

Title: Methods and means for the production of Ig-like molecules

FIELD

The invention relates to the fields of molecular biology, medicine and biological therapeutics. It particularly relates to the field of therapeutic antibodies for the treatment of various diseases.

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BACKGROUND

Many currently used biological therapeutics are isolated recombinant, human or humanized monoclonal antibodies that enhance the ability of the body's immune system to neutralize or eliminate cells and/or molecules involved in disease processes or to eradicate invading pathogens or infectious agents. Monoclonal antibodies bind to a single specific area, or epitope, of an antigen and, for use in therapy, are often selected for a desirable functional property such as for example killing of tumor cells, blocking of receptor-ligand interactions or virus neutralization. Nowadays, there are about 30 FDA approved monoclonal antibodies, which are typically produced at large quantities and their biophysical and biochemical characteristics can be analyzed in great detail to ensure batch-to-batch consistency, which facilitates regulatory acceptability. Despite these favorable characteristics, monoclonal antibodies have several disadvantages, some of which relate to their monospecific nature and the complexity of diseases. Diseases processes are often multifactorial in nature, and involve redundant or synergistic action of disease mediators or up-regulation of different receptors, including crosstalk between their signaling networks. Consequently, blockade of multiple, different factors and pathways involved in pathology may result in improved therapeutic efficacy. By nature of their monospecificity, monoclonal antibodies can only interfere with a single step within the complex disease processes which often does not have an optimal effect. In addition to not fully addressing multiple aspects of a disease process, it has become clear that targeting a single epitope on a single cellular or soluble protein or pathogen often will not suffice to efficiently treat disease because the target epitope may no longer be available for the monoclonal antibody to bind to and exert the desired effect. As an example, tumor cells often escape from monoclonal antibody therapy by down-

regulation, mutation or shielding of the target epitope present on a growth factor receptor. By activating alternative receptors and/or their ligands, tumor cells than may exploit a different path leading to continued growth and metastasis. Similarly, viruses and other pathogens frequently mutate and lose or shield the target epitope, thereby escaping monoclonal antibody treatment. Monoclonal antibodies that bind to a single epitope often do not recruit the full spectrum of effector mechanisms evoked by polyclonal antibodies, including, amongst other things, opsonization (enhancing phagocytosis of antigens), steric hindrance (antigens coated with antibodies are prevented from attaching to host cells or mucosal surfaces), toxin neutralization, agglutination or precipitation (antibodies binding several soluble antigens cause aggregation and subsequent clearance), activation of complement and antibody-dependent cellular cytotoxicity (antibodies enable the killing of target cells by natural killer cells and neutrophils).

15 Polyclonal antibodies for therapeutic applications may be obtained from pooled human serum. Such serum-derived therapeutic polyclonal antibodies may for example be used to treat or prevent infections caused by viruses such as the rabies virus, cytomegalovirus and respiratory syncytial virus, to neutralize toxins such as tetanus toxin and botulinum toxin or to prevent Rhesus D allograft immunization. A more

20 widespread use of serum-derived polyclonal antibody preparations has been prevented by the fact that source plasma is only available for a limited range of targets such as infectious diseases and toxins. Moreover, the products are highly dependent on donor blood availability, both in terms of quantity and suitability, resulting in considerable variation between batches. In addition, screening technologies fail to keep up with

25 constantly evolving viruses, thus, immunoglobulin products carry a potential risk of infectious disease transmission. Finally, the long process of blood collection, screening and immunoglobulin purification means plasma-derived immunoglobulins are expensive to produce.

30 Mixtures of monoclonal antibodies may improve the efficacy of monoclonal antibodies while avoiding the limitations associated with serum-derived polyclonal antibodies. In the art, combinations of two human or humanized monoclonal antibodies have been tested in preclinical models and in clinical trials (for example mixtures of 2

monoclonal antibodies against the HER2 receptor, mixtures of 2 antibodies against the EGFR receptor and , 2 monoclonal antibodies against the rabies virus). In the art, it has been shown that combinations of 2 monoclonal antibodies may have additive or synergistic effects and recruit effector mechanisms that are not associated with either antibody alone. For example, mixtures of 2 monoclonal antibodies against the EGFR or HER2 were shown to more potently kill tumor cells based on a combination of activities including enhanced receptor internalization, improved blockade of signalling pathways downstream of the receptors as well as enhanced immune effector-mediated cytotoxicity. For combination therapies based on 2 monoclonal antibodies, the component antibodies may be produced separately and combined at the protein level. A drawback of this approach is the staggering cost of developing the 2 antibodies individually in clinical trials and (partially) repeating that process with the combination. This would lead to unacceptable cost of treatments based on antibody combinations. Alternatively, the 2 recombinant cell lines producing the component monoclonal antibodies may be mixed in a fermentor and the resultant mixture of antibodies may be purified as a single preparation (WO 2004/061104). A drawback of this approach is the poor control over the composition and hence reproducibility of the resulting recombinant polyclonal antibody preparation, especially when considering that such compositions may change over time as the cells are being cultured.

During the past decade, bispecific antibodies have emerged as an alternative to the use of combinations of 2 antibodies. Whereas a combination of 2 antibodies represents a mixture of 2 different immunoglobulin molecules that bind to different epitopes on the same or different targets, in a bispecific antibody this is achieved through a single immunoglobulin molecule. By binding to 2 epitopes on the same or different targets, bispecific antibodies may have similar effects as compared to a combination of 2 antibodies binding to the same epitopes. Furthermore, since bispecific antibodies of the IgG format combine 2 different monovalent binding regions in a single molecule and mixtures of 2 IgG antibodies combine 2 different bivalent binding molecules in a single preparation, different effects of these formats have been observed as well. From a technological and regulatory perspective, this makes development of a single bispecific antibody less complex because manufacturing,

preclinical and clinical testing involve a single, molecule. Thus, therapies based on a single bispecific antibody are facilitated by a less complicated and cost-effective drug development process while providing more efficacious antibody therapies.

5 Bispecific antibodies based on the IgG format, consisting of 2 heavy and two light chains have been produced by a variety of methods. For instance, bispecific antibodies may be produced by fusing two antibody-secreting cell lines to create a new cell line or by expressing two antibodies in a single cell using recombinant DNA technology. These approaches yield multiple antibody species as the respective heavy chains from
10 each antibody may form monospecific dimers (also called homodimers), which contain two identical paired heavy chains with the same specificity, and bispecific dimers (also called heterodimers) which contain two different paired heavy chains with different specificity. In addition, light chains and heavy chains from each antibody may randomly pair to form inappropriate, non-functional combinations. This problem,
15 known as heavy and light chain miss-pairings, can be solved by choosing antibodies that share a common light chain for expression as bispecific. But even when a common light chain is used, expression of two heavy chains and one common light chain in a single cell will result in 3 different antibody species, i.e. two monospecific 'parental' antibodies and the bispecific antibody so that the bispecific antibody of
20 interest needs to be purified from the resulting antibody mixture. Although technologies have been employed to further increase the percentage of bispecific antibodies in the mixtures of parental and bispecific antibodies and to decrease the percentage of miss-paired heavy and light chains, there remains a need for bispecific formats that eliminate or minimize some of the disadvantages mentioned above.

25 Taken together, the art provides a variety of technologies and methods for generating monoclonal antibodies, bispecific antibodies, mixtures of monoclonal antibodies, or mixtures of monospecific and bispecific antibodies that can subsequently be used for therapeutic application in patients. However, as discussed above, each of these
30 existing technologies and methods have their drawbacks and limitations. There is thus a need for improved and/or alternative technologies for producing biological therapeutics in the form of mixtures or bispecific approaches for targeting multiple disease-modifying molecules

DESCRIPTION OF THE INVENTION

5 The invention provides methods and means for improved and/or alternative technologies for producing biological therapeutics in the form of mixtures or bispecific approaches for targeting multiple disease-modifying molecules, as well as products and uses resulting from these methods and means.

Various approaches are described in the art in order to promote the formation of a
10 certain bispecific antibody of interest, thereby reducing the content of undesired antibodies in the resulting mixture.

For antibodies, it is well-known that the CH3-CH3 interaction is the primary driver for Fc dimerization (Ellerson JR., et al., J. Immunol 1976 (116) 510-517; Deisenhofer J. biochemistry 1981 (20) 2361-2370). It is furthermore well-known that when two
15 CH3 domains interact with each other they meet in a protein-protein interface which comprises "contact" residues (also called contact amino acids, interface residues or interface amino acids). Contact amino acids of a first CH3 domain interact with one or more contact amino acids of a second CH3 domain. Contact amino acids are typically within 5.5 Å (preferably within 4.5 Å) of each other in the three-dimensional structure
20 of an antibody. The interaction between contact residues from one CH3 domain and contact residues from a different CH3 domain may for instance be via Van der Waals forces, hydrogen bonds, water-mediated hydrogen bonds, salt bridges or other electrostatic forces, attractive interactions between aromatic side chains, disulfide bonds, or other forces known to one skilled in the art. It was previously shown that
25 approximately one-third of the contact amino acid side chains at the human IgG1 CH3 domain interface can account for the majority of contributions to domain folding and association. It can further be envisaged that other (neighbouring) amino acid residues may affect the interactions in the protein-protein interface.

30 Approaches to interfere with the dimerization of antibody heavy chains have been employed in the art. Specific engineering in the CH3 domains was applied in order to favour heterodimerization over homodimerization. Examples of such engineering of the CH3-CH3 interface include the introduction of complementary protuberance and

cavity mutations, also known as 'knob-into-hole' approaches as described for instance in WO1998/050431, Ridgeway et al., 1996 and Merchant et al. 1998.

Generally, the method involves introducing a protuberance at the interface of a first polypeptide and a corresponding cavity in the interface of a second polypeptide, such
5 that the protuberance can be positioned in the cavity so as to promote heteromultimer formation and hinder homomultimer formation. "Protuberances" or "knobs" are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" or "holes" of identical or similar size to the protuberances are created in the
10 interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). The protuberance and cavity can be made by synthetic means such as altering the nucleic acid encoding the polypeptides or by peptide synthesis.

Using the knob-into-hole technology alone, the proportion of a bispecific antibody of
15 interest is at best 87% of the mixture of the 2 parental and bispecific antibodies. Merchant et al., succeeded in raising the proportion of bispecific antibodies to 95% of the mixture by introduction of an additional disulfide bond between the two CH3 domains in the CH3-CH3 interface. Still, in order to use such bispecific antibody as a medicament, the bispecific antibody has to be purified (separated) from the
20 homodimers and formulated into a pharmaceutically acceptable diluent or excipient. Purification of heterodimers from such mixtures poses a major challenge because of the similarity in physico-chemical properties of the homodimers and heterodimers. It is one object of the present invention to provide methods for producing a bispecific antibody in a single cell clone with a further improved proportion of the bispecific
25 antibody in the mixture. According to the invention, knob-into-hole technology can thus be used as one of the means, alone or together with other means, to achieve said further improved bispecific proportion in a mixture.

Another example of such engineering of the CH3-CH3 interface is provided by a heterodimeric Fc technology that supports the design of bispecific and asymmetric
30 fusion proteins by devising strand-exchange engineered domain (SEED) CH3 heterodimers. These SEED CH3 heterodimers are derivatives of human IgG and IgA CH3 domains that are composed of alternating segments of human IgA and IgG

CH3 sequences which results in pairs of complementary human SEED CH3 heterodimers, the so-called SEED-bodies (Davis JH. Et al., Protein Engineering, Design & Selection 2010(23)195-202; WO2007/110205).

Yet another approach for the production of a given bispecific antibody of interest is
5 based on electrostatic engineering of contact residues within the CH3-CH3 interface that are naturally charged, as for example described in EP01870459 or US2010/0015133, WO2007/147901, WO2010/129304, Gunasekaran et al (2010) and WO 2009/089004. These publications describe mutations in the CH3 domains of heavy chains wherein naturally occurring charged amino acid contact residues are replaced
10 by amino acid residues of opposite charge. This creates an altered charge polarity across the Fc dimer interface such that co-expression of electrostatically matched Fc chains support favorable attractive interactions thereby promoting desired Fc heterodimer formation, whereas unfavorable repulsive charge interactions suppress unwanted Fc homodimer formation.

15 It was described that within the CH3-CH3 interface four unique charges residue pairs are involved in the domain-domain interaction. These are D356/K439', E357/K370', K392/D399' and D399/K409' (numbering according to Kabat (1991) where residues in the first chain are separated from residues in the second chain by '/' and where the prime (') indicates the residue numbering in the second chain). As the
20 CH3-CH3 interface displays a 2-fold symmetry, each unique charge pair is represented twice in intact IgG (i.e., also K439/D356', K370/E357', D399/K392' and K409/D399' charge interactions are present in the interface). Taking advantage of this two-fold symmetry, it was demonstrated that a single charge reversion, e.g. K409D in the first chain, or D399'K in the second chain resulted in diminished homodimer
25 formation due to repulsion of identical charges. Combining different charge reversions further enhanced this repulsive effect. It was demonstrated that expression of different CH3 domains comprising different, complementary charge reversions, could drive heterodimerization, resulting in an increased proportion of the bispecific species in the mixture.

30 Using the approach described above, it is possible to produce a bispecific antibody in a single cell with proportions ranging between about 76% and about 96%. It is an object of the present invention to provide methods for producing a bispecific antibody in a single cell with a further improved percentage of desired bispecific antibodies.

According to the present invention, electrostatic engineering technology can be used as one of the means, alone or together with other means, e.g knob-into-hole approaches, to achieve said further improved percentages of desired (bispecific) antibodies.

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In one aspect, the present invention provides a method for producing at least two different Ig-like molecules from a single host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

- 10 a) a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain,
b) a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,
c) a third nucleic acid molecule encoding a 3rd CH3 domain-comprising polypeptide
15 chain, and
d) a fourth nucleic acid molecule encoding a 4th CH3 domain-comprising polypeptide chain,

wherein at least two of said nucleic acid molecules are provided with means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said
20 3rd and 4th CH3-domain comprising polypeptides, said method further comprising the step of culturing said host cell and allowing for expression of said at least four nucleic acid molecules and harvesting said at least two different Ig-like molecules from the culture.

25 It is often desired to produce more than one (bispecific) antibody, for instance in order to more efficiently interfere with multiple biological pathways involved in a disease process or with the invasion, replication and/or spreading of a pathogen.

A mixture of more than one bispecific antibody is also particularly useful for the
30 treatment of certain diseases. For example, tumor cells use many different strategies to develop resistance during treatment with antibodies or small molecule drugs. Resistance may involve multiple cell surface receptors and soluble molecules and it is considered beneficial to develop antibody-based treatments for cancers that address

multiple such disease- and escape-associated molecules simultaneously. In case more than 2 such disease- and escape-related target molecules or epitopes are involved, a mixture of bispecific antibodies provides an innovative and attractive therapeutic format. Preferably, such mixtures of bispecific antibodies are produced by a single cell
5 to facilitate a drug development process that is less complicated from a regulatory point of view and cost-effective and feasible from a drug manufacturing and clinical development point of view. In a single cell-based approach, it is desirable to use methods that allow controlled and efficient production of the bispecific antibodies, thus reducing or even completely abrogating the need of separating the desired
10 mixture of bispecific IgG molecules from non-desired monospecific IgG molecules. In the prior art, mixtures of monospecific and bispecific antibodies have been produced by a single cell (WO2004/009618), but these mixtures represent complex concoctions of several different bispecific and monospecific antibody species.. It is a further object of the present invention to provide means and methods for producing defined
15 mixtures of bispecific antibodies in single cells. Preferably, methods are provided which result in mixtures of (bispecific) antibodies with a proportion of at least 95%, at least 97% or even more than 99% of dimeric IgG molecules, irrespective of the amount of monomeric by-products, see herein below. Typically, in a cell where multiple intact IgG molecules are produced, half molecules (monomeric by-products) may be present
20 that can be simply removed by size exclusion chromatography known in the art.

The present invention provides methods for producing a defined mixture of at least two different Ig-like molecules in single cells, instead of a single (bispecific) antibody of interest, wherein the formation of other, undesired dimeric antibody species is
25 diminished or even absent. The resulting mixture is well defined and its composition is controlled by the design of CH3 domain mutants. Furthermore, regulation of expression levels and/or different transfection ratios used for expression affects the composition of the mixture. In a method according to the invention, a first nucleic acid molecule encodes a CH3 domain which preferentially pairs with a CH3 domain
30 encoded by a second nucleic acid molecule, and a third nucleic acid molecules encodes a CH3 domain which preferentially pairs with a CH3 domain encoded by a fourth nucleic acid molecule.

As used herein, the term “preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides” means that essentially all the resulting dimers comprising the 1st CH3 domain-comprising polypeptide and/or the 2nd CH3 domain-comprising polypeptide will be dimers consisting of one 1st CH3 domain-comprising polypeptide paired with one 2nd CH3 domain-comprising polypeptide. Likewise, the term “preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides” means that essentially all of the resulting dimers comprising the 3rd CH3 domain-comprising polypeptide and/or the 4th CH3 domain-comprising polypeptide will be dimers consisting of one 3rd CH3 domain-comprising polypeptide paired with one 4th CH3 domain-comprising polypeptide. As a result, when nucleic acid molecules encoding four different (A, B, C, D) CH3 domain-comprising polypeptides are introduced in a single cell, instead of a mixture of 10 different Ig-like dimers (AA, AB, AC, AD, BB, BC, BD, CC, CD and DD), a mixture of predominantly two specific Ig-like molecules is produced.

In a method according to the present invention, each of the CH3-domain comprising polypeptide chains preferably further comprises a variable region recognizing a target epitope. In one particularly preferred embodiment, each of said 4 variable regions of the 4 CH3-domain comprising polypeptide chains recognizes a different target epitope. For instance, if the first nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen A, the second nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen B, the third nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen C, and the fourth nucleic acid molecule encodes a heavy chain that further contains a variable domain with specificity for antigen D, a mixture will then be produced containing bispecific Ig-like molecules that are specific for AB and bispecific Ig-like molecules that are specific for CD. The formation of monospecific antibodies (with AA, BB, CC or DD specificity) or bispecific antibodies with specificity for AC, AD, BC or BD is lowered or even absent due to the means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said 3rd and 4th CH3 domain-comprising polypeptides. It is, of course, possible to use further nucleic acid molecules, for instance encoding a 5th and a 6th CH3 domain-comprising polypeptide, in order to produce defined mixtures comprising more than two different Ig-like molecules.

Of note, the ratio of the nucleic acids used in a method according to the invention does not need to be 1:1:1:1 and the ratio of the resulting Ig-like molecules that are expressed does not need to be 1:1. It is possible to use means known in the art to produce mixtures of antibodies with optimized ratios. For instance, expression levels
5 of nucleic acid molecules and hence the ratios of the resulting Ig-like molecules produced may be regulated by using different genetic elements such as promoters, enhancers and repressors or by controlling the genomic integration site of copy number of the DNA constructs encoding antibodies .

Said means for preferential pairing preferably may comprise engineered
10 complementary knob-into-hole mutations, disulfide bridges, charge mutations or combinations thereof. The skilled person will appreciate that said means for preferential pairing may be chosen within a certain type of mutations, i.e. all at least 4 nucleic acid molecules encoding CH3-domain comprising polypeptide chains may for example comprise charge mutations as means for preferential pairing. Additionally,
15 also non-engineered wildtype CH3 may in certain instances be used for preferential pairing of two wildtype CH3-domain comprising polypeptide chains. In a particularly preferred embodiment, said means for preferential pairing comprise at least one CH3 mutation selected from Table B, as explained elsewhere in this application.

20 One aspect of the present invention provides a method according to the invention, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides are different from said means for preferential pairing of said 3rd and 4th CH3-domain comprising polypeptides. By 'different' it is meant that the means for preferential pairing of said 1st and 2nd CH3 domain comprising polypeptides
25 are designed such that preferential pairing of the 1st and 2nd chain is favoured. The design is such that essentially no interaction between the 1st and the 3rd and/or 4th CH3 domain comprising polypeptide chain will take place. In other words, dimerization between said 1st CH3 domain comprising polypeptide and said 3rd or 4th polypeptide is reduced to essentially zero and so forth. The 3rd and the 4th CH3
30 domain-comprising polypeptides may either be wildtype or may comprise means for preferential pairing that are different from the means for preferential pairing of the 1st and 2nd CH3 domains. Current studies have focused on the production of a single bispecific antibody, using for instance the knob-into-hole technology or mutations of

charged contact amino acids present in CH3 domains. Production of defined mixtures of at least two (bispecific) Ig-like molecules, without significant co-production of other dimeric by-products, has, however, not been realized prior to the present invention. The present invention provides methods for the efficient and controlled production of
5 a well-defined mixture of Ig-like molecules, with a high proportion of bispecifics in the mixture. Even a proportion of (two) bispecifics of at least 95%, at least 97% or more is obtained in a system where two bispecifics are desired. This means that only at most 5%, at most 3% or less monospecific bivalent by-products are obtained. Of note, the amount of monomeric by-products, i.e. half molecules, is less important since these
10 half-molecules are easily separated from dimers using their size difference.

In another preferred embodiment, the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize different target epitopes, whereas the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains
15 recognize the same target epitopes. This will result in the predominant production of one kind of bispecific Ig-like molecule and one kind of monospecific Ig-like molecule. For instance, if the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize different target epitopes and if the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains both recognize the
20 same target epitope which is different from the target epitopes recognized by the 1st and the 2nd CH3-domains, a mixture of Ig-like molecules having specificity for AB or CC will be formed. Further provided is therefore a method according to the invention, wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is the same, but different from the target
25 epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain.

Alternatively, when the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize different target epitopes and when the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains both recognize the
30 same epitope as the 1st or the 2nd CH3-domain comprising polypeptide chains, a mixture of Ig-like molecules having specificity for AB and AA, or AB and BB will be formed. A method according to the invention, wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is

the same as the target epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain is therefore also herewith provided.

It is another object of the present invention to provide means and methods for
5 producing defined mixtures of bispecific antibodies and monospecific antibodies in a single cell culture. A non-limiting example of such well-defined mixture is a mixture of bispecific antibodies with specificity AB and monospecific antibodies with specificity AA. Another example is a mixture of bispecific antibodies with specificity AB and monospecific antibodies with specificity BB. Yet another example is a mixture of
10 bispecific antibodies with specificity AB and monospecific antibodies with specificity CC. Again, preferably means and methods are provided which yield mixtures of antibodies of interest with at least 90%, more preferably at least 95% and most preferably at least 97% or even more than 99% of desired antibodies.

15 In yet another embodiment, a method according to the invention is provided wherein the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize the same target epitope, whereas the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains recognize a second target epitope which differs from the target epitope recognized by said 1st and 2nd variable regions. This
20 will result in the predominant production of monospecific Ig-like molecules having either specificity for AA or specificity for BB. The formation of bispecific Ig-like molecules is diminished or even avoided. In several embodiments it is preferred to produce mixtures of monospecific antibodies in a single cell, rather than mixtures of bispecific antibodies. For instance when cross-linking of two identical target molecules
25 is desired, or when two targets are located too far away from each other so that they cannot be bound by a single bispecific antibody. It can also be advantageous to produce mixtures of monospecific antibodies in a single cell as the mixture can be regarded as a single therapeutic product. In the art, the therapeutic efficacy and safety of various monospecific antibodies has already been proven and market
30 authorisation has been obtained. Production of mixtures of monospecific antibodies in a single cell will thus facilitate the testing for efficacy and safety of several of such mixtures and will reduce the efforts and costs for regulatory approval and manufacturing. There are, however, currently no methods available for producing

specific mixtures of monospecific antibodies in a single cell wherein the formation of bispecific by-products is reduced to below 5%. It is another object of the present invention to provide means and methods for producing such well-defined homodimeric antibody mixtures in single cells wherein the formation of bispecific antibodies is
5 reduced to below 5%.

Hence, a method according to the present invention is suitable for the production of any desired mixture of bispecific and/or monospecific Ig-like molecules. Again, it is possible to use further nucleic acid molecules, for instance encoding a 5th and a 6th
10 (and 7th and 8th and so forth) CH3 domain-comprising polypeptide, in order to produce defined mixtures comprising more than two different Ig-like molecules.

Preferably, in a method according to the present invention at least two CH3 domains are used that comprise at least one combination of mutations provided by the present
15 invention. Through these mutations novel specific interactions are formed between two CH3 domains. These mutations according to the present invention are discussed below in more detail.

The term 'Ig-like molecule' as used herein means a proteinaceous molecule that
20 possesses at least one immunoglobulin (Ig) domain. Said Ig-like molecule comprises a sequence comprising the function of at least an immunoglobulin CH3 domain, preferably the sequence comprises an IgG1 CH3 domain. In a more preferred embodiment, said Ig-like molecule comprises a full length Fc backbone. In a most preferred embodiment, the Ig-like molecules are antibodies. The term 'antibody' as
25 used herein means a proteinaceous molecule belonging to the immunoglobulin class of proteins, containing one or more domains that bind an epitope on an antigen, where such domains are derived from or share sequence homology with the variable region of an antibody. Antibodies are known in the art and include several isotypes, such as IgG1, IgG2, IgG3, IgG4, IgA, IgD, IgE, and IgM. An antibody according to the
30 invention may be any of these isotypes, or a functional derivative and/or fragment of these. In a preferred embodiment, Ig-like molecules are produced that are antibodies of the IgG isotype because IgG antibodies e.g. have a longer half life as compared to antibodies of other isotypes.

Antibodies produced with methods according to the present invention can have sequences of any origin, including murine and human sequences. Antibodies can consist of sequences from one origin only, such as fully human antibodies, or they can have sequences of more than one origin, resulting for instance in chimeric or
5 humanized antibodies. Antibodies for therapeutic use are preferably as close to natural antibodies of the subject to be treated as possible (for instance human antibodies for human subjects). Antibody binding can be expressed in terms of specificity and affinity. The specificity determines which antigen or epitope thereof is bound by the binding domain. The affinity is a measure for the strength of binding to
10 a particular antigen or epitope. Specific binding is defined as binding with affinities (K_D) of at least 1×10^{-5} M, more preferably 1×10^{-7} M, more preferably higher than 1×10^{-9} M. Typically, monoclonal antibodies for therapeutic applications have affinities of up to 1×10^{-10} M or even higher. The term 'antigen' as used herein means a substance or molecule that, when introduced into the body, triggers the production of an antibody
15 by the immune system. An antigen, among others, may be derived from pathogenic organisms, tumor cells or other aberrant cells, from haptens, or even from self structures. At the molecular level, an antigen is characterized by its ability to be bound by the antigen-binding site of an antibody. Also mixtures of antigens can be regarded as 'antigen', i.e. the skilled person would appreciate that sometimes a lysate
20 of tumor cells, or viral particles may be indicated as 'antigen' whereas such tumor cell lysate or viral particle preparation exists of many antigenic determinants. An antigen comprises at least one, but often more, epitopes. The term 'epitope' as used herein means a part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. Although epitopes are usually thought to be derived
25 from non-self proteins, sequences derived from the host that can be recognized are also classified as epitopes.

The term 'CH3 domain' is well known in the art. The IgG structure has four chains, two light and two heavy chains; each light chain has two domains, the variable and
30 the constant light chain (VL and CL) and each heavy chain has four domains, the variable heavy chain (VH) and three constant heavy chain domains (CH1, CH2, CH3). The CH2 and CH3 domain region of the heavy chain is called Fc (Fragment crystallizable) portion, Fc fragment, Fc backbone or simply Fc. The IgG molecule is a

heterotetramer having two heavy chains that are held together by disulfide bonds (-S-S-) at the hinge region and two light chains. The heavy chains dimerize through interactions at the CH3-CH3 domain interface and through interactions at the hinge region. The number of hinge disulfide bonds varies among the immunoglobulin subclasses (Papadea and Check 1989). The Fc fragment of an immunoglobulin molecule is a dimer of the two C-terminal constant regions, i.e. CH2 and CH3 domains, of the heavy chain. Among its physiological functions are interactions with the complement system and with specific receptors on the surface of a variety of cells. Interactions between the CH3 domains of two individual heavy chains are known to play an important role in driving heavy chain dimerization. Thus, CH3 domains direct the association of antibody heavy chains, and it is known that the interface between CH3 domains contains more than 20 contact residues from each chain that play a role in the CH3-CH3 interaction (Deisenhofer J., *Biochemistry* 1981(20)2361-2370; Miller S., *J. Mol. Biol.* 1990(216)965-973; Padlan, *Advances in Protein Chemistry* 1996 (49) 57-133).

The terms 'contact residue', 'contact amino acid', 'interface residue' and 'interface amino acid' as used herein typically refers to any amino acid residue present in the CH3 domain that can be involved in interdomain contacts, as can be calculated by technologies known in the art, including calculating solvent accessible surface area (ASA) of the CH3 domain residues in the presence and absence of the second chain (Lee and Richards *J. Mol. Biol.* 1971(55)379) where residues that show difference ($> 1\text{\AA}^2$) in ASA between the two calculations are identified as contact residues. Contact residues that have been identified include residues at positions 347, 349, 350, 351, 352, 353, 354, 355, 356, 357, 360, 364, 366, 368, 370, 390, 392, 394, 395, 397, 399, 400, 405, 407, 409, 439 according to the EU numbering system (Table A).

Table A: List of CH3 domain interface residues

Interface residue in chain A	Contacting residues in chain B
Q347	K360
Y349	S354, D356, E357, K360
T350	S354, R355
L351	L351, P352, P353, S354, T366
S354	Y349, T350, L351
R355	T350
D356	Y349, K439
E357	Y349, K370
K360	Q347, Y349
S364	L368, K370
T366	L351, Y407
L368	S364, K409
K370	E357, S364
N390	S400
K392	L398, D399, S400, F405
T394	T394, V397, F405, Y407
P395	V397
V397	T394, P395
D399	K392, K409
S400	N390, K392
F405	K392, T394, K409
Y407	T366, T394, Y407, K409
K409	L368, D399, F405, Y407
K439	D356

Contact residues within the CH3-CH3 interface can either be amino acids that are charged, or amino acid residues that are neutral. The term ‘charged amino acid residue’ or ‘charged residue’ as used herein means amino acid residues with electrically charged side chains. These can either be positively charged side chains, such as present in arginine (Arg, R), histidine (His, H) and lysine (Lys, K) or can be negatively charged side chains, such as present in aspartic acid (Asp, D) and glutamic acid (Glu, E). The term ‘neutral amino acid residue’ or neutral residue as used herein refers to all other amino acids that do not carry electrically charged side chains. These neutral residues include serine (Ser, S), threonine (Thr, T), asparagine (Asn, N), glutamine (Glu, Q), Cysteine (Cys, C), glycine (Gly, G), proline (Pro, P), alanine (Ala, A), valine (Val, V), isoleucine (Ile, I), leucine (Leu, L), methionine (Met, M), phenylalanine (Phe, F), tyrosine (Tyr, Y), and tryptophan (Trp, T).

The term ‘CH3-CH3 domain interface’, or ‘CH3 interface’, ‘CH3-CH3 pairing’, ‘domain interface’ or simply ‘interface’, as used herein, refers to the association between two

CH3 domains of separate CH3-domain comprising polypeptides that is a result of interacting amino acid residues, i.e. at least one interaction between an amino acid of a first CH3 domain and an amino acid of a second CH3 domain. Such interaction is for instance via Van der Waals forces, hydrogen bonds, water-mediated hydrogen bonds, salt bridges or other electrostatic forces, attractive interactions between aromatic side chains, the formation of disulfide bonds, or other forces known to one skilled in the art.

As used herein, said means for preferential pairing of the first and second CH3 domain-comprising polypeptides and said third and fourth CH3 domain-comprising polypeptide can be any means known in the art. In one embodiment, at least one nucleic acid molecule encodes a CH3 domain which contains at a contact residue position a large amino acid residue (i.e. a “knob” or “protuberance”) such as for instance R, F, Y, W, I or L, whereas at least one other nucleic acid molecule encodes a CH3 domain which contains at a complementary contact residue position a small amino acid residue (i.e. a “hole” or “cavity”) such as for instance G, A, S, T or V. The resulting CH3 domains will preferentially pair with each other due to the steric conformation of said contact amino acids. The knob-into-hole technology is described herein before in more detail. In a further embodiment of the present invention, at least one nucleic acid molecule encodes a CH3 domain which contains at a contact residue position that is naturally charged, i.e. a naturally occurring K, H, R, D or E, an amino acid that now carries the opposite charge as compared to wildtype, whereas at least one other nucleic acid molecule encodes a CH3 domain which contains at a complementary contact residue position that is naturally charged, an amino acid that now carries the opposite charge as compared to wildtype. The resulting engineered CH3 domains will preferentially pair with each other due to the opposite charges of said contact amino acids, whereas pairing of identical CH3 domains will be diminished due to electrostatic repulsion. In one embodiment, CH3 mutations as described in EP01870459, WO 2009/089004, Gunasekaran et al (2010), are used. In one embodiment, the means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides are “knob” and “hole” amino acid residues and the means for preferential pairing of said 3th and 4th CH3 domain-comprising polypeptides are charge-engineered amino acids. Preferably, both said means for preferential pairing of

said 1st and 2nd CH3 domain-comprising polypeptides and said 3th and 4th CH3 domain-comprising polypeptides are charge-engineered amino acids. In one embodiment, different amino acid residues are engineered for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides as compared to the amino acid residues that are engineered for preferential pairing of said 3th and 4th CH3 domain-comprising polypeptides. In a particularly preferred embodiment at least a first and a second nucleic acid molecule encode CH3 domains with novel mutations as provided by the present invention. As described herein below in more detail, the present invention provides novel CH3 mutations which enable the production of certain bispecific Ig-like molecules of interest without a significant amount of undesired (dimeric) by-products. The present invention also provides novel CH3 mutations which enable the production of certain monospecific Ig-like molecules of interest without a significant amount of undesired (dimeric) by-products. The use of at least one of these CH3 mutations according to the present invention is, therefore, preferred.

The term 'polypeptide', 'polypeptide molecule' or 'polypeptide chain' as used herein refers to a chain of amino acids that are covalently joined together through peptide bonds. Proteins are typically made up of one or more polypeptide molecules. One end of every polypeptide, called the amino terminal or N-terminal, has a free amino group. The other end, with its free carboxyl group, is called the carboxyl terminal or C-terminal. Polypeptides according to the present invention may have gone through post-translational modification processes and may e.g. be glycosylated. The CH3 domain-comprising polypeptide chains of the present invention thus refer to polypeptide chains that at least encompass an Ig CH3 domain and that may have gone through post-translational modification processes.

The term "nucleic acid molecule" as used herein is defined as a molecule comprising a chain of nucleotides, more preferably DNA and/or RNA. In one embodiment, double-stranded RNA is used. In other embodiments a nucleic acid molecule of the invention comprises other kinds of nucleic acid structures such as for instance a DNA/RNA helix, peptide nucleic acid (PNA), locked nucleic acid (LNA) and/or a ribozyme. Hence, the term "nucleic acid molecule" also encompasses a chain comprising non-natural

nucleotides, modified nucleotides and/or non-nucleotide building blocks which exhibit the same function as natural nucleotides.

A "host cell" according to the invention may be any host cell capable of expressing
5 recombinant DNA molecules, including bacteria such as for instance *Escherichia* (e.g. *E. coli*), *Enterobacter*, *Salmonella*, *Bacillus*, *Pseudomonas*, *Streptomyces*, yeasts such as *S. cerevisiae*, *K. lactis*, *P. pastoris*, *Candida*, or *Yarrowia*, filamentous fungi such as *Neurospora*, *Aspergillus oryzae*, *Aspergillus nidulans* and *Aspergillus niger*, insect cells such as *Spodoptera frugiperda* SF-9 or SF-21 cells, and preferably mammalian
10 cells such as Chinese hamster ovary (CHO) cells, BHK cells, mouse cells including SP2/0 cells and NS-0 myeloma cells, primate cells such as COS and Vero cells, MDCK cells, BRL 3A cells, hybridomas, tumor-cells, immortalized primary cells, human cells such as W138, HepG2, HeLa, HEK293, HT1080 or embryonic retina cells such as PER. C6, and the like. Often, the expression system of choice will involve a
15 mammalian cell expression vector and host so that the antibodies can be appropriately glycosylated. A human cell line, preferably PER.C6, can advantageously be used to obtain antibodies with a completely human glycosylation pattern. The conditions for growing or multiplying cells (see e. g. Tissue Culture, Academic Press, Kruse and Paterson, editors (1973)) and the conditions for expression of the
20 recombinant product may differ somewhat, and optimization of the process is usually performed to increase the product proportions and/or growth of the cells with respect to each other, according to methods generally known to the person skilled in the art. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell
25 Biotechnology: a Practical Approach (M. Butler, ed., IRL Press, 1991). Expression of antibodies in recombinant host cells has been extensively described in the art (see e.g. EP0120694; EP0314161; EP0481790; EP0523949; US patent 4,816,567; WO 00/63403). The nucleic acid molecules encoding the light and heavy chains may be present as extrachromosomal copies and/or stably integrated into the chromosome of
30 the host cell, the latter is preferred.

To obtain expression of nucleic acid sequences encoding the CH3 domain-comprising polypeptides, it is well known to those skilled in the art that sequences capable of

driving such expression can be functionally linked to the nucleic acid sequences encoding the CH3 domain-comprising polypeptides. Functionally linked is meant to describe that the nucleic acid sequences encoding the CH3 domain-comprising polypeptides or precursors thereof is linked to the sequences capable of driving
5 expression such that these sequences can drive expression of the CH3 domain-comprising polypeptides or precursors thereof. Useful expression vectors are available in the art, e.g. the pcDNA vector series of Invitrogen. Where the sequence encoding the polypeptide of interest is properly inserted with reference to sequences governing the transcription and translation of the encoded polypeptide, the resulting expression
10 cassette is useful to produce the polypeptide of interest, referred to as expression. Sequences driving expression may include promoters, enhancers and the like, and combinations thereof. These should be capable of functioning in the host cell, thereby driving expression of the nucleic acid sequences that are functionally linked to them. Promoters can be constitutive or regulated, and can be obtained from various sources,
15 including viruses, prokaryotic, or eukaryotic sources, or artificially designed. Expression of nucleic acids of interest may be from the natural promoter or derivative thereof or from an entirely heterologous promoter. Some well-known and much used promoters for expression in eukaryotic cells comprise promoters derived from viruses, such as adenovirus, e.g. the E1A promoter, promoters derived from cytomegalovirus
20 (CMV), such as the CMV immediate early (IE) promoter, promoters derived from Simian Virus 40 (SV40), and the like. Suitable promoters can also be derived from eukaryotic cells, such as methallothionein (MT) promoters, elongation factor 1 α (EF-1 α) promoter, actin promoter, an immunoglobulin promoter, heat shock promoters, and the like. Any promoter or enhancer/promoter capable of driving expression of the
25 sequence of interest in the host cell is suitable in the invention. In one embodiment the sequence capable of driving expression comprises a region from a CMV promoter, preferably the region comprising nucleotides -735 to +95 of the CMV immediate early gene enhancer/promoter. The skilled artisan will be aware that the expression sequences used in the invention may suitably be combined with elements that can
30 stabilize or enhance expression, such as insulators, matrix attachment regions, STAR elements (WO 03/004704), and the like. This may enhance the stability and/or levels of expression.

Protein production in recombinant host cells has been extensively described, e.g. in Current Protocols in Protein Science, 1995, Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT, ISBN 0-471-11184-8; Bendig, 1988. Culturing a cell is done to enable it to metabolize, and/or grow and/or divide and/or produce recombinant

5 proteins of interest. This can be accomplished by methods well known to persons skilled in the art, and includes but is not limited to providing nutrients for the cell. The methods comprise growth adhering to surfaces, growth in suspension, or combinations thereof. Several culturing conditions can be optimized by methods well known in the art to optimize protein production yields. Culturing can be done for

10 instance in dishes, roller bottles or in bioreactors, using batch, fed-batch, continuous systems, hollow fiber, and the like. In order to achieve large scale (continuous) production of recombinant proteins through cell culture it is preferred in the art to have cells capable of growing in suspension, and it is preferred to have cells capable of being cultured in the absence of animal- or human-derived serum or animal- or

15 human-derived serum components. Thus purification is easier and safety is enhanced due to the absence of additional animal or human proteins derived from the culture medium, while the system is also very reliable as synthetic media are the best in reproducibility.

20 Ig-like molecules are expressed in host cells and are harvested from the cells or, preferably, from the cell culture medium by methods that are generally known to the person skilled in the art. After harvesting, these Ig-like molecules may be purified by using methods known in the art. Such methods may include precipitation, centrifugation, filtration, size-exclusion chromatography, affinity chromatography,

25 cation- and/or anion-exchange chromatography, hydrophobic interaction chromatography, and the like. For a mixture of antibodies comprising IgG molecules, protein A or protein G affinity chromatography can be suitably used (see e.g. US patents 4,801,687 and 5,151,504).

30 Ig-like molecules, and/or mixtures thereof, produced with methods according to the present invention preferably have a common light chain. Further provided is, therefore, a method according to the invention, further comprising providing said host cell with a nucleic acid molecule encoding a common light chain. This is a light chain

that is capable of pairing with at least two different heavy chains, thereby forming functional antigen binding domains. A functional antigen binding domain is capable of specifically binding to an antigen. Preferably, a common light chain is used that is capable of pairing with all heavy chains produced with a method according to the invention, thereby forming functional antigen binding domains, so that mispairing of unmatched heavy and light chains is avoided. In one aspect, only common light chains with one identical amino acid sequence are used. Alternatively, those of skill in the art will recognize that “common” also refers to functional equivalents of the light chain of which the amino acid sequence is not identical. Many variants of said light chain exist wherein mutations (deletions, substitutions, additions) are present that do not materially influence the formation of functional binding regions. Such variants are thus also capable of binding different heavy chains and forming functional antigen binding domains. The term ‘common light chain’ as used herein thus refers to light chains which may be identical or have some amino acid sequence differences while retaining the binding specificity of the resulting antibody after pairing with a heavy chain. It is for instance possible to prepare or find light chains that are not identical but still functionally equivalent, e.g. by introducing and testing conservative amino acid changes, and/or changes of amino acids in regions that do not or only partly contribute to binding specificity when paired with the heavy chain, and the like. A combination of a certain common light chain and such functionally equivalent variants is encompassed within the term “common light chain”. Reference is made to WO 2004/009618 for a detailed description of the use of common light chains. Alternatively, the skilled person may select, as an alternative to using a common light chain and to avoid mispairing of unmatched heavy and light chains, means for forced pairing of the heavy and light chain, such as for example described in WO2009/080251, WO2009/080252 and/or WO2009/080253.

The present invention provides novel engineered CH3 domains as well as novel combinations of CH3 mutations. Before the present invention, charged contact amino acids of CH3 domains that were known to be involved in CH3-CH3 pairing were substituted by amino acids of opposite charge, thereby influencing the CH3-CH3 pairing. The mutations according to the present invention are an inventive alternative to this approach, because now CH3 amino acids that are non-charged or

neutral in wildtype CH3 are substituted with charged residues. The present invention in this embodiment does not exchange charged contact amino acids by amino acids of opposite charge but substitutes non-charged CH3 amino acids for charged ones. The approach of the present invention provides not only a method for efficiently steering
5 the dimerization of CH3 domains but also has the advantage that at least one additional charge-charge interaction in the CH3 interface is created. In view of this additional charge-charge interaction on top of the existing charge-pairs in the CH3-CH3 interface, the dimers according to the invention are generally more stable as compared to the wild type dimers. Moreover, it has surprisingly become possible to
10 increase the proportion of one or more Ig-like molecules of interest in a mixture even further. As described herein before, methods known in the art for preferential production of a bispecific antibody typically involves the production of some undesired dimeric side products. For instance, the proportion of a bispecific antibody of interest using the knob-into-hole technology is at best 87%, whereas the electrostatic
15 engineering approach wherein charged contact amino acids are substituted by amino acids of opposite charge, also results in proportions of up to 96% (see for instance Example 11). Quite surprisingly, the present inventors have succeeded in introducing mutations that further enhance the proportion of an Ig-like molecule of interest in a mixture. For instance, Example 17 discloses a method using mutations according to
20 the present invention, wherein the proportion of a bispecific antibody of interest was raised to such extent that no dimeric by-product was detectable in the resulting mixture at all. Unpaired half-molecules consisting of only a single heavy chain paired with a common light chain were present to some extent in the mixtures, but these are the result of unbalanced expression of the heavy chains and can be easily separated
25 from the mixture by size exclusion chromatography. Hence, with such mutations according to the present invention, a bispecific Ig-like molecule can be produced in a single cell with a high proportion with essentially no contaminating dimeric by-products being present, which is particularly suitable for the production of a pharmaceutical composition.

30

The amino acids at position 366 of one CH3 domain and position 351 of a second CH3 domain have been reported to be a pair of contact residues in the CH3-CH3 interface, meaning that they are located sufficiently close to each other in the three-dimensional

conformation of the resulting Ig-like molecule in order to be capable of interacting with each other. Hence, the first CH3 domain will preferentially pair with the second CH3 domain.

5 In one embodiment, threonine (T) at position 366 of a first CH3 domain is replaced by a first charged amino acid and leucine (L) at position 351 of a second CH3 domain is replaced by a second charged amino acid, wherein said first and second charged amino acids are of opposite charge. If the first CH3 domain-comprising polypeptide, that carries a charged residue at position 366, further comprises a variable domain which has specificity for antigen A, and if the second CH3 domain-comprising polypeptide,
10 that carries an oppositely charged residue at position 351, further comprises a variable domain which has specificity for antigen B, bispecific Ig-like molecules with an AB specificity will be predominantly formed. Further provided is therefore a method according to the present invention, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides or said means for preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides are a
15 substitution of threonine at position 366 of said 1st or 3rd CH3 domain by a first charged amino acid and substitution of leucine at position 351 of said 2nd or 4th CH3 domain by a second charged amino acid, wherein said first and second charged amino acids are of opposite charge.

20

One preferred combination of mutations according to the present invention is the substitution of threonine (T) by lysine (K) at position 366 of a first CH3 domain-comprising polypeptide which further comprises a variable domain (for instance with specificity A) and the substitution of leucine (L) by aspartic acid (D) at position 351 of
25 a second CH3 domain-comprising polypeptide which further comprises a variable domain (for instance with specificity B). This is denoted as a T366K/L351'D pair mutation. As explained before, the amino acids at position 366 of one CH3 domain and position 351 of a second CH3 domain have been reported to be a pair of contact residues in the CH3-CH3 interface. The lysine that is introduced at position 366 and
30 the aspartic acid introduced at position 351 have opposite charges, so that these amino acids will electrostatically attract each other. Hence, the first CH3 domain will preferentially attract the second CH3 domain and Ig-like molecules comprising a first CH3 domain containing lysine at position 366 paired with a second CH3 domain

containing aspartic acid at position 351 will be predominantly formed. If the first CH3 domain-comprising polypeptide has specificity for antigen A, and if the second CH3 domain-comprising polypeptide has specificity for antigen B, bispecific Ig-like molecules with 'AB' specificity will be predominantly formed. As mentioned above, one of the advantages of the mutations according to the present invention is the fact that a novel interaction between newly introduced charged amino acids is created, instead of replacing existing charged amino acid interactions. This was not previously disclosed or suggested. One embodiment therefore provides a method for producing a single Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein said Ig-like molecule is a heterodimer, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitution L351D, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said single Ig-like molecules from the culture.

Preferably, the above mentioned T366K/L351'D mutations according to the present invention are further combined with the substitution of leucine (L) by glutamic acid (E) at position 368 of the second CH3 domain. This is denoted as a T366K/L351'D,L368'E mutation. As shown in Example 17, introduction of this mutation according to the invention into a first CH3 domain-comprising polypeptide with specificity for antigen A, and a second CH3 domain-comprising polypeptide with specificity for antigen B results in a particular good proportion of bispecific Ig-like molecules with dual AB specificity. With this mutation it has even become possible to obtain bispecific antibody without any detectable amount of homodimers formed. A particularly preferred embodiment therefore provides a method for producing a single Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3

domains that are capable of forming an interface and wherein said Ig-like molecule is a heterodimer and wherein contaminating homodimers are less than 5%, preferably less than 2%, more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

5 - a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and

- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and L368E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said single Ig-like molecules from the culture.

15

In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and tyrosine (Y) is substituted by glutamic acid (E) at position 349 of said second CH3 domain. This is denoted as a

20 T366K/L351'D,Y349'E mutation. Residue Y349 is a neighboring residue of the residue at position 351 that may contribute to dimer interactions. According to in silico data, Y349E adds to the stability of the heterodimer (lower in silico scores) as well as to the destabilization of the monodimer (higher in silico scores) and glutamic acid (E) on position 349 is more favorable than aspartic acid (D). Thus, introduction of a second amino acid substitution in the second CH3 domain comprising polypeptide, comprising already the amino acid substitution at position 351, favors heterodimerization further.

A particularly preferred embodiment therefore provides a method for producing a single Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two
30 CH3 domains that are capable of forming an interface and wherein said Ig-like molecule is a heterodimer and wherein contaminating homodimers are less than 5%, more preferably less than 2%, even more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
 - a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,
- 5 wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and Y349E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said single Ig-like molecules from the
- 10 culture.

In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and tyrosine (Y) is substituted by glutamic acid (E) at position 349 of said second CH3 domain and leucine (L) is substituted by glutamic acid (E) at position 368 of said second CH3 domain. This is denoted as a T366K/L351'D,Y349'E,L368'E mutation. The two residues Y349 and L368 are residues that may contribute to dimer interactions. According to the in silico data, Y349E and L368E add to the stability of the heterodimer (lower in silico scores) as well as to the destabilization of the BB dimer (higher in silico scores) and glutamic acids (E) on positions 349 and 368 are more favorable than aspartic acids (D). Thus, introduction of a second and third amino acid substitution in the B-chain, which already comprises the amino acid substitution at position 351, favors heterodimerization further. A particularly preferred embodiment therefore provides a method for producing a single Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein said Ig-like molecule is a heterodimer and wherein contaminating homodimers are less than 5%, more preferably less than 2%, even more preferably less than 1%, and most preferably essentially absent, said method comprising

20 providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and

30

- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide
5 chain comprises the amino acid substitutions L351D and Y349E and L368E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said single Ig-like molecules from the culture.

10 In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by lysine (K) at position 351 of said first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and leucine (L) is substituted by glutamic acid (E) at position 368 of said second CH3 domain. This is denoted as a
15 T366K,L351K/L351'D,L368'E mutation. This mutation also enhances the proportion of the (bispecific) antibody of interest, as shown in the Examples. Also with this mutation it has become possible to obtain bispecific antibody without any detectable amount of homodimers formed. Further provided is therefore a method for producing a single Ig-like molecule from a single cell, wherein said Ig-like molecule comprises
20 two CH3 domains that are capable of forming an interface and wherein said Ig-like molecule is a heterodimer and wherein contaminating homodimers are less than 5%, preferably less than 2%, more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

25 - a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and

- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitutions T366K and L351K, and wherein said second CH3 domain

30 comprising polypeptide chain comprises the amino acid substitutions L351D and L368E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said single Ig-like molecules from the culture.

In yet another preferred embodiment, threonine (T) is substituted by lysine (K) at position 366 of a first CH3 domain and leucine (L) is substituted by lysine (K) at position 351 of said first CH3 domain and leucine (L) is substituted by aspartic acid (D) at position 351 of a second CH3 domain and tyrosine (Y) is substituted by aspartic acid (D) at position 349 of said second CH3 domain and arginine (R) is substituted by aspartic acid (D) at position 355 of said second CH3 domain. This is denoted as a T366K,L351K/L351'D,Y349'D,R355'D mutation. The T366K-L351K/L351'D-Y349'D pair may be further improved by the R355'D mutation in the B-chain, which results in a higher BB-in silico score, but also the AB in silico score is slightly higher. Further provided is therefore a method for producing a single Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein said Ig-like molecule is a heterodimer and wherein contaminating homodimers are less than 5%, more preferably less than 2%, even more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell:

- a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain, and
- a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,

wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitutions T366K and L351K, and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and Y349D and R355D, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said single Ig-like molecules from the culture.

Table B provides an overview of mutations that can be introduced in CH3 domains as preferred means for preferential pairing to create either heterodimers or homodimers.

Table B:

AA substitutions in CH3	Construct #	Preferentially pairs with
- (wildtype)	-	Wildtype
E356K, D399K	1	Construct 2 or 3
K392D, K409D	2	Construct 1
K392D, K409D, K439D	3	Construct 1
K392D, D399K, K409D	4	Construct 4
E356K, E357K, K439D, K370D	5	Construct 5
T366W	6	Construct 7
T366S, L368A, Y407V	7	Construct 6
T366K	43	Construct 63, 69, 70, 71, 73
L351D	63	Construct 43, 68
T366K, L351K	68	Construct 63, 69, 70, 71, 72, 75
L351D, L368E	69	Construct 43, 68
L351E, Y349E	70	Construct 43, 68
L351D, Y349E	71	Construct 43, 68
L351D, R355D	72	Construct 43, 68
L351D, Y349E, L368E	73	Construct 43
L351D, Y349D, R355D	75	Construct 68

A method according to the present invention, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and/or said means for preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides comprise at least one combination of mutations as depicted in Table B is therefore also provided herewith. Preferably, said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said means for preferential pairing of said 3rd and 4th CH3 domain-comprising polypeptides comprise at least two combinations of mutations as depicted in Table B.

The present invention also provides novel combinations of CH3 mutations with which it has become possible to produce a mixture of at least two monospecific Ig-like molecules in a single cell, wherein contaminating bispecific Ig-like molecules are less than 5%, preferably more than 2%, even more preferably less than 1%, and most
5 preferably even essentially absent. These mutations according to the invention are, therefore, particularly suitable for the production of a mixture of monospecific antibodies, which is for instance advantageous when a high level of crosslinking of two identical target molecules is desired, when the density of antibodies on a target cells needs to be high enough to recruit certain effector functions such as complement-
10 mediated lysis of a tumor cell, or when two targets are located too far away from each other so that they cannot be bound by as single bispecific antibody, or in order to simplify regulatory approval procedures. In such cases, it is often desired to optimize the production platform for such monospecific antibodies. As shown in Example 10, the present invention provides the insight that when lysine (K) at position 392 of a
15 first CH3 domain-comprising polypeptide (for instance having specificity A) is substituted by aspartic acid (D) and when aspartic acid (D) at position 399 of said first CH3 domain-comprising polypeptide is substituted by lysine (K) and when lysine (K) at position 409 of said first CH3 domain-comprising polypeptide is substituted by
20 aspartic acid (D), it has become possible to produce a mixture of at least two different monospecific Ig-like molecules in a single cell, including monospecific Ig-like molecules with specificity AA, wherein the formation of bispecific by-products (bispecific Ig-like molecules) is reduced to below 5%, or even to below 3%, or even essentially not detectable at all. Hence, the above mentioned combination of mutations (denoted herein as K392D, D399K, K409D) is particularly preferred for the
25 production of a mixture of monospecific Ig-like molecules. The skilled person will appreciate that functional variants thereof, i.e., K392E, D399R, K409E, may result in similar effects. Additionally, double mutants comprising D399K and K409D substitutions, or other functional variants such as e.g. K392D and K409D, D399R and K409E and so forth, may also result in similar effects.

30 The same holds true for a combination of mutations wherein glutamic acid (E) at position 356 of a first CH3 domain-comprising polypeptide is substituted by lysine (K) and wherein glutamic acid (E) at position 357 of said first CH3 domain-comprising polypeptide is substituted by lysine (K) and wherein lysine (K) at position 439 of said

first CH3 domain-comprising polypeptide is substituted by aspartic acid (D) and wherein lysine (K) at position 370 of said first CH3 domain-comprising polypeptide is substituted by aspartic acid (D). This combination of mutations (denoted herein as E356K, E357K, K439D, K370D) is also particularly preferred for the production of a mixture of monospecific Ig-like molecules. The skilled person will appreciate that functional variants thereof, i.e., E356R, E357R, K439E, K370E, may result in similar effects. Additionally, triple or double mutants comprising E356K and K439D, and E357K and K370D substitutions, or other functional variants may also result in similar effects. A further embodiment therefore provides a method for producing at least two different monospecific Ig-like molecules from a single host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

- a) a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain having a specificity A,
- b) a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain having a specificity B,

wherein said first CH3 domain-comprising polypeptide chain comprises a K392D, D399K, K409D mutation and said second CH3 domain-comprising polypeptide chain comprises either a wildtype CH3 domain or comprises a E356K, E357K, K439D, K370D mutation, said method further comprising the step of culturing said host cell and allowing for expression of said nucleic acid molecules and harvesting said at least two different Ig-like molecules from the culture.

An alternative embodiment provides a method for producing at least two different monospecific Ig-like molecules from a single host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that are capable of forming an interface, said method comprising providing in said cell

- a) a first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain having a specificity A,
- b) a second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain having a specificity B,

wherein said first CH3 domain-comprising polypeptide chain comprises either a wildtype CH3 domain or comprises a K392D, D399K, K409D mutation and said second CH3 domain-comprising polypeptide chain comprises a E356K, E357K,

K439D, K370D mutation, said method further comprising the step of culturing said host cell and allowing for expression of said nucleic acid molecules and harvesting said at least two different Ig-like molecules from the culture.

5 As shown in Example 10, two monospecific Ig-like molecules can be produced in a single cell, wherein the formation of bispecific Ig-like molecules is essentially undetectable. The skilled person may select a 3rd nucleic acid molecule encoding a wildtype or engineered CH3 domain-comprising polypeptide chain to provide to said host cell such that a mixture of 3 monospecific antibodies is produced, and so forth.

10

In one aspect of the invention, a method according to the invention is provided wherein each of the CH3-domain comprising polypeptide chains further comprises a variable region recognizing a different target epitope, wherein the target epitopes are located on the same molecule. This often allows for more efficient counteraction of the (biological) function of said target molecule as compared to a situation wherein only one epitope is targeted. For example, any combination of at least two Ig-like molecules may simultaneously bind to 2, 3 or 4 epitopes present on growth factor receptors critical for tumors cells to proliferate, thereby effectively blocking several independent signalling pathways leading to uncontrolled proliferation.

15

20 In a preferred embodiment, the target molecule is a soluble molecule. In another preferred embodiment, the target molecule is a membrane-bound molecule.

In another aspect of the invention, a method according to the invention is provided wherein each of the CH3-domain comprising polypeptide chains further comprises a variable region recognizing a target epitope, wherein the target epitopes are located on different molecules. In this case, each of the different target molecules may either be a soluble molecule or a membrane-bound molecule. In one embodiment, the different target molecules are soluble molecules. Alternatively, one target molecule is a soluble molecule whereas the second target molecule is a membrane bound molecule. In yet another alternative, both target molecules are membrane bound molecules. In one embodiment the different target molecules are expressed on the same cells, whereas in other embodiments the different target molecules are expressed on different cells. As a non-limiting example, any combination of at least

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two Ig-like molecules may be suitable for simultaneously blocking multiple membrane-bound receptors, neutralizing multiple soluble molecules such as cytokines or growth factors for tumor cells or for neutralizing different viral serotypes or viral strains.

5

One preferred embodiment provides a method according to the invention, wherein at least one of said target epitopes is located on a tumor cell. Alternatively, or additionally, at least one of said target epitopes is located on the surface of an effector cell. This is for instance suitable for recruitment of T cells or NK cells for tumor cell killing. For instance, at least one Ig-like molecule is produced with a method according to the invention that is capable of recruiting immune effector cells, preferably human immune effector cells, by specifically binding to a target molecule located on immune effector cells. In a further embodiment, said immune effector cell is activated upon binding of the Ig-like molecule to the target molecule. Recruitment of effector mechanisms may for instance encompass the redirection of immune modulated cytotoxicity by administering an Ig-like molecule produced by a method according to the invention that is capable of binding to a cytotoxic trigger molecule such as the T cell receptor or an Fc gamma receptor, thereby activating downstream immune effector pathways. The term 'immune effector cell' or 'effector cell' as used herein refers to a cell within the natural repertoire of cells in the mammalian immune system which can be activated to affect the viability of a target cell. Immune effector cells include cells of the lymphoid lineage such as natural killer (NK) cells, T cells including cytotoxic T cells, or B cells, but also cells of the myeloid lineage can be regarded as immune effector cells, such as monocytes or macrophages, dendritic cells and neutrophilic granulocytes. Hence, said effector cell is preferably an NK cell, a T cell, a B cell, a monocyte, a macrophage, a dendritic cell or a neutrophilic granulocyte. Target antigens present on immune effector cells may include CD3, CD16, CD25, CD28, CD64, CD89, NKG2D and Nkp46. Further provided is therefore a method according to the invention, wherein said target epitope is located on a CD3, CD16, CD25, CD28, CD64, CD89, NKG2D or a Nkp46 molecule.

The viability of a target cell may include cell survival, proliferation and/or ability to interact with other cells.

In a preferred embodiment, a method according to the invention is provided, wherein said at least two different Ig-like molecules are antibodies, most preferably antibodies of the IgG isotype, as described herein above.

5 Further provided is an Ig-like molecule, or a mixture of at least two Ig-like molecules, obtainable by a method according to the present invention. Said Ig-like molecule or mixture of Ig-like molecules preferably comprises at least one CH3 mutation as depicted in Table B. An Ig-like molecule or a mixture of at least two Ig-like molecules, comprising at least one mutation as depicted in Table B is therefore also herewith
10 provided, as well as a pharmaceutical composition comprising at least one Ig-like molecule, or a mixture of at least two Ig-like molecules, according to the present invention. In one embodiment said Ig-like molecule is a bispecific Ig-like molecule, such as a bispecific antibody. In another embodiment said Ig-like molecule is a monospecific Ig-like molecule, such as a monospecific antibody. Also provided is a
15 pharmaceutical composition comprising a mixture of at least two Ig-like molecules obtainable by a method according to the invention. Said at least two Ig-like molecules according to the invention are preferably antibodies. Said pharmaceutical composition may comprise a mixture comprising monospecific or bispecific Ig-like molecules, or a combination of monospecific and bispecific Ig-like molecules.
20 A nucleic acid molecule encoding a CH3 domain-comprising polypeptide chain that comprises at least one mutation as depicted in Table B is also provided herewith, as well as a recombinant host cell comprising at least one nucleic acid molecule encoding a CH3 domain-comprising polypeptide chain that comprises at least one mutation as depicted in Table B.

25 The invention is further illustrated by the following examples. These examples are not limiting the invention in any way, but merely serve to clarify the invention.

Brief description of the drawings

- Figure 1: A) schematic representation of construct vector MV1057. The stuffer region is the region into which an antibody VH region is cloned. B) schematic representation of phage display vector MV1043.
- 5
- Figure 2: amino acid sequence of wildtype IgG1 Fc, as present in construct vector MV1057 (EU numbering scheme applied).
- Figure 3: nucleotide and amino acid sequences of VH regions used for cloning into the various constructs.
- 10
- Figure 4: mass spec data of transfections A, G and H.
- Figure 5: mass spec data of transfections M and U.
- Figure 6: mass spec data of transfection O.
- Figure 7: prevention of homodimerisation by substitution of neutral amino acids for charged amino acids.
- 15
- Figure 8: Native MS spectrum of transfection sample ZO (T366K/L351'D) (A) and Convolved MS spectrum of transfection sample ZO (T366K/L351'D). The second/main peak represents the bispecific molecule (B).
- Figure 9: HADDOCK scores on experimentally verified mutation pairs
- Figure 10: Cartoons of interactions in the CH3-CH3 interface; A) K409D:K392D/D399'K:E356'K, B) D399K:E356K/D399'K:E356'K, C) K409D:K392D/
- 20
- K409'D:K392'D
- Figure 11: HADDOCK scores for various 366/351' charge mutants
- Figure 12: Cartoons of interactions in the CH3-CH3 interface ; A) L351D/L351'D, B) L351D:S354A:R355D/ L351'D:S354'A:R355'D
- 25
- Figure 13: HADDOCK scores for additional charge mutations around position L351
- Figure 14: HADDOCK scores for additional charge mutations around position T366 in chain A and position L351 in chain B.
- Figure 15: Cartoons of interactions in the CH3-CH3 interface
- Figure 16: HADDOCK scores for variants around T366/L351
- 30
- Figure 17: HADDOCK scores for additional variants around T366/L351

Examples

5 Example 1: amino acid substitutions to create various different CH3-domains

In order to have a wide variety of Ig-like molecules that differ in their CH3 domains such that pairing of CH3-domain comprising Ig-like molecules is preferentially promoted or inhibited, a number of amino acid substitutions that were known to promote heterodimer formation, as well as a number of alternative amino acid substitutions that were not previously reported nor tested but that were chosen to promote homodimer formation, were introduced into a construct vector (construct vector MV1057; Figure 1A). The construct vector MV1057 comprises nucleic acid sequences encoding the normal wildtype IgG1 Fc part, as depicted in figure 2. Table 1 lists the amino acid substitutions that were introduced in this wildtype Fc, resulting in a series of seven constructs. All constructs were made at Genentech. Constructs 1, 2 and 3, or alternatives thereof, have previously been described to drive heterodimerization (EP01870459, WO2009/089004) as have constructs 6 and 7 (WO98/50431). Constructs 4 and 5 are new and are designed to promote homodimerization.

Table 1

AA substitutions in CH3	construct #	Will pair with	% bispecific product reported
- (wildtype)	-	- (wildtype)	~50%
E356K, D399K	1	Construct 2 or 3	~100%
K392D, K409D	2	Construct 1	~100%
K392D, K409D, K439D	3	Construct 1	~100%
K392D, D399K, K409D	4	Construct 4	
E356K, E357K, K439D, K370D	5	Construct 5	
T366W	6	Construct 7	~86,7%
T366S, L368A, Y407V	7	Construct 6	~86,7%

Example 2: cloning of VH into constructs with CH3 mutations

Several antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain were used for cloning into these constructs. Figure 3 provides full sequences and specificities of the antibody VH regions used throughout the studies. The MF coding refers to internal Merus designation for various VHs, e.g. 5 VH MF1337 has specificity for tetanus toxoid, MF1025 for porcine thyroglobulin, MF1122 for bovine fibrinogen.

VH regions present in phage display vector MV1043 (Figure 1B) were digested with restriction enzymes SfiI and BstEII (New England Biolabs/ cat# R0123L and R0162L/ 10 according to manufacturer's instructions) that release the VH fragment from this vector. Vector MV1057 was digested with SfiI and BstEII according to standard procedures (according to manufacturer's instructions). Fragments and vector were purified over gel (Promega/ cat# V3125/ according to manufacturer's instructions) to 15 isolate the cut vector and VH gene inserts. Both were combined by ligation after which the ligation was transformed into E. coli DH5 α (Invitrogen/ cat# 12297-016/ according to manufacturer's instructions). After overnight selection single colonies were picked and vectors with a correct insert identified by sequencing.

Example 3: transfection and expression of full IgG in HEK293T cells

20 Transfection of the various plasmids encoding the recloned VH variants, and further encoding the common light chain huIGKV1-39, in HEK293T cells was performed according to standard procedures such that IgG could express (de Kruif et al Biotech Bioeng. 2010). After transfection, IgG expression levels in supernatants were measured using the ForteBIO Octet-QK system, which is based on Bio-Layer 25 Interferometry (BLI) and which enables real-time quantitation and kinetic characterization of biomolecular interactions; for details see www.fortebio.com. When expression levels exceeding 5 μ g/ml were measured, the IgG was purified using Protein A affinity purification.

30 **Example 4: purification of IgG**

Culture supernatants were purified using protein A columns (GE Healthcare/ cat# 11-0034-95/ according to manufacturer's instructions) and eluted in 0,1 M citrate buffer pH 3.0 and immediately neutralized in an equal volume of 1,0 M Tris-HCL pH 8.0 or

directly rebuffed to PBS using a desalting column. Alternatively one could purify IgG using protein A beads (sepharose beads CL-4B, GE healthcare cat #170780-01)

Example 5: Ag-specific ELISA's

- 5 Antigen specific ELISAs were performed to establish binding activity against the antigens and capture ELISAs were carried out to demonstrate binding activity of the bispecific antibodies. Biotinylated second antigen was used for detection of the complex. (de Kruif et al Biotech Bioeng. 2010)

10 Example 6: SDS-PAGE

The purified IgG mixtures were analysed by SDS-PAGE (NuPAGE® 4-12% bis-tris gel/ Invitrogen/ cat# NP0323BOX) under reduced and non-reducing conditions according to standard procedures, and staining of proteins in gel was carried out with colloidal blue (PageBlue™ protein staining solution/ Fermentas/ cat# RO571).

15

Example 7: Enzymatic deglycosylation of IgG1

- As there is heterogeneity in the glycosylation of the IgGs, the proteins were deglycosylated in order to create a single product with a distinct mass, suitable for mass spectrometric analysis. One unit of N-glycosidase F (PNGase F; Roche
- 20 Diagnostics, Mannheim, Germany) was incubated per 10 µg of IgG1, overnight at 37°C. Buffer exchange using 10 kDa MWCO centrifugal filter columns (Millipore) was performed to remove the original purification buffer (0,1 M citrate buffer pH 3.0 / 1,0 M Tris-HCL pH 8.0) and to rebuffer to PBS. Similar buffer exchange procedures were performed to remove the detached glycan chains, and to change the buffer to 150 mM
- 25 ammonium acetate pH 7.5. Filters were washed with 200 µl 150 mM ammonium acetate pH 7.5, for 12 min 11,000 rpm and 4°C. After washing 50 µl deglycosylated IgG was loaded on the filter and 450 µl of 150 mM ammonium acetate pH 7.5 was added, subsequently followed by another centrifugation round of 12 min at 11,000 rpm at 4°C. In total the centrifugation was repeated 5 times, each time fresh 150 mM
- 30 ammonium acetate pH 7.5 buffer was added to a total volume of 500 µl. After the last centrifugation step the remaining buffer exchanged deglycosylated IgG1, approximately 25 µl, was collected and transferred to an eppendorf tube, ready for mass spectrometric analysis.

Example 8: Native mass spectrometric analysis

Mass Spectrometry was used to identify the different IgG species in the purified IgG mixtures and to establish in what ratios these IgG species are present. Briefly, 2-3 μ l
5 at a 1 μ M concentration in 150 mM ammonium acetate pH 7.5 of IgG's were loaded into gold-plated borosilicate capillaries made in-house (using a Sutter P-97 puller [Sutter Instruments Co., Novato, CA, USA] and an Edwards Scancoat six sputter-coater [Edwards Laboratories, Milpitas, CA, USA]) for analysis on a LCT 1 mass spectrometer (Waters Corp., Milford, MA, USA), adjusted for optimal performance in
10 high mass detection (Tahallah et al., RCM 2001). A capillary voltage of 1300 V was used and a sampling cone voltage of 200 V; however, these settings were adjusted when a higher resolution of the 'signal-to-noise' ratio was required. The source backing pressure was elevated in order to promote collisional cooling to approximately 7.5 mbar. To measure the IgG1's under denaturing conditions the proteins were
15 sprayed at a 1 μ M concentration in 5% formic acid.

Example 9: Data processing and quantification

Processing of the acquired spectra was performed using MassLynx 4.1 software (Waters Corp., Milford, MA, USA). Minimal smoothing was used, after which the
20 spectra were centered. The mass of the species was calculated using each charge state in a series. The corresponding intensities of each charge state were assigned by MassLynx and summed. This approach allowed the relative quantification of all species in a sample. Alternatively, quantification of the peaks can be performed using area-under-the-curve (AUC) methods, known in the art. All analyses were repeated
25 three times to calculate standard deviations of both the masses of the IgG's as well as their relative abundance.

Example 10: mixtures of 2 or 3 monospecific antibodies from a single cell

Several antibody VH regions with known specificities and known ability to pair with
30 the human IGKV1-39 light chain (Figure 3) were used for recloning into the wildtype construct vector MV1057, or in construct 4 or construct 5 of Table 1, resulting in vectors I-III (Table 2). The resulting vectors I, II and III, each containing nucleic acid sequences encoding for the common human light chain as well as an Ig heavy chain

with different CH3 region and different VH specificity, were subsequently transfected into cells, either alone to demonstrate formation of intact monospecific antibodies only, or in combination with one or two other construct vectors to obtain mixtures of two monospecific or three monospecific antibodies. Table 3 depicts the transfection schedule and results.

Table 2: **VH specificity inserted in different constructs**

Vector	VH gene	Antigen specificity	VH mass (Da)	Merus designation	Cloned in construct #
I	IGHV 1.08	Tetanus (A)	13703	MF1337	wildtype
II	IGHV 3.23	Thyroglobulin (B)	12472	MF1025	4
III	IGHV 3.30	Fibrinogen (C)	12794	MF1122	5

Table 3: transfection schedule and results

#	Transfection of	Transfection code and ratio	Expected species	Calculated mass - 2LYS	Experimental mass	AA found (%)	BB found (%)	CC found (%)	Other molecules (%)
1	Only vector I	A	AA	146521	146503	100			
1	Only vector II	G	BB	144032	144087		100		
1	Only vector III	H	CC	144647	144656			100	
2	Vector I and II	M (I:II=1:1)	AA BB	146521 144032	146518 144030	51	45		4
2	Vector I and III	N (I:III=1:1)	AA CC	146521 144647	146509 144633	88		9	3
		U (I:III=1:5)	AA CC	146521 144647	146522 144643	47		48	5
2	Vector II and III	nd	BB CC						
3	Vector I, II and III	O (I:II:III=1:1:1)	AA BB CC	146521 144032 144647	146525 144032 144650	66	4	30	
		V (I:II:III=1:1:10)	AA BB CC	146521 144032 144647	146531 144043 144654	8	81	9	2

nd= not done.

It was observed that transfections A, G and H resulted in formation of homodimers only, and 100% of bivalent monospecific AA, BB or CC was retrieved from cells transfected with any one of vectors I, II or III (Fig. 4). Although this was to be expected and previously demonstrated for transfection A, it is actually now shown for the first time that homodimerisation of CH3-engineered Ig heavy chains containing either the triple amino acid substitution of construct 4 (i.e., K392D, D399K, K409D) or the quadruple amino acid substitution of construct 5 (i.e., E356K, E357K, K439D, K370D) occurs (transfections G and H).

Next, co-expression experiments of two vectors in a single cell were performed. Interestingly, transfections M and N show that wildtype and CH3 engineered Ig heavy chains can be co-expressed in a single cell together with a common light chain resulting in mixtures of two species of monospecific antibodies without the presence of undesired bispecific antibodies and with as little as 4-5% contaminating 'other molecules' present in the mixture. 'Other molecules' is defined as all molecules that do not have the mass of an intact IgG, and includes half molecules consisting of a single heavy and light chain pair. Importantly, the fraction 'other' does not include bispecific product. In transfection M, the ratio of AA:BB was close to 1:1 upon transfection of equal ratios of vector DNA. However, transfection N resulted in an almost 10:1 ratio of AA:CC. Therefore, this transfection was repeated with adjusted ratios of DNA (transfection U). Indeed, a 1:5 ratio of vector DNA I:III equalized the ratio of AA:CC antibody product in the mixture towards an almost 1:1 ratio. Thus, transfections M and U show that it is possible to express two different, essentially pure, monospecific antibodies in a single cell, without undesired by products (i.e., no abundant presence of AC or half molecules A or C) (fig. 5). The novel CH3 modifications of constructs 4 and 5 differ substantially from wildtype CH3 such that heterodimerization between wildtype and 4, or wildtype and 5, does not occur, which is advantageous for application in large scale production of mixtures of monospecific antibodies from single cells.

Analogous to these results, also transfection of two different CH3 engineered Ig heavy chains (constructs 4 and 5) are expected to result in mixtures of two different monospecific antibodies only, without further undesired species present. It is reasoned that the CH3 modifications of construct 4 differ substantially from the CH3

modifications of constructs 5 such that heterodimerization does not occur. In that case, co-expression of CH3-engineered heavy chains of constructs 4 and 5, together with wildtype CH3 heavy chains in a single cell would result in 3 monospecific antibodies only.

5 Indeed, this was observed to be the case as it was found that also a mixture of three pure monospecific antibodies could be obtained by expression of three different Ig heavy chains, designed to form homodimers over heterodimers, together with a common light chain in a single cell, with no contaminations present in the mixture (transfection O) (Fig. 6). As is clear from Table 3, with equal ratios of vector DNA
10 used during transfection O, no 1:1:1 ratio of AA:BB:CC antibodies was obtained. Transfections with altered vector DNA ratios (1:1:10, transfection V) demonstrated that ratios of AA:BB:CC in the mixtures can be steered towards desired ratios. Taken together, these experiments show that two or three essentially pure monospecific antibodies can be expressed in a single cell without undesired by
15 products, offering advantages for large scale production of mixtures of therapeutic monospecific antibodies.

Example 11: mixtures of 2 bispecific antibodies from a single cell

Whereas use of CH3-engineered heavy chains for production of single bispecific
20 antibodies has been reported elsewhere, this experiment was designed to investigate whether it is feasible to produce mixtures of 2 different bispecific antibodies from a single cell.

Antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain (fig. 3) were used for recloning into vectors containing
25 constructs 1-3 or 6-7 of Table 1 resulting in vectors IV-X (Table 4). Vectors IV-X, each containing nucleic acid sequences encoding the common human light chain as well as an Ig heavy chain with different CH3 region and different VH specificity, were subsequently transfected into cells, either alone to demonstrate that formation of intact monospecific antibodies was hampered, or in combination with another
30 construct vector to obtain bispecific antibodies or mixtures of two bispecific antibodies. Table 5 depicts the transfection schedule and results.

Table 4: **VH specificity inserted in different constructs**

Vector	VH gene	Antigen specificity	VH mass (Da)	Cloned in construct #
IV	IGHV 3.23	Thyroglobulin (B)	12472	1
V	IGHV 3.30	Fibrinogen (C)	12794	2
VI	IGHV 1.08	Tetanus (A)	13703	2
VII	IGHV 3.30	Fibrinogen (C)	12794	3
VIII	IGHV 1.08	Tetanus (A)	13703	3
IX	IGHV 1.08	Tetanus (A)	13703	6
X	IGHV 3.23	Thyroglobulin (B)	12472	7

Table 5:

# different bispecifics produced	Transfection of	Transfection code and ratio	Expected species	Calculated mass - 2LYS	Experimental mass	Half molecules found (%)	Full IgG found (%)	Bispecific found (%)	Other molecules (%)
0	vector IV	B	Half B	144082	144066	40	60		
0	vector V	C	Half C	144651	144622	77	23		
0	vector VI	D	Half A	146469	146459	23	77		
0	vector VII	E	Half C	144625	144643	76	24		

0	vector VIII	F	Half A	146443	146468	64	36		
0	vector IX	P	Half A	146691	146677	82	18		
0	vector X	Q	Half B	143818	143844	58	42		
1	Vector IV and V	I (1:1)	BC	144367	144352			96	4
1	Vector IV and VII	J (1:1)	BC	144354	144382			96	4
2	Vector IV, V and VI	K(1:1:1)	BC + AB	144367 + 145276	144351+ 145260			38 + 47	15 (A + C)
		S(2:1:1)	BC + AB	144367 + 145276	144371 + 145277			42 + 55	3 (BB)
2	Vector IV, VII and VIII	L (1:1:1)	BC + AB	144354 + 145263	144346 + 145255			16 + 60	24 (A + C)
		T (2:1:1)	BC + AB	144354 + 145263	144385 + 145292			58 + 39	3 (BB)

It was previously demonstrated that CH3-engineered Ig heavy chains encoded by constructs 1 and 2 are still able to form homodimers when expressed alone in single cells (WO2009/089004). However, WO2009/089004 further reports that CH3 domains that are engineered to comprise triple charge pair mutations, such as present in construct 3, are no longer capable of forming homodimers when expressed alone.

In the present study, these findings were only partly confirmed. Indeed, the results of transfections B, C and D demonstrated the presence of full IgGs, in addition to a high proportion of unpaired half molecules, demonstrating some homodimerization of CH3 domains encoded by constructs 1 and 2. Transfections E and F also resulted in
5 production of full IgGs in addition to unpaired half molecules, demonstrating that the triple charge mutations of construct 3 do not fully impair homodimerisation. It was furthermore demonstrated that also the 'knob' and 'hole' CH3 variants of constructs 6 and 7 form homodimers (18% homodimers for 'knob-knob' and 42% homodimers for 'hole-hole').

10 CH3 variants that fully prevent homodimerisation when expressed alone are preferred, to prevent or minimize undesired byproducts (homodimers) upon co-expression with a second CH3 variant for heterodimerization. Interestingly, the present experiments demonstrate for the first time that also mixtures of bispecific antibodies can be expressed in single cells with virtually no
15 homodimers in the mixture. Transfections K and L clearly show that the expected bispecific species BC + AB are indeed obtained (38% + 47% in transfection K, and 16% + 60% in transfection L). In both transfections a relatively high percentage of undesired half molecules was observed (15% half molecule A + half molecule C in transfection K, and 24% half molecule A + half molecule C in transfection L). The
20 relatively high percentage of half molecules still present was attributed to low amounts of matching heavy chains of vector IV due to unbalanced expression of heavy chains in a matched pair. Therefore, transfections were repeated with an adjusted ratio of vector DNA, 2:1:1, in transfections S and T. This resulted in equal amounts of IgG heavy chains constituting a matched pair and pure mixtures of bispecific IgG
25 without the presence of half IgG molecules and with as little as 3% homodimeric BB present. Ideally, this low proportion of contaminating monospecific product should be reduced to essentially zero. It is therefore desired to find additional CH3-mutants that would result in mixtures of bispecific antibodies with minimal contaminating monospecific antibodies present.

30 The present study demonstrates for the first time that essentially pure mixtures of two bispecific antibodies recognizing 3 different target epitopes can be produced in a single cell, with minimal presence of monospecific antibodies in the mixture.

Example 12: varieties of mixtures

As it was demonstrated that production of mixtures of 2 bispecific antibodies recognizing 3 epitopes from a single cell, or production of mixtures of 2 or 3 monospecific antibodies from a single cell is technically feasible, we next explored the feasibility of controlled production of a variety of other mixtures. A fourth antibody VH region with known specificity and known ability to pair with the human IGKV1-39 light chain will be used for recloning into vectors containing constructs 1-3 or 7 of Table 1, resulting in vectors I', II', III' or X' (the ' indicating a different specificity as compared to corresponding vector numbers). The resulting vectors I'-III', X' and IV-IX, each containing nucleic acid sequences encoding for the common human light chain as well as an Ig heavy chain with different CH3 region and different VH specificity, will subsequently be transfected into cells, in combination with other construct vectors to obtain a variety of mixtures of bispecific and/or monospecific antibodies. The variety of mixtures that will be obtained include mixtures of 2 bispecific antibodies recognizing 4 epitopes, 2 bispecific antibodies and one monospecific antibody, or mixtures of 1 bispecific and one monospecific antibody from a single cell. Table 6 depicts the transfection schedule and expected results.

Table 6

Variety of mixture	Transfection of	Transfection code and ratio	Expected species	Expected % monoclonal IgG	Expected % Bispecific
2 BsAbs, 4 epitopes	IV+V+IX+X'	ZA (1:1:1:1)	BC + AD	0	50 + 50
2 BsAbs, 4 epitopes	IV+VII+IX+X'	ZB (1:1:1:1)	BC + AD	0	50 + 50
2 bsAbs + 1 mAb	IV+V+VI+wt'	ZC (2:1:1:2)	BC + AB + DD	33	33 + 33
2 bsAbs + 1 mAb	IV+V+VI+II'	ZD (2:1:1:2)	BC + AB + DD	33	33 + 33
2 bsAbs + 1 mAb	IV+V+VI+III'	ZE (2:1:1:2)	BC + AB + DD	33	33 + 33

1 bsAb + 1 mAb	IV+V+wt'	ZF (1:1:2)	BC + DD	50	50
1 bsAb + 1 mAb	IV+V+II'	ZG(1:1:2)	BC + DD	50	50
1 bsAb + 1 mAb	IV+V+III'	ZH(1:1:2)	BC + DD	50	50
1 bsAb + 1 mAb	IV+VII+wt ,	ZI (1:1:2)	BC + DD	50	50
1 bsAb + 1 mAb	IV+VII+II'	ZJ (1:1:2)	BC + DD	50	50
1 bsAb + 1 mAb	IV+VII+III ,	ZK (1:1:2)	BC + DD	50	50
1 bsAb + 1 mAb	IX+X+wt'	ZL (1:1:2)	AB + DD	50	50
1 bsAb + 1 mAb	IX+X+II'	ZM (1:1:2)	AB + DD	50	50
1 bsAb + 1 mAb	IX+X+III'	ZN (1:1:2)	AB + DD	50	50

Although, theoretically, production of all mixtures should be feasible, it is known from previous work by others that large scale production of classical knob-into-hole variants is hampered by instability issues. Mixtures resulting from transfections ZA,
5 ZB, ZL, ZM and ZN are thus expected to become problematic when transferred to larger scale production.

Thus, the current set of constructs present in Table 1 would not allow production of all theoretical mixtures from single cells at a larger scale, as knob-into-hole variants are reported to be unstable, and it cannot be excluded that CH3 domains comprising a
10 'knob' or a 'hole' will dimerize with either charge variants or wildtype CH3 domains. It is thus desired to design new CH3-variants that are engineered to preferentially form homodimers or heterodimers only and which will not homo- or heterodimerize with constructs 1-5 of Table 1 as to allow for co-expression in single cells.

Example 13: identification of novel charge pair mutants

The objective of this study was to engineer the IgG CH3 region to result in the production of only heterodimers or only homodimers upon mixed expression of different IgG heavy chains in a single cell, wherein the novel engineered CH3

5 domains will not homo- or heterodimerize with known engineered CH3 domains, or with wildtype CH3 domains. Therefore, as a first step in identifying novel engineered CH3 domains that would meet the criteria, many interface contact residues in the IgG CH3 domain were scanned one by one or in groups for substitutions that would result in repulsion of identical heavy chains – i.e., reduced homodimer formation - via

10 electrostatic interactions. The objective was to obtain a list of residues that, when substituted by a charged residue, would result in repulsion of identical chains such that these mutations may be used to drive homo- and/or heterodimer formation upon mixed expression of different IgG heavy chains, whereby the obtained full length IgGs are stable and are produced with high proportions. In a follow up, the identified

15 substitutions will be used to generate bispecific antibodies or mixtures of bispecific or monospecific antibodies by engineering matched pairs of CH3 residues in one or more IgG heavy chains - CH3 regions. Additionally, newly identified charge mutant pairs may be combined with existing pairs, such that multiple nucleic acid molecules encoding different heavy chains, all carrying different and complementing CH3

20 mutations, can be used for expression in cells such that mixtures of monospecific antibodies only, or bispecific antibodies only, or mixtures of defined monospecific and bispecific antibodies can preferentially be obtained. The residues to be tested in the present study are contact residues as previously identified (Deisenhofer J., 1981; Miller S., 1990; Padlan, 1996, Gunasekaran, 2010). The rationale for this approach is

25 that repulsive charges are engineered into each available pair of contacting residues. Samples are subsequently analyzed on non-reducing SDS-PAGE to identify pairs in which dimer formation is reduced, as visualized by the presence of bands of approximately 72 kD. All available pairs will be screened as single mutations or in combination with a single other mutation as the repulsive electrostatic interaction

30 between one non-matching pair may or may not be sufficient to result in sufficient amounts of half-molecules for detection by this method, the mutations are also combined.

Amino acid substitutions were introduced in construct vector MV1057 by Geneart according to the table 7 and expression of constructs was performed by transfection in HEK293T cells, according to standard procedures. IgG expression levels were measured in Octet. When production failed twice, the mutation was considered to be detrimental to expression and the mutation was not pursued further.

Table 7: list of amino acid substitutions in the various constructs that were made (EU numbering)

AA substitutions in CH3	construct #	Effect on homodimer formation (- = no effect; +++ = max. inhibition; NT= not tested on gel)
Q347K	8	-
Y349D	9	+.
Y349K	10	+.
T350K	11	-
T350K, S354K	12	+.
L351K, S354K	13	+.
L351K, T366K	14	++
L351K, P352K	15	+.
L351K, P353K	16	++
S354K, Y349K	17	++
D356K	18	-
E357K	19	-
S364K	20	++
T366K, L351K	21	++
T366K, Y407K	22	+++
L368K	23	NT
L368K, S364K	24	++
N390K, S400K	25	+.
T394K, V397K	26	+

AA substitutions in CH3	construct #	Effect on homodimer formation (- = no effect; +++ = max. inhibition; NT= not tested on gel)
T394K, F405K	27	+++
T394K, Y407K	28	+++
P395K, V397K	29	+.
S400K	30	-
F405K	31	+++
Y407K	32	++
Q347K, V397K, T394K	33	+
Y349D, P395K, V397K	34	+
T350K, T394K, V397K	35	NT
L351K, S354K, S400K	36	+
S354K, Y349K, Y407K	37	+.
T350K, N390K, S400K	38	+.
L368K, F405K	39	++
D356K, T366K, L351K	40	+++
Q347K, S364K	41	+++
L368D, Y407F	42	+
T366K	43	+
L351K, S354K, T366K	44	+
Y349D, Y407D	45	+
Y349D, S364K,	46	+

AA substitutions in CH3	construct #	Effect on homodimer formation (- = no effect; +++ = max. inhibition; NT= not tested on gel)
Y407D		
Y349D, S364K, S400K, T407D	47	+
D399K	48	+.
D399R	49	+.
D399H	50	+.
K392D	51	+.
K392E	52	+.
K409D	53	+

Supernatants containing ≥ 5 $\mu\text{g/ml}$ IgG were analyzed in SDS-PAGE and IgG was purified using protein A. The proteins were stained using colloidal blue. Homodimers were visible as a band of approximately 150 kD. Smaller bands of approx 75 kD
5 represented the presence of half molecules (see negative control: K392D, K409D). Blots are shown in Figure 7.

The results of SDS-PAGE gels were analyzed and scored as presented in table 7, right hand column. A number of residues were considered promising for further testing in combination, including residues Q347, S354, Y349, L351, K360, T366, T394, and
10 V397. The choice was based on high scores in the inhibition of formation of homodimers combined with the availability of contacting residues that can be modified without running into issues such as other non-complementary charges. For example, it is known that residues F405 and Y407 have multiple interactions at the CH3-CH3 interface, including interactions with residues that are already charged,
15 which may be problematic after introduction of multiple charge mutations among these interacting residues (see Table A). New constructs were made in vector MV1057 (Table 8), and antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain were used for recloning into vectors containing

these new constructs (see Table 9) such that combinations could further be tested. Table 10 depicts the transfection schedules and results.

Table 8:

AA substitutions in CH3	construct #
L351K	61
T394K	62
L351D	63
T366D	64
S354D, Y349D	65
V397D	66
K360D	67

Table 9: VH specificity inserted in different constructs

Vector	VH gene	Antigen specificity	VH mass (Da)	Cloned in construct #
XI	IGHV 1.08	Tetanus (A)	13703	8
XII	IGHV 1.08	Tetanus (A)	13703	17
XIII	IGHV 1.08	Tetanus (A)	13703	43
XIV	IGHV 1.08	Tetanus (A)	13703	61
XV	IGHV 1.08	Tetanus (A)	13703	62
XVI	IGHV 3.30	Fibrinogen (C)	12794	63
XVII	IGHV 3.30	Fibrinogen (C)	12794	64
XVIII	IGHV 3.30	Fibrinogen (C)	12794	65
XIX	IGHV 3.30	Fibrinogen (C)	12794	66
XX	IGHV 3.30	Fibrinogen (C)	12794	67

Table 10:

Transfection of	Transfection code (ratio)	Expected species	AA found (%)	AC found (%)	CC found (%)	Half A found (%)	Half C found (%)	other (%)
XIII + XVI	ZO (1:1)	AC	0	69	7	24	0	0
	ZT (3:1)	AC	10	45	16	27	0	0
	ZU (1:1)	AC	5	61	10	13	0	0
	ZV (1:3)	AC	3	61	23	13	0	0
	ZW (1:1)	AC	0	88.3	2.4	7	0	2.3
XIV + XVII	ZP	AC	30	52	13	0	0	5
XII + XVIII	ZQ	AC	4	51	33	2	1	8
XV + XIX	ZR	AC	20	42	11	0	1	26
XI + XX	ZS	AC	34	41	15	0	0	10

Combinations of CH3 variants were expressed, and analyzed in SDS-PAGE (data not shown) and in native mass spectrometry (MS). Results are summarized in Table 10.

- 5 The ZO transfection resulted in the highest proportion of heterodimers in the mixtures (69% AC). Interestingly, in the ZO transfection, the AA homodimer was not present whereas the CC homodimer comprised a small proportion (7%). Mass spectrometric analysis unveiled that the remaining protein in the mixture consisted of half A molecules, probably resulting from unequal expression of the A and C heavy chains. The raw MS data from transfection sample ZO are shown in Figure 8.
- 10 Surprisingly, whereas transfection ZO resulted in fair amounts of bispecific product, the reverse charge pair of transfection ZP (L351K/T366'D versus T366K/L351'D of ZO) did not result in similar results, and only 52% of bispecific product was observed, with considerable amounts of the two homodimers being present (30% AA and 13% CC). An explanation for this may be that the negatively charged D structurally closely resembles T, hence the T366D may not be potent enough to repulse itself and T366D may thus still form homodimers as was indeed observed.
- 15 It can be envisaged that subtle variants of the newly found T366K/L351'D pair (e.g. by testing all permutations including new constructs T366R and L351E) may result in similar percentages of BsAbs.
- 20

Example 14: HADDOCK for design of new CH3 mutants to drive efficient heterodimerization.

As described in example 13, the newly found charge pair T366K/L351'D increases the
5 proportion of heterodimers in the mixture (69 %) with a small fraction of undesired
CC homodimers (7%) (L351D/L351'D) and a substantial fraction of half A molecules
(24%) 'contaminating' the mixture. In this example, an in silico approach was used to
generate further insight in amino acid residues involved in CH3 interface
interactions, to test complementary substitutions in opposing CH3 regions and to find
10 novel CH3 pairs containing complementary substitutions that further increase
efficient heterodimerization while preventing efficient formation of homodimers of the
two heavy chains.

HADDOCK (High Ambiguity Driven protein-protein DOCKing) is an information-
driven flexible docking approach for the modeling of biomolecular complexes.

15 HADDOCK distinguishes itself from ab-initio docking methods in the fact that it
encodes information from identified or predicted protein interfaces in ambiguous
interaction restraints (AIRs) to drive the docking process. (de Vries et al., 2010).
The input for the HADDOCK web server consists of a protein structure file, which can
be a crystal structure, NMR structure cluster or a modeled structure. After the
20 docking or refinement, HADDOCK returns a so-called HADDOCK score, which is a
weighted average of VanderWaals energy, electrostatic energy, buried surface area
and desolvation energy. The HADDOCK score can be interpreted as an indication of
binding energy or affinity, even though a direct translation to experimental data is
often hard to achieve. In addition to this, HADDOCK provides structure files for the
25 'top four' structures that resulted from the docking run. These structure files can be
downloaded and visualized, enabling the detailed analysis of the interactions of the
individual residues.

In this example, the interactions between the CH3-domains of the IgG1 heavy chains
were studied. A high-resolution crystal structure of the Fc part of the IgG (structure
30 1L6X) was used as starting structure
(<http://www.rcsb.org/pdb/explore/explore.do?structureId=1l6x> ; Idusogie, E.E. et al.,
J.I. 2000(164)4178-4184).

In example 13, it was found that co-transfection of vectors XIII and XVI resulted in the formation of the CC homodimeric contaminant (Table 10). HADDOCK was used to search for additional mutations to the T366K/L351'D pair that prevent homodimerization.

- 5 The HADDOCK output consists of a set of calculated energies, a HADDOCK score (which is a weighted average of the energies) and four structure files corresponding to the four lowest-energy structures found by the program. The HADDOCK-scores are used to compare different structures; the other energies are merely used to get an indication about what is happening in the structures (e.g. good electrostatic
- 10 interactions, smaller buried surface, high Van der Waals energy). The lower the HADDOCK score, the better. For each mutation pair, the scores were calculated for the AA, AB and BB dimers.

Sets of mutation pairs from example 12 were run in HADDOCK to see whether the calculated energies would correlate to the experimental data. Table 11 presents all

15 theoretical energies, which are visualized in Figure 9.

Table 11:

Construct combinations	HADDOCK Score	VdW energy	Electrostatic energy	Desolvation energy	Buried surface area
Wildtype-wildtype	-208.2	-62.8	-773	9.2	2505.8
1-2 (E356KD399K - K392DK409D)	-225.8	-56.4	-862	3	2458.3
2-2 (K392DK409D - K392DK409D)	-180.3	-67.9	-562.1	0.1	2312.5
1-1 (E356KD399K - E356KD399K)	-176.7	-75.5	-469.3	-7.3	2349.6
1-3 (E356KD399K - K392DK409DK439D)	-220.6	-67.9	-793.8	6.1	2499.8
3-3 (K392DK409DK439D - K392DK409DK439D)	-150.1	-76.6	-387.6	4.1	2261.2
6-7 (T366W -)	-221.3	-65.8	-735.5	-8.3	2509.0

T366SL368AY407V)					
6-6 (T366W – T366W)	1916.9*	2072.3	-681.3	-19.2	2499.9
7-7 (T366SL368AY407V – T366SL368AY407V)	-191.9	-55.0	-683.2	-0.2	2427.2
43-63 (T366K – L351D)	-210.6	-64	-758.4	5.1	2456.5
43-43 (T366K – T366K)	-191.7	-71.2	-634.1	6.3	2533.5
63-63 (L351D – L351D)	-212.5	-60.4	-774	2.6	2445.6

*this value is unusually high due to high VanderWaals energy score, probably due to steric clash of T366W/T366'W

With 2 wildtype CH3 domains, the HADDOCK scores are the same for AA, AB and
 5 BB because the A and B CH3 regions are identical. In most other cases, the AB pair has the lowest score, which is as expected. For the T366K/L351D pair the BB score is slightly better than the AB score (-210.6 vs. -212.5), but this difference is within the error of the calculations. Using HADDOCK, the structures of the heterodimers of these pairs were visualized. For example, the construct combinations 1-2, 1-1 and 2-2
 10 are presented in Figure 10. From these visualizations it is apparent that salt bridges are formed in the heterodimer (Figure 10A *left hand panel*) whereas electrostatic repulsion occurs between residues of identical chains (Figure 10B and C, *middle and right hand panel*). The higher HADDOCK scores for the homodimers can thus be explained by the electrostatic repulsion of the mutated interface residues. These
 15 residues have to bend away from each other and don't have interaction with residues on the other chain, causing a drop in the affinity.

Table 11 and Figure 9 confirm what was observed in example 13. The T366K/L351'D AC heterodimer and the L351D/L351'D CC homodimer form with a similar energy, explaining the presence of both the heterodimer and homodimer in the mixture. The
 20 T366K/T366'K AA homodimer, on the other hand, is barely detectable in the mixture although T366K half A molecules are present. Table 11 and Figure 9 indeed show that the HADDOCK score for the T366K/T366'K CC homodimer is higher than the score for the AC heterodimer; hence formation of this homodimer is energetically less favorable.

25

Example 15: 366/351 variations

In example 13, it is hypothesized that alternatives for the T366K/L351'D mutant charge pair can be designed that may have similar results in terms of percentage of bispecific antibodies in the mixture. Alternatives may include substitutions T366R, T366D, T366E, L351E, L351K and L351R. The proportion of CC homodimers of L351D/L351'D may be diminished by creating variants of the 366/351 pair. All possible mutation pairs were run in HADDOCK and the resulting scores are presented in Table 12 and visualized in Figure 11.

10 Table 12

Construct combinations	HADDOCK Score	VdW energy	Electrostatic energy	Desolvation energy	Buried surface area
T366K – L351D	-210.6	-64	-758.4	5.1	2456.5
T366K – T366K	-191.7	-71.2	-634.1	6.3	2533.5
L351D – L351D	-212.5	-60.4	-774	2.6	2445.6
T366K – L351E	-216.9	-55.7	-854.7	9.8	2532.7
L351E – L351E	-217.9	-65.5	-802.2	8	2532
T366R – L351D	-210.5	-68.8	-760.8	10.4	2514.5
T366R – T366R	-201.8	-77.4	-626.4	0.9	2608
T366R – L351E	-225.8	-56.2	-874.8	5.4	2579.2
T366D – L351R	-211.2	-71.3	-723.6	4.8	2455.6
T366D – T366D	-198.1	-58.1	-713.4	2.1	2477
L351R – L351R	-220.7	-75.5	-806.5	16.1	2552.2
T366D – L351K	-223.9	-62.1	-810.1	0.3	2487.8
L351K – L351K	-224.4	-75.6	-812.1	13.6	204.5
T366E – L351R	-222.3	-69	-783	3.4	2557.2
T366E – T366E	-201.9	-57.6	-741	4	2487.5
T366E – L351K	-215.9	-58.4	-808.9	4.3	2486

When looking at the HADDOCK scores, it was observed that some of the mutations have a similar 'pattern' when compared to T366K/L351'D. For most permutations the AA homodimer was found to have a higher HADDOCK-score than the AB

heterodimer, but the BB homodimer appeared as favorable as the AB heterodimer. Even though the 351 residue is known to be a 'neighbor' to itself on the other chain, i.e. residue 351 of chain A pairs with residue 351 of chain B at the CH3-CH3 interface, there is barely a negative influence of the identical charges when the BB dimer is formed. Looking at the L351D/L351'D structure this is explained by the aspartic acids bending away from each other and the stabilizing influence of at least the naturally occurring Arginine at position 355 and also some stabilization of negative charge by the naturally occurring Serine at position 354 (see Figure 12A). Mutation of these residues (S354A and R355D) provides only little improvement. From figure 12B it is clear that the backbone-hydrogen of A354 causes stabilization of the homodimer. From this series, the T366R/L351'E pair seems to be the most favorable, with the lowest HADDOCK score for the bispecific molecule.

Example 16: mutations around T366K/L351'D

In the series of HADDOCK analyses in this example, the T366K/L351'D or T366K/L351'E pair were taken as a starting structure. In order to identify additional mutations that would further increase the predicted percentage of bispecifics of these A and B chains, additional mutations on the B-chain were used to calculate the HADDOCK-scores and energies. When the structure of the CH3 domain is studied using a viewer for visualization of protein structures at a molecular level (YASARA, www.yasara.org), one can calculate the distances between individual residues. While doing so, it was observed that the two residues Y349 and L368 are neighboring residues that may contribute positively or negatively to dimer interactions and these have been mutated in this example –in addition to the L351D mutation– to study the result on dimer formation of the homo- and heterodimers (see figure 13). Both residues seem to add to the stability of the heterodimer (lower HADDOCK scores) as well as to the destabilization of the BB dimer (higher HADDOCK scores). Glutamic acids (E) on positions 349 and 368 seem to be more favorable than aspartic acids (D). Thus, introduction of a second amino acid substitution in the B-chain, comprising already the amino acid substitution at position 351, seems to favor heterodimerization further.

In a next set of HADDOCK analyses, the T366K/L351'D pair was again taken as starting structure. In addition to the substitutions in the B chain that further

increased heterodimerization (i.e. Y349D/E and L368E), additional mutations were added to the A-chain which already comprises the T366K substitution. As shown in Figure 14, there are several mutation pairs that seem favorable towards the formation of bispecific heterodimers. In the T366K-L351K/L351'D-Y349'D pair, all
5 four mutated residues are involved in the heterodimeric pairing, which is not de case for T366K-L351K/L351'E-L368'E in which K351 is not directly involved in the binding. However, the HADDOCK-score for this latter heterodimer is -228.9; significantly lower than the -214.2 for the T366K/ L351'E-L368'E, which can be explained by hydrogen bonding interactions of the K at position 351 (see Figure 15).
10 The T366K-L351K/L351'D-Y349'D pair may be further improved by the R355'D mutation in the B-chain, which results in a higher BB-HADDOCK score, but also the AB HADDOCK score is slightly higher. Overall the additional L351K results in lower AB scores and similar AA and BB scores when compared to the sole T366K mutation in the A chain. Theoretically this would result in higher amounts of bispecific
15 heterodimers in the samples.

As is apparent from figure 11, having an R rather than a K at position 366 may be more potent in driving heterodimerization. Therefore, some of the HADDOCK analyses shown in figure 13 were repeated but now with T366R rather than T366K in the A-chain. It was demonstrated that it is not favourable to combine an R366 in
20 chain A with double mutations in chain B (figure 16). This may be due to the large size of this residue, interfering with other interface interactions, even though all the expected salt-bridges with R366 are present in the structures. Also, the HADDOCK score for the AA homodimer is lower for R366 than for K366, which also doesn't contribute favorably to heterodimer formation. Therefore no further HADDOCK
25 analyses were performed using R366 in the interface.

A total of 14 best performing pairs, according to HADDOCK predictions, have been selected (see Table 13 and Figure 17). In some pairs, an R355D substitution is included to remove the stabilizing influence of the naturally occurring R355 on the L351/L351'D interaction.

30

Table 13:

Construct combinations	HADDOCK Score AB	HADDOCK Score AA	HADDOCK Score BB
Wildtype-wildtype	-208.2	-208.2	-208.2
T366K – L351D	-210.6	-191.7	-212.5
T366K – L351E	-216.9	-191.7	-217.9
T366R – L351E	-225.8	-201.8	-217.9
T366E – L351R	-222.3	-201.9	-220.3
T366K – L351DY349E	-215.9	-191.7	-190
T366K – L351DL368E	-223.3	-191.7	-198.9
T366K – L351EY349E	-214.5	-191.7	-187.5
T366KL351K – L351D	-233.2	-205	-212.5
T366K – L351DY349EL368E	-207.5	-191.7	-179.5
T366KL351K – L351DY349D	-255.2	-205	-204.3
T366KL351K – L351DY349E	-227.2	-205	-190
T366KL351K – L351DL368E	-243.9	-205	-198.9
T366KL351K – L351DR355D	-233.6	-205	-211.9
T366KL351K – L351DY349DR355D	-242.8	-205	-183.5
T366D – L351KY349K	-237.9	-198.1	-228.4

Example 17: in vitro expression of bispecifics using CH3 mutants based on HADDOCK predictions

- The analysis in example 16 suggested that some CH3 variants with additional mutations around the T366K/L351D pair would yield mixtures with higher proportions of the bispecific component and potentially lower proportions of the homodimeric component. These best performing pairs were selected for production and further analysis. Table 14 lists the constructs that were made and which were

used for recloning antibody VH regions with known specificities and known ability to pair with the human IGKV1-39 light chain.

Table 14:

AA substitutions in CH3	Construct #
T366K, L351K	68
L351D, L368E	69
L351E, Y349E	70
L351D, Y349E	71
L351D, R355D	72
L351D, Y349E, L368E	73
L351D, Y349D	74
L351D, Y349D, R355D	75
L351K, L368K	76
L351R	77
T366E	78

5

The results of expression of two different heavy chains carrying the amino acid substitutions shown in Table 14 or heavy chains carrying the amino acid substitutions of previous constructs are presented in Table 15. Expression of two different heavy chains comprising the amino acid substitutions T366K and L351'D-L368E respectively resulted in approximately 87% of the bispecific AB heterodimer in the mixture with no AA or BB homodimers present (combination nr. 3 of Table 15). About 12% half molecules (half A) comprising the T366K substitution was observed. Furthermore, it was found that the percentage of bispecific AB heterodimer increased when the additional amino acid substitution L351K was introduced in the first heavy chain. Expression of two different heavy chains comprising the amino acid substitutions T366K-L351K and L351'D-L368'E respectively resulted in approximately 92% of bispecific AB heterodimer and AA and BB homodimers are essentially absent in the mixture (combination nr. 12 of Table 15). The absence of homodimers is advantageous, because the fraction containing the intact IgG molecules is composed of AB heterodimer only. For purification and subsequent

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therapeutic application, the half molecules can be removed by standard approaches such as size exclusion chromatography. Hence, applying these newly identified charge mutants in the production process for generating bispecific antibodies provides advantages over known charge mutants and knobs-into-holes mutants where the presence of 'contaminating' homodimeric antibodies is not excluded. In addition, the T366K/L351'D:L368'E and T366K:L351K/ L351'D:L368'E charge pairs have an additional advantage over the previously described E356K:D399K/K392'D:K409'D and E356K:D399K/K392'D:K409'D:K439'D charge pairs, in that the previously described charge variants are based on the reversal of existing charges within the CH3-CH3 interface whereas our newly identified charge variants are adding additional charge pairs to the CH3-CH3 interface. The introduction of additional charge pairs in the CH3-CH3 interface may further increase the stability of the interface and thereby of the intact antibody. The same holds true for the mutations used in combinations nrs. 4, 5, 6, 9, 10, and 11, which also resulted in favorable proportions of bispecific heterodimer with exceedingly low proportions of AA and BB homodimers present in the mixtures.

Table 15*:

Combination of 2 different heavy chains	Mutations A (construct #)	Mutations B (construct #)	% AA found	% AB found	% BB found	% half A found	% half B found
1	T366E (78)	L351R (77)	3	81	2	13	0
2	T366K (43)	L351D (63)	0	88	3	9	0
3	T366K (43)	L351D,L368E (69)	0	87	0	12	0
4	T366K (43)	L351E,Y349E (70)	2	85	0	11	0
5	T366K (43)	L351D,Y349E (71)	2	92	1	5	0
6	T366K (43)	L351D,Y349E,L368E (73)	0	96	1	4	0
7	T366K,L351K (68)	L351D (63)	0	77	12	10	1
8	T366K,L351K (68)	L351D,R355D (72)	0	79	8	10	1
9	T366K,L351K	L351D,Y349D,R355D	1	93	2	4	1

	(68)	(75)					
10	T366K,L351K (68)	L351D,Y349D (74)	1	95	1	3	0
11	T366K,L351K (68)	L351D,Y349E (71)	1	95	0	3	1
12	T366K,L351K (68)	L351D,L368E (69)	0	92	0	8	0

*The greyed cells in this Table represent results of the preferred combinations of novel charge variants for driving heterodimerization.

5 Example 18: IgG stability analyses

In this study, a series of CH3 mutation pairs that resulted in high proportions of bispecific heterodimers in the intact IgG fraction and very low amounts (<5%) of parental IgGs will be further analyzed for stability of the Fc part of the IgG molecule. The mutated CH3 domains that are used to promote the heterodimerization of the heavy chains may have unexpected destabilizing effects on the Fc region of the IgG, that may result in undesirable properties such as a reduction of *in vivo* half life, reduction in effector function and an increase in immunogenicity. The newly identified charge pairs will be compared to wildtype bispecifics and a bispecific containing previously identified charge mutations (chain A comprising construct 1 and chain B comprising construct 2). All bispecifics in this study will contain the same heavy and light chain variable regions, ensuring that the observed effects are caused by mutations in the Fc-part of the molecule and not by variation in the variable regions.

A method well known in the art for determining protein-protein interactions and stability is native PAGE gel electrophoresis. Proteins with a lower stability may unfold in native PAGE resulting in different migration patterns compared to stable molecules. Since the heavy chains of the IgG are not only interacting through the CH3 regions, but are also bound by sulfide bridges, the electrophoresis should be performed under reducing conditions. The samples will thus be analyzed with SDS-PAGE, reduced native PAGE using the NativePAGE™ Novex® Bis-Tris Gel System according to manufacturer's instructions (user manual NativePAGE™ Novex® Bis-Tris Gel System). Additionally, native MS analyses will be performed.

Differential Scanning Calorimetry and stability studies

Differential Scanning Calorimetry (DSC) is a well known analysis method to determine the stability of the various regions of the IgG; variants/regions with a lower stability may have lower melting temperatures. In previous experiments (data not shown) it was observed that the melting peak of the Fab largely overlaps with the melting peak of the CH3 domain, in such a way that the latter is often not distinguishable as a peak or shoulder. To prevent this, the enzyme papain can be used to cleave the Fab fragments from the IgG, so that only the Fc part is left. This can be purified using protein A, resulting in pure IgG-Fc if the cleavage is 100% complete. The protein samples will be digested using papain using the Pierce® Fab preparation kit, followed by SDS-PAGE to confirm the cleavage and purification of the Fc-region. DSC analysis will be performed according to standard procedures (Ionescu et al., J. Pharm. Sci. 2008 (97)1414).

A series of stability studies will be performed on these bispecifics. These studies include spectroscopic (UV-Vis absorbance, fluorescence and light-scatter) and microscopic (light and fluorescence microscopy with Nile Red staining) analyses that provide information on the aggregation state of the CH3 variants.

The UV-Vis absorbance spectra will be recorded with a double beam, two monochromators Cary 300 Bio spectrophotometer at 25°C. The spectra will be monitored between 250 and 400 nm using a path length of 1 cm. The absorbance at wavelengths of 320 nm and longer provides information on the aggregation state of the IgG.

Intrinsic fluorescence spectra will be monitored at 25°C using a FluoroMax spectrofluorimeter. The fluorescence method will be optimized. The fluorescence emission will provide information on conformation and aggregation properties.

90° light-scattering spectra will be monitored at 25°C using a FluoroMax spectrofluorimeter by running a synchronous scan ($\lambda_{em} = \lambda_{ex}$) between 400 nm and 750 nm with an integration time of 0.01s. Excitation and emission slits will be optimized.

For example, right angle light-scattering can distinguish between IgG samples that have no and 5% dimers.

For fluorescence microscopy with Nile Red staining, just prior to measurements, Nile Red in ethanol will be added to the sample. The samples will be filled in an

microscopy slide and analyzed by fluorescence microscopy. Particles will be counted. The lower size limit of the particles that can be observed by fluorescence microscopy is approximately 0.5 μm .

Application of stress such as temperature, pH, mechanical stress, denaturants on
5 proteins might result in a conformation change (e.g. unfolding) and/or aggregation. As
it was previously reported that charge-engineered bispecific antibodies have reduced
melting temperature of the modified CH3 (Gunasekaran 2010), these studies aim to
discriminate between the novel charge mutants of the present invention and existing
known charge mutants.

10 Thermo-stability studies using the Octet are explored, both with Protein A biosensors
and by using FcRn binding to IgG. To examine the thermal stability of CH3-
engineered IgGs, the samples will be incubated at a concentration of 100 $\mu\text{g/ml}$ (in
PBS) at 4, 50, 55, 60, 65, 70 and 75°C for 1 hour using a PCR machine. Following this
the samples will be cooled down slowly during a period of 15 minutes to 25°C and kept
15 at this temperature for 2 hours, after which they will be stored overnight at 4°C.
Precipitated antibodies will be removed by centrifugation, after which the total IgG
concentration of soluble antibodies will be determined by Octet using the protein A
Biosensor (1/10 dilution in PBS). Assays that measure binding of the CH3 engineered
IgG to FcRn using the Octet are being explored. Either protein L biosensors are used
20 to bind the light chain of IgG to the sensor, followed by incubation with FcRn in
solution, or anti-penta-HIS biosensors are used to bind His-tagged FcRn protein,
followed by incubation with the IgG of interest. These methods may be more sensitive
than using the protein A Biosensor and can also be used for thermal stability studies.
All samples will also be analyzed for serum stability. Briefly, (engineered) IgG
25 samples will be incubated at 37°C in human serum, control samples will be kept at
4°C. After 1, 2, 3 and 4 weeks, samples are centrifuged to remove precipitated IgG.
Subsequently the sample is titrated in antigen-specific ELISA to determine the
relative amounts of functional IgG. Purified control antibody freshly spiked in human
serum will be used as a reference.

30

**Example 20: improved mixtures of two bispecific antibodies recognizing 4
different epitopes (AB and CD) from a single cell**

In example 12 it is hypothesized that mixtures resulting from transfections ZA or ZB are expected to become problematic when transferred to larger scale production, as knob-into-hole variants are reported to be unstable and it cannot be excluded that CH3 domains comprising a 'knob' or a 'hole' will dimerize with charge-engineered CH3 domains. As it is demonstrated in the above that novel charge pair mutants have been found that preferentially drive heterodimerization, with virtually no formation of homodimers, CH3 domain-comprising polypeptide chains comprising these novel charge pair mutants can be expressed in cells together with previously known charge-engineered CH3 domain-comprising polypeptide chains or SEED bodies, resulting in the preferential formation of two bispecific molecules only.

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Claims

1. A method for producing at least two different Ig-like molecules from a single
5 host cell, wherein each of said two Ig-like molecules comprises two CH3 domains that
are capable of forming an interface, said method comprising providing in said cell
 - a. A first nucleic acid molecule encoding a 1st CH3 domain-comprising
polypeptide chain,
 - b. A second nucleic acid molecule encoding a 2nd CH3 domain-comprising
10 polypeptide chain,
 - c. A third nucleic acid molecule encoding a 3rd CH3 domain-comprising
polypeptide chain, and
 - d. A fourth nucleic acid molecule encoding a 4th CH3 domain-comprising
polypeptide chain,
15 wherein at least two of said nucleic acid molecules are provided with means for
preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said
3rd and 4th CH3-domain comprising polypeptides, said method further comprising the
step of culturing said host cell and allowing for expression of said at least four nucleic
acid molecules and harvesting said at least two different Ig-like molecules from the
20 culture.

2. The method of claim 1, further comprising providing said host cell with a
nucleic acid molecule encoding a common light chain.

- 25 3. The method of claim 1 or 2, wherein each of the CH3-domain comprising
polypeptide chains further comprises a variable region recognizing a target epitope.

4. The method of claim 3, wherein each of the 4 variable regions of the 4 CH3-
domain comprising polypeptide chains recognize different target epitopes.
30

5. The method of claim 3, wherein the variable regions of the 1st and the 2nd CH3-
domain comprising polypeptide chains recognize different target epitopes, whereas

the variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains recognize the same target epitopes.

6. The method of claim 5 wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is the same as
5 the target epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain.

7. The method of claim 5 wherein the target epitope recognized by the variable regions of the 3rd and 4th CH3 domain comprising polypeptide chain is different from
10 the target epitope recognized by the variable region of the 1st or the 2nd CH3-domain comprising polypeptide chain.

8. The method of claim 3, wherein the variable regions of the 1st and the 2nd CH3-domain comprising polypeptide chains recognize the same target epitope, whereas the
15 variable regions of the 3rd and the 4th CH3-domain comprising polypeptide chains recognize a second target epitope which differs from the target epitope recognized by said 1st and 2nd variable regions.

9. The method of any one of claims 3-8 wherein the target epitopes are located on
20 the same target molecule.

10. The method of claim 9 wherein the target molecule is a soluble molecule.

11. The method of claim 9 wherein the target molecule is a membrane-bound
25 molecule.

12. The method of any one of claims 3-8 wherein the target epitopes are located on different target molecules.

30 13. The method of claim 12, wherein the different target molecules are expressed on the same cells.

14. The method of claim 12, wherein the different target molecules are expressed on different cells.
15. The method of claim 12, wherein the target molecules are soluble molecules.
- 5
16. The method of claim 12, wherein one target molecule is a soluble molecule whereas the second target molecule is a membrane bound molecule.
17. The method according to any one of claims 1-16, wherein said at least two
- 10 different Ig-like molecules are antibodies.
18. The method of claim 1, wherein said means for preferential pairing comprises engineered complementary knob-into-hole mutations, disulfide bridges, charge mutations or combinations thereof.
- 15
19. The method of claim 18, wherein said means for preferential pairing are selected from Table B.
20. The method of claim 1, wherein all 4 of said nucleic acid molecules are provided with means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides and said 3rd and 4th CH3-domain comprising polypeptides, wherein said means for preferential pairing of said 1st and 2nd CH3 domain-comprising polypeptides are different from those means for preferential pairing of said 3rd and 4th CH3-domain comprising polypeptides.
- 25
21. The method of any one of claims 3-8, 12-14 or 16, wherein at least one of said target epitopes is located on a tumor cell.
22. The method of any one of claims 3-8, 12-14 or 16, wherein at least one of said
- 30 target epitopes is located on an effector cell.
23. The method of claim 22, wherein said effector cell is an NK cell, a T cell, a B cell, a monocyte, a macrophage, a dendritic cell or a neutrophilic granulocyte.

24. The method of any one of claims 22-23, wherein said target epitope is located on a CD3, CD16, CD25, CD28, CD64, CD89, NKG2D or a NKp46 molecule.

5

25. A method for producing a single Ig-like molecule from a single cell, wherein said Ig-like molecule comprises two CH3 domains that are capable of forming an interface and wherein said Ig-like molecule is a heterodimer and wherein contaminating homodimers are less than 5%, preferably less than 2%, more preferably less than 1%, and most preferably essentially absent, said method comprising providing in said cell

- a. A first nucleic acid molecule encoding a 1st CH3 domain-comprising polypeptide chain,
 - b. A second nucleic acid molecule encoding a 2nd CH3 domain-comprising polypeptide chain,
- wherein said first CH3 domain-comprising polypeptide chain comprises the amino acid substitution T366K and wherein said second CH3 domain comprising polypeptide chain comprises the amino acid substitutions L351D and L368E, said method further comprising the step of culturing said host cell and allowing for expression of said at two nucleic acid molecules and harvesting said single Ig-like molecules from the culture.

26. The method of claim 25, wherein said first CH3-domain-comprising polypeptide chain further comprises the amino acid substitution L351K.

25

Title: Methods and means for the production of Ig-like molecules

Abstract

The invention provides means and methods for producing one or more Ig-like molecules in a single host cell. Novel CH3 mutations enabling the production of monospecific and/or bispecific Ig-like molecules of interest are also provided.

Provisional Application for Patent Cover Sheet

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

Inventor(s)

Inventor 1					Remove
Given Name	Middle Name	Family Name	City	State	Country ;
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All Inventors Must Be Listed – Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. **Add**

Title of Invention	Methods and means for the production of Ig-like molecules
Attorney Docket Number (if applicable)	P88706US00

Correspondence Address

Direct all correspondence to (select one):

<input checked="" type="radio"/> The address corresponding to Customer Number	<input type="radio"/> Firm or Individual Name
Customer Number	76637

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.	
<input checked="" type="radio"/> No.	
<input type="radio"/> Yes, the name of the U.S. Government agency and the Government contract number are:	

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Entity Status

Applicant claims small entity status under 37 CFR 1.27

- Yes, applicant qualifies for small entity status under 37 CFR 1.27
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Please see 37 CFR 1.4(d) for the form of the signature.

Signature	/Tamara Elmore/			Date (YYYY-MM-DD)	2012-04-20
First Name	Tamara	Last Name	Elmore	Registration Number (If appropriate)	61088

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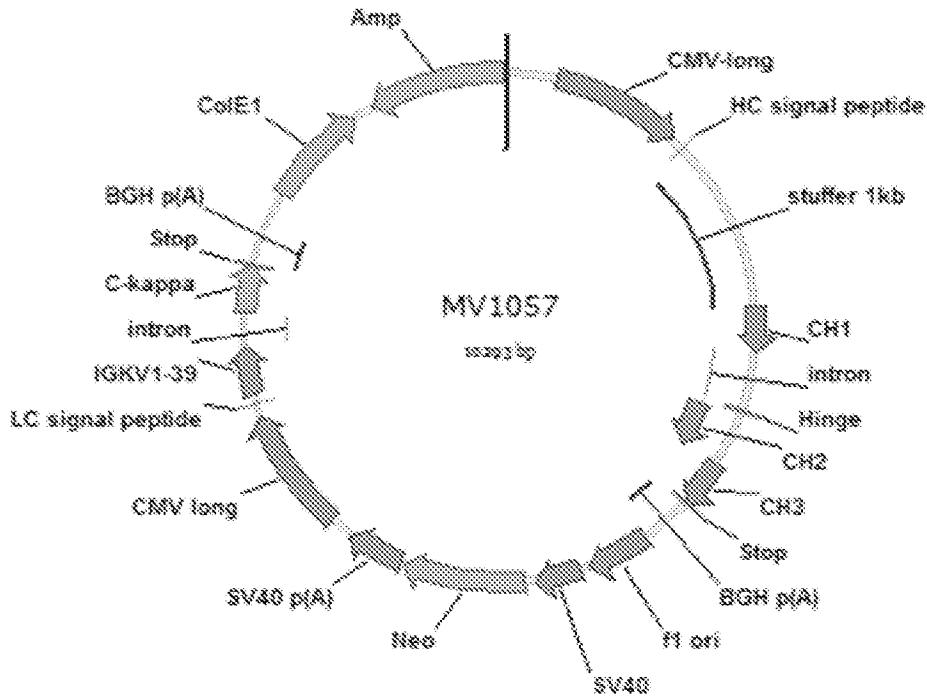
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Figure 1:

A) schematic representation of construct vector MV1057



B) schematic representation of phage display vector MV1043

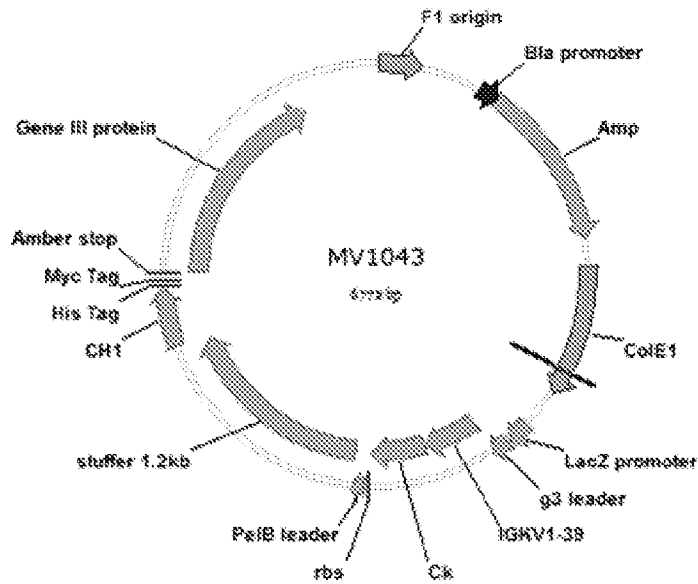


Figure 2: amino acid sequence of wildtype IgG1 Fc, as present in construct vector MV1057 (EU numbering scheme applied)

131	141	151	161	171	181
SSKSTSGGTA	ALGCLVKDYF	PEPVTVSWNS	GALTSGVHTF	PAVLQSSGLY	SLSSVTVPS
191	201	211	221	231	241
SSLGTQTYIC	NVNHKPSNTK	VDKRVEPKSC	DKTHTCPPCP	APELLGGPSV	FLFPPKPKDT
251	261	271	281	291	301
LMISRTPEVT	CVVVDVSHED	PEVKFNWYVD	GVEVHNAKTK	PREEQYNSTY	RVVSVLTVLH
311	321	331	341	351	361
QDWLNGKEYK	CKVSNKALPA	PIEKTISKAK	GQPREPQVYT	LPPSREEMTK	NQVSLTCLVK
371	381	391	401	411	421
GFYPSDIAVE	WESNGQPENN	YKTTTPVLDS	DGSFFLYSKL	TVDKSRWQQG	NVFSCSVMHE
431	441				
ALHNHYTQKS	LSLSPGK				

Figure 3: nucleotide and amino acid sequences of VH regions used

MF1025_VH

gaggtgcagctggtggagtctgggggaggcttggtacagcctgggggtccctgagactctcctgtgcag
cctctggattcacctttagcagctatgccatgagctgggtccgccaggctccagggaggggctggagtg
ggtctcagctattagtggtagtggtgtagcacatactacgcagactccgtgaagggccggttcaccatc
tccagagacaattccaagaacacgctgtatctgcaaatgaacagcctgagagccgaggacacggccgtgt
attactgtgcaagggccgattggtgggcgacttttgactactggggccaaggtaccctggtcacc

MF1025_VH

EVQLVESGGGLVQPGGSLRLSCLAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTI
SRDNSKNTLYLQMNSLRAEDTAVYYCARADWWATFDYWGQGLVT

MF1122_VH

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cctctggattcaccttcagtagctatggcatgcaactgggtccgccaggctccaggcaagggctggagtg
ggtggcagttatatcatatgatggaagtaataaatactatgcagactccgtgaagggccgattcaccatc
tccagagacaattccaagaacacgctgtatctgcaaatgaacagcctgagagctgaggacacggccgtgt
attactgtgcaagagccctcttcacgaccatcgccatggactattggggccaaggtaccctggtcacc

MF1122_VH


EVQLVESGGGVVQPGRSLRLSCLAASGFTFSSYGMHWVRQAPGKLEWVAVISYDGSNKYYADSVKGRFTI
SRDNSKNTLYLQMNSLRAEDTAVYYCARALFTTIAMDYWGQGLVT

MF1337_VH

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cttttcaagacagagacggcgccctactatcacttcgctctggacgtctggggccaagggaccacggtcacc

MF1337

VHEVQLVETGAEVKKPGASVKVSKASDYIFTKYDINWVRQAPGQGLEWGWMSANTGNTGYAQKFQGRVTMTRD
TSINTAYMELSSLTSGDTAVYFCARSSLFKTEAPYYHFALDVWGQGTITV

H	MF1122	
	Construct 5: E356K	
	E357K	
	K439D K370D	

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10-10-2010_11_0100_11_0100_11_0100_11_0100_11_0100

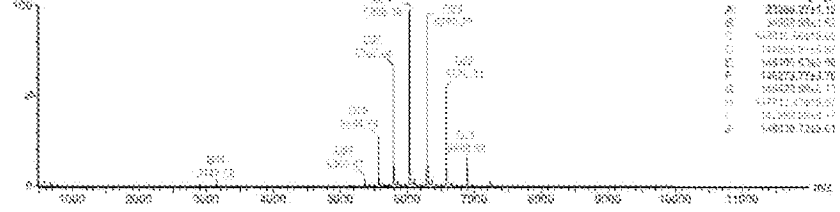


Figure 5

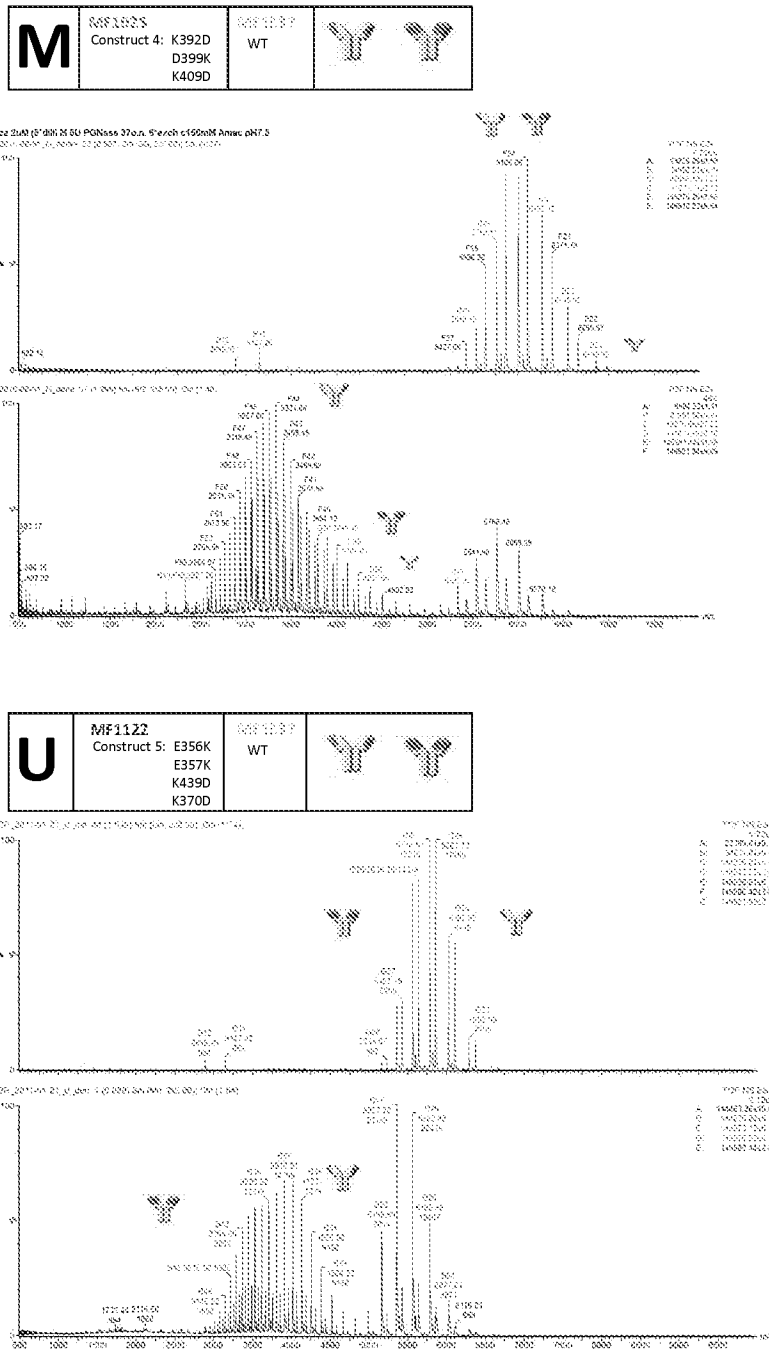


Figure 6:

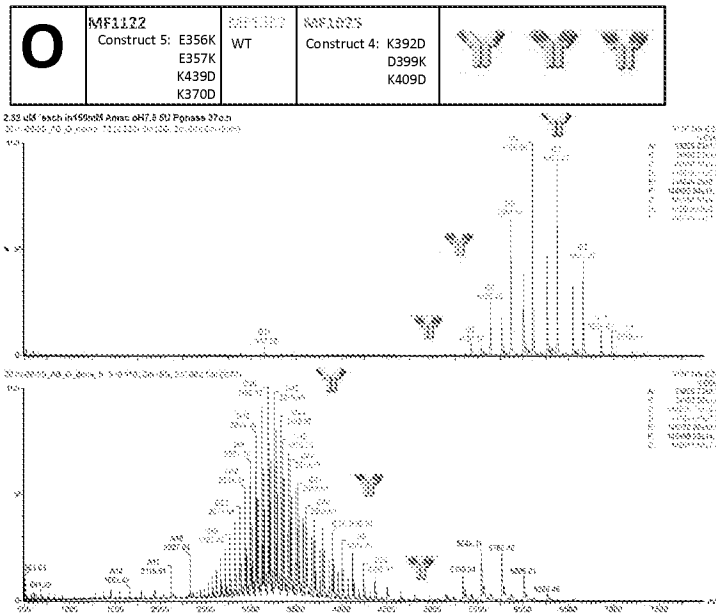
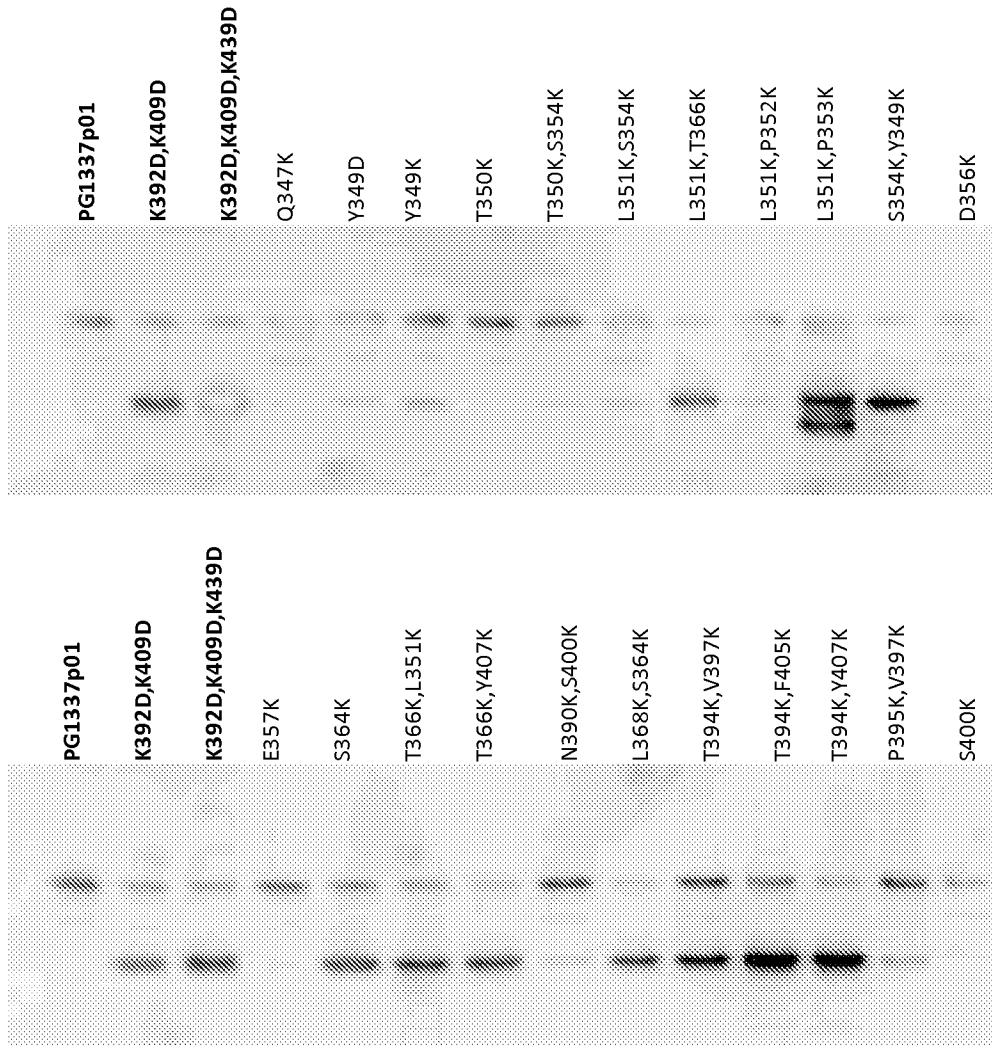


Figure 7:



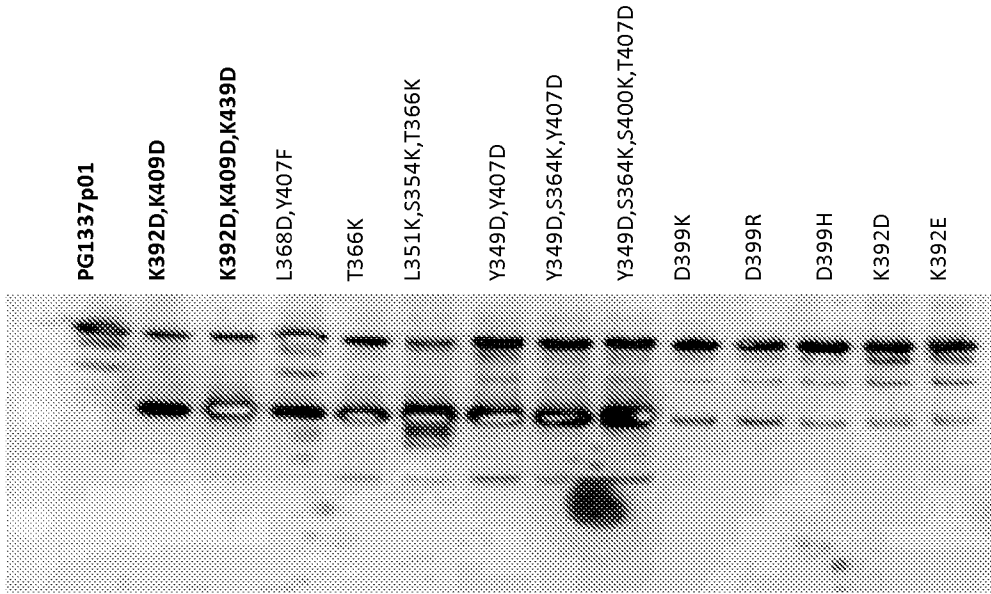
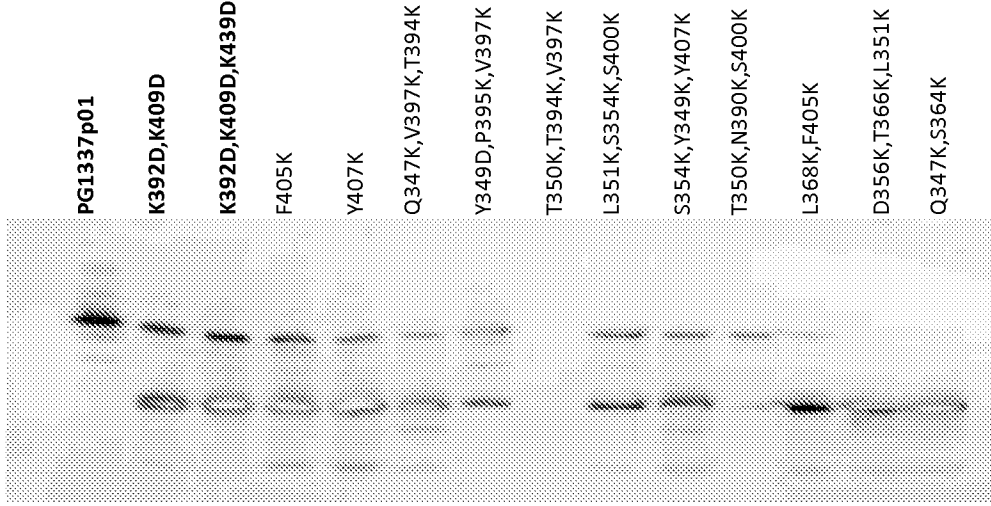
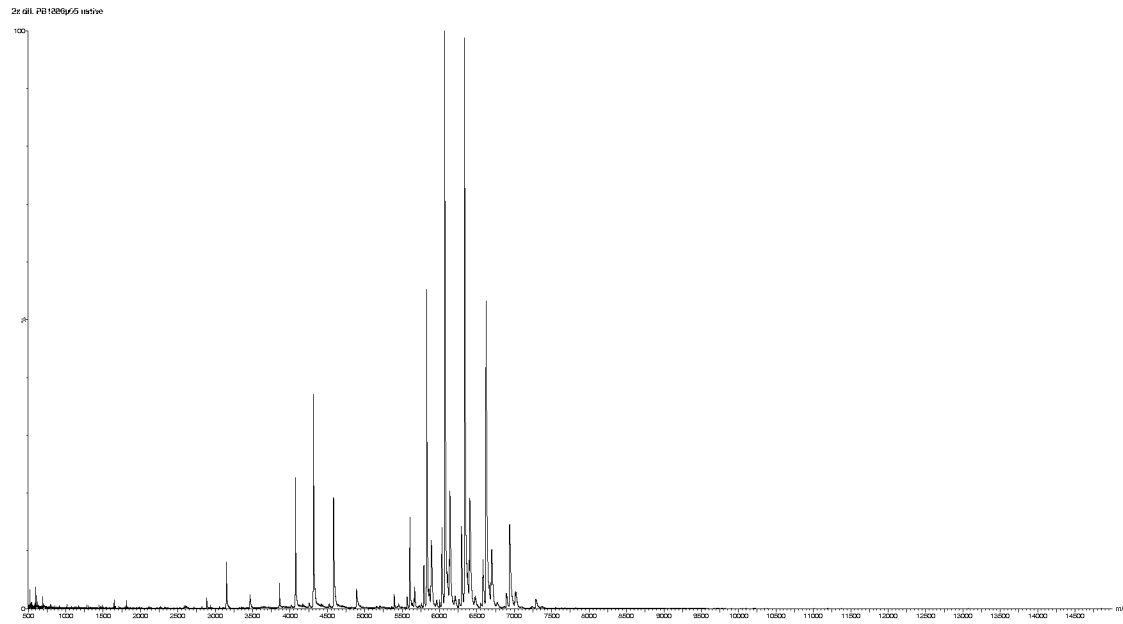
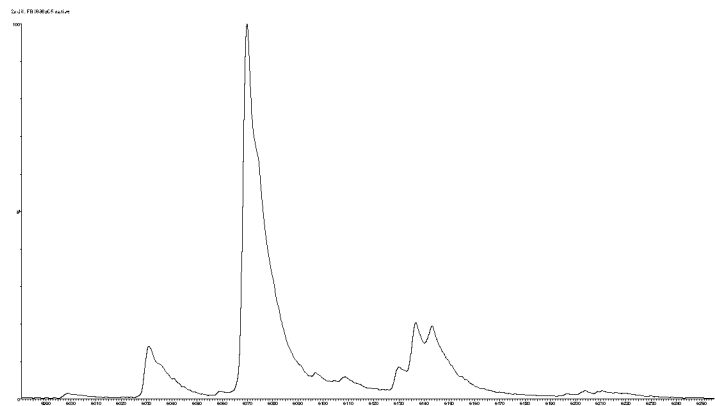


Figure 8:



A) Native MS spectrum of transfection sample ZO (T366K/L351'D)



B) Convolved MS spectrum of transfection sample ZO (T366K/L351'D). The second/main peak represents the bispecific molecule.

Figure 9:

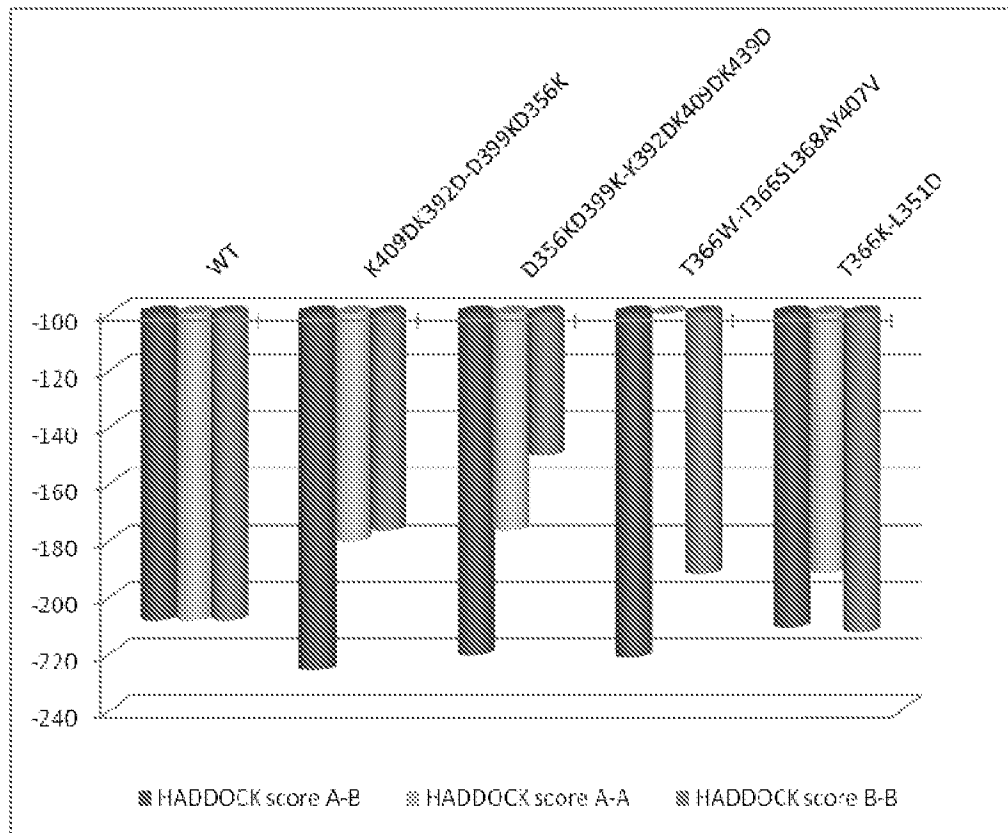
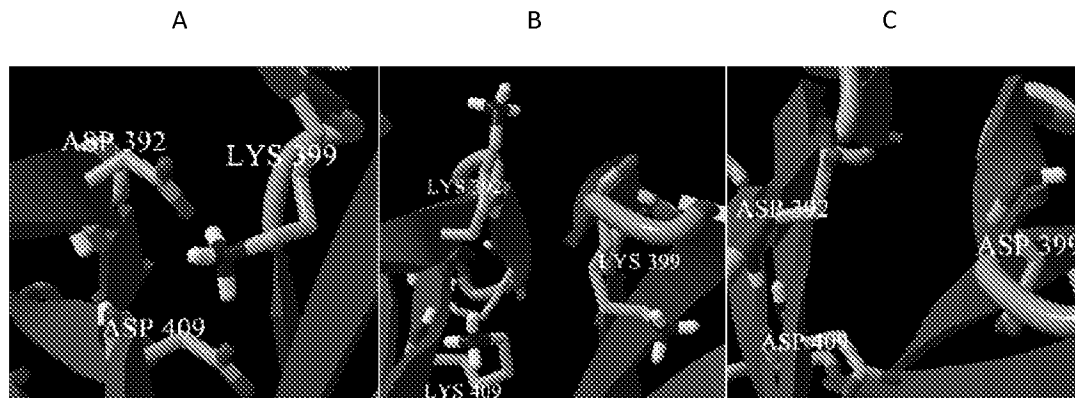


Figure 10



- A K409D:K392D/D399'K:E356'K
B D399K:E356K/D399'K:E356'K
C K409D:K392D/K409'D:K392'D

Figure 11:

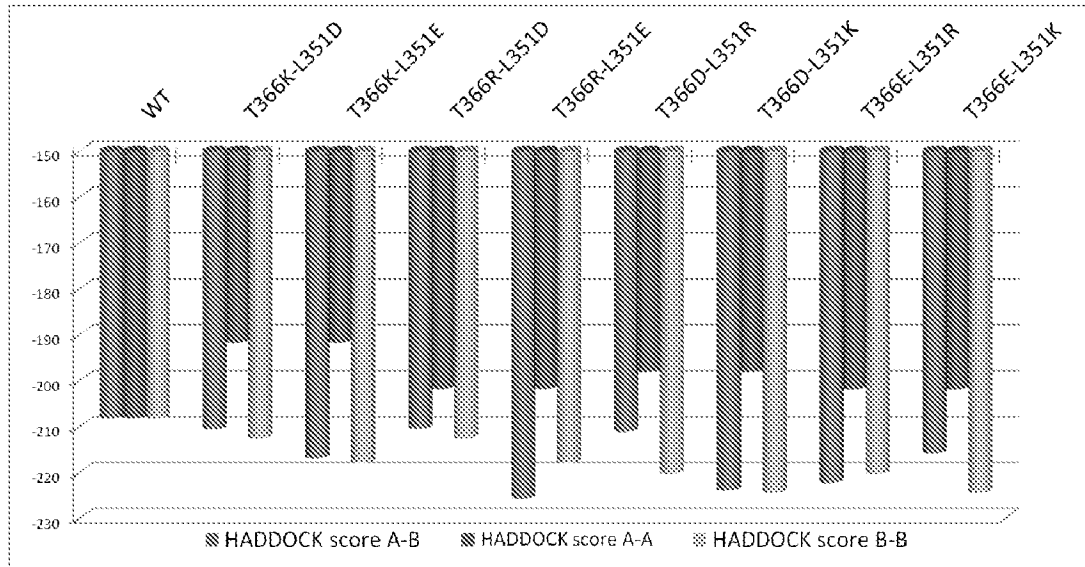
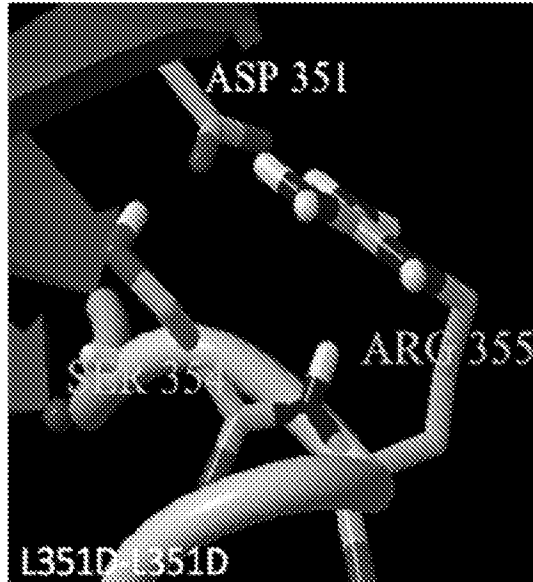


Fig 12:

A



B

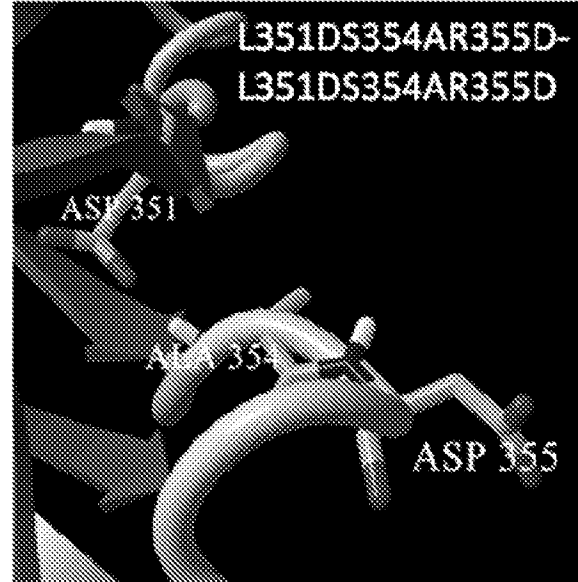


Figure 13:

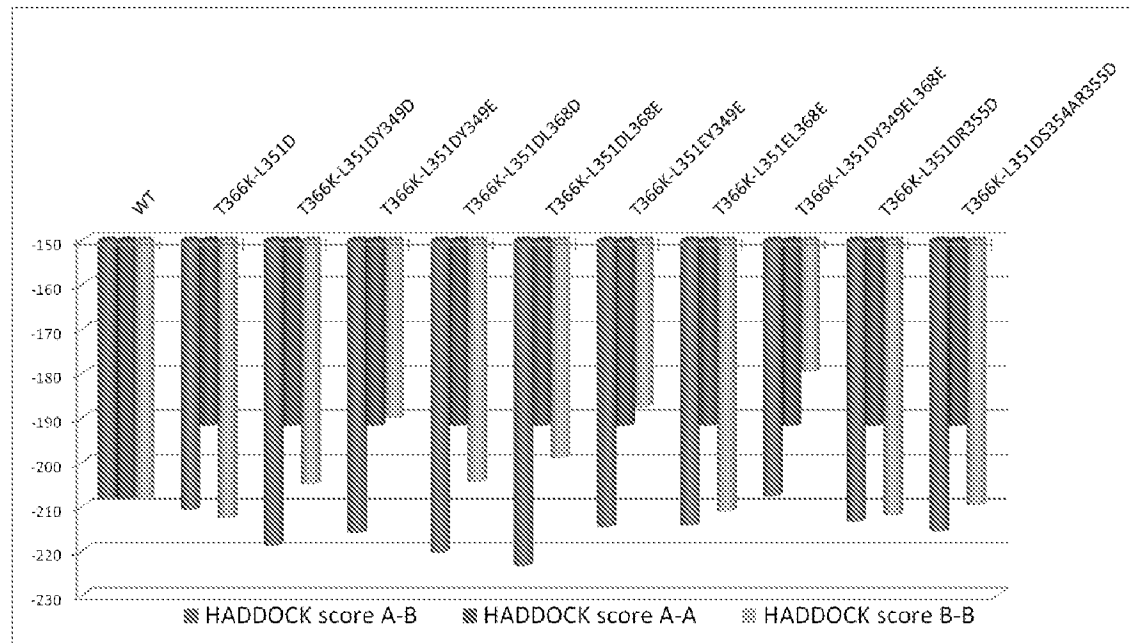


Figure 14:

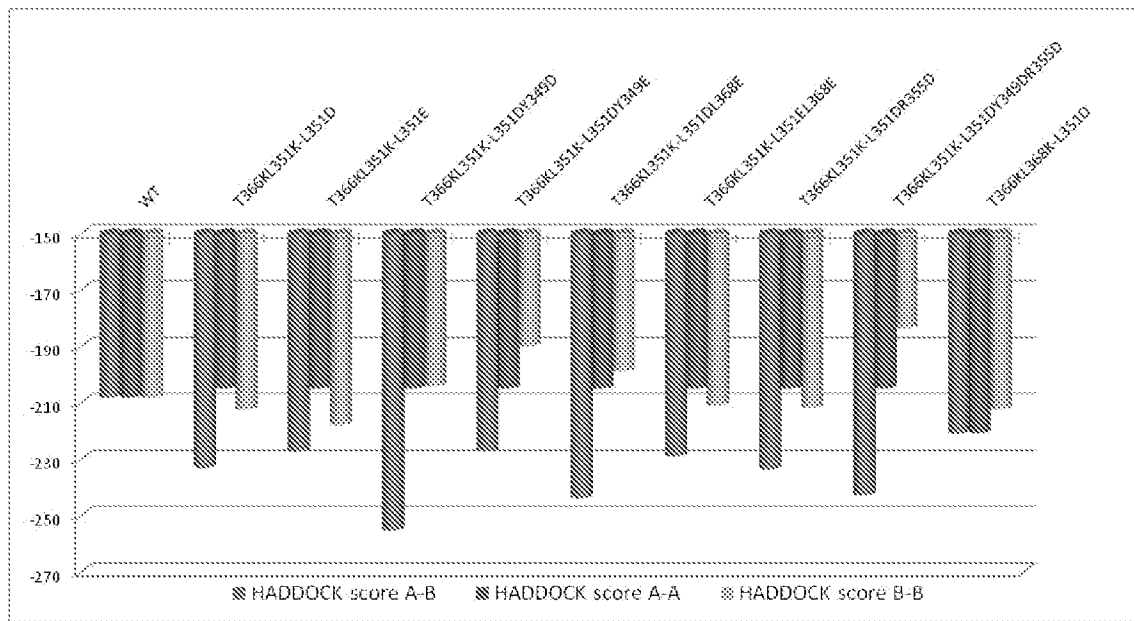


Figure 15:

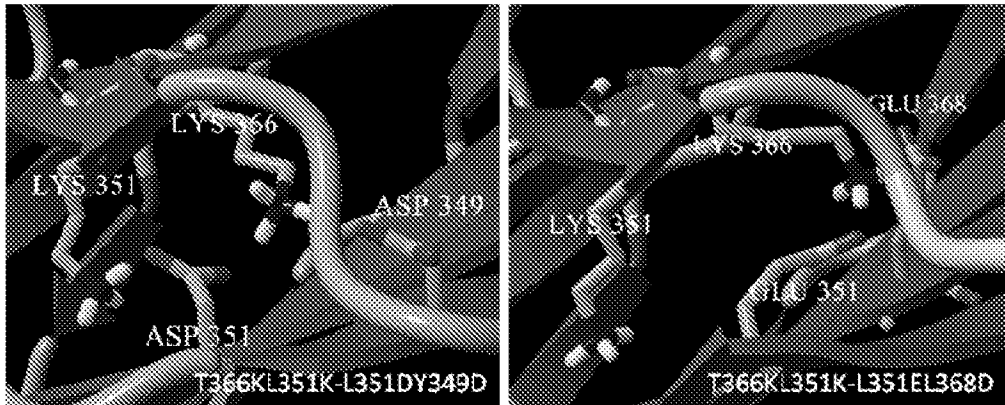


Figure 16:

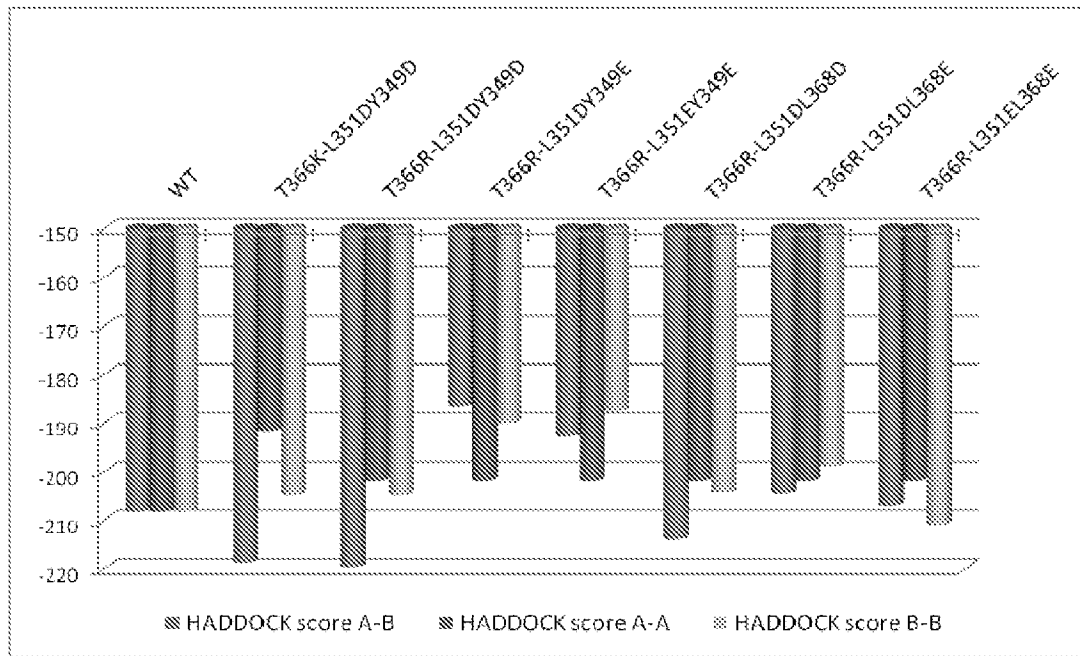
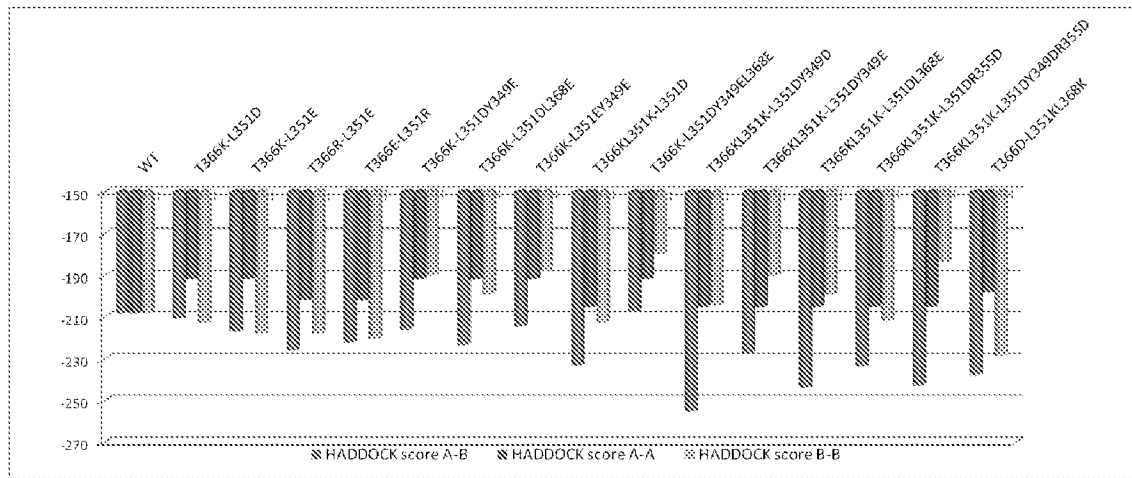


Figure 17:



Electronic Patent Application Fee Transmittal

Application Number:				
Filing Date:				
Title of Invention:	Methods and means for the production of Ig-like molecules			
First Named Inventor/Applicant Name:	Cornelis Adriaan de Kruif			
Filer:	Tamara J. Elmore/Diana Tabben			
Attorney Docket Number:	P88706US00			
Filed as Small Entity				
Provisional Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Provisional Application filing fee	2005	1	125	125
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				125

Electronic Acknowledgement Receipt

EFS ID:	12589004
Application Number:	61635935
International Application Number:	
Confirmation Number:	7304
Title of Invention:	Methods and means for the production of Ig-like molecules
First Named Inventor/Applicant Name:	Cornelis Adriaan de Kruif
Customer Number:	76637
Filer:	Tamara J. Elmore/Diana Tabben
Filer Authorized By:	Tamara J. Elmore
Attorney Docket Number:	P88706US00
Receipt Date:	20-APR-2012
Filing Date:	
Time Stamp:	11:11:30
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$125
RAM confirmation Number	8686
Deposit Account	505610
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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1	Specification	P88706US00_Specification.pdf	409795 cc7d1aefe41a13244d94234400af5bbda196ed06	no	76
Warnings:					
Information:					
2	Provisional Cover Sheet (SB16)	P88706US00_Provisional_Cover_Sheet.pdf	1522782 6cf199484c5a7340a536bd8327dc7c9cd0f87f91	no	3
Warnings:					
Information:					
3	Drawings-other than black and white line drawings	P88706US00_Figures.pdf	3213792 092cd465143f9ccdc7a313e046e2e18c652757f1	no	19
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	29399 7dab5a33a3acfb1959c85297aa6e5b0b54966e10	no	2
Warnings:					
Information:					
Total Files Size (in bytes):				5175768	

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Application Number: 61635935

Document Date: 4/20/2012

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- Drawings

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