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Fortsættes ...

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CHRISTIAN KLEIN ET AL: "Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies", MABS, vol. 4, no. 6, November 2012 (2012-11), pages 653-663, XP055106060, ISSN: 1942-0862, DOI: 10.4161/mabs.21379

DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention relates to novel heterodimeric proteins such as bispecific antibodies of primarily rodent origin.

BACKGROUND OF THE INVENTION

[0002] Monoclonal antibodies have in recent years become successful therapeutic molecules, in particular for the treatment of cancer. Unfortunately, however, monoclonal antibodies are often unable to cure diseases when used as monotherapy. Bispecific antibodies can potentially overcome some of the limitations of monoclonal antibody therapy, e.g. they could be used as mediators to target a drug or toxic compound to target cells, as mediators to retarget effector mechanisms to disease-associated sites or as mediators to increase specificity for tumor cells, for example by binding to a combination of targets molecules that is exclusively found on tumor cells. Different formats and uses of bispecific antibodies have been reviewed by Chames and Baty ((2009) Curr Opin Drug Disc Dev 12: 276) and by Kontermann and Brinkman ((2015) Drug Discovery Today 20: 838).

[0003] An *in vitro* method for producing bispecific antibodies is described in WO 2008119353 (Genmab). Herein is described that a bispecific antibody is formed by "Fab-arm" or "half-molecule" exchange (swapping of a heavy chain and attached light chain) between two monospecific antibodies having IgG4-like CH3 regions upon incubation under reducing conditions. This Fab-arm exchange reaction is the result of a disulfide-bond isomerization reaction and dissociation-association of CH3 domains wherein heavy-chain disulfide bonds in the hinge regions of the parent (originally monospecific) antibodies are reduced and the resulting free cysteines form an inter heavy-chain disulfide bond with cysteine residues of another parent antibody molecule (originally with a different specificity), and simultaneously CH3 domains of the parent antibodies dissociate and re-associate with the CH3 domains of the other parent antibody. The resulting product is a bispecific antibody having two Fab arms which potentially are composed of different sequences. WO2011131746 (Genmab) describes that by introducing asymmetrical, matching mutations in the CH3 regions of the two monospecific starting proteins, the Fab-arm exchange reaction can be forced to become directional and thereby yield highly stable heterodimeric proteins. This so-called controlled Fab-arm exchange (cFAE) involves the mixing of two separately expressed parental IgGs under controlled reducing conditions to allow the recombination of antibody half-molecules.

[0004] The recombination is driven by two matching point-mutations, such as F405L and K409R (EU-numbering index), one in either parental IgG, which weaken the non-covalent CH3-CH3 interaction in the parental Abs enough to allow dissociation of half-molecules, but at the

same time, strongly favour heterodimerization, thus promoting bispecific IgG endproduct yield and post-exchange stability upon re-oxidation of the hinge disulfides. The document by Gunasekaran K et al (2010), J. Biol. Chem., vol.285 pp.19637-19646 also describes enhancement of antibody Fc heterodimer formation through electrostatic steering effects.

[0005] Except for their dual specificity, it was found that bispecific antibodies generated from human IgGs using cFAE behaved as regular, monospecific human IgGs. Thus, Fc-effector functions of these bispecific human IgGs were preserved and the pharmacokinetics were similar as regular IgG (Labrijn et al. 2013. Proc Natl Acad Sci U S A 110(13):5145-50).

[0006] To predict the efficacy and safety of bispecific IgGs for further development in the clinic, extensive preclinical research is required. The use of animal models is essential for the understanding of the mechanisms of action of therapeutic bispecific human IgGs. Proof-of-concepts for therapeutic antibodies are often established in pre-clinical xenograft models using immunodeficient mice. For many therapeutic concepts, however, the use of surrogate antibodies in rodent disease models such as a mouse model is warranted. The use of mouse or other rodent antibodies enables optimal cross-talk with mouse or other rodent effector cells and proteins, it allows the bispecific antibodies to be assessed in rodent disease models and in addition limits potential immunogenicity which is likely to be a problem for human antibodies applied in rodents, thus allowing long-term treatment of the rodent.

[0007] In the early 1980s it has been shown that fusion of two mouse hybridomas (quadroma) allows for the production of murine hybrid immunoglobulin molecules containing two different binding sites (Milstein C, Cuello AC. Nature. 1983 Oct 6-12;305(5934):537-40). Because the various H and L chains expressed by the quadroma will randomly pair, the yield of the desired bispecific pair of H/L chains was very low. Therefore, another bispecific antibody format involving cross-rodent species IgGs has been developed by Lindhofer et al. (1995 J Immunol 155:219). Using this method, rat and mouse hybridomas producing different antibodies are fused, leading to enrichment of functional bispecific antibodies, because of preferential species-restricted heavy/light chain pairing. The bispecific antibodies are then purified from the mixture that also contains monospecific antibodies. Thus, half of the resulting bispecific antibody is derived from mouse IgG and the other half is derived from rat IgG. Such bispecific antibodies will most likely be immunogenic in mouse or rat as half of the molecule will be foreign.

[0008] Thus, there is a need for surrogate bispecific rodent antibodies for facilitating studies in immunocompetent mouse or other rodent models, in which antibodies will have optimal cross-talk with mouse or other rodent effector cells and proteins, which can be used in rodent disease models and will have limited immunogenicity.

[0009] It is thus an object of the present invention to provide heterodimeric proteins such as bispecific antibodies which can be used in rodent disease models as surrogate for human bispecific antibodies e.g. for investigating mechanisms of action, effector functions, toxicity, unwanted mechanisms and side effects, anti-tumor efficacy, treatment efficacy, T-cell mediated

anti-tumor efficacy, immune modulation, pharmacodynamics and/or pharmacokinetics of the heterodimeric protein, which antibodies have limited immunogenicity in the rodent compared to fully human or humanized antibodies. It is a further object of the present invention to provide rodent heterodimeric proteins which can be used as research tools.

SUMMARY OF THE INVENTION

[0010] The invention is as defined in the claims.

[0011] The present inventors surprisingly found, that the two matching point mutations of the prior art bispecifics were less optimal in driving rodent homodimeric proteins in the direction that favours heterodimerization.

[0012] The inventors found that at least one further amino acid modification on the first homodimer was needed for providing at least 85% of the desired rodent heterodimeric proteins. Accordingly, the present invention relates in one aspect to heterodimeric proteins comprising a first polypeptide of a first homodimeric protein, said first polypeptide comprising a first variable region having a first binding specificity and a first Fc region, said first Fc region comprising a first CH3 domain; and

a second polypeptide of a second homodimeric protein, said second polypeptide comprising a second variable region having a second binding specificity and a second Fc region, said second Fc region comprising a second CH3 domain,

wherein the variable regions may originate from any species and the Fc regions are rodent species IgG Fc regions, and wherein the first CH3 domain comprises a lysine at position 370 and a substitution of the amino acid residue at position 409 to an arginine and the second CH3 domain comprises a substitution of the amino acid residue at position 405 to a leucine, relative to the wild type IgG isotype from said rodent species when using EU numbering index.

[0013] The invention further relates to the use of such heterodimeric proteins and to the method of production of such proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

Figure 1: A) Principle of controlled Fab-arm exchange for the generation of human IgG1-based bispecific antibodies. Alignment of human (*Homo sapiens*; hs) and rodent (*Mus musculus*; mm, *Rattus norvegicus*; rn) sequences of (B) hinge regions and (C) CH3 regions. EU-numbering convention is used to annotate amino acid residues.

Figure 2: Efficiency of controlled Fab-arm exchange (cFAE) as measured by dual-binding ELISA (solid bars) or Hydrophobic Interaction Chromatography (HIC) (open bars) of mixtures

of 2F8-derived and 7D8-derived (A) hslgG1-CH3(mmG1), (B) hslgG1-CH3(mmG2a) or (C) hslgG1-CH3(mmG2b) parental antibodies. The 2F8-derived and 7D8-derived parental antibodies contained the indicated mutations, respectively. Data represent mean $\pm$ SEM. nd = not done. As the presence of aggregates can influence quantification by ELISA (overestimation), the HIC values are considered more accurate.

Figure 3: Exemplary Hydrophobic Interaction Chromatography (HIC) characterization of bispecific antibodies (containing mouse-derived CH3 domains). A) Overlay of HIC profiles of 2F8 and 7D8-derived parental antibodies (black dotted and gray dashed lines, respectively) and the bispecific antibody product (black solid line) generated by controlled Fab-arm exchange. B) Exemplary HIC profiles of bispecific antibodies generated by controlled Fab-arm exchange using the indicated (above and left of panels) combination of parental antibodies. Numbers indicate percentages of bispecific antibody product (middle peaks) and residual 2F8 and 7D8-derived parental antibodies (left and right peaks, respectively). Vertical lines correspond with the retention times of the HIC analyses of the individual parental antibodies.

Figure 4: A) Schematic representation of chimeric IgG1 molecules containing mouse-derived constant regions having the F405L and K409R matching mutations alone or in combination with additional N/R411T and V/T370K mutations, respectively. B) and C) Efficiency of controlled Fab-arm exchange as measured by dual-binding ELISA (solid bars) or HIC (open bars) of mixtures of 2F8-derived and 7D8-derived mmlgG1, mmlgG2a, mmlgG2b or mmlgG3 parental antibodies without mutations (WT) (B; left panel), with the F405L or K409R mutation, respectively, (B; right panel), with the F405L or T/V370K-K409R mutations, respectively, (C; left panel), or with the F405L-N/R411T or T/V370K-K409R mutations, respectively, (C; right panel). Data represent mean $\pm$ SEM. nd = not done.

Figure 5: Exemplary Hydrophobic Interaction Chromatography (HIC) characterization of bispecific antibodies (containing mouse-derived constant regions) generated by controlled Fab-arm exchange using the indicated (above and left of panels) combination of parental antibodies. Numbers indicate percentages of bispecific antibody product (middle peaks) and residual 2F8 and 7D8-derived parental antibodies (left and right peaks, respectively). Vertical lines correspond with the retention times of the HIC analyses of the individual parental antibodies.

Figure 6: A) Isothermal titration calorimetry (ITC) analysis of the binding of mmlgG2b variants to the Z-domain. Isotherms resulting from the binding of Z-domain to mmlgG2b variants. Top panels show the isotherms representing power input over time. In the bottom panels total energy required for equilibration as a function of the molar ratio of injectant (Z-domain) to titrant (IgG). B) MAbSelect Sure separation of mmlgG2b variants and the corresponding bispecific antibody product by 3-step pH gradient elution. Chromatograms show the absorption at 280 nm (solid line; left y-axis) and the pH profile (dashed grey line; right y-axis) during the purification. C) Fractions from each elution step: pH 7.2, 4.0, and 3.4 for the mixed sample containing both parental Abs and the bispecific Ab (dotted hashed trace in panel B) were analyzed by hydrophobic interaction chromatography (HIC). Dashed lines indicate the elution volume of the two parental Abs and the bispecific Ab. Note that the elution volume of the

bispecific Ab is intermediate between that of the parental Abs.

Figure 7: A) Schematic representation of chimeric IgG1 molecules containing rat-derived constant regions having the F405L and K409R matching mutations alone or in combination with additional N/S411T and S/T370K mutations, respectively. B) and C) Efficiency of controlled Fab-arm exchange as measured by dual-binding ELISA (solid bars) or HIC (open bars) of mixtures of 2F8-derived and 7D8-derived rnlGg1, rnlGg2a, rnlGg2b or rnlGg2c parental antibodies without mutations (WT) (B; left panel), with the F405L or K409R mutation, respectively, (B; right panel), with the F405L or S/T370K-K409R mutations, respectively, (C; left panel), or with the F405L-N/S411T or S/T370K-K409R mutations, respectively, (C; right panel). Data represent mean $\pm$ SEM. nd = not done. na = not applicable.

Figure 8: Antibody-Dependent Cellular Phagocytosis (ADCP) of Daudi cells (target cell; T) by bone marrow-derived mouse macrophages (effector cell; E) incubated with a fixed 1 μ g/mL concentration of the indicated (A) mmlGg1-7D8, (B) mmlGg2a-7D8 and (C) mmlGg2b-7D8 variant, with an E:T ratio of 1:1. Data represent mean $\pm$ SD of triple measurements of a representative experiment.

Figure 9: T-cell mediated cytotoxicity of AU565 cells (target cells; T) cocultured for 3 days at 37°C with mouse splenocytes (effector cells; E) in the presence of serial diluted (A) mmlGg1-derived or (B) mmlGg2a-derived bispecific antibodies and parental control antibodies (as indicated), with a E:T ratio of 2:1. Data represents mean of a representative experiment (n=2).

Figure 10: Total antibody plasma concentration over time (A) and plasma clearance rate (B) of mmlGg1-derived or mmlGg2a-derived bispecific antibodies in C57Bl/6J mice. Six groups of mice (3 mice per group) were injected with the indicated antibodies (100 μ g/mice). Blood samples were drawn at different times and plasma concentrations were determined by antigen-specific ELISA. Data represent mean $\pm$ SD.

Figure 11: Evaluation of the *in vivo* efficacy of mmlGg2a-derived 145-2C11xCTA99 bispecific antibodies in a syngeneic xenograft model with gp75-expressing B16/F10 tumor cells. On day 6, when average tumor size was $\sim$ 200-400 mm³, mice were randomized (n = 8 or 9 per group) and treated intravenously with the indicated doses, followed by a second dose at day 8 (A) or 9 (B) (arrowheads indicate treatment days). Data represent mean tumor volumes $\pm$ SEM.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The invention is as defined in the claims.

Definitions

[0016] The term "immunoglobulin" refers to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) chains and one pair of heavy (H) chains, all four inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterized. See for instance Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Briefly, each heavy chain typically is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain "constant region" typically is comprised of three domains, CH1, CH2, and CH3. The heavy chains are inter-connected via disulfide bonds in the so-called "hinge region". Each light chain typically is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region typically is comprised of one domain, CL. The VH and VL regions may be further subdivided into regions of hypervariability (or hypervariable regions which may be hypervariable in sequence and/or form of structurally defined loops), also termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (see also Chothia and Lesk J. Mol. Biol. 196, 901 917 (1987)).

[0017] Herein, the numbering of amino acid residues in the constant region is performed according to the "EU numbering index" as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). The variable regions are numbered according to the IMGT numbering system as described in "IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains": Lefranc, M.-P. et al., Dev. Comp. Immunol., 27, 55-77 (2003)

When used herein, the term "Fab-arm" is used interchangeably with the term "half molecule" and refers to one heavy chain-light chain pair.

[0018] When used herein, the term "Fc region" refers to an antibody region comprising at least the hinge region, the CH2 domains and the CH3 domains. Thus, the Fc region is part of the constant region.

[0019] When used herein, the term "constant region" refers to constant regions of an immunoglobulin, i.e. the regions CH1, hinge, CH2 and CH3.

[0020] The term "antibody" (Ab) in the context of the present invention refers to an immunoglobulin molecule, a fragment of an immunoglobulin molecule, or a derivative of either thereof, which has the ability to specifically bind to an antigen under typical physiological conditions with a half-life of significant periods of time, such as at least about 30 min, at least about 45 min, at least about one hour, at least about two hours, at least about four hours, at least about 8 hours, at least about 12 hours (h), about 24 hours or more, about 48 hours or more, about 3, 4, 5, 6, 7 or more days, etc., or any other relevant functionally-defined period (such as a time sufficient to induce, promote, enhance, and/or modulate a physiological response associated with antibody binding to the antigen and/or time sufficient for the antibody

to recruit an effector activity). The variable regions of the heavy and light chains of the immunoglobulin molecule contain a binding domain that interacts with an antigen. The constant regions of the antibodies (Abs) may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (such as effector cells) and components of the complement system such as C1q, the first component in the classical pathway of complement activation. An antibody may also be a bispecific antibody, diabody, or similar molecule. The term "bispecific antibody" refers to antibodies having specificities for at least two different epitopes, typically non-overlapping epitopes. As indicated above, the term antibody herein, unless otherwise stated or clearly contradicted by the context, includes fragments of an antibody that retain the ability to specifically bind to the antigen. Such fragments may be provided by any known technique, such as enzymatic cleavage, peptide synthesis and recombinant expression techniques. It has been shown that the antigen-binding function of an antibody may be performed by fragments of a full-length antibody, e.g. a F(ab')₂ fragment. It also should be understood that the term antibody, unless specified otherwise, also includes polyclonal antibodies, monoclonal antibodies (mAbs), antibody-like polypeptides, such as chimeric antibodies and humanized antibodies. An antibody as generated can possess any isotype.

[0021] The term "full-length antibody" when used herein, refers to an antibody which contains all heavy and light chain constant and variable domains that are normally found in an antibody of that isotype.

[0022] As used herein, "isotype" refers to the immunoglobulin class (for humans for instance IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM) that is encoded by heavy chain constant region genes, including all allotypes. Isotypes of *Mus musculus* (mm) include for instance IgG1, IgG2a, IgG2b, IgG2c and IgG3. Isotypes of *Rattus norvegicus* (rn) include for instance IgG1, IgG2a, IgG2b, and IgG2c.

[0023] The term "allotype" when used herein, refers to variations in the amino acid sequences which are found naturally within a population.

[0024] The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0025] The term "rodent" as used herein is intended to include all members of the order of Rodentia (Carleton, M. D.; Musser, G. G. (2005). "Order Rodentia", In Wilson, Don E.; Reeder, DeeAnn M. Mammal Species of the World: A Taxonomic and Geographic Reference, Volume 12. JHU Press. pp. 745-752. ISBN 978-0-8018-8221-0.)

[0026] The term "rodent antibody" or "rodent heterodimeric protein" as used herein, is intended to include antibodies or heterodimeric proteins having variable and constant regions derived from a rodent species immunoglobulin sequences, such as e.g. a mouse or a rat. The rodent antibodies or heterodimeric proteins of the invention may include amino acid residues not encoded by rodent immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). The term "rodent antibody" or "rodent heterodimeric protein", as used herein, is also intended to include antibodies or heterodimeric proteins in which CDR sequences or the variable region sequences are derived from another species such as e.g. a human or another mammalian species.

[0027] When used herein, the term "heavy chain antibody" or "heavy-chain antibody" refers to an antibody which consists only of two heavy chains and lacks the two light chains usually found in antibodies. Heavy chain antibodies, which naturally occur in e.g. camelids, can bind antigens despite having only VH domains.

[0028] The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. The epitope may comprise amino acid residues directly involved in the binding (also called immunodominant component of the epitope) and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked by the specific antigen binding peptide (in other words, the amino acid residue is within the footprint of the specific antigen binding peptide).

[0029] As used herein, the term "binding" in the context of the binding of an antibody to a predetermined antigen typically is a binding with an affinity corresponding to a KD of about 10^{-6} M or less, e.g. 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or even less when determined by for instance surface plasmon resonance (SPR) technology in a BIAcore 3000 instrument using the antigen as the ligand and the antibody as the analyte, and binds to the predetermined antigen with an affinity corresponding to a KD that is at least ten-fold lower, such as at least 100 fold lower, for instance at least 1,000 fold lower, such as at least 10,000 fold lower, for instance at least 100,000 fold lower than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen. The amount with which the affinity is lower is dependent on the KD of the antibody, so that when the KD of the antibody is very low (that is, the antibody is highly specific), then the amount with which the affinity for the antigen is lower than the affinity for a non-specific antigen may be at least 10,000 fold. The term "KD" (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction.

[0030] When used herein the term "heterodimeric interaction between the first and second

CH3 regions" refers to the interaction between the first CH3 region and the second CH3 region in a first-CH3/second-CH3 heterodimeric protein.

[0031] When used herein the term "homodimeric interactions of the first and second CH3 regions" refers to the interaction between a first CH3 region and another first CH3 region in a first-CH3/first-CH3 homodimeric protein and the interaction between a second CH3 region and another second CH3 region in a second-CH3/second-CH3 homodimeric protein.

[0032] An "isolated antibody," as used herein, denotes that the material has been removed from its original environment (e.g., the natural environment if it is naturally occurring or the host cell if it is recombinantly expressed). It is also advantageous that the antibodies be in purified form. The term "purified" does not require absolute purity; rather, it is intended as a relative definition, indicating an increase of the antibody concentration relative to the concentration of contaminants in a composition as compared to the starting material.

[0033] The term "host cell", as used herein, is intended to refer to a cell into which an expression vector has been introduced, e.g. an expression vector encoding an antibody of the invention. Recombinant host cells include, for example, transfectomas, such as CHO cells, HEK293 cells, NS/O cells, and lymphocytic cells.

[0034] When used herein, the term "co-expression" of two or more nucleic acid constructs, refers to expression of the two constructs in a single host cell.

[0035] The term "tumor cell protein" refers to a protein located on the cell surface of a tumor cell.

[0036] As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, for instance lymphocytes (such as B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, polymorphonuclear cells, such as neutrophils, granulocytes, mast cells, and basophils. Some effector cells express specific Fc receptors and carry out specific immune functions. In some embodiments, an effector cell is capable of inducing antibody-dependent cellular cytotoxicity (ADCC), such as a natural killer cell, capable of inducing ADCC. In some embodiments, an effector cell may phagocytose a target antigen or target cell.

[0037] The term "reducing conditions" or "reducing environment" refers to a condition or an environment in which a substrate, here a cysteine residue in the hinge region of an antibody, is more likely to become reduced than oxidized.

[0038] The term "disulfide bond isomerization" refers to an exchange of disulfide bonds between different cysteines, i.e., the shuffling of disulfide bonds.

Embodiments of the invention:

[0039] In a first aspect the invention relates to a heterodimeric protein comprising:

a first polypeptide of a first homodimeric protein, said first polypeptide comprising a first variable region having a first binding specificity and a first Fc region, said first Fc region comprising a first CH3 domain; and

a second polypeptide of a second homodimeric protein, said second polypeptide comprising a second variable region having a second binding specificity and a second Fc region, said second Fc region comprising a second CH3 domain,

wherein the variable regions may originate from any species and the Fc regions are rodent species IgG Fc regions, and wherein the first CH3 domain comprises a lysine at position 370 and a substitution of the amino acid residue at position 409 to arginine and the second CH3 domain comprises a substitution of the amino acid residue at position 405 to leucine, relative to the wild type IgG isotype from said rodent species when using EU numbering index for position numbering.

[0040] In one aspect of the invention the heterodimeric protein is a bispecific antibody.

[0041] In one embodiment the Fc regions of the heterodimeric protein are from mouse, rat, hamster, cotton rat or guinea pig origin.

[0042] In one embodiment the Fc regions of the heterodimeric protein are from mouse origin.

[0043] In one embodiment the Fc regions of the heterodimeric protein are from rat origin.

[0044] In one embodiment the Fc regions of the heterodimeric protein are from hamster origin.

[0045] In one embodiment the Fc regions of the heterodimeric protein are from cotton rat origin.

[0046] In one embodiment the Fc regions of the heterodimeric protein are from guinea pig origin.

[0047] In a preferred embodiment the Fc regions of the heterodimeric protein are of mouse or rat origin. In one embodiment they are of *Mus musculus* (*mm*) origin. In another embodiment they are of *Rattus norvegicus* (*rn*) origin. The first and second Fc regions may be obtained from the same species but they may also be of two different species, so that the first Fc region is of one species and the second Fc region is of another species. It is preferred that they are of the same species. In another embodiment the Fc regions are chimeric so that e.g. the first Fc

region may be of two different species. In one embodiment the first and second Fc regions both origin from *Mus Musculus*. In another embodiment the first and second Fc regions both origin from *Rattus Norvegicus*.

[0048] In one aspect of the invention, the variable regions of the heterodimeric protein are of rodent origin. In one embodiment the variable regions are of the same origin as the Fc regions so that the heterodimeric protein is e.g. fully mouse protein, such as fully mouse bispecific antibody. In another embodiment it is a fully rat bispecific antibody. Such bispecific fully mouse or rat antibodies may be useful as surrogate antibodies for use in a mouse or rat model for predicting e.g. the efficacy and safety of human or humanized bispecific antibodies binding the same targets. These bispecific antibodies may also be useful for further preclinical research where the use of animal models is essential for the understanding of the mechanisms of action of therapeutic bispecific human IgGs. Proof-of-concepts for therapeutic antibodies are often established in pre-clinical xenograft models using immune deficient mice. For many therapeutic concepts, however, the use of surrogate antibodies in rodent disease models such as a mouse model is warranted. The use of mouse or other rodent antibodies enables optimal cross-talk with mouse or other rodent effector cells and proteins, and in addition limits potential immunogenicity which is likely to be a problem for human antibodies applied in rodents, thus allowing long-term treatment of the rodent. Thus, in certain applications it is an advantage that the bispecific antibody is fully rodent.

[0049] For other applications it may be an advantage that the variable regions originate from a human antibody. Accordingly, in one embodiment the variable regions of the heterodimeric protein are of human origin. In another embodiment they are humanized rodent variable regions. In yet another embodiment the first and second polypeptides of the heterodimeric protein comprise human variable regions and rodent Fc regions. In yet another embodiment the first and second polypeptides of the heterodimeric protein comprise human variable regions and mouse Fc regions, such as *Mus musculus* derived Fc regions.

[0050] Hereby chimeric heterodimeric proteins are provided which at least recognize human targets but which preferably have no or very limited immunogenicity in rodents. In this way, mechanisms of human heterodimeric protein clinical candidates, such as bispecific antibody clinical candidates may be tested in a mouse or other rodent model. Accordingly, the present invention provides chimeric bispecific antibodies that may comprise human variable regions and rodent constant regions. Using human variable regions which are identical to variable regions of a lead clinical candidate and the rest of the molecule being of rodent origin, the present invention provides a format by which e.g. mechanisms of action, effector functions, toxicity, unwanted mechanisms and/or side effects, anti-tumor efficacy, treatment efficacy, T-cell mediated anti-tumor efficacy, immune modulation, pharmacodynamics and/or pharmacokinetics of said heterodimeric protein may be investigated in a rodent model. This information may be very useful and predictive of the corresponding functions and effects in humans of the fully human antibody counterpart having identical variable regions but human constant regions.

[0051] In some embodiments, the stable heterodimeric protein can be obtained at high yield using the method of the invention on the basis of two homodimeric starting proteins containing only a few, fairly conservative, asymmetrical mutations in the CH3 regions.

[0052] Thus, in one embodiment, the sequences of said first and second CH3 regions contain amino acid substitutions at non-identical positions.

[0053] In one embodiment, said first homodimeric protein has no more than two amino acid substitutions in the CH3 region, and the second homodimeric protein has no more than one amino acid substitution in the CH3 region relative to the relevant wild-type CH3 regions.

[0054] In certain aspects of the disclosure the first CH3 domain of the heterodimeric protein comprise a Lysine (K) at amino acid position 370 and a substitution of the amino acid residue at position 409 selected from Gly, Ala, Val, Ile, Ser, Arg, His, Asp, Asn, Glu, Gln, Trp, Phe, Tyr, and Thr and the second CH3 domain comprises a substitution of the amino acid residue at position 405 selected from Ala, Val, Leu, Ile, Ser, Lys, His, Asp, Asn, Glu, Gln, Trp, Tyr and Thr, relative to wild type mmlgG1 when using EU numbering index.

[0055] According to the invention, the first CH3 domain of the heterodimeric protein comprises a Lysine (K) at amino acid position 370 and a substitution of the amino acid residue at position 409 with an Arginine (R) and the second CH3 domain comprises a substitution of the amino acid residue at position 405 with a Leucine (L). In one embodiment these mutations are the only mutations compared to the relevant wild type CH3 domain.

[0056] In another embodiment the second CH3 domain further comprises an amino acid selected from the group comprising: Gly, Ala, Val, Ile, Ser, His, Asp, Glu, Gln, Trp, Phe, Tyr, Met and Thr at position 411.

[0057] In certain embodiments the second CH3 domain further comprises a Threonine (T) at position 411 so that the second CH3 domain contains a Leucine at position 405 and a Threonine at position 411.

[0058] According to the present invention, the amino acid sequences of the first and second CH3 regions of the homodimeric starting proteins are different from each other and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions.

[0059] In one embodiment, the increased strength of the heterodimeric interaction as compared to each of the homodimeric interactions is due to CH3 modifications other than the introduction of covalent bonds, cysteine residues or charged residues.

[0060] In certain embodiments of the invention the Fc regions of the first and second polypeptides are from the same species. In other embodiments they are of the same species

and isotype. In another embodiment they are of different species and/or different isotypes.

[0061] In an embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG1 isotype, preferably *Mus musculus* IgG1.

[0062] In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG2a isotype, preferably *Mus musculus* IgG2a.

[0063] In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG2b isotype, preferably *Mus musculus* IgG2b.

[0064] In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG2c isotype, preferably *Mus musculus* IgG2c.

[0065] In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG3 isotype, preferably *Mus musculus* IgG3.

[0066] In an embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG1 isotype, preferably *Rattus norvegicus* IgG1.

[0067] In another embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG2a isotype, preferably *Rattus norvegicus* IgG2a.

[0068] In another embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG2b isotype, preferably *Rattus norvegicus* IgG2b.

[0069] In another embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG2c isotype, preferably *Rattus norvegicus* IgG2c.

[0070] In further embodiments, the invention relates to heterodimeric protein wherein the first homodimeric protein and the second homodimeric protein are selected from the group consisting of (i) an antibody (ii) a fusion protein comprising an Fc region, such as an Fc region fused to a receptor, cytokine or hormone, (iii) an antibody conjugated to a prodrug, peptide, drug or a toxin, and (iv) a fusion protein comprising an Fc region conjugated to a prodrug, peptide, drug or a toxin.

[0071] In one embodiment the invention relates to heterodimeric protein wherein the first homodimeric protein and the second homodimeric protein are fusion proteins each comprising an Fc region which further comprises an antigen binding region, such as a Fragment antigen-binding (Fab) region or a single-chain variable fragment (scFv).

[0072] In some embodiments, said first and/or second homodimeric protein comprise, in addition to the Fc region, one or more or all of the other regions of an antibody, i.e. a CH1 region, a VH region, a CL region and/or a VL region. Thus, in one embodiment, said first

homodimeric protein is a full-length antibody. In another embodiment, said second homodimeric protein is a full-length antibody.

[0073] In other embodiments, however, only one of the homodimeric proteins is a full-length antibody and the other homodimeric protein is not a full-length antibody, e.g. an Fc region without a variable region, expressed in conjunction to another protein or peptide sequence like a receptor, cytokine or hormone, or conjugated to a prodrug, peptide, a drug or a toxin.

[0074] In a further embodiment, neither of the homodimeric proteins is a full-length antibody. For example, both homodimeric proteins may be Fc regions that are fused to another protein or peptide sequence like a receptor, cytokine or hormone, or conjugated to a prodrug, peptide, a drug or a toxin.

[0075] In preferred embodiments both the first and second homodimeric proteins are antibodies, preferably full-length antibodies. Preferably the first and second homodimeric proteins bind different epitopes. The epitopes may be on the same or different antigens. Preferably the epitopes are not overlapping so that the heterodimeric protein may bind both epitopes at the same time. In such embodiments, the heterodimeric proteins that are generated are bispecific antibodies. Thus, the bispecific format may be used in many ways to generate desired combinations of bispecific antibodies. In addition to being able of combining antibodies targeting different antigens in a very selective way it can be used to change a desired property, e.g. to increase CDC, by combining two different antibodies targeting the same antigen. Furthermore, it can be used to remove partial agonistic activity of an antagonistic antibody or convert an agonistic antibody into an antagonistic antibody by making a bispecific antibody thereof with an irrelevant (inactive) antibody.

[0076] In a further embodiment, one or both of the homodimeric proteins is glyco-engineered to reduce fucose and thus enhance ADCC, e.g. by addition of compounds to the culture media during antibody production as described in US2009317869 or as described in van Berkel et al. (2010) *Biotechnol. Bioeng.* 105:350 or by using FUT8 knockout cells, e.g. as described in Yamane-Ohnuki et al (2004) *Biotechnol. Bioeng.* 87:614. ADCC may alternatively be optimized using the method described by Umaña et al. (1999) *Nature Biotech* 17:176. In a further embodiment, one or both of the homodimeric proteins has been engineered to enhance complement activation, e.g. as described in Natsume et al. (2009) *Cancer Sci.* 100:2411 or Diebolder et al. (2014) *Science* 343:1260.

[0077] In a further embodiment, one or both of the homodimeric proteins has been engineered to reduce or increase the binding to the neonatal Fc receptor (FcRn) in order to manipulate the serum half-life of the heterodimeric protein. In one embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to P257A or L225Q, T256N and P257A or P307H and M309Q in mmlgG1 which substitution decreases FcRn binding of those homodimers. In another embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to T252L, T254S and T256F in mmlgG1 which substitution increases FcRn binding and serum half-life of those homodimers.

In a further embodiment, one of the homodimeric starting proteins has been engineered to not bind Protein A, thus allowing the separation of the heterodimeric protein from said homodimeric starting protein by passing the product over a protein A column. This may in particular be useful for embodiments wherein an excess of one homodimeric protein is used relative to the other homodimeric protein as starting material. In such embodiments, it may be useful to engineer the homodimeric protein that is in excess so that it loses its ability to bind protein A. Following the heterodimerization reaction, the heterodimeric protein may then be separated from a surplus of unexchanged homodimeric protein by passage over a protein A column. In one embodiment, the one homodimer starting protein is engineered by a substitution at a position corresponding to I253D in mmlgG2b which substitution decreases Protein A binding of that homodimer. In another embodiment the other homodimer is engineered by substituting the amino acid positions corresponding to P307T and Q309L of mmlgG2b which substitutions increases Protein A binding of that other homodimer. In a preferred embodiment, the first homodimer comprises the substitution I253D and the second homodimer comprises the substitutions P307T and Q309L. These substitutions may be made in any homodimeric starting protein such as for example mmlgG2a or mmlgG2b at corresponding amino acid positions. In another embodiment the other homodimer is engineered by substituting the amino acid positions corresponding to T252M and/or T254S of mmlgG1 which substitutions increases Protein A binding of those homodimers.

In a further embodiment, one of the homodimeric proteins is an Fc region or a full-length antibody recognizing a non-relevant epitope or a full-length antibody containing germline-derived sequences that have not undergone somatic hypermutation and do not bind self-antigens. In such an embodiment the heterodimeric protein functions as a monovalent antibody. In another embodiment, both homodimeric proteins comprises the same heavy chain, but only one of the homodimeric proteins contains a light chain which forms a functional antigen-binding site with said heavy chain, whereas the other homodimeric protein contains a non-functional light chain, which does not bind any antigen in combination with said heavy chain. In such an embodiment, the heterodimeric protein functions as a monovalent antibody. Such a non-functional light chain can e.g. be a germline-derived sequence that has not undergone somatic hypermutation and does not bind self-antigens.

[0078] In a further embodiment, one or both of the homodimeric proteins has been engineered to inhibit or abolish the interaction of the proteins according to the invention with Fc Receptors (FcRs) present on a wide range of effector cells, such as monocytes, or with C1q to activate the complement pathway in order to reduce unwanted effects. In one embodiment the homodimer starting proteins are engineered by substitutions at positions corresponding to D265A in mmlgG1, mmlgG2a or mmlgG2b which substitution decrease FcR and C1q binding of those homodimers. In another embodiment, the homodimer starting proteins are engineered by a substitution at a position corresponding to L235E in mmlgG2a which substitution decreases FcR binding of those homodimers. In a further embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to L234A and L235A in mmlgG2a which substitutions decrease FcR binding in those homodimers. In another embodiment, the homodimer starting proteins are engineered by a substitution at a position corresponding to N324D in mmlgG2a which substitution decreases C1q binding of those

homodimers. In a further embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to N297A or E318A in mmlgG2b which substitutions decrease FcR binding and C1q binding in those homodimers. In another embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to E318V, K320A, K320Q, K322A, K322Q or E318A in mmlgG2b which substitutions decrease C1q binding in those homodimers. In a further embodiment, the homodimer starting proteins are engineered by deletion of amino acids at positions corresponding to 233, 234 and 235 in mmlgG2b which deletion decreases FcR binding and C1q binding in those homodimers.

[0079] In one embodiment, the first and/or second homodimeric protein is conjugated to a drug, a prodrug or a toxin or contains an acceptor group for the same. Such acceptor group may e.g. be an unnatural amino acid.

[0080] In another aspect, the invention relates to the use of a heterodimeric protein according to the invention as a research tool for exploring functions of corresponding fully human heterodimeric proteins having similar or identical binding specificity in a rodent model. Thus, the invention in one aspect relates to use of rodent, such as mouse or rat, bispecific antibodies for use in a rodent model wherein the binding part (the variable region or the CDR regions) of the molecule is obtained from a human antibody such as a human bispecific antibody for which further characterization in an animal model is of interest. In other embodiments, the binding part of the molecule may be obtained from another species or may be humanized rodent variable regions or CDR regions.

[0081] In another aspect, the invention relates to the use of a heterodimeric protein according to the invention for exploring functions such as mechanisms of action, effector functions, toxicity, unwanted mechanisms and side effects, anti-tumor efficacy, treatment efficacy, T-cell mediated anti-tumor efficacy, immune modulation, pharmacodynamics and/or pharmacokinetics of said heterodimeric protein. When the heterodimeric protein is a rodent model of a human bispecific antibody this information may be valuable for the characterization of the antibodies and for selection of clinical candidates.

[0082] In yet another aspect, the invention relates to an in vitro method for generating a heterodimeric protein of the invention, said method comprising the following steps:

1. a) providing a first homodimeric protein comprising a first variable region having a first binding specificity, a hinge region, and a first Fc region, said first Fc region comprising a first CH3 domain; and
 2. b) providing a second homodimeric protein comprising a second variable region having a second binding specificity, a hinge region, and a second Fc region, said second Fc region comprising a second CH3 domain,
- wherein the variable regions may originate from any species and the Fc regions are rodent species IgG Fc region, and wherein the first CH3 domain comprises a lysine at position 370 and a substitution of the amino acid residue at position 409 with arginine, and the second CH3 domain comprises a substitution of the amino acid residue at

position 405 with leucine, relative to the wild type IgG1 isotype from said rodent when using EU-index numbering and wherein the sequences of said first and second CH3 regions are different and are such that a heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions,

3. c) incubating said first homodimeric protein together with said second homodimeric protein under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide-bond isomerization, and
4. d) obtaining said heterodimeric protein.

[0083] The sequence of the hinge region of the homodimeric starting proteins may vary. In one embodiment both hinge regions are wild type hinge regions of the same isotype and species as the rest of the Fc regions. Thus, where the Fc region is obtained from mmlgG1, the hinge region is likewise obtained from mmlgG1.

[0084] In many embodiments wherein the first and said second homodimeric proteins are antibodies, said antibodies further comprise light chains. Said light chains may be different, i.e. differ in sequence and each form a functional antigen-binding domain with only one of the heavy chains. In another embodiment, however, said first and second homodimeric proteins are heavy-chain antibodies, which do not need a light chain for antigen binding, see e.g. Hamers-Casterman (1993) Nature 363:446.

[0085] As described above, step c) of the method of the invention comprises incubating said first protein together with said second protein under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide-bond isomerisation. Examples of suitable conditions are given herein. The minimal requirements for the cysteines in the hinge region for undergoing disulfide-bond isomerisation may differ depending on the homodimeric starting proteins, in particular depending on the exact sequence in the hinge region. It is important that the respective homodimeric interactions of said first and second CH3 regions are sufficiently weak to allow cysteines in the hinge region to undergo disulfide-bond isomerisation under the given conditions.

[0086] In one embodiment, the reducing conditions in step c) comprise the addition of a reducing agent, e.g. a reducing agent selected from the group consisting of: 2-mercaptoethylamine (2-MEA), dithiothreitol (DTT), dithioerythritol (DTE), glutathione (GSH), tris(2-carboxyethyl)phosphine (TCEP), L-cysteine and beta-mercapto-ethanol, preferably a reducing agent selected from the group consisting of: 2-mercaptoethylamine, dithiothreitol and tris(2-carboxyethyl)phosphine.

[0087] In a further embodiment, step c) comprises incubation for at least 90 min at a temperature of at least 20°C in the presence of at least 25 mM 2-mercaptoethylamine or in the presence of at least 0.5 mM dithiothreitol. The incubation may be performed at a pH of from 5

to 8, such as at pH 7.0 or at pH 7.4.

[0088] In a further embodiment, step d) comprises restoring the conditions to become nonreducing or less reducing, for example by removal of a reducing agent, e.g. by desalting.

[0089] In some embodiments, the method of the invention yields an antibody product wherein more than 80%, such as more than 90%, e.g. more than 95%, such as more than 99% of the antibody molecules are the desired bispecific antibodies, when measured by HIC analysis. HIC analysis may be performed as described in example 8.

EXAMPLES

[0090]

Table 1: Sequences

SEQ ID	SEQ Desc.	SEQ.
SEQ ID NO: 1	Human IgG1 (Accession number P01857) CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICN VNHKPSNTKVDKRV
SEQ ID NO: 2	Human IgG1 CH2	ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAK
SEQ ID NO: 3	Human kappa (Accession number J00241) CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC
SEQ ID NO: 4	Mouse IgG1 (Accession number J00453) CH1	AKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWN SGSLSSGVHTFPAVLESDLYTLSSSVTVPSRPSETVTCNV AHPASSTKVDKKI
SEQ ID NO: 5	Mouse IgG2a (Accession number V00825) CH1	AKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWN SGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNV AHPASSTKVDKKI
SEQ ID NO: 6	Mouse IgG2b (Accession number V00763) CH1	AKTTPPSVYPLAPGCGDTTGSSVTSGCLVKGYFPEPVTVTWN SGSLSSSVHTFPALLQSGLYTMSSSVTVPSSTWPSQTVTCSV AHPASSTTVDKKL
SEQ ID NO: 7	Mouse IgG3 (Accession number X00915) CH1	ATTTAPSVYPLVPGCDTSGSSVTLGCLVKGYFPEPVTVKWN YGALSSGVRTVSSVLQSGFYSLSSLVTVPSSTWPSQTVICNV AHPASKTELIKRI

SEQ ID	SEQ Desc.	SEQ.
SEQ ID NO: 8	Mouse IgG1 CH2	VPEVSSVFIFPPKPKDVLTTITLTPKVTCVVVDISKDDPEVQF SWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGK EFKCRVNSAAFPAPIEKTISKTK
SEQ ID NO: 9	Mouse IgG2a CH2	APNLLGGPSVFIFPPKIKDVLMISSLPIVTCVVVDVSEDDPD VQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM SGKEFKCKVNNKDLPAPIERTISKPK
SEQ ID NO: 10	Mouse IgG2b CH2	APNLEGGPSVFIFPPNIKDVLMISSLTPKVTCVVVDVSEDDPD VQISWFVNNVEVHTAQTQTHREDYNSTIRVVSTLPIQHQDWM
		SGKEFKCKVNNKDLPSPIERTISKIK
SEQ ID NO: 11	Mouse IgG3 CH2	PGNILGGPSVFIFPPKPKDALMISSLTPKVTCVVVDVSEDDPD VHVSWFVDNKEVHTAWTQPREAQYNSTFRVVSALPIQHQDWM RGKEFKCKVNNKALPAPIERTISKPK
SEQ ID NO: 12	Mouse kappa (Accession number V00807) CL	RADAAPTVSIFPPSSEQLTSGGASVVCFLNMFYPKDINVKWK IDGSEKQNGVLNSWTDQDSKDYMSSTLTTLTKDEYERHNS YTCEATHKTSTSPIVKSENRNEC
SEQ ID NO: 13	Rat IgG1 (Accession number AABR03048905) CH1	AETTAPSVYPLAPGTALKSNSMVTLGCLVKGYFPEPVTVTN SGALSSGVHTFPAVLQSGLYTLTSSVTVPSSTWPSQTVTCNV AHPASSTKVDDKI
SEQ ID NO: 14	Rat IgG2a (Accession number AABR03049560) CH1	AETTAPSVYPLAPGTALKSNSMVTLGCLVKGYFPEPVTVTN SGALSSGVHTFPAVLQSGLYTLTSSVTVPSSTWSSQAVTCNV AHPASSTKVDDKI
SEQ ID NO: 15	Rat IgG2b (Accession number AABR03048905) CH1	AQTTAPSVYPLAPGCGDTTSSVTLGCLVKGYFPEPVTVTN SGALSSDVHTFPAVLQSGLYTLTSSVTSSTWPSQTVTCNV PASSTKVDDKV
SEQ ID NO: 16	Rat IgG2c (Accession number AABR03049912) CH1	ARTTAPSVYPLVPGCSGTSGSLVTLGCLVKGYFPEPVTKWN SGALSSGVHTFPAVLQSGLYTLSSSVTVPSSTWSSQTVTCNV AHPATKSNLIKRI
SEQ ID NO: 17	Rat IgG1 CH2	GSEVSSVFIFPPKPKDVLTTITLTPKVTCVVVDISQDDPEVHF SWFVDDVEVHTAQTRPPEEQFNSTFRSVSELPILHQDWLNGR TFRCKVTSAAFPSPIEKTISKPE
SEQ	Rat IgG2a CH2	

SEQ ID	SEQ Desc.	SEQ.
ID NO: 18		GSEVSSVFIFPPKTKDVLTTITLTPKVTCVVVDISQNDPEVRF SWFIDDVEVHTAQTHAPEKQSNSTLRVSELPIVHRDWLNGK TFKCKVNSGAFFPAPIEKSIKPE
SEQ ID NO: 19	Rat IgG2b CH2	VPELLGGPSVFIFPPKPKDILLISQNAKVTCVVVDVSEEEPD VQFSWFVNNVEVHTAQTPREEQYNSTFRVVSALPIQHQDWM SGKEFKCKVNNKALPSPIEKTISKPK
SEQ ID NO: 20	Rat IgG2c CH2	CDDNLGRPSVFIFPPKPKDILMITLTPKVTCVVVDVSEEEPD VQFSWFVDNVRVFTAQTQPHEEQNLNGTFRVVSTLHIQHQDWM SGKEFKCKVNNKDLPSPIEKTISKPR
SEQ ID NO: 21	Rat kappa (Accession number V01241) CL	RADAAPTVSIFFPPSTEQLATGGASVVCLMNNFYPRDISVKWK IDGTERRDGVLDSDVTDQDSKDYSTYSMSSTLSLTKADYESHNL YTCEVVHKTSSSPVVKSENRNEC
SEQ ID NO: 22	Mouse IGHG1	KTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNS GSLSSGVHTFFPAVLESDLYTLSSSVTVPSSPRPSETVTCNVA HPASSTKVDDKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDV LTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTPR EEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEK TISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPED ITVEWQWNGQPAENYKNTQPIMNNTNGSYFVYSKLVNQSNWE AGNTFTCSVLHEGLHNHHTTEKSLSHSPGK
SEQ ID NO: 23	Mouse IGHG2A	AKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWN SGSLSSGVHTFFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNV AHPASSTKVDDKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIF PPKIKDVLMIISLPIVTCVVVDVSEDDPDVQISWFVNNVEVH TAQTQTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNNKD
		LPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMV TDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLR VEKKNWVERNYSYSCSVVHEGLHNHHTTKSFSRTPGK
SEQ ID NO: 24	Mouse IGHG2B	AKTTPPSVYPLAPGCGDTTGSSVTSGCLVKGYFPEPVTVTWN SGSLSSSVHTFPALLQSGLYTMSSSVTVPSSTWPSQTITCSV AHPASSTTVDDKLEPSGPISTINPCPPCKECKCPAPNLEGG PSVFIFPPNIKDVLMIISLTPKVTCVVVDVSEDDPDVQISWV NNVEVHTAQTPQTHREDYNSTIRVVSTLPIQHQDWMMSGKEFKC KVNNKDLPSPIERTISKIKGLVRAPQVYTLPPPAEQLSRKDV SLTCLVVGFNPGDISVEWTSNGHTEENYKDTAPVLDSDGSYF IYSKLVNMKTSKWEKTDSCNVRHEGLKNYYLKKTISRSPGK
SEQ ID NO: 25	Mouse IGHG2C	KTTAPSVYPLAPVCGGTTGSSVTLGCLVKGYFPEPVTLTWNS GSLSSGVHTFPALLQSGLYTLSSSVTVTSNTWPSQTITCNVA HPASSTKVDDKIEPRVPITQNPCPLKECPPCAAPDLLGGPS VFIFPPKIKDVLMIISLSPMVTCTVVVDVSEDDPDVQISWFVNN VNNVEVHTAQTPQTHREDYNSTIRVVSTLPIQHQDWMMSGKEFKC

SEQ ID	SEQ Desc.	SEQ.
		VEVHTAQTTQTHREDYNSTLRVVSALPTQHQDWMMSGKEFKCKV NNRALPSPIEKTISKPRGPVRAPQVYVLPPPAEEMTKKEFSL TCMITGFLPAELIADVWTSNGRTEQNYKNTATVLDSGDGSYFMY SKLRVQKSTWERGSLFACSVVHEVLHNHLLTKTISRSLGK
SEQ ID NO: 26	Mouse IGHG3	ATTAPSVYPLVPGCSDTSGSSVTLGCLVKGYFPEPVTVKWN YGALSSGVRTVSSVLQSGFYSLSSLVTVPSSSTWPSQTVICNV AHPASKTELIKRIEPRIPKPSTPPGSSCPPGNILGGPSVFIF PPKPKDALMISLTPKVTCVVVDVSEDDPDVHVSWFVDNKEVH TAWTQPREAQYNSTFRVVSALPIQHQDWMRGKEFKCKVNKA LPAPIERTISKPKGRAQTPQVYTIPPPREQMSKKKVSLTCLV TNFFSEAI SVEWERNGELEQDYKNTPPILDSGDGTYFLYSKLT VDTDSWLQGEIFTCSVVHEALHNHHTQKNLSRSPGK
SEQ ID NO: 27	Mouse IGKC	ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKI DGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTCKDEYERHNSY TCEATHKTSTSPIVKSFNRNEC
SEQ ID NO: 28	Rat IGHG1	AETTAPSVYPLAPGTALKSNSMVTLGCLVKGYFPEPVTVTWN SGALSSGVHTFPAVLQSGLYTLTSSVTVPSSSTWPSQTVTCNV AHPASSTKVDDKIVPRNCGGDCPCICTGSEVSSVFIFPPKP KDVLTITLTPKVTCVVVDISQDDPEVHFSWFVDDVEVHTAQ RPPEEQFNSTFRSVSELPILHQDWLNGRTFRCKVTSAAFPSP IEKTISKPEGRTQVPHVYTMSPTEEMTQNEVSITCMVKGFY PPDIYVEWQMNGQPQENYKNTPTMDTDGSYFLYSKLNVKKE KWQQGNTFTCSVLHEGLHNHHTEKSLSHSPGK
SEQ ID NO: 29	Rat IGHG2A	AETTAPSVYPLAPGTALKSNSMVTLGCLVKGYFPEPVTVTWN SGALSSGVHTFPAVLQSGLYTLTSSVTVPSSSTWSSQAVTCNV AHPASSTKVDDKIVPRECNPCGCTGSEVSSVFIFPPKTKDVL TITLTPKVTCVVVDISQNDPEVRFSWFIDDVEVHTAQTHAPE KQSNSTLRSVSELPIVHRDWLNGKTFKCKVNSGAFFAPIEKS ISKPEGTPRGPQVYTMAPPKEEMTQSQVSITCMVKGFYPPDI YTEWKMNQGPQENYKNTPTMDTDGSYFLYSKLNVKKETWQQ GNTFTCSVLHEGLHNHHTEKSLSHSPGK
SEQ ID NO: 30	Rat IGHG2B	AQTTAPSVYPLAPGCGDTSSTVTLGCLVKGYFPEPVTVTWN SGALSSDVHTFPAVLQSGLYTLTSSVTSSSTWPSQTVTCNVAH PASSTKVDDKVERRNGGIGHKCPTCPTCHKCPVPELLGGPSV FIFPPKPKDILLISQNAKVTCVVVDVSEEEPVDVQFSWFVNNV EVHTAQTPREEQYNSTFRVVSALPIQHQDWMMSGKEFKCKVN NKALPSPIEKTISKPKGLVRKPQVYVMGPPTEQLTEQTVSLT CLTSGFLPNDIGVEWTSNGHIEKNYKNTPEVMDSDGSFFMYS KLNVERSRWDSRAPFVCSVVHEGLHNHHHVEKSISRPPGK
SEQ ID NO: 31	Rat IGHG2C	ARTTAPSVYPLVPGCSGTSGSLVTLGCLVKGYFPEPVTVKWN SGALSSGVHTFPAVLQSGLYTLSSSVTVPSSTWSSQTVTCSV AHPATKSNLIKRIEPRRPKPRPPTDICSCDDNLGRPSVFIFP PKPKDILMITLTPKVTCVVVDVSEEEPVDVQFSWFVDNVRVFT AQTPHEEQLNGTFRVVSTLHIQHQDWMMSGKEFKCKVNNKDL PSPIEKTISKPRGKARTPQVYTIPPPREQMSKNKVSLTCMVT SFYPASISVEWERNGELEQDYKNTLPVLDSDESYFLYSKLSV DTDSWMRGDIYTCSVVHEALHNHHTQKNLSRSPGK

SEQ ID	SEQ Desc.	SEQ.
SEQ ID NO: 32	Rat IGKC	RADAAPTVSIFPPSTEQLATGGASVVCLMNNFYPRDISVKWK IDGTERRDGVLDSDVTDQDSKDYMSSTLSLTKADYESHNL YTCEVVHKTSSSPVVKSENRNEC
SEQ ID NO: 33	Mouse IGHG1 CH1	AKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWN SGSLSSGVHTFPAVLESPLYTLSSSVTVPSPPRPSETVTCNV AHPASSTKVDDKI
SEQ ID NO: 34	Mouse IGHG2A CH1	AKTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWN SGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNV AHPASSTKVDDKI
SEQ ID NO: 35	Mouse IGHG2B CH1	AKTTPPSVYPLAPGCGDTTGSSVTSGCLVKGYFPEPVTVTWN SGSLSSSVHTFPALLQSGLYTMSSSVTVPSSTWPSQTVTCSV AHPASSTTVDDKL
SEQ ID NO: 36	Mouse IGHG2c CH1	AKTAPSVYPLAPVCGGTTGSSVTLGCLVKGYFPEPVTLTWN SGSLSSGVHTFPALLQSGLYTLSSSVTVTSNTWPSQTITCNV AHPASSTKVDDKI
SEQ ID NO: 37	Mouse IGHG3 CH1	ATTTAPSVYPLVPGCSDTSGSSVTLGCLVKGYFPEPVTVKWN YGALSSGVRTVSSVLQSGFYSLSSLVTVPSSSTWPSQTVICNV AHPASKTELIKRI
SEQ ID NO: 38	Mouse IGHG1 HINGE	VPRDCGCKPCICT
SEQ ID NO: 39	Mouse IGHG2A HINGE	EPRGPTIKPCPPCKCP
SEQ ID NO: 40	Mouse IGHG2B HINGE	EPSGPISTINPCPPCKECKCP
SEQ ID NO: 41	Mouse IGHG2C HINGE	EPRVPITQNPCPPLKECPPCA
SEQ ID	Mouse IGHG3 HINGE	EPRIKPKSTPPGSSCP

SEQ ID	SEQ Desc.	SEQ.
NO: 42		
SEQ ID NO: 43	Mouse IGHG2c CH2	APDLLGGPSVFIFPPKIKDVLMI SLSPMVT CVVVDVSEDDPD VQISWFVNNVEVHTAQ TQTHREDYNSTLRVVSALPIQH QDWM SGKEFKCKVNNRALPSPIEKTISKPR
SEQ ID NO: 44	Mouse IGHG1 CH3	GRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQ WNGQPAENYKNTQPIMNTNGSYFVYSKLVQKSNWEAGNTFT CSVLHEGLHNHHTEKSLSHSPGK
SEQ ID NO: 45	Mouse IGHG2A CH3	GSVRAPQVYVLPPEEEMTKKQVTLTCMVTD FMPEDIYVEWT NNGKTELNYKNTEPVLDS DGSYFMYSKLRVEKKNWVERNSYS CSVVHEGLHNHHTTKSFSRTPGK
SEQ ID NO: 46	Mouse IGHG2B CH3	GLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNPGDISVEWT SNGHTEENYKDTAPVLDS DGSYFIYSKLVNMKTSKWEKTD SFS CNVRHEGLKNYYLKKTISRSPGK
SEQ ID NO: 47	Mouse IGHG2c CH3	GPVRAPQVYVLPPPAEEMTKKEFSLTCMITGFLPAEIAVDWT SNGRTEQNYKNTATVLDS DGSYFMYSKLRVQKSTWERGSLFA CSVVHEVLHNHLTTKTISRSLGK
SEQ ID NO: 48	Mouse IGHG3 CH3	GRAQTPQVYTIPPPREQMSKKKVSLTCLVTNFFSEAISVEWE RNGELEQDYKNTPPILDS DGTYFLYSKLVTD TDSWLQGEIFT CSVVHEALHNHHTQKNLSRSPGK
SEQ ID NO: 49	Rat IGHG1 CH1	AETTAPSVYPLAPGTALKSNSMVT LGCLVKGYFPEPVTVTWN SGALSSGVHTFPAVLQSGLYTLTSSVTVPSS TWPSQTVTCNV AHPASSTKVDDKKI
SEQ ID NO: 50	Rat IGHG2A CH1	AETTAPSVYPLAPGTALKSNSMVT LGCLVKGYFPEPVTVTWN SGALSSGVHTFPAVLQSGLYTLTSSVTVPSS TWSSQAVTCNV AHPASSTKVDDKKI
SEQ ID NO: 51	Rat IGHG2B CH1	AQTTAPSVYPLAPGCGDTTSSTVTLGCLVKGYFPEPVTVTWN SGALSSDVHTFPAVLQSGLYTLTSSVTSS TWPSQTVTCNV PASSTKVDDKKV
SEQ ID NO: 52	Rat IGHG2c CH1	ARTTAPSVYPLVPGCSGTSGSLVTLGCLVKGYFPEPVTVKWN SGALSSGVHTFPAVLQSGLYTLSSSVTVPSSTWSSQTVTCSV AHPATKSNLIKRI
SEQ	Rat IGHG1 HINGE	VPRNCGGDCKPCICT

SEQ ID	SEQ Desc.	SEQ.
ID NO: 53		
SEQ ID NO: 54	Rat IGHG2A HINGE	VPRECNPCGCT
SEQ ID NO: 55	Rat IGHG2B HINGE	ERRNGGIGHKCPTCPTCHKCP
SEQ ID NO: 56	Rat IGHG2c HINGE	EPRRPKPRPPTDICS
SEQ ID NO: 57	Rat IGHG1 CH3	GRTQVPHVYTMSPKEEMTQNEVSITCMVKGFYPPDIYVEWQ MNGQPQENYKNTPTMDTDGSYFLYSKLNVKKEKWQQGNTFT CSVLHEGLHNHHTKSLSHSPGK
SEQ ID NO: 58	Rat IGHG2A CH3	GTPRGPQVYTMAPPKEEMTQSQVSITCMVKGFYPPDIYTEWK MNGQPQENYKNTPTMDTDGSYFLYSKLNVKKETWQQGNTFT CSVLHEGLHNHHTKSLSHSPGK
SEQ ID NO: 59	Rat IGHG2B CH3	GLVRKPQVYVMGPPTEQLTEQTVSLTCLTSGFLPNDIGVEWT SNGHIEKNYKNTPEVMDSDGSFFMYSKLNVERSRWDSRAPFV CSVVHEGLHNHHVEKSISRPP
SEQ ID NO: 60	Rat IGHG2c CH3	GKARTPQVYTIPPPREQMSKNKVSLTCMVTSFYPAISVEWE RNGELEQDYKNTLPVLDSDESYFLYSKLSVDTDSWMRGDIYT CSVVHEALHNHHTQKNLSRSPGK
SEQ ID NO: 61	Human IgG1 Hinge	EPKSCDKTHTCPPCP
SEQ ID NO: 62	VH HER2-169	QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPG QGLEWMGWLSAYSGNTIYAQKLQGRVTMTTDTSTTTAYMELR SLRSDDTAVYYCARDRIVVRPDYFDYWGQGTLVTVSS
SEQ ID NO: 63	VL HER2-169	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQ APRLLIYDASNRAIGIPARFSGSGSGTDFTLTISSELEPEDFA VYYCQQRSNWPRTEFGQGTKVEIK
SEQ	VH 145-2C11	

SEQ ID	SEQ Desc.	SEQ.
ID NO: 64		EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGMHWVRQAPG RGLSVAYITSSSINIKYADAVKGRFTVSRDNAKNLLFLQMN ILKSEDTAMYYCARFDWDKNYWGQGTMTVTVSS
SEQ ID NO: 65	VL 145-2C11	DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNWYQQKPGK APKLLIYYTNKLADGVPSRFSGSGSGRDSSEFTISSLESEDIG SYYCQQYYNYPWTFGPGTKLEIK
SEQ ID NO: 66	VH CTA99	EVQLQQSGAELVRPGALVKLSCKTSGFNIKDYFLHWVRQRPD QGLEWIGWINPDNGNTVYDPKFQGTASLTADTSSNTVYLQLS GLTSEDTAVYFCTRRDYTEKAALDYWGQGASVIVSS
SEQ ID NO: 67	VL CTA99	AIQMSQSPASLSASVGETVTITCRASGNIYNLAWYQQKQGK SPHLLVYDAKTLADGVPSRFSGSGSGTQYSLKISSLQTEDSG NYYCQHFWSLPFTFGSGTKLEIK
SEQ ID NO: 68	VH b12	QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFIHWVRQAPG QRFEGWGWINPYNGNKEFSAKFQDRVTFTADTSANTAYMELR SLRSADTAVYYCARVGPYSWDDSPQDNYMDVWGKGTTVIVS S
SEQ ID NO: 69	VL b12	EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRAVWYQHKPG QAPRLVIHGVSNRASGISDRFSGSGSGTDFTLTITRVEPEDF ALYYCQVYGASSYTFGQGTKLERK

Example 1: Expression vectors for the expression of human IgG1-2F8, IgG1-7D8 and variants thereof

[0091] The VH and VL coding regions of HuMab 2F8 (WO 02/100348) and HuMab 7D8 (WO 04/035607) were cloned in the expression vector pConGlf (containing the genomic sequence of the human IgGlf allotype constant region (Lonza Biologics)) for the production of the human IgG1 heavy chain and pConKappa (containing the human kappa light chain constant region, Lonza Biologics) for the production of the kappa light chain. Alternatively, in follow-up constructs, vectors were used containing the fully codon-optimized coding regions of the heavy chain in the pcDNA3.3 vector (Invitrogen) or the human kappa light chain of HuMab 2F8 or HuMab 7D8 in the pcDNA3.3 vector. The heavy chain constant region amino acid sequences as used were the following:

Human IgG1 (Accession number P01857) CH1, SEQ ID NO: 1:
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
TVPSSSLGTQTYICNVNHKPSNTKVKDRV

Human IgG1 Hinge sequence is described in **Table 1** (SEQ ID NO: 61) and **Figure 1B**.

Human IgG1 CH2, SEQ ID NO: 2:

ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK

Human IgG1 CH3 sequences are described in **Figure 1C**.

Human kappa (Accession number J00241) CL, SEQ ID NO: 3

RTVAAPSVFIFPPSDEQLKSGTASVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC

[0092] To introduce mutations in the CH3 regions of the antibody heavy chains, i.e. F405L or K409R (EU-numbering convention as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991) is used throughout), Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's recommendations. Alternatively the constructs were fully synthesized or VH regions were cloned in a vector already containing the specific amino acid encoding substitutions.

Example 2: Expression vectors for the expression of chimeric IgG1-2F8-CH3(mmG1), IgG1-2F8-CH3(mmG2a), IgG1-2F8-CH3(mmG2b), IgG1-2F8-CH3(mmG3), IgG1-7D8-CH3(mmG1), IgG1-7D8-CH3(mmG2a), IgG1-7D8-CH3(mmG2b) and IgG1-7D8-CH3(mmG3) constructs and variants thereof

[0093] Vectors containing the coding regions for the human IgG1 CH1-hinge-CH2 regions, mouse CH3 (IgG1, IgG2a, IgG2b or IgG3) regions and human kappa light chains constant regions and the human VH and VL regions of Humab 2F8 and 7D8 were synthesized, fully codon-optimized and inserted in separate (heavy chain and light chain) pcDNA3.3 vectors. The heavy chain constant region sequences as used were the following:

Human IgG1 CH1-Hinge-CH2 sequences (SEQ ID Nos: 1, 61 and 2).

Mouse IgG1, IgG2a, IgG2b and IgG3 CH3 sequences (SEQ ID NOs: 44, 45, 46 and 48).

[0094] To introduce mutations in the CH3 regions of the antibody heavy chains, i.e. F405L, K409R, V370K, T370K, N411T or R411T, Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's recommendations. Alternatively the constructs were fully synthesized or VH regions were cloned in a vector already containing the specific amino acid encoding substitutions.

Example 3: Expression vectors for the expression of chimeric mmlgG1-2F8, mmlgG2a-2F8 mmlgG2b-2F8, mmlgG3-2F8, mmlgG1-7D8, mmlgG2a-7D8 mmlgG2b-7D8 and

mmlgG3-7D8 constructs and variants thereof

[0095] Vectors containing the coding regions for the mouse (*Mus musculus*) IgG1, mouse IgG2a, mouse IgG2b, mouse IgG3 heavy and kappa light chains constant regions and the human VH and VL regions of Humab 2F8 and 7D8 were synthesized, fully codon-optimized and inserted in separate (heavy chain and light chain) pcDNA3.3 vectors. The heavy chain constant region amino acid sequences as used were the following:

Mouse IgG1 (Accession number J00453) CH1, SEQ ID NO: 4

AKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDLYTLSSSVTV
PSSPRPSETVTCNVAHPASSTKVDDKI

Mouse IgG2a (Accession number V00825) CH1, SEQ ID NO: 5

AKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTV
TSSTWPSQSITCNVAHPASSTKVDDKI

Mouse IgG2b (Accession number V00763) CH1, SEQ ID NO: 6

AKTTPPSVYPLAPGCGDTTGSSVTSGCLVKGYFPEPVTVTWNSGSLSSSVHTFPALLQSGLYTMSSSVT
VPSSTWPSQTVTCSVAHPASSTTVDDKL

Mouse IgG3 (Accession number X00915) CH1, SEQ ID NO: 7

ATTTAPSVYPLVPGCSDTSGSSVTLGCLVKGYFPEPVTVKWNYGALSSGVRTVSSVLQSGFYSLSSLVTV
PSSTWPSQTVICNVAHPASKTELIKRI

Mouse IgG1, IgG2a, IgG2b and IgG3 Hinge sequences are described in **Table 1** and **Figure 1B**.

Mouse IgG1 CH2, SEQ ID NO: 8

VPEVSSVFIFPPKPKDVLITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSV
SELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTK

Mouse IgG2a CH2, SEQ ID NO: 9

APNLLGGPSVFIFPPKIKDVLMLISLPIVTCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHREDYNSTL
RVVSALPIQHQQDWMSGKEFKCKVNNKDLPAPIERTISKPK

Mouse IgG2b CH2, SEQ ID NO: 10

APNLEGGPSVFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHREDYNSTI
RVVSTLPIQHQQDWMSGKEFKCKVNNKDLPSPIERTISKIK

Mouse IgG3 CH2, SEQ ID NO: 11

PGNILGGPSVFIFPPKPKDALMISLTPKVTCVVVDVSEDDPDVHVSFVDNKEVHTAWTQPREAQYNST
FRVVSALPIQHQQDWMRGKEFKCKVNNKALPAPIERTISKPK

Mouse IgG1, IgG2a, IgG2b and IgG3 CH3 sequences are described in **Figure 1C**.

Mouse kappa (Accession number V00807) CL, SEQ ID NO: 12

RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKKWKIDGSRQNGVLNSWTDQDSKDYSTYSM
SSTLTLTKEDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

[0096] To introduce mutations in the CH3 regions of the antibody heavy chains, i.e. F405L, K409R, V370K, T370K, N411T or R411T, Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's recommendations. Alternatively the constructs were fully synthesized or VH regions were cloned in a vector already containing the specific amino acid encoding substitutions. Likewise, mutations were introduced in the CH2 regions of the heavy chains, i.e. L234A-L235A, L235E, D265A, N324D, in order to inactivate the antibodies effector functions (Nimmerjahn et al, Immunity 2005, 23:41, Clynes et al, Nature Med 2000, 6:443, Duncan et al, Nature 1988, 332:563, Baudino et al, J Immunol 2008, 180:1948, Li et al, Int Immunopharm 2006, 6:880, Baudino et al, J Immunol 2008, 181: 6664, Nose et al, Eur J Immunol 1989, 19:2179).

Example 4: Expression vectors for the expression of chimeric rnlG1-2F8, rnlG2a-2F8, rnlG2b-2F8, rnlG2c-2F8, rnlG1-7D8, rnlG2a-7D8, rnlG2b-7D8 and rnlG2c-7D8 constructs and variants thereof

[0097] Vectors containing the coding regions for the rat (*Rattus norvegicus*) IgG1, rat IgG2a, rat IgG2b, rat IgG2c heavy and kappa light chains constant regions and the VH and VL regions of Humab 2F8 and 7D8 were synthesized, fully codon-optimized and inserted in separate (heavy chain and light chain) pcDNA3.3 vectors. The heavy chain constant region amino acid sequences as used were the following:

Rat IgG1 (Accession number AABR03048905) CH1, SEQ ID NO: 13

AETTAPSVYPLAPGTALKSNSMVTLGCLVKGYFPEPVTVTWNSGALSSGVHTFPAVLQSGLYTLTSSVTV
PSSTWPSQTVTCNVAHPASSTKVDKKI

Rat IgG2a (Accession number AABR03049560) CH1, SEQ ID NO: 14

AETTAPSVYPLAPGTALKSNSMVTLGCLVKGYFPEPVTVTWNSGALSSGVHTFPAVLQSGLYTLTSSVTV
PSSTWSSQAVTCNVAHPASSTKVDKKI

Rat IgG2b (Accession number AABR03048905) CH1, SEQ ID NO: 15

AQTTAPSVYPLAPGCGDTSSTVTLGCLVKGYFPEPVTVTWNSGALSSDVHTFPAVLQSGLYTLTSSVTS
STWPSQTVTCNVAHPASSTKVDKKV

Rat IgG2c (Accession number AABR03049912) CH1, SEQ ID NO: 16

ARTTAPSVYPLVPGCSGTSGSLVTLGCLVKGYFPEPVTVKWNSGALSSGVHTFPAVLQSGLYTLSSSVTV
PSSTWSSQTVTCSVAHPATKSNLIKRI

Rat IgG1, IgG2a, IgG2b and IgG2c Hinge sequences are described in **Table 1** and **Figure 1B**.

Rat IgG1 CH2, SEQ ID NO: 17

GSEVSSVFIFPPKPKDVLITITLTPKVTCTVVDISQDDPEVHFSWFVDDVEVHTAQTRPPEEQFNSTFRSV
SELPILHQDWLNGRTFRCKVTSAAFPSPIEKTISKPE

Rat IgG2a CH2, SEQ ID NO: 18

GSEVSSVFIFPPKTKDVLITITLTPKVTCTVVDISQNDPEVRFSWFIDDVEVHTAQTHAPEKQSNSTLRSV
SELPILHQDWLNGRTFRCKVTSAAFPSPIEKTISKPE

SELPVVRKDWLNGKIFKCKVNSGAPPAPIEKSDSKPE

Rat IgG2b CH2, SEQ ID NO: 19

VPELLGGPSVFIFPPKPKDILLISQNAKVTCVVVDVSEEEPDVQFSWVNNVEVHTAQTQPREEQYNSTF
RVVSALPIQHQQDWMSGKEFKCKVNNKALPSPIEKTISKPK

Rat IgG2c CH2, SEQ ID NO: 20

CDDNLGRPSVFIFPPKPKDILMITLTPKVTCVVVDVSEEEPDVQFSWFDNVRVFTAQTQPHEEQNLNGTF
RVVSTLHIQHQQDWMSGKEFKCKVNNKDLPSPIEKTISKPR

Rat IgG1, IgG2a, IgG2b and IgG2c CH3 sequences are described in table 1 (SEQ ID NOs 57-60).

Rat kappa (Accession number V01241) CL, SEQ ID NO: 21

RADAAPTVSIFPPSTEQLATGGASVVCLMNNFYPRDISVKWKIDGTERRDGVLDSDVTDQDSKDSTYSM
SSTLSLTKADYESHNLTYTCEVVHKTSSSPVVKSFNRNEC

[0098] To introduce mutations in the CH3 regions of the antibody heavy chains, i.e. F405L, K409R, S370K, T370K, N411T or S411T, Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's recommendations. Alternatively the constructs were fully synthesized or VH regions were cloned in a vector already containing the specific amino acid encoding substitutions.

Example 5: Antibody production by transient expression in Freestyle™ 293-F or Expi293F™ cells

[0099] Antibodies were produced, under serum-free conditions, by co-transfecting relevant heavy and light chain expression vectors in Freestyle™ 293-F cells (LifeTechnologies), using 293fectin™ (LifeTechnologies), according to the manufacturer's instructions. Alternatively, antibodies were produced, under serum-free conditions, by co-transfecting relevant heavy and light chain expression vectors in Expi293F™ cells (LifeTechnologies), using ExpiFectamine™ 293 (LifeTechnologies), according to the manufacturer's instructions.

Example 6: Purification of antibodies

[0100] Antibodies were purified by protein A affinity chromatography. In short, culture supernatant was filtered over 0.2 µm dead-end filters, loaded on 5 mL MabSelect SuRe columns (GE Health Care) and eluted with 0.1 M sodium citrate-NaOH, pH 3. The eluate was immediately neutralized with 2M Tris-HCl, pH 9 and dialyzed overnight to 12.6 mM NaH₂PO₄, 140 mM NaCl, pH 7.4 (B.Braun). Alternatively, subsequent to purification, the eluate was loaded on a HiPrep Desalting column and the antibody was exchanged into 12.6 mM NaH₂PO₄, 140 mM NaCl, pH 7.4 (B.Braun) buffer. After dialysis or exchange of buffer, samples

were sterile filtered over 0.2 µm dead-end filters. Alternatively, antibodies were purified by protein G affinity chromatography.

Purity was determined by SDS-PAGE/CE-SDS and concentration was measured by absorbance at 280 nm. Batches of purified antibody were tested by high-performance size-exclusion chromatography (HP-SEC) for aggregates or degradation products. Purified antibodies were stored at 2-8°C.

Example 7: Dual-binding ELISA to determine efficiency of bispecific antibodies generation

[0101] The presence of bispecific antibodies was tested by determination of bispecific antigen binding using a sandwich enzyme-linked immunosorbent assay (ELISA). ELISA plates (Greiner bio-one, Frickenhausen, Germany) were coated overnight with 2 µg/mL (100 µL/well) of recombinant extracellular domain of EGFR in PBS at 4 °C. The plates were washed once with PBST. Dilution series of the antibody samples (0-1 µg/mL in 3-fold dilutions) in PBST/0.2 % BSA (PBSTB) were transferred to the coated ELISA plates (100 µL/well) and incubated on a plate shaker (300 rpm) for 60 min at room temperature (RT). Samples were discarded and the plates were washed once with PBS/0.05 % Tween 20 (PBST). Next, the plates were incubated on a plate shaker (300 rpm) with 2 µg/mL mouse anti-idiotypic monoclonal antibody 2F2 SAB1.1 (directed against 7D8; Genmab) in PBTB (100 µL/well) for 60 min. The plates were washed once with PBS/0.05 % Tween 20 (PBST). Next, the plates were incubated on a plate shaker (300 rpm) with an HRP-conjugated goat anti-mouse IgG (15G; Jackson ImmunoResearch Laboratories, Westgrove, PA, USA; 1:5.000) in PBSTB (100 µL/well) for 60 min at RT. The plates were washed once with PBS/0.05 % Tween 20 (PBST). ABTS (50 mg/mL; Roche Diagnostics GmbH, Mannheim, Germany) was added (100 µL/well) and incubated protected from light for 30 min at RT. The reaction was stopped with 2% oxalic acid (100 µL/well; Riedel de Haen, Seelze, Germany). After 10 min at RT, absorbance at 405 nm was measured in an ELISA plate reader. Percentage (%) Bispecifics is defined as ((absorbance at 405 nm of the sample)-(absorbance at 405 nm of assay diluent only))/(absorbance at 405 nm of reference bsIgG1-7D8x2F8)-(absorbance at 405 nm of assay diluent only))x100.

Example 8: Hydrophobic interaction chromatography to determine efficiency of bispecific antibody generation

[0102] Hydrophobic interaction chromatography (HIC) can be used to separate both parental antibodies from the bispecific antibody product, based on the inherent hydrophobic properties of the different proteins. Here, High Pressure Liquid Chromatography (HPLC)-HIC was applied to quantify the efficiency of the cFAE reaction. For this, samples of the parental antibodies and the bispecific antibody product, generated by cFAE, were diluted twofold with HIC eluent A (15.4 mM K₂HPO₄, 9.6 mM KH₂PO₄, 1,5 M (NH₄)₂SO₄; pH 7.0) to a final concentration of 1

mg/mL for injection into the HPLC. The IgG molecules with different hydrophobic properties were separated by using a Butyl-NPR, 2.5 μ m, 4.6 x 35 mm HIC-HPLC column (Tosoh Bioscience) with a flow rate of 1 mL/min. 50 μ L was injected and elution was performed with a 12-min gradient of HIC eluent A to HIC eluent B (15.4 mM K_2HPO_4 , 9.6 mM KH_2PO_4 ; pH 7.0) and detection occurred at 280 nm. Empower 3 software (Waters) was used to assign and integrate peak areas. Chromatograms of the parental antibodies were used as reference to identify their position in the end-product. The relative peak areas of the bispecific antibody and residual parental antibodies were used to calculate the efficiency of the cFAE reaction.

Example 9: Generation of bispecific chimeric antibodies, containing mouse-derived CH3 domains that include matching mutations F405L and K409R in combination with additional CH3 mutations by controlled Fab-arm exchange

[0103] Bispecific antibodies were generated in vitro using the DuoBody® platform technology, i.e. controlled Fab-arm exchange (cFAE) or 2-MEA-induced Fab-arm exchange as described in WO 2011/131746; Labrijn et al., PNAS 2013, 110: 5145-50; Gramer et al., MAbs 2013, 5: 962-973 and Labrijn et al., Nat Biotechnol 2014, 9: 2450-63 (summarized in **Figure 1A**). Mixtures of constructs were subjected to controlled Fab-arm exchange (cFAE):

To generate bispecific antibodies, 100 μ g of each parental antibody was mixed and incubated with 75 mM 2-mercaptoethylamine-HCl (2-MEA) in a total volume of 400 μ L PBS (B.Braun, product #3623140) at 31°C for 5 hours. The reduction reaction was stopped when the reducing agent 2-MEA was removed by using Amicon Ultra 0.5 ml centrifugal units (10 kD MWCO, Millipore, product #UFC501096) and washing 4x with 400 μ L PBS by centrifuging 10 min at 13000x g. Samples were collected in a new tube by inverting the filter and centrifuging 2 min at 1000x g. Volumes were adjusted to 200 μ L (when needed) with PBS. The absorbance of 280nm (A280) of bispecific products was measured to determine the final concentration. Samples were stored at 2-8 °C until further analysis. Dual-binding ELISA (as described in **Example 7**) and HIC analysis (as described in **Example 8**) was performed to determine the amount of bispecific product.

[0104] **Figure 2** shows that introduction of the matching mutations F405L and K409R in the chimeric constructs containing mouse IgG1 and mouse IgG2a CH3 regions, i.e. hslgG1-CH3(mmG1) and hslgG1-CH3(mmG2a), had no effect, whereas their introduction in chimeric constructs containing the mouse IgG2b CH3 region, i.e. hslgG1-CH3(mmG2b) enabled cFAE. To find additional CH3 domain mutations for increasing the efficiency of heterodimerization of parental rodent homodimers into the bispecific product, the following constructs were generated:

Table 2: Chimeric constructs containing mouse-derived CH3 domains	
hslgG1-CH3(mmG1)-7D8-K409R	hslgG1-CH3(mmG1)-2F8-F405L
hslgG1-CH3(mmG1)-7D8-M368L-K409R	hslgG1-CH3(mmG1)-2F8-M368L-F405L
hslgG1-CH3(mmG1)-7D8-T370K-K409R	hslgG1-CH3(mmG1)-2F8-T370K-F405L

Table 2: Chimeric constructs containing mouse-derived CH3 domains	
hslgG1-CH3(mmG1)-7D8-K409R-N411T	hslgG1-CH3(mmG1)-2F8-F405L-N411T
hslgG1-CH3(mmG1)-7D8-M368L-T370K-K409R	hslgG1-CH3(mmG1)-2F8-M368L-T370K-F405L
hslgG1-CH3(mmG1)-7D8-M368L-K409R-N411T	hslgG1-CH3(mmG1)-2F8-M368L-F405L-N411T
hslgG1-CH3(mmG1)-7D8-T370K-K409R-N411T	hslgG1-CH3(mmG1)-2F8-T370K-F405L-N411T
hslgG1-CH3(mmG2a)-7D8-K409R	hslgG1-CH3(mmG2a)-2F8-F405L
hslgG1-CH3(mmG2a)-7D8-M368L-K409R	hslgG1-CH3(mmG2a)-2F8-M368L-F405L
hslgG1-CH3(mmG2a)-7D8-T370K-K409R	hslgG1-CH3(mmG2a)-2F8-T370K-F405L
hslgG1-CH3(mmG2a)-7D8-K409R-R411T	hslgG1-CH3(mmG2a)-2F8-F405L-R411T
hslgG1-CH3(mmG2a)-7D8-M368L-T370K-K409R	hslgG1-CH3(mmG2a)-2F8-M368L-T370K-F405L
hslgG1-CH3(mmG2a)-7D8-M368L-K409R-R411T	hslgG1-CH3(mmG2a)-2F8-M368L-F405L-R411T
hslgG1-CH3(mmG2a)-7D8-T370K-K409R-R411T	hslgG1-CH3(mmG2a)-2F8-T370K-F405L-R411T
hslgG1-CH3(mmG2b)-7D8-K409R	hslgG1-CH3(mmG2b)-2F8-F405L
hslgG1-CH3(mmG2b)-7D8-V370K-K409R	hslgG1-CH3(mmG2b)-2F8-V370K-F405L
hslgG1-CH3(mmG2b)-7D8-K409R-N411T	hslgG1-CH3(mmG2b)-2F8-F405L-N411T
hslgG1-CH3(mmG2b)-7D8-V370K-K409R-N411T	hslgG1-CH3(mmG2b)-2F8-V370K-F405L-N411T

[0105] Mixtures of constructs were subjected to cFAE (as described above) and dual-binding ELISA (as described in **Example 7**) and HIC analysis (as described in **Example 8**) was performed to determine the amount of bispecific product.

[0106] **Figure 2A** and **2B** show that, whereas introduction of the M368L mutation alone had no significant effect, introducing mutations T370K and N/R411T, either alone or together, in both parental antibodies, increased the cFAE efficiency in IgG1-CH3(mmG1) (A) and IgG1-CH3(mmG2a) (B) backbones as measured by Dual-binding ELISA and HIC. Introducing M368L together with T370K or N/R411T had no additional effect compared to the introduction of T370K or N/R411T alone. The T370K-F405L x T370K-K409R, T370K-F405L-N/R411T x T370K-K409R-N/R411T, M368L-T370K-F405L x M368L-T370K-K409R and M368L-F405L-N/R411T x M368L-K409R-N/R411T mixtures in the IgG1-CH3(mmG1) (A) and IgG1-

CH3(mmG2a) (B) backbones reached higher cFAE efficiencies. Surprisingly, the highest cFAE efficiencies in IgG1-CH3(mmG1) and IgG1-CH3(mmG2a) backbones, as measured by both dual-binding ELISA and HIC (see also **Figure 3** for exemplary chromatograms), were obtained when the T370K mutation alone was introduced in the K409R parental antibody only, and mixed with parental antibodies containing the F405L mutation only or the combination of F405L with the N/R411T mutation, i.e. F405L x T370K-K409R or F405L-N/R411T x T370K-K409R.

[0107] **Figure 2C** and **Figure 3B** show that although the combination of F405L x K409R in a IgG1-CH3(mmG2b) backbone was already able to undergo cFAE, the introduction of mutations in the equivalent positions could still increase the cFAE efficiency. F405L x V370K-K409R or F405L-N411T x T370K-K409R were the most efficient combinations.

Example 10: Generation of bispecific mouse antibodies, containing mouse-derived constant regions that include matching mutations F405L and K409R alone or in combination with additional CH3 mutations, N/R411T and T/V370K, respectively, by controlled Fab-arm exchange

[0108] To verify that the identified combinations also supported efficient cFAE in the context of an entire mouse constant domain, they were introduced in chimeric constructs containing mouse-derived constant regions (**Figure 4A**). This resulted in the following constructs:

Table 3: Chimeric constructs containing mouse-derived constant regions	
mmlgG1-7D8	mmlgG1-2F8
mmlgG1-7D8-K409R	mmlgG1-2F8-F405L
mmlgG1-7D8-T370K-K409R	mmlgG1-2F8-F405L-N411T
mmlgG2a-7D8	mmlgG2a-2F8
mmlgG2a-7D8-K409R	mmlgG2a-2F8-F405L
mmlgG2a-7D8-T370K-K409R	mmlgG2a-2F8-F405L-R411T
mmlgG2b-7D8	mmlgG2b-2F8
mmlgG2b-7D8-K409R	mmlgG2b-2F8-F405L
mmlgG2b-7D8-T370K-K409R	mmlgG2b-2F8-F405L-N411T
mmlgG3-7D8	mmlgG3-2F8
mmlgG3-7D8-K409R	

[0109] Mixtures of constructs were subjected to cFAE (as described in **Example 9**) and dual-binding ELISA (as described in **Example 7**) and HIC analysis (as described in **Example 8**) was performed to determine the amount of bispecific product.

[0110] **Figure 4** shows that, like for the chimeric constructs containing mouse CH3 domains,

introduction of the matching mutations F405L and K409R in the chimeric constructs containing the entire mouse constant regions, only resulted in sub-efficient cFAE (approx. 80%) in a mmlgG2b backbone (**Figure 4B**) and almost no cFAE in mmlgG1 and mmlgG2a backbones. Surprisingly, the constructs containing the entire constant region of mmlgG3 enabled sub-efficient cFAE (approx. 80%) (**Figure 4A**).

High cFAE efficiencies such as 95-100% and 86-95%, as measured by both dual-binding ELISA and HIC, respectively, were observed in the chimeric constructs containing the entire mouse constant regions when the T/V370K mutation was introduced in the K409R parental antibody (mmlgG1-T370K-K409R, mmlgG2a-T370K-K409R and mmlgG2b-V370K-K409R), and mixed with parental antibodies containing the F405L mutation only (mmlgG1-F405L, mmlgG2a-F405L and mmlgG2b-F405L) or the combination of F405L with the N/R411T mutation (mmlgG1-F405L-N411T, mmlgG2a-F405L-R411T and mmlgG2b-F405L-N411T) (**Figure 4C**). **Figure 5** shows exemplary HIC chromatograms.

Example 11: differential protein A purification to polish bispecific mouse antibodies

[0111] In order to facilitate differential protein A purification for cases where the presence of residual parental antibodies are undesired, a set of mutations in the CH2 domain were designed that disrupt (I253D) or enhance (P307T, Q309L) binding of mmlgG2b to protein A. These mutations were introduced into a set of parental mmlgG2b antibodies to yield mmlgG2b-I253D-F405L and mmlgG2b-P307T-Q309L-K409R and purified proteins were assessed for their ability to bind the Z-domain (an engineered analogue of the IgG-binding domain of protein A) by isothermal titration calorimetry. **Figure 6A** shows that compared to mmlgG2b-F405L, the mmlgG2b-I253D-F405L construct does not bind the Z-domain and mmlgG2b-P307T-Q309L-K409R binds the Z-domain 5-fold tighter.

[0112] Mixtures of constructs were subjected to cFAE (as described in **Example 9**) and purified by protein A affinity chromatography, basically as described in **Example 6**, with the exception that a 3-step pH gradient was used for elution (pH 7.2, pH 4.0, pH 3.4). The flow-through and elution fractions were subsequently analyzed by HIC. **Figure 6B** shows that the mmlgG2b-I253D-F405L parental antibody does not bind to protein A and can only be detected in the flow through. In contrast, mmlgG2b-P307T-Q309L-K409R parental antibody binds tightly to protein A and is mainly detected after the pH 3.4 elution. The bispecific mouse antibody mixture was resolved into three fractions (flowthrough, pH elution 4.0 and pH elution 3.4) which contained, respectively, the residual mmlgG2b-I253D-F405L parental antibody, the bs-mmlgG2b-I253D-F405LxP307T-Q309L-K409R and the residual mmlgG2b-P307T-Q309L-K409R parental antibody (with a trace of bispecific mouse antibody) (**Figure 6B and C**). Thus highly pure bispecific molecules could be obtained using differential protein A purification from a mixture of parental and bispecific mmlgG2b antibodies. Similar results are expected for mmlgG2a antibodies with corresponding mutations.

Example 12: Generation of bispecific rat antibodies, containing rat-derived constant regions that include mutations F405L and K409R alone or in combination with

additional CH3 mutations, N/S411T and S/T370K, respectively, by controlled Fab-arm exchange

[0113] To assess whether these identified combinations of mutations were also relevant for other rodent species, like rats (*Rattus norvegicus*), they were introduced in chimeric constructs containing rat-derived constant regions (**Figure 7A**). This resulted in the following constructs:

Table 4: Chimeric constructs containing rat-derived constant regions	
rnlgG1-7D8	rnlgG1-2F8
rnlgG1-7D8-K409R	rnlgG1-2F8-F405L
rnlgG2a-7D8	rnlgG1-2F8-F405L-N411T
rnlgG2a-7D8-K409R	rnlgG2a-2F8
rnlgG2b-7D8	rnlgG2a-2F8-F405L
rnlgG2b-7D8-K409R	rnlgG2a-2F8-F405L-N411T
rnlgG2b-7D8-S370K-K409R	rnlgG2b-2F8
rnlgG2c-7D8	rnlgG2b-2F8-F405L
rnlgG2c-7D8-K409R	rnlgG2b-2F8-F405L-N411T
rnlgG2c-7D8-T370K-K409R	rnlgG2c-2F8
	rnlgG2c-2F8-F405L
	rnlgG2c-2F8-F405L-S411T

[0114] Mixtures of constructs were subjected to cFAE (as described in **Example 9**) and dual-binding ELISA (as described in **Example 7**) and HIC analysis (as described in **Example 8**) was performed to determine the amount of bispecific product.

[0115] **Figure 7** shows that, introduction of the matching mutations F405L and K409R in the chimeric constructs containing the entire rat constant regions, resulted in highly efficient cFAE (such as 95-100% and 86-95%, as measured by both dual-binding ELISA and HIC, respectively) in the rnlG1, rnlG2a and rnlG2c backbones (**Figure 7B**) and almost no cFAE in rnlG2b backbones. High cFAE efficiencies were observed in the chimeric constructs containing the entire rat constant regions when the K409R parental antibody naturally contained the K370 (rnlG1-K409R and rnlG2a-K409R) or when the S/T370K mutation was introduced in the K409R parental antibody (rnlG2b-S370K-K409R and mmlG2c-T370K-K409R), and mixed with parental antibodies containing the F405L mutation only (rnlG1-F405L, rnlG2a-F405L, rnlG2b-F405L and rnlG2c-F405L) or the combination of F405L with the N/R411T mutation (rnlG1-F405L-N411T, rnlG2a-F405L-N411T, rnlG2b-F405L-N411T and rnlG2c-F405L-S411T) (**Figure 7C**).

Example 13: Culturing bone marrow-derived macrophages

[0116] Bone marrow was isolated from the hind legs of BALB/c mice (Charles Rivers Laboratories) by flushing the femoral and tibial bones using an insulin syringe filled with bone marrow medium (DMEM (Cambrex, product # BE12-709F) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin (Cambrex, product #DE17-603E), 10% (v/v) heat-inactivated donor bovine serum (Gibco, product #10371-029) and 2 mM L-glutamine (Cambrex, product #US17-905C)) until the bones turned white. The cells were collected in a 50 ml tube and the volume was adjusted to 30 ml with bone marrow medium. After passing through a cell strainer, the cells were seeded in petri dishes at a cell concentration of 1.25×10^5 cells/mL in 10 mL. Cells were cultured for 7-8 days at 37°C/ 5% CO₂ in the presence of 50 U/mL M-CSF (PeproTech Inc., product #315-02). After 3-4 days of incubation, 5 ml/dish fresh bone marrow medium with M-CSF was added.

Example 14: Antibody-dependent Cellular Phagocytosis (ADCP)

[0117] On day 7-8, bone-marrow derived macrophages were washed with PBS and harvested by incubating the cells for 10 min at 37°C with 2 mL Versene (Gibco, product #15040-033). Detached cells were collected in a 50 ml tube and Versene was inactivated by adding bone marrow medium. Cells were washed twice and resuspended in 5 mL ADCP working medium (DMEM (Cambrex, product # BE12-917F) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin, 10% (v/v) donor bovine serum, 2 mM L-glutamin) and 2,5% HEPES (Sigma Aldrich, product #H0887)). Cells were counted and the concentration was adjusted to $0,5 \times 10^6$ cells/mL with ADCP working medium. The bone-marrow derived macrophages were seeded into 96-well culture plates (200 µL/well) and allowed to adhere overnight at 37°C, 5% CO₂.

The following day, Daudi cells (ATCC; CCL-213)(cultured in RPMI 1640 (Lonza) supplemented with 10% (v/v) heat-inactivated donor bovine serum, 2 mM L-glutamine (Lonza), 1mM sodium pyruvate (Lonza), 50 IU/mL penicillin, and 50 µg/mL streptomycin) were harvested and labeled for 25 min at 37°C with 0.01 nM Calcein-AM (Molecular probes, product #C-3100), according to the manufacturer's instructions. Labeled Daudi cells were washed twice with ADCP working medium, counted and the concentration was adjusted to 1×10^6 cells/mL. Supernatant from the bone-marrow derived macrophages culture plates was removed and Daudi target cells (T) were added to the macrophage effector cells (E) at an E:T ratio of 1:1 in the presence of a fixed antibody concentration of 1 µg/mL. After a 4 h incubation at 37°C/ 5% CO₂, target cells were washed away with PBS and macrophages were detached with Trypsin-EDTA (Gibco, product #15400-054) and stained with F4/80-PE (AbD Serotec) and CD19-APC (DAKO, Glostrup, Denmark). ADCP was evaluated on a FACSCanto II flow cytometer (BD Biosciences) and defined as percentage of macrophages that had phagocytized. Percentage of phagocytosis was calculated using the following gate settings: the percentage of calcein-AM-positive and CD19-negative cells within the F4/80-positive cells.

[0118] Figure 8 shows that the K409R mutation alone or in combination with T370K (in

mmlgG1 and mmlgG2a) or V370K (in mmlgG2b) had no effect on the phagocytic activity of the mouse antibody backbones. Introduction of point-mutation described in literature to affect effector functions did, in some cases, influence the phagocytic activity. For example, introducing D265A diminished the phagocytic activity in both mmlgG1-T370K-K409R and mmlgG2a-T370K-K409R backbones, and completely inactivated the phagocytic activity in a mmlgG2b-V370K-K409R backbone. Introduction of the L235E and N324D point-mutations, either alone or combined, had no effect on the phagocytic activity in mmlgG2a-T370K-K409R, whereas introducing L235E in combination with D265A completely inactivated the phagocytic activity. The mutation-combinations L234A-L235A, L234A-L235A-D265A or L234A-L235A-N324D also completely inactivated the phagocytic activity in mmlgG2a-T370K-K409R. Introduction of the L234F and K322A point-mutations alone had no effect on the phagocytic activity in mmlgG2b-V370K-K409R. The mutation-combinations L234F-D265A or L234F-D265A-K322A also completely inactivated the phagocytic activity in mmlgG2b-V370K-K409R.

Example 15: Isolation of mouse splenocytes

[0119] Splenocytes were extracted from freshly removed spleens of untreated BALB/cJ mice (Charles River Laboratories) by using the mouse spleen dissociation kit (Miltenyi Biotec, product # 130-095-926) and the GentleMACS dissociator (Miltenyi Biotec, product # 130-093-235) as per the manufacturer's instructions. In short, spleens were added to GentleMACS C tubes (Miltenyi Biotec, product # 130-093-237) each containing 2.4 mL 1x Buffer S, 50 μ L Enzyme D and 15 μ L Enzyme A and placed on the GentleMACS dissociator. GentleMACS programs m_spleen_02 was run, followed by incubation for 15 minutes at 37°C in a water bath. Subsequently GentleMACS programs m_spleen_03 was run, cells were collected and a single cell suspension was made by passing the cells through a cellstrainer. The cell strainer was washed with 2.5 ml 1x buffer S and cells were pelleted at 300 g for 10 min and resuspend in 10 ml PEB buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA). Splenocytes were counted using Burker turk solution and counting chamber.

Example 16 *In vitro* cytotoxicity induced by HER2xmmCD3 bispecific mouse antibodies using mouse splenocytes as effector cells

[0120] CD3 is a protein complex that is associated with the T cell receptor α and β chain expressed on mature T cells. Combination of a CD3 specific antibody Fab-arm with a tumor antigen specific antibody Fab-arm in a bispecific antibody would result in the specific retargeting of T cells to tumor cells, leading to T cell mediated tumor cell lysis. Likewise, CD3 positive T cells could be targeted to other derailed cells in the body, to infected cells or directly to pathogens. To assess whether mouse T-cells could be retargeted to tumor cell lines *in vitro*, various bispecific antibodies were generated using anti-human HER2 (HER2-169) and anti-mouse CD3 ϵ (145-2C11) antibody heavy and light chain variable region sequences.

[0121] HER2-169 (Labrijn et al. 2013. Proc Natl Acad Sci U S A 110(13):5145-50, WO2012143524 (Genmab)) including sequence disclosures.

VH HER2-169, SEQ ID NO: 62:
QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPGQGLEWMGWLSAYSGNTIYAQKLQGR
VTMTTDTSTTTAYMELRSLRSDDTAVYYCARDRIVRPDYFDYWGGQGLTVTVSS

VL HER2-169, SEQ ID NO: 63:
EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTD
FTLTISSELPEDFAVYYCQQRSNWPRTFGQGTKVEIK

[0122] 145-2C11 (Alegre et al. 1995. J Immunol. 155(3): 1544-55) including sequence disclosures.

VH 145-2C11, SEQ ID NO: 64:
EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGMHWVRQAPGRGLESVAYITSSSINIKYADAVKGRFT
VSRDNAKNLLFLQMNILKSEDTAMYYCARFDWDKNYWGQGTMTVTVSS

VL 145-2C11, SEQ ID NO: 65:
DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNWYQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGR
DSSFTISSELEDIGSYYCQQYYNYPWTFGPGTKLEIK

[0123] The VH and VL sequences were introduced into chimeric constructs containing mouse-derived constant regions that contained F405L, T370K-K409R, D265A-F405L-N411T, or D265A-T370K-K409R mutations. This resulted in the following constructs:

Table 5: HER2 and mmCD3 chimeric constructs	
mmlgG1-HER2-169-T370K-K409R	mmlgG1-145-2C11-F405L
mmlgG1-HER2-169-D265A-T370K-K409R	mmlgG1-145-2C11-D265A-F405L-N411T
mmlgG2a-HER2-169-T370K-K409R	mmlgG2a-145-2C11-F405L

[0124] Bispecific antibodies using these constructs (Table 5) and mmlgG1-7D8-T370K-K409R (Table 3) were generated (as described in Example 9) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

[0125] The HER2xmmCD3 bispecific antibodies (and CD20xmmCD3 included as control) were then tested in an *in vitro* cytotoxicity assay using AU565 cells as target cells (T) and mouse splenocytes as effector cells (E). For this, AU565 cells (ATCC; CRL-2351) were cultured to near confluency. Cells were washed twice with PBS, and trypsinized for 5 minutes at 37°C. 12 mL culture medium was added to inactivate trypsin. After being collected and washed, the cells were resuspended in 10 mL culture medium and a single cell suspension was made by

passing the cells through a cellstrainer. 100 μ L of a 4×10^5 cells/mL suspension was added to each well of a 96-well culture plate, and cells were incubated at least 3 hrs at 37°C, 5% CO₂ to allow adherence to the plate. Mouse splenocytes were isolated as described in **Example 15**. Isolated splenocytes were resuspended in culture medium to a final concentration of 8×10^6 cells/mL. Culture medium was removed from the adhered AU565 cells, and replaced with 50 μ L/well 2x concentrated antibody-dilution and 50 μ L/well splenocyte suspension (final ratio E:T = 10:1). Plates were incubated for 3 days at 37°C, 5% CO₂. Supernatants were removed and plates were washed twice with PBS. To each well 150 μ L culture medium and 15 μ L Alamar blue was added. Plates were incubate for 4 hours at 37°C, 5% CO₂, and absorbance (OD 590 nm) was measured (Envision, Perkin Elmer).

[0126] **Figure 9A and B** show that all bispecific HER2xmmCD3 antibodies induced dose-dependent T-cell mediated killing of AU565 cells in an *in vitro* cytotoxicity assay with splenocytes. Killing was critically dependent on the presence of a tumor-targeting Fab-arm (bs-mmlgG1-145-2C11-F405LxHER2-169-T370K-K409R, bs-mmlgG1-145-2C11-D265A-F405L-N411TxHER2-169-D265A-T370K-K409R, bs-mmlgG2a-145-2C11-F405LxHER2-169-T370K-K409R), whereas control antibodies (CD3 monospecific (mmlgG1-145-2C11-F405L, mmlgG1-145-2C11-D265A-F405L-N411T, mmlgG2a-145-2C11-F405L), HER2 monospecific (mmlgG1-HER2-169-T370K-K409R, mmlgG1-HER2-169-D265A-T370K-K409R, mmlgG2a-HER2-169-T370K-K409R), irrelevant monospecific (mmlgG1-7D8-T370K-K409R) and irrelevant bispecific (bs-mmlgG1-145-2C11-F405Lx7D8-T370K-K409R)) did not induce T cell cytotoxicity. As shown in **Figure 9A**, the presence of the D265A mutation in the mmlgG1 backbone, and thus the inability to interact with Fc-receptors, did not impact the potential to induce dose-dependent cytotoxicity of AU565 cells using splenocytes.

Example 17: Pharmacokinetic (PK) analysis of bispecific mouse antibody variants

[0127] The mice in this study were housed in a barrier unit of the Central Laboratory Animal Facility (Utrecht, The Netherlands) and kept in filter-top cages with water and food provided ad libitum. All experiments were approved by the Utrecht University animal ethics committee. 8-11 weeks old, female C57Bl/6J mice (Charles River Laboratories) were injected intravenously with 100 μ g wild type antibody (mmlgG1-2F8 or mmlgG2a-b12), mmlgG1-based bispecific antibody (F405L-N411TxT370K-K409R), mmlgG2a-based bispecific antibody (F405L-R411TxT370K-K409R), mmlgG1-based bispecific antibody with additional D265A mutations or mmlgG2a-based bispecific antibody with additional L234A-L235A mutations, using 3 mice per group. 50 μ L blood samples were collected from the saphenous vein at 10 minutes, 4 hours, 1 day, 2 days, 7 days, 14 days and 21 days after antibody administration. Blood was collected into heparin containing vials and centrifuged for 5 minutes at 10,000 x g. Plasma was stored at -20°C until determination of antibody concentrations.

Antibody concentrations were determined using an EGFR ELISA. For this assay, EGFR-ECDHis (Genmab), coated to 96-well Microton ELISA plates (Greiner, Germany) at a concentration of 2 μ g/mL was used as capturing antigen. Alternatively, antibody concentrations

were determined using a gp120 ELISA, for which, gp120-JRFL (Progenies), coated to 96-well Microton ELISA plates at a concentration of 0.5 µg/mL was used as capturing antigen. After blocking plates with PBS supplemented with 0.2% bovine serum albumin, the plates were washed and samples were added, serially diluted with ELISA buffer (PBS supplemented with 0.05% Tween 20 and 0.2% bovine serum albumin), and incubated on a plate shaker for 1 h at room temperature (RT). Plates were subsequently washed and incubated with HRP-labeled goat anti-mouse IgG immunoglobulin (#115-036-072, Jackson, West Grace, PA) and developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Roche, Mannheim, Germany). The reaction was stopped after 30 min by adding 2% oxalic acid to the wells. Absorbance was measured in a microplate reader (Biotek, Winooski, VT) at 405 nm.

Plasma clearance rates (mL/day/kg) were calculated based on the area under the curve (AUC), according to the following equation:

$$\text{Plasma clearance} = \text{Dose } (\mu\text{g/kg}) / \text{AUC } (\mu\text{g/mL/day})$$

Data analysis was performed using Graphpad prism software.

[0128] Figure 10 A shows total antibody plasma concentrations over time. The shape of the PK profiles is identical in all groups. Figure 10B shows plasma clearance rates. No statistically significant differences in the clearance rates were observed between all groups. These data indicate that introduction of the matching mutations F405L-N411T and T370K-K409R in mmlgG1 or F405L-R411T and T370K-K409R in mmlgG2a, nor the D265A mutation in mmlgG1 or the L234A-L235A mutations in mmlgG2a had any effect on the pharmacokinetic properties.

Example 18: *in vivo* anti-tumor efficacy of bispecific mouse antibody variants in T-cell redirection model

[0129] To assess whether mouse T-cells could be redirected to tumor cells *in vivo*, various bispecific antibodies were generated using anti-gp75 (CTA99; tumor-specific arm), anti-gp120 (b12; irrelevant arm) and anti-mouse CD3ε (145-2C11) antibody heavy and light chain variable region sequences.

[0130] CTA99 (WO 2009114585 A1) including sequence disclosures.

VH CTA99, SEQ ID NO: **66**:

EVQLQQSGAELVRPGALVKLSCKTSGFNIKDYFLHWVRQRPDQGLEWIGWINPDNGNTVYDPKFQGTASLTADTSSNTVYLQLSGLTSEDVAVYFCTRRDYTYEKAALDYWGQGASVIVSS

VL CTA99, SEQ ID NO: **67**:

AIQMSQSPASLSASVGETVTITCRASGNIYNYLAWYQQKQKSPHLLVYDAKTLADGVPSRFSGSGSGTQYSLKISSLQTEDSGNYCQHFWSLPFTFGSGTKLEIK

[0131] b12 (Barbas, CF. J Mol Biol. 1993 Apr 5;230(3):812-23.) including sequence disclosures.

VH b12, SEQ ID NO: **68**:
QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFWIHWVRQAPGQRFQWVGWINPYNGNKEFSAKFQD
RVTFTADTSANTAYMELRSLRSADTAVYYCARVGPYSWDDSPQDNYYMDVWGKGTTVIVSS

VL b12, SEQ ID NO: **69**:
EIVLTQSPGTLSPGERATFSCRSSHISIRSRVAWYQHKPGQAPRLVIHGVSNRASGISDRFSGSGSG
TDFTLTITRVEPEDFALYYCQVYGASSYTFGQGTKLERK

[0132] The VH and VL sequences were introduced into chimeric constructs containing mouse IgG2a-derived constant regions that contained L234A-L235A-T370K-K409R or L234A-L235A-F405L-R411T mutations. This resulted in the following constructs:

Table 6: gp75 and mmCD3 chimeric constructs	
mmlgG2a-CTA99-L234A-L235A-T370K-K409R	mmlgG2a-145-2C11-L234A-L235A-F405L-R411T
mmlgG2a-b12-L234A-L235A-T370K-K409R	mmlgG2a-b12-L234A-L235A-F405L-R411T

[0133] Bispecific antibodies using these constructs (Table 6) and mmlgG1-7D8-T370K-K409R (Table 3) were generated (as described in Example 9) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

[0134] The mice in this study were housed in a barrier unit of the Central Laboratory Animal Facility (Utrecht, The Netherlands) and kept in filter-top cages with water and food provided ad libitum. All experiments were approved by the Utrecht University animal ethics committee. 8-11 weeks old, female C57Bl/6J mice (Charles River Laboratories) were injected subcutaneously into the right flank with 1 x 10⁵ B16/F10 cells (ATCC; CRL-6475) in PBS. The B16/F10 cells were previously cultured to 70% confluency in IMDM medium supplemented with HEPES, L-Glutamine (Lonza, cat #: BE12-722F), 10% Donor Bovine Serum with Iron (Life Technologies, 10371-029) and Pen/Strep (Lonza, cat DE17-603E).

[0135] The mice received intravenous injections of mmlgG2a-derived 145-2C11xCTA99 bispecific antibodies (bs-mmlgG2a-L234A-L235A-F405L-R411TxL234A-L235A-T370K-K409R) or their monovalent counterparts (145-2C11xb12 and b12xCTA99) at 6 and 8 (or 9) days after tumor cell injection. The antibodies were dosed at concentrations ranging from 0.005 mg/kg to 5 mg/kg. Tumor-size was subsequently followed over time.

[0136] Figure 11 shows that whereas doses of 0.005 mg/kg and 0.05 mg/kg mmlgG2a-derived 145-2C11xCTA99 bispecific antibody did not inhibit B16/F10 tumor growth, doses of 0.5 mg/kg or 5 mg/kg mmlgG2a-derived 145-2C11xCTA99 bispecific antibody could inhibit tumor growth, compared to equal doses of their monovalent counterparts (145-2C11xb12 and b12xCTA99).

SEQUENCE LISTING

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Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser
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Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser
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Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr
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Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp

35

40

45

Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
50 55 60

Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
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Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
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20 25 30

Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
35 40 45

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
50 55 60

Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
65 70 75 80

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
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Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
100 105

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Ser	Ser	Ser	Val	Thr	Val	Pro	Ser	Ser	Pro	Arg	Pro	Ser	Glu	Thr	Val		
65					70					75					80		
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Phe	Pro	Glu	Pro	Val	Thr	Leu	Thr	Trp	Asn	Ser	Gly	Ser	Leu	Ser	Ser
		35				40						45			

Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Asp	Leu	Tyr	Thr	Leu
	50					55					60				

Ser	Ser	Ser	Val	Thr	Val	Thr	Ser	Ser	Thr	Trp	Pro	Ser	Gln	Ser	Ile
65					70					75					80

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35 40 45

Gly Val Arg Thr Val Ser Ser Val Leu Gln Ser Gly Phe Tyr Ser Leu
50 55 60

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85 90 95

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35 40 45

Val Glu Val His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn
50 55 60

Ser Thr Phe Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp
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Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys
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35 40 45

Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
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Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
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Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
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35 40 45

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35 40 45

Val Asp Asn Lys Glu Val His Thr Ala Trp Thr Gln Pro Arg Glu Ala
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Gln Tyr Asn Ser Thr Phe Arg Val Val Ser Ala Leu Pro Ile Gln His
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35 40 45

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Gln Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser
50 55 60

Thr Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu
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35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
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35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
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35 40 45

Asp Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

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35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

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 35 40 45

Val Glu Val His Thr Ala Gln Thr Arg Pro Pro Glu Glu Gln Phe Asn
 50 55 60

Ser Thr Phe Arg Ser Val Ser Glu Leu Pro Ile Leu His Gln Asp Trp
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 35 40 45

Val Glu Val His Thr Ala Gln Thr His Ala Pro Glu Lys Gln Ser Asn
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Ser Thr Leu Arg Ser Val Ser Glu Leu Pro Ile Val His Arg Asp Trp
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35 40 45

Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Pro Arg Glu Glu
50 55 60

Gln Tyr Asn Ser Thr Phe Arg Val Val Ser Ala Leu Pro Ile Gln His
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50 55 60

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50 55 60

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Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys Ile
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Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu
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Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr
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Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser
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Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro
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1		5		10		15											
Asp	Thr	Thr	Gly	Ser	Ser	Val	Thr	Leu	Gly	Cys	Leu	Val	Lys	Gly	Tyr		
			20					25					30				
Phe	Pro	Glu	Pro	Val	Thr	Leu	Thr	Trp	Asn	Ser	Gly	Ser	Leu	Ser	Ser		
		35					40					45					
Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Asp	Leu	Tyr	Thr	Leu		
	50					55					60						
Ser	Ser	Ser	Val	Thr	Val	Thr	Ser	Ser	Thr	Trp	Pro	Ser	Gln	Ser	Ile		
65					70					75					80		
Thr	Cys	Asn	Val	Ala	His	Pro	Ala	Ser	Ser	Thr	Lys	Val	Asp	Lys	Lys		
				85					90					95			
Ile	Glu	Pro	Arg	Gly	Pro	Thr	Ile	Lys	Pro	Cys	Pro	Pro	Cys	Lys	Cys		
			100					105					110				
Pro	Ala	Pro	Asn	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro		
		115					120					125					
Lys	Ile	Lys	Asp	Val	Leu	Met	Ile	Ser	Leu	Ser	Pro	Ile	Val	Thr	Cys		
	130					135					140						
Val	Val	Val	Asp	Val	Ser	Glu	Asp	Asp	Pro	Asp	Val	Gln	Ile	Ser	Trp		
145					150					155					160		
Phe	Val	Asn	Asn	Val	Glu	Val	His	Thr	Ala	Gln	Thr	Gln	Thr	His	Arg		
				165						170					175		
Glu	Asp	Tyr	Asn	Ser	Thr	Leu	Arg	Val	Val	Ser	Ala	Leu	Pro	Ile	Gln		
			180					185					190				
His	Gln	Asp	Trp	Met	Ser	Gly	Lys	Glu	Phe	Lys	Cys	Lys	Val	Asn	Asn		
		195					200					205					
Lys	Asp	Leu	Pro	Ala	Pro	Ile	Glu	Arg	Thr	Ile	Ser	Lys	Pro	Lys	Gly		
	210					215					220						
Ser	Val	Arg	Ala	Pro	Gln	Val	Tyr	Val	Leu	Pro	Pro	Pro	Glu	Glu	Glu		
225					230					235					240		
Met	Thr	Lys	Lys	Gln	Val	Thr	Leu	Thr	Cys	Met	Val	Thr	Asp	Phe	Met		
				245					250					255			
Pro	Glu	Asp	Ile	Tyr	Val	Glu	Trp	Thr	Asn	Asn	Gly	Lys	Thr	Glu	Leu		
			260					265					270				
Asn	Tyr	Lys	Asn	Thr	Glu	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Tyr	Phe		
		275					280					285					
Met	Tyr	Ser	Lys	Leu	Arg	Val	Glu	Lys	Lys	Asn	Trp	Val	Glu	Arg	Asn		

Asp Tyr Ser Asp Leu Arg Val Glu Asp Asp Asn Asp Val Glu Arg Asn
290 295 300

Ser Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr
305 310 315 320

Thr Lys Ser Phe Ser Arg Thr Pro Gly Lys
325 330

<210> 24

<211> 336

<212> PRT

<213> Mus musculus

<400> 24

Ala Lys Thr Thr Pro Pro Ser Val Tyr Pro Leu Ala Pro Gly Cys Gly
1 5 10 15

Asp Thr Thr Gly Ser Ser Val Thr Ser Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser
35 40 45

Ser Val His Thr Phe Pro Ala Leu Leu Gln Ser Gly Leu Tyr Thr Met
50 55 60

Ser Ser Ser Val Thr Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val
65 70 75 80

Thr Cys Ser Val Ala His Pro Ala Ser Ser Thr Thr Val Asp Lys Lys
85 90 95

Leu Glu Pro Ser Gly Pro Ile Ser Thr Ile Asn Pro Cys Pro Pro Cys
100 105 110

Lys Glu Cys His Lys Cys Pro Ala Pro Asn Leu Glu Gly Gly Pro Ser
115 120 125

Val Phe Ile Phe Pro Pro Asn Ile Lys Asp Val Leu Met Ile Ser Leu
130 135 140

Thr Pro Lys Val Thr Cys Val Val Val Asp Val Ser Glu Asp Asp Pro
145 150 155 160

Asp Val Gln Ile Ser Trp Phe Val Asn Asn Val Glu Val His Thr Ala
165 170 175

Gln Thr Gln Thr His Arg Glu Asp Tyr Asn Ser Thr Ile Arg Val Val
180 185 190

Ser Thr Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe
195 200 205

Lys Cys Lys Val Asn Asn Lys Asn Leu Pro Ser Pro Ile Glu Arg Thr

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Lys  Lys  Lys  Val  Asn  Asn  Lys  Asp  Asn  Lys  Ser  Lys  Lys  Lys  Lys  Lys
210                                215                                220

Ile  Ser  Lys  Ile  Lys  Gly  Leu  Val  Arg  Ala  Pro  Gln  Val  Tyr  Thr  Leu
225                                230                                235                                240

Pro  Pro  Pro  Ala  Glu  Gln  Leu  Ser  Arg  Lys  Asp  Val  Ser  Leu  Thr  Cys
                                245                                250                                255

Leu  Val  Val  Gly  Phe  Asn  Pro  Gly  Asp  Ile  Ser  Val  Glu  Trp  Thr  Ser
                                260                                265                                270

Asn  Gly  His  Thr  Glu  Glu  Asn  Tyr  Lys  Asp  Thr  Ala  Pro  Val  Leu  Asp
                                275                                280                                285

Ser  Asp  Gly  Ser  Tyr  Phe  Ile  Tyr  Ser  Lys  Leu  Asn  Met  Lys  Thr  Ser
                                290                                295                                300

Lys  Trp  Glu  Lys  Thr  Asp  Ser  Phe  Ser  Cys  Asn  Val  Arg  His  Glu  Gly
305                                310                                315                                320

Leu  Lys  Asn  Tyr  Tyr  Leu  Lys  Lys  Thr  Ile  Ser  Arg  Ser  Pro  Gly  Lys
                                325                                330                                335

<210> 25
<211> 334
<212> PRT
<213> Mus musculus

<400> 25
Lys  Thr  Thr  Ala  Pro  Ser  Val  Tyr  Pro  Leu  Ala  Pro  Val  Cys  Gly  Gly
1                                5                                10                                15

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

Pro  Glu  Pro  Val  Thr  Leu  Thr  Trp  Asn  Ser  Gly  Ser  Leu  Ser  Ser  Gly
                                35                                40                                45

Val  His  Thr  Phe  Pro  Ala  Leu  Leu  Gln  Ser  Gly  Leu  Tyr  Thr  Leu  Ser
                                50                                55                                60

Ser  Ser  Val  Thr  Val  Thr  Ser  Asn  Thr  Trp  Pro  Ser  Gln  Thr  Ile  Thr
65                                70                                75                                80

Cys  Asn  Val  Ala  His  Pro  Ala  Ser  Ser  Thr  Lys  Val  Asp  Lys  Lys  Ile
                                85                                90                                95

Glu  Pro  Arg  Val  Pro  Ile  Thr  Gln  Asn  Pro  Cys  Pro  Pro  Leu  Lys  Glu
                                100                                105                                110

Cys  Pro  Pro  Cys  Ala  Ala  Pro  Asp  Leu  Leu  Gly  Gly  Pro  Ser  Val  Phe
115                                120                                125

Ile  Phe  Pro  Pro  Lys  Ile  Lys  Asp  Val  Leu  Met  Ile  Ser  Leu  Ser  Pro
130                                135                                140

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Met Val Thr Cys Val Val Val Asp Val Ser Glu Asp Asp Pro Asp Val
145 150 155 160

Gln Ile Ser Trp Phe Val Asn Asn Val Glu Val His Thr Ala Gln Thr
165 170 175

Gln Thr His Arg Glu Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala
180 185 190

Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys
195 200 205

Lys Val Asn Asn Arg Ala Leu Pro Ser Pro Ile Glu Lys Thr Ile Ser
210 215 220

Lys Pro Arg Gly Pro Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro
225 230 235 240

Pro Ala Glu Glu Met Thr Lys Lys Glu Phe Ser Leu Thr Cys Met Ile
245 250 255

Thr Gly Phe Leu Pro Ala Glu Ile Ala Val Asp Trp Thr Ser Asn Gly
260 265 270

Arg Thr Glu Gln Asn Tyr Lys Asn Thr Ala Thr Val Leu Asp Ser Asp
275 280 285

Gly Ser Tyr Phe Met Tyr Ser Lys Leu Arg Val Gln Lys Ser Thr Trp
290 295 300

Glu Arg Gly Ser Leu Phe Ala Cys Ser Val Val His Glu Val Leu His
305 310 315 320

Asn His Leu Thr Thr Lys Thr Ile Ser Arg Ser Leu Gly Lys
325 330

<210> 26

<211> 330

<212> PRT

<213> Mus musculus

<400> 26

Ala Thr Thr Thr Ala Pro Ser Val Tyr Pro Leu Val Pro Gly Cys Ser
1 5 10 15

Asp Thr Ser Gly Ser Ser Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Lys Trp Asn Tyr Gly Ala Leu Ser Ser
35 40 45

Gly Val Arg Thr Val Ser Ser Val Leu Gln Ser Gly Phe Tyr Ser Leu
50 55 60

Ser Ser Leu Val Thr Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val
65 70 75 80

Ile Cys Asn Val Ala His Pro Ala Ser Lys Thr Glu Leu Ile Lys Arg
85 90 95

Ile Glu Pro Arg Ile Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Cys
100 105 110

Pro Pro Gly Asn Ile Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro
115 120 125

Lys Pro Lys Asp Ala Leu Met Ile Ser Leu Thr Pro Lys Val Thr Cys
130 135 140

Val Val Val Asp Val Ser Glu Asp Asp Pro Asp Val His Val Ser Trp
145 150 155 160

Phe Val Asp Asn Lys Glu Val His Thr Ala Trp Thr Gln Pro Arg Glu
165 170 175

Ala Gln Tyr Asn Ser Thr Phe Arg Val Val Ser Ala Leu Pro Ile Gln
180 185 190

His Gln Asp Trp Met Arg Gly Lys Glu Phe Lys Cys Lys Val Asn Asn
195 200 205

Lys Ala Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly
210 215 220

Arg Ala Gln Thr Pro Gln Val Tyr Thr Ile Pro Pro Pro Arg Glu Gln
225 230 235 240

Met Ser Lys Lys Lys Val Ser Leu Thr Cys Leu Val Thr Asn Phe Phe
245 250 255

Ser Glu Ala Ile Ser Val Glu Trp Glu Arg Asn Gly Glu Leu Glu Gln
260 265 270

Asp Tyr Lys Asn Thr Pro Pro Ile Leu Asp Ser Asp Gly Thr Tyr Phe
275 280 285

Leu Tyr Ser Lys Leu Thr Val Asp Thr Asp Ser Trp Leu Gln Gly Glu
290 295 300

Ile Phe Thr Cys Ser Val Val His Glu Ala Leu His Asn His His Thr
305 310 315 320

Gln Lys Asn Leu Ser Arg Ser Pro Gly Lys
325 330

<210> 27

<211> 106

<212> PRT

<213> Mus musculus

<400> 27

Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln
1 5 10 15

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

Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln
35 40 45

Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr
50 55 60

Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg
65 70 75 80

His Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro
85 90 95

Ile Val Lys Ser Phe Asn Arg Asn Glu Cys
100 105

<210> 28

<211> 326

<212> PRT

<213> Rattus Norvegicus

<400> 28

Ala Glu Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Gly Thr Ala
1 5 10 15

Leu Lys Ser Asn Ser Met Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ala Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Thr Ser Ser Val Thr Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val
65 70 75 80

Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
85 90 95

Ile Val Pro Arg Asn Cys Gly Gly Asp Cys Lys Pro Cys Ile Cys Thr
100 105 110

Gly Ser Glu Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp
115 120 125

Val Leu Thr Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp
130 135 140

Ile Ser Glu Asn Asn Pro Glu Val His Phe Ser Thr Phe Val Asn Asn

Ile Ser Gln Asp Asp Pro Glu Val His Phe Ser Trp Phe Val Asp Asp
145 150 155 160

Val Glu Val His Thr Ala Gln Thr Arg Pro Pro Glu Glu Gln Phe Asn
165 170 175

Ser Thr Phe Arg Ser Val Ser Glu Leu Pro Ile Leu His Gln Asp Trp
180 185 190

Leu Asn Gly Arg Thr Phe Arg Cys Lys Val Thr Ser Ala Ala Phe Pro
195 200 205

Ser Pro Ile Glu Lys Thr Ile Ser Lys Pro Glu Gly Arg Thr Gln Val
210 215 220

Pro His Val Tyr Thr Met Ser Pro Thr Lys Glu Glu Met Thr Gln Asn
225 230 235 240

Glu Val Ser Ile Thr Cys Met Val Lys Gly Phe Tyr Pro Pro Asp Ile
245 250 255

Tyr Val Glu Trp Gln Met Asn Gly Gln Pro Gln Glu Asn Tyr Lys Asn
260 265 270

Thr Pro Pro Thr Met Asp Thr Asp Gly Ser Tyr Phe Leu Tyr Ser Lys
275 280 285

Leu Asn Val Lys Lys Glu Lys Trp Gln Gln Gly Asn Thr Phe Thr Cys
290 295 300

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

Ser His Ser Pro Gly Lys
325

<210> 29

<211> 322

<212> PRT

<213> Rattus Norvegicus

<400> 29

Ala Glu Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Gly Thr Ala
1 5 10 15

Leu Lys Ser Asn Ser Met Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ala Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Thr Ser Ser Val Thr Val Pro Ser Ser Thr Trp Ser Ser Gln Ala Val
65 70 75 80

Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
85 90 95

Ile Val Pro Arg Glu Cys Asn Pro Cys Gly Cys Thr Gly Ser Glu Val
100 105 110

Ser Ser Val Phe Ile Phe Pro Pro Lys Thr Lys Asp Val Leu Thr Ile
115 120 125

Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Gln Asn
130 135 140

Asp Pro Glu Val Arg Phe Ser Trp Phe Ile Asp Asp Val Glu Val His
145 150 155 160

Thr Ala Gln Thr His Ala Pro Glu Lys Gln Ser Asn Ser Thr Leu Arg
165 170 175

Ser Val Ser Glu Leu Pro Ile Val His Arg Asp Trp Leu Asn Gly Lys
180 185 190

Thr Phe Lys Cys Lys Val Asn Ser Gly Ala Phe Pro Ala Pro Ile Glu
195 200 205

Lys Ser Ile Ser Lys Pro Glu Gly Thr Pro Arg Gly Pro Gln Val Tyr
210 215 220

Thr Met Ala Pro Pro Lys Glu Glu Met Thr Gln Ser Gln Val Ser Ile
225 230 235 240

Thr Cys Met Val Lys Gly Phe Tyr Pro Pro Asp Ile Tyr Thr Glu Trp
245 250 255

Lys Met Asn Gly Gln Pro Gln Glu Asn Tyr Lys Asn Thr Pro Pro Thr
260 265 270

Met Asp Thr Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Asn Val Lys
275 280 285

Lys Glu Thr Trp Gln Gln Gly Asn Thr Phe Thr Cys Ser Val Leu His
290 295 300

Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser Pro
305 310 315 320

Gly Lys

<210> 30

<211> 333

<212> PRT

<213> Rattus Norvegicus

<400> 30

Ala Gln Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Gly Cys Gly
1 5 10 15

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1          2          3          4          5          6          7          8          9          10         11         12         13         14         15
Asp Thr Thr Ser Ser Thr Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20                25                30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ala Leu Ser Ser
35                40                45

Asp Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50                55                60

Thr Ser Ser Val Thr Ser Ser Thr Trp Pro Ser Gln Thr Val Thr Cys
65                70                75                80

Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys Val Glu
85                90                95

Arg Arg Asn Gly Gly Ile Gly His Lys Cys Pro Thr Cys Pro Thr Cys
100               105               110

His Lys Cys Pro Val Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Ile
115               120               125

Phe Pro Pro Lys Pro Lys Asp Ile Leu Leu Ile Ser Gln Asn Ala Lys
130               135               140

Val Thr Cys Val Val Val Asp Val Ser Glu Glu Glu Pro Asp Val Gln
145               150               155               160

Phe Ser Trp Phe Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln
165               170               175

Pro Arg Glu Glu Gln Tyr Asn Ser Thr Phe Arg Val Val Ser Ala Leu
180               185               190

Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys
195               200               205

Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu Lys Thr Ile Ser Lys
210               215               220

Pro Lys Gly Leu Val Arg Lys Pro Gln Val Tyr Val Met Gly Pro Pro
225               230               235               240

Thr Glu Gln Leu Thr Glu Gln Thr Val Ser Leu Thr Cys Leu Thr Ser
245               250               255

Gly Phe Leu Pro Asn Asp Ile Gly Val Glu Trp Thr Ser Asn Gly His
260               265               270

Ile Glu Lys Asn Tyr Lys Asn Thr Glu Pro Val Met Asp Ser Asp Gly
275               280               285

Ser Phe Phe Met Tyr Ser Lys Leu Asn Val Glu Arg Ser Arg Trp Asp
290               295               300

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Ser Arg Ala Pro Phe Val Cys Ser Val Val His Glu Gly Leu His Asn
305 310 315 320

His His Val Glu Lys Ser Ile Ser Arg Pro Pro Gly Lys
325 330

<210> 31

<211> 329

<212> PRT

<213> Rattus Norvegicus

<400> 31

Ala Arg Thr Thr Ala Pro Ser Val Tyr Pro Leu Val Pro Gly Cys Ser
1 5 10 15

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

Phe Pro Glu Pro Val Thr Val Lys Trp Asn Ser Gly Ala Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Ser Ser Ser Val Thr Val Pro Ser Ser Thr Trp Ser Ser Gln Thr Val
65 70 75 80

Thr Cys Ser Val Ala His Pro Ala Thr Lys Ser Asn Leu Ile Lys Arg
85 90 95

Ile Glu Pro Arg Arg Pro Lys Pro Arg Pro Pro Thr Asp Ile Cys Ser
100 105 110

Cys Asp Asp Asn Leu Gly Arg Pro Ser Val Phe Ile Phe Pro Pro Lys
115 120 125

Pro Lys Asp Ile Leu Met Ile Thr Leu Thr Pro Lys Val Thr Cys Val
130 135 140

Val Val Asp Val Ser Glu Glu Glu Pro Asp Val Gln Phe Ser Trp Phe
145 150 155 160

Val Asp Asn Val Arg Val Phe Thr Ala Gln Thr Gln Pro His Glu Glu
165 170 175

Gln Leu Asn Gly Thr Phe Arg Val Val Ser Thr Leu His Ile Gln His
180 185 190

Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
195 200 205

Asp Leu Pro Ser Pro Ile Glu Lys Thr Ile Ser Lys Pro Arg Gly Lys
210 215 220

Ala Arg Thr Pro Gln Val Tyr Thr Ile Pro Pro Pro Arg Glu Gln Met
225 230 235 240

Ser Lys Asn Lys Val Ser Leu Thr Cys Met Val Thr Ser Phe Tyr Pro
245 250 255

Ala Ser Ile Ser Val Glu Trp Glu Arg Asn Gly Glu Leu Glu Gln Asp
260 265 270

Tyr Lys Asn Thr Leu Pro Val Leu Asp Ser Asp Glu Ser Tyr Phe Leu
275 280 285

Tyr Ser Lys Leu Ser Val Asp Thr Asp Ser Trp Met Arg Gly Asp Ile
290 295 300

Tyr Thr Cys Ser Val Val His Glu Ala Leu His Asn His His Thr Gln
305 310 315 320

Lys Asn Leu Ser Arg Ser Pro Gly Lys
325

<210> 32

<211> 107

<212> PRT

<213> Rattus Norvegicus

<400> 32

Arg Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Thr Glu
1 5 10 15

Gln Leu Ala Thr Gly Gly Ala Ser Val Val Cys Leu Met Asn Asn Phe
20 25 30

Tyr Pro Arg Asp Ile Ser Val Lys Trp Lys Ile Asp Gly Thr Glu Arg
35 40 45

Arg Asp Gly Val Leu Asp Ser Val Thr Asp Gln Asp Ser Lys Asp Ser
50 55 60

Thr Tyr Ser Met Ser Ser Thr Leu Ser Leu Thr Lys Ala Asp Tyr Glu
65 70 75 80

Ser His Asn Leu Tyr Thr Cys Glu Val Val His Lys Thr Ser Ser Ser
85 90 95

Pro Val Val Lys Ser Phe Asn Arg Asn Glu Cys
100 105

<210> 33

<211> 97

<212> PRT

<213> Mus musculus

<400> 33

Ala Lys Thr Thr Pro Pro Ser Val Tyr Pro Leu Ala Pro Gly Ser Ala
1 5 10 15

Ala Gln Thr Asn Ser Met Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Glu Ser Asp Leu Tyr Thr Leu
50 55 60

Ser Ser Ser Val Thr Val Pro Ser Ser Pro Arg Pro Ser Glu Thr Val
65 70 75 80

Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
85 90 95

Ile

<210> 34

<211> 97

<212> PRT

<213> Mus musculus

<400> 34

Ala Lys Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Val Cys Gly
1 5 10 15

Asp Thr Thr Gly Ser Ser Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Leu Thr Trp Asn Ser Gly Ser Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu
50 55 60

Ser Ser Ser Val Thr Val Thr Ser Ser Thr Trp Pro Ser Gln Ser Ile
65 70 75 80

Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
85 90 95

Ile

<210> 35

<211> 97

<212> PRT

<213> Mus musculus

<400> 35

Ala Lys Thr Thr Pro Pro Ser Val Tyr Pro Leu Ala Pro Gly Cys Gly
1 5 10 15

Asp Thr Thr Gly Ser Ser Val Thr Ser Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser
35 40 45

Ser Val His Thr Phe Pro Ala Leu Leu Gln Ser Gly Leu Tyr Thr Met
50 55 60

Ser Ser Ser Val Thr Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val
65 70 75 80

Thr Cys Ser Val Ala His Pro Ala Ser Ser Thr Thr Val Asp Lys Lys
85 90 95

Leu

<210> 36

<211> 97

<212> PRT

<213> Mus musculus

<400> 36

Ala Lys Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Val Cys Gly
1 5 10 15

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

Phe Pro Glu Pro Val Thr Leu Thr Trp Asn Ser Gly Ser Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Leu Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Ser Ser Ser Val Thr Val Thr Ser Asn Thr Trp Pro Ser Gln Thr Ile
65 70 75 80

Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
85 90 95

Ile

<210> 37

<211> 97

<212> PRT

<213> Mus musculus

<400> 37

Ala Thr Thr Thr Ala Pro Ser Val Tyr Pro Leu Val Pro Gly Cys Ser
1 5 10 15

Asp Thr Ser Gly Ser Ser Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Lys Trp Asn Tyr Gly Ala Leu Ser Ser
35 40 45

Gly Val Arg Thr Val Ser Ser Val Leu Gln Ser Gly Phe Tyr Ser Leu
50 55 60

Ser Ser Leu Val Thr Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val
65 70 75 80

Ile Cys Asn Val Ala His Pro Ala Ser Lys Thr Glu Leu Ile Lys Arg
85 90 95

Ile

<210> 38

<211> 13

<212> PRT

<213> Mus musculus

<400> 38

Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr
1 5 10

<210> 39

<211> 16

<212> PRT

<213> Mus musculus

<400> 39

Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro
1 5 10 15

<210> 40

<211> 22

<212> PRT

<213> Mus musculus

<400> 40

Glu Pro Ser Gly Pro Ile Ser Thr Ile Asn Pro Cys Pro Pro Cys Lys
1 5 10 15

Glu Cys His Lys Cys Pro
20

<210> 41

<211> 21

<212> PRT

<213> Mus musculus

<400> 41

Glu Pro Arg Val Pro Ile Thr Gln Asn Pro Cys Pro Pro Leu Lys Glu
1 5 10 15

Cys Pro Pro Cys Ala
20

<210> 42
<211> 16
<212> PRT
<213> Mus musculus

<400> 42
Glu Pro Arg Ile Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Cys Pro
1 5 10 15

<210> 43
<211> 110
<212> PRT
<213> Mus musculus

<400> 43

Ala	Pro	Asp	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Lys
1				5					10					15	

Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Met Val Thr Cys Val
20 25 30

Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
35 40 45

Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu

50 55 60

Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
65 70 75 80

Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Arg
85 90 95

Ala Leu Pro Ser Pro Ile Glu Lys Thr Ile Ser Lys Pro Arg
100 105 110

<210> 44
<211> 107
<212> PRT
<213> Mus musculus

<400> 44

Gly	Arg	Pro	Lys	Ala	Pro	Gln	Val	Tyr	Thr	Ile	Pro	Pro	Pro	Lys	Glu
1				5					10					15	

Gln Met Ala Lys Asp Lys Val Ser Leu Thr Cys Met Ile Thr Asp Phe
20 25 30

Phe Pro Glu Asp Ile Thr Val Glu Trp Gln Trp Asn Gly Gln Pro Ala
35 40 45

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
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Glu Asn Tyr Lys Asn Thr Gln Pro Ile Met Asn Thr Asn Gly Ser Tyr
50 55 60

Phe Val Tyr Ser Lys Leu Asn Val Gln Lys Ser Asn Trp Glu Ala Gly
65 70 75 80

Asn Thr Phe Thr Cys Ser Val Leu His Glu Gly Leu His Asn His His
85 90 95

Thr Glu Lys Ser Leu Ser His Ser Pro Gly Lys
100 105

<210> 45

<211> 107

<212> PRT

<213> Mus musculus

<400> 45

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

Glu Met Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe
20 25 30

Met Pro Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu
35 40 45

Leu Asn Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr
50 55 60

Phe Met Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg
65 70 75 80

Asn Ser Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His
85 90 95

Thr Thr Lys Ser Phe Ser Arg Thr Pro Gly Lys
100 105

<210> 46

<211> 107

<212> PRT

<213> Mus musculus

<400> 46

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

Gln Leu Ser Arg Lys Asp Val Ser Leu Thr Cys Leu Val Val Gly Phe
20 25 30

Asn Pro Gly Asp Ile Ser Val Glu Trp Thr Ser Asn Gly His Thr Glu
35 40 45

Glu Asn Tyr Lys Asp Thr Ala Pro Val Leu Asp Ser Asp Gly Ser Tyr
-- -- --

50

55

60

Phe Ile Tyr Ser Lys Leu Asn Met Lys Thr Ser Lys Trp Glu Lys Thr
65 70 75 80

Asp Ser Phe Ser Cys Asn Val Arg His Glu Gly Leu Lys Asn Tyr Tyr
85 90 95

Leu Lys Lys Thr Ile Ser Arg Ser Pro Gly Lys
100 105

<210> 47

<211> 107

<212> PRT

<213> Mus musculus

<400> 47

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

Glu Met Thr Lys Lys Glu Phe Ser Leu Thr Cys Met Ile Thr Gly Phe
20 25 30

Leu Pro Ala Glu Ile Ala Val Asp Trp Thr Ser Asn Gly Arg Thr Glu
35 40 45

Gln Asn Tyr Lys Asn Thr Ala Thr Val Leu Asp Ser Asp Gly Ser Tyr
50 55 60

Phe Met Tyr Ser Lys Leu Arg Val Gln Lys Ser Thr Trp Glu Arg Gly
65 70 75 80

Ser Leu Phe Ala Cys Ser Val Val His Glu Val Leu His Asn His Leu
85 90 95

Thr Thr Lys Thr Ile Ser Arg Ser Leu Gly Lys
100 105

<210> 48

<211> 107

<212> PRT

<213> Mus musculus

<400> 48

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

Gln Met Ser Lys Lys Lys Val Ser Leu Thr Cys Leu Val Thr Asn Phe
20 25 30

Phe Ser Glu Ala Ile Ser Val Glu Trp Glu Arg Asn Gly Glu Leu Glu
35 40 45

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

Phe Leu Tyr Ser Lys Leu Thr Val Asp Thr Asp Ser Trp Leu Gln Gly
65 70 75 80

Glu Ile Phe Thr Cys Ser Val Val His Glu Ala Leu His Asn His His
85 90 95

Thr Gln Lys Asn Leu Ser Arg Ser Pro Gly Lys
100 105

<210> 49

<211> 97

<212> PRT

<213> Rattus Norvegicus

<400> 49

Ala Glu Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Gly Thr Ala
1 5 10 15

Leu Lys Ser Asn Ser Met Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ala Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Thr Ser Ser Val Thr Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val
65 70 75 80

Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
85 90 95

Ile

<210> 50

<211> 97

<212> PRT

<213> Rattus Norvegicus

<400> 50

Ala Glu Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Gly Thr Ala
1 5 10 15

Leu Lys Ser Asn Ser Met Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ala Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Thr Ser Ser Val Thr Val Pro Ser Ser Thr Trp Ser Ser Gln Ala Val
65 70 75 80

Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
85 90 95

Ile

<210> 51

<211> 95

<212> PRT

<213> Rattus Norvegicus

<400> 51

Ala Gln Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Gly Cys Gly
1 5 10 15

Asp Thr Thr Ser Ser Thr Val Thr Leu Gly Cys Leu Val Lys Gly Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ala Leu Ser Ser
35 40 45

Asp Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Thr Ser Ser Val Thr Ser Ser Thr Trp Pro Ser Gln Thr Val Thr Cys
65 70 75 80

Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys Val
85 90 95

<210> 52

<211> 97

<212> PRT

<213> Rattus Norvegicus

<400> 52

Ala Arg Thr Thr Ala Pro Ser Val Tyr Pro Leu Val Pro Gly Cys Ser
1 5 10 15

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

Phe Pro Glu Pro Val Thr Val Lys Trp Asn Ser Gly Ala Leu Ser Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Gly Leu Tyr Thr Leu
50 55 60

Ser Ser Ser Val Thr Val Pro Ser Ser Thr Trp Ser Ser Gln Thr Val

65

70

75

80

Thr	Cys	Ser	Val	Ala	His	Pro	Ala	Thr	Lys	Ser	Asn	Leu	Ile	Lys	Arg
				85					90					95	

Ile

<210> 53

<211> 15

<212> PRT

<213> Rattus Norvegicus

<400> 53

Val	Pro	Arg	Asn	Cys	Gly	Gly	Asp	Cys	Lys	Pro	Cys	Ile	Cys	Thr
1				5					10					15

<210> 54

<211> 11

<212> PRT

<213> Rattus Norvegicus

<400> 54

Val	Pro	Arg	Glu	Cys	Asn	Pro	Cys	Gly	Cys	Thr
1				5					10	

<210> 55

<211> 21

<212> PRT

<213> Rattus Norvegicus

<400> 55

Glu	Arg	Arg	Asn	Gly	Gly	Ile	Gly	His	Lys	Cys	Pro	Thr	Cys	Pro	Thr
1				5					10					15	

Cys	His	Lys	Cys	Pro
			20	

<210> 56

<211> 17

<212> PRT

<213> Rattus Norvegicus

<400> 56

Glu	Pro	Arg	Arg	Pro	Lys	Pro	Arg	Pro	Pro	Thr	Asp	Ile	Cys	Ser	Cys
1				5					10					15	

Asp

<210> 57

<211> 107

<212> PRT

<213> Rattus Norvegicus

<400> 57

Gly	Arg	Thr	Gln	Val	Pro	His	Val	Tyr	Thr	Met	Ser	Pro	Thr	Lys	Glu
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1 5 10 15

Glu Met Thr Gln Asn Glu Val Ser Ile Thr Cys Met Val Lys Gly Phe
 20 25 30

Tyr Pro Pro Asp Ile Tyr Val Glu Trp Gln Met Asn Gly Gln Pro Gln
 35 40 45

Glu Asn Tyr Lys Asn Thr Pro Pro Thr Met Asp Thr Asp Gly Ser Tyr
 50 55 60

Phe Leu Tyr Ser Lys Leu Asn Val Lys Lys Glu Lys Trp Gln Gln Gly
 65 70 75 80

Asn Thr Phe Thr Cys Ser Val Leu His Glu Gly Leu His Asn His His
 85 90 95

Thr Glu Lys Ser Leu Ser His Ser Pro Gly Lys
 100 105

<210> 58

<211> 107

<212> PRT

<213> Rattus Norvegicus

<400> 58

Gly Thr Pro Arg Gly Pro Gln Val Tyr Thr Met Ala Pro Pro Lys Glu
 1 5 10 15

Glu Met Thr Gln Ser Gln Val Ser Ile Thr Cys Met Val Lys Gly Phe
 20 25 30

Tyr Pro Pro Asp Ile Tyr Thr Glu Trp Lys Met Asn Gly Gln Pro Gln
 35 40 45

Glu Asn Tyr Lys Asn Thr Pro Pro Thr Met Asp Thr Asp Gly Ser Tyr
 50 55 60

Phe Leu Tyr Ser Lys Leu Asn Val Lys Lys Glu Thr Trp Gln Gln Gly
 65 70 75 80

Asn Thr Phe Thr Cys Ser Val Leu His Glu Gly Leu His Asn His His
 85 90 95

Thr Glu Lys Ser Leu Ser His Ser Pro Gly Lys
 100 105

<210> 59

<211> 105

<212> PRT

<213> Rattus Norvegicus

<400> 59

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

Gln Leu Thr Glu Gln Thr Val Ser Leu Thr Cys Leu Thr Ser Gly Phe
 20 25 30

Leu Pro Asn Asp Ile Gly Val Glu Trp Thr Ser Asn Gly His Ile Glu
 35 40 45

Lys Asn Tyr Lys Asn Thr Glu Pro Val Met Asp Ser Asp Gly Ser Phe
 50 55 60

Phe Met Tyr Ser Lys Leu Asn Val Glu Arg Ser Arg Trp Asp Ser Arg
 65 70 75 80

Ala Pro Phe Val Cys Ser Val Val His Glu Gly Leu His Asn His His
 85 90 95

Val Glu Lys Ser Ile Ser Arg Pro Pro
 100 105

<210> 60

<211> 107

<212> PRT

<213> Rattus Norvegicus

<400> 60

Gly Lys Ala Arg Thr Pro Gln Val Tyr Thr Ile Pro Pro Pro Arg Glu
 1 5 10 15

Gln Met Ser Lys Asn Lys Val Ser Leu Thr Cys Met Val Thr Ser Phe
 20 25 30

Tyr Pro Ala Ser Ile Ser Val Glu Trp Glu Arg Asn Gly Glu Leu Glu
 35 40 45

Gln Asp Tyr Lys Asn Thr Leu Pro Val Leu Asp Ser Asp Glu Ser Tyr
 50 55 60

Phe Leu Tyr Ser Lys Leu Ser Val Asp Thr Asp Ser Trp Met Arg Gly
 65 70 75 80

Asp Ile Tyr Thr Cys Ser Val Val His Glu Ala Leu His Asn His His
 85 90 95

Thr Gln Lys Asn Leu Ser Arg Ser Pro Gly Lys
 100 105

<210> 61

<211> 15

<212> PRT

<213> Homo Sapiens

<400> 61

Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro
1			5						10					15

<210> 62

<211> 121

<212> PRT

<213> Homo Sapiens

<400> 62

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

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

Gly	Ile	Ser	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Met
		35					40						45		

Gly	Trp	Leu	Ser	Ala	Tyr	Ser	Gly	Asn	Thr	Ile	Tyr	Ala	Gln	Lys	Leu
	50					55					60				

Gln	Gly	Arg	Val	Thr	Met	Thr	Thr	Asp	Thr	Ser	Thr	Thr	Thr	Ala	Tyr
65					70					75					80

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

Ala	Arg	Asp	Arg	Ile	Val	Val	Arg	Pro	Asp	Tyr	Phe	Asp	Tyr	Trp	Gly
			100					105					110		

Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser
		115					120	

<210> 63

<211> 107

<212> PRT

<213> Homo Sapiens

<400> 63

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

Glu	Arg	Ala	Thr	Leu	Ser	Cys	Arg	Ala	Ser	Gln	Ser	Val	Ser	Ser	Tyr
			20					25					30		

Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro	Arg	Leu	Leu	Ile
		35					40					45			

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

Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Glu	Pro
65					70					75				80	

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Pro Arg
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

<210> 64

<211> 116

<212> PRT

<213> Cricetulus migratorius

<400> 64

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

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

Gly Met His Trp Val Arg Gln Ala Pro Gly Arg Gly Leu Glu Ser Val
35 40 45

Ala Tyr Ile Thr Ser Ser Ser Ile Asn Ile Lys Tyr Ala Asp Ala Val
50 55 60

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

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

Ala Arg Phe Asp Trp Asp Lys Asn Tyr Trp Gly Gln Gly Thr Met Val
100 105 110

Thr Val Ser Ser
115

<210> 65

<211> 107

<212> PRT

<213> Cricetulus migratorius

<400> 65

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

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

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

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

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

Glu Asp Ile Gly Ser Tyr Tyr Cys Gln Gln Tyr Tyr Asn Tyr Pro Trp
85 90 95

Thr Phe Gly Pro Gly Thr Lys Leu Glu Ile Lys
100 105

<210> 66

<211> 121

<212> PRT

<213> Mus musculus

<400> 66

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

Leu Val Lys Leu Ser Cys Lys Thr Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30

Phe Leu His Trp Val Arg Gln Arg Pro Asp Gln Gly Leu Glu Trp Ile
35 40 45

Gly Trp Ile Asn Pro Asp Asn Gly Asn Thr Val Tyr Asp Pro Lys Phe
50 55 60

Gln Gly Thr Ala Ser Leu Thr Ala Asp Thr Ser Ser Asn Thr Val Tyr
65 70 75 80

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

Thr Arg Arg Asp Tyr Thr Tyr Glu Lys Ala Ala Leu Asp Tyr Trp Gly
100 105 110

Gln Gly Ala Ser Val Ile Val Ser Ser
115 120

<210> 67

<211> 107

<212> PRT

<213> Mus musculus

<400> 67

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

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

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

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

Ser Gly Ser Gly Thr Gln Tyr Ser Leu Lys Ile Ser Ser Leu Gln Thr
65 70 75 80

Glu Asp Ser Gly Asn Tyr Tyr Cys Gln His Phe Trp Ser Leu Pro Phe
85 90 95

Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105

<210> 68

<211> 127

<212> PRT

<213> Homo Sapiens

<400> 68

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

Ser Val Lys Val Ser Cys Gln Ala Ser Gly Tyr Arg Phe Ser Asn Phe
20 25 30

Val Ile His Trp Val Arg Gln Ala Pro Gly Gln Arg Phe Glu Trp Met
35 40 45

Gly Trp Ile Asn Pro Tyr Asn Gly Asn Lys Glu Phe Ser Ala Lys Phe
50 55 60

Gln Asp Arg Val Thr Phe Thr Ala Asp Thr Ser Ala Asn Thr Ala Tyr
65 70 75 80

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

Ala Arg Val Gly Pro Tyr Ser Trp Asp Asp Ser Pro Gln Asp Asn Tyr
100 105 110

Tyr Met Asp Val Trp Gly Lys Gly Thr Thr Val Ile Val Ser Ser
115 120 125

<210> 69

<211> 108

<212> PRT

<213> Homo Sapiens

<400> 69

Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
1 5 10 15

Glu Arg Ala Thr Phe Ser Cys Arg Ser Ser His Ser Ile Arg Ser Arg
20 25 30

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

Ile His Gly Val Ser Asn Arg Ala Ser Gly Ile Ser Asp Arg Phe Ser
 50 55 60

Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Thr Arg Val Glu
 65 70 75 80

Pro Glu Asp Phe Ala Leu Tyr Tyr Cys Gln Val Tyr Gly Ala Ser Ser
 85 90 95

Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Arg Lys
 100 105

REFERENCES CITED IN THE DESCRIPTION

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KRAV

1. Et heterodimerisk protein omfattende:

5 et første polypeptid af et første homodimerisk protein, hvor det første polypeptid omfatter en første variabel region med en første bindingsspecificitet og en første Fc-region, idet den første Fc-region omfatter et første CH3-domæne; og
et andet polypeptid af et andet homodimerisk protein, hvor det andet polypeptid omfatter en anden variabel region med en anden bindingsspecificitet og en anden Fc-region, hvor den
10 anden Fc-region omfatter et andet CH3-domæne,
hvor de variable regioner kan stamme fra en hvilken som helst art, og Fc-regionerne er IgG Fc-regioner fra gnavere, og hvor det første CH3-domæne omfatter et lysin i position 370 og en substitution af aminosyreresten i position 409 til arginin, og det andet CH3 domæne omfatter en substitution af aminosyreresten i position 405 til leucin, i forhold til vildtype-IgG-
15 isotypen fra gnaverarten ved anvendelse af EU-nummereringsindeks.

2. Heterodimerisk protein ifølge krav 1, hvor Fc-regionerne stammer fra mus eller rotter, fortrinsvis *Mus musculus* eller *Rattus norvegicus*.

20 **3.** Heterodimerisk protein ifølge et hvilket som helst af de foregående krav, hvor de variable regioner er af human oprindelse eller humaniserede variable regioner fra gnavere.

4. Heterodimerisk protein ifølge et hvilket som helst af de foregående krav, hvor det første og det andet polypeptid omfatter humane variable regioner og gnaver Fc-regioner.

25

5. Heterodimerisk protein ifølge et hvilket som helst af de foregående krav, hvor det andet CH3-domæne yderligere omfatter en Thr i position 411.

30 **6.** Heterodimerisk protein ifølge et hvilket som helst af de foregående krav, hvor aminosyreresten i position 253 i den første homodimer er substitueret med Asp (D).

7. Det heterodimeriske protein ifølge et hvilket som helst af de foregående krav, hvor aminosyreresterne i positionerne 307 og/eller 309 i den anden homodimer er substitueret med henholdsvis Thr (T) og Leu (L).

35

- 8.** Heterodimerisk protein ifølge et hvilket som helst af de foregående krav, hvor det første homodimeriske protein og det andet homodimeriske protein er valgt fra gruppen bestående af (i) et antistof (ii) et fusionsprotein omfattende en IgG Fc-region, såsom en Fc-region fusioneret med en receptor, et cytokin eller et hormon, (iii) et antistof konjugeret til et prodrug, peptid, medikament eller et toksin, og (iv) et fusionsprotein omfattende en IgG Fc-region konjugeret til et prodrug, peptid, medikament eller et toksin.
- 9.** Heterodimerisk protein ifølge et hvilket som helst af de foregående krav, hvor det første og/eller det andet homodimeriske protein er et antistof i fuld længde.
- 10.** Heterodimerisk protein ifølge et hvilket som helst af de foregående krav, hvor Fc-regionen i det første polypeptid er af en isotype og art valgt fra gruppen bestående af *Mus musculus* IgG1, *Mus musculus* IgG2a, *Mus musculus* IgG2b, *Mus musculus* IgG2c, *Mus musculus* IgG3, *Rattus norvegicus* IgG1, *Rattus norvegicus* IgG2a, *Rattus norvegicus* IgG2b og *Rattus norvegicus* IgG2c og Fc-regionen i det andet polypeptid er af en isotype valgt fra gruppen bestående af *Mus musculus* IgG1, *Mus musculus* IgG2a, *Mus musculus* IgG2b, *Mus musculus* IgG2c, *Mus musculus* IgG3, *Rattus norvegicus* IgG1, *Rattus norvegicus* IgG2a, *Rattus norvegicus* IgG2b og *Rattus norvegicus* IgG2c, og hvor Fc-regionerne i både den første og den anden polypeptid eventuelt er af den samme isotype og art.
- 11.** Det heterodimeriske protein ifølge et hvilket som helst af de foregående krav, hvor Fc-regionerne i både det første og det andet polypeptid stammer fra *Mus musculus* og begge er af den samme isotype, såsom IgG1, IgG2a eller IgG2b-isotype.
- 12.** Anvendelse af et heterodimerisk protein ifølge et hvilket som helst af de foregående krav til udforskning af funktioner i en gnavermodel med tilsvarende fuldt ud humane heterodimeriske proteiner, hvor det humane heterodimeriske protein og det heterodimeriske protein ifølge opfindelsen har tilsvarende bindingsspecificiteter.
- 13.** Anvendelse ifølge krav 12, hvor den udforskede funktion er valgt fra listen omfattende; virkningsmekanismer, effektorfunktioner, toksicitet, uønskede mekanismer og bivirkninger, antitumoreffektivitet, behandlingseffektivitet, T-celle-medieret antitumoreffektivitet, immunmodulation, farmakodynamik og/eller farmakokinetik for det heterodimeriske protein.
- 14.** In vitro-metode til generering af et heterodimerisk protein ifølge et hvilket som helst af kravene 1-11, hvilken fremgangsmåde omfatter følgende trin:

- a) tilvejebringelse af et første homodimerisk protein omfattende en første variabel region med en første bindingsspecificitet, en hinge-region og en første Fc-region, hvor den første Fc-region omfatter et første CH3-domæne; og
- 5 b) tilvejebringelse af et andet homodimerisk protein omfattende en anden variabel region med en anden bindingsspecificitet, en hinge-region og en anden Fc-region, hvor den anden Fc-region omfatter et andet CH3-domæne,
- 10 hvor de variable regioner kan stamme fra en hvilken som helst art, og Fc-regionerne er IgG Fc-region fra gnaverarter, og hvor det første CH3-domæne omfatter en lysin i position 370 og en substitution af aminosyreresten i position 409 til en arginin, og det andet CH3-domæne omfatter en substitution af aminosyreresten i position 405 til en leucin, i forhold til vildtype-IgG-isotypen fra gnaverarterne ved anvendelse af EU-indeksnummerering, og hvor sekvenserne af den første og anden CH3-region er forskellige og er af sådan karakter, at en heterodimerisk interaktion mellem den første og den anden CH3-region er stærkere end hver
- 15 af de homodimeriske interaktioner mellem den første og den anden CH3-region,
- c) inkubering af det første homodimeriske protein sammen med det andet homodimeriske protein under reducerende betingelser, der er tilstrækkelige for at cysteinerne i hinge-regionen gennemgår disulfidbinding-isomerisering, og
- d) opnåelse af det heterodimeriske protein.
- 20

DRAWINGS

FIG. 1

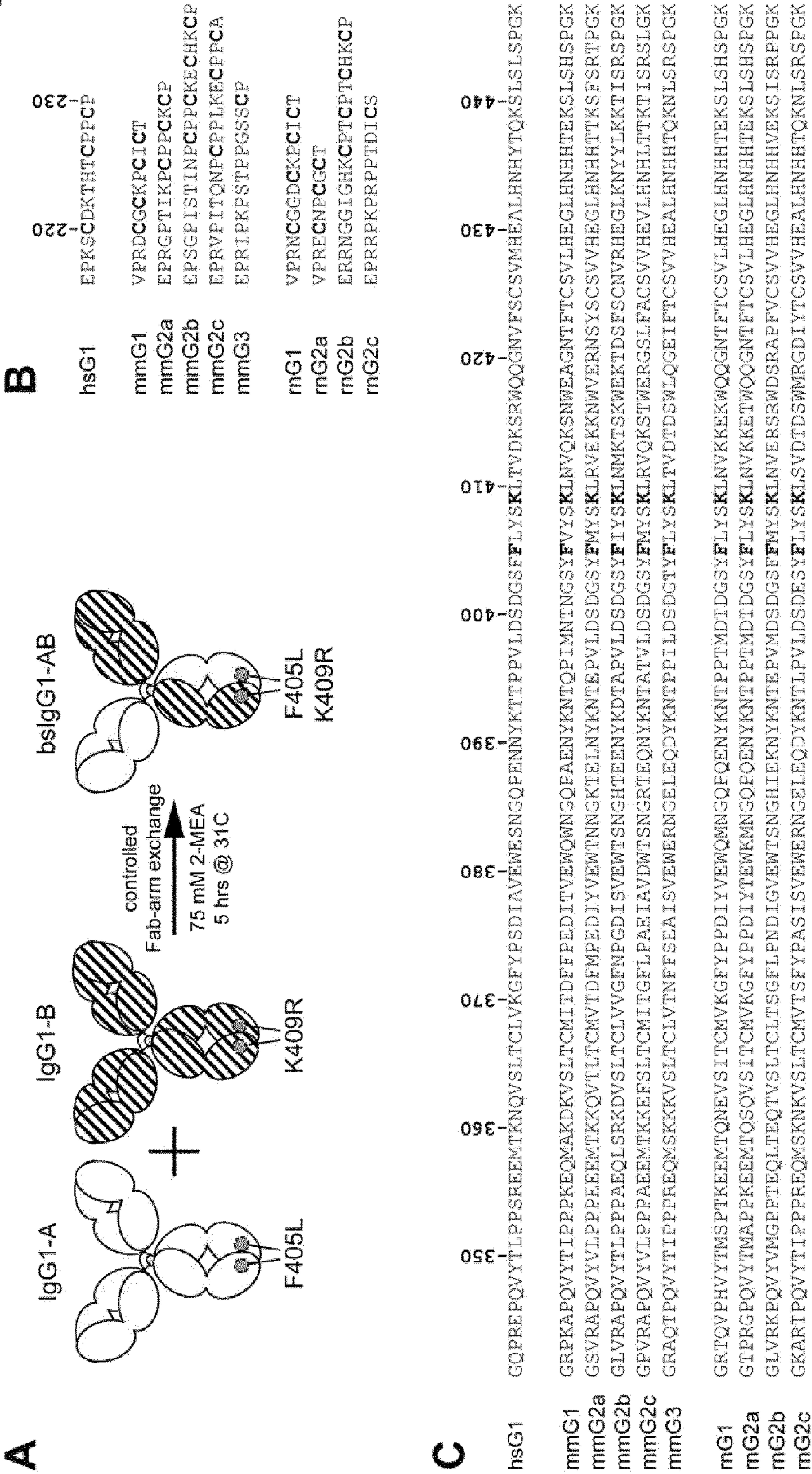


FIG. 2

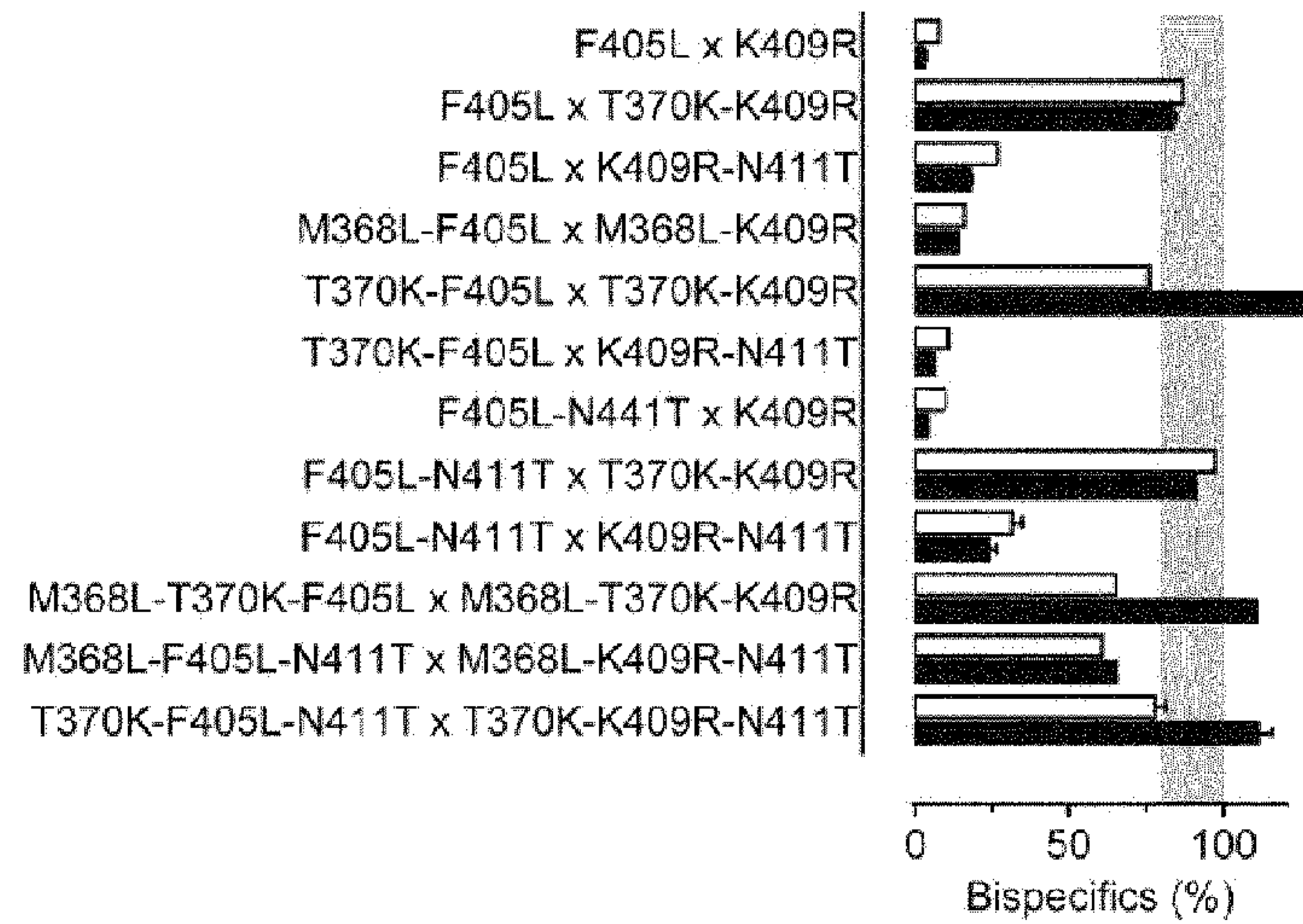
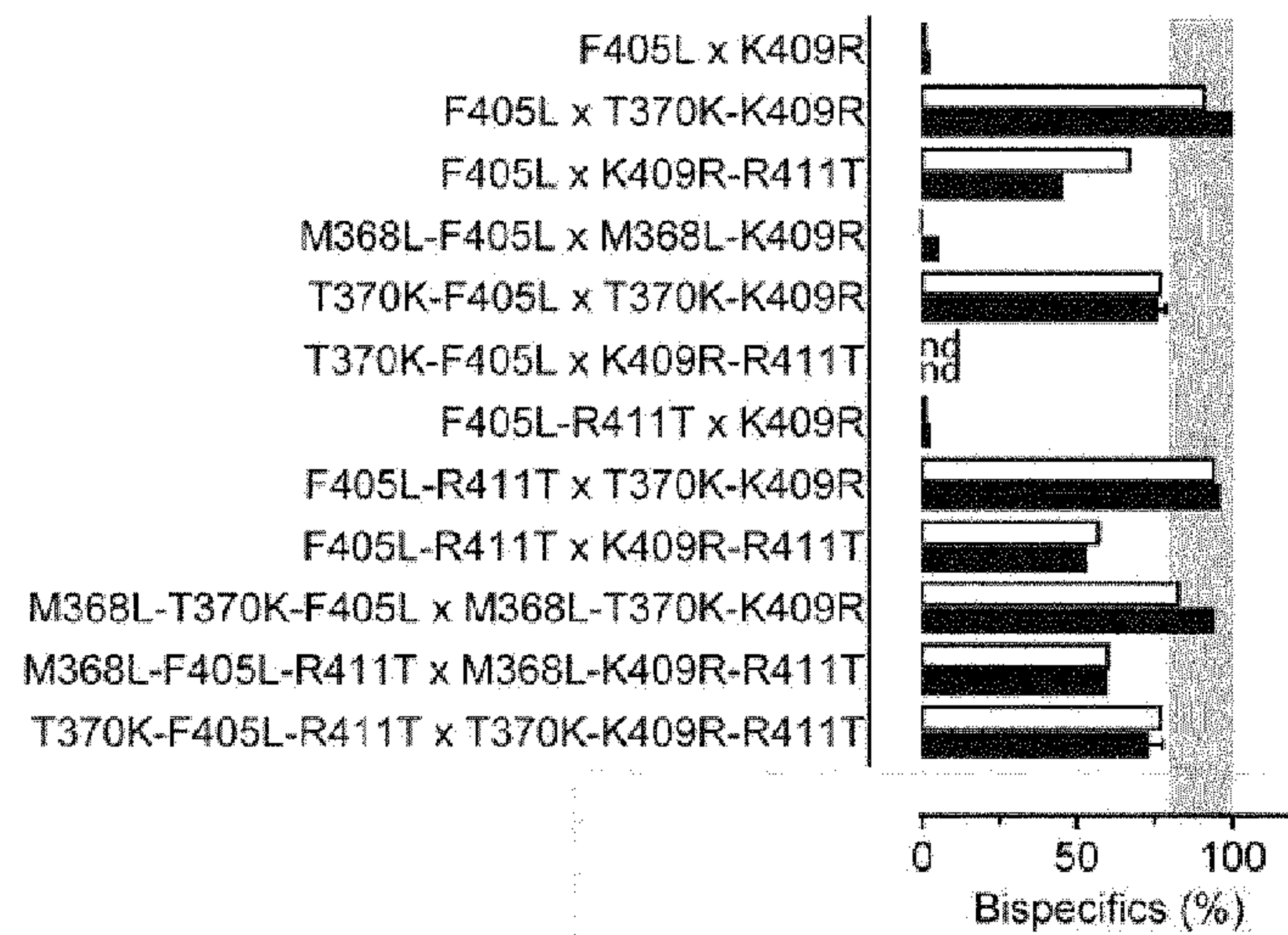
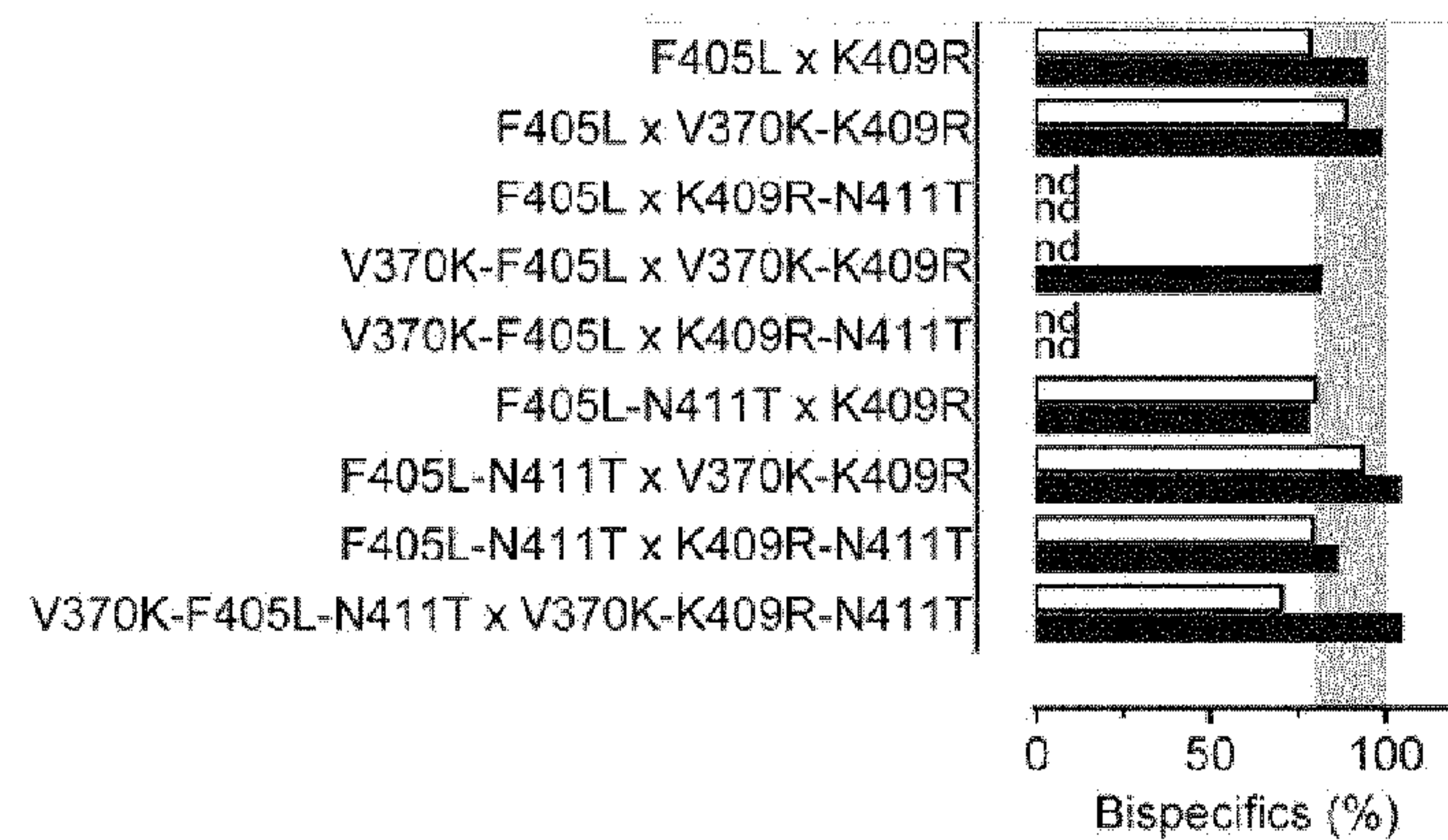
A**B****C**

FIG. 3

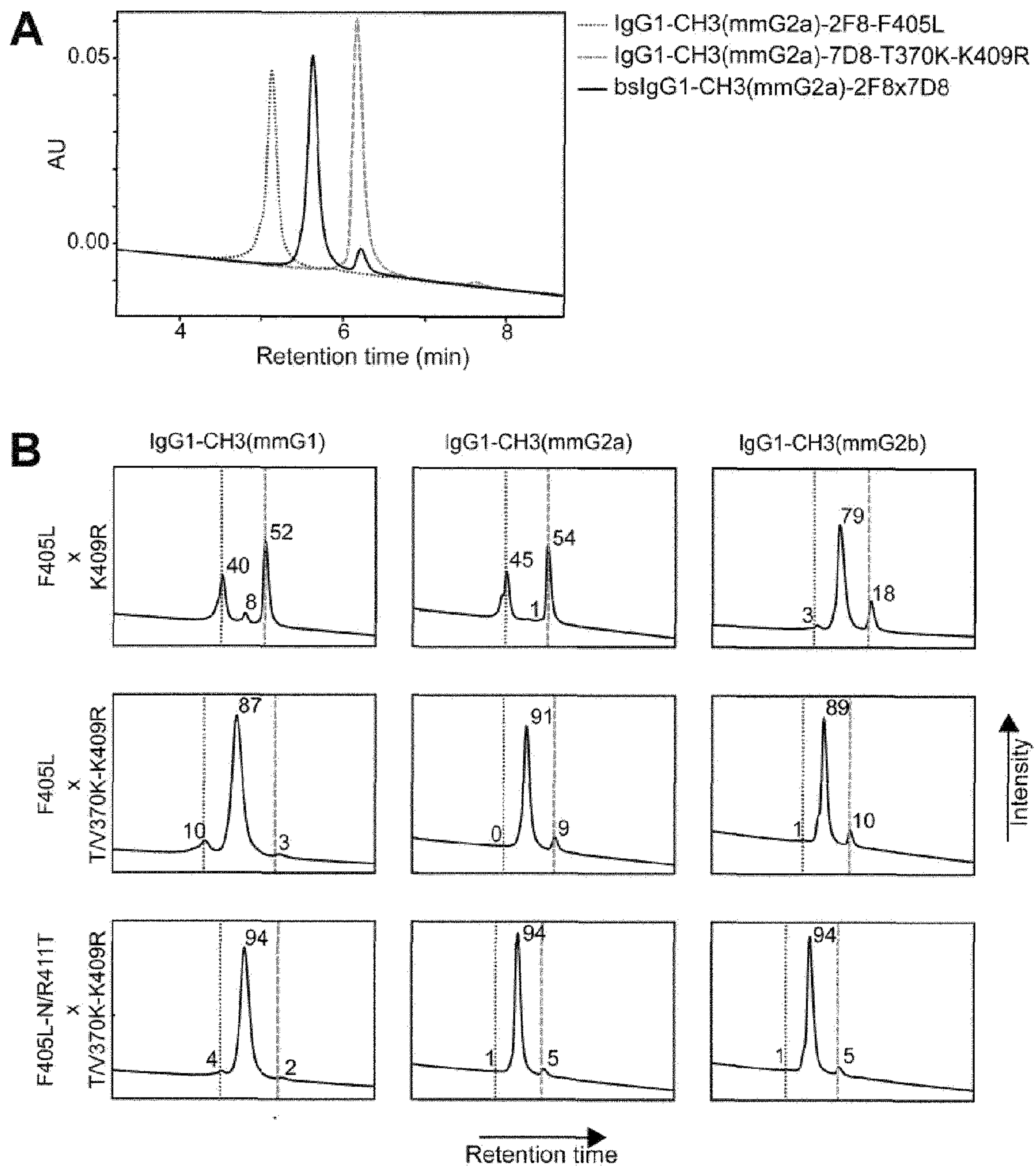


FIG. 4

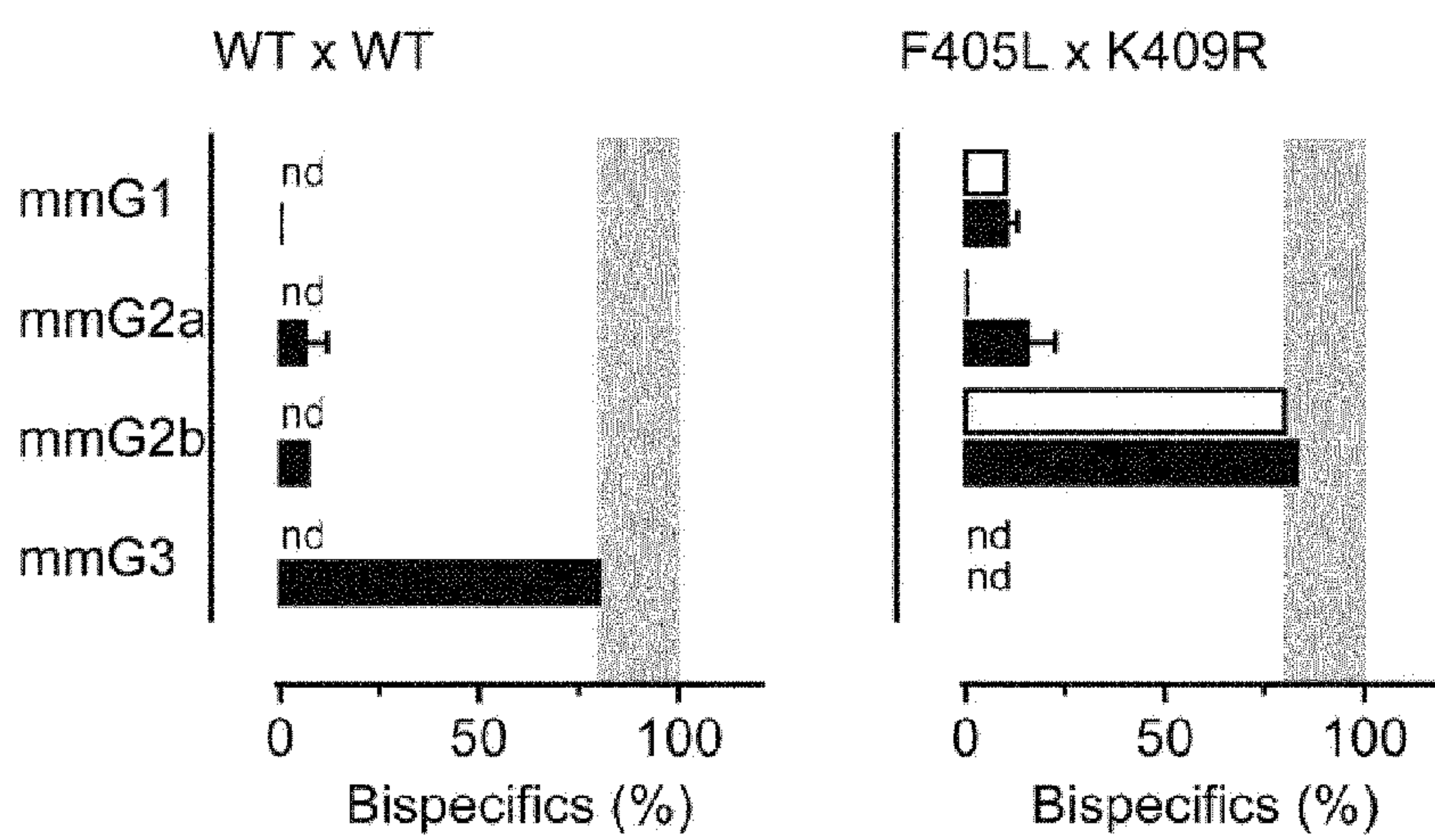
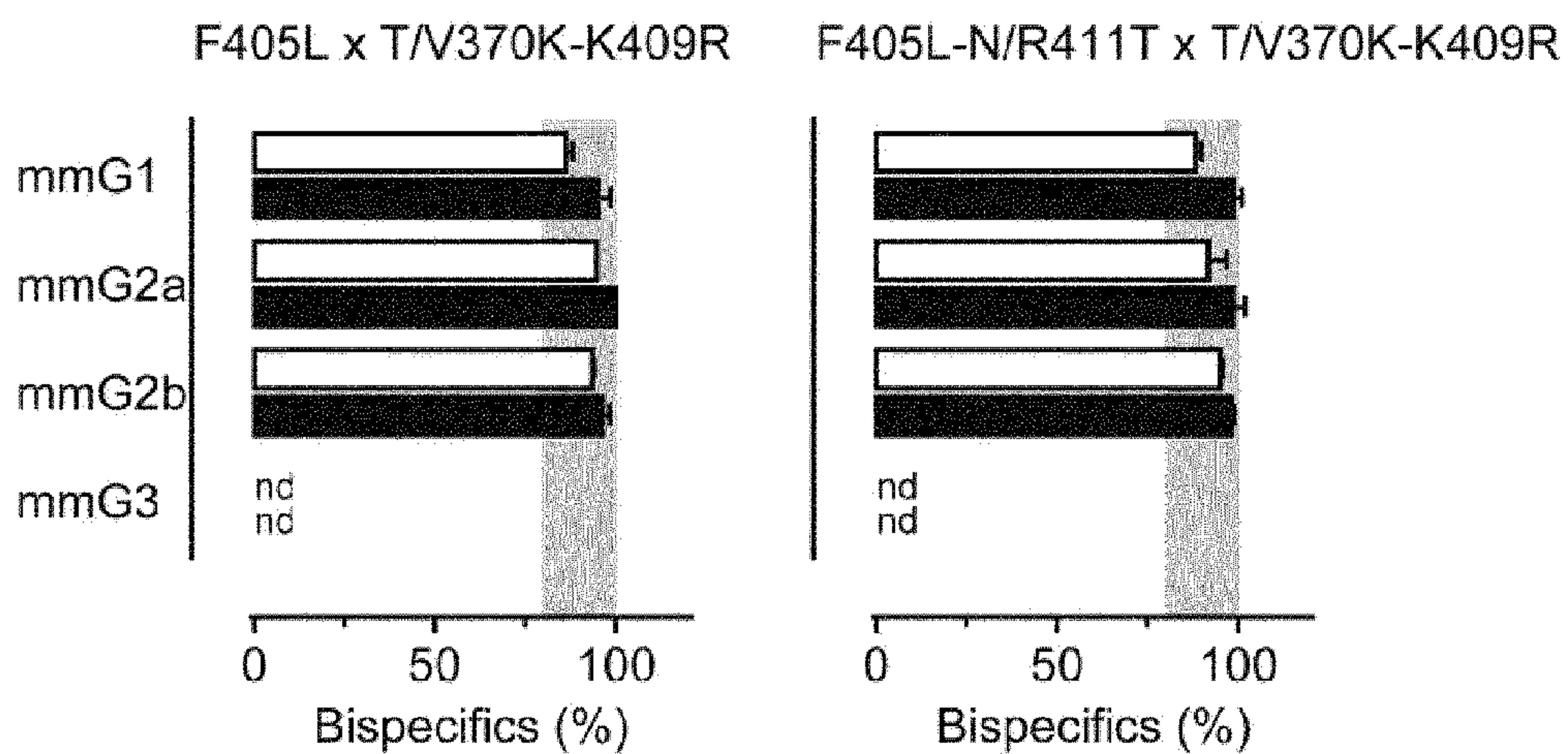
A**B****C**

FIG. 5

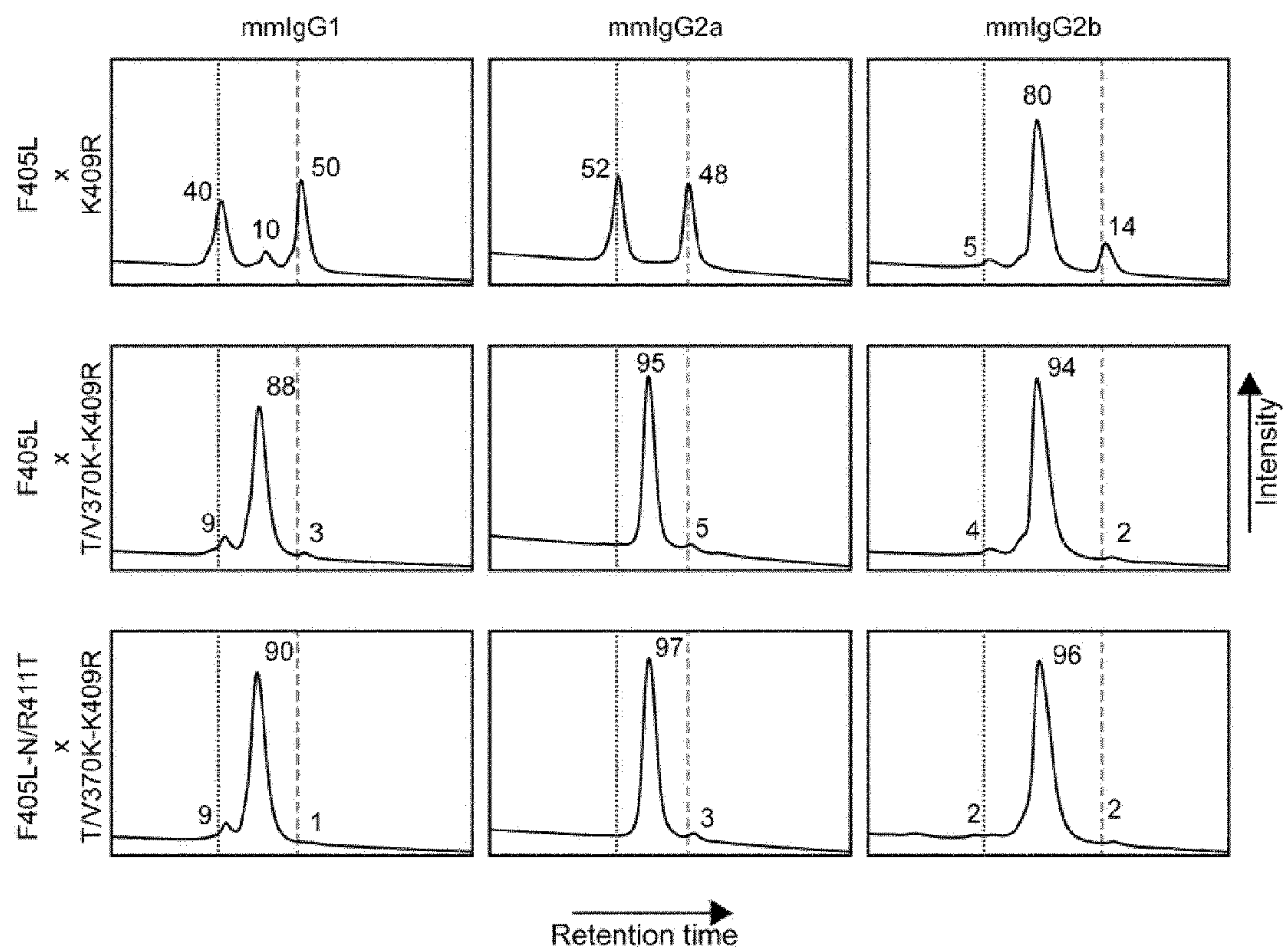


FIG. 6

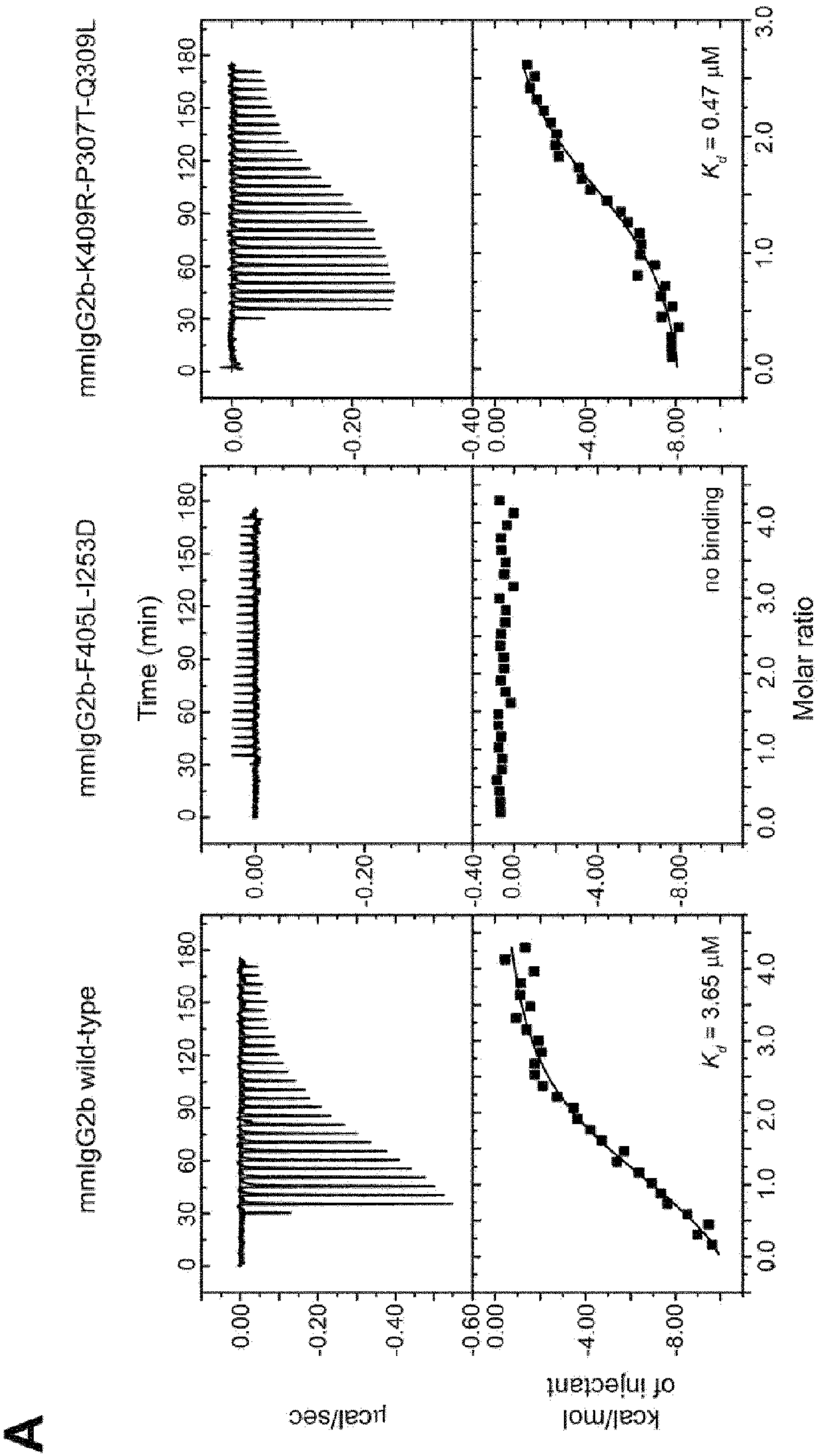


FIG. 6 (CONTINUED)

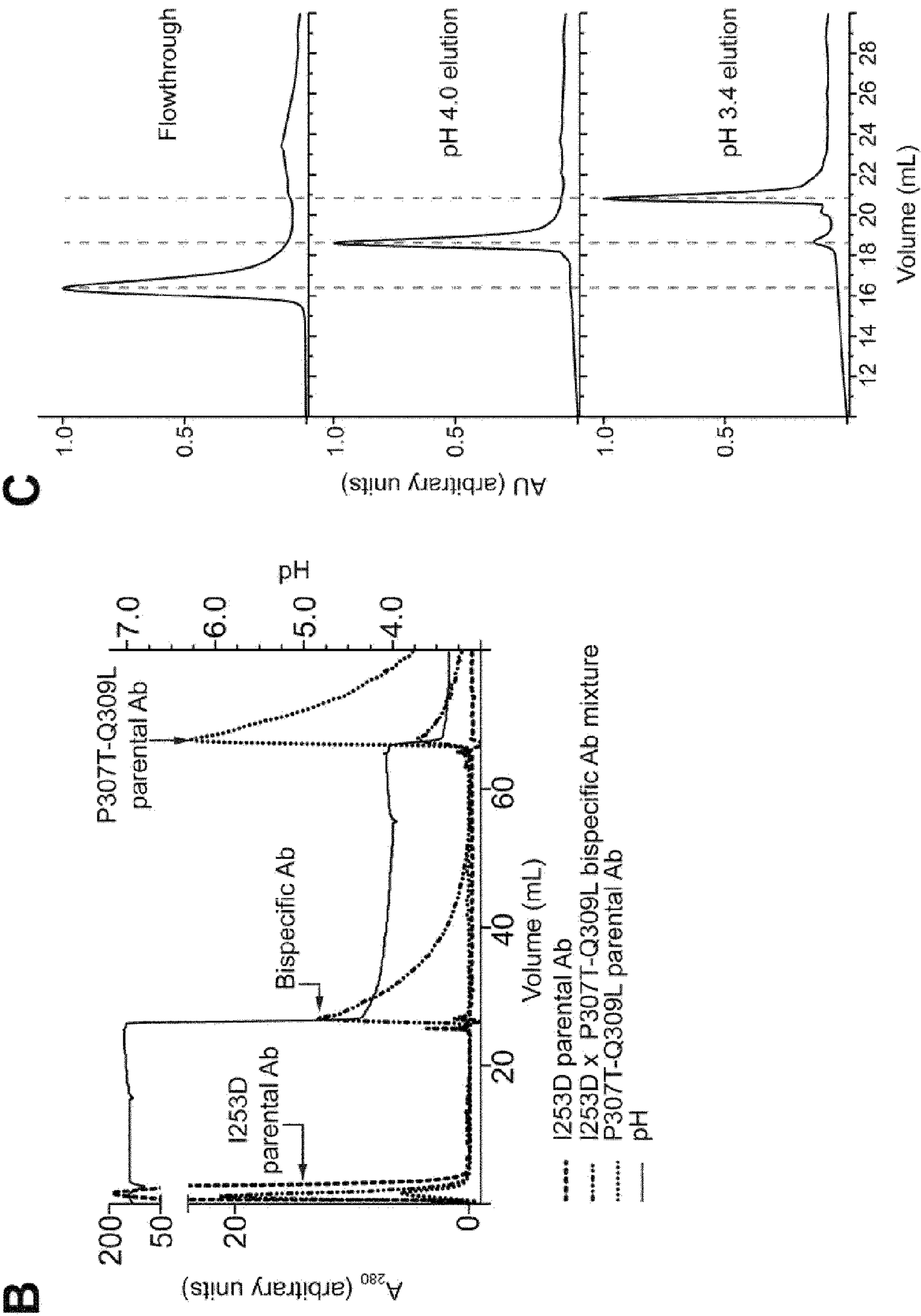


FIG. 7

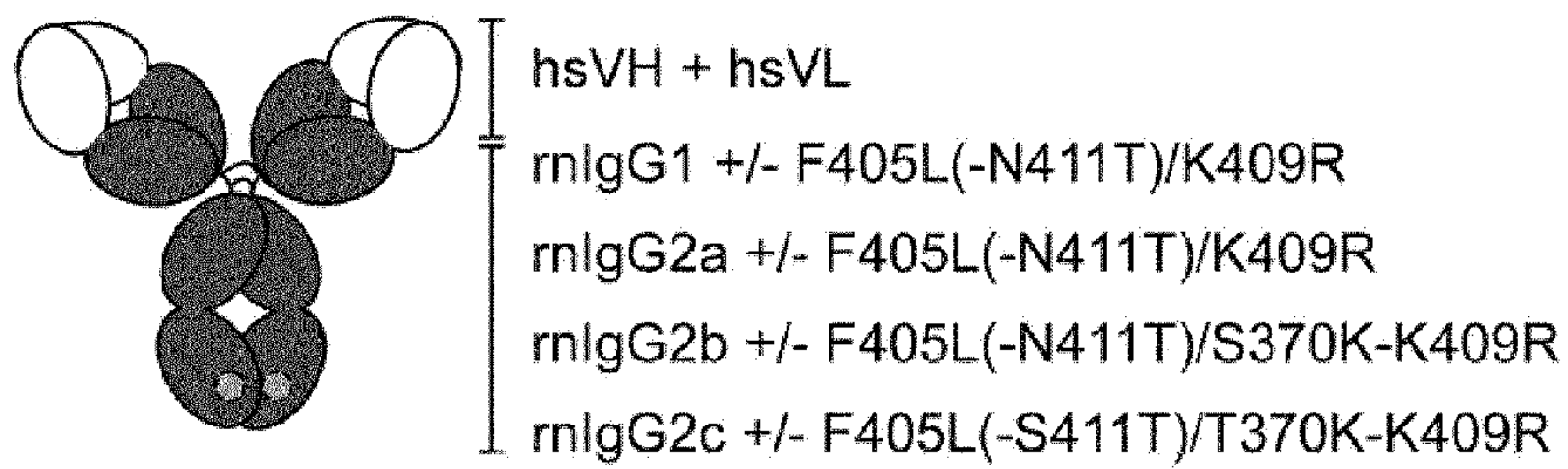
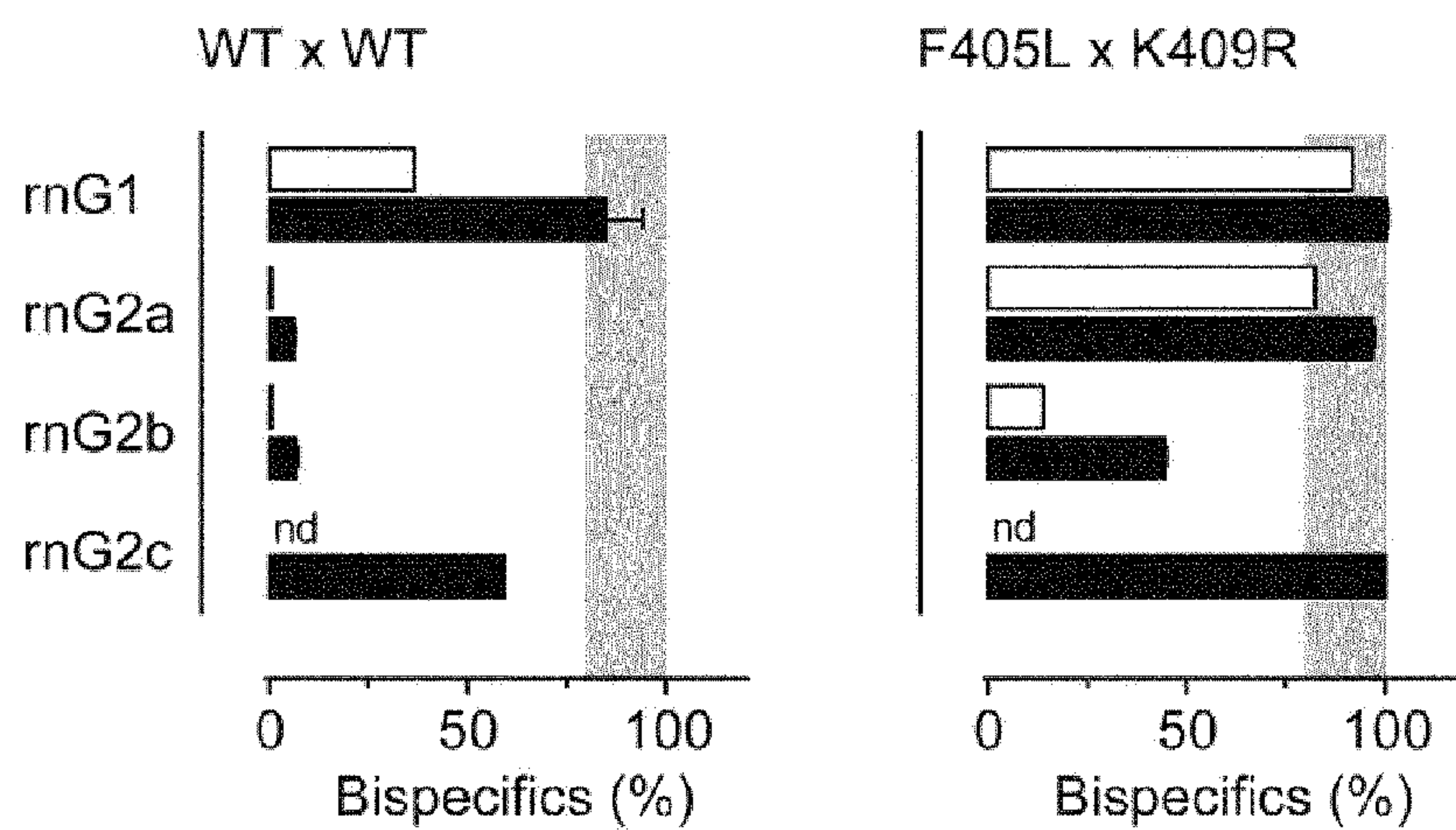
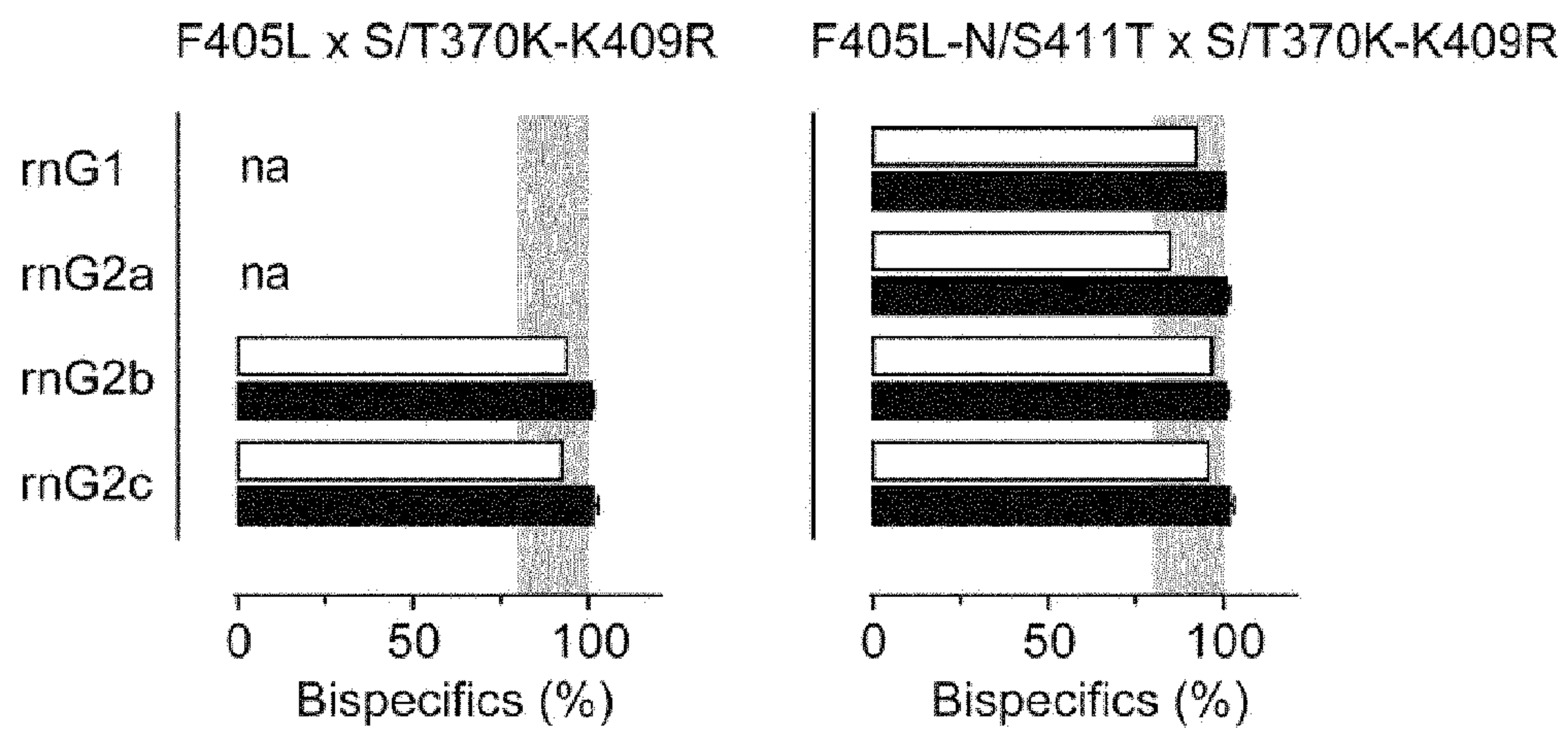
A**B****C**

FIG. 8

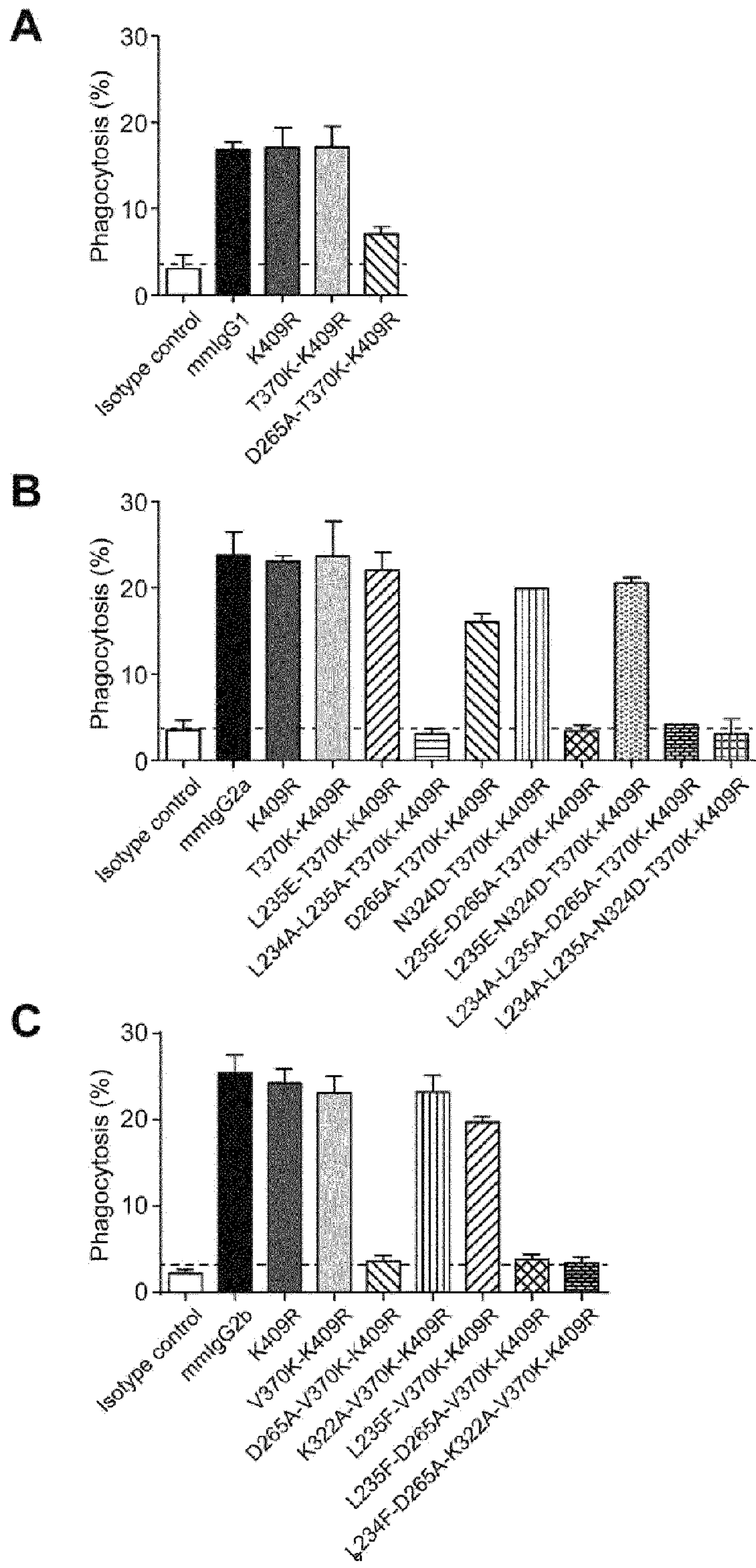


FIG. 9

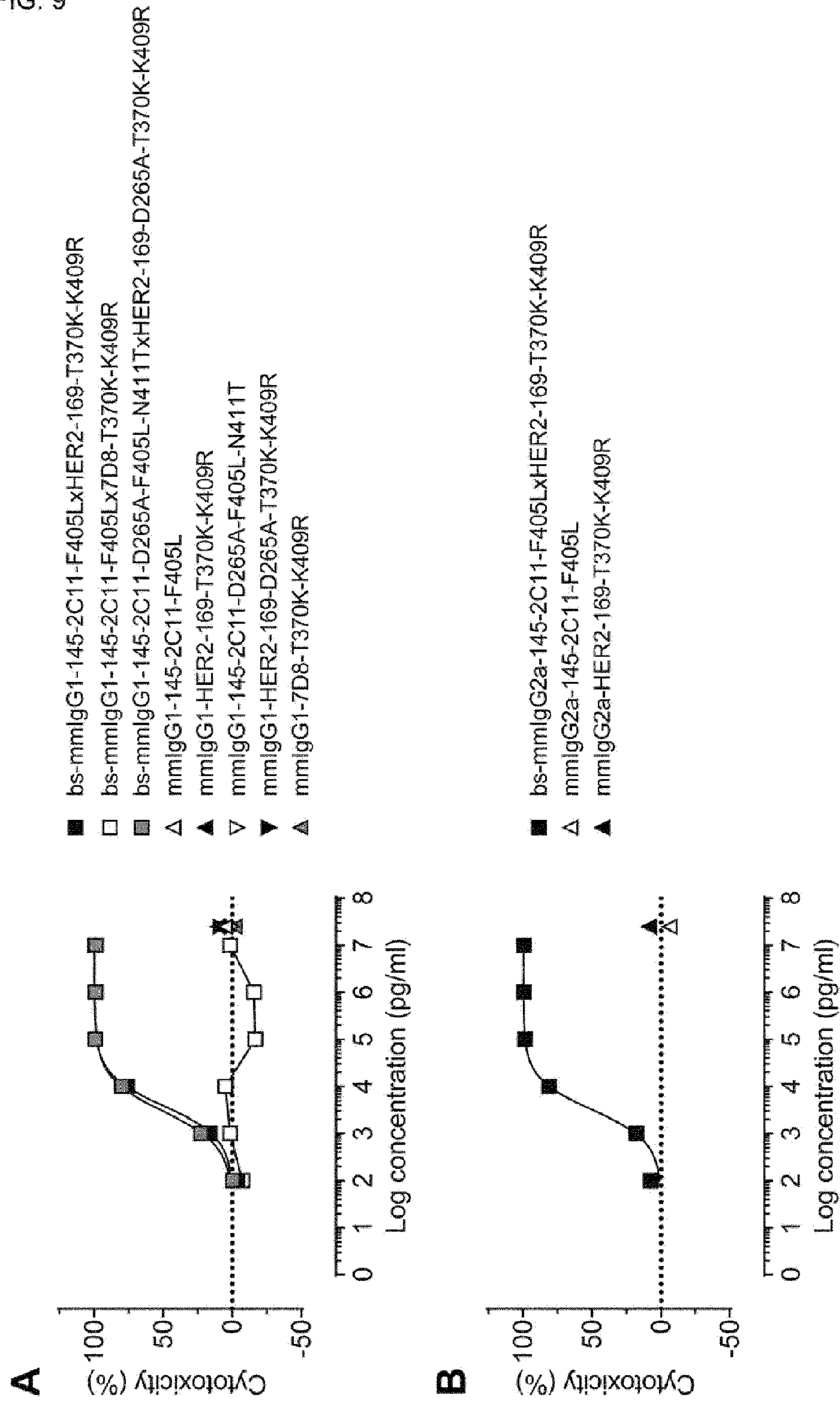


FIG. 10

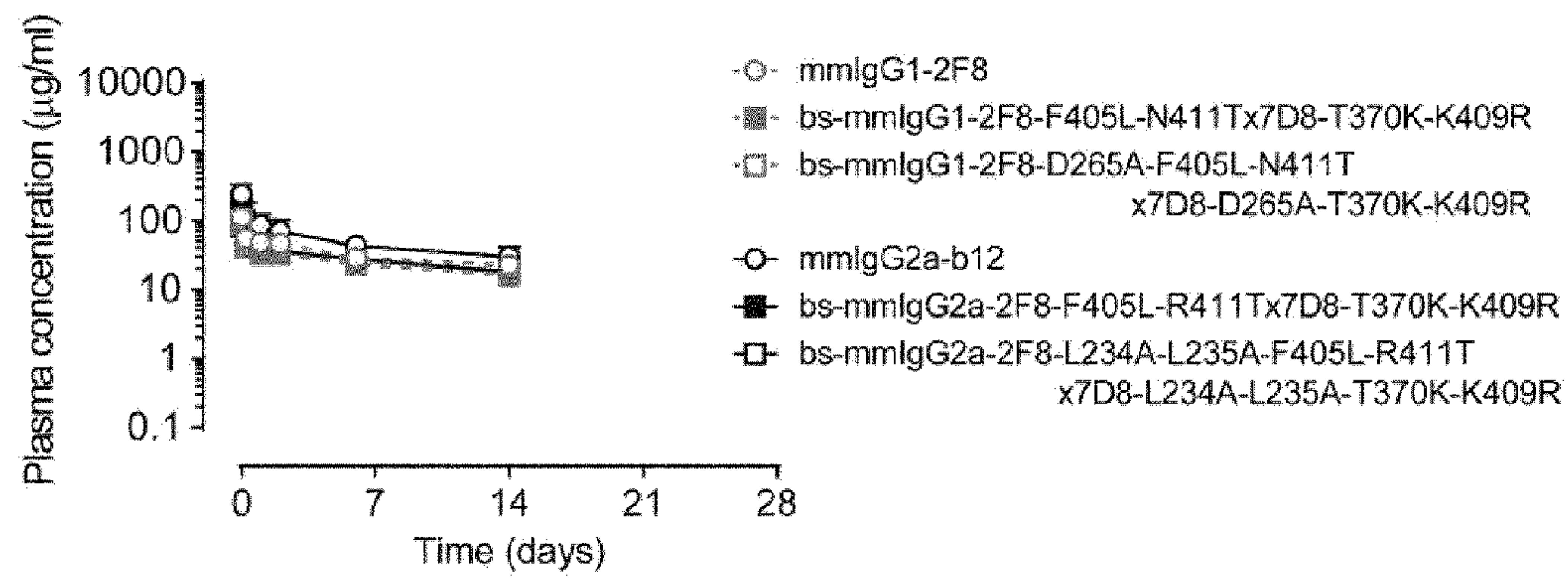
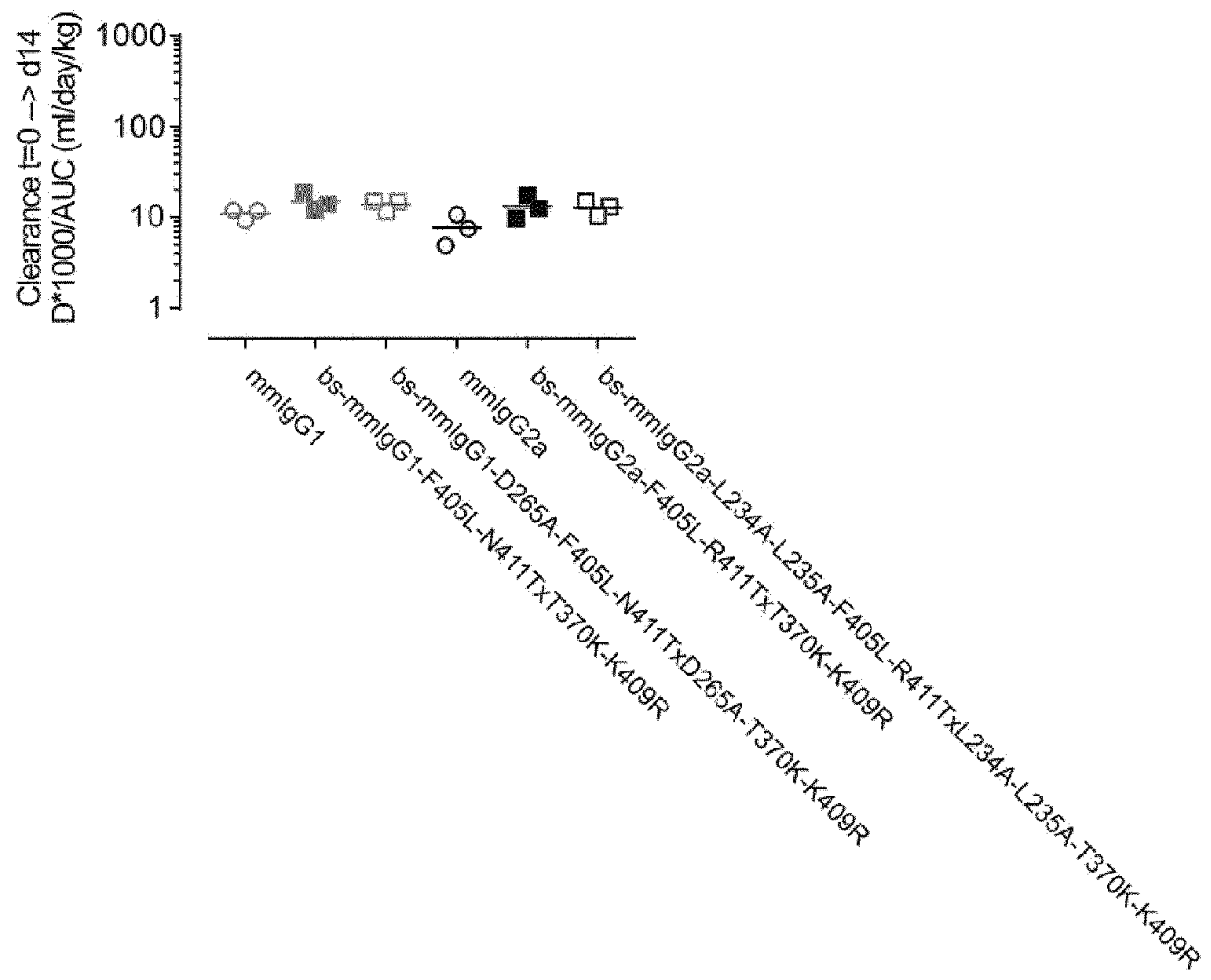
A**B**

FIG. 11

