodiments, host cells comprising the bispecific antibody or the expression vectors are provided. In yet other embodiments, provided are methods of making the bispecific antibody comprising culturing the host cells described above under conditions that result in the expression of the bispecific antibody and isolating the bispecific antibody from the cell culture. In certain embodiments, the isolated bispecific antibody is formulated as a sterile composition for administration to a human subject in need thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the exemplary methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of unwanted side products from mispairing in an asymmetric antibody. This figure shows that several unwanted side products can form by mispairing of light and/or heavy chains when two antibodies are co-expressed. The desired asymmetric antibody may represent a minor fraction, and purification of that desired antibody may present difficulties.

Figure 2 is a diagrammatic representation of CH3 heterodimerization by lysine repositioning. Lysine 370 and Lysine 409 are stacked in the normal head-to-tail homodimer of wild-type CH3 domains of human IgG1. The mutations S364K/K409S in monomer A and K370S/F405K in monomer B reverse the orientation of the stacked on lysines, enabling the formation of the A/B heterodimer but leading to clashes in the homodimers (bottom). Thus, lysine repositioning provides an effective strategy for heavy chain heterodimerization. The amino acid positions referred to above are based on EU numbering.

Figure 3A is a depiction of SDS-PAGE analysis of IgG/Fc heterodimers. Aliquots of protein A-purified heterodimers were analyzed by SDS page. The heterodimer between IgG1 and Fc is the predominant species. Two fractions of Knobs-into-Holes were loaded. The half-design MP1 shows high amount of heterodimer, but lacks mutations to prevent formation of the Fc dimer and thus contains no Fc monomer.

Figure 3B is a bar graph showing molar ratios and relative amounts of the components that were estimated from LC-MS.

Figure 3C is a depiction of the melting temperature of purified heterodimers that was determined by DSC.

Figure 4A shows the expression of heterodimerization mutants and half-antibody formation. CHO cells were transfected with various ratios of mp4a and mp4b heavy chains, which contain the same Fab (M60-A02 anti EGFR). Half-antibody formation in the expressed proteins was analyzed by SDS PAGE

Figure 4B shows a series of mass spectra that was used to quantify half-antibody formation. The MS spectra of the samples showed maximal amount of heterodimer and minimal homodimers are present at transfection ratio 1:1.

Figure 5A is a diagrammatic representation of one of the application's solution to the light chain pairing problem, the creation of an E-fab. In an E-fab, the CH1 and CL domains are substituted by IgE CH2 (Cε2) domains, which form a natural dimer.

Figure 5B is a superimposition of structures of the human Cε2 domain (white) and a human IgG1 CH1/kappa constant domains (black) which shows overall similarity of the Ig-folds. The Cε2 domains pair at a different angle and with greater distance in the upper portion of the interface, but tighter packing in the lower interface. The Pro at the beginning of β-strand A (represented as a sphere) is in a very similar position and orientation in the CH1/CL and Cε2 domains.

Figure 5C is a sequence alignment of human IgG1 CH1 and kappa constant with the Cε2 domain and the E-Fab heavy and light chain. The sequences are aligned with IMGT numbering shown above, the 7 β-strands of the Ig-fold are indicated by arrows, the conserved Pro at the start of β-strand A is in bold and underlined. The agly mutation (N38Q) in E-Fabs and interchain disulfide cysteines are also in bold and underlined.

Figure 5D is a sequence alignment of IgE CH2 domains from human, chimpanzee, mouse, rat, and rabbit. Identical residues are shown as dots.

Figure 6A is a schematic representation of the strategy to test light chain pairing. The heavy chain of the Fab A (HCA) is fused to HSA, while the light chain of Fab B (LCB) was tagged at the N-terminus with GFP. Correct pairing of the chains can be checked by molecular weight of the Fabs.

Figure 6B is an analysis of Fab-pairing by SDS-page. The indicated HSA-tagged anti-EGFR M60-A02 Fabs or E-Fabs were co-expressed with a GFP-tagged Fab anti-IGF-1R C06 (see also, Table 3). Correct pairing of heavy and light chains produces bands at 114 and 74 kDa under non-reducing conditions, while bands at 47 or 140 kDa indicate mispairing.

Figure 6C is an analysis of Fab-pairing by SDS-page. The anti-IGF-1R Fab C06 was built as E-fab and light chain pairing with another anti-IGF-1R Fab (G11) was tested as in Figure 6B.

Figure 6D is a graph depicting binding of the anti-IGF-1R E-Fab as tested by Octet. The Fabs were expressed individually, purified on protein A spin columns, and used in Octet binding (300 nM, 100 nM, and 30 nM) to soluble IGF1-R loaded onto His-tips.

Figure 7 is a schematic representation of an asymmetric IgG with E-Fab and lysine repositioning. The constant domains of one Fab arm are replaced with the E-Fab, while the other Fab arms contains wild-type IgG CH1/CL domains to solve the light chain pairing problem. The lysine repositioning mutations are included in the CH3 domains of the two heavy chains to enforce heterodimerization.

Figure 8A is an analysis of EGFR/IGF-1R bispecific antibodies expressed in CHO-S cells and supernatants by SDS-PAGE. The E-bodies E0 and E2 contain the anti-EGFR as E-Fab, while the IgG1-heterodimer control contains no light chain solution.

Figure 8B is a representation of a mass spectrometry analysis of the asymmetric IgGs from Figure 8A which shows no light chain mispairing in the E-body but significant amounts of antibody with mispaired LC in the control. Heavy chain homodimers were not detectable in either sample.

Figure 8C is a graph depicting binding of the bispecific antibodies to His-tagged EGFR. Undiluted supernatants from CHO-S cells were used in Octet binding after loading of his-tagged ligands (5 μg/mL).

Figure 8D is a graph depicting binding of the bispecific antibodies to His-tagged IGF-1R. Undiluted supernatants from CHO-S cells were used in Octet binding after loading of his-tagged ligands (5 μg/mL).

Figure 9A is a graph depicting simultaneous binding of two antigens by a Trastuzumab/Cetuximab bispecific antibody. His-tagged soluble HER2 (5 μg/ml) was loaded onto Octet tips allowing capture of the bispecific antibody (200 nM) in the next step. Subsequent binding of EGFR (15 μg/ml) demonstrates simultaneous binding of both ligands by the bispecific.

Figure 9B is a graph depicting the results of the reverse of the experiment shown in Figure 9A. EGFR was loaded first, followed by binding of the bispecific antibody and HER2.

Figure 10A shows Efab constructs with various elbow linker sequences. The anti-HER2 antibody Trastuzumab was engineered as Efab. Various elbow linker sequences that were included in the light chain are listed in the table. The sequences include the last 5 amino acids of the variable domain as well as the first 5 amino acids of the IgE CH2 domain in addition to the elbow sequence (bold).

Figure 10B is a graph of Octet binding studies. The Efabs were expressed as asymmetric IgG with Cetuximab in CHO cells (as in Figure 9), purified and used in Octet binding studies (100 nM) with His-tagged HER2 (5 μg/ml) loaded onto anti-His tips.

Figure 10C is an analysis of Fab-pairing by SDS-page. To assess whether the IgM CH2 domain could solve the light chain pairing equally well as the IgE CH2 domain, two Fabs (anti-EGFR M60 and anti-IGF-1R C06) were co-expressed: with one Fab fused to HSA and another Fab fused to GFP. The kappa and CH1 constant domains of the Fab were replaced with IgM CH2 domains (the resulting molecule was named M-Fab). Analysis of the chain pairing by SDS-PAGE showed that the M-Fab solved the light chain mispairing between M60 and C06 that was prominently observed in the control.

Figure 10D is a graph of Octet binding studies. The Efabs and the M-Fab were expressed in CHO-S as Fabs (without Fc), and the supernatants were used for testing binding to HER2 by Octet.

Figure 11A shows a photograph of a gel of a bispecific antibody comprising an anti-EGFR M60-A02 Efab and the anti-IGF1R M13.C06 as a normal Fab with an IgG1 agly (T299A)

constant region containing mp3 heterodimer mutations expressed in CHO cells and purified by protein A.

Figure 11B shows the results of Octet studies where the bispecific was tested for simultaneous binding. In the upper panel, His-tagged soluble IGF1R (5 μg/ml) was bound to Octet tips, followed by binding of the bispecific antibody or the respective mAbs. In the third step the second antigen (EGFR) was bound to the antibody-antigen complex as indicated. A positive signal compared to the no EGFR control in the third step demonstrates simultaneous binding. In the lower panel, His-tagged soluble EGFR (5 μg/ml) was bound to Octet tips, followed by binding of the bispecific antibody or the respective mAbs. In the third step the second antigen (IGF1R) was bound to the antibody-antigen complex as indicated. A positive signal compared to the no IGF1R control in the third step demonstrates simultaneous binding.

Figure 12A is a schematic drawing of a bispecific antibody with an Efab and an IgG4P/IgG1 constant region containing mp4 heterodimer. The antibody also comprises an N297Q substitution in the constant domain (IgG4P/IgG1 agly).

Figure 12B is a photograph of a gel of bispecific antibodies as shown in (A) that were generated with the anti-HER2 antibody Pertuzumab and the anti-IGF1R antibody C06 in both orientations, with the antibody named first always being an Efab. Aliquots of the protein-A-purified material from CHO cells were analyzed by SDS-PAGE.

Figure 12C shows the results of binding when tested by Octet using soluble IGF1R and HER2 protein. In the upper panel, His-tagged soluble IGF1R (5 μg/ml) was bound to Octet tips, followed by binding of the bispecific antibody or the respective mAbs. In the third step the second antigen (HER2) was bound to the antibody-antigen complex as indicated. A positive signal compared to the no HER2 control in the third step demonstrates simultaneous binding. In the lower panel, His-tagged soluble HER2 (5 μg/ml) was bound to Octet tips, followed by binding of the bispecific antibody or the respective mAbs. In the third step the second antigen (IGF1R) was bound to the antibody-antigen complex as indicated. A positive signal compared to the no IGF1R control in the third step demonstrates simultaneous binding.

Figure 13A is a schematic drawing of an IgG with an Efab fused to the C-terminus of the heavy chain (an "Mab-Fab").

Figure 13B is a photograph of a gel of Mab-Fabs of Trastuzumab and the anti-IGF-1R antibody C06. Mab-Fabs of Trastuzumab and the anti-IGF-1R antibody C06 were transiently

expressed in CHO cells and purified by protein A. SDS-PAGE shows that the proteins assembled correctly forming the ~240 kDa Mab-Fabs.

Figure 13C shows the results of binding when tested by Octet. His-tagged soluble IGF1R or HER2 (5 μg/ml) was loaded onto Octet tips allowing capture of the bispecific antibody (200 nM) in the next step. Subsequent binding of the second ligand (15 μg/ml) demonstrates simultaneous binding of both ligands by the bispecific antibody.

Figure 14A is a schematic drawing of a monovalent antibody containing a LC-Fc fusion and Fc heterodimer mutations.

Figure 14B is a photograph of a gel with the LC-Fc monovalent antibody and the corresponding bivalent IgG1 (anti-EGFR M60-A02) that were produced from CHO cells. SDS-PAGE of protein-A purified material shows clean assembly of the monovalent LC-Fc antibody.

Figure 14C are graphs depicting monovalent LC-Fc and the bivalent IgG in Octet binding studies at various concentrations. The monovalent LC-Fc lacks avid binding and dissociation of the antigen-antibody complex is seen.

Figure 15A provides schematic drawings of bispecific antibodies with an Efab linked to a regular Fab via HAS (left) a peptide linker (right).

Figure 15B are photographs of gels of the two examples of bispecifics as shown in Figure 15A that were expressed in CHO cells, and protein A-purified, and analyzed by SDS-PAGE. The anti-EGFR and anti IGF1R antibodies M60-A02 and M13.C06 were used in both orientations, as Efab or as regular Fab, respectively.

Figure 15C is a graph of purified bispecifics used in Octet binding studies. Simultaneous binding of both antigens by these bispecifics was observed only when the bispecific was bound to EGFR first followed by IGF-1R binding.

Figure 15D is a graph of purified bispecifics used in Octet binding studies. Simultaneous binding of both antigens by these bispecifics was not observed when the bispecific was bound to IGF-1R first followed by EGFR binding.

Detailed Description

Bispecific antibodies are an emerging class of biologics drugs that hold the potential of achieving new therapeutic mechanisms of action. However, there are several challenges associated with expressing and properly forming a bispecific antibody. Specifically, two

problems must be solved to efficiently express a bispecific antibody in the form of an asymmetric IgG composed of four different chains: the so-called heavy chain mispairing problem and the light chain mispairing problem. When there are two different heavy chains and two different light chains, they can mispair in several different permutations (*see*, **Figure 1**). Thus, to form a properly paired bispecific antibody, the two heavy chains must form a heterodimer, and each heavy chain must pair with its cognate light chain. This disclosure provides a bispecific antibody platform that can convert any two antibodies that each bind different epitopes into a bispecific antibody in the form of an asymmetric IgG. The platform is based on a heavy chain heterodimerization strategy termed lysine repositioning, and a solution to the light chain mispairing problem that does not require engineering of the variable domains of antibodies. In one embodiment, the platform contains an Fc region, which is engineered in the CH3 domains by lysine repositioning to drive heterodimerization of the two heavy chains of a bispecific antibody. In another embodiment, the CH1 and CL domains of one of the Fab arms are substituted with an IgE CH2 domain (or an IgM CH2 domain), or a fragment thereof that can still pair with the IgE CH2 domain (or the IgM CH2 domain). This engineering of the Fabs can reduce or prevent mispairing of light chains in the bispecific antibody. This disclosure describes the design, engineering, and testing of a bispecific antibody platform, which can efficiently generate an asymmetric IgG from 2 antibodies by co-expression.

Solution to Light Chain Mispairing Problem

In order to achieve the correct assembly of the light chain (LC): heavy chain (HC) pairs in a bispecific antibody as illustrated in Figure 1, the solution disclosed herein is to modify the amino acid sequence of the light chain and heavy chain such that the constant domains (*i.e.*, the CL and CH1 domains) of these chains are replaced with an Ig-fold domain (or fragments thereof that can still form stable disulfide-linked dimers between the Ig-fold domains of the light and heavy chains).

In one instance the Ig-fold domain that replaces the CH1 and CL domains is the CH2 domain of IgE ("CH2E"), or a fragment thereof that can form stable disulfide-linked dimers with another CH2E. The amino acid sequence of an exemplary CH2E domain is provided below:

```
1   VCSRDFTPPT VKILQSSCDG GGHFPPTIQL LCLVSGYTPG TINITWLEDG
51  QVMDVDLSTA STTQEGELAS TQSELTLSQK HWLSDRTYTC QVTYQGHTFE
101 DSTKKCA  (SEQ ID NO:1)
```

In the sequence shown above, the two cysteines that can be involved in forming interchain disulfide bonds are boldened; the two cysteines that can form intradomain disulfide bonds are boldened and italicized; and the N-linked glycosylation site is underlined.

In another instance, the Ig-fold domain that replaces the CH1 and CL domains of the heavy and light chain is the CH2 domain of IgM ("CH2M"), or a fragment thereof that can form stable disulfide-linked dimers. The amino acid sequence of an exemplary CH2M domain is provided below:

1 VIAELPPKVS VFVPPRDGFF GNPRKSKLI*C* QATGFSPRQI QVSWLREGKQ
51 VGSGVTTDQV QAEAKESGPT TYKVTSTLTI KESDWLGQSM FT*C*RVDHRGL
101 TFQQNASSM**C** VP (**SEQ ID NO:2**)

In the sequence shown above, the cysteine that can be involved in forming interchain disulfide bonds is boldened; the two cysteines that can form intradomain disulfide bonds are italicized; and the N-linked glycosylation site is underlined.

This disclosure provides several examples of antibodies making use of the solution to the light chain pairing problem. In one embodiment, the antibody features amino acid sequences having the formulas:

VH1 construct: VH1-L-X-CH2E (or CH2M); and

VL1 construct: VL1-L-X-CH2E (or CH2M),

wherein "VH1" and "VL1" are the heavy chain variable domain and light chain variable domain that pair to form a first antigen-binding site for a first epitope; wherein "L" is an optional linker (described further below); wherein "X" is an optional elbow region (described further below); and wherein "CH2E" refers to SEQ ID NO:1 or a fragment thereof that can form stable disulfide-linked dimers with SEQ ID NO:1 (e.g., amino acids 9-107 of SEQ ID NO:1), and wherein "CH2M" refers to SEQ ID NO:2 or a fragment thereof that can form stable disulfide-linked dimers with SEQ ID NO:2 (e.g., amino acids 7-112 of SEQ ID NO:2). In certain embodiments, one or both "L" and "X" are absent in the VH1 and VL1 constructs.

In another embodiment, the antibody features amino acid sequences having the formulas:

VH1 construct: VH1-X-L-CH2E (or CH2M), and

VL1 construct: VL1-X-L-CH2E (or CH2M).

In a further embodiment, the antibody features amino acid sequences having the formulas:

VH1 construct: VH1-L-X-L-CH2E (or CH2M), and

VL1 construct: VL1-L-X-L-CH2E (or CH2M).

It is to be understood that when CH2E is used in a VH1 construct described above, CH2E is also used in the corresponding VL1 construct. Similarly, when CH2M is used in a VH1 construct described above, CH2M is also used in the corresponding VL1 constructs. The CH2E and CH2M domains in the paired VH1 and VL1 constructs described above may be identical in amino acid sequence; however, they need not be identical. They may, *e.g.,* differ at 12 or fewer, 11 or fewer, 10 or fewer, 9 or fewer, 8 or fewer, 7, or fewer, 6 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, 2 or fewer, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acids. In certain embodiments, the CH2E domains of the VH1 and VL1 constructs described above are different at 12 or fewer, 11 or fewer, 10 or fewer, 9 or fewer, 8 or fewer, 7, or fewer, 6 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, 2 or fewer, or 1 amino acid position(s) compared with SEQ ID NO:1. In certain embodiments, the CH2M domains of the VH1 and VL1 constructs described above are different at 12 or fewer, 11 or fewer, 10 or fewer, 9 or fewer, 8 or fewer, 7, or fewer, 6 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, 2 or fewer, or 1 amino acid position(s) compared with SEQ ID NO:2. These differences may be the result of amino acid substitutions, deletions, and/or insertions. For example, the N-glycosylation site in SEQ ID NOs.: 1 or 2 can be modified (*e.g.,* in the NIT sequence of SEQ ID NO:1, the N residue can be changed to Q or the T residue changed to A or C; in the NAS sequence in SEQ ID NO:2, the N residue can be changed to Q or the S residue changed to A or C). Alternatively, or in addition, one or more of the cysteines that form intradomain disulfide bonds may be mutated only in the CH2E (or CH2M) domain of one of the VH1 and VL1 constructs. In certain instances, the cysteine or cysteines involved in interchain disulfide bond formation in both the CH2E domains (or CH2M domains) of the VH1 and VL1 constructs are substituted (*e.g.,* with a conservative amino acid). Also, mutations to prevent homodimerization of the CH2E domains (or CH2M domains) may be made (*e.g.,* replacing serine at position 17 of SEQ ID NO:1 with e.g., isoleucine or threonine; and replacing threonine at position 103 of SEQ ID NO:1 with *e.g.,* glycine or serine). The alignment of the IgE CH2 domains from human, chimpanzee, mouse, rat, and rabbit (**Figure 5D**) identifies amino acid residues that are not conserved between all species and can likely be substituted without eliminating bioactivity.

In some instances the amino acid substitutions to the CH2E domain can be conservative. A conservative substitution is the substitution of one amino acid for another with similar characteristics. Conservative substitutions include substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and glutamic acid; asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The non-polar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic or acidic groups by another member of the same group can be deemed a conservative substitution.

In some instances the amino acid substitutions to the CH2E domain can be non-conservative. Non-conservative substitutions include those in which (i) a residue having an electropositive side chain (e.g., Arg, His or Lys) is substituted for, or by, an electronegative residue (e.g., Glu or Asp), (ii) a hydrophilic residue (e.g., Ser or Thr) is substituted for, or by, a hydrophobic residue (e.g., Ala, Leu, Ile, Phe or Val), (iii) a cysteine or proline is substituted for, or by, any other residue, or (iv) a residue having a bulky hydrophobic or aromatic side chain (e.g., Val, Ile, Phe or Trp) is substituted for, or by, one having a smaller side chain (e.g., Ala, Ser) or no side chain (e.g., Gly).

In certain embodiments, the CH2E domains of the VH1 and VL1 constructs described above are at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the amino acid sequence set forth in SEQ ID NO:1 and the CH2E domains of the VH1 and VL1 constructs can still pair together. In certain embodiments, the CH2E domains of the VH1 and VL1 constructs described above are at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the amino acid sequence set forth in SEQ ID NO:3 and the CH2E domains of the VH1 and VL1 constructs can still pair together.

In certain embodiments, if CH2M domains are employed in the VH1 and VL1 constructs described above, the CH2M domains are at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the amino acid sequence set forth in SEQ ID NO:2 and the CH2M domains of the VH1 and VL1 constructs can still pair together.

Percent identity between amino acid sequences can be determined using the BLAST 2.0 program. Sequence comparison can be performed using an ungapped alignment and using the default parameters (Blossom 62 matrix, gap existence cost of 11, per residue gap cost of 1, and a lambda ratio of 0.85). The mathematical algorithm used in BLAST programs is described in Altschul et al., *Nucleic Acids Research*, 25:3389-3402 (1997).

In certain embodiments, the CH2E domain that is used in both the VH1 and VL1 constructs described above is 100% identical to SEQ ID NO:1. In some embodiments, the CH2E domain that is used in the VH1 and VL1 constructs described above is a fragment of SEQ ID NO:1, *e.g.*, missing amino acids at the N and/or C-terminal of SEQ ID NO:1, and which can form stable disulfide-linked dimers with a polypeptide encoded by SEQ ID NO:1. For example, the fragments of SEQ ID NO:1 may be missing 20, 19, 18, 17, 16, 15, 14, 13, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid(s) at the N- and/or C-terminus of SEQ ID NO:1. In certain embodiments, the CH2E domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 2-107, 3-107, 4-107, 5-107, 6-107, 7-107, 8-107, 9-107, 10-107, 11-107, 12-107, 13-107, 14-107, 15-107, 16-107, 17-107, 18-107, 19-107, or 20-107 of SEQ ID NO:1. In other embodiments, the CH2E domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 2-106, 3-106, 4-106, 5-106, 6-106, 7-106, 8-106, 9-106, 10-106, 11-106, 12-106, 13-106, 14-106, 15-106, 16-106, 17-106, 18-106, 19-106, or 20-106 of SEQ ID NO:1. In yet other embodiments, the CH2E domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 2-105, 3-105, 4-105, 5-105, 6-105, 7-105, 8-105, 9-105, 10-105, 11-105, 12-105, 13-105, 14-105, 15-105, 16-105, 17-105, 18-105, 19-105, or 20-105 of SEQ ID NO:1. In certain embodiments, the CH2E domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 9-107, 9-106, 9-105, 9-104, 9-103, 9-102, 9-101, 9-100, 9-99, 9-98, or 9-97 of SEQ ID NO:1. In all of these embodiments, there may be one to twelve (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12)

substitutions compared to the amino sequence set forth in SEQ ID NO:1 in one or both CH2E domains used in the VH1 and VL1 constructs described above. For example, the N-linked glycosylation site may be mutated (*e.g.*, asparagine of the NIT site may be substituted by glutamine or the threonine of the NIT site may be substituted by alanine or cysteine); one or both of the cysteines involved in interchain disulfide bond formation may be substituted with another amino acid (*e.g.*, a conservative amino acid); or mutations may be introduced into CH2E that prevent formation of heavy chain:heavy chain or light chain:light chain dimers (when the CH2E domain is part of the heavy chain and light chain).

In certain embodiments, the CH2M domain that is used in VH1 and VL1 constructs described above is 100% identical to SEQ ID NO:2. In some embodiments, the CH2M domain that is used in VH1 and VL1 constructs described above is a fragment of SEQ ID NO:2, *e.g.*, missing amino acids at the N and/or C-terminal of SEQ ID NO:2, and which can form stable disulfide-linked dimers with a polypeptide encoded by SEQ ID NO:2. For example, the fragments of SEQ ID NO:2 may be missing 20, 19, 18, 17, 16, 15, 14, 13, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid(s) at the N- and/or C-terminus of SEQ ID NO:2. In certain embodiments, the CH2M domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 2-112, 3-112, 4-112, 5-112, 6-112, 7-112, 8-112, 9-112, 10-112, 11-112, 12-112, 13-112, 14-112, 15-112, 16-112, 17-112, 18-112, 19-112, or 20-112 of SEQ ID NO:2. In other embodiments, the CH2M domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 2-111, 3-106, 4-111, 5-111, 6-111, 7-111, 8-111, 9-111, 10-111, 11-111, 12-111, 13-111, 14-111, 15-111, 16-111, 17-111, 18-111, 19-111, or 20-111 of SEQ ID NO:2. In yet other embodiments, the CH2M domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 2-110, 3-110, 4-110, 5-110, 6-110, 7-110, 8-110, 9-110, 10-110, 11-110, 12-110, 13-110, 14-110, 15-110, 16-110, 17-110, 18-110, 19-110, or 20-110 of SEQ ID NO:2. In certain embodiments, the CH2M domain that is used in VH1 and VL1 constructs described above comprises or consists of amino acids 7-112, 7-111, 7-110, 7-109, 7-108, 7-107, 7-105, 7-104, 7-103, 7-102, 7-101, 7-100, or 7-99 of SEQ ID NO:1. In all of these embodiments, there may be one to twelve (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12) substitutions compared to the amino sequence set forth in SEQ ID NO:2 in one or both CH2M domains used in the VH1 and VL1 constructs described above. For example, the N-linked glycosylation site may be mutated (*e.g.*, asparagine of the NAS site may be substituted by

glutamine or the serine of the NAS site may be substituted by alanine or cysteine); one or both of the cysteines involved in interchain disulfide bond formation may be substituted with another amino acid (*e.g.*, a conservative amino acid); or mutations may be introduced into CH2M that prevent formation of heavy chain:heavy chain or light chain:light chain dimers (when the CH2M domain is part of the heavy chain and light chain).

In one embodiment, the CH2 domain used in the VH1 constructs described above is a CH2 domain of human immunoglobulin E, in which the N-glycosylation site is mutated to glutamine, and the first 8 amino acids are replaced with the first 5 amino acids of a human IgG1 CH1 domain. The amino acid sequence of this CH2E domain is provided below:

1 ASTKGPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:3**)

In one embodiment, the CH2 domain used in the VL1 constructs described above is a CH2 domain of human immunoglobulin E, in which the N-glycosylation site is mutated to glutamine, and the first 8 amino acids are replaced with the first 5 amino acids of a human kappa domain. The amino acid sequence of this CH2E domain is provided below:

1 RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:4**)

In another embodiment, the amino acid sequence of an exemplary CH2 domain of human immunoglobulin E to be used in a VH1 construct described above, and which is engineered to form a heterodimer with a VL1 construct described above, is made by replacing serine at position 17 of SEQ ID NO:1 with isoleucine. The amino acid sequence of this CH2E domain is provided below (note that in this sequence the N-linked glycosylation site is also mutated and the first eight amino acids of SEQ ID NO:1 are replaced with the first 5 amino acids of IgG1 CH1):

1 ASTKGPTVKI LQSICDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:5**)

The amino acid sequence of an exemplary CH2 domain of human immunoglobulin E to be connected with a VL domain of a VL1 construct described above, and which is engineered to form a heterodimer with a VH1 construct described above, is made by replacing threonine at position 103 of SEQ ID NO:1 with glycine. The amino acid sequence of this CH2E domain is provided below (note that in this sequence the N-linked glycosylation site is also mutated and the

first eight amino acids of SEQ ID NO:1 are replaced with the first 5 amino acids of the kappa chain):

```
1   RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51  DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDSG
101 KKCA  (SEQ ID NO:6)
```

In certain embodiments, the CH2E domain that is used in VH1 and VL1 constructs described above include C-terminal truncations of SEQ ID NOs.: 5 and/or 6. In certain instances, the C-terminal most 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid of SEQ ID NO:5 and/or SEQ ID NO:6 is deleted. In a specific embodiment, the CH2E domain that is used in the VH1 constructs described above comprise or consist of amino acids 1-103, 1-102, 1-101, 1-100, 1-99, 1-98, 1-97, 1-96, or 1-95 of SEQ ID NO:5. In another specific embodiment, the CH2E domain that is used in the VL1 constructs described above comprise or consist of amino acids 1-103, 1-102, 1-101, 1-100, 1-99, 1-98, 1-97, 1-96, or 1-95 of SEQ ID NO:6. In yet other embodiments, the CH2E domain that is used in the VH1 constructs described above comprises or consists of amino acids 6-103, 7-103, 8-103, 9-103, or 10-103 of SEQ ID NO:5. In yet other embodiments, the CH2E domain that is used in the VL1 constructs described above comprises or consists of amino acids 6-103, 7-103, 8-103, 9-103, or 10-103 of SEQ ID NO:6. In certain embodiments, SEQ ID NO:5 and/or SEQ ID NO:6, or the fragments thereof described above, may additionally contain five or fewer, four or fewer, three or fewer, two or fewer or one further mutation relative to SEQ ID NO:1.

In certain embodiments, the antibodies described above further comprise a heavy chain variable domain "VH2" and a light chain variable domain "VL2", wherein the VH2 is connected to a CH1 domain and the VL2 domain is connected to a CL domain, and wherein the VH2 and VL2 pair to form a second antigen-binding site for a second epitope. In some embodiments, the antibodies described above further comprise two Fc domains. The Fc domains comprises a hinge region, a CH2 domain and a CH3 domain of an antibody. In certain instances, the hinge, CH2, and CH3 domains are from IgG1. In certain instances, the hinge and CH3 domains are from IgG4 and the CH2 domain from IgG1. In specific embodiments, when the hinge region is from IgG4, it includes the S228P (EU numbering) mutation. One of the two Fc domains of the antibody may be directly linked to one of the CH2E domains (or CH2M domain) of one of the two Fabs of the antibody, or may be linked via a linker. The Fc region may be linked to the VH1 construct or the VL1 construct. The Fc region may comprise any mutation(s) that increase

heterodimerization between the heavy chains of the bispecific antibody relative to the same Fc regions without the mutation(s). For example, the Fc regions may comprise knobs-into-holes mutations, electrosteering mutations, or other mutations described in Table 2 of Example 1 of this application. In a specific embodiment, the Fc regions of the bispecific antibody include lysine repositioning mutations described herein.

In certain embodiments, the antibodies described above further comprise a heavy chain variable domain "VH2" and a light chain variable domain "VL2", wherein the VH2 is connected to a CH1 domain and the VL2 domain is connected to a CL domain, and wherein the VH2 and VL2 pair to form a second antigen-binding site for a second epitope, but the antibody lacks an Fc domain(s). The CH2E domain (or CH2M domain) linked to the VH1 or VL1 domain of the constructs described above can be linked to the antibody comprising the VH2 and VL2 domains. For example, the C-terminus of the CH2E domain (or CH2M domain) of the VH1 construct may be linked to the C-terminus of the CH1 domain that is connected to VH2, or the C-terminus of the CH2E domain (or CH2M domain) of the VH1 construct may be linked to the N-terminus of the VH2 domain. Other exemplary configurations are for the C-terminus of the CH2E domain (or CH2M domain) of the VL1 construct may be linked to the C-terminus of the CH1 domain that is connected to VH2, or the C-terminus of the CH2E domain (or CH2M domain) of the VL1 construct may be linked to the N-terminus of the VH2 domain. Other exemplary configurations include the C-terminus of the CH2E domain (or CH2M domain) of the VH1 construct linked to the C-terminus of the CL domain that is connected to VL2, or the C-terminus of the CH2E domain (or CH2M domain) of the VH1 construct linked to the N-terminus of the VL2 domain. Further exemplary configurations are for the C-terminus of the CH2E domain (or CH2M domain) of the VL1 construct to be linked to the C-terminus of the CL domain that is connected to VL2, or the C-terminus of the CH2E domain (or CH2M domain) of the VL1 construct to be linked to the N-terminus of the VL2 domain. In some instances, the linker between the CH2E domain (or CH2M domain) of the VH1 or VL1 construct and the second Fab is a peptide linker. In other instances, the linker between the CH2E domain (or CH2M domain) of the VH1 or VL1 construct and the second Fab is human serum albumin (HSA). In some instances, the linker between the CH2E domain (or CH2M domain) of the VH1 or VL1 construct and the second Fab is polyethylene glycol. In yet other instances, the linker between the CH2E domain of the VH1 or VL1 construct and the second Fab is an XTEN molecule (*e.g.*, AE-144, AE-288).

In certain instances, the VH1 and VL1 constructs are part of a tetravalent bispecific antibody. These tetravalent antibodies comprise (i) a whole antibody, the variable domains of which bind one epitope of an antigen, and (ii) two Fabs that each bind another epitope of the same antigen or a different antigen. In some embodiments, the whole antibody is an IgG1. In other embodiments, the whole antibody is an IgG4(G1) – *i.e.,* an antibody comprising the hinge and CH2 regions of IgG4 but the CH3 domain of IgG1. In certain embodiments, the whole antibody is an IgG4(G1)P – i.e., where the antibody is an IgG4(G1) except that the hinge region has the S228P (EU numbering) mutation. The two Fabs are linked to the C-terminus of the CH3 domains of the whole antibody. The two Fabs may be linked to the CH3 domain of the whole antibody either via the N-terminus of one of the two variable domains (*i.e.,* VH or VL) of each Fab or via the C-terminus of one of the constant domains of each of the two Fabs. If the Fab constant domain is not replaced by CH2E or CH2M domains, the linkage may be to either the C-terminus of the CH1 domain or the C-terminus of the CL domain. If the Fab constant domains are replaced with the CH2E domains (or CH2M domains), the linkage can be to the C-terminus of the CH2E domain (or CH2M domain)). The tetravalent bispecific antibodies can include the CH2E domains (or CH2M domains) detailed above either in the two arms of the whole antibody or in the two Fabs. Note that, in this instance, the term "whole antibody" is used differently than its usual meaning (*i.e.,* an antibody comprising four chains: VL1-CL, VH1-CH1-hinge-CH2-CH3, VL2-CL, and VH2-CH1-hinge-CH2-CH3) to also include an antibody comprising four chains: VL1-CH2E (or CH2M), VH1-CH2E(or CH2M)-hinge-CH2-CH3, VL2-CH2E(or CH2M), and VH2-CH2E(or CH2M)-hinge-CH2-CH3.

There is no particular limitation on the linkers that can be used in the constructs described above. In some embodiments, the linker is a peptide linker. Any arbitrary single-chain peptide comprising about one to 25 residues (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids) can be used as a linker. In certain instances, the linker contains only glycine and/or serine residues. Examples of such peptide linkers include: Gly, Ser; Gly Ser; Gly Gly Ser; Ser Gly Gly; Gly Gly Gly Ser (**SEQ ID NO:10**); Ser Gly Gly Gly (**SEQ ID NO:55**); Gly Gly Gly Gly Ser (**SEQ ID NO:56**); Ser Gly Gly Gly Gly (**SEQ ID NO:57**); Gly Gly Gly Gly Gly Ser (**SEQ ID NO:58**); Ser Gly Gly Gly Gly Gly (**SEQ ID NO:59**); Gly Gly Gly Gly Gly Gly Ser (**SEQ ID NO:60**); Ser Gly Gly Gly Gly Gly Gly (**SEQ ID NO:61**); (Gly Gly Gly Gly Ser)n (**SEQ ID NO:56**)n, wherein n is an integer of one or more; and (Ser Gly Gly Gly Gly)n

(**SEQ ID NO:57**)n, wherein n is an integer of one or more. In other embodiments, the linker peptides are modified such that the amino acid sequence GSG (that occurs at the junction of traditional Gly/Ser linker peptide repeats) is not present. For example, the peptide linker comprise an amino acid sequence selected from the group consisting of: $(GGGXX)_nGGGGS$ (**SEQ ID NO:62**) and $GGGGS(XGGGS)_n$ (**SEQ ID NO:63**), where X is any amino acid that can be inserted into the sequence and not result in a polypeptide comprising the sequence GSG, and n is 0 to 4. In one embodiment, the sequence of a linker peptide is $(GGGX_1X_2)_nGGGGS$ and X_1 is P and X_2 is S and n is 0 to 4 (**SEQ ID NO:64**). In another embodiment, the sequence of a linker peptide is $(GGGX_1X_2)_nGGGGS$ and X_1 is G and X_2 is Q and n is 0 to 4 (**SEQ ID NO:65**). In another embodiment, the sequence of a linker peptide is $(GGGX_1X_2)_nGGGGS$ and X_1 is G and X_2 is A and n is 0 to 4 (**SEQ ID NO:66**). In yet another embodiment, the sequence of a linker peptide is $GGGGS(XGGGS)_n$, and X is P and n is 0 to 4 (**SEQ ID NO:67**). In one embodiment, a linker peptide of the invention comprises or consists of the amino acid sequence $(GGGGA)_2GGGGS$ (**SEQ ID NO:68**). In another embodiment, a linker peptide comprises or consists of the amino acid sequence $(GGGGQ)_2GGGGS$ (**SEQ ID NO:69**). In yet another embodiment, a linker peptide comprises or consists of the amino acid sequence $(GGGPS)_2GGGGS$ (**SEQ ID NO:70**). In a further embodiment, a linker peptide comprises or consists of the amino acid sequence $GGGGS(PGGGS)_2$ (**SEQ ID NO:71**).

In certain embodiments, the linker is a synthetic compound linker (chemical cross-linking agent). Examples of cross-linking agents that are available on the market include N-hydroxysuccinimide (NHS), disuccinimidylsuberate (DSS), bis(sulfosuccinimidyl)suberate (BS3), dithiobis(succinimidylpropionate) (DSP), dithiobis(sulfosuccinimidylpropionate) (DTSSP), ethyleneglycol bis(succinimidylsuccinate) (EGS), ethyleneglycol bis(sulfosuccinimidylsuccinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES), and bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone (sulfo-BSOCOES).

The elbow region for the VH1 construct can be, *e.g.,* a fragment of an IgG CH1 domain (*e.g.,* one to ten, one to nine, one to eight, one to seven, one to six, one to five, one to four, one to three, or one to two consecutive amino acids from an IgG CH1 domain). In one embodiment the elbow domain is from IgG1 and comprises or consists of one to ten, one to nine, one to eight, one to seven, one to six, one to five, one to four, one to three, or one to two consecutive amino acids

from the N or C-terminus of an IgG1 CH1 domain. The amino acid sequence of an IgG1 CH1 domain is provided below:

ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKV (**SEQ ID NO:72**)

A non-limiting example of an elbow region for the VH1 constructs described above is ASTKG (**SEQ ID NO:7**). In one embodiment the VH1 construct comprises the following amino acid sequence:

1 ASTKGPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIN ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:45**)

wherein the first 8 amino acids of CH2E (VCSRDFTP (**SEQ ID NO:8**) are replaced with the first 5 amino acids of a human IgG1 CH2 domain (elbow region ASTKG (**SEQ ID NO:7**)). In another embodiment, the elbow region for the VH1 constructs described above is SRDFT (**SEQ ID NO:77**). In certain embodiments, the VH1 constructs have a linker but no elbow region. In such instances, the linker can be, e.g., **SEQ ID NO:56** or **SEQ ID NO:58**.

The elbow region for the VL1 construct can be, *e.g.,* a fragment of a kappa or lambda CL domain (*e.g.*, one to ten, one to nine, one to eight, one to seven, one to six, one to five, one to four, one to three, one to two consecutive amino acids from the kappa or lambda domains). In one embodiment the elbow domain is from the kappa domain and comprises or consists of one to ten, one to nine, one to eight, one to seven, one to six, one to five, one to four, one to three, or one to two consecutive amino acids from the N or C-terminus of a kappa domain. The amino acid sequence of the human kappa CL domain is provided below:

RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGEC (**SEQ ID NO:73**)

The amino acid sequence of the human lambda CL domain is provided below (the elbow region is the first six amino acids, shown as bold/underlined):

GQPKAAPSVT LFPPSSEELQ ANKATLVCLI SDFYPGAVTV AWKADSSPVK AGVETTTPSK QSNNKYAASS YLSLTPEQWK SHRSYSCQVT HEGSTVEKTV APTECS (**SEQ ID NO:74**)

A non-limiting example of an elbow region for the VL1 constructs described above is RTVAA (**SEQ ID NO:9**). In one embodiment the VL1 construct comprises the following amino acid sequence:

```
1   RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIN ITWLEDGQVM
51  DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (SEQ ID NO:46)
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wherein the first 8 amino acids of CH2E (VCSRDFTP (**SEQ ID NO:8**)) are replaced with the first 5 amino acids of a human kappa domain (elbow region RTVAA (**SEQ ID NO:9**)). In another embodiment, the elbow region for the VL1 constructs described above is GQPKAA (**SEQ ID NO:78**). In certain embodiments, the VL1 constructs have a linker but no elbow region. In such instances, the linker can be, e.g., SEQ ID NO:56 or SEQ ID NO:58.

Solution to Heavy Chain Mispairing Problem

This application also discloses lysine repositioning as an effective strategy for heavy chain heterodimerization. Based on structural analysis of the CH3 domains, modeling, and analysis of potential interface mutations, a strategy of lysine repositioning was devised to engineer asymmetric complementary CH3 interfaces (*see*, **Figure 2**). Specifically, substitution of Lys409 (EU numbering) with Ser and Ser364 (EU numbering) with Lys in CH3 monomer A repositions the lysine from β-strand E to the adjacent antiparallel β-strand B. Conversely, substitution of Lys370 (EU numbering) with Ser and Phe405 (EU numbering) with Lys in CH3 monomer B repositions the Lysine from β-strand B to β-strand E. In other embodiments, the lysine repositioning involves the mutations of MP4 of Table 2. The repositioned lysines are stacked in the heterodimer, but impose steric and charge clashes in the homodimers, which prevent their formation, and therefore drive heterodimerization of the CH3 domains. Thus, by incorporating these changes into the CH3 domain of an antibody (*e.g.*, CH3 of an IgG1 antibody) one can increase heterodimerization of the heavy chain of an antibody relative to an antibody without these mutations. This strategy was found to be a highly effective strategy for heavy chain heterodimerization with superior or comparable efficiency to published Fc heterodimerization mutations.

The amino acid sequence of a wild type human IgG1 CH3 domain is provided below:

```
1   GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN
51  YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS
101 LSLSPGK (SEQ ID NO:11)
```

The amino acid sequence of a wild type human IgG4 CH3 domain is provided below:

1 GQPREPQVYT LPPSQEEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN
51 YKTTPPVLDS DGSFFLYSRL TVDKSRWQEG NVFSCSVMHE ALHNHYTQKS
101 LSLSLGK (**SEQ ID NO:12**)

In one embodiment one of the two CH3 domains of an Fc region of an antibody comprises the amino acid sequence set forth below:

1 GQPREPQVYT LPPSRDELTK NQVKLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS
61 DGSFFLYSLL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:13**)

In this embodiment, the second of the two CH3 domains of an Fc region of an antibody comprises the amino acid sequence set forth below:

1 GQPREPQVYT LPPSRDELTK NQVSLTCLVS GFYPSDIAVE WESNGQPENN YKTTPPILDS
61 DGSFKLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:14**)

In some embodiments, the above-mentioned two CH3 domains may include five or fewer, four or fewer, three or fewer, two or fewer, or one amino acid substitution in one or both CH3 domains. For example, the CH3 domain can be mutated to alter one or more effector functions of an antibody. Non-limiting examples of positions (according to EU numbering) that can be modified in a CH3 domain to alter one or more effector functions include positions 342, 344, 356, 358, 359, 360, 361, 362, 373, 375, 376, 378, 380, 382, 383, 384, 386, 388, 389, 398, 414, 416, 419, 428, 430, 433, 434, 435, 437, 438, and 439. See also examples of possible sites for substitutions and examples of mutations in U.S. Patent No.: US 8,586,713 B2 at column 10, lines 32-64. Other non-limiting examples of substitutions (listed according to EU numbering) that can be made in a CH3 domain to alter one or more effector functions include E345R, H433A, N434A, H435A, Y436D, Q438D, K439E, S440K, and/or K439E/S440K (see, Diebolder CA et al., *Science*, 343(6176): 1260–1263 (2014)). Other substitutions that can be made to a CH3 domain include those that affect binding to protein A. Non-limiting examples of such substitutions include H435R, H435R, and/or Y436F (US Patent No.: US 8,586,713 B2) (all EU numbering). Substitutions may also be made to introduce an artificial disulfide bond. Non-limiting examples of such substitutions include P445G, G446E, and K447C; P343C and A431C; and S375C and P396C (WO2011/003811 A1) (all EU numbering). The CH3 domain may be mutated to make changes to surface residues. This can be, e.g., for altering the isoelectric point (pI) of the antibody. Non-limiting examples of such substitutions include E345K, Q347E/K/R,

R355E, R355Q, K392E, K392N, Q419E (US 2014/0294835 A1) (all EU numbering). In some instances, the C-terminal of the CH3 domain can be truncated and/or modified. For example, one can delete K447 (EU numbering) and/or add the peptide DEDE or other amino acids to the C-terminus of the CH3 domain. In certain instances, the CH3 domain can be mutated to affect glycosylation of the domain. A non-limiting example of such a mutation is Y407E (EU numbering) (Rose et al., *MAbs*, 5(2):219-28 (2013)).

The CH3 domains described above can be part of an Fc domain. The Fc domain of a heavy chain of an antibody includes the hinge region, a CH2 domain and a CH3 domain. The CH3 domain may include mutations in addition to the lysine repositioning mutations discussed above.

The hinge region in the Fc domain can be a hinge region of any antibody class. The hinge region of the IgG1, IgG2, and IgG4 antibodies generally extend from the amino acid at position 216 to the amino acid at position 230 (position numbering according to EU numbering). In certain embodiments, the hinge region is a hinge from an IgG1 antibody. In other embodiments, the hinge region is a hinge from an IgG4 antibody. When the hinge region is from the IgG4 class it may contain the S228P (EU numbering) mutation. Below are exemplary hinge regions that can be employed either in whole or in part (e.g., there can be N- and/or C-terminal truncations).

Human IgG1 hinge: EPKSCDKTHTCPPCP (**SEQ ID NO:15**)

Human IgG4 hinge: ESKYGPPCPSCP (**SEQ ID NO:16**)

Mutant human IgG4 (S228P) hinge: ESKYGPPCP**P**CP (**SEQ ID NO:17**)

In certain instances, 1, 2, 3, 4, or 5 amino acids may be deleted at the N- and/or C-terminus of the above hinge sequences. In certain instances, there could be four or fewer, three or fewer, two or fewer, one, two, three, or four amino acid substitutions, deletions, and/or insertions in the hinge sequences or N- and/or C-terminal truncations thereof.

The CH2 domain can be from any class of antibody. In certain embodiments, the CH2 domain is from an IgG1 antibody. In other embodiments, the CH2 domain is from an IgG4 antibody. The CH2 domains may contain one or mutations. For example, the CH2 domain may have a mutation of the N-linked glycosylation site such that that site is not glycosylated. In certain embodiments, the asparagine in the N-linked glycosylation site of a CH2 domain is mutated to glutamine (e.g., Asn297Gln). In other embodiments, the threonine in the N-linked glycosylation site of a CH2 domain is mutated to alanine or cysteine (e.g.., Thr299Ala or Thr299Cys). In other examples, the CH2 domain may be mutated to change the effector

function, e.g., Leu234Ala/Leu235Ala, Pro329Gly, and/or Pro331Ser. The CH2 domain can also be mutated to change binding to FcRn.

In certain embodiments, the constant domain is an IgG4P/IgG1 hybrid. In certain instances, the constant domain is an IgG4P/IgG1 (agly) hybrid. These hybrids include the hinge region and CH2 domain of IgG4 and the CH3 domain of IgG1. The hinge region of IgG4 has the S228P mutation. In the agly construct, it further includes one of an N297Q, T299A, or T299C mutation.

In one embodiment, one of the two Fc regions comprises the amino acid sequence set forth below (hinge region italicized; N-linked glycosylation site underlined; CH2 region in regular font; CH3 domain boldened):

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK **GQPREPQVYT LPPSRDELTK NQV*K*LTCLVK**
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYS*LL* TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:18**)

In this embodiment, the second of the two Fc regions comprises the amino acid sequence set forth below (hinge region italicized; N-linked glycosylation site underlined; CH2 region in regular font; CH3 domain boldened):

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK **GQPREPQVYT LPPSRDELTK NQVSLTCLV*S***
GFYPSDIAVE WESNGQPENN YKTTPP*I*LDS DGSF*K*LYS*K*L TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:19**)

In another embodiment, one of the two Fc regions comprises the amino acid sequence set forth below (hinge region italicized; the mutated N-linked glycosylation site underlined; CH2 region in regular font; CH3 domain boldened):

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYQSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK **GQPREPQVYT LPPSRDELTK NQV*K*LTCLVK**
GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYS*L*L TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:20**)

In this embodiment, the second of the two Fc regions comprises the amino acid sequence set forth below (hinge region italicized; CH2 region in regular font with the mutated N-linked glycosylation site of the CH2 domain underlined; CH3 domain boldened):

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
PEVKFNWYVD GVEVHNAKTK PREEQYQSTY RVVSVLTVLH QDWLNGKEYK
CKVSNKALPA PIEKTISKAK **GQPREPQVYT LPPSRDELTK NQVSLTCLVS**
GFYPSDIAVE WESNGQPENN YKTTPPILDS DGSFKLYSKL TVDKSRWQQG
NVFSCSVMHE ALHNHYTQKS LSLSPG (SEQ ID NO:21)

These Fc regions can include nine or fewer, eight or fewer, seven or fewer, six or fewer, five or fewer, four or fewer, three or fewer, two or fewer, one, two, three, four, five, six, seven eight, or nine amino acid substitutions, insertions, and/or deletions so long as the Fc domains can heterodimerize. The amino acid substitutions may be conservative or non-conservative amino acid substitutions.

Any of the CH3 domains and Fc domains described above can be part of a heavy chain that pairs with the light chains described above that have their CL domains replaced with a CH2E domain (or a CH2M domain). Specifically, the heavy chains can include a second CH2 domain that is directly linked, or linked via an intervening sequence, to a VH domain, wherein the second CH2 domain is a CH2E domain, or a fragment thereof that can dimerize with a CH2E domain. In certain instances, the heavy chains can include a second CH2 domain that is directly linked to or linked via an intervening sequence to a VH domain, wherein the second CH2 domain is a CH2M domain, or a fragment thereof that can dimerize with a CH2M domain. Any of the CH3 domains and Fc domains described above can also be part of a heavy chain that pairs with the light chains described above that have the CH1 and CL domains. In certain instances, any of the CH3 domains and Fc domains described above are part of a first heavy chain that pairs with the light chains described above that have their CL domains replaced with a CH2E domain (or a CH2M domain) and any of the CH3 domains and Fc domains described above are part of a second heavy chain that pairs with the light chains described above that include the CH1 and CL domains.

In one embodiment, the heavy chain comprises the following amino acid sequence:

ASTKGPTVKI LQS**I**CDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM DVDLSTASTT
QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST KKCAS*DKTHT CPPCP*APELL
GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ
YQSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAK**GQPRE PQVYTLPPSR**
DELTKNQVKL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF LYSILTVDKS
RWQQGNVFSC SVMHEALHNH YTQKSLSLSP G (SEQ ID NO:22)

In one embodiment, this heavy chain heterodimerizes with a light chain comprising the following amino acid sequence:

RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDSG KKCA (**SEQ ID NO:6**)

These heavy and light chain sequences can each include nine or fewer, eight or fewer, seven or fewer, six or fewer, five or fewer, four or fewer, three or fewer, two or fewer, one, two, three, four, five, six, seven eight, or nine amino acid substitutions, insertions, and/or deletions so long as the heavy and light chains can heterodimerize. The amino acid substitutions may be conservative or non-conservative amino acid substitutions.

In some embodiments, the CH3 domains and Fc domains described above that heterodimerize can be part of a monovalent antibody. The monovalent antibody comprises an Fab that is linked directly or via an intervening linker sequence to one of the two Fc regions of the antibody; the other Fc region does not have an Fab region attached to it.

In other embodiments, the CH3 domains and Fc domains described above that heterodimerize can be part of a single chain Fc (scFc). In certain instances, the CH1 or CL domains of an Fab are linked directly or via an intervening linker to a first Fc region and the Fc region is linked to the second Fc region (*e.g.,* the C-terminus of the CH3 domain of the first Fc region is linked to the N-terminus of the hinge region of the second Fc region).

In certain embodiments, the Fc domains described above that heterodimerize are directly linked, or linked via linker to an Fab. That is, the VH/CH1 containing part of the Fab is linked via its C-terminus to the N-terminus of the hinge region of a first Fc and the VL/CL containing part of the Fab is linked via its C-terminus to the N-terminus of the hinge region of a second Fc region.

In yet other embodiments, the CH3 domains and Fc domains described above that heterodimerize are part of an antibody comprising a first heavy chain and a second heavy chain, wherein the first and second heavy chains pair with a common light chain.

Nucleic Acids

This disclosure also encompasses nucleic acids encoding the heavy and light chains of the heterodimeric bispecific and monovalent antibodies described herein. Many nucleic acid sequences encoding immunoglobulin regions including the VH, VL, hinge, CH1, CH2, CH3, and

CH4 regions are known in the art. *See, e.g.,* Kabat et al. in SEQUENCES OF IMMUNOLOGICAL INTEREST, Public Health Service N.I.H., Bethesda, Md., 1991. Using the guidance provided herein, one of skill in the art could combine such nucleic acid sequences and/or other nucleic acid sequence known in the art to create nucleic acid sequences encoding the heterodimeric bispecific antibodies described herein.

In addition, nucleic acid sequences encoding heterodimeric bispecific antibodies described herein can be determined by one of skill in the art based on the amino acid sequences provided herein and knowledge in the art. Besides more traditional methods of producing cloned DNA segments encoding a particular amino acid sequence, companies now routinely produce chemically synthesized, gene-sized DNAs of any desired sequence to order, thus streamlining the process of producing such DNAs.

Methods of Making Bispecific Antibodies

The bispecific and monovalent antibodies described herein can be made using methods well known in the art. For example, nucleic acids encoding the four polypeptide chains of a bispecific antibody can be introduced into a host cell by a variety of known methods, *e.g.,* transformation, transfection, electroporation, bombardment with nucleic acid-coated microprojectiles, etc. In some instances, the nucleic acids encoding the bispecific antibody can be inserted into a vector appropriate for expression in the host cells before being introduced into the host cells. Typically, such vectors can contain sequence elements enabling expression of the inserted nucleic acids at the RNA and protein levels. Such vectors are well known in the art, and many are commercially available. The host cells containing the nucleic acids can be cultured under conditions so as to enable the cells to express the nucleic acids. The resulting heterodimeric bispecific antibodies can be collected from the cell mass or the culture medium. Alternatively, the heterodimeric bispecific antibodies can be produced *in vivo*, for example in plant leaves (*see, e.g.,* Scheller et al., *Nature Biotechnol.*, 19: 573-577 (2001) and references cited therein), bird eggs (*see, e.g.,* Zhu et al. (2005), *Nature Biotechnol.*, 23: 1159-1169 (2005) and references cited therein), or mammalian milk (*see, e.g.,* Laible et al., *Reprod. Fertil. Dev.* 25(1): 315 (2012)). The isolated antibodies can be formulated as a sterile composition for administration to a human subject.

Several kinds of host cells can be used including, *e.g.*, bacterial cells such as *Escherichia coli* or *Bacillus stearothermophilus*, fungal cells such as *Saccharomyces cerevisiae* or *Pichia pastoris*, insect cells such as lepidopteran insect cells including *Spodoptera frugiperda* cells, or mammalian cells such as Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, monkey kidney cells, HeLa cells, human hepatocellular carcinoma cells, or 293 cells, among many others.

The following are examples of the practice of the invention. They are not to be construed as limiting the scope of the invention in any way.

Examples

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art can develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

Example 1: Engineering Heavy Chain Heterodimerization

The CH3 domain is one of the main points of contact between the two heavy chains of an IgG. The two CH3 domains bind each other in a head-to-tail orientation with tight packing of contact residues at the interface. Heavy chain heterodimerization can be achieved by engineering the hydrophobic surface at the core of the contact between the two CH3 domains or by altering charged residues at the perimeter of the contact surface. In order to maintain the tight packing of hydrophobic contact residues, the perimeter of the CH3 interface was chosen for engineering asymmetry. Further design criteria for mutation of the CH3 domains included: (i) efficient drivers of heterodimerization; (ii) few mutations; and (iii) overall charge-neutral changes. Structural analysis of the CH3 domain and design of mutations was done using a crystal structure of human IgG1 Fc (PDB code 3AVE). A comparative analysis has shown high identity between multiple published structures of human Fc domains suggesting the choice of structure may not be critical for engineering the CH3. Based on modeling and analysis of potential interface mutations, a strategy of lysine repositioning was devised to engineer asymmetric complementary

CH3 interfaces (**Figure 2**). Substitution of Lys409 (EU numbering) with Ser and Ser364 (EU numbering) with Lys in monomer A repositions the Lysine from β-strand E to the adjacent antiparallel β-strand B. Conversely, substitution of Lys370 (EU numbering) with Ser and Phe405 (EU numbering) with Lys in monomer B repositions the Lysine from β-strand B to β-strand E. The repositioned lysines are stacked in the heterodimer, but impose steric and charge clashes in the homodimers, which should prevent their formation, and therefore drive heterodimerization.

To experimentally test the effectiveness of the heterodimerization by lysine repositioning, test antibodies were constructed as mono-mAbs, where one side is a full human IgG1 heavy chain and the other is a free Fc without Fab. The resulting heterodimer can easily be distinguished from the homodimers due to the significant difference in molecular weight, and heterodimerization can be evaluated by separation on SDS-PAGE. The anti-EGFR antibody M60-A02 was used in these test constructs. The mutations in the CH3 domains were generated by PCR-based mutagenesis using the primers listed in Table 1.

Table 1. *Primers for generating the CH3 mutations S364K/K409S and K370S/F405K in human IgG1 by overlapping PCR.*

PCR	forward primer		reverse primer	
S364K 5'	CO2-966	TGGGCTGCCTGGTCAAG (**SEQ ID NO:23**)	CO2-968	gtcagTTTgacctggttcttg (**SEQ ID NO:24**)
S364K 3'	CO2-967	caagaaccaggtcAAActgac (**SEQ ID NO:25**)	AV1-177	GAATTGGCCGCCCTAGATG (**SEQ ID NO:26**)
K409S 5'	CO2-966	TGGGCTGCCTGGTCAAG (**SEQ ID NO:27**)	CO2-972	ggtgagGCTgctgtagagGA (**SEQ ID NO:28**)
K409S 3'	CO2-971	TCctctacagcAGCctcacc (**SEQ ID NO:29**)	AV1-177	GAATTGGCCGCCCTAGATG (**SEQ ID NO:30**)
K370S 5'	CO2-548	GCTCTGGGTTCCAGGTTC (**SEQ ID NO:31**)	CO2-970	gatagaagccGCTgaccagg (**SEQ ID NO:32**)
K370S 3'	CO2-969	cctggtcAGCggcttctatc (**SEQ ID NO:33**)	AV1-177	GAATTGGCCGCCCTAGATG (**SEQ ID NO:34**)
F405K 5'	CO2-548	GCTCTGGGTTCCAGGTTC (**SEQ ID NO:35**)	CO2-974	tgtagagTTTgaaggagccgt (**SEQ ID NO:36**)
F403K 3'	CO2-973	acggctccttcAAActctaca (**SEQ ID NO:37**)	AV1-177	GAATTGGCCGCCCTAGATG (**SEQ ID NO:38**)

The amplification was carried out using Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493L), and the PCR products were used with the HiFi Gibson assembly kit (SGI #GA1100-50) to build the constructs.

In order the gain some insight into the individual effects of the repositioned lysines, constructs with individual mutations were built as well. These half-designs were named MP1 and MP2, respectively (Table 2).

Table 2. *Constructs for testing Fc heterodimerization.*

Protein	Monomer A (anti-EGFR M60-A02)			Monomer B (human IgG1 Fc)		
MP1	pMP233	M60-A02 mp1a	S364K	pMP234	Fc mp1b	K370S
MP2	pMP235	M60-A02 mp2a	K409S	pMP236	Fc mp2b	F405K
MP3	pMP237	M60-A02 mp3a	S364K/K409S	pMP238	Fc mp3b	K370S/F405K
MP4	pMP254	M60-A02 mp4a	S364K/K409L	pMP259	Fc mp4b	K370S/V397I/ F405K
ZW1	pMP205	M60-A02 zw1a	T350V/L351Y/ F405A/T407V	pMP208	Fc mzw1b	T350V/T366L/ K392L/T394W
Knobs/ Holes	pMP209	M60-A02 hole	T366S/L368A/ Y407V	pMP212	Fc knob	T366W
electrost. steering	pMP213	M60-A02 KD	K409D	pMP216	Fc DK	D399K
wt IgG1	pMP221	M60-A02 wt IgG1	none	pMP222	Fc wt hu IgG1	none

The complete design has both lysines repositioned (pMP237 has the mutations S364K/K409S, and pMP238 has K370S/F405K) and should be most effective in driving heterodimerization. This design was named MP3. The peptide sequences of the CH3 domains of MP3 are shown below:

Amino Acid Sequence of the CH3 domain MP3 Monomer A (mutations are bold and underlined)

1 GQPREPQVYT LPPSRDELTK NQV**K**LTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS
61 DGSFFLYS**S**L TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:39**)

Amino Acid Sequence of the CH3 domain MP3 Monomer B (mutations are bold and underlined)

1 GQPREPQVYT LPPSRDELTK NQVSLTCLV**S** GFYPSDIAVE WESNGQPENN YKTTPPVLDS
61 DGSF**K**LYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:40**)

Additionally, several published CH3 heterodimerization mutations were generated for comparison (Atwell et al., *J. Mol. Biol.*, *270*:26-35, 1997; Gunasekaran et al., J. *Biol. Chem.*, 285:19637-19646, 2010; Von Kreudenstein et al., *mAbs* 5:646-654, 2013). Furthermore, the

constructs pMP221 and pMP222 were built to represent a wild-type IgG1 control. All constructs that were generated for this initial test of heterodimerization are summarized in Table 2, and all plasmids were verified by DNA sequencing.

The antibodies were expressed in CHO cells by transient transfection. All antibodies expressed well, producing titers between 100 and 200 mg/L after 9 days expression using CHO cells transfected at 6 X 10^6 cells/ml with Fectopro and DNA at a 2:1 ratio and temperature shift to 28^0C the next day. Supernatants were purified on a protein A column and aliquots were analyzed by SDS-PAGE (**Figure 3A**). The heterodimer of IgG1 heavy chain and free Fc was predominantly found in all samples. In addition, the co-transfection of wild-type IgG1 and Fc also produced strong bands corresponding to both homodimers. These bands were significantly weaker in the published mutants and all lysine repositioning mutants. Thus, lysine repositioning and published mutations drive the formation of heavy chain heterodimers and reduce homodimerization. The protein A-purified material was further analyzed by mass spectrometry and the relative amounts of major components estimated. The amount of heterodimer in the samples ranged from 35% to 80%, being slightly higher in the lysine repositioning mutants MP1, MP2 and MP3 than in published designs (**Figure 3B**). However, varying expression levels of the individual chains may influence these results making direct comparisons difficult. Importantly, the designs MP2 and MP3 as well as ZW1 and Knobs-into-holes mutations all contained monomeric Fc. The presence of monomeric Fc demonstrates that Fc dimers are destabilized by the mutations in CH3, and are therefore less like to form. Altogether, these results show that lysine repositioning is an efficient heavy chain heterodimerization strategy, which prevents homodimerization as efficiently as several published mutations.

After this initial testing, additional structural modeling was done to identify mutations that could improve the packing of interface residues. Such mutations could potentially increase the efficiency of heterodimerization or the stability of dimers, and would therefore be beneficial. Two changes were identified that could improve the original lysine repositioning design MP3. This new design was named MP4 and the amino acid changes are shown in Table 2. IgG1/Fc heterodimers with the MP4 mutations were expressed in CHO cells as described above and purified from supernatants by Protein A. Mass spectrometry analysis demonstrated that MP4 produced heterodimers between the IgG1 heavy chain and the free Fc as efficiently as MP3 (**Figure 3B**).

All heterodimeric proteins were further purified on KappaSelect columns to remove the Fc dimers and monomers, followed by a polishing step on Tandem Superdex S200. This three-step purification yielded relatively pure heterodimers that were used for differential scanning calorimetry (DSC) to determine melting temperatures. The wild type IgG1 control showed the expected Tm of the CH3 domain of ~83°C (**Figure 3C**). The ZW1 design has a similar CH3 melting temperature (**Figure 3C**), as it was extensively engineered in an iterative process to regain the thermal stability of wild type IgG1 (Von Kreudenstein et al., *mAbs* 5:646-654, 2013). The knobs-into-holes and electrostatic steering comparator molecules showed only one peak in DSC profiles, suggesting that the Tm of the CH3 domain is decreased to ~70°C and the domain is melting with the CH2 and Fab domains, producing the single peak on DSC (**Figure 3C**). The MP3 molecule showed only one peak at ~70°C in DSC profiles as well, indicating the Tm of CH3 has similarly dropped (**Figure 3C**). The proteins MP4 and MP4a3b (MP4 Monomer A and MP3 Monomer B) showed a shoulder on the right of the CH2/Fab peak (**Figure 3C**), suggesting the mutation K409L confers slightly increased stability over the original mutation K409S, and that it may be possible to improve the Tm by further engineering. Nevertheless, the design MP4 has a decreased Tm compared to a wild-type IgG1 CH3 domain.

The peptide sequences of the CH3 domains of MP4 are shown below:

Sequence of the CH3 domain MP4 Monomer A (mutations are bold and underlined)

1 GQPREPQVYT LPPSRDELTK NQV**K**LTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS
61 DGSFFLYS**L**L TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:41**)

Sequence of the CH3 domain MP4 Monomer B (mutations are bold and underlined)

1 GQPREPQVYT LPPSRDELTK NQVSLTCLV**S** GFYPSDIAVE WESNGQPENN YKTTPP**I**LDS
61 DGSF**K**LYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG (**SEQ ID NO:42**)

The DNA sequences of the plasmids (pMP254 and pMP259) encoding MP4 are provided below:

DNA sequence of pMP254, anti-EGFR M60-A02 IgG1 heavy chain with MP4A mutations (S634K, K409L):

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   1 agcttgacat tgattattga ctagttatta atagtaatca attacggggt cattagttca
  61 tagcccatat atggagttcc gcgttacata acttacggta aatggcccgc ctggctgacc
 121 gcccaacgac ccccgcccat tgacgtcaat aatgacgtat gttcccatag taacgccaat
 181 agggactttc cattgacgtc aatgggtgga gtatttacgg taaactgccc acttggcagt
 241 acatcaagtg tatcatatgc caagtacgcc ccctattgac gtcaatgacg gtaaatggcc
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 301 cgcctggcat tatgcccagt acatgacctt atgggacttt cctacttggc agtacatcta
 361 cgtattagtc atcgctatta ccatggtgat gcggttttgg cagtacatca atgggcgtgg
 421 atagcggttt gactcacggg gatttccaag tctccacccc attgacgtca atgggagttt
 481 gttttggcac caaaatcaac gggactttcc aaaatgtcgt aacaactccg ccccattgac
 541 gcaaatgggc ggtaggcgtg tacggtggga ggtctatata agcagagctc gtttagtgaa
 601 ccgtcagatc gcctggagac gccatccacg ctgttttgac ctccatagaa gacaccggga
 661 ccgatccagc ctccgcggcc gggaacggtg cattggaacg cggattcccc gtgccaagag
 721 tgacgtaagt accgcctata gagtctatag gcccaccccc ttggcttctt atgcatgcta
 781 tactgttttt ggcttggggt ctatacaccc ccgcttcctc atgttatagg tgatggtata
 841 gcttagccta taggtgtggg ttattgacca ttattgacca ctcccctatt ggtgacgata
 901 ctttccatta ctaatccata acatggctct ttgccacaac tctctttatt ggctatatgc
 961 caatacactg tccttcagag actgacacgg actctgtatt tttacaggat ggggtctcat
1021 ttattattta caaattcaca tatacaacac caccgtcccc agtgcccgca gtttttatta
1081 aacataacgt gggatctcca cgcgaatctc gggtacgtgt tccggaacgg tggagggcag
1141 tgtagtctga gcagtactcg ttgctgccgc gcgcgccacc agacataata gctgacagac
1201 taacagactg ttcctttcca tgggtctttt ctgcagtcac cgtccttgac acgggatccg
1261 cggccgccac cATGGGTTGG AGCCTCATCT TGCTCTTCCT TGTCGCTGTT GCTACGCGTG
1321 TCCTGTCCGA GGTGCAGCTG TTGGAGTCTG GGGGAGGCTT GGTCCAGCCT GGGGGGTCCC
1381 TGAGACTCTC CTGTGCAGCC TCTGGATTCA CCTTCAGTGA CTATATTATG CACTGGGTCC
1441 GCCAGGCTCC AGGGAAGGGG CTGGAGTGGG TCTCAGTTAT TAGTAGTTCT GGTGGCGACA
1501 CATCCTACGC AGACTCCGTG AAGGGCCGAT TCACCATCTC CAGAGACAAT TCCAAGAACA
1561 CGCTGTATCT GCAAATGAAC AGCCTGAGAG CCGAGGACAC GGCCGTGTAT TACTGTGCGA
1621 AAGTCCTCGC GGGTTACTTC GACTGGTTAC CCTTTGACTA CTGGGGCCAG GGAACCCTGG
1681 TCACCGTCTC GAGCGCCTCC ACCAAGGGCC CATCGGTCTT CCCCCTGGCA CCCTCCTCCA
1741 AGAGCACCTC TGGGGGCACA GCGGCCCTGG GCTGCCTGGT CAAGGACTAC TTCCCCGAAC
1801 CGGTGACGGT GTCGTGGAAC TCAGGCGCCC TGACCAGCGG CGTGCACACC TTCCCGGCTG
1861 TCCTACAGTC CTCAGGACTC TACTCCCTCA GCAGCGTGGT GACCGTGCCC TCCAGCAGCT
1921 TGGGCACCCA GACCTACATC TGCAACGTGA ATCACAAGCC CAGCAACACC AAGGTGGACA
1981 AGAAAGTTGA GCCCAAATCT TGTGACAAGA CTCACACATG CCCACCGTGC CCAGCACCTG
2041 AACTCCTGGG GGGACCGTCA GTCTTCCTCT TCCCCCCAAA ACCCAAGGAC ACCCTCATGA
2101 TCTCCCGGAC CCCTGAGGTC ACATGCGTGG TGGTGGACGT GAGCCACGAA GACCCTGAGG
2161 TCAAGTTCAA CTGGTACGTG GACGGCGTGG AGGTGCATAA TGCCAAGACA AAGCCGCGGG
2221 AGGAGCAGTA CAACAGCACG TACCGTGTGG TCAGCGTCCT CACCGTCCTG CACCAGGACT
2281 GGCTGAATGG CAAGGAGTAC AAGTGCAAGG TCTCCAACAA AGCCCTCCCA GCCCCCATCG
2341 AGAAAACCAT CTCCAAAGCC AAAGGGCAGC CCCGAGAACC ACAGGTGTAC ACCCTGCCCC
2401 CATCCCGGGA TGAGCTGACC AAGAACCAGG TCAAACTGAC CTGCCTGGTC AAAGGCTTCT
2461 ATCCCAGCGA CATCGCCGTG GAGTGGGAGA GCAATGGGCA GCCGGAGAAC AACTACAAGA
2521 CCACGCCTCC CGTGTTGGAC TCCGACGGCT CCTTCTTCCT CTACAGCCTC CTCACCGTGG
2581 ACAAGAGCAG GTGGCAGCAG GGGAACGTCT TCTCATGCTC CGTGATGCAT GAGGCTCTGC
2641 ACAACCACTA CACGCAGAAG AGCCTCTCCC TGTCTCCGGG TTAGtaatta attaatgcat
2701 ctagggcggc caattccgcc cctctccctc ccccccccct aacgttactg gccgaagccg
2761 cttggaataa ggccggtgtg cgtttgtcta tatgtgattt tccaccatat tgccgtcttt
2821 tggcaatgtg agggcccgga aacctggccc tgtcttcttg acgagcattc ctaggggtct
2881 ttcccctctc gccaaaggaa tgcaaggtct gttgaatgtc gtgaaggaag cagttcctct
2941 ggaagcttct tgaagacaaa caacgtctgt agcgaccctt tgcaggcagc ggaacccccc
3001 acctggcgac aggtgcctct gcggccaaaa gccacgtgta taagatacac ctgcaaaggc
3061 ggcacaaccc cagtgccacg ttgtgagttg gatagttgtg gaaagagtca aatggctctc
3121 ctcaagcgta ttcaacaagg ggctgaagga tgcccagaag gtaccccatt gtatgggatc
3181 tgatctgggg cctcggtgca catgctttac atgtgtttag tcgaggttaa aaaaacgtct
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6961 actggaaagc gggcagtgag cgcaacgcaa ttaatgtgag ttagctcact cattaggcac
7021 cccaggcttt acactttatg cttccggctc gtatgttgtg tggaattgtg agcggataac
7081 aatttcacac aggaaacagc tatgaccatg attacgcca (**SEQ ID NO:43**)

DNA Sequence of pMP259, IgG1 Fc with MP4B mutations (K370S, V397I, F405K):

```
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1621 AGTACAAGTG CAAGGTCTCC AACAAAGCCC TCCCAGCCCC CATCGAGAAA ACCATCTCCA
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6421 gtatgttgtg tggaattgtg agcggataac aatttcacac aggaaacagc tatgaccatg
6481 attacgcca  (SEQ ID NO:44)
```

Example 2: Testing of Heavy Chain Heterodimerization Mutants

To further characterize the lysine repositioning designs, the mutations were incorporated into full IgG1 heavy chains. These constructs contained the same Fab on both heavy chains (anti-EGFR M60-A02) to limit potential influences and avoid the light chain pairing problem. Equal amounts of the two heavy chains and the light chain of M60-A02 were expressed in CHO transients and the supernatant analyzed by SDS-PAGE. All supernatants contained considerable amounts of half-antibodies composed of 1 heavy and 1 light chain, but the design MP4 produced a lower amount of half-antibodies than MP3 as shown by quantification of LC-MS peaks (data not shown). The mass spectrometry analysis also revealed that half-antibodies were formed only by heavy chain B in these samples (data not shown). This suggests that half-antibody formation may be a consequence of over-expression of heavy chain B, which opens up the possibility to reduce the level of half-antibodies by optimizing the ratio between the two heavy chains. Therefore, the heavy chains of design MP4 with identical Fabs (plasmids pMP254 and pMP399) were transfected into CHO-S cells in various ratios from 1:5 to 5:1. The total amount of heavy chains (14 µg) and the amount of light chain were kept constant in these transfections. The over-expression of chain A reduced the formation of half-antibodies and increased the amount of full IgG in the supernatants, confirming that half-antibody formation is influenced by differential expression between the chains (**Figure 4A**). Since both heavy chains in these experiments contained the same Fab (anti-EGFR M60), a differential expression of the two heavy chains might be due to the CH3 mutation. However, mass spectrometry analysis also demonstrated that over-expression of either chain lead to increased formation of the respective homodimer (**Figure 4B**). Thus, an equal ratio between the two heavy chains is a good compromise to minimize unwanted side products.

Example 3: Developing a Solution to the Light Chain Pairing Problem

In order to drive correct assembly of light chain: heavy chain (LC:HC) pairs in a bispecific antibody as illustrated in **Figure 1**, the interface between the chains might be engineered so that steric clashes or repelling charges prevent incorrect assembly. The light chain of an antibody makes contact with the heavy chain in the variable domain and the constant domain, and both contacts (VL:VH and CL:CH1) contribute to recognition and engagement. Consequently, point mutations in the constant domains may not be sufficient to steer each light

chain towards correct pairing, and engineering of the variable domains may become necessary. Therefore, an alternative strategy for solving the light chain pairing was pursued.

The variable domains of an antibody can maintain their binding properties in the absence of constant regions or when they are fused to heterologous proteins. We hypothesized that Ig-fold domains may be used in place of CH1/CL as a strategy to prevent mispairing of light chains and chose the CH2 domain of IgE (Cε2) as a candidate Ig-fold domain to substitute for the CH1:CL domain (**Figure 5A**). The CH2 domain of IgE (Cε2) forms homodimers that are linked by two inter-chain disulfides. This domain serves as dimerization interface between IgE heavy chains instead of a hinge region, which is not present in IgE. Also, unlike the CH2 domain of IgG the Cε2 domain is not involved in effector function and makes no contact with the FcεRIα (Holdom et al., *Nature Struct. & Mol. Biol.*, 18:571-576, 2011).

The structure of the Cε2 domain is similar to other Ig-fold domains such as CL and CH1 domains, but the interface and the angle between the units of a dimer are distinct (**Figure 5B**). The two Cε2 domains in a dimer have less extensive interaction than other Ig-fold dimers, and the interaction is dominated by polar rather than the usual nonpolar residues. Thus, Cε2 and CH1:CL are overall so different that cross-pairing of the Ig-fold domains is unlikely. However, the two prolines that mark the start of the first β-strand of the Ig-fold (highlighted in bold and underlined in **Figure 5C**) are in a similar position in the structures of Cε2 and CH1/kappa constant domains. Thus, it appears possible to fuse the variable domains of an antibody onto Cε2 domains, and maintain the geometry of the VH:VL pair and therefore its binding properties. This hybrid Fab construct composed of the variable domains of an antibody fused to Cε2 domains was named "E-Fab" (**Figure 5C**). The amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) is provided below (the N-linked glycosylation site is underlined):

```
1   VCSRDFTPPT VKILQSSCDG GGHFPPTIQL LCLVSGYTPG TINITWLEDG
51  QVMDVDLSTA STTQEGELAS TQSELTLSQK HWLSDRTYTC QVTYQGHTFE
101 DSTKKCA  (SEQ ID NO:1)
```

To fuse the VH domain to the Cε2 domain, the connector sequence (ASTKG (**SEQ ID NO:7**)) of the IgG1 CH1 constant region was joined to the first β-strand of the Cε2 domain starting with Pro2 (IMGT unique numbering of C-domains, **Figure 5C**). The amino acid

sequence of the CH2 domain of human immunoglobulin E (IgE) in which the first 8 amino acids (VCSRDFTP (**SEQ ID NO:8**)) are replaced with the first 5 amino acids of a human IgG1 CH1 domain (elbow region ASTKG (**SEQ ID NO:7**) is underlined) is provided below:

1 ASTKGPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTI**N ITW**LEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:45**)

The same strategy was used for fusing the VL to the Cε2 domain with the connector from the kappa constant region (RTVAA (**SEQ ID NO:9**)). The amino acid sequence of the CH2 domain of human immunoglobulin E, in which the first 8 amino acids (VCSRDFTP (**SEQ ID NO:8**)) are replaced with the first 5 amino acids of a human kappa domain (elbow region RTVAA (**SEQ ID NO:9**) is underlined) is provided below:

1 RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTI**N ITW**LEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:46**)

The human Cε2 domain has one N-linked glycosylation site at Asn38 (IMGT numbering), which was mutated to Gln to prevent glycosylation of this site in the E-Fab. In this initial design, the variable domains were grafted onto a Cε2 domain, which is unaltered except for the agly mutation and identical between heavy and light chain. This first design was named E-Fab E0. The amino acid sequences of the E0 design of the heavy chain of an E-Fab is provided below (the N-glycosylation site is mutated to glutamine):

1 ASTKGPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:3**)

The amino acid sequences of the E0 design of the light chain of an E-Fab is provided below (the N-glycosylation site is mutated to glutamine):

1 RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:4**)

However, since the Cε2 domain normally homodimerizes, additional mutations were introduced into the domains to prevent the formation of HC:HC or LC:LC dimers, and these

designs were named E-Fab E1 through E3 (Table 3; positions according to IMGT numbering system).

Table 3. Constructs for E-Fab testing.

Fab A (anti-EGFR M60-A02)	**light chain construct**	**heavy chain construct (HSA-fusion)**
wt IgG1	pXWU174	pMP363
E-Fab E0	pMP386 (**N38Q**)	pMP390 (**N38Q**)
E-Fab E1	pMP387 (**N38Q, L22G**)	pMP391 (**N38Q, L7W**)
E-Fab E2	pMP463 (**N38Q, T121G**)	pMP392 (**N38Q, S10I**)
E-Fab E3	pMP464 (**N38Q, T121S**)	pMP465 (**N38Q, S10T**)
Fab B (anti-IGF-1R C06)	**light chain (GFP fusion)**	**heavy chain**
wt IgG1 (no mutations)	pMP340	pMP341

The amino acid sequences of the E1 design of the heavy chain of an E-Fab is provided below:

1 ASTKGPTVKI WQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:47**)

The amino acid sequences of the E1 design of the light chain of an E-Fab is provided below:

1 RTVAAPTVKI LQSSCDGGGH FPPTIQLGCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:48**)

The amino acid sequences of the E2 design of the heavy chain and the light chain of an E-Fab are provided at the end of this Example.

The amino acid sequences of the E3 design of the heavy chain of an E-Fab is provided below:

1 ASTKGPTVKI LQSTCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST
101 KKCA (**SEQ ID NO:49**)

The amino acid sequences of the E3 design of the light chain of an E-Fab is provided below:

1 RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM
51 DVDLSTASTT QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDSS
101 KKCA (**SEQ ID NO:50**)

To evaluate the E-Fab designs in controlling light chain pairing, test constructs were built using the two Fabs M60-A02 anti-EGFR and M13.C06 anti-IGF1R. Both heavy chains belong to the subgroup HV3, and can therefore be purified using protein A. The test molecules were tagged with GFP (30 kDa, LC of Fab B anti IGF-1R) or HSA (66 kDa, HC of Fab A or E-Fab, anti-EGFR) to enable simple differentiation of correct vs. incorrect pairing by migration on SDS page (**Figure 6A**). The E-Fab constructs were built from gBlocks. Additional mutations were introduced into the Cε2 domain by PCR-based mutagenesis, and products cloned into CHO expression vectors derived from pV90/pV100 by restriction enzyme-based methods (**Table 3**). The Fabs were co-expressed by transient transfection in CHO-S cells at 40 ml scale. Cells were shifted to 28^0C for expression 24 hours after transfection, and supernatants harvested after 8 days and cleared by centrifugation and filtration (0.22 μm).

Analysis of the expressed proteins by SDS-PAGE showed that the E-Fab composed of the variable domains of a regular antibody fused to the Cε2 domain was highly efficient in controlling the light chain pairing. When the two Fabs M60-A02 and M13-C036 were co-expressed as wild-type IgG1/kappa Fab, mispairing of the M60-A02 light chain with the M13-C06 heavy chain was readily detected (**Figure 6B**). However, when the M60-A02 was made as E-Fab, no mispairing was seen. In contrast, point mutations in the CH1/CL domains failed to abrogate light chain mispairing in this experiment (data not shown). Similarly, when the anti-IGF-1R M13-C06 was constructed as E-Fab, no mispairing was detected when it was co-expressed with another Fab (anti IGF-1R G11) (**Figure 6C**). Importantly when the Fabs were expressed individually and binding to antigens tested by Octet, the E-fabs and wild-type Fabs similarly bound to His-tagged IGF-1R, which was loaded onto anti-His tips (**Figure 6D**).

Thus, based on the above data the E-Fab is an excellent strategy to solve the light chain pairing problem in a bispecific antibody, as it enforces strictly correct chain pairing and the binding properties of the Fab are maintained. Interestingly, the Cε2 domain did not strongly induce the formation of homodimers between the chains of the E-Fab. Nevertheless, the designs E2 and E3, which are engineered to form heterodimers between heavy and light chain, were carried forward and tested in the context of full bispecifics. Only the design E1, which appeared to induce some dimerization between heavy chains (**Figure 6B**) was not tested further. The peptide sequence of the constant domains of the E-Fab design E2 are shown below:

Amino Acid Sequence of E-Fab light chain constant region, agly N38Q (design E2, T121G, pMP463)

1 RTVAAPTVKI LQSSCDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM DVDLSTASTT
61 QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDSG KKCA (**SEQ ID NO:6**)

Amino Acid Sequence of E-Fab heavy chain constant region, agly N38Q (design E2, S10I, pMP392)

1 ASTKGPTVKI LQSICDGGGH FPPTIQLLCL VSGYTPGTIQ ITWLEDGQVM DVDLSTASTT
61 QEGELASTQS ELTLSQKHWL SDRTYTCQVT YQGHTFEDST KKCA (**SEQ ID NO:5**)

Example 4: Building and testing a bispecific asymmetric IgG

The E-Fab and lysine repositioning constructs were combined to generate full IgG-like bispecific antibodies. Such a bispecific antibody is composed of 4 different chains, and contains both light chain pairing and heavy chain hererodimerization solutions (**Figure 7**). Using again the anti-EGFR antibody M60-A02, and anti-IGF1R M13.C06 or G11, the two different IgG1 heavy chains were built with the heterodimerization mutations MP3.

Amino Acid Sequence of mature M60-A02 Ebody heavy chain (EFab E2, mp3a heterodimerization, pMP401))(CH2 domain underlined; CH3 domain boldened)

EVQLLESGGG LVQPGGSLRL SCAASGFTFS DYIMHWVRQA PGKGLEWVSV ISSSGGDTSY ADSVKGRFTI
SRDNSKNTLY LQMNSLRAED TAVYYCAKVL AGYFDWLPFD YWGQGTLVTV SSASTKGPTV KILQSICDGG
GHFPPTIQLL CLVSGYTPGT IQITWLEDGQ VMDVDLSTAS TTQEGELAST QSELTLSQKH WLSDRTYTCQ
VTYQGHTFED STKKCASDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV
KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE KTISKAK**GQP**
REPQVYTLPP SRDELTKNQV KLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS FFLYSSLTVD
KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPG (**SEQ ID NO:51**)

Amino Acid Sequence of mature M60-A02 Ebody light chain (EFab E2, pMP463)(CH2 domain underlined)

DIQMTQSPAT LSLSPGETAT LSCRASQSVS YYLAWYQQKP GQAPRLLIYD TFNRATGIPA RFSGSGSGTD
FTLTISRLEP EDFAVYYCQQ YGSSPPWLTF GGGTKVEIKR TVAAPTVKIL QSSCDGGGHF PPTIQLLCLV
SGYTPGTIQI TWLEDGQVMD VDLSTASTTQ EGELASTQSE LTLSQKHWLS DRTYTCQVTY QGHTFEDSGK
KCA (**SEQ ID NO:52**)

Amino Acid Sequence of mature M13.C06 IgG1 heavy chain (mp3b heterodimerization, pMP404) (CH3 domain boldened)

EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYRMQWVRQA PGKGLEWVSG ISPSGGTTWY ADSVKGRFTI
SRDNSKNTLY LQMNSLRAED TAVYYCARWS GGSGYAFDIW GQGTMVTVSS ASTKGPSVFP LAPSSKSTSG
GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS

NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW
YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAK**GQPREPQ**
VYTLPPSRDE LTKNQVSLTC LVSGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFKLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPG (**SEQ ID NO:53**)

Amino Acid Sequence of mature M13.C06 kappa light chain (pMP407)

DIQMTQSPLS LSASVGDRVT ITCQASRDIR NYLNWYQQKP GKAPKLLIYD ASSLQTGVPS RFGGSGSGTD
FSFTIGSLQP EDEATYYCQQ FDSLPHTFGQ GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY
PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN
RGEC (**SEQ ID NO:54**)

To control the pairing of the different light chains, the anti-EGFR Fab was constructed as Efab (**Figure 7**). A control antibody was built with the lysine repositioning mutations to generate an IgG1 heavy chain heterodimer that lacks a light chain pairing solution.

The antibodies were again expressed in CHO-S cells by transient transfection and the supernatants harvested and cleared after 8 days of culture. Separation by SDS-PAGE showed a band migrating at the size of a full IgG and an additional band corresponding to half-antibodies (**Figure 8A**). Mass spectrometry analysis demonstrated that half-antibodies were again formed by the heavy chain B in these experiments. Importantly, no light chain mispairing was detected in the E-Fabs by LC-MS. The IgG1 heterodimer showed some correct paring of heavy chains with their light chains, but a strong peak of heavy chain heterodimer with two anti-EGFR light chains was also detectable (**Figure 8B**). These results confirm again that the E-Fab solves the light chain pairing problem.

Importantly, homodimers between the heavy chains were not detectable in the mass-spec profiles, demonstrating that the heavy chain heterodimerization by lysine repositioning is highly efficient when combined with an E-Fab (**Figure 8B**). However, mass spectrometry also revealed small levels of O-glycans that were present on Efab bispecifics but not on the IgG1 control.

Next, crude supernatants were used in Octet binding experiments with soluble His-tagged EGFR (**Figure 8C**) and IGF-1R (**Figure 8D**). The IgG1 control as well as the bispecific Ebody bound to both EGFR and IGF-1R, but the binding appeared somewhat different between Ebody and control. However, the interpretation of these results is complex due to the different expression, amounts of half antibody and light chain mispairing in these crude samples.

Altogether, the analysis by mass spectrometry together with the binding data demonstrate that the Ebody correctly assembled into an asymmetric IgG. This is simply achieved by co-

expression of the four different chains (2HC and 2 LC) in CHO cells. The E-fab fully solved light chain pairing, and the lysine repositioning strictly enforced heavy chain heterodimerization in these experiments.

Example 5: Further Testing of the Bispecific Platform

To further test the bispecific platform using Efab and Fc heterodimerization, another IgG-like bispecific antibody was generated from two therapeutic antibodies, Trastuzumab and Cetuximab, which are approved for the treatment of various cancers. To generate the bispecific, the anti-HER antibody Trastuzumab was cloned using the light chain pairing-solution Efab, and was then combined with the anti-EGFR antibody Cetuximab using the mp4 heavy chain heterodimerization mutations (S364K/K409L and K370S/V397I/F405K). The four plasmids (pMP521, pMP526, pMP528, and pMP530) encoding the two heavy chains and two light chains of the antibody were then co-expressed in CHO-S cells by means of transient transfection, and the resulting bispecific antibody purified by protein A. This antibody was then used in Octet binding studies with soluble versions of the two antigens, HER2 and EGFR (see, **Figures 9A** and **9B**). As shown in Figures 9A and 9B, the bispecific antibody is capable of simultaneous binding to both its antigens.

pMP530 Trastuzumab Efab IgG1 mp4a

1 EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR
51 IYPTNGYTRY ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSRWG
101 GDGFYAMDYW GQGTLVTVSS ASTKGPTVKI LQSICDGGGH FPPTIQLLCL
151 VSGYTPGTIQ ITWLEDGQVM DVDLSTASTT QEGELASTQS ELTLSQKHWL
201 SDRTYTCQVT YQGHTFEDST KKCASDKTHT CPPCPAPELL GGPSVFLFPP
251 KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ
301 YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE
351 PQVYTLPPSR DELTKNQVKL TCLVKGFYPS DIAVEWESNG QPENNYKTTP
401 PVLDSDGSFF LYSLLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP
451 G (**SEQ ID NO:79**)

pMP528 Trastuzumab Efab light chain

1 DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS
51 ASFLYSGVPS RFSGSRSGTD FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ
101 GTKVEIKRTV AAPTVKILQS SCDGGGHFPP TIQLLCLVSG YTPGTIQITW
151 LEDGQVMDVD LSTASTTQEG ELASTQSELT LSQKHWLSDR TYTCQVTYQG
201 HTFEDSGKKC A (**SEQ ID NO:80**)

pMP521 Cetuximab IgG1 mp4b

```
1   DILLTQSPVI LSVSPGERVS FSCRASQSIG TNIHWYQQRT NGSPRLLIKY
51  ASESISGIPS RFSGSGSGTD FTLSINSVES EDIADYYCQQ NNNWPTTFGA
101 GTKLELKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151 DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201 LSSPVTKSFN RGEC (SEQ ID NO:81)
```

pMP526 Cetuximab kappa

```
1   QVQLKQSGPG LVQPSQSLSI TCTVSGFSLT NYGVHWVRQS PGKGLEWLGV
51  IWSGGNTDYN TPFTSRLSIN KDNSKSQVFF KMNSLQSNDT AIYYCARALT
101 YYDYEFAYWG QGTLVTVSAA STKGPSVFPL APSSKSTSGG TAALGCLVKD
151 YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
201 ICNVNHKPSN TKVDKKVEPK SCDKTHTCPP CPAPELLGGP SVFLFPPKPK
251 DTLMISRTPE VTCVVVDVSH EDPEVKFNWY VDGVEVHNAK TKPREEQYNS
301 TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIEKTISK AKGQPREPQV
351 YTLPPSRDEL TKNQVSLTCL VSGFYPSDIA VEWESNGQPE NNYKTTPPIL
401 DSDGSFKLYS KLTVDKSRWQ QGNVFSCSVM HEALHNHYTQ KSLSLSPG
    (SEQ ID NO:82)
```

Example 6: Further Testing of Light Chain Pairing Solutions

To further optimize the construction of Efabs, the linkers between the variable domains and the CH2 domain of IgE were investigated further. In previous examples the elbow regions from human kappa and CH1 domains were used as linkers between the variable domains and IgE CH2. However, other linker sequences could potentially alter the geometry or provide a different degree of flexibility, and thus affect the correct assembly of the chains or the binding of antigen by the Efab. To test this, the anti-HER2 antibody Trastuzumab was engineered as Efab and included various elbow sequences within the light chain. The linkers used were from a lambda light chain or from the human IgE CH2 domain, or flexible Gly-Ser linkers of different lengths (**Figure 10**). The Efabs were expressed as asymmetric IgG with Cetuximab (as in **Figure 9**) in CHO cells by transient transfection. The material was purified by protein A and tested in Octet binding studies. Interestingly, the various elbow linker sequences had little effect on the ability of Trastuzumab to bind to HER2, and all construct showed similar binding compared to a wild-type Trastuzumab Fab.

The IgM class of antibodies contains a CH2 domain similarly to IgE, which acts in place of a hinge region to pair two heavy chains by a disulfide bridge. Thus, it was tested whether using the IgM CH2 domain could solve the light chain pairing equally well as the IgE CH2

domain. To test this, two Fabs (M60 and C06) were again co-expressed as shown in **Figure 6A**, with one Fab fused to HSA and another Fab fused to GFP. In this case, however, the kappa and CH1 constant domain of one Fab were replaced with IgM CH2 domains, and the resulting molecule was named M-Fab (**Figure 10C**). Analysis of the chain pairing by SDS-PAGE showed that the M-Fab solved the light chain mispairing between M60 and C06 that was prominently observed in the control (**Figure 10C**). Thus, the M-Fab appears to function as efficiently as the Efab in solving the light chain pairing problem.

Next, the ability of an M-Fab to bind antigen was compared to Efabs with various elbow regions in the light chain. These Efab and the M-Fab were again constructed using the variable domains of the anti-HER2 antibody Trastuzumab. The mature peptide sequences of the heavy and light chain of the M-Fab construct are shown below.

Amino Acid Sequence of a Trastuzumab M-fab heavy chain with 6x His tag (pMP623) (elbow region boldened; IgM CH2 domain underlined; 6xHis italicized)

EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR IYPTNGYTRY ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSRWG GDGFYAMDYW GQGTLVTVSS **ASTKGP**KVSV FVPPRDGFFG NPRKSKLICQ ATGFSPRQIQ VSWLREGKQV GSGVTTDQVQ AEAKESGPTT YKVTSTLTIK ESDWLGQSMF TCRVDHRGLT FQQQASSMC*H HHHHH* (**SEQ ID NO:75**)

Amino Acid Sequence of a Trastuzumab M-fab light chain (pMP596) (elbow region boldened; IgM CH2 domain underlined

DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS RFSGSRSGTD FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ GTKVEIK**RTV AAP**KVSVFVP PRDGFFGNPR KSKLICQATG FSPRQIQVSW LREGKQVGSG VTTDQVQAEA KESGPTTYKV TSTLTIKESD WLGQSMFTCR VDHRGLTFQQ QASSMC (**SEQ ID NO:76**)

The Efabs and the M-Fab were expressed in CHO-S as Fabs (without Fc), and the supernatants used for testing binding to HER2 by Octet. In this binding assay the Mfab and the various Efab constructs showed very similar binding (**Figure 10D**).

Together these results show that using the IgM CH2 domains as constant domains of a Fab (M-fab) solves the light chain pairing problem and maintains the binding characteristics of the Fab. Thus, the M-Fab is another useful light chain pairing solution.

Example 7: Additional Applications of Heavy and Light Chain Pairing Technologies

Next, the heavy and light chain pairing solutions were tested further in the context of various other bispecific and monospecific formats to explore how versatile and functional of these technologies are.

First, the Fc heterodimerization mutations were introduced into the CH3 domain of other Fc regions and the functionality of such bispecific antibodies was tested in binding studies. To this end, the mp3 heterodimer mutations (S364K/K409S and K370S/F405K) were cloned into an IgG1 agly T299A scaffold, which lacks the N-linked glycosylation at Asn297 and therefore has reduced effector function compared to glycosylated IgG1. A bispecific antibody with this Fc region in the form of an asymmetric IgG with an Efab of the anti-EGFR antibody M60-A02 and a regular Fab of the anti-IGF-1R antibody M13.C06 was produced by transient expression in CHO cells (**Figure 11A**). The resulting bispecific antibody showed robust simultaneous binding to both ligands in a sandwich-format Octet binding assay (**Figure 11B**), demonstrating that the bispecific antibody efficiently formed from the four co-expressed chains (plasmids pMP463, pMP402, pMP405, pMP407). Thus, the Fc heterodimerization strategy by lysine repositioning appears to function independent of the Fc glycosylation.

pMP402 M60 Efab IgG1 agly T299A mp3a S364K/K409S

```
1   EVQLLESGGG LVQPGGSLRL SCAASGFTFS DYIMHWVRQA PGKGLEWVSV
51  ISSSGGDTSY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKVL
101 AGYFDWLPFD YWGQGTLVTV SSASTKGPTV KILQSICDGG GHFPPTIQLL
151 CLVSGYTPGT IQITWLEDGQ VMDVDLSTAS TTQEGELAST QSELTLSQKH
201 WLSDRTYTCQ VTYQGHTFED STKKCASDKT HTCPPCPAPE LLGGPSVFLF
251 PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE
301 EQYNSAYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE KTISKAKGQP
351 REPQVYTLPP SRDELTKNQV KLTCLVKGFY PSDIAVEWES NGQPENNYKT
401 TPPVLDSDGS FFLYSSLTVD KSRWQQGNVF SCSVMHEALH NHYTQKSLSL
451 SPG  (SEQ ID NO:83)
```

pMP405 C06 IgG1 agly T299A mp3b K370S/F405K

```
1   EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYRMQWVRQA PGKGLEWVSG
51  ISPSGGTTWY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARWS
101 GGSGYAFDIW GQGTMVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK
151 DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT
201 YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP
251 KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
```

```
301 SAYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
351 VYTLPPSRDE LTKNQVSLTC LVSGFYPSDI AVEWESNGQP ENNYKTTPPV
401 LDSDGSFKLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPG
```

(**SEQ ID NO:84**)

Second, to test the heavy chain heterodimerization in another Fc format, the mp4 heterodimer mutations (S364K/K409L and K370S/V397I/F405K) were cloned into an IgG4P/IgG1 agly (N297Q) hybrid constant domain, which pairs the minimal effector function of an aglycosylated IgG4 CH2 domain with the stability of an IgG1 CH3 domain (**Figure 12**). This Fc also contains the hinge-stabilizing mutation S228P. Hence, bispecific antibodies with this hybrid IgG4P/IgG1 constant domain and the variable domains of the anti-HER2 antibody Pertuzumab and the anti-IGF-1R antibody C06 were generated. The variable domains were used in both orientations, on the chain A as Efab or on the chain B as regular Fab, respectively. The antibody was again produced in CHO cells, and protein A-purified material was used in Octet binding studies (**Figure 12**). Interestingly, the purified bispecific antibodies showed simultaneous binding to both antigens only when IGF1R was loaded first followed by antibody and HER2 (**Figure 12C**), but not when tested in the reverse order, which might be due to the different location of tags on the soluble antigens.

Third, the bispecific technology was considered in the context of a Mab-Fab. A different way to construct a bispecific antibody is the Mab-Fab, which contains an IgG with a Fab fused to the C-terminus of the heavy chain (**Figure 13A**). This format resembles a symmetric molecule with two identical heavy chains, making the Fc heterodimer technology unnecessary. However, the presence of two different Fabs, each containing its unique light chain, requires a light chain pairing solution. To test whether an Efab would function in this context to promote correct light chain assembly, a Mab-Fab was constructed with the anti-HER2 antibody Trastuzumab as IgG1 with an Efab of the anti-IGF-1R antibody C06 fused to its C-terminus (**Figure 13**). Additionally, the inverse construct of C06 as IgG1 with an Efab of Trastuzumab fused to the C-terminus was also generated. In both cases the Efab was linked at the N-terminus of its heavy chain with a single G4S(**SEQ ID NO:56**)-linker to the C-terminus of the IgG heavy chain. The Mab-Fabs were again produced in CHO transients by co-expression of three plasmids (one heavy and two light chains, pMP759/pMP519/pMP511 or pMP760/pMP528/pMP407), and purified by protein A. Analysis of the purified material by SDS-PAGE showed that the three chains of the Mab-Fab assembled correctly into the 250 kDa protein (**Figure 13B**). Furthermore, the bispecific

antibodies showed some degree of simultaneous binding in a sandwich Octet binding assay (**Figure 13C**). However, this simultaneous binding was only observed when the Efab was bound first, irrespective of whether C06 or Trastuzumab was the Efab, suggesting some form of steric hindrance in this format could affect the simultaneous binding of both antigens.

pMP759 Trastuzumab IgG1-C06 EFab

```
1   EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR
51  IYPTNGYTRY ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSRWG
101 GDGFYAMDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK
151 DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT
201 YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP
251 KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
301 STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
351 VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV
401 LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGS
451 GGGGSEVQLL ESGGGLVQPG GSLRLSCAAS GFTFSIYRMQ WVRQAPGKGL
501 EWVSGISPSG GTTWYADSVK GRFTISRDNS KNTLYLQMNS LRAEDTAVYY
551 CARWSGGSGY AFDIWGQGTM VTVSSASTKG PTVKILQSIC DGGGHFPPTI
601 QLLCLVSGYT PGTIQITWLE DGQVMDVDLS TASTTQEGEL ASTQSELTLS
651 QKHWLSDRTY TCQVTYQGHT FEDSTKKCA (SEQ ID NO:85)
```

pMP511 C06 Efab light chain

```
1   DIQMTQSPLS LSASVGDRVT ITCQASRDIR NYLNWYQQKP GKAPKLLIYD
51  ASSLQTGVPS RFGGSGSGTD FSFTIGSLQP EDEATYYCQQ FDSLPHTFGQ
101 GTKLEIKRTV AAPTVKILQS SCDGGGHFPP TIQLLCLVSG YTPGTIQITW
151 LEDGQVMDVD LSTASTTQEG ELASTQSELT LSQKHWLSDR TYTCQVTYQG
201 HTFEDSGKKC A (SEQ ID NO:86)
```

pMP519 Trastuzumab kappa light chain

```
1   DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS
51  ASFLYSGVPS RFSGSRSGTD FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ
101 GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151 DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201 LSSPVTKSFN RGEC (SEQ ID NO:87)
```

pMP760 C06-IgG1-Trastuzumab Efab

```
1   EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYRMQWVRQA PGKGLEWVSG
51  ISPSGGTTWY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARWS
101 GGSGYAFDIW GQGTMVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK
151 DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT
201 YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP
```

```
251 KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
301 STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
351 VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV
401 LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGS
451 GGGGSEVQLV ESGGGLVQPG GSLRLSCAAS GFNIKDTYIH WVRQAPGKGL
501 EWVARIYPTN GYTRYADSVK GRFTISADTS KNTAYLQMNS LRAEDTAVYY
551 CSRWGGDGFY AMDYWGQGTL VTVSSASTKG PTVKILQSIC DGGGHFPPTI
601 QLLCLVSGYT PGTIQITWLE DGQVMDVDLS TASTTQEGEL ASTQSELTLS
651 QKHWLSDRTY TCQVTYQGHT FEDSTKKCA (SEQ ID NO:88)
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Fourth, in addition to the various applications in generating bispecifics, the Fc-heterodimerization technology can also be utilized to generate a monospecific monovalent antibody. A heterodimer between a half antibody and a free Fc as shown in Figure 3 is one such example of a monospecific monovalent. Another example is the direct fusion of a LC to an Fc, which is then co-expressed with the matching heavy chain (**Figure 14A**). An Fc heterodimerization strategy is required in this format to prevent the formation of homodimers of light chains or heavy chains. To test whether the mp4 Fc heterodimer would also be suitable to construct this type of monovalent monospecific antibody, a LC-Fc fusion construct was generated with the variable domain of the anti-EGFR antibody M60-A02. Expression of this plasmid (pMP533) together with the heavy chain of M60-A02 containing the corresponding Fc heterodimer mutation (plasmid pMP254) in CHO cells produced a single protein species (**Figure 14B**). When this protein was analyzed in Octet binding it showed the characteristics of monovalent binding (**Figure 14C**). Altogether, this data shows that the Fc heterodimerization by lysine repositioning appears to be a versatile and efficient way of generating monovalent monospecific antibodies.

Lastly, the use of the Efab light chain pairing solution was also tested in bispecific antibodies, which lack the Fc portion of an IgG. Such bispecifics can be generated for example by direct fusion of two Fabs with a peptide linker (**Figure 15A, right diagram**). Alternatively, the peptide that links the two Fabs could be a heterologous protein such as human serum albumin (HSA) (**Figure 15A, left diagram**). In either case, the pairing of the light and heavy chains of the two Fabs needs to be correct for the bispecific to function. Thus, the Efab light chain pairing solution could enable such a fusion of two Fabs into bispecific molecules. To test this, the M60-A02 and M13.C06 antibodies were again used, and bispecifics were again generated with the two Fabs in both orientations. When these bispecifics were expressed in CHO cells they produced a single protein suggesting again that the antibody chains had assembled correctly (**Figure 15B**).

When the antibodies were tested in Octet binding studies, simultaneous binding was again observed only in one particular orientation (**Figure 15C**). Nonetheless, the present data shows that the Efab light chain pairing solution can be employed to efficiently generate bispecifics from two Fabs in various formats that lack the Fc of a normal IgG.

Other Embodiments

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

Claims

1. An antibody or antigen-binding fragment thereof comprising a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (**SEQ ID NO:1**), wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), and wherein the first polypeptide and the second polypeptide pair to form a dimer.

2. The antibody of claim 1, wherein the first polypeptide is directly linked to an Fc domain.

3. The antibody of claim 1, wherein the second polypeptide is directly linked to an Fc domain.

4. The antibody or antigen-binding fragment thereof of any one of claims 1 to 3, wherein the first polypeptide and/or the second polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:1**.

5. The antibody or antigen-binding fragment thereof of any one of claims 1 to 3, wherein the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

6. The antibody or antigen-binding fragment thereof of any one of claims 1 to 3, wherein the first polypeptide and/or the second polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

7. The antibody or antigen-binding fragment thereof of any one of claims 1 to 6, wherein the first polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:5**.

8. The antibody or antigen-binding fragment thereof of any one of claims 1 to 6, wherein the first polypeptide comprises the amino acid sequence set forth in **SEQ ID NO:5**.

9. The antibody or antigen-binding fragment thereof of any one of claims 1 to 8, wherein the second polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:6**.

10. The antibody or antigen-binding fragment thereof of any one of claims 1 to 8, wherein the second polypeptide comprises the amino acid sequence set forth in **SEQ ID NO:6**.

11. The antibody or antigen-binding fragment thereof of any one of claims 1 to 3, wherein the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in amino acids 9-107 of **SEQ ID NO:1** at twelve or fewer amino acid residues.

12. The antibody or antigen-binding fragment thereof of any one of claims 1 to 3, wherein the first polypeptide and/or the second polypeptide contain an amino acid other than cysteine at at least one of the two cysteine residues of **SEQ ID NO:1** that do not form an intrachain disulfide bond.

13. The antibody or antigen-binding fragment thereof of any one of claims 1 to 3, 5, 11, or 12, wherein:

(i) the first polypeptide and the second polypeptide each contain an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:1** such that the first polypeptide and the second polypeptide are not N-glycosylated; or

(ii) the first polypeptide or the second polypeptide contains an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:1** such that the first polypeptide or the second polypeptide is not N-glycosylated.

14. The antibody or antigen-binding fragment thereof of any one of claims 1 to 13, wherein the antibody or antigen-binding fragment thereof comprises a second heavy chain variable domain

(second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen.

15. The antibody or antigen-binding fragment thereof of claim 14, wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain.

16. An antibody or antigen-binding fragment thereof comprising a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (**SEQ ID NO:2**), wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), and wherein the first polypeptide and the second polypeptide pair to form a dimer.

17. The antibody of claim 16, wherein the first polypeptide is directly linked to an Fc domain.

18. The antibody of claim 16, wherein the second polypeptide is directly linked to an Fc domain.

19. The antibody or antigen-binding fragment thereof of any one of claims 16 to 18, wherein the first polypeptide and/or the second polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:2**.

20. The antibody or antigen-binding fragment thereof of any one of claims 16 to 18, wherein the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

21. The antibody or antigen-binding fragment thereof of any one of claims 16 to 18, wherein the first polypeptide and/or the second polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

22. The antibody or antigen-binding fragment thereof of any one of claims 16 to 18, wherein the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in amino acids 7-112 of **SEQ ID NO:2** at at least twelve amino acid residues.

23. The antibody or antigen-binding fragment thereof of any one of claims 16 to 18, 20, or 22, wherein:

(i) the first polypeptide and the second polypeptide each contain an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:2** such that the first polypeptide and the second polypeptide are not N-glycosylated; or

(ii) the first polypeptide or the second polypeptide contains an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:2** such that the first polypeptide or the second polypeptide is not N-glycosylated.

24. The antibody or antigen-binding fragment thereof of any one of claims 16 to 23, wherein the antibody or antigen-binding fragment thereof comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen.

25. The antibody or antigen-binding fragment thereof of claim 24, wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain.

26. A bispecific antibody comprising:

(i) a first fragment antigen-binding (first Fab) comprising a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region, wherein the first VH is either (i) directly linked or

(ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (**SEQ ID NO:1**), and wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**),; and

(ii) a second Fab comprising a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region, and wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain,

wherein the first Fab and the second Fab specifically bind to different antigens or to different epitopes of the same antigen, and

wherein the first Fab is connected to the second Fab.

27. The bispecific antibody of claim 26, wherein the first Fab is connected to the second Fab by a linker.

28. The bispecific antibody of claim 26, wherein the first Fab is connected to the second Fab by a heterologous polypeptide.

29. The bispecific antibody of claim 28, wherein the heterologous polypeptide is human serum albumin.

30. The bispecific antibody of claim 28, wherein the heterologous polypeptide is an XTEN.

31. The bispecific antibody of claim 26, wherein the first Fab is connected to the second Fab by polyethylene glycol (PEG).

32. The bispecific antibody of any one of claims 26 to 31, wherein the first polypeptide and/or the second polypeptide each comprise the amino acid sequence set forth in **SEQ ID NO:1**.

33. The bispecific antibody of any one of claims 26 to 31, wherein the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

34. The bispecific antibody of any one of claims 26 to 31, wherein the first polypeptide and/or the second polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

35. The bispecific antibody of any one of claims 26 to 34, wherein the first polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:5**.

36. The bispecific antibody of any one of claims 26 to 34, wherein the first polypeptide comprises the amino acid sequence set forth in **SEQ ID NO:5**.

37. The bispecific antibody of any one of claims 26 to 36, wherein the second polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:6**.

38. The bispecific antibody of any one of claims 26 to 36, wherein the second polypeptide comprises the amino acid sequence set forth in **SEQ ID NO:6**.

39. The bispecific antibody of any one of claims 26 to 31, wherein the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in amino acids 9-107 of **SEQ ID NO:1** at twelve amino acid residues or fewer.

40. The bispecific antibody of any one of claims 26 to 31, wherein the first polypeptide and/or the second polypeptide contain an amino acid other than cysteine at at least one of the two cysteine residues of **SEQ ID NO:1** that can form an interchain disulfide bond.

41. The bispecific antibody of any one of claims 26 to 31, 33, 39, or 40, wherein

(i) the first polypeptide and the second polypeptide each contain an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:1** such that the first polypeptide and the second polypeptide are not N-glycosylated; or

(ii) the first polypeptide or the second polypeptide contains an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:1** such that the first polypeptide or the second polypeptide is not N-glycosylated.

42. A bispecific antibody comprising:

(i) a first fragment antigen-binding (first Fab) comprising a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region, wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (**SEQ ID NO:2**), and wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**); and

(ii) a second Fab comprising a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region, and wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain and the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain,

wherein the first Fab and the second Fab specifically bind to different antigens or to different epitopes of the same antigen, and

wherein the first Fab is connected to the second Fab.

43. The bispecific antibody of claim 42, wherein the first Fab is connected to the second Fab by a linker.

44. The bispecific antibody of claim 43, wherein the linker is a peptide linker.

45. The bispecific antibody of claim 42, wherein the first Fab is connected to the second Fab by a heterologous polypeptide.

46. The bispecific antibody of claim 45, wherein the heterologous polypeptide is human serum albumin.

47. The bispecific antibody of claim 45, wherein the heterologous polypeptide is an XTEN.

48. The bispecific antibody of claim 42, wherein the first Fab is connected to the second Fab by polyethylene glycol (PEG).

49. The bispecific antibody of any one of claims 42 to 48, wherein the first polypeptide and/or the second polypeptide each comprise the amino acid sequence set forth in **SEQ ID NO:2**.

50. The bispecific antibody of any one of claims 42 to 48, wherein the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

51. The bispecific antibody of any one of claims 42 to 48, wherein the first polypeptide and/or the second polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

52. The bispecific antibody of any one of claims 42 to 48, wherein the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in **SEQ ID NO:2** at at least twelve amino acid residues.

53. The bispecific antibody of any one of claims 42 to 48, wherein the first polypeptide and/or the second polypeptide contain an amino acid other than cysteine at the cysteine residue of **SEQ ID NO:2** that can form an interchain disulfide bond.

54. The bispecific antibody of any one of claims 42 to 48, 50, 52, or 53, wherein

(i) the first polypeptide and the second polypeptide each contain an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:2** such that the first polypeptide and the second polypeptide are not N-glycosylated; or

(ii) the first polypeptide or the second polypeptide contains an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:2** such that the first polypeptide or the second polypeptide is not N-glycosylated.

55. A tetravalent bispecific antibody comprising:

(i) a whole IgG antibody that specifically binds to a first epitope of a first antigen, the whole IgG antibody comprising a first CH3 domain and a second CH3 domain; and

(ii) a first Fab and a second Fab,

wherein the first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a second epitope of the first antigen or to a second antigen, and

wherein the second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the same epitope as the first Fab;

wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (**SEQ ID NO:1**), the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), the second VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), and the second VL is either (i) directly linked or (ii) linked via a linker to a fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**); and

wherein the first Fab is connected to the C-terminus of the first CH3 domain of the whole IgG antibody and the second Fab is connected to the C-terminus of the second CH3 domain of the whole IgG antibody.

56. The tetravalent antibody of claim 55, wherein the first Fab is connected to the C-terminus of the first CH3 domain of the whole antibody through a first linker and the second Fab is connected to the C-terminus of the second CH3 domain of the whole antibody through a second linker.

57. The tetravalent antibody of claim 56, wherein the first and second linkers are peptide linkers.

58. The tetravalent antibody of any one of claims 55 to 57, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:1**.

59. The tetravalent antibody of any one of claims 55 to 57, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

60. The tetravalent antibody of any one of claims 55 to 57, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

61. The tetravalent antibody of any one of claims 55 to 60, wherein the first polypeptide and the third polypeptide each comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:5**.

62. The tetravalent antibody of any one of claims 55 to 60, wherein the first polypeptide and the third polypeptide each comprise the amino acid sequence set forth in **SEQ ID NO:5**.

63. The tetravalent antibody of any one of claims 55 to 62, wherein the second polypeptide and the fourth polypeptide each comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:6**.

64. The tetravalent antibody of any one of claims 55 to 62, wherein the second polypeptide and the fourth polypeptide each comprise the amino acid sequence set forth in **SEQ ID NO:6**.

65. The tetravalent antibody of any one of claims 55 to 57, wherein the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in amino acids 9-107 of **SEQ ID NO:1** at at least twelve amino acid residues.

66. A tetravalent bispecific antibody comprising:

(i) a whole IgG antibody that specifically binds to a first epitope of a first antigen, the whole IgG antibody comprising a first CH3 domain and a second CH3 domain; and

(ii) a first Fab and a second Fab,

wherein the first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a second epitope of the first antigen or to a second antigen, and

wherein the second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the same epitope as the first Fab;

wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (**SEQ ID NO:2**), the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), the second VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an

amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), and the second VL is either (i) directly linked or (ii) linked via a linker to a fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**); and

wherein the first Fab is connected to the C-terminus of the first CH3 domain of the whole IgG antibody and the second Fab is connected to the C-terminus of the second CH3 domain of the whole IgG antibody.

67. The tetravalent antibody of claim 66, wherein the first Fab is connected to the C-terminus of the first CH3 domain of the whole antibody through a first linker and the second Fab is connected to the C-terminus of the second CH3 domain of the whole antibody through a second linker.

68. The tetravalent antibody of any one of claims 65 to 67, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide each comprise the amino acid sequence set forth in **SEQ ID NO:2**.

69. The tetravalent antibody of claim 66 or 67, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

70. The tetravalent antibody of claim 66 or 67, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

71. The tetravalent antibody of claim 66 or 67, wherein the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in amino acids 7-112 of **SEQ ID NO:2** at at least twelve amino acid residues.

72. A tetravalent bispecific antibody comprising:

(i) a first Fab and a second Fab,

wherein the first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, and

wherein the second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the first epitope of the first antigen; and

(ii) a whole antibody comprising a first heavy chain comprising a first IgG CH2 domain and a first IgG CH3 domain, a second heavy chain comprising a second IgG CH2 domain and a second IgG CH3 domain, a first light chain, and a second light chain, wherein the antibody comprises a third VH and third VL and a fourth VH and a fourth VL, wherein the third VH and the third VL pair to form a third variable region that binds specifically to an epitope of a second antigen, and wherein the fourth VH and the fourth VL pair to form a fourth variable region that binds specifically to the same epitope of the second antigen,

wherein the third VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (**SEQ ID NO:1**), the third VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), the fourth VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), and the fourth VL is either (i) directly linked or (ii) linked via a linker to a fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), wherein the first polypeptide is connected to the N-terminus of the first IgG CH2 domain and the third polypeptide is connected to the N-terminus of the second IgG CH2 domain; and

wherein the first Fab is connected to the C-terminus of the first IgG CH3 domain and the second Fab is connected to the C-terminus of the second IgG CH3 domain.

73. The tetravalent antibody of claim 72, wherein the first Fab is connected to the C-terminus of the first IgG CH3 domain through a first linker and the second Fab is connected to the C-terminus of the second IgG CH3 domain through a second linker.

74. The tetravalent antibody of claim 73, wherein the first and second linkers are peptide linkers.

75. The tetravalent antibody of any one of claims 72 to 74, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:1**.

76. The tetravalent antibody of any one of claims 72 to 74, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

77. The tetravalent antibody of any one of claims 72 to 74, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

78. The tetravalent antibody of any one of claims 72 to 77, wherein the first polypeptide and/or the third polypeptide comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:5**.

79. The tetravalent antibody of any one of claims 72 to 77, wherein the first polypeptide and/or the third polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:5**.

80. The tetravalent antibody of any one of claims 72 to 79, wherein the second polypeptide and/or the fourth polypeptide comprise an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:6**.

81. The tetravalent antibody of any one of claims 72 to 79, wherein the second polypeptide and/or the fourth polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:6**.

82. The tetravalent antibody of any one of claims 72 to 74, wherein the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in **SEQ ID NO:1** at at least twelve amino acid residues.

83. A tetravalent bispecific antibody comprising:

(i) a first Fab and a second Fab,

wherein the first Fab comprises a first heavy chain variable domain (first VH) and a first light chain variable domain (first VL), wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, and

wherein the second Fab comprises a second heavy chain variable domain (second VH) and a second light chain variable domain (second VL), wherein the second VH and the second VL pair to form a second variable region that binds specifically to the first epitope of the first antigen; and

(ii) a whole antibody comprising a first heavy chain comprising a first IgG CH2 domain and a first IgG CH3 domain, a second heavy chain comprising a second IgG CH2 domain and a second IgG CH3 domain, a first light chain, and a second light chain, wherein the antibody comprises a third VH and third VL and a fourth VH and a fourth VL, wherein the third VH and the third VL pair to form a third variable region that binds specifically to an epitope of a second antigen, and wherein the fourth VH and the fourth VL pair to form a fourth variable region that binds specifically to the same epitope of the second antigen,

wherein the third VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (**SEQ ID NO:2**), the third VL is either (i) directly linked or (ii) linked via a linker to a second

polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), the fourth VH is either (i) directly linked or (ii) linked via a linker to a third polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), and the fourth VL is either (i) directly linked or (ii) linked via a linker to a fourth polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), wherein the first polypeptide is connected to the N-terminus of the first IgG CH2 domain and the third polypeptide is connected to the N-terminus of the second IgG CH2 domain; and

wherein the first Fab is connected to the C-terminus of the first IgG CH3 domain and the second Fab is connected to the C-terminus of the second IgG CH3 domain.

84. The tetravalent antibody of claim 83, wherein the first Fab is connected to the C-terminus of the first IgG CH3 domain through a first linker and the second Fab is connected to the C-terminus of the second IgG CH3 domain through a second linker.

85. The tetravalent antibody of claim 84, wherein the first and second linkers are peptide linkers.

86. The tetravalent antibody of any one of claims 83 to 85, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:2**.

87. The tetravalent antibody of any one of claims 83 to 85, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

88. The tetravalent antibody of any one of claims 83 to 85, wherein the first polypeptide, the second polypeptide, the third polypeptide, and/or the fourth polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

89. The tetravalent antibody of any one of claims 83 to 85, wherein the first polypeptide, the second polypeptide, the third polypeptide and/or the fourth polypeptide differ from the amino acid sequence set forth in **SEQ ID NO:2** at at least twelve amino acid residues.

90. A heterodimerization module comprising:

(i) a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a serine; and

(ii) a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acid at position 370 is a serine and the amino acids at positions 405 and 409 are lysines,

wherein the amino acid positions in (i) and (ii) are based on the EU numbering system, and

wherein the first IgG1 CH3 domain and the second IgG1 CH3 pair to form a heterodimer.

91. The heterodimerization module of claim 90, further comprising a first IgG1 CH2 domain and a second IgG1 CH2 domain, wherein the first IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain.

92. The heterodimerization module of claim 91, further comprising a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is linked to the N-terminus of a first hinge region, and wherein the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain.

93. The heterodimerization module of claim 92, further comprising a linker that links the C-terminus of the first IgG1 CH3 domain to the N-terminus of a second hinge region that is linked to the N-terminus of the second IgG1 CH2 domain.

94. The heterodimerization module of claim 92, further comprising a second Fab linked via a second hinge region to the N-terminus of the second IgG1 CH2 domain.

95. The heterodimerization module of claim 91, further comprising a VH domain, a CH1 domain, a VL domain, and a CL domain, wherein (i) the C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first hinge region, and the C-terminus of the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain that is directly linked to the first IgG1 CH3 domain, (ii) the C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second hinge region, and the C-terminus of the second hinge region is linked to the N-terminus of the second IgG1 CH2 domain that is directly linked to the second IgG1 CH3 domain, and (iii) the VH domain and the VL domain pair to form a variable region that binds specifically to an antigen.

96. The heterodimerization module of claim 91, further comprising a first VH and a first VL and a second VH and a second VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first antigen, and wherein the second VH and the second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a second antigen.

97. The heterodimerization module of claim 96, wherein the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

98. The heterodimerization module of claim 90, further comprising a first IgG4 CH2 domain and a second IgG4 CH2 domain, wherein the first IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain.

99. The heterodimerization module of claim 98, further comprising a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is

linked to the N-terminus of a first IgG4 hinge region, and wherein the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain.

100. The heterodimerization module of claim 99, wherein the first IgG4 hinge region comprises the S228P mutation (EU numbering).

101. The heterodimerization module of claim 98, further comprising a linker that links the C-terminus of the first IgG1 CH3 domain to the N-terminus of a second IgG4 hinge region that is linked to the N-terminus of the second IgG4 CH2 domain.

102. The heterodimerization module of claim 101, wherein the second IgG4 hinge region comprises the S228P mutation (EU numbering).

103. The heterodimerization module of claim 99, further comprising a second Fab linked via a second IgG4 hinge region to the N-terminus of the second IgG4 CH2 domain.

104. The heterodimerization module of claim 103, wherein the second IgG4 hinge region comprises the S228P mutation (EU numbering).

105. The heterodimerization module of claim 98, further comprising a VH domain, a CH1 domain, a VL domain, and a CL domain, wherein (i) the C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first IgG4 hinge region, and the C-terminus of the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain that is directly linked to the first IgG1 CH3 domain, (ii) the C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second IgG4 hinge region, and the C-terminus of the second IgG4 hinge region is linked to the N-terminus of the second IgG4 CH2 domain that is directly linked to the second IgG1 CH3 domain, and (iii) the VH domain and the VL domain pair to form a variable region that binds specifically to an antigen.

106. The heterodimerization module of claim 105, wherein the first IgG4 hinge region and the second IgG4 hinge region each comprise the S228P mutation (EU numbering).

107. The heterodimerization module of claim 98, further comprising a first VH and a first VL and a second VH and a second VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first antigen, and wherein the second VH and the second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a second antigen.

108. The heterodimerization module of claim 107, wherein the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

109. A heterodimerization module comprising:

(i) a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a leucine; and

(ii) a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acid at position 370 is a serine, the amino acid at position 397 is an isoleucine, and the amino acids at positions 405 and 409 are lysines,

wherein the amino acid positions in (i) and (ii) are based on the EU numbering system, and

wherein the first IgG1 CH3 domain and the second IgG1 CH3 pair to form a heterodimer.

110. The heterodimerization module of claim 109, further comprising a first IgG1 CH2 domain and a second IgG1 CH2 domain, wherein the first IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG1 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain.

111. The heterodimerization module of claim 110, further comprising a first IgG1 CH2 domain and a second IgG1 CH2 domain, wherein the first IgG1 CH2 domain is directly linked to the N-terminus of the first IgG1 CH3 domain and the second IgG1 CH2 domain is directly linked to the N-terminus of the second IgG1 CH3 domain.

112. The heterodimerization module of claim 110, further comprising a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is linked to the N-terminus of a first hinge region, and wherein the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain.

113. The heterodimerization module of claim 112, further comprising a linker that links the C-terminus of the first IgG1 CH3 domain to the N-terminus of a second hinge region that is linked to the N-terminus of the second IgG1 CH2 domain.

114. The heterodimerization module of claim 112, further comprising a second Fab linked via a second hinge region to the N-terminus of the second IgG1 CH2 domain.

115. The heterodimerization module of claim 110, further comprising a VH domain, a CH1 domain, a VL domain, and a CL domain, wherein (i) the C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first hinge region, and the C-terminus of the first hinge region is linked to the N-terminus of the first IgG1 CH2 domain that is directly linked to the first IgG1 CH3 domain, (ii) the C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second hinge region, and the C-terminus of the second hinge region is linked to the N-terminus of the second IgG1 CH2 domain that is directly linked to the second IgG1 CH3 domain, and (iii) the VH domain and the VL domain pair to form a variable region that binds specifically to an antigen.

116. The heterodimerization module of claim 110, further comprising a first VH and a first VL and a second VH and a second VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first antigen, and wherein the second VH and the

second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a second antigen.

117. The heterodimerization module of claim 116, wherein the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

118. The heterodimerization module of claim 109, further comprising a first IgG4 CH2 domain and a second IgG4 CH2 domain, wherein the first IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the first IgG1 CH3 domain and the second IgG4 CH2 domain is either (i) directly linked or (ii) linked via a linker to the N-terminus of the second IgG1 CH3 domain.

119. The heterodimerization module of claim 118, further comprising a first Fab comprising two polypeptide chains, wherein the C-terminus of one of the two polypeptide chains of the Fab is linked to the N-terminus of a first IgG4 hinge region, and wherein the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain.

120. The heterodimerization module of claim 119, wherein the first IgG4 hinge region comprises the S228P mutation (EU numbering).

121. The heterodimerization module of claim 118, further comprising a linker that links the C-terminus of the first IgG1 CH3 domain to the N-terminus of a second IgG4 hinge region that is linked to the N-terminus of the second IgG4 CH2 domain.

122. The heterodimerization module of claim 121, wherein the second IgG4 hinge region comprises the S228P mutation (EU numbering).

123. The heterodimerization module of claim 119, further comprising a second Fab linked via a second IgG4 hinge region to the N-terminus of the second IgG4 CH2 domain.

124. The heterodimerization module of claim 123, wherein the second IgG4 hinge region comprises the S228P mutation (EU numbering).

125. The heterodimerization module of claim 118, further comprising a VH domain, a CH1 domain, a VL domain, and a CL domain, wherein (i) the C-terminus of the VH domain is linked to the N-terminus of the CH1 domain, the C-terminus of the CH1 domain is linked to the N-terminus of a first IgG4 hinge region, and the C-terminus of the first IgG4 hinge region is linked to the N-terminus of the first IgG4 CH2 domain that is directly linked to the first IgG1 CH3 domain, (ii) the C-terminus of the VL domain is linked to the N-terminus of the CL domain, the C-terminus of the CL domain is linked to the N-terminus of a second IgG4 hinge region, and the C-terminus of the second IgG4 hinge region is linked to the N-terminus of the second IgG4 CH2 domain that is directly linked to the second IgG1 CH3 domain, and (iii) the VH domain and the VL domain pair to form a variable region that binds specifically to an antigen.

126. The heterodimerization module of claim 125, wherein the first IgG4 hinge region and the second IgG4 hinge region each comprise the S228P mutation (EU numbering).

127. The heterodimerization module of claim 118, further comprising a first VH and a first VL and a second VH and a second VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first antigen, and wherein the second VH and the second VL pair to form a second variable region that binds specifically to a different epitope of the first antigen or to a second antigen.

128. The heterodimerization module of claim 127, wherein the amino acid sequence of the first VL is identical to the amino acid sequence of the second VL.

129. A bispecific antibody comprising:

(i) a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the

CH2 domain of human immunoglobulin E (IgE) (**SEQ ID NO:1**), wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), and wherein the first polypeptide and the second polypeptide pair to form a dimer;

(ii) a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen, wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain, wherein the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain, and wherein the CH1 domain and the CL domain pair to form a dimer; and

(iii) a heterodimerization module comprising:

(a) a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a serine; and

(b) a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acid at position 370 is a serine and the amino acids at positions 405 and 409 are lysines, wherein the amino acid positions in (a) and (b) are based on the EU numbering system.

130. A bispecific antibody comprising:

(i) a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human immunoglobulin E (IgE) (**SEQ ID NO:1**), wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 9-107 of the amino acid sequence of the CH2 domain of human IgE (**SEQ ID NO:1**), and wherein the first polypeptide and the second polypeptide pair to form a dimer;

(ii) a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to

a second antigen, wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain, wherein the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain, and wherein the CH1 domain and the CL domain pair to form a dimer; and

(iii) a heterodimerization module comprising:

(a) a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a leucine; and

(b) a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acid at position 370 is a serine, the amino acid at position 397 is an isoleucine, and the amino acids at positions 405 and 409 are lysines,

wherein the amino acid positions in (a) and (b) are based on the EU numbering system.

131. The bispecific antibody of claim 129 or 130, further comprising two IgG1 CH2 domains.

132. The bispecific antibody of claim 129 or 130, further comprising two IgG4 CH2 domains.

133. The bispecific antibody of any one of claims 129 to 132, wherein a single polypeptide chain comprises the first VH, the first polypeptide, and the first IgG1 CH3 domain.

134. The bispecific antibody of any one of claims 129 to 132, wherein a single polypeptide chain comprises the first VH, the first polypeptide, and the second IgG1 CH3 domain.

135. The bispecific antibody of any one of claims 129 to 132, wherein a single polypeptide chain comprises the first VL, the second polypeptide, and the first IgG1 CH3 domain.

136. The bispecific antibody of any one of claims 129 to 132, wherein a single polypeptide chain comprises the first VL, the second polypeptide, and the second IgG1 CH3 domain.

137. The bispecific antibody of claim 133, wherein a second single polypeptide chain comprises the second VH, the CH1 domain, and the second IgG1 CH3 domain.

138. The bispecific antibody of claim 134, wherein a second single polypeptide chain comprises the second VH, the CH1 domain, and the first IgG1 CH3 domain.

139. The bispecific antibody of claim 135, wherein a second single polypeptide chain comprises the second VL, the CL domain, and the second IgG1 CH3 domain.

140. The bispecific antibody of claim 136, wherein a second single polypeptide chain comprises the second VL, the CL domain, and the first IgG1 CH3 domain.

141. The bispecific antibody of any one of claims 129 to 140, wherein the first polypeptide and/or the second polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:1**.

142. The bispecific antibody of any one of claims 129 to 140, wherein the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

143. The bispecific antibody of any one of claims 129 to 140, wherein the first polypeptide and/or the second polypeptide comprise amino acids 9-107 of the amino acid sequence set forth in **SEQ ID NO:1**.

144. The bispecific antibody of any one of claims 129 to 140, wherein the first polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:5**.

145. The bispecific antibody of any one of claims 129 to 140, wherein the first polypeptide comprises the amino acid sequence set forth in **SEQ ID NO:5**.

146. The bispecific antibody of any one of claims 129 to 145, wherein the second polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence set forth in **SEQ ID NO:6**.

147. The bispecific antibody of any one of claims 129 to 145, wherein the second polypeptide comprises the amino acid sequence set forth in **SEQ ID NO:6**.

148. The bispecific antibody of any one of claims 129 to 140, wherein the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in **SEQ ID NO:1** at at least twelve amino acid residues.

149. The bispecific antibody of any one of claims 129 to 140, wherein the first polypeptide and the second polypeptide each contain an amino acid other than cysteine at at least one of the two cysteine residues of **SEQ ID NO:1** that do not form intrachain disulfide bond.

150. The bispecific antibody of any one of claims 129 to 140 or 142, wherein:

(i) the first polypeptide and the second polypeptide each contain an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:1** such that the first polypeptide and the second polypeptide are not N-glycosylated; or

(ii) the first polypeptide or the second polypeptide contains an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:1** such that the first polypeptide or the second polypeptide is not N-glycosylated.

151. A bispecific antibody comprising:

(i) a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (**SEQ ID NO:2**), wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), and wherein the first polypeptide and the second polypeptide pair to form a dimer;

(ii) a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen, wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain, wherein the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain, and wherein the CH1 domain and the CL domain pair to form a dimer; and

(iii) a heterodimerization module comprising:

(a) a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a serine; and

(b) a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acid at position 370 is a serine and the amino acids at positions 405 and 409 are lysines, wherein the amino acid positions in (a) and (b) are based on the EU numbering system.

152. A bispecific antibody comprising:

(i) a first VH and a first VL, wherein the first VH and the first VL pair to form a first variable region that binds specifically to a first epitope of a first antigen, wherein the first VH is either (i) directly linked or (ii) linked via a linker to a first polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human immunoglobulin M (IgM) (**SEQ ID NO:2**), wherein the first VL is either (i) directly linked or (ii) linked via a linker to a second polypeptide comprising an amino acid sequence that is at least 80% identical to amino acids 7-112 of the amino acid sequence of the CH2 domain of human IgM (**SEQ ID NO:2**), and wherein the first polypeptide and the second polypeptide pair to form a dimer;

(ii) a second VH and a second VL, wherein the second VH and the second VL pair to form a second variable region that binds specifically to a second epitope of the first antigen or to a second antigen, wherein the second VH is either (i) directly linked or (ii) linked via a linker to a CH1 domain, wherein the second VL is either (i) directly linked or (ii) linked via a linker to a CL domain, and wherein the CH1 domain and the CL domain pair to form a dimer; and

(iii) a heterodimerization module comprising:

(a) a first IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acids at positions 364 and 370 are lysines and the amino acid at position 409 is a leucine; and

(b) a second IgG1 CH3 domain having an amino acid sequence that is at least 80% identical to the sequence set forth in **SEQ ID NO:11**, wherein the amino acid at position 370 is a serine, the amino acid at position 397 is an isoleucine, and the amino acids at positions 405 and 409 are lysines,

wherein the amino acid positions in (a) and (b) are based on the EU numbering system.

153. The bispecific antibody of claim 151 or 152, further comprising two IgG1 CH2 domains.

154. The bispecific antibody of claim 151 or 152, further comprising two IgG4 CH2 domains.

155. The bispecific antibody of any one of claims 151 to 154, wherein a single polypeptide chain comprises the first VH, the first polypeptide, and the first IgG1 CH3 domain.

156. The bispecific antibody of any one of claims 151 to 154, wherein a single polypeptide chain comprises the first VH, the first polypeptide, and the second IgG1 CH3 domain.

157. The bispecific antibody of any one of claims 151 to 154, wherein a single polypeptide chain comprises the first VL, the second polypeptide, and the first IgG1 CH3 domain.

158. The bispecific antibody of any one of claims 151 to 154, wherein a single polypeptide chain comprises the first VL, the second polypeptide, and the second IgG1 CH3 domain.

159. The bispecific antibody of claim 155, wherein a second single polypeptide chain comprises the second VH, the CH1 domain, and the second IgG1 CH3 domain.

160. The bispecific antibody of claim 156, wherein a second single polypeptide chain comprises the second VH, the CH1 domain, and the first IgG1 CH3 domain.

161. The bispecific antibody of claim 157, wherein a second single polypeptide chain comprises the second VL, the CL domain, and the second IgG1 CH3 domain.

162. The bispecific antibody of claim 158, wherein a second single polypeptide chain comprises the second VL, the CL domain, and the first IgG1 CH3 domain.

163. The bispecific antibody of any one of claims 151 to 162, wherein the first polypeptide and/or the second polypeptide comprise the amino acid sequence set forth in **SEQ ID NO:2**.

164. The bispecific antibody of any one of claims 151 to 162, wherein the first polypeptide and/or the second polypeptide comprise an amino acid sequence that is at least 90% identical to amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

165. The bispecific antibody of any one of claims 151 to 162, wherein the first polypeptide and/or the second polypeptide comprise amino acids 7-112 of the amino acid sequence set forth in **SEQ ID NO:2**.

166. The bispecific antibody of any one of claims 151 to 162, wherein the first polypeptide and/or the second polypeptide differ from the amino acid sequence set forth in **SEQ ID NO:2** at at least twelve amino acid residues.

167. The bispecific antibody of any one of claims 151 to 162, wherein the first polypeptide and the second polypeptide each contain an amino acid other than cysteine at the cysteine residue of **SEQ ID NO:2** that does not form an intrachain disulfide bond.

168. The bispecific antibody of any one of claims 151 to 162 or 166, wherein:

(i) the first polypeptide and the second polypeptide each contain an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:2** such that the first polypeptide and the second polypeptide are not N-glycosylated; or

(ii) the first polypeptide or the second polypeptide contains an amino acid other than asparagine and/or threonine or serine in the N-glycosylation motif in **SEQ ID NO:2** such that the first polypeptide or the second polypeptide is not N-glycosylated.

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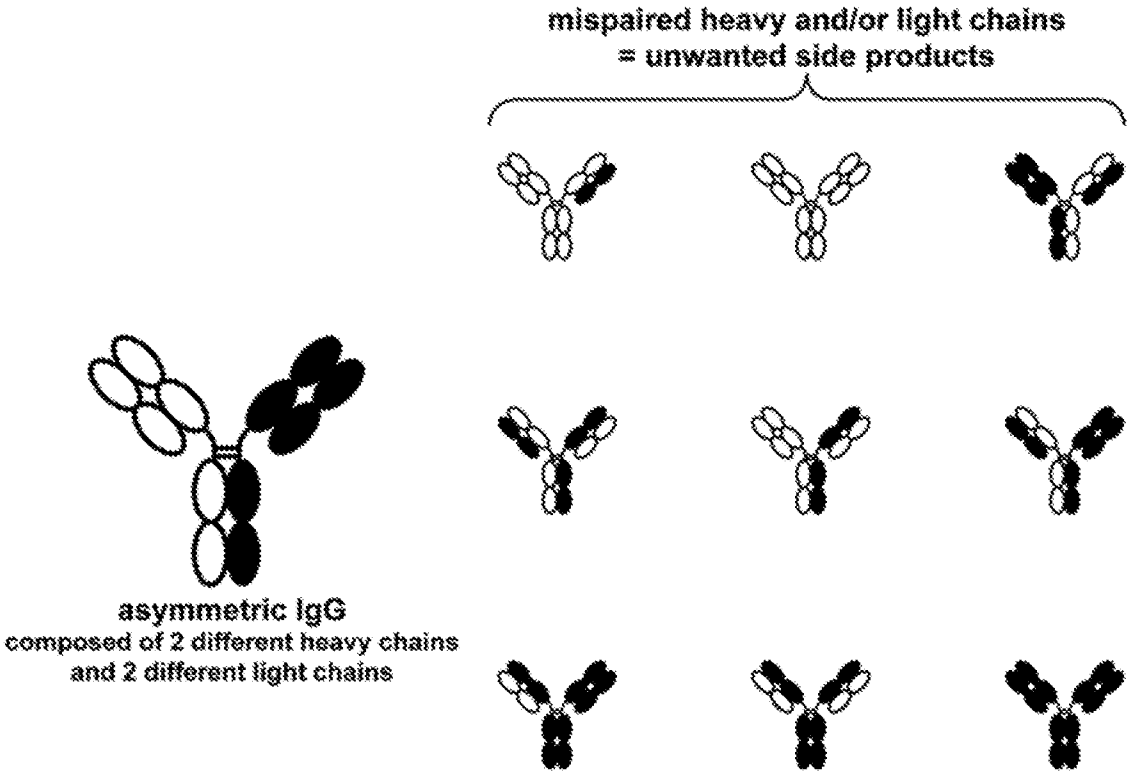
Figure 1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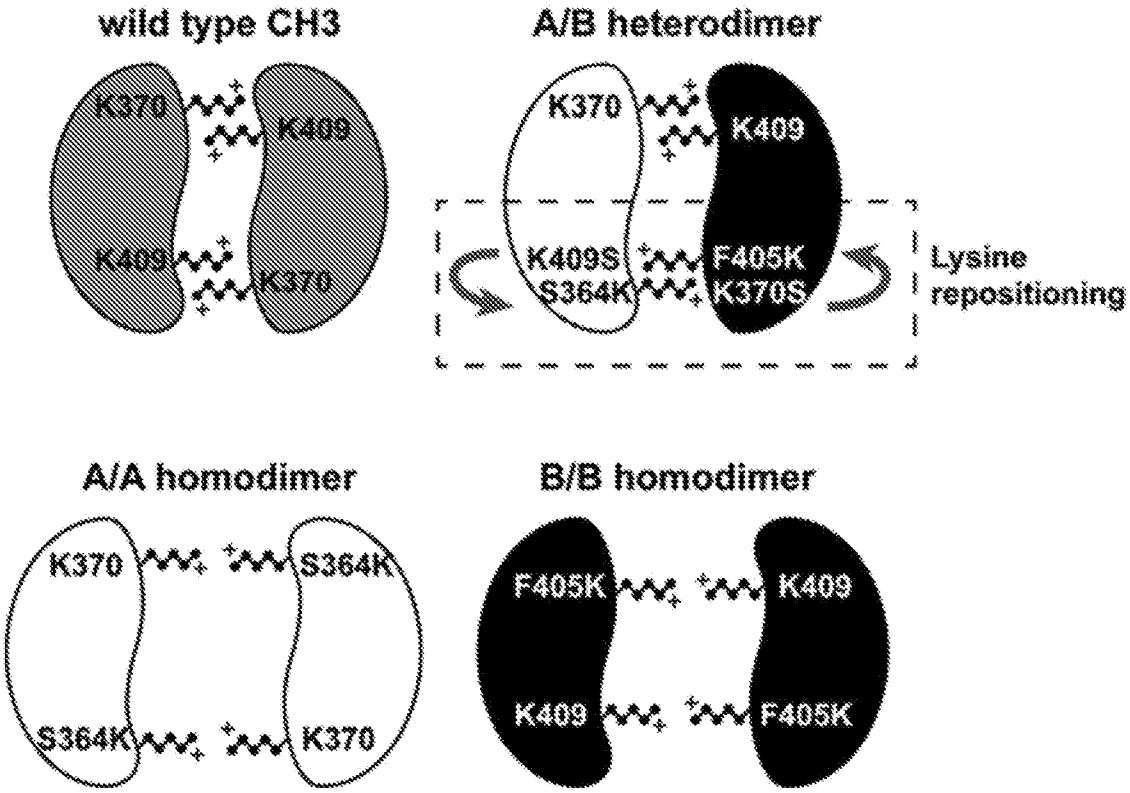
= unwanted side products
asymmetric IgG
composed of 2 different heavy chains
and 2 different light chains

Figure 2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K409
K409
K370
A/B heterodimer
K370
K409
K409S
S364K
F405K
K370S
Lysine repositioning
A/A homodimer
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S364K
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K370
B/B homodimer
F405K
K409
K409
F405K

Figure 3A

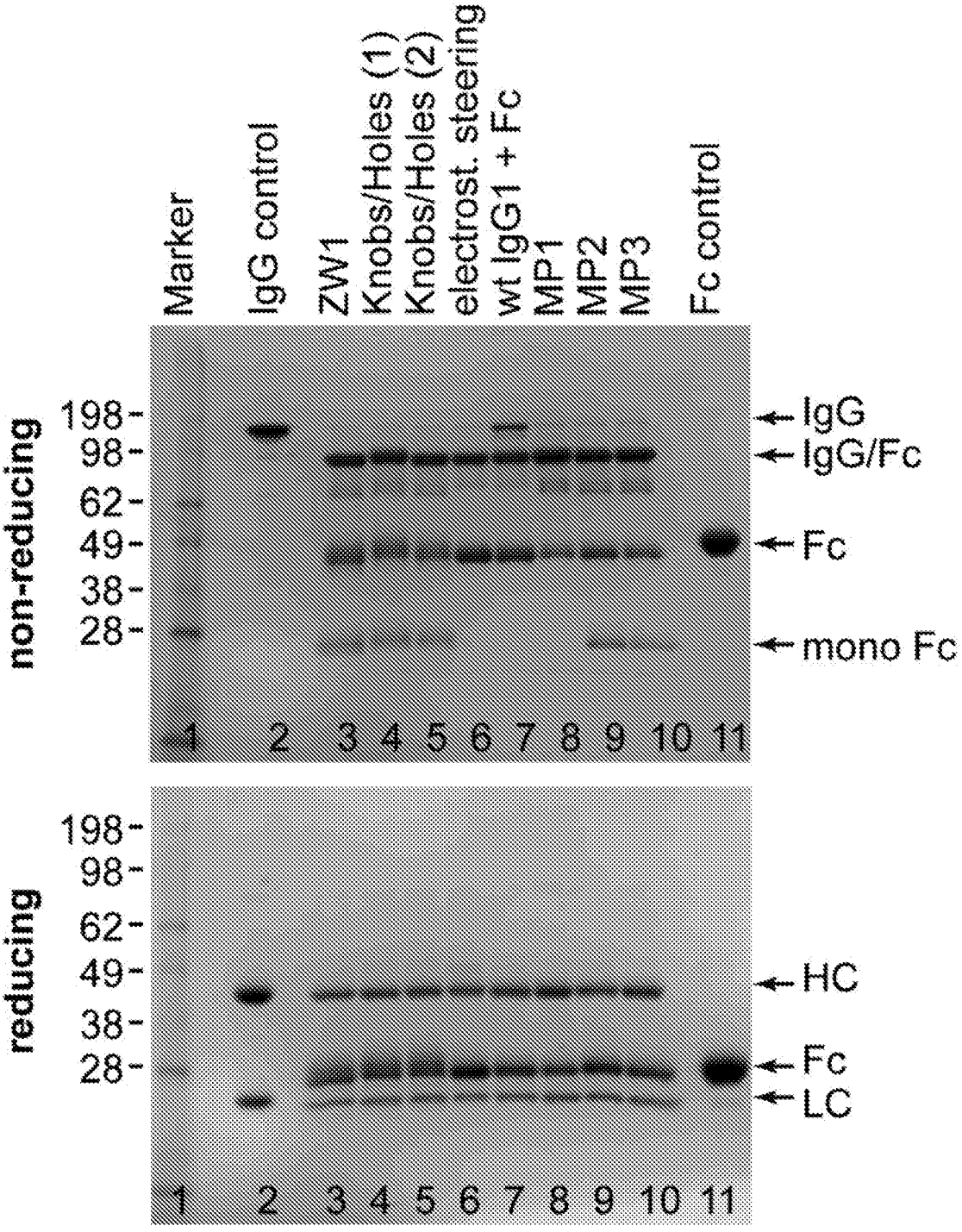

Marker
IgG control
ZW1
Knobs/Holes (1)
Knobs/Holes (2)
electrost. steering
wt IgG1 + Fc
MP1
MP2
MP3
Fc control
non-reducing
198
98
62
49
38
28
IgG
IgG/Fc
Fc
mono Fc
1 2 3 4 5 6 7 8 9 10 11
reducing
198
98
62
49
38
28
HC
Fc
LC
1 2 3 4 5 6 7 8 9 10 11

Figure 3B

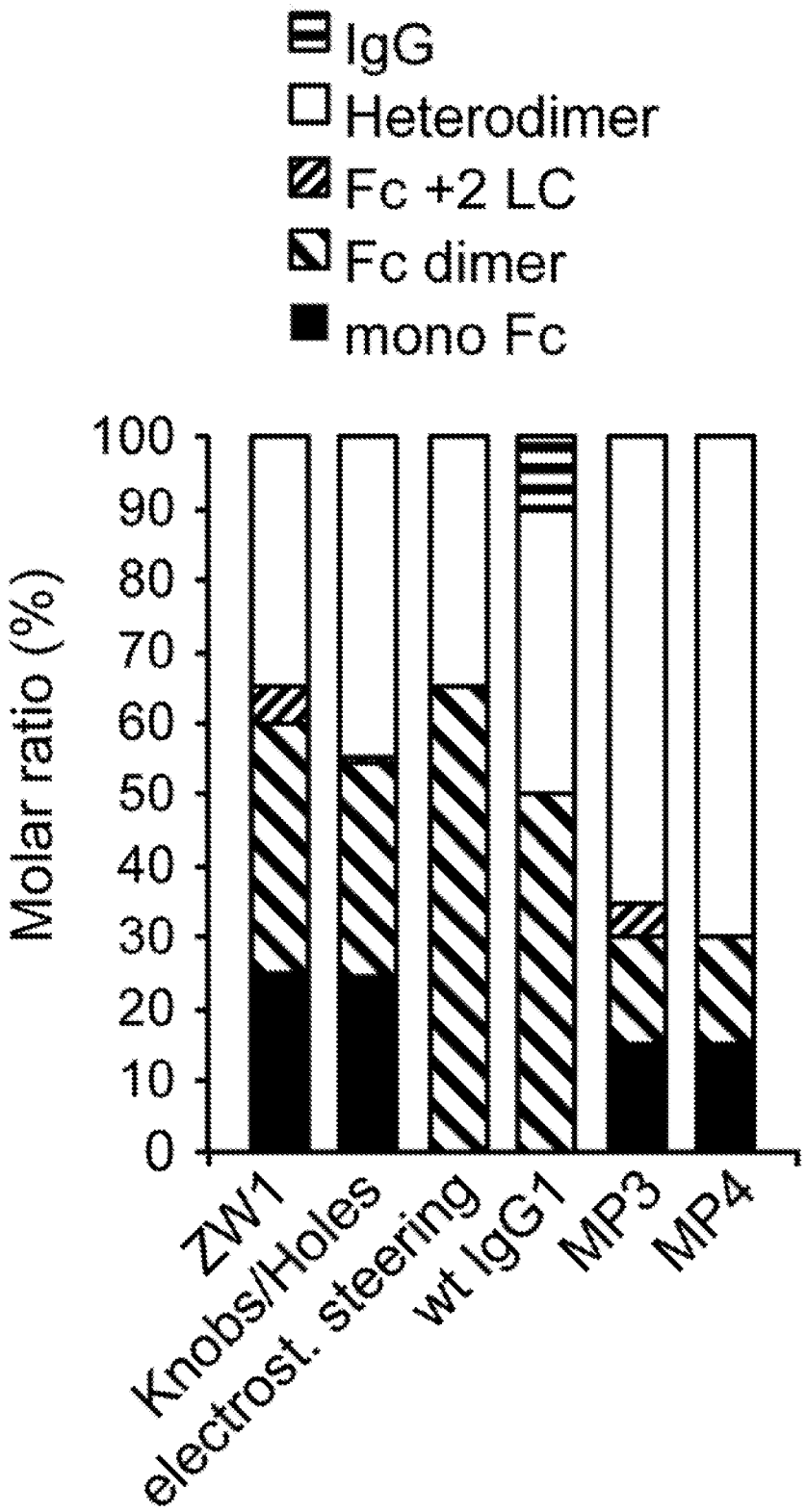

IgG
Heterodimer
Fc +2 LC
Fc dimer
mono Fc
Molar ratio (%)
100
90
80
70
60
50
40
30
20
10
0
ZW1
Knobs/Holes
electrost. steering
wt IgG1
MP3
MP4

Figure 3C

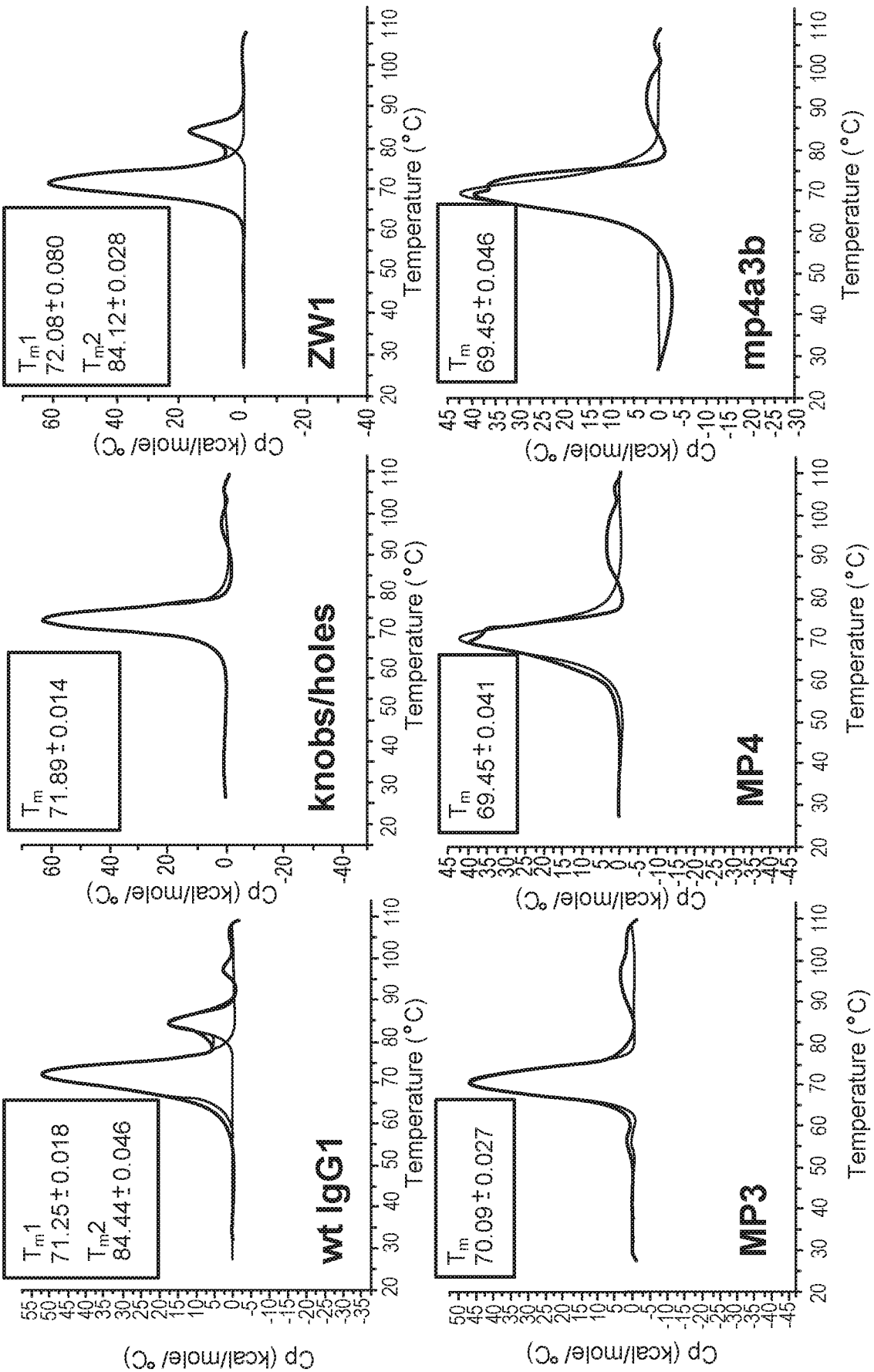

wt IgG1
Tm1 71.25 ± 0.018
Tm2 84.44 ± 0.046
knobs/holes
Tm 71.89 ± 0.014
ZW1
Tm1 72.08 ± 0.080
Tm2 84.12 ± 0.028
MP3
Tm 70.09 ± 0.027
MP4
Tm 69.45 ± 0.041
mp4a3b
Tm 69.45 ± 0.046
Cp (kcal/mole/°C)
Temperature (°C)

Figure 4A

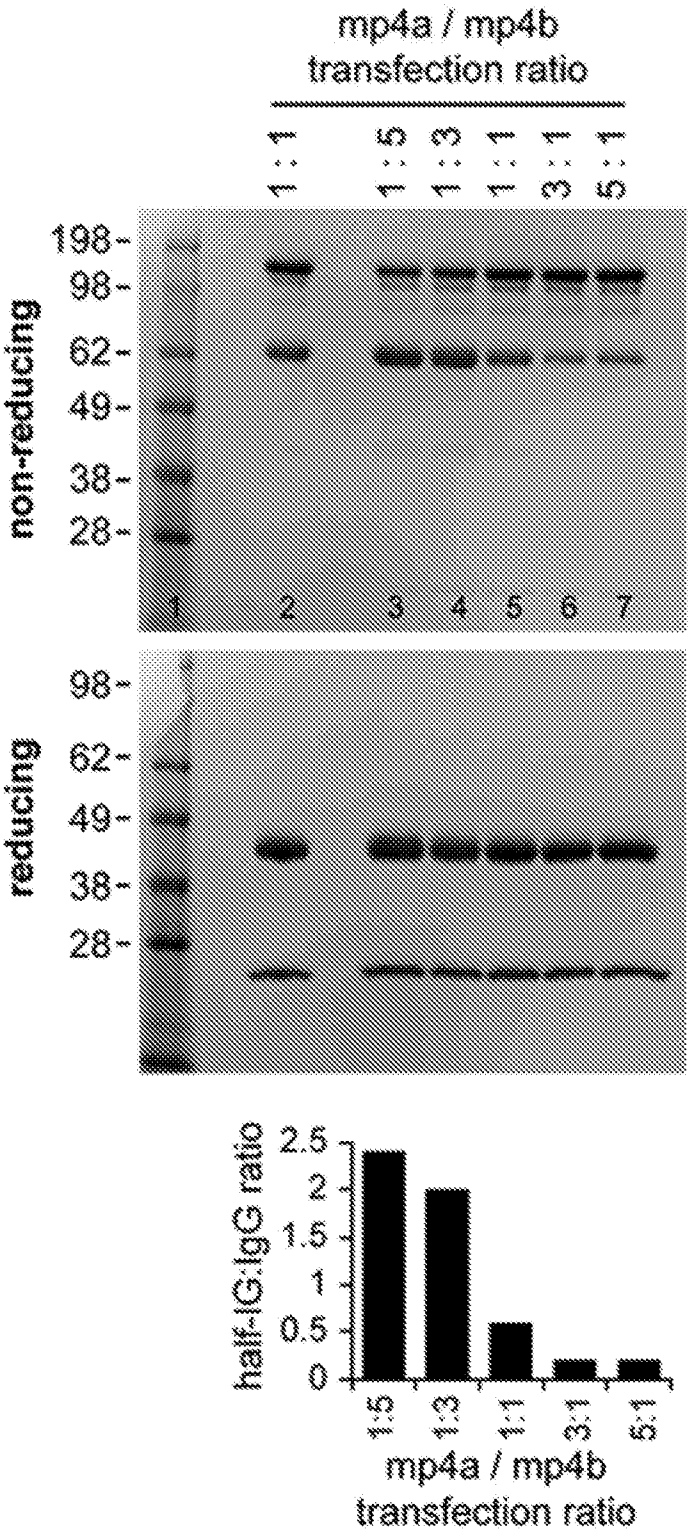

mp4a / mp4b
transfection ratio
1 : 1
1 : 5
1 : 3
1 : 1
3 : 1
5 : 1
non-reducing
198
98
62
49
38
28
1
2
3
4
5
6
7
reducing
98
62
49
38
28
half-IG:IgG ratio
2.5
2
1.5
1
0.5
0
1:5
1:3
1:1
3:1
5:1
mp4a / mp4b
transfection ratio

Figure 4B

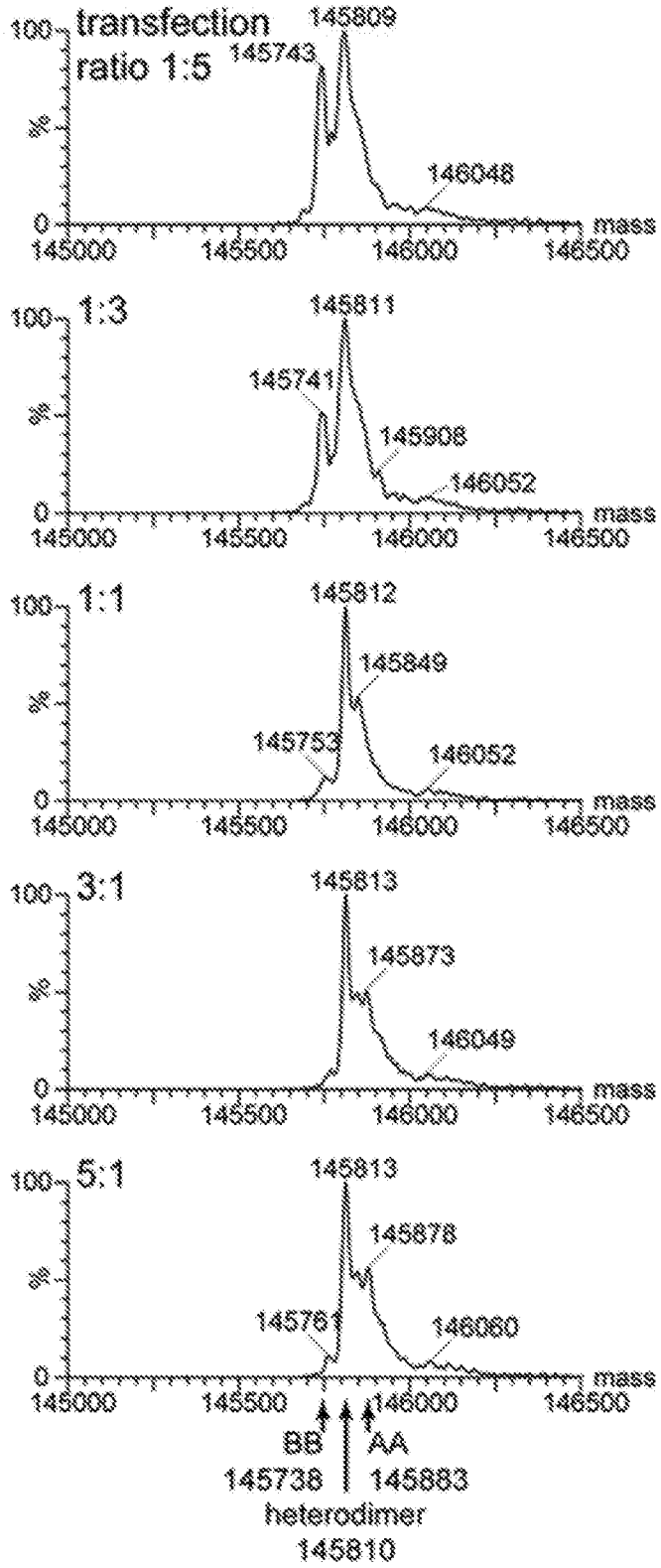

transfection ratio 1:5
145809
145743
146048
100
%
0
145000
145500
146000
146500
mass
1:3
145811
145741
145908
146052
1:1
145812
145849
145753
146052
3:1
145813
145873
146049
5:1
145813
145878
145761
146060
BB
145738
AA
145883
heterodimer
145810

Figure 5A

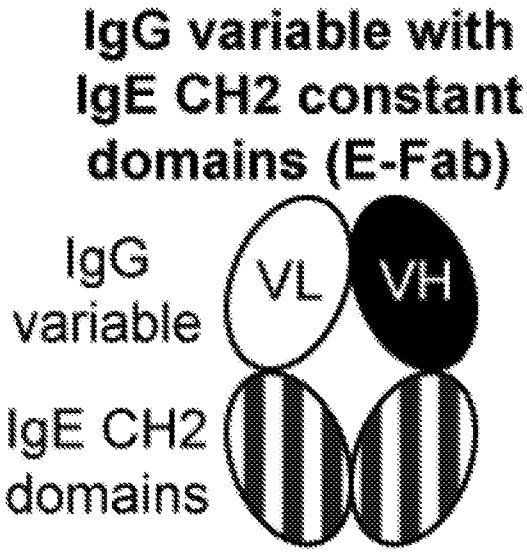

IgG variable with IgE CH2 constant domains (E-Fab)
IgG variable
VL
VH
IgE CH2 domains

Figure 5B

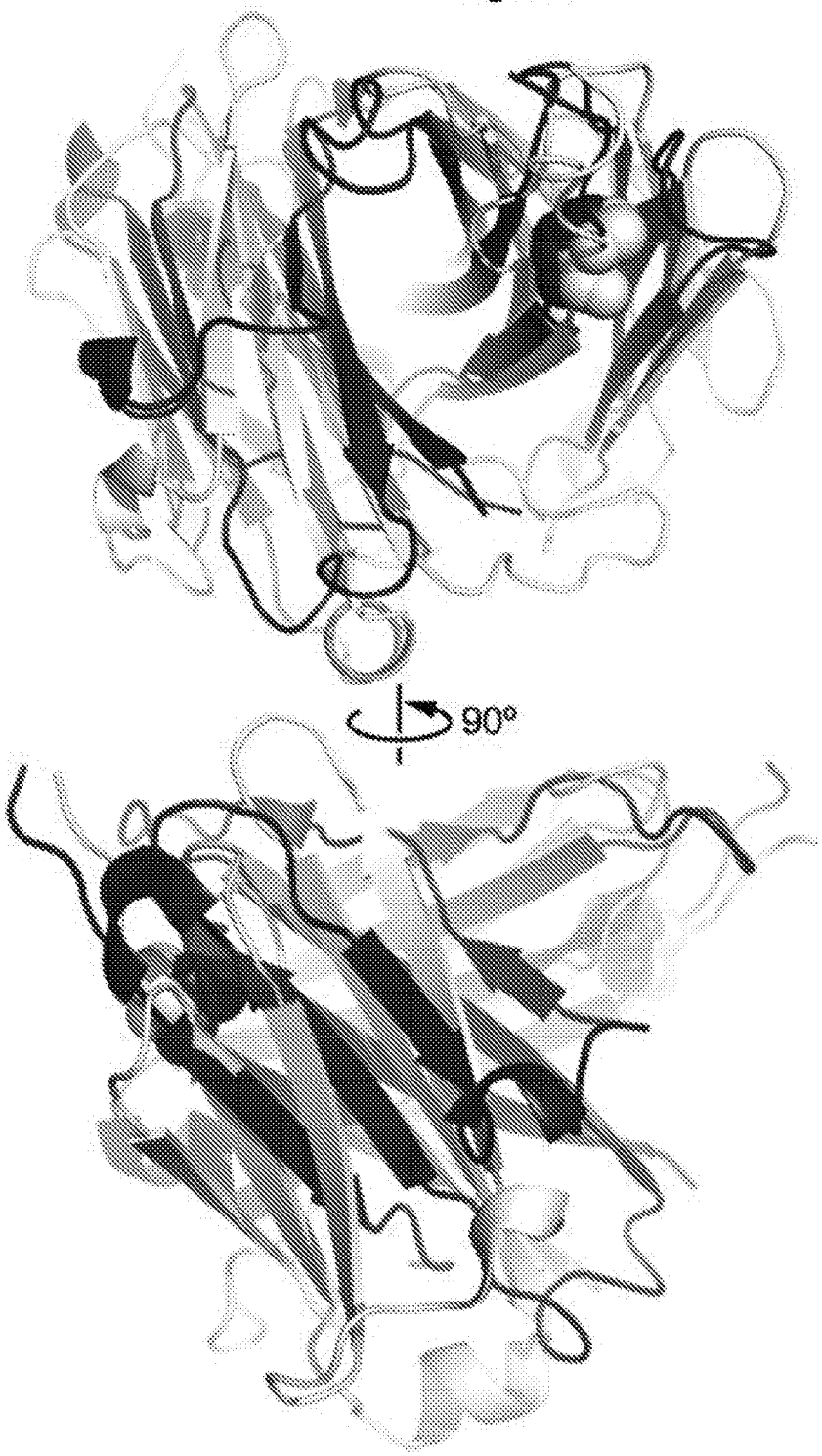


Figure 5C


A
B
1 10 15 16 23 26 27 38
7654321|.....|....|123|.....|..| |.....|
CH1/hinge ...ASTKGPSVFPLAPSSKSTS...GGTAALGCLVK DYFP..EPVT
Ckappa ...RTVAAPSVFIFPPSDEQLK...SGTASVVCLLN NFYP..REAK
IgE CH2 VCSRDFTPPTVKILQSSCDGGGHF.PPTIQLLCLVS GYTP..GTIN
EFab HC ...ASTKGPTVKILQSSCDGGGHF.PPTIQLLCLVS GYTP..GTIQ
Efab LC ...RTVAAPTVKILQSSCDGGGHF.PPTIQLLCLVS GYTP..GTIQ
C
D
E
39 45 77 84 85 89 96
|.....|1234567|.....|123454321|...|.....|12
CH1/hinge VSWNSGALTS....GVHTFPAVLQSS.GLYSLSSVVTVPSSSL..
Ckappa VQWKVDNALQSG..NSQESVTEQDSKDSTYSLSSTLTLSKADY..
IgE CH2 ITWLEDGQVMD...VDLSTASTTQEG.ELASTQSELTLSQKHW..
EFab HC ITWLEDGQVMD...VDLSTASTTQEG.ELASTQSELTLSQKHW..
Efab LC ITWLEDGQVMD...VDLSTASTTQEG.ELASTQSELTLSQKHW..
F
G
97 104 105 117 118
|.....| |.....| |.....
CH1/hinge .GTQTYIC NVNHKP..SNTKV DKKVEPKSCDKTHTCPPCP
Ckappa EKHKVYAC EVTHQG..LSSPV TKSFNRGEC
IgE CH2 LSDRTYTC QVTYQ....GHTF EDSTKKCA
EFab HC LSDRTYTC QVTYQ....GHTF EDSTKKCA
Efab LC LSDRTYTC QVTYQ....GHTF EDSTKKCA

Figure 5D

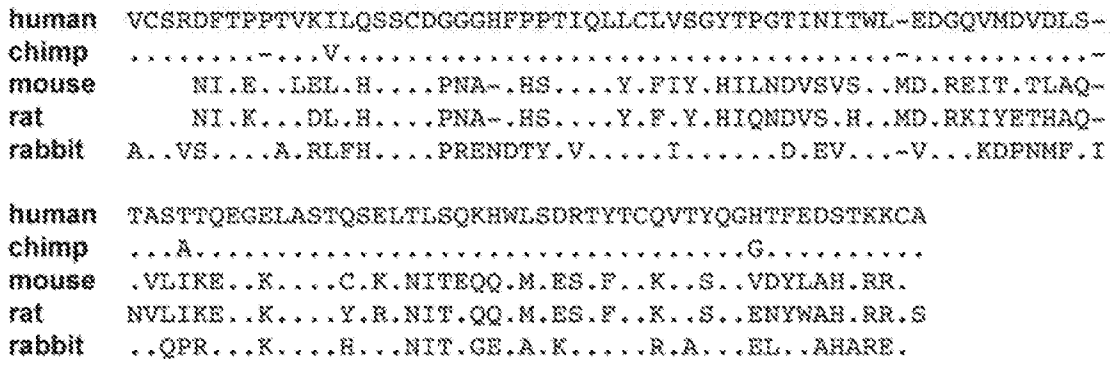
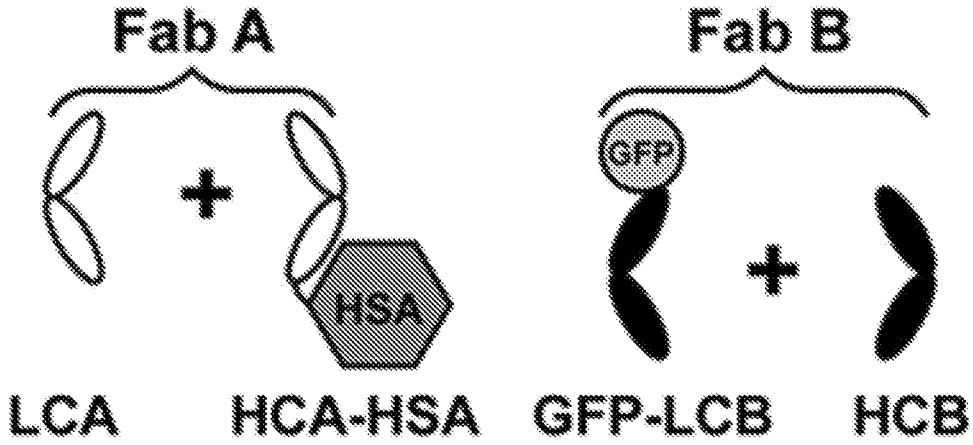

human VCSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWL~EDGQVMDVDLS~
chimp .....~...V.....~.....~
mouse NI.E..LEL.H....PNA~.HS....Y.FIY.HILNDVSVS..MD.REIT.TLAQ~
rat NI.K...DL.H....PNA~.HS....Y.F.Y.HIQMDVS.H..MD.RKIYETHAQ~
rabbit A..VS....A.RLFH....PRENDTY.V.....I......D.EV...~V...KDPNMF.I
human TASTTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKCA
chimp ...A.....G.....
mouse .VLIKE..K....C.K.NITEQQ.M.ES.F..K..S..VDYLAH.RR.
rat NVLIKE..K....Y.R.NIT.QQ.M.ES.F..K..S..ENYWAH.RR.S
rabbit ..QPR...K....H...NIT.GE.A.K.....R.A...EL..AHARE.

Figure 6A


Fab A
Fab B
GFP
HSA
LCA
HCA-HSA
GFP-LCB
HCB

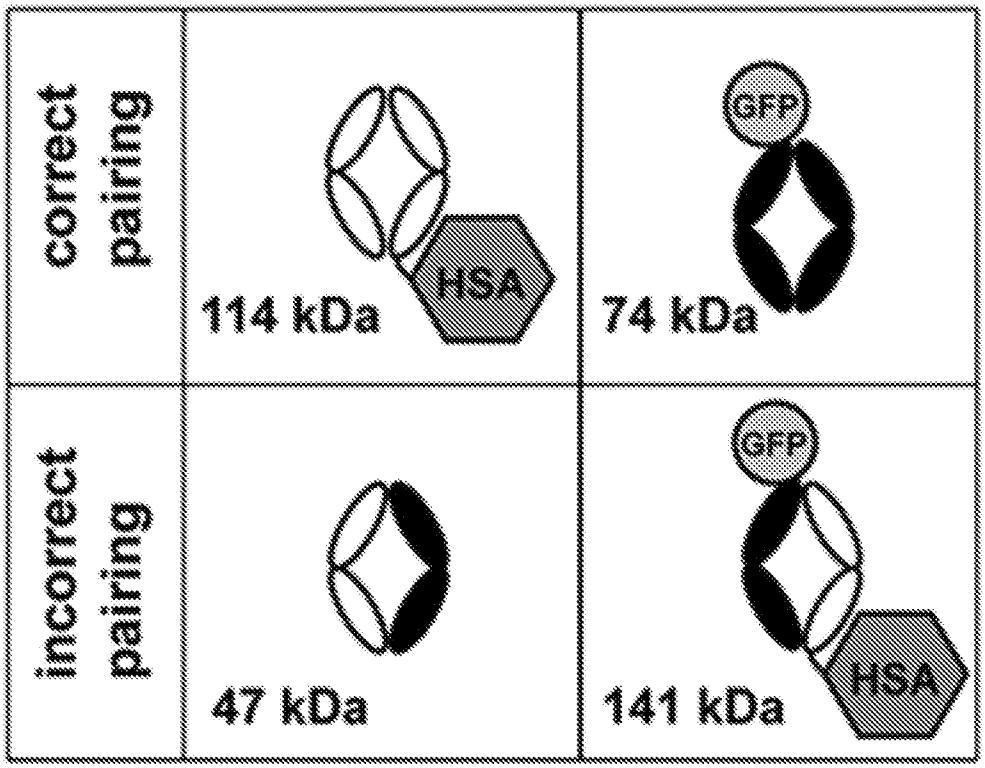

correct pairing
incorrect pairing
HSA
114 kDa
GFP
74 kDa
47 kDa
GFP
HSA
141 kDa

Figure 6B

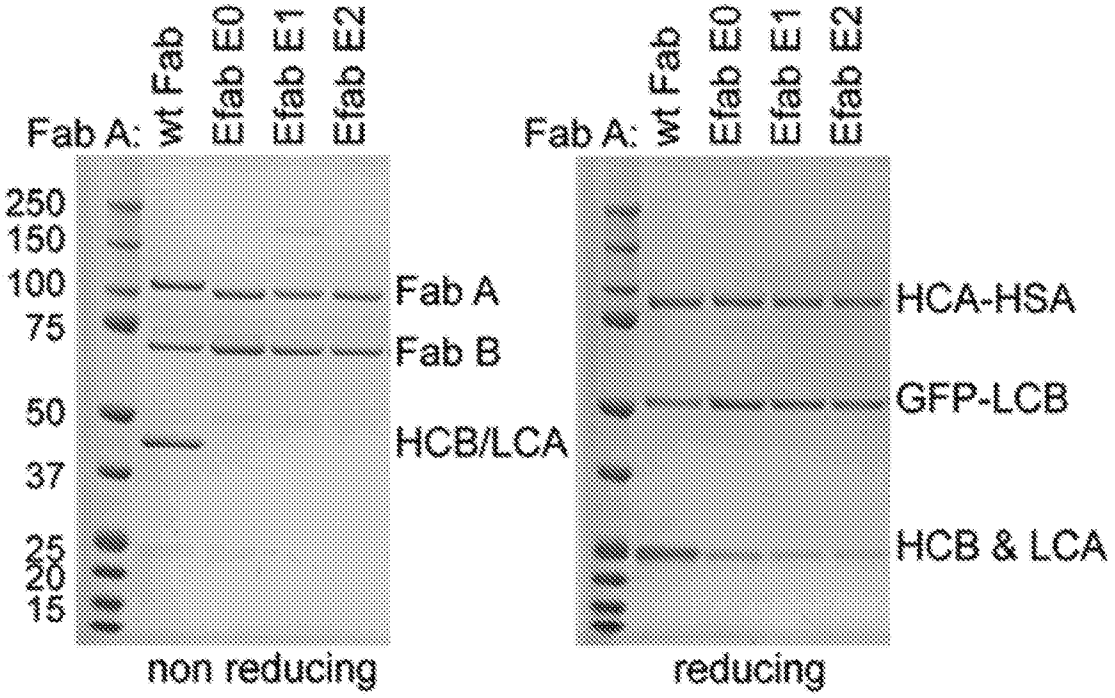

Fab A:
wt Fab
Efab E0
Efab E1
Efab E2
250
150
100
75
50
37
25
20
15
Fab A
Fab B
HCB/LCA
non reducing
Fab A:
wt Fab
Efab E0
Efab E1
Efab E2
HCA-HSA
GFP-LCB
HCB & LCA
reducing

Figure 6C

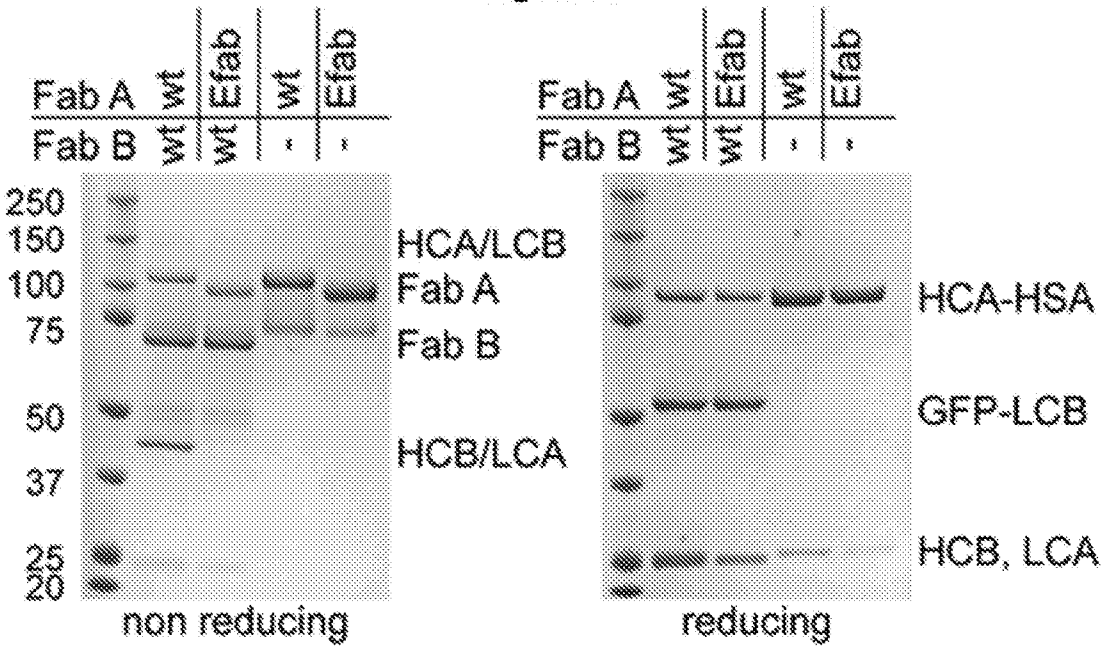

Fab A wt Efab wt Efab
Fab B wt wt - -
250
150
100
75
50
37
25
20
HCA/LCB
Fab A
Fab B
HCB/LCA
non reducing
HCA-HSA
GFP-LCB
HCB, LCA
reducing

Figure 6D

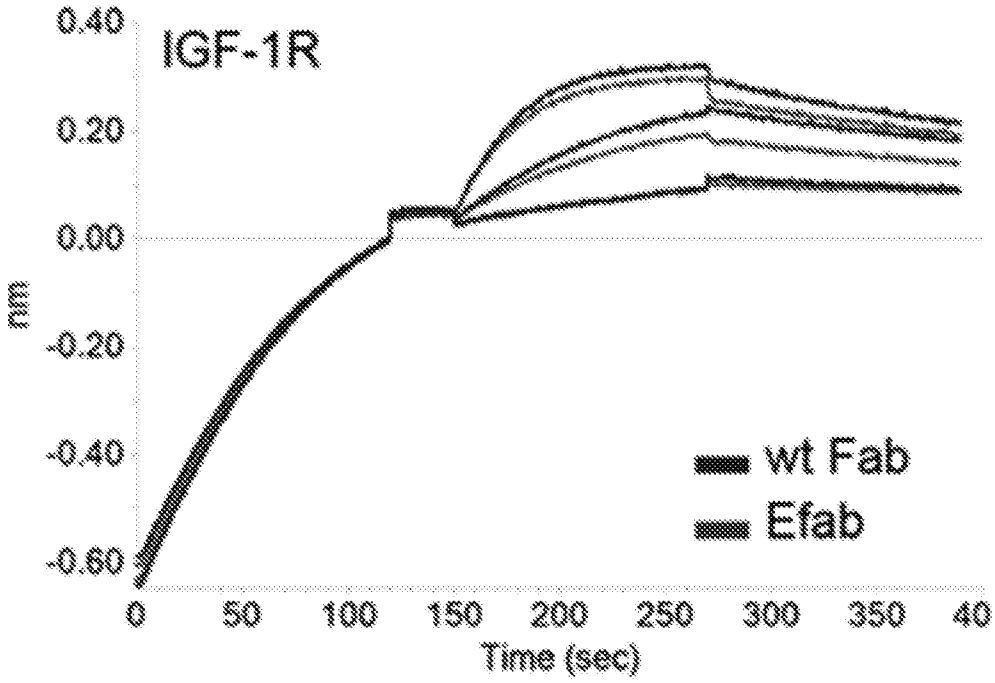

IGF-1R
0.40
0.20
0.00
-0.20
-0.40
-0.60
nm
0
50
100
150
200
250
300
350
40
Time (sec)
wt Fab
Efab

Figure 7

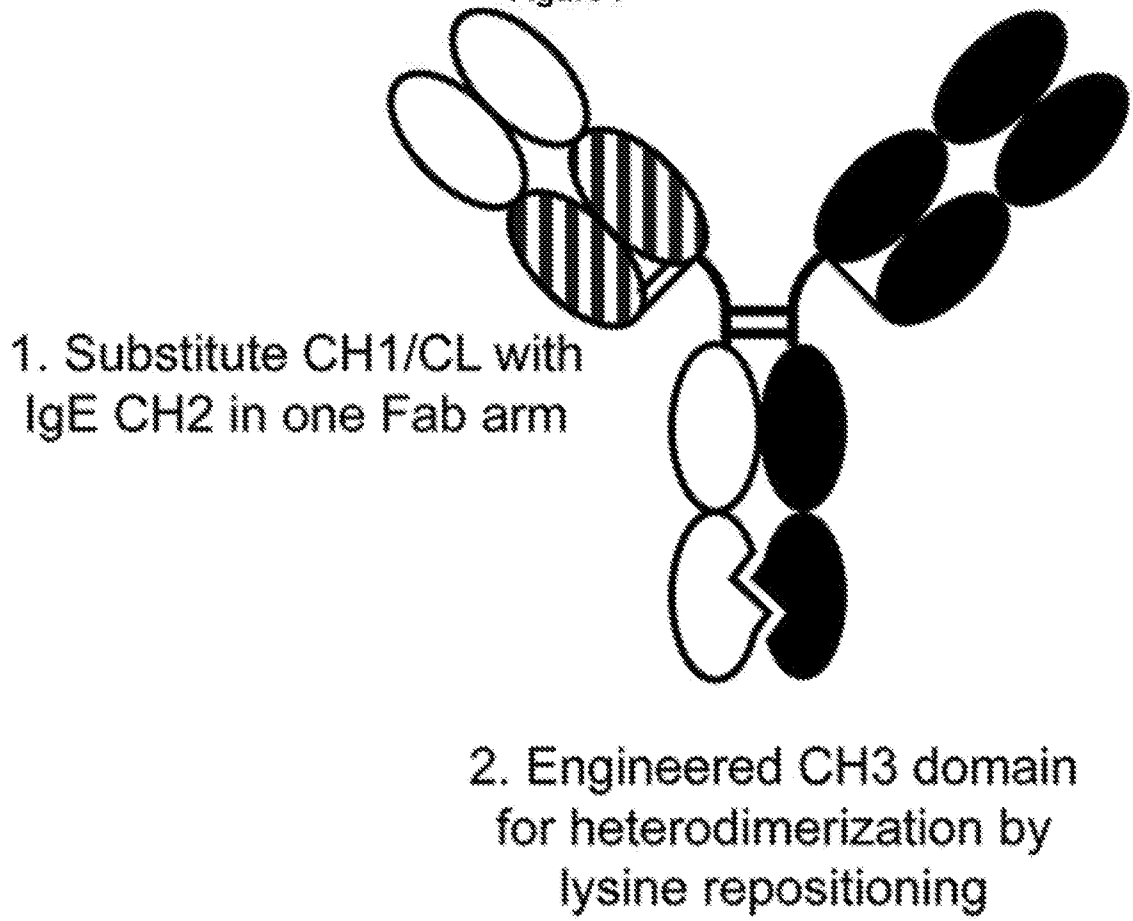

1. Substitute CH1/CL with IgE CH2 in one Fab arm
2. Engineered CH3 domain for heterodimerization by lysine repositioning

Figure 8A

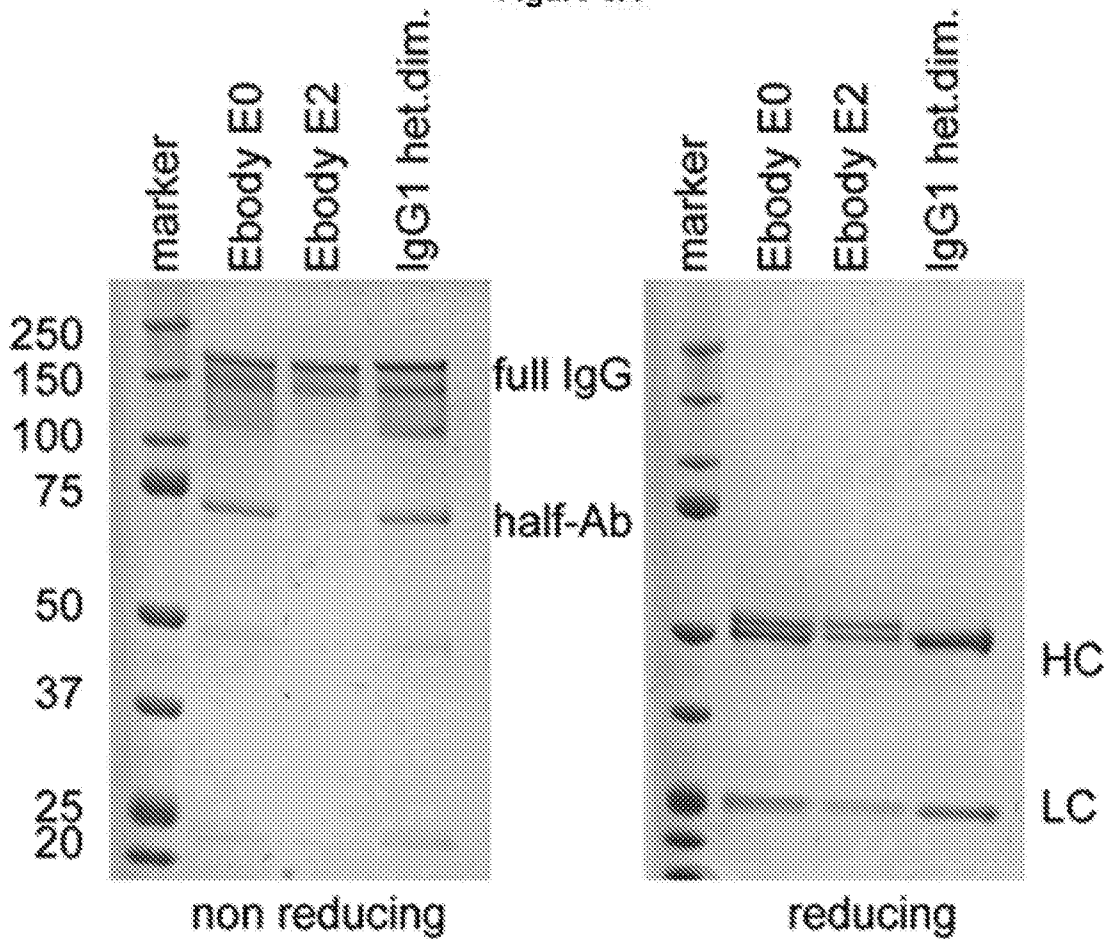

marker
Ebody E0
Ebody E2
IgG1 het.dim.
marker
Ebody E0
Ebody E2
IgG1 het.dim.
250
150
100
75
50
37
25
20
full IgG
half-Ab
HC
LC
non reducing
reducing

Figure 8B

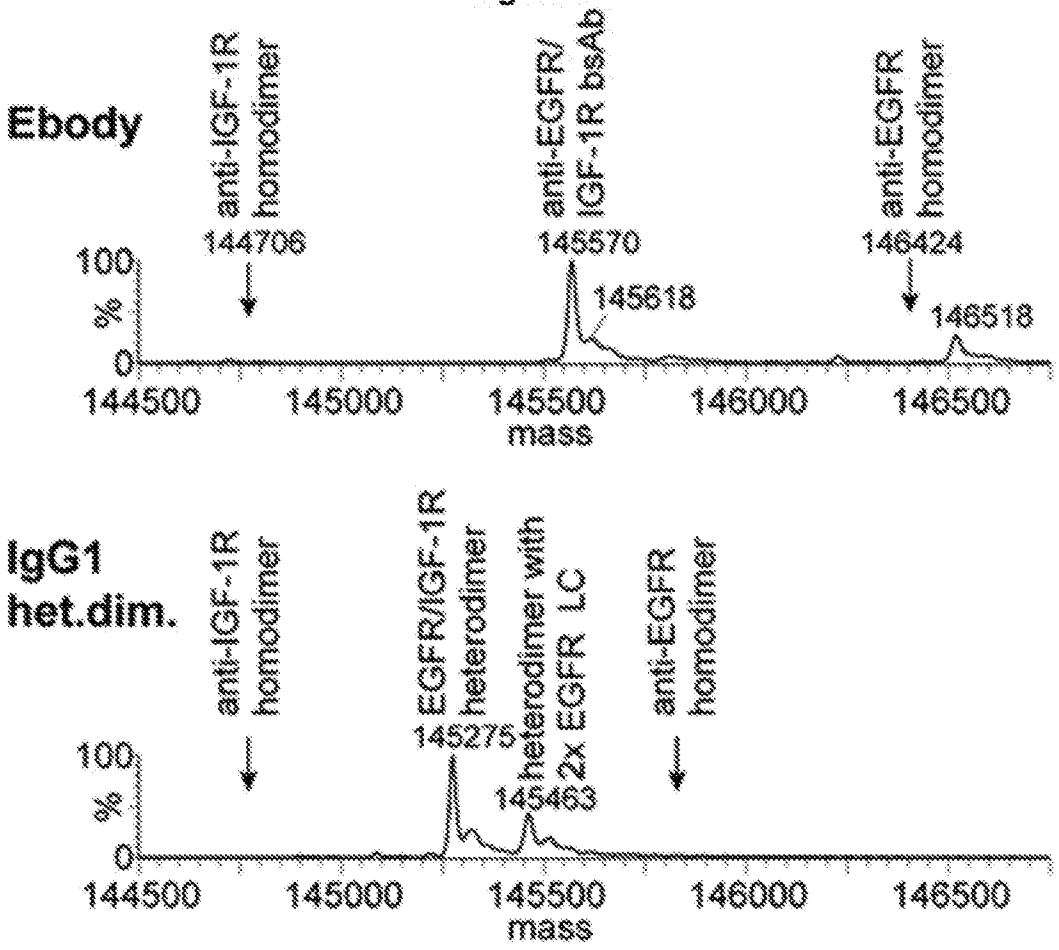

Ebody
anti-IGF-1R homodimer
144706
anti-EGFR/ IGF-1R bsAb
145570
145618
anti-EGFR homodimer
146424
146518
100
%
0
144500
145000
145500
146000
146500
mass
IgG1 het.dim.
anti-IGF-1R homodimer
EGFR/IGF-1R heterodimer
145275
heterodimer with 2x EGFR LC
145463
anti-EGFR homodimer
100
%
0
144500
145000
145500
146000
146500
mass

Figure 8C

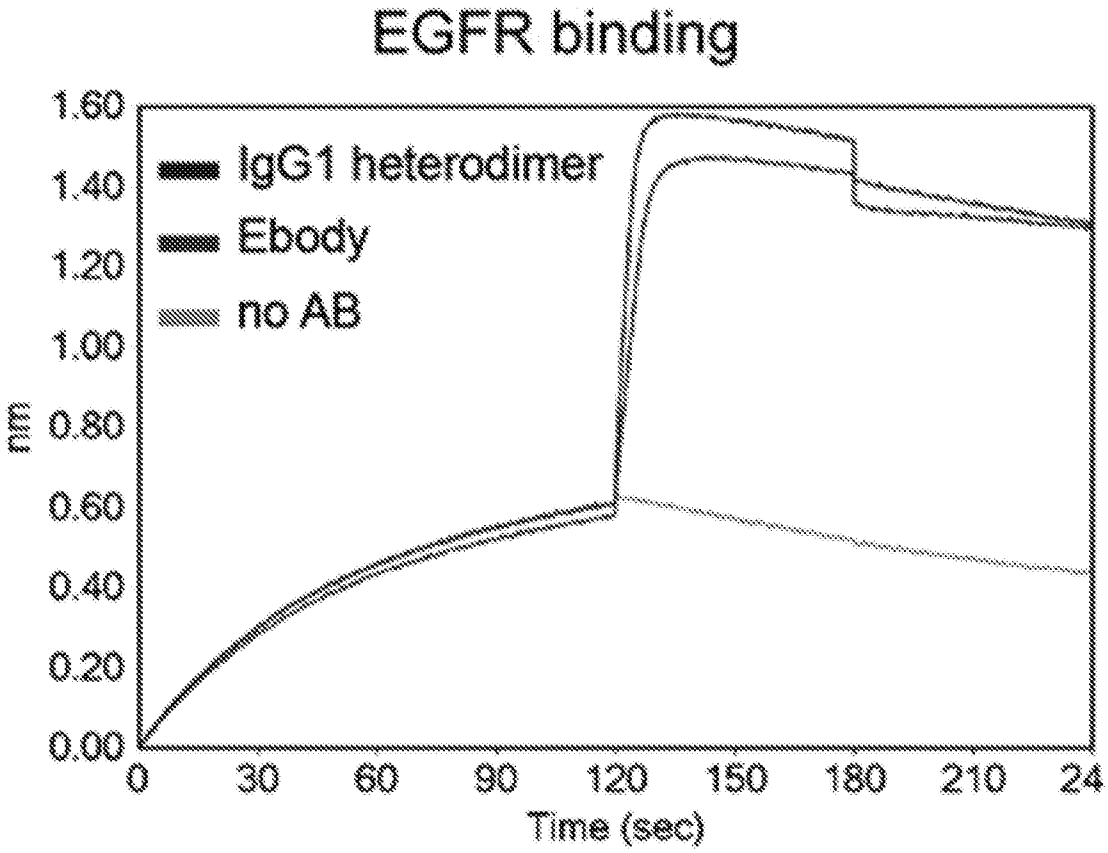

EGFR binding
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
nm
IgG1 heterodimer
Ebody
no AB
0
30
60
90
120
150
180
210
24
Time (sec)

Figure 8D

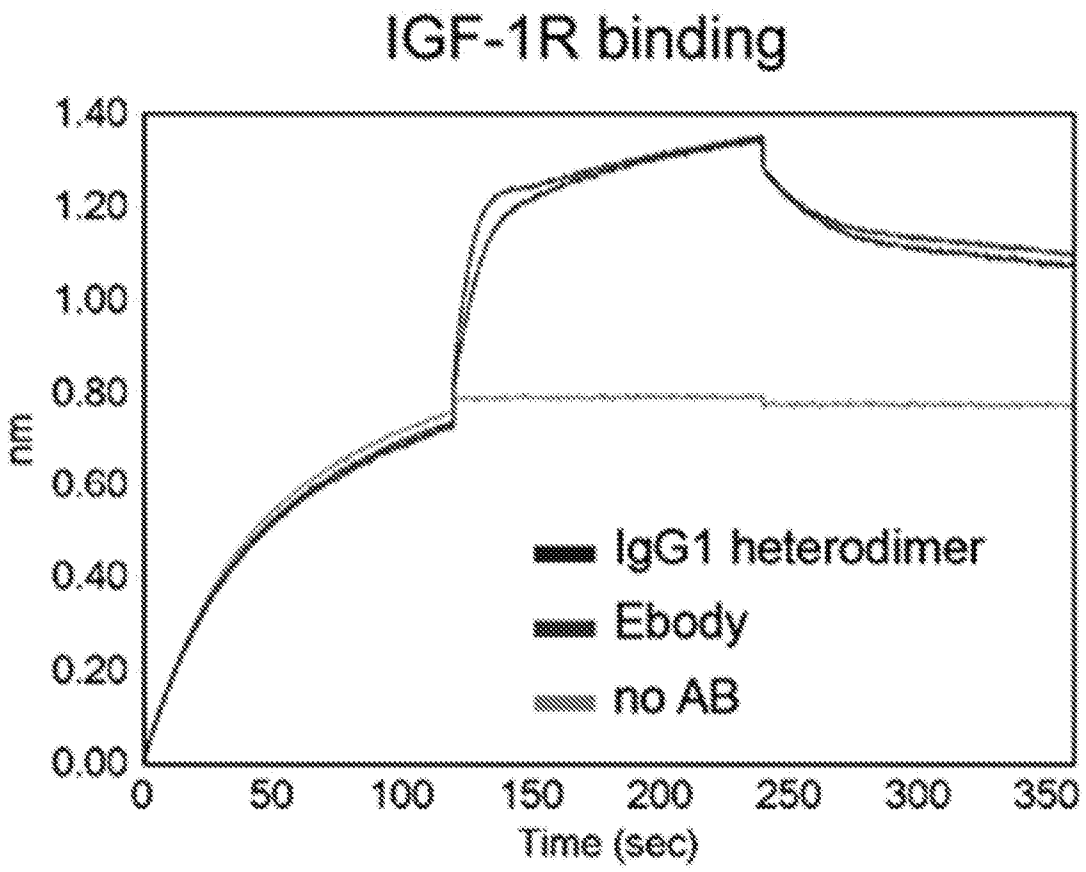

IGF-1R binding
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
nm
0
50
100
150
200
250
300
350
Time (sec)
IgG1 heterodimer
Ebody
no AB

Figure 9

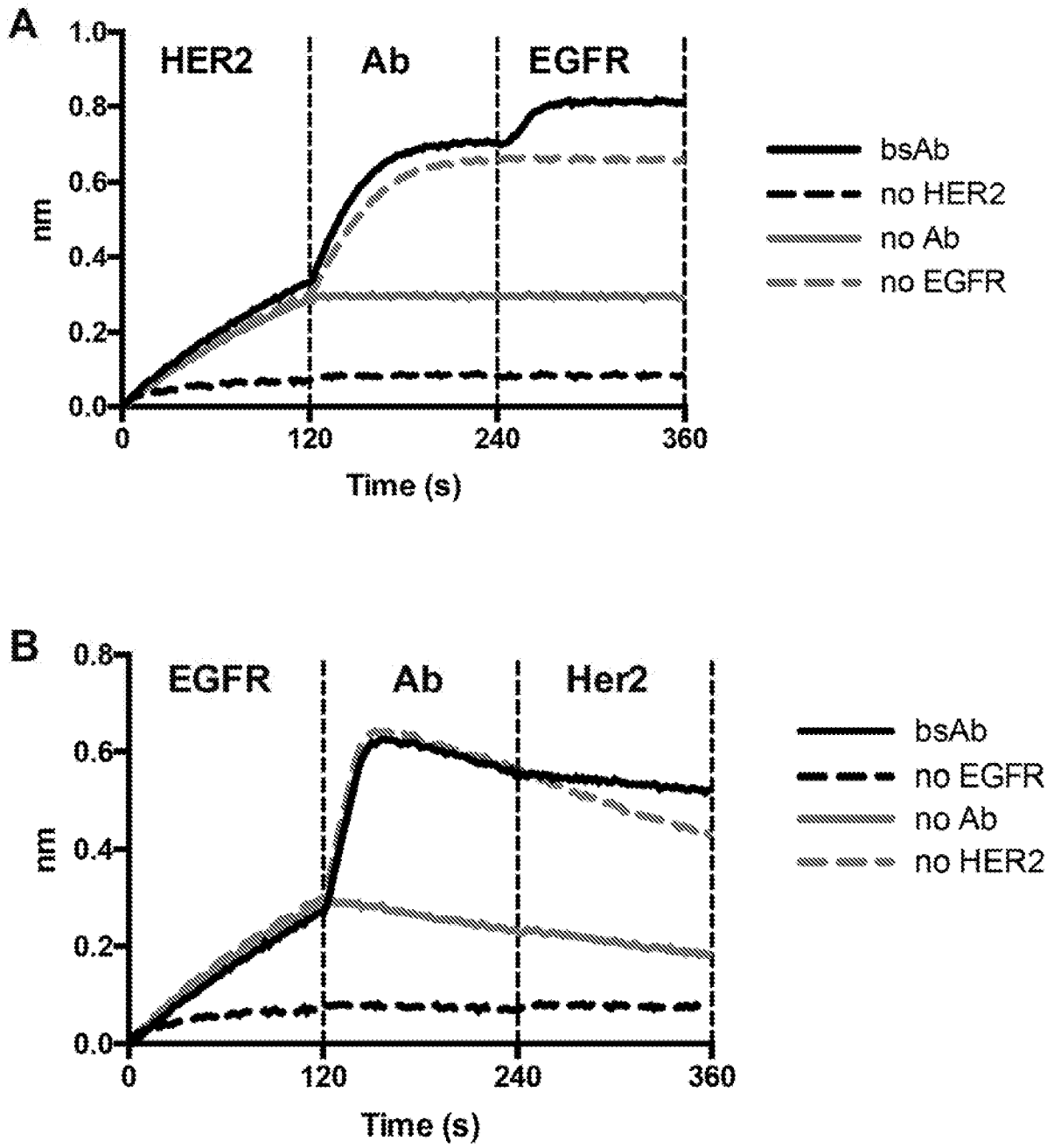

A
HER2
Ab
EGFR
nm
1.0
0.8
0.6
0.4
0.2
0.0
0
120
240
360
Time (s)
bsAb
no HER2
no Ab
no EGFR
B
EGFR
Ab
Her2
nm
0.8
0.6
0.4
0.2
0.0
0
120
240
360
Time (s)
bsAb
no EGFR
no Ab
no HER2

Figure 10

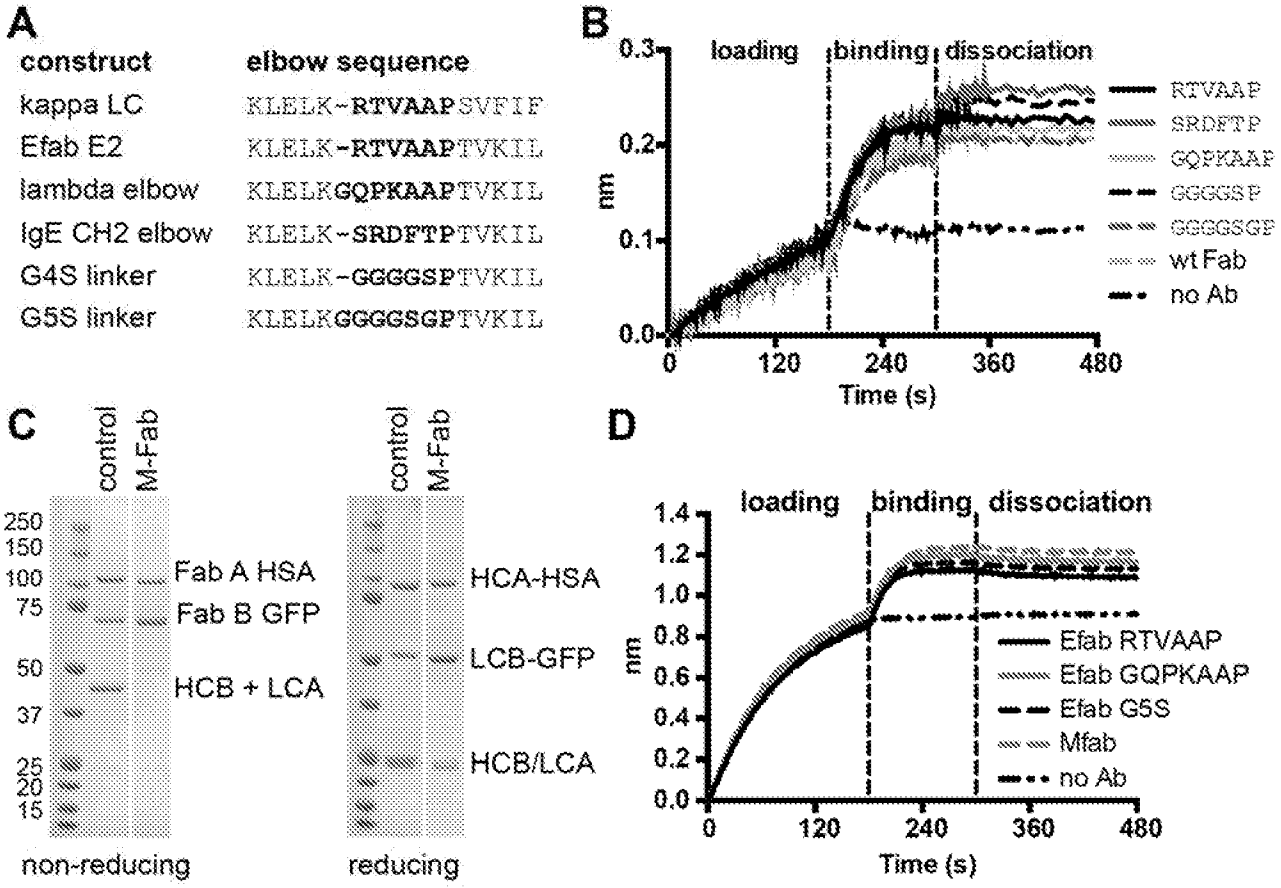

A
construct
elbow sequence
kappa LC
KLELK-RTVAAPSVFIF
Efab E2
KLELK-RTVAAPTVKIL
lambda elbow
KLELKGQPKAAPTVKIL
IgE CH2 elbow
KLELK-SRDFTPTVKIL
G4S linker
KLELK-GGGGSPTVKIL
G5S linker
KLELKGGGGSGPTVKIL
B
loading
binding
dissociation
nm
Time (s)
RTVAAP
SRDFTP
GQPKAAP
GGGGSP
GGGGSGP
wt Fab
no Ab
C
control
M-Fab
Fab A HSA
Fab B GFP
HCB + LCA
HCA-HSA
LCB-GFP
HCB/LCA
non-reducing
reducing
D
loading
binding
dissociation
nm
Time (s)
Efab RTVAAP
Efab GQPKAAP
Efab G5S
Mfab
no Ab

Figure 11

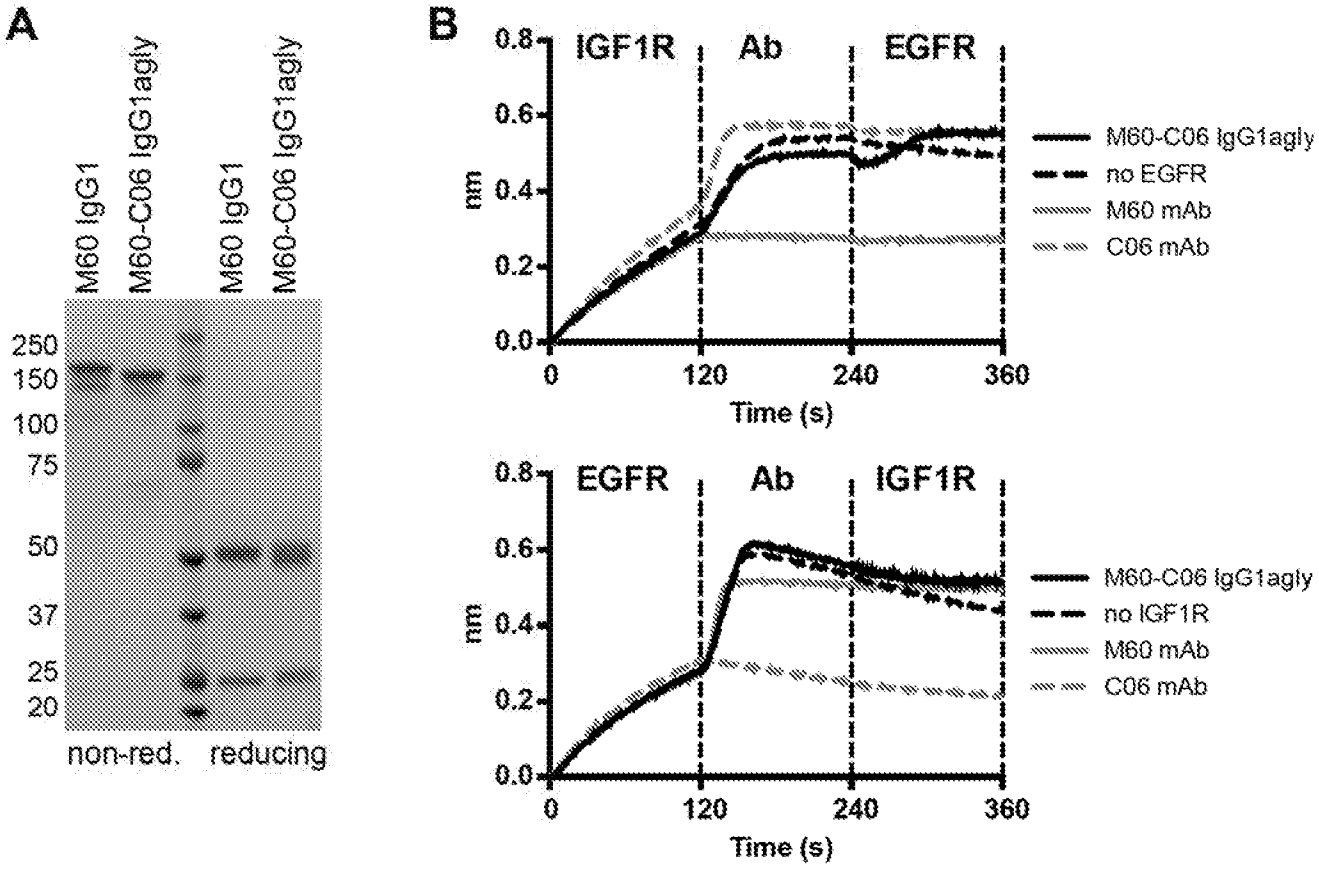

A
M60 IgG1
M60-C06 IgG1agly
M60 IgG1
M60-C06 IgG1agly
250
150
100
75
50
37
25
20
non-red.
reducing
B
IGF1R
Ab
EGFR
nm
0.8
0.6
0.4
0.2
0.0
0
120
240
360
Time (s)
M60-C06 IgG1agly
no EGFR
M60 mAb
C06 mAb
EGFR
Ab
IGF1R
M60-C06 IgG1agly
no IGF1R
M60 mAb
C06 mAb

Figure 12

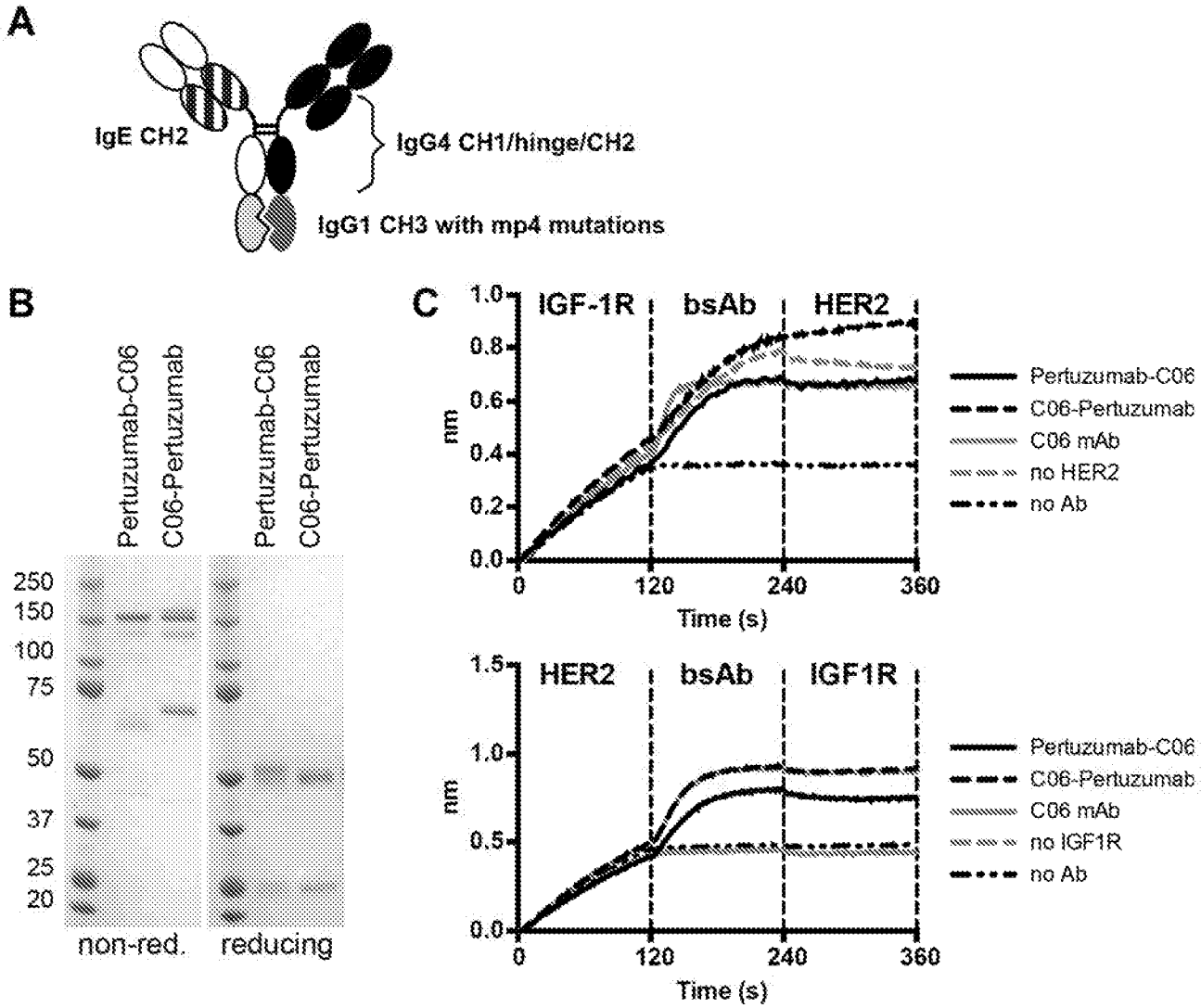

A
IgE CH2
IgG4 CH1/hinge/CH2
IgG1 CH3 with mp4 mutations
B
Pertuzumab-C06
C06-Pertuzumab
Pertuzumab-C06
C06-Pertuzumab
250
150
100
75
50
37
25
20
non-red.
reducing
C
IGF-1R
bsAb
HER2
nm
1.0
0.8
0.6
0.4
0.2
0.0
0
120
240
360
Time (s)
Pertuzumab-C06
C06-Pertuzumab
C06 mAb
no HER2
no Ab
HER2
bsAb
IGF1R
1.5
1.0
0.5
0.0
Pertuzumab-C06
C06-Pertuzumab
C06 mAb
no IGF1R
no Ab

Figure 13

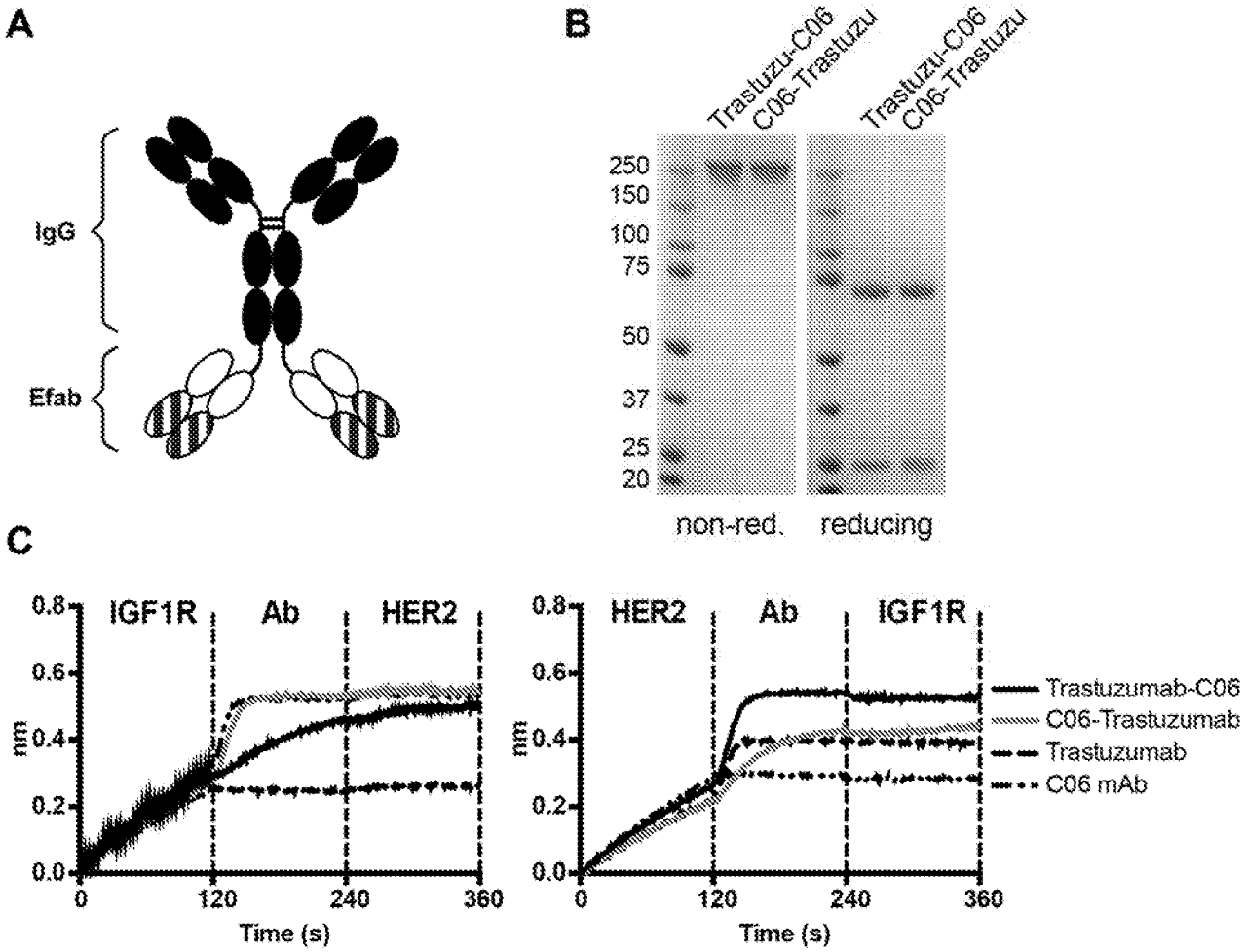

A
IgG
Efab
B
Trastuzu-C06
C06-Trastuzu
Trastuzu-C06
C06-Trastuzu
250
150
100
75
50
37
25
20
non-red.
reducing
C
IGF1R
Ab
HER2
HER2
Ab
IGF1R
nm
0.8
0.6
0.4
0.2
0.0
0
120
240
360
Time (s)
Trastuzumab-C06
C06-Trastuzumab
Trastuzumab
C06 mAb

Figure 14

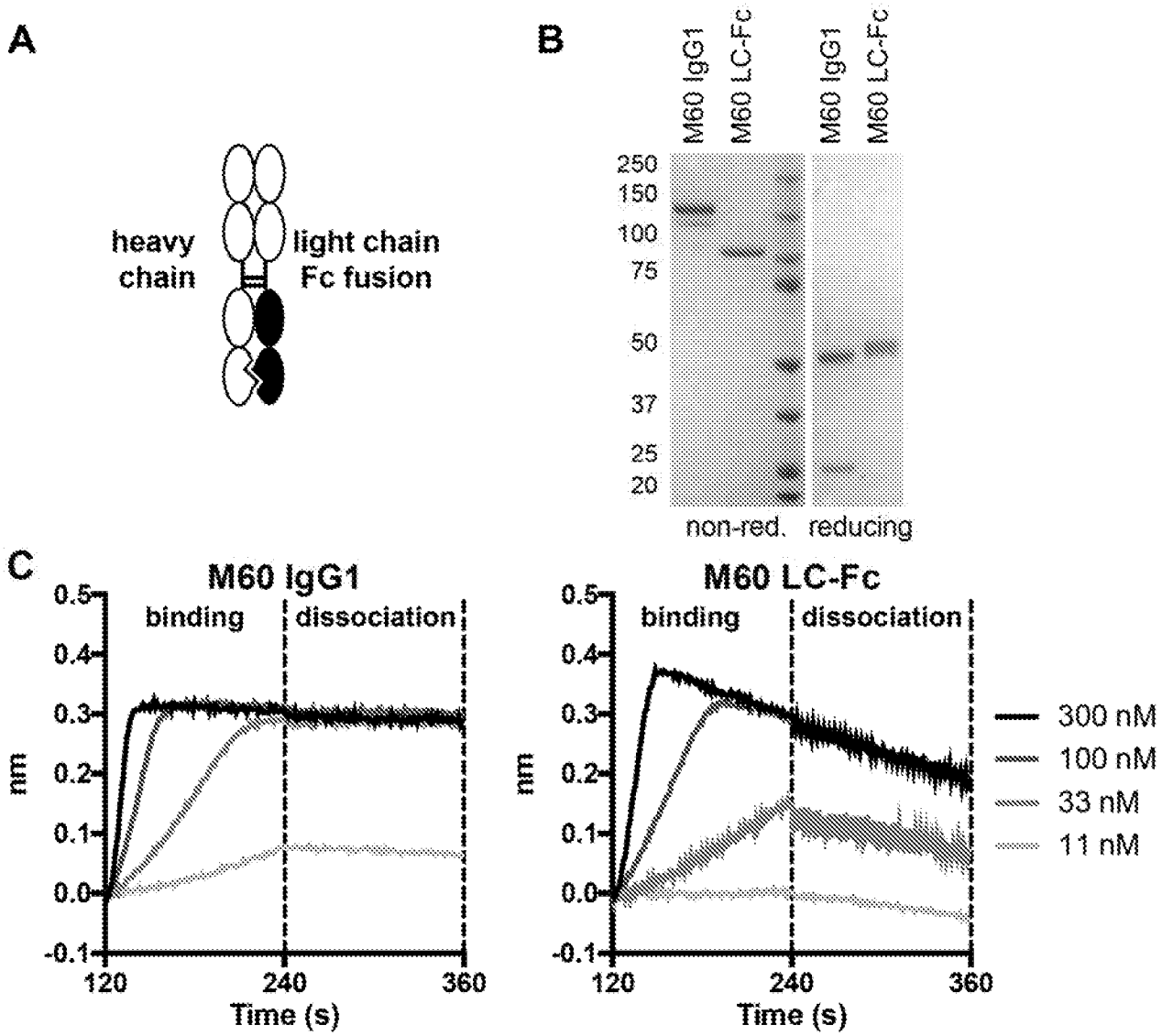
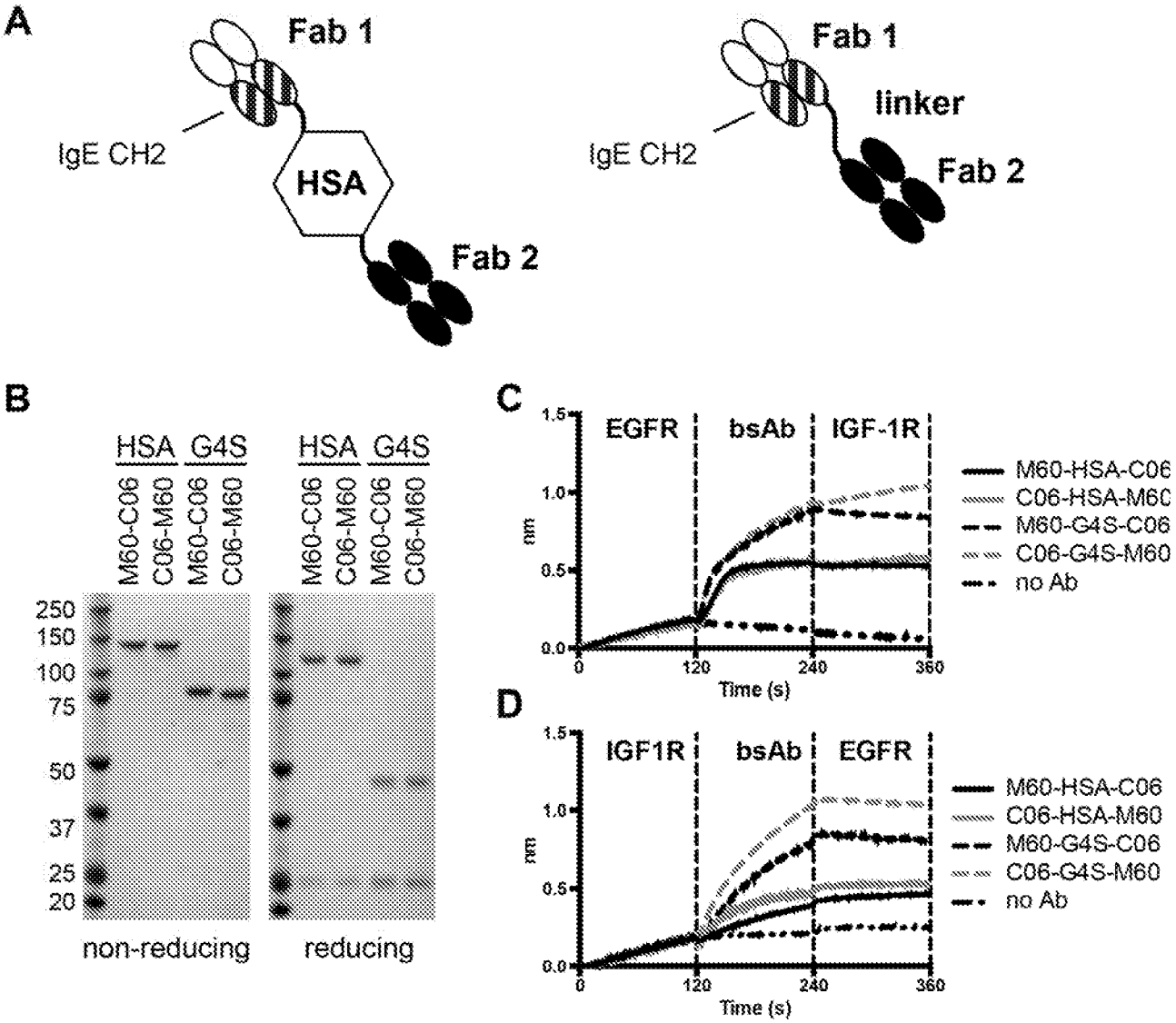

A
heavy chain
light chain Fc fusion
B
M60 IgG1
M60 LC-Fc
M60 IgG1
M60 LC-Fc
250
150
100
75
50
37
25
20
non-red.
reducing
C
M60 IgG1
binding
dissociation
nm
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
120
240
360
Time (s)
M60 LC-Fc
binding
dissociation
nm
Time (s)
300 nM
100 nM
33 nM
11 nM

Figure 15


A
Fab 1
IgE CH2
HSA
Fab 2
Fab 1
linker
IgE CH2
Fab 2
B
HSA
G4S
HSA
G4S
M60-C06
C06-M60
M60-C06
C06-M60
M60-C06
C06-M60
M60-C06
C06-M60
250
150
100
75
50
37
25
20
non-reducing
reducing
C
EGFR
bsAb
IGF-1R
nm
1.5
1.0
0.5
0.0
0
120
240
360
Time (s)
M60-HSA-C06
C06-HSA-M60
M60-G4S-C06
C06-G4S-M60
no Ab
D
IGF1R
bsAb
EGFR
nm
1.5
1.0
0.5
0.0
0
120
240
360
Time (s)
M60-HSA-C06
C06-HSA-M60
M60-G4S-C06
C06-G4S-M60
no Ab