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(54) **HEAT-STABLE RESPIRATORY SYNCYTIAL VIRUS F PROTEIN OLIGOMERS AND THEIR USE IN IMMUNOLOGICAL COMPOSITIONS**

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 CPC **C07K 14/135**; **A61K 31/155**
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(57) **ABSTRACT**

Heat-stable oligomeric recombinant polypeptides, presenting at least one antigenic epitope of the pre-fusion Respiratory Syncytial Virus (RSV) F protein, comprising the RSV F protein ectodomain, functionally deleted in the HRB region, transmembrane and cytoplasmic domains replaced with a heterologous trimerization domain, and absent two functional multibasic furin cleavage sites, are useful as antigenic components in immunogenic compositions useful in methods of inducing an immune response and vaccinate against RSV infections.

25 Claims, 27 Drawing Sheets

Specification includes a Sequence Listing.

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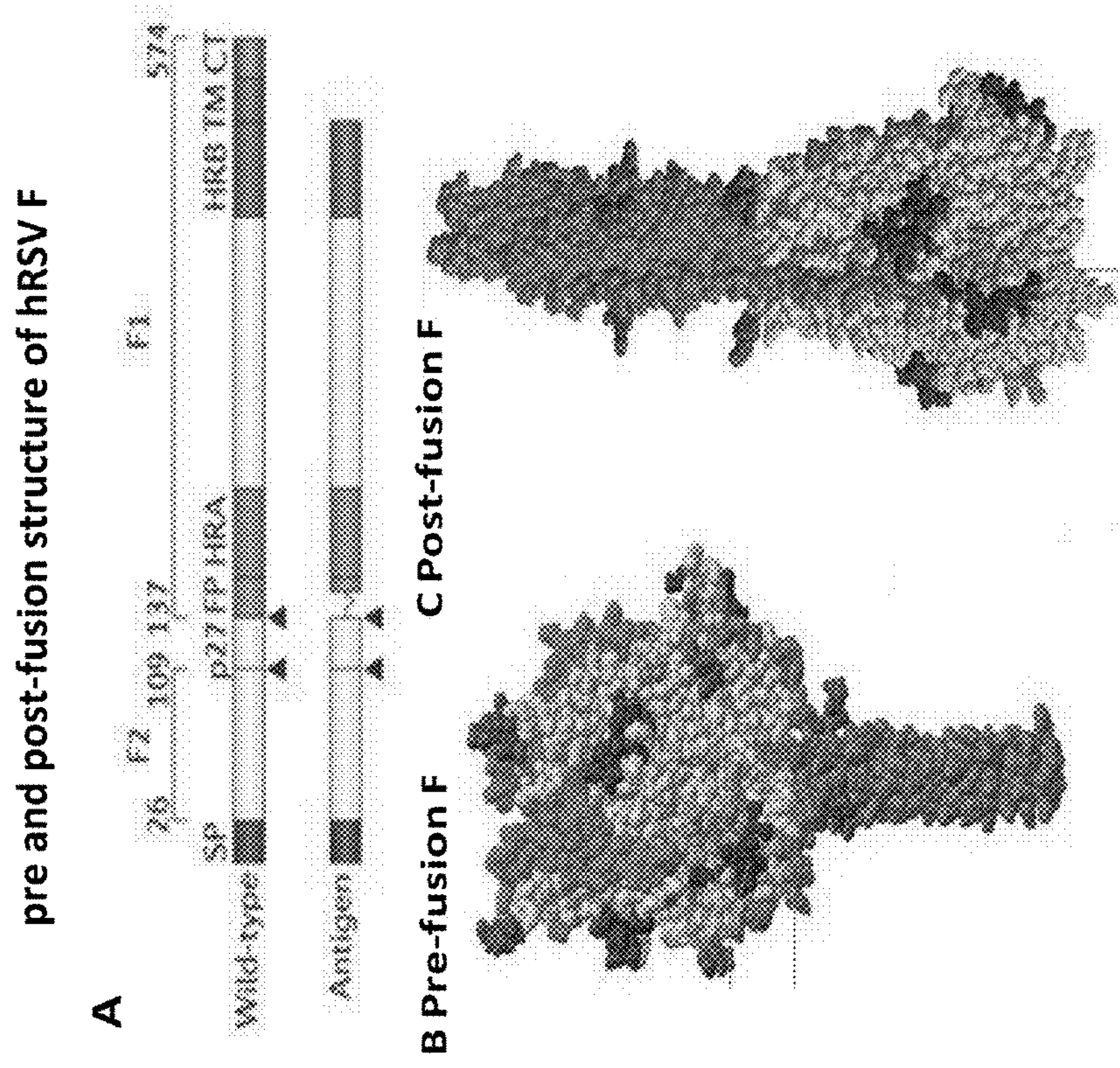


Fig. 1

antigenic sites in pre- and post-fusion hRSV F

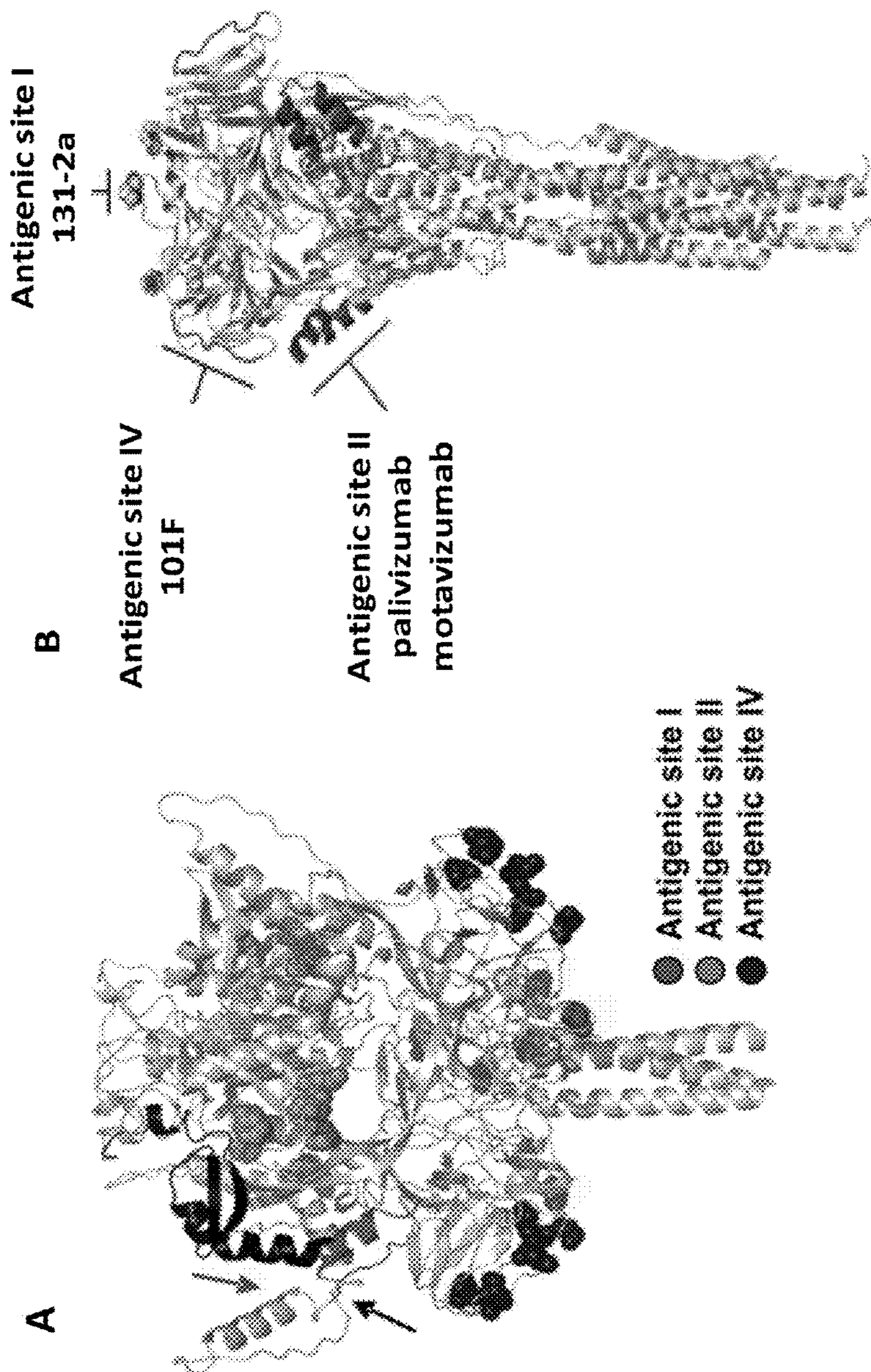
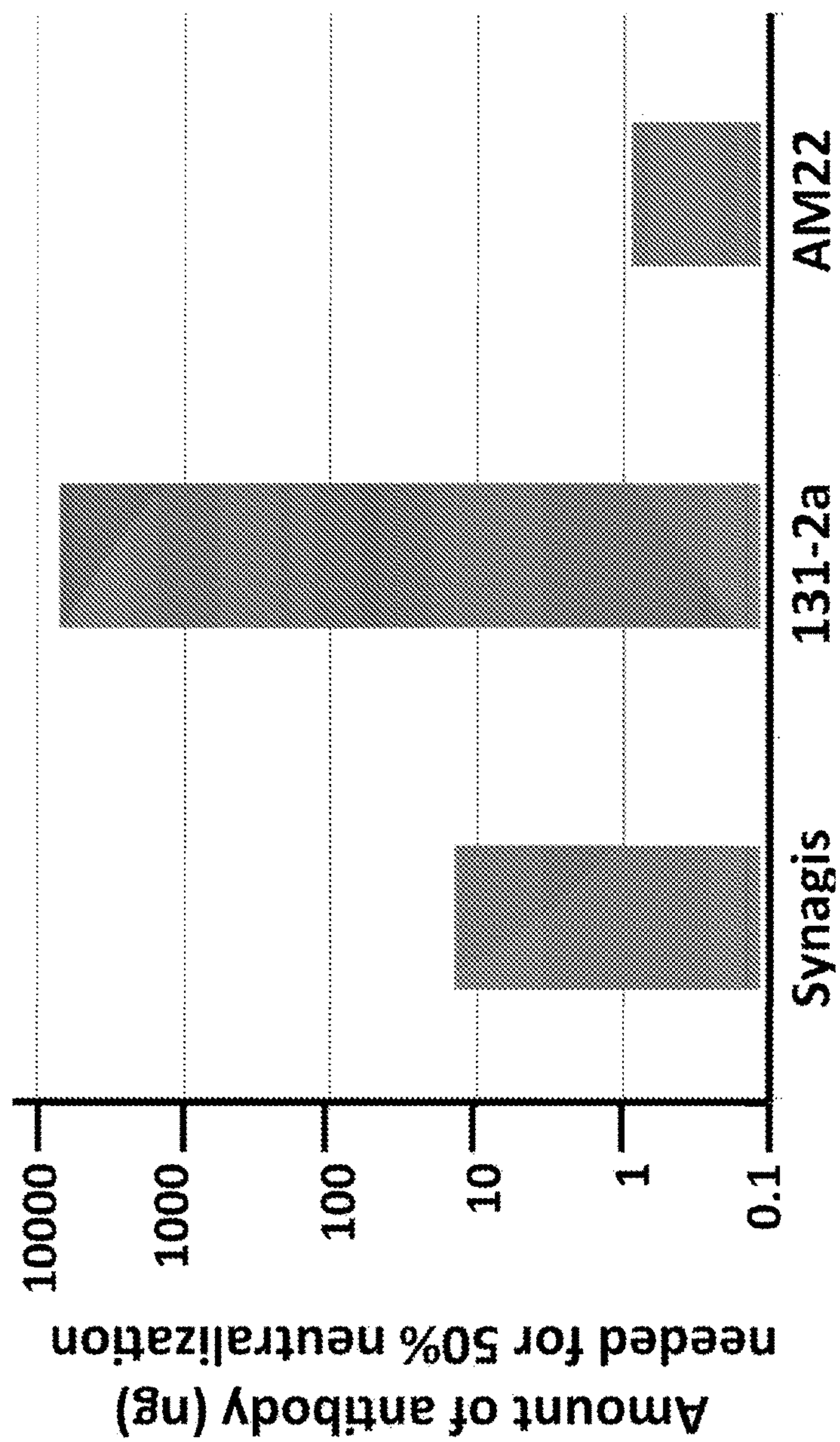
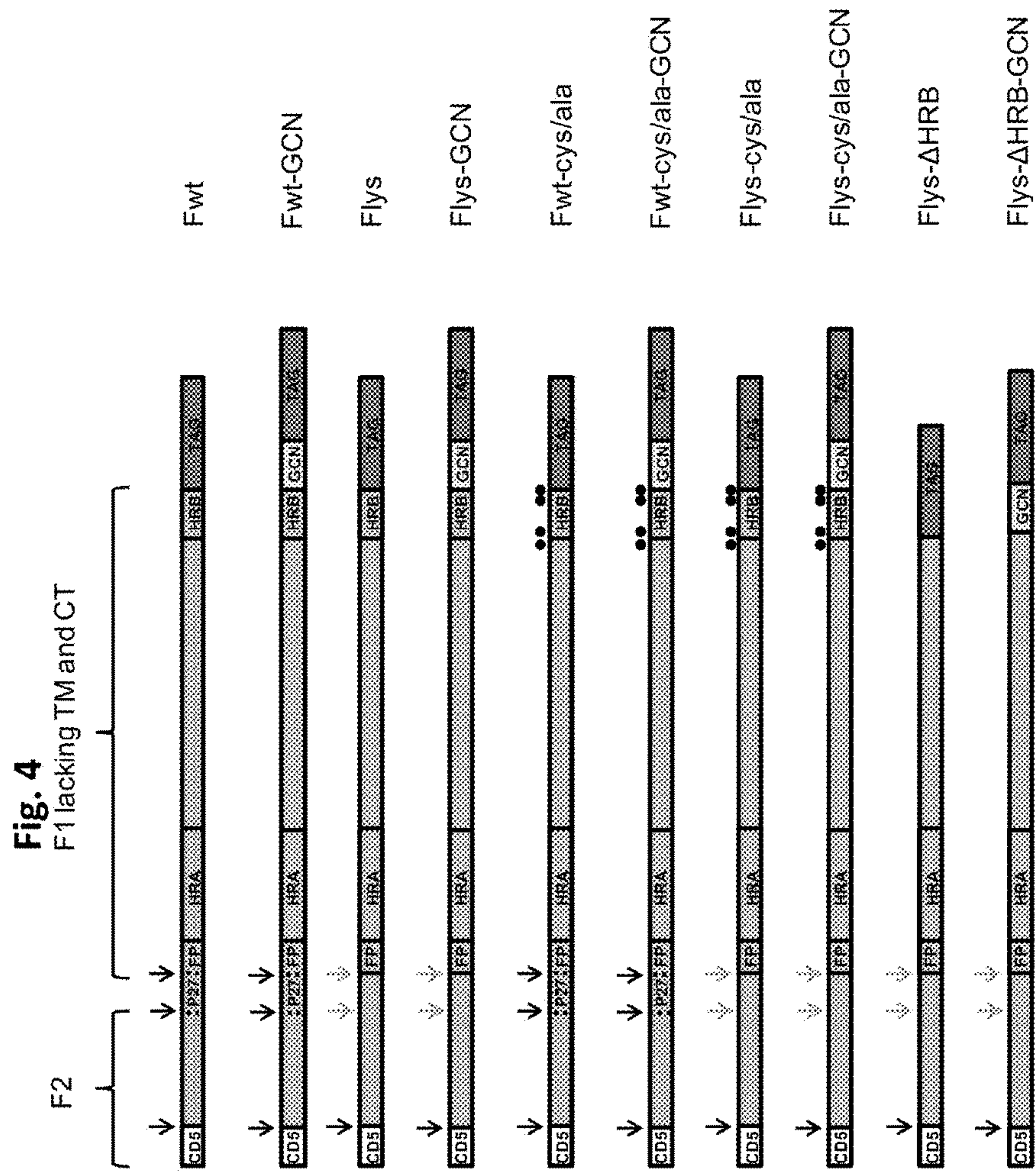


Fig. 2

Fig. 3





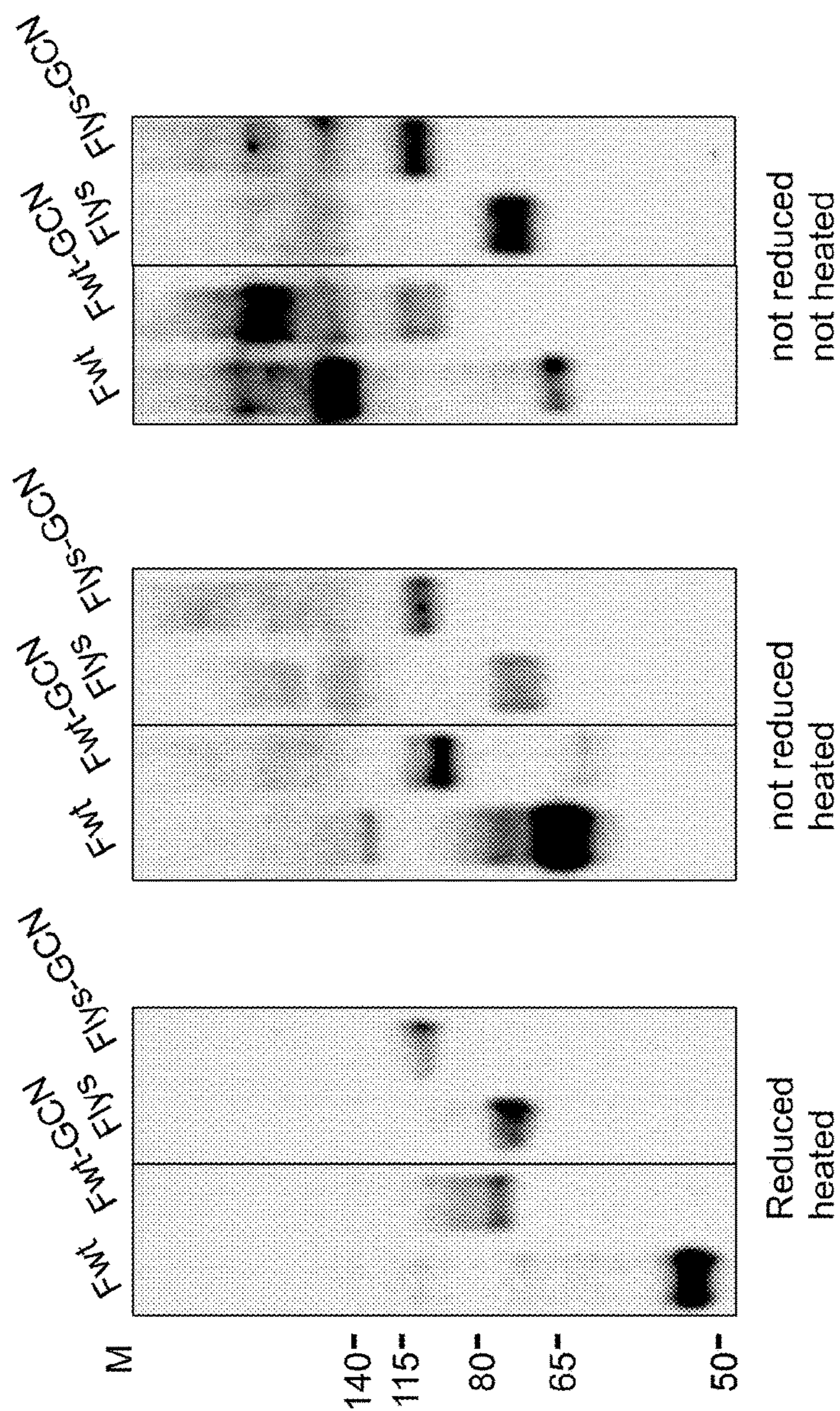


Fig. 5

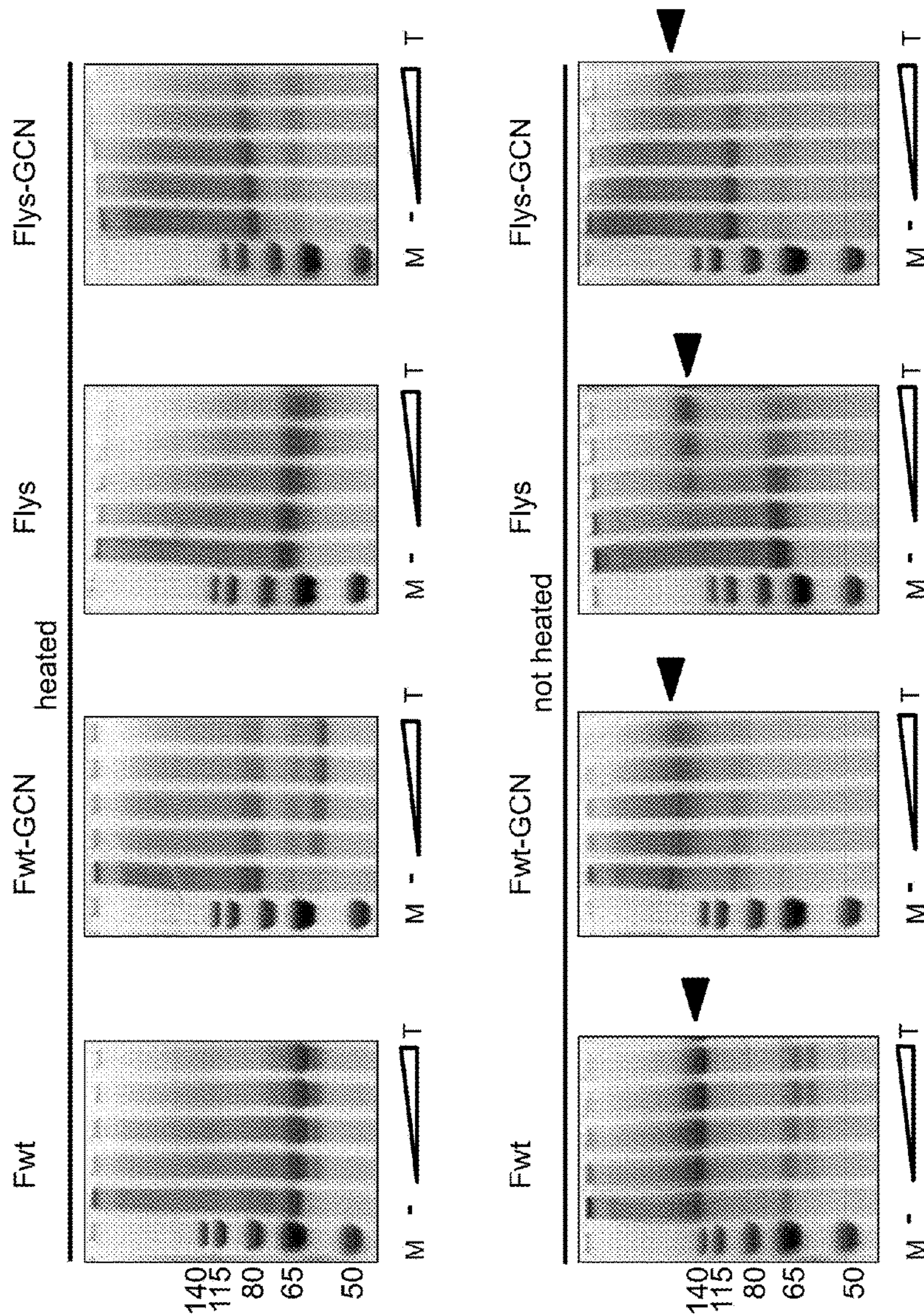


Fig. 6

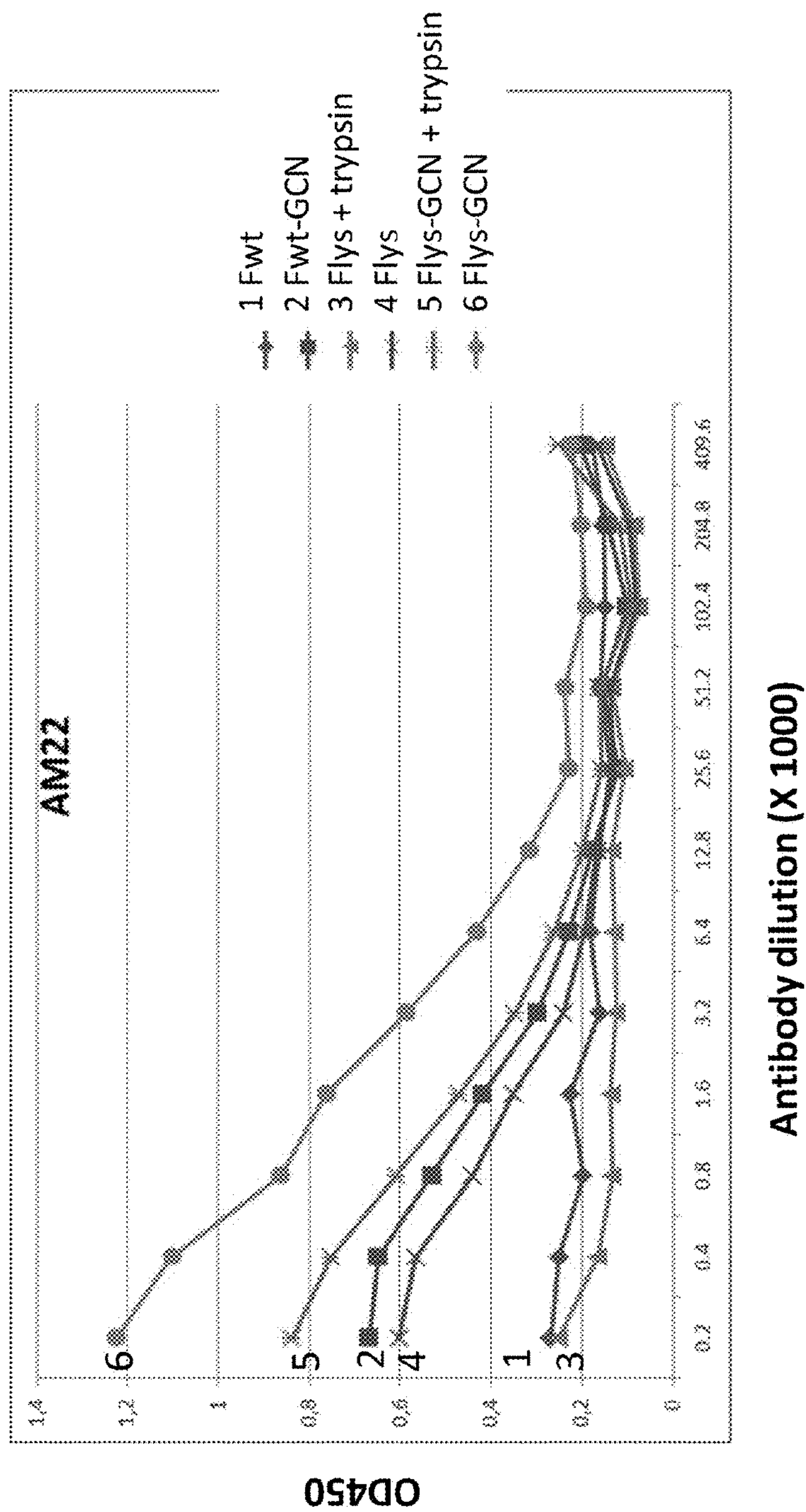


Fig. 7A

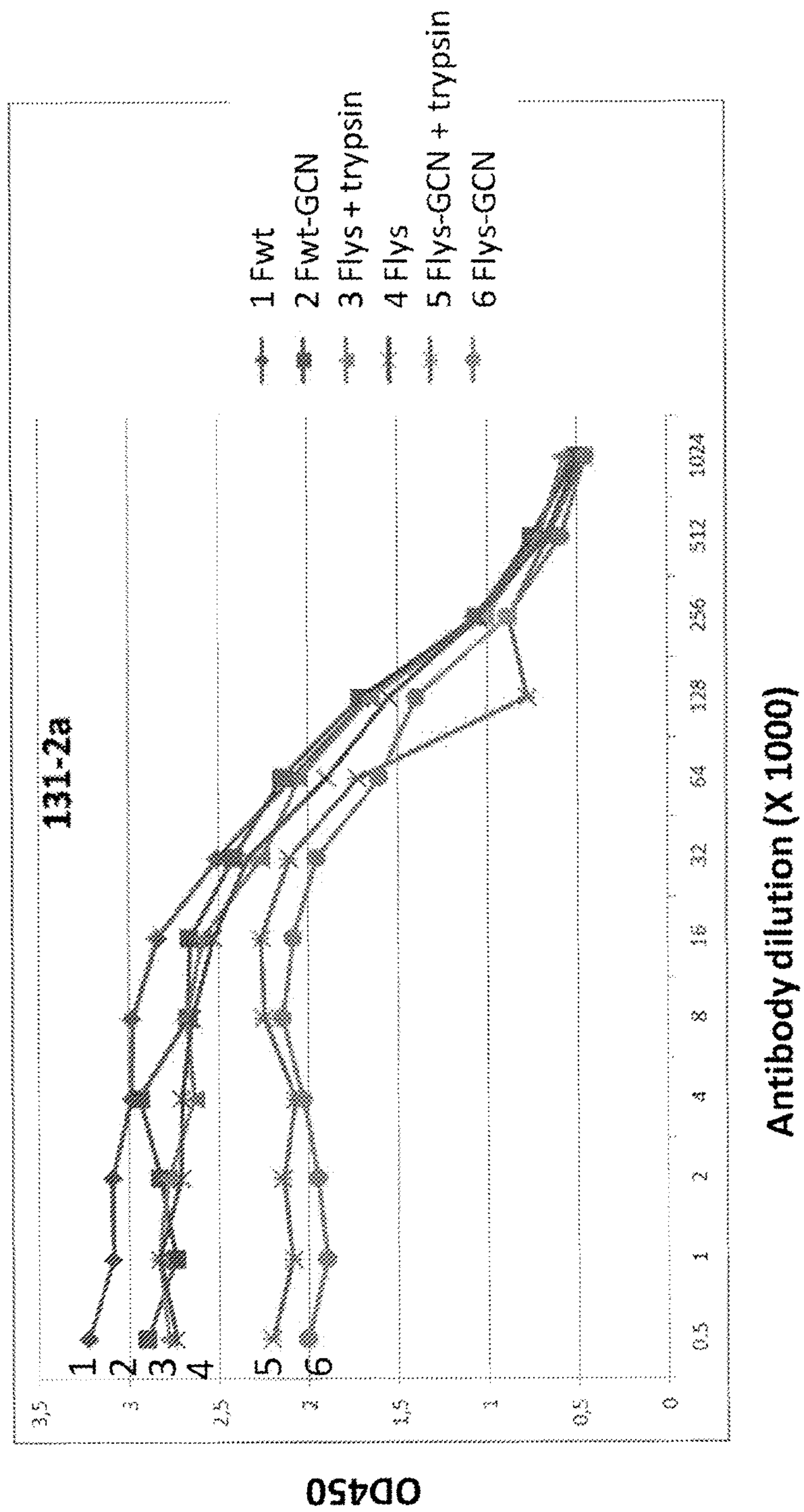
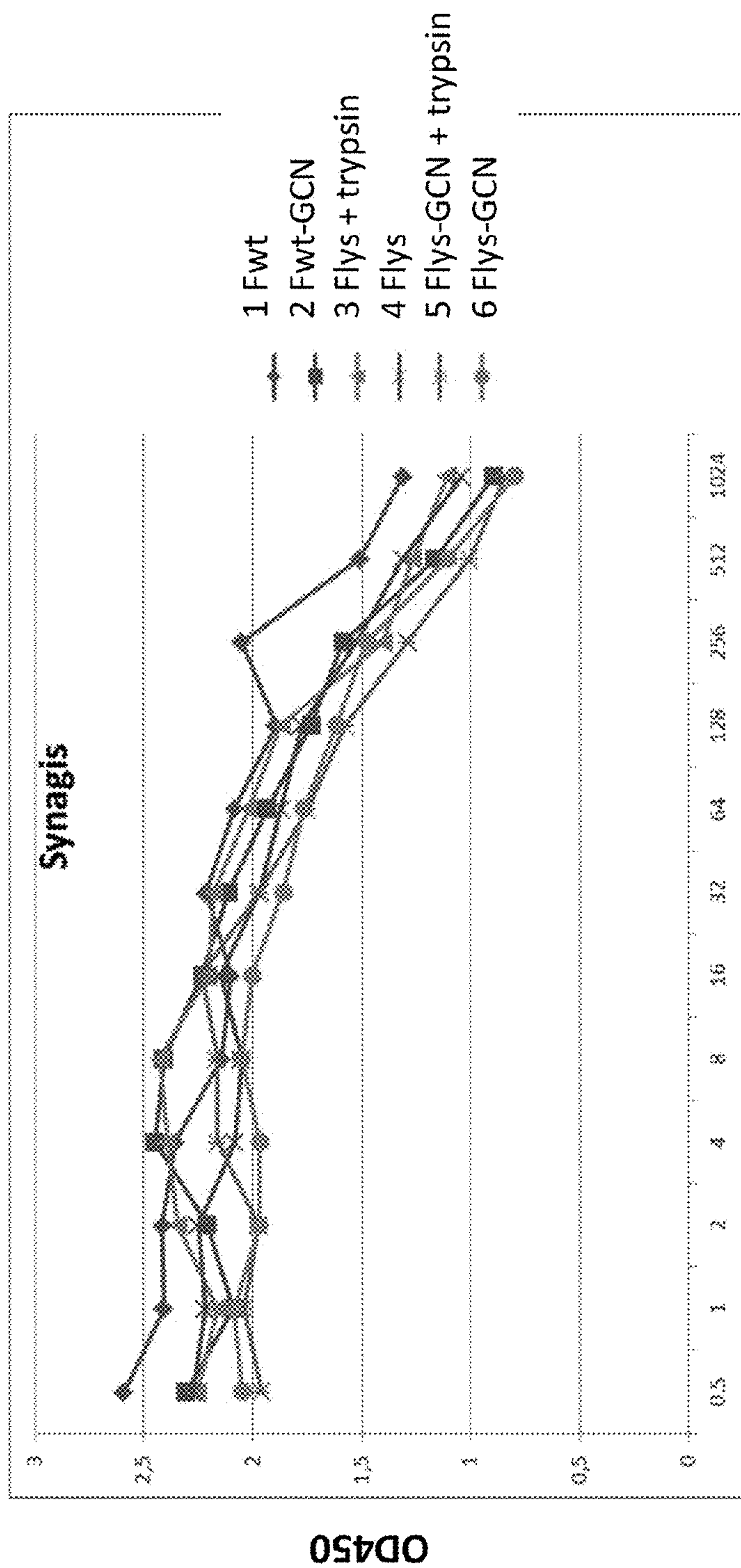


Fig. 7B



Antibody dilution (X 1000)

Fig. 7C

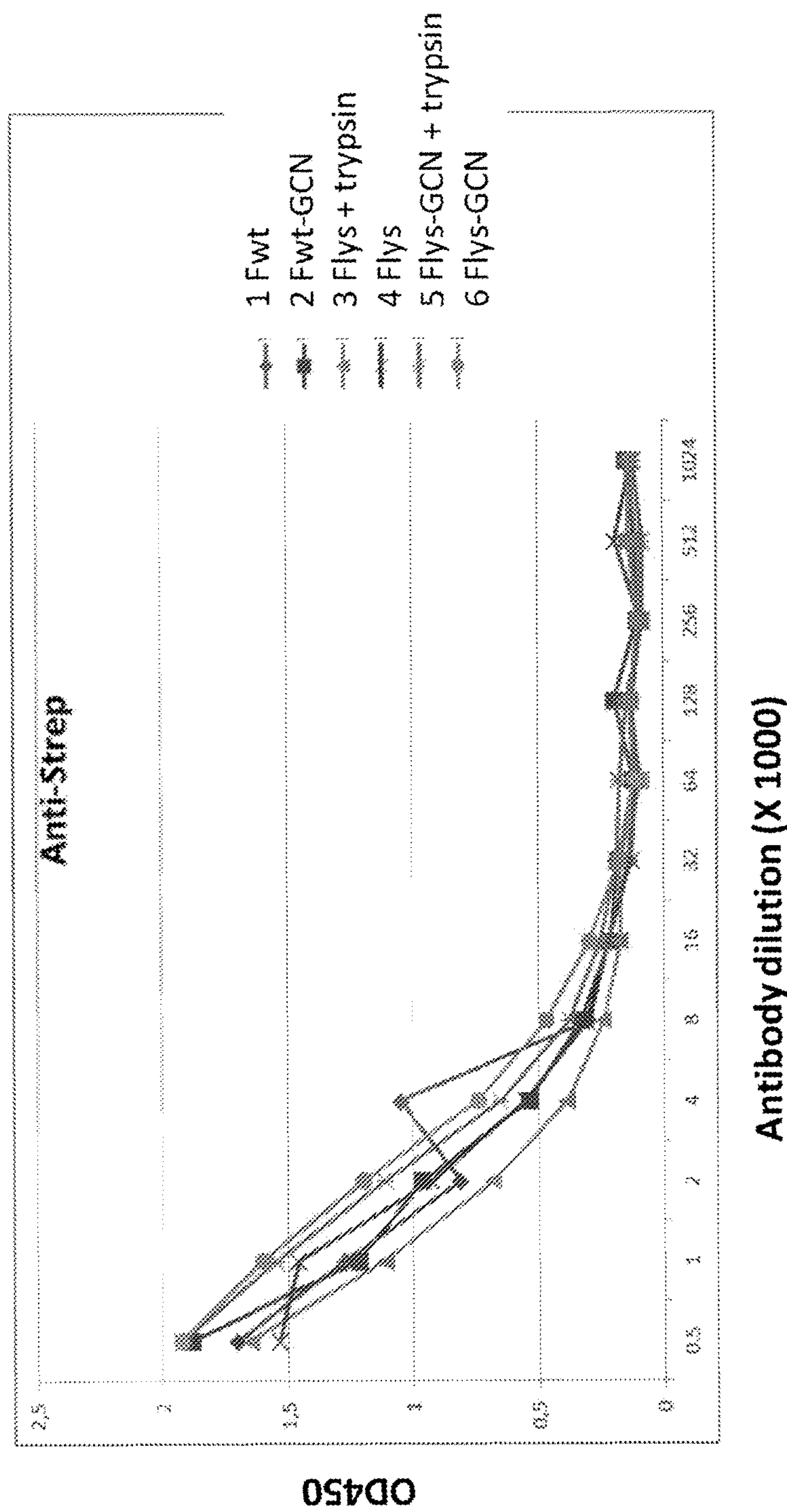


Fig. 7D

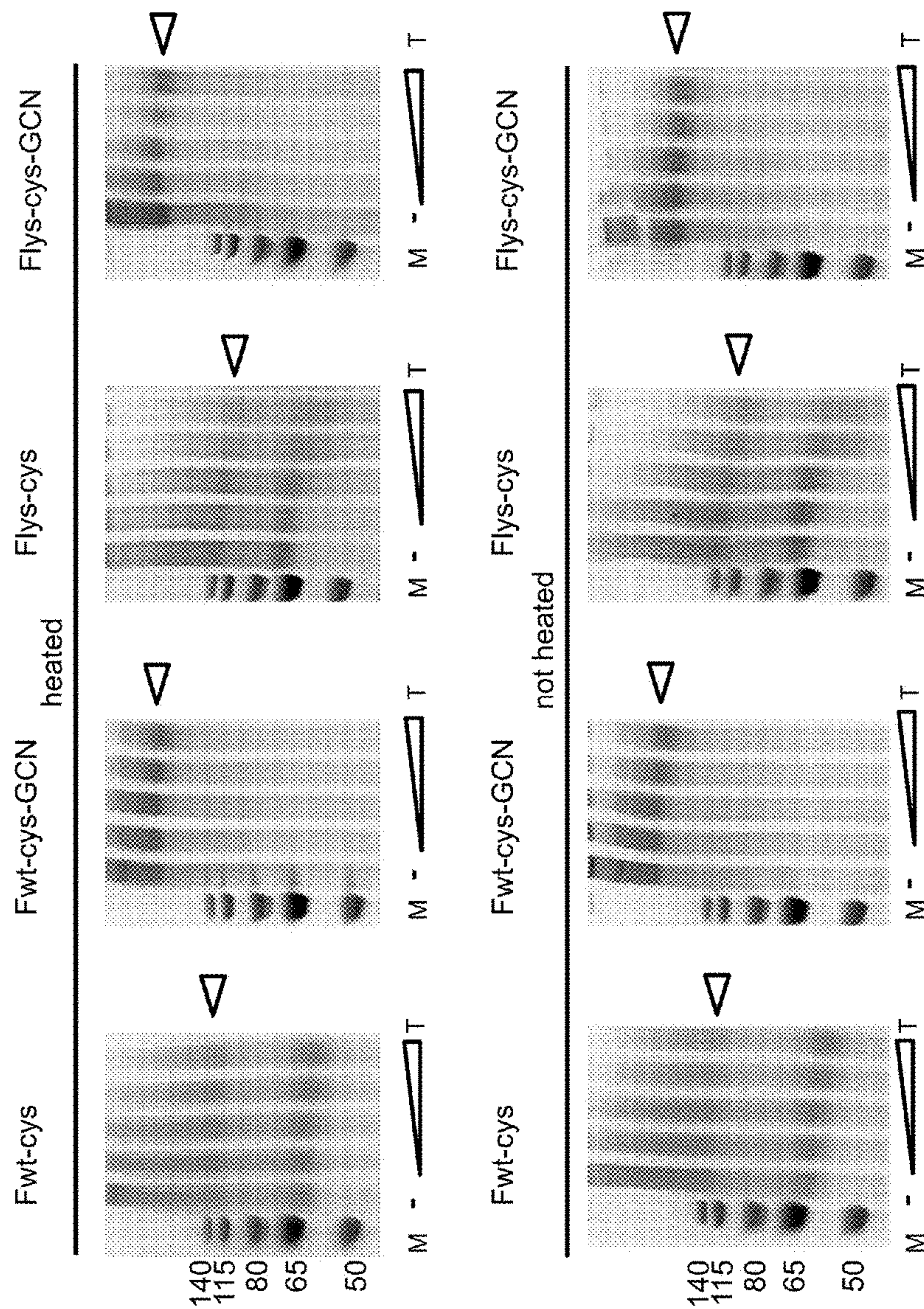


Fig. 9

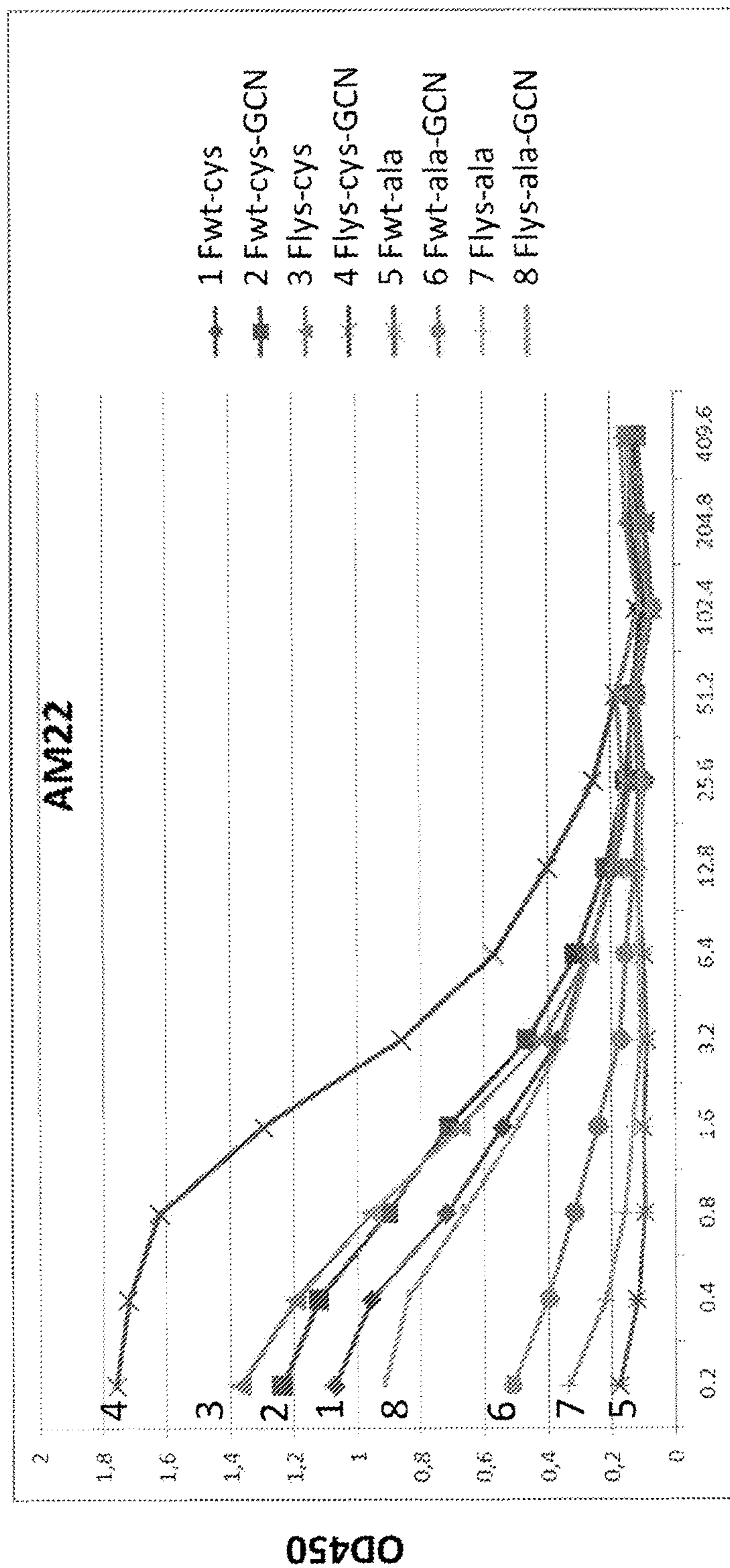


Fig. 10A

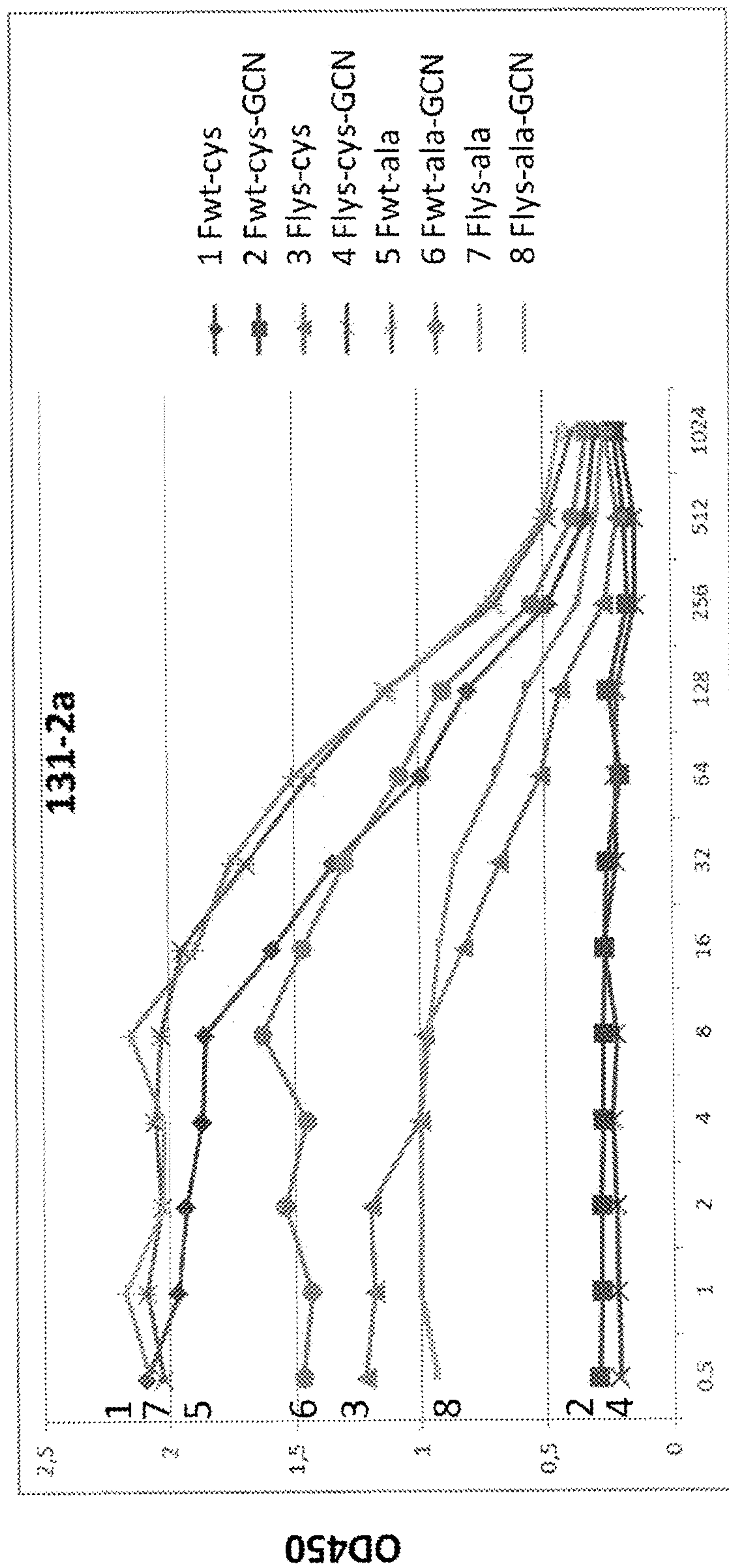
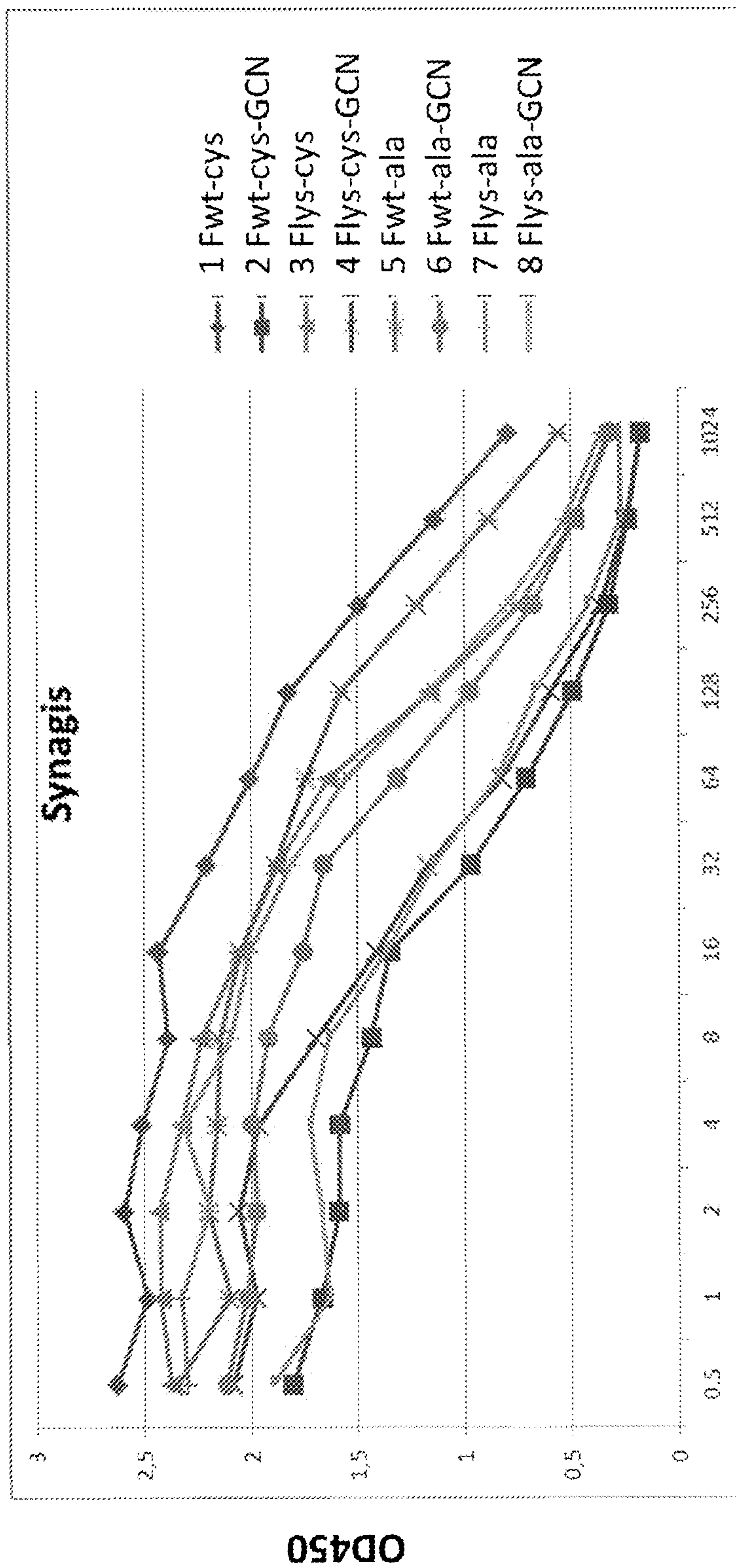
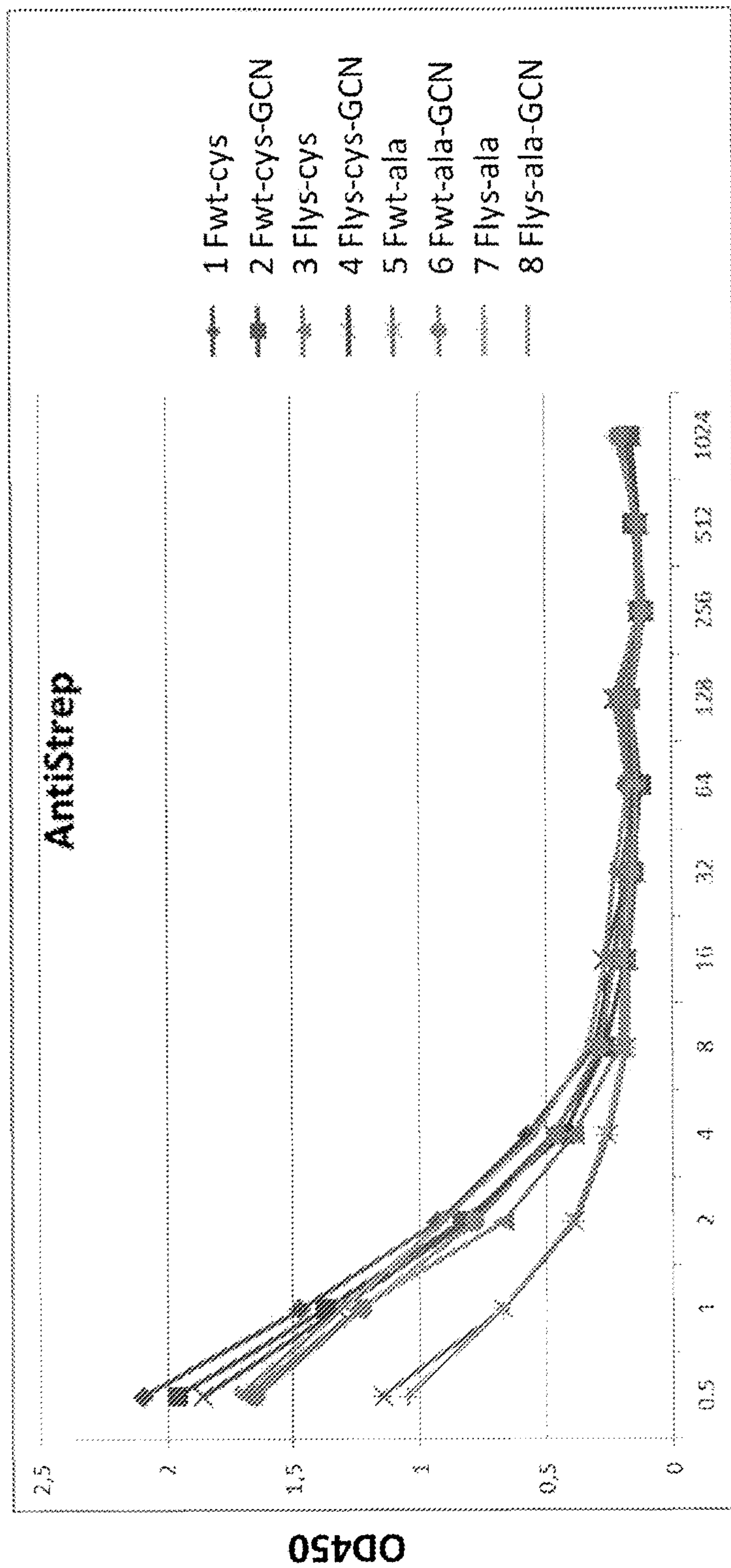


Fig. 10B



Antibody dilution (X 1000)

Fig. 10C



Antibody dilution (X 1000)

Fig. 10D

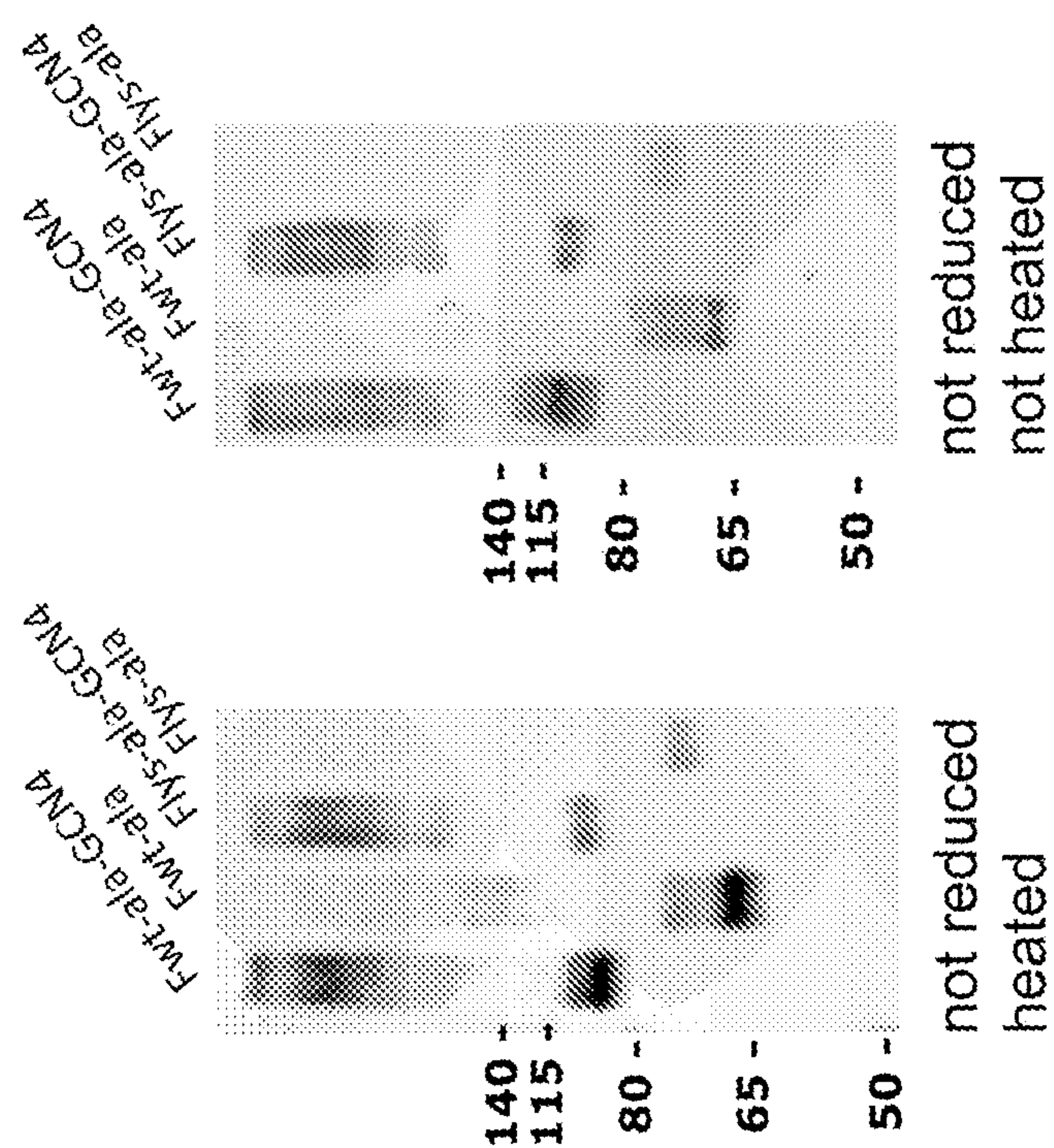


Fig. 11

Fig. 12A

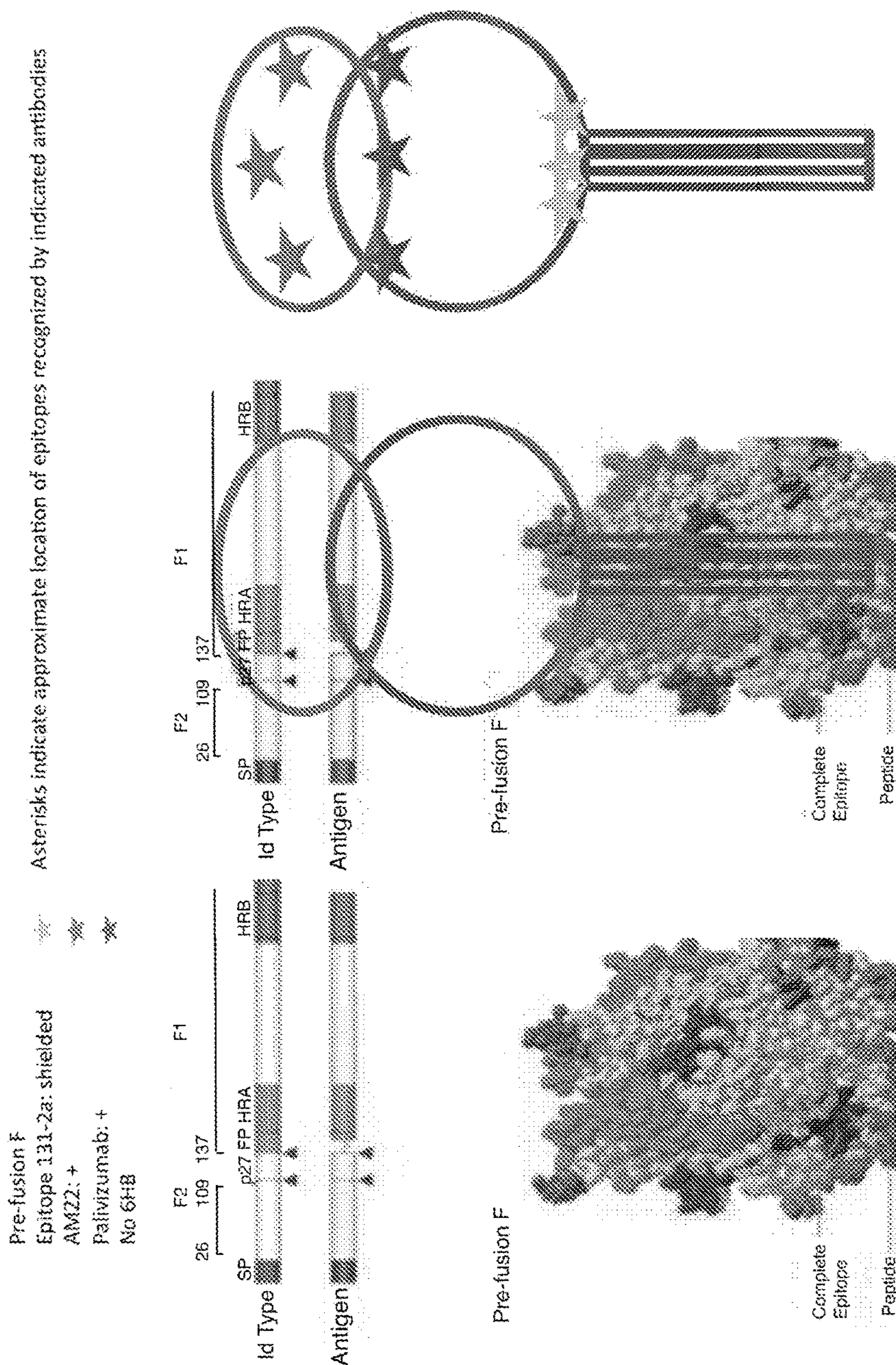


Fig. 12B

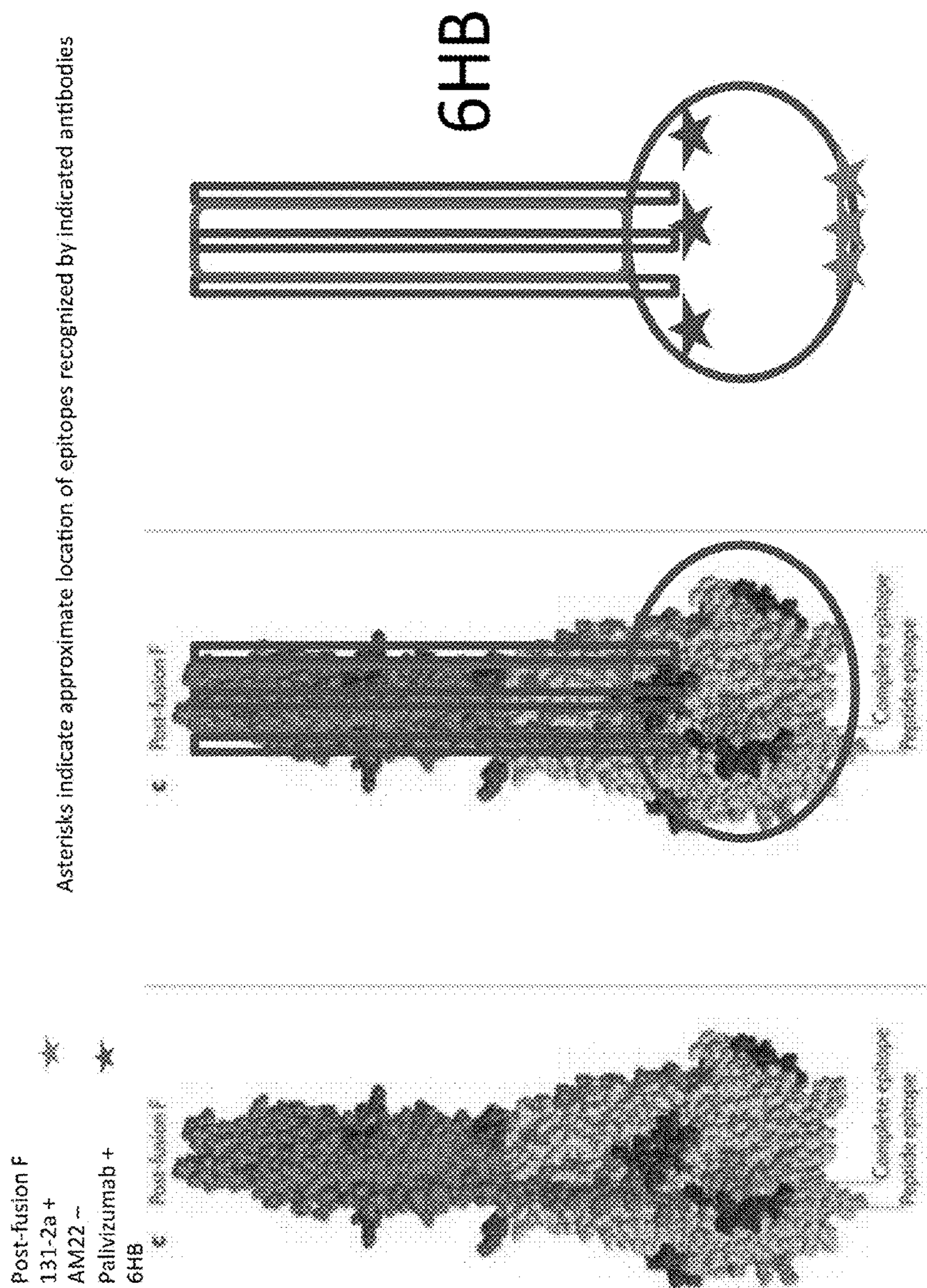
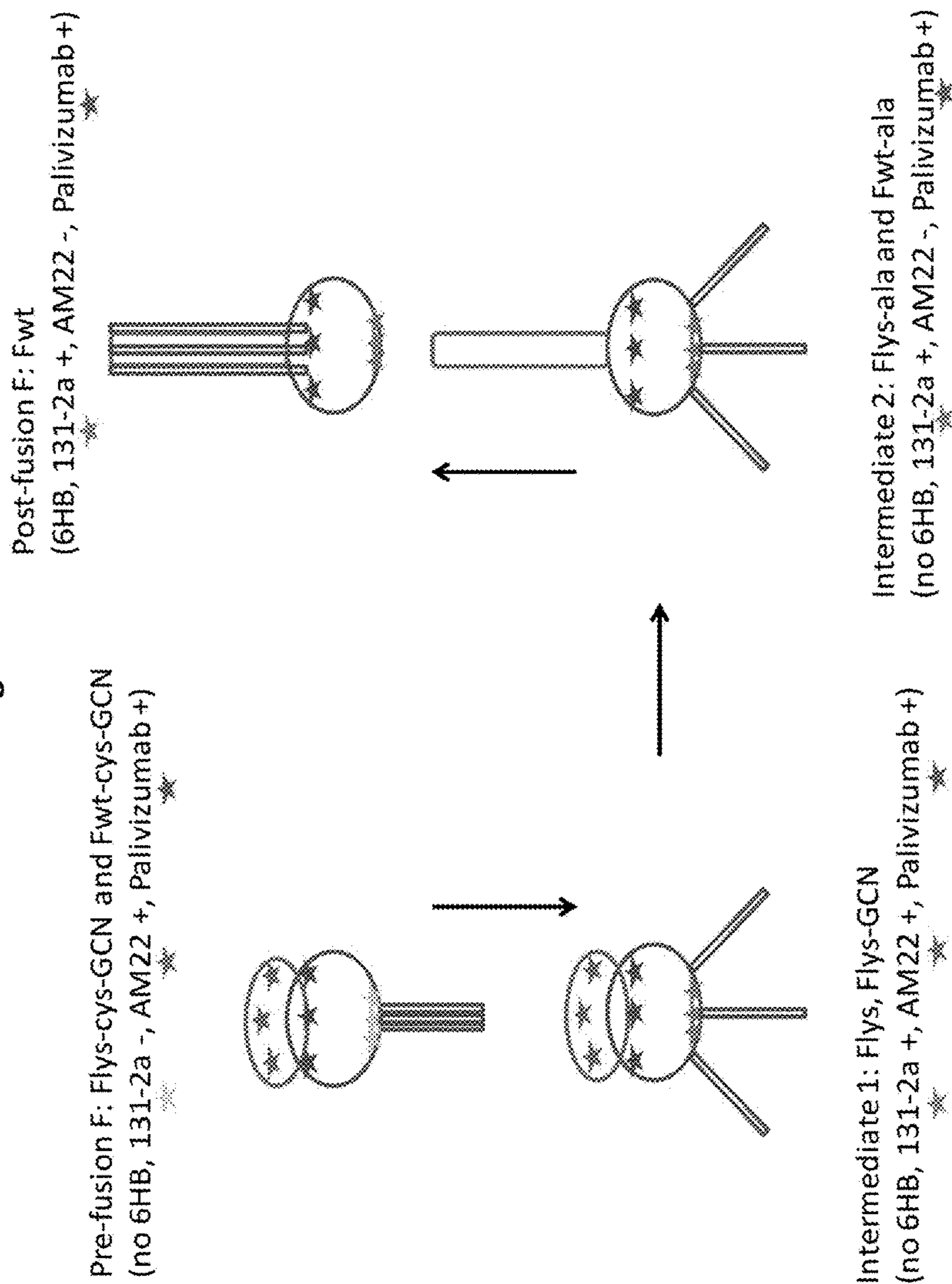


Fig. 12C



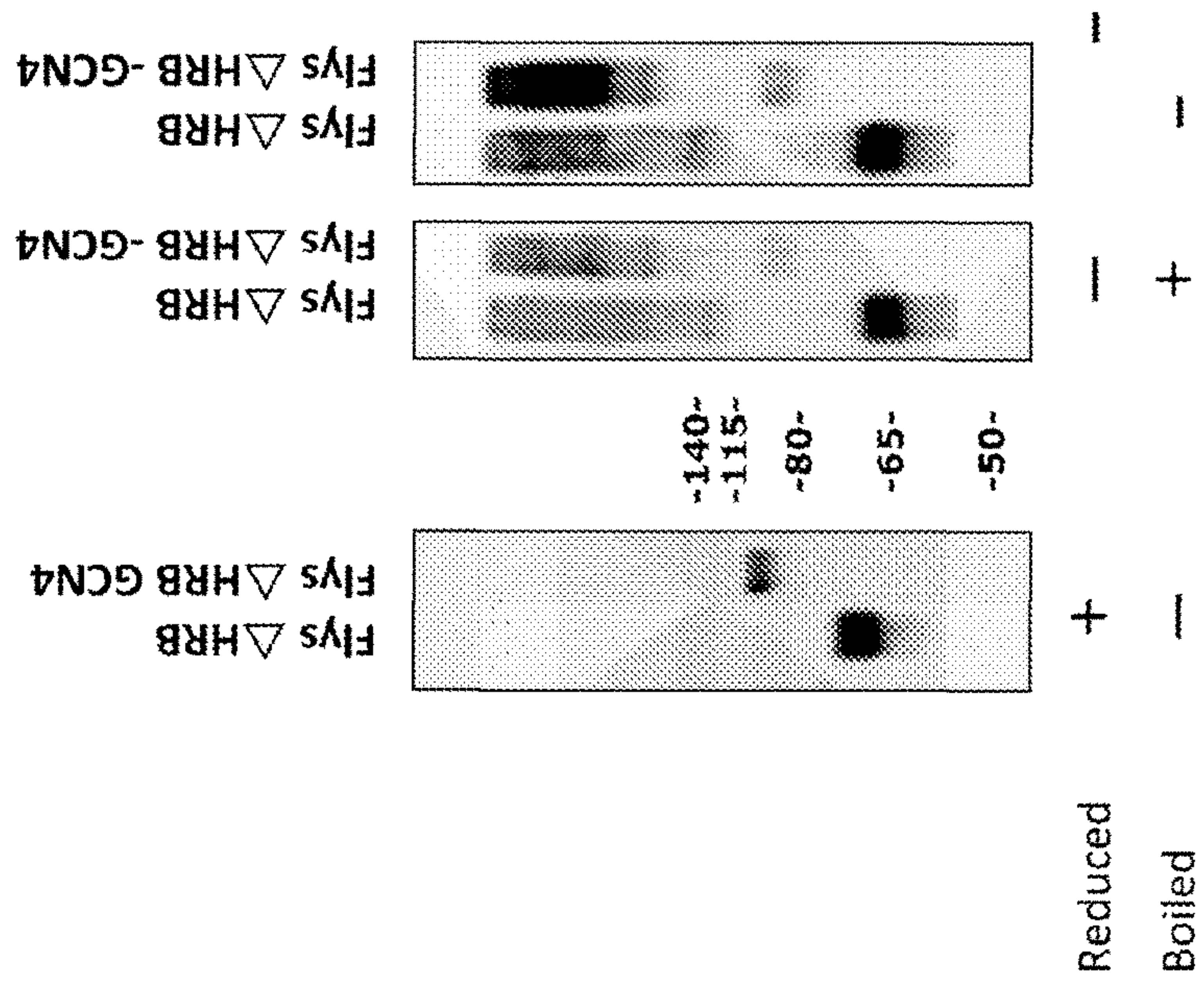


Fig. 13

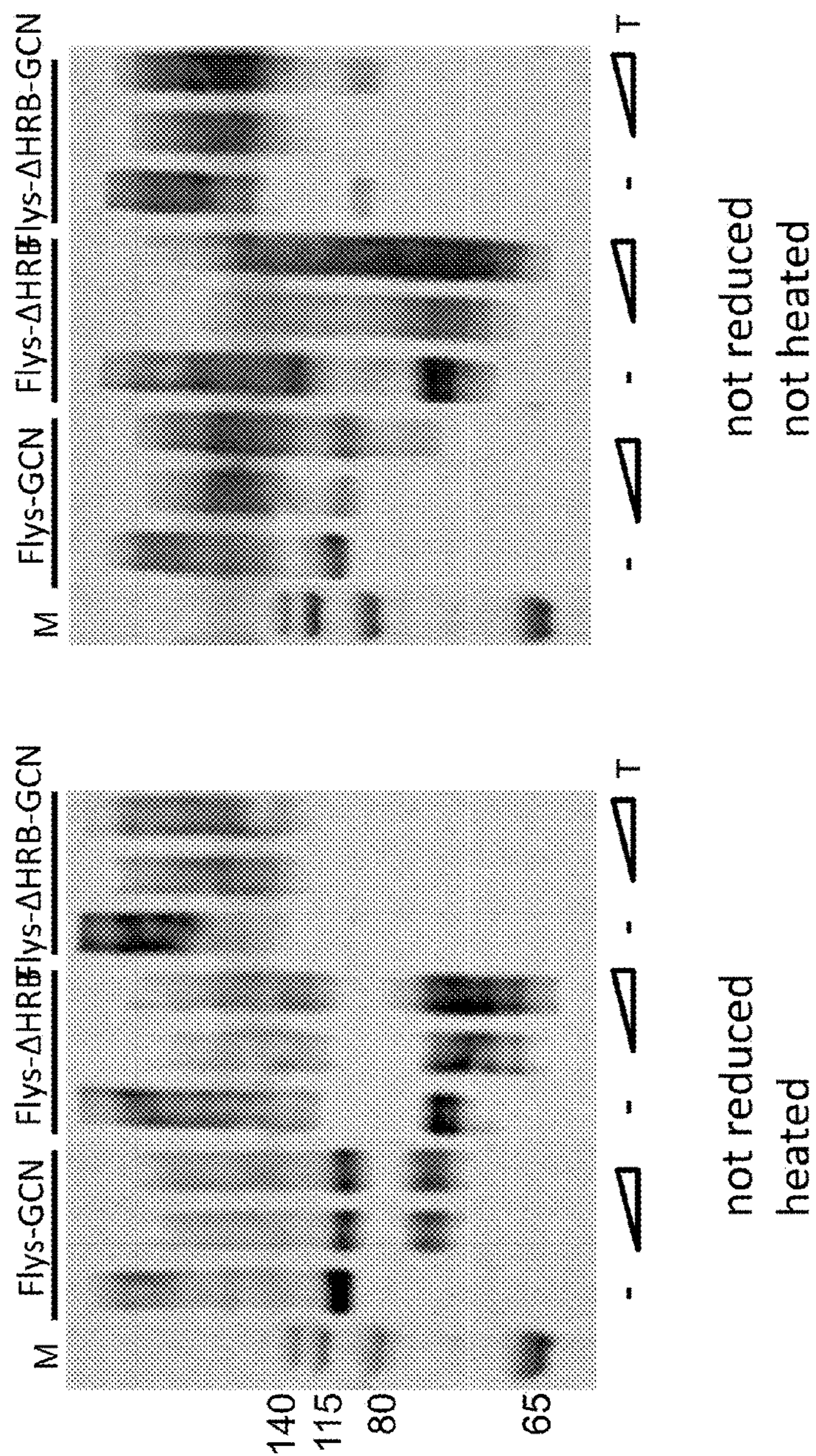


Fig. 14

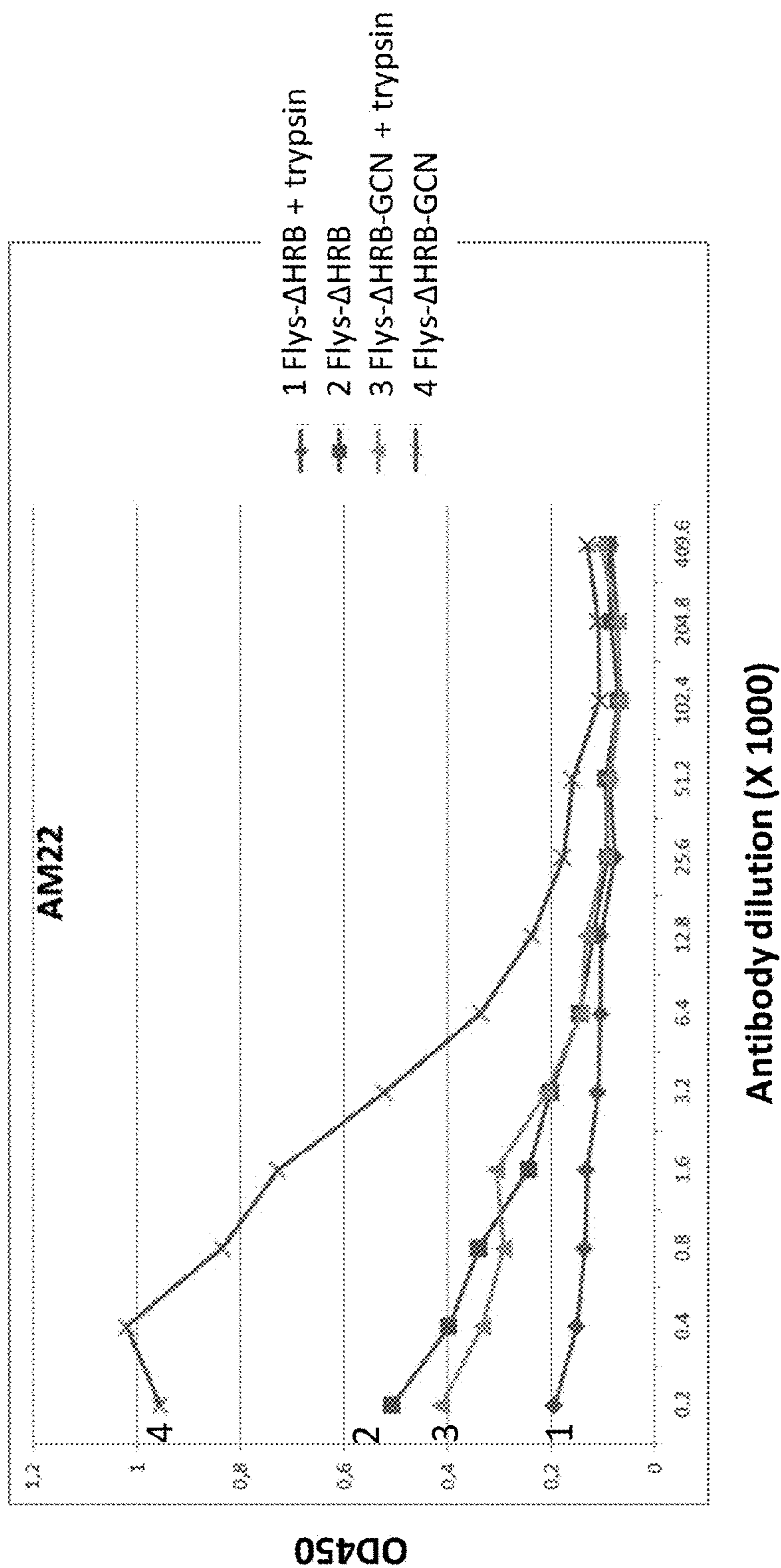
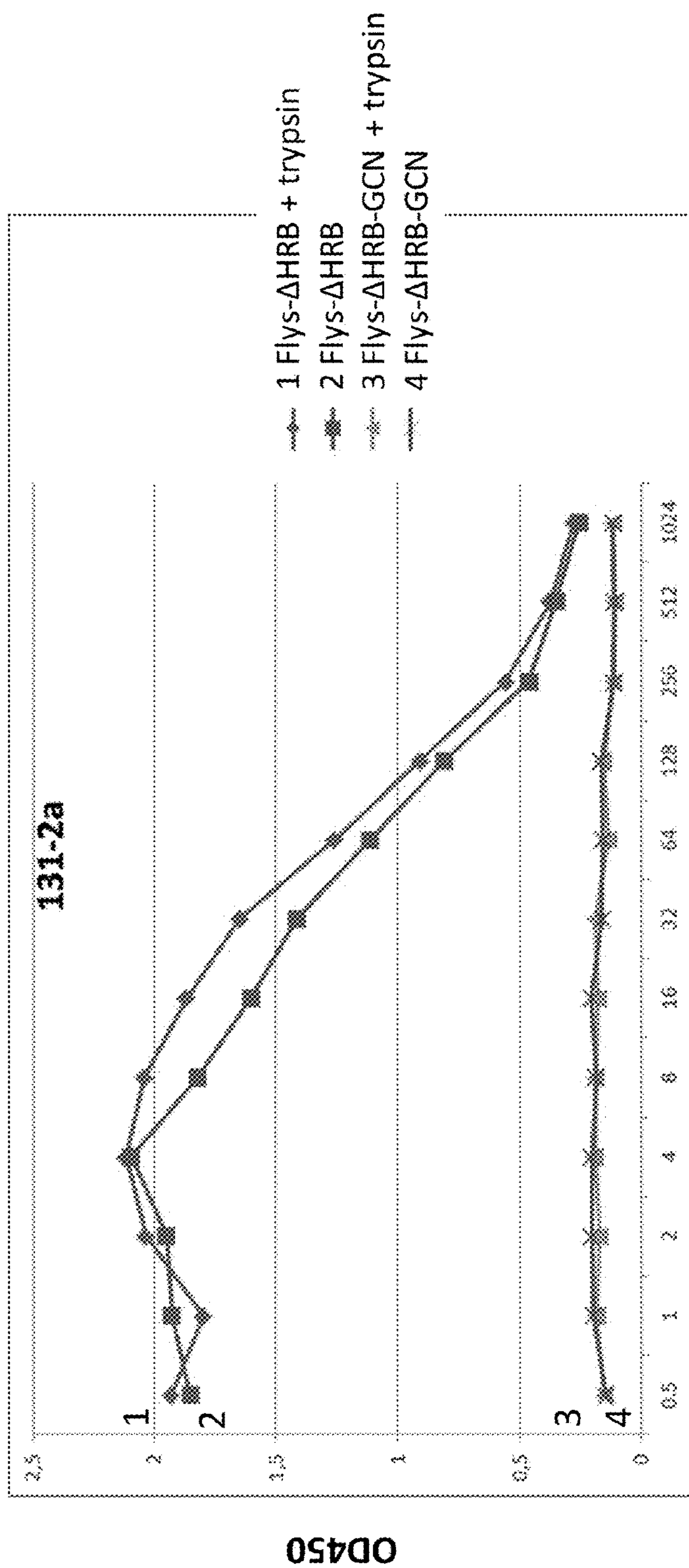


Fig. 15A



Antibody dilution (X 1000)

Fig. 15B

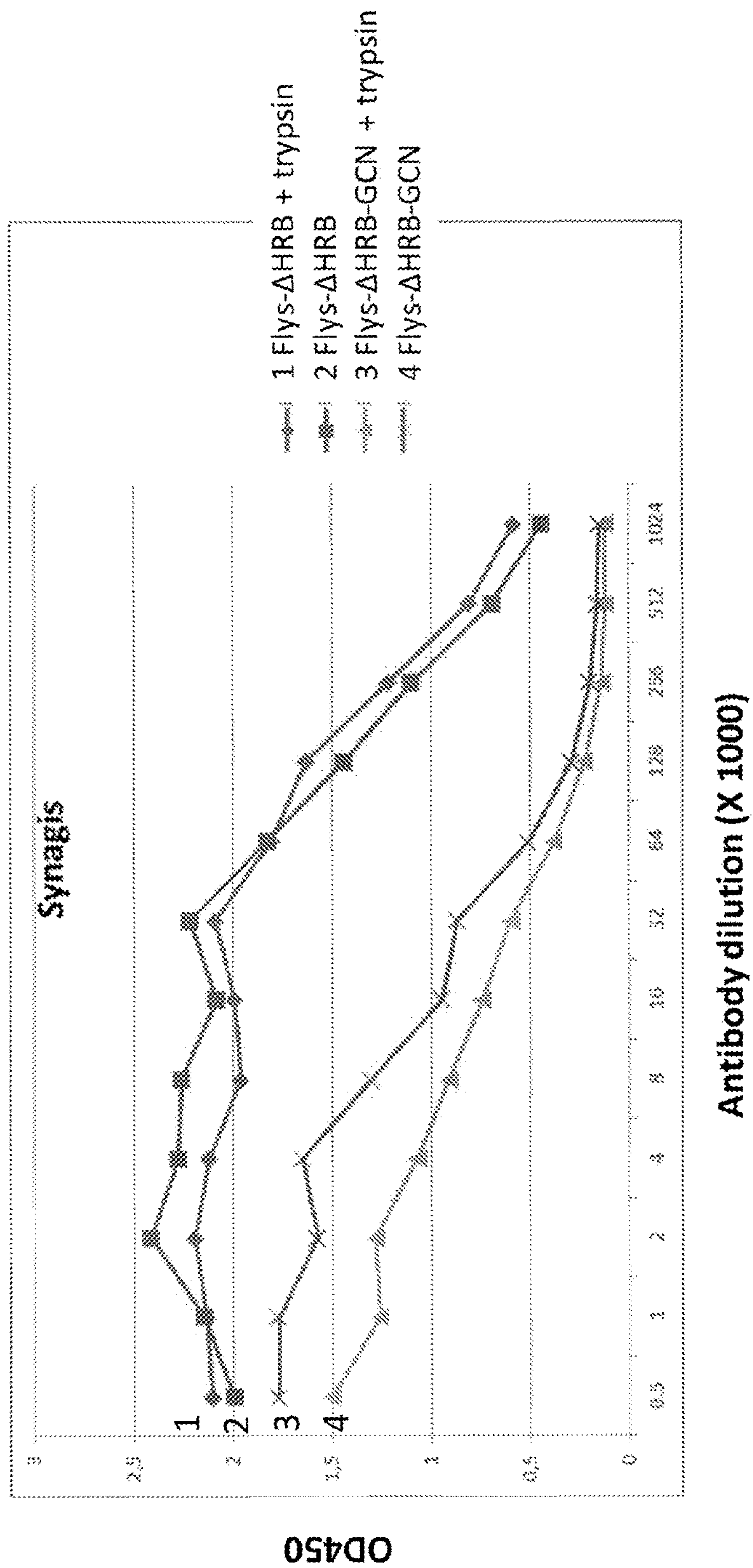


Fig. 15C

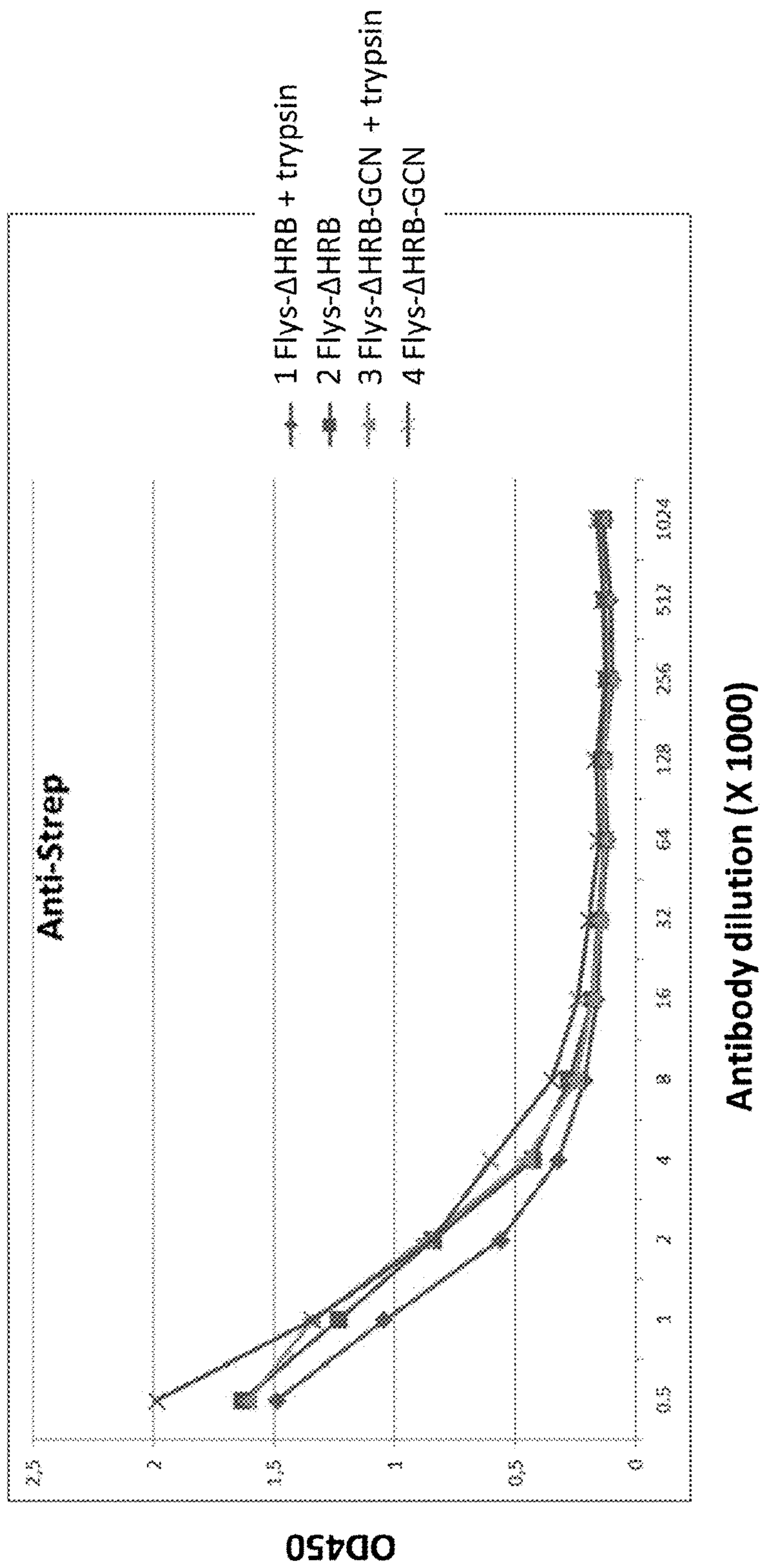
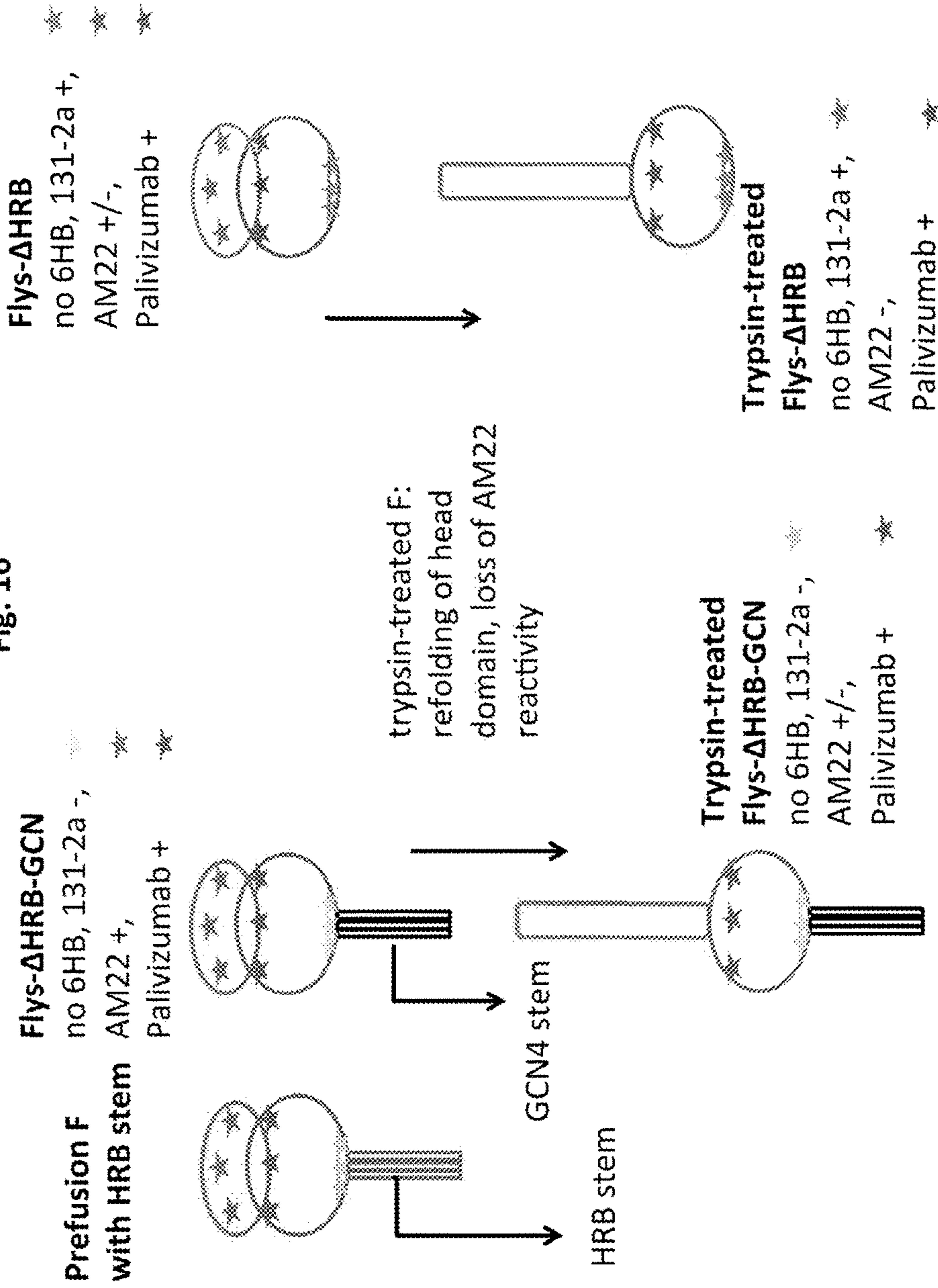


Fig. 15D

Fig. 16



**HEAT-STABLE RESPIRATORY SYNCYTIAL
VIRUS F PROTEIN OLIGOMERS AND
THEIR USE IN IMMUNOLOGICAL
COMPOSITIONS**

Matter enclosed in heavy brackets [] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue; a claim printed with strikethrough indicates that the claim was canceled, disclaimed, or held invalid by a prior post-patent action or proceeding.

FIELD OF THE INVENTION

The invention relates to the field of medicine and in particular to vaccines, and, more particularly, recombinant proteins that are useful in vaccines to immunize against Respiratory Syncytial Virus (RSV).

BACKGROUND OF THE INVENTION

Human Respiratory Syncytial Virus (hRSV) causes acute upper and lower respiratory tract infections and is a major cause for hospitalization of infants in the first year of life. Re-infection with RSV occurs frequently and sterilizing immunity is never firmly established. RSV also causes a significant disease burden and mortality in the elderly, comparable to influenza.

hRSV is an enveloped negative strand RNA virus belonging to the subfamily Pneumovirinae of the family Paramyxoviridae. Other members of this subfamily are bovine RSV (bRSV) and human metapneumovirus (hMPV). The hRSV particle contains two major glycoproteins, which are the key targets of neutralizing antibodies: the attachment protein G and the fusion protein F (review by Collins P L and J A Melero. 2011. Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. *Virus Res* 162:80-99). There are two RSV serotypes (A and B), which differ more in their G than F proteins. The F protein appears to be a more efficient neutralizing and protective antigen compared to G. This may be related to the high carbohydrate content of the G protein, which may shield the protein from immune recognition. In addition, the G protein is also secreted from infected cells, in which form it may function as an antigen decoy. The F protein not only functions to fuse viral and host membranes, but also plays a major role in virus-cell attachment. Neutralizing antibodies targeting F may therefore interfere with virus-cell attachment and/or with virus-cell fusion.

The RSV F protein is a type I membrane protein that is synthesized as an inactive precursor protein (named 'F0') that assembles into trimers. This precursor protein is cleaved by furin-like proteases into the forms named 'F2', 'p27' and 'F1' during its transport through the secretory route. Homotrimers of F2 and F1, which are covalently linked via disulfide bridges, form the metastable pre-fusion active structure. The F1 contains heptad repeats A and B (referred to as HRA and HRB), the fusion peptide (FP) and the transmembrane (TM) domain, the latter two positioned at opposite sides of the molecule. Upon virus-cell attachment, conformational changes in the RSV F protein lead to the insertion of the hydrophobic fusion peptide into a host cell membrane. Subsequently, this fusion intermediate refolds into a highly stable post-fusion structure. The assembly of this latter structure is dictated by the assembly of a six-helix bundle (6HB). This 6HB contains HRA and HRB of each

monomer in an anti-parallel conformation, as a result of which the transmembrane domain, located downstream of HRB, and the fusion peptide, located upstream of HRA, are positioned in adjacent positions and fusion of the viral and host membranes is achieved. Recent studies have elucidated the structure of the F protein in its post-fusion conformation (McLellan J S et al. 2011. *J Virol* 85:7788-96; Swanson K A et al. 2011. *Proc Natl Acad Sci USA* 108:9619-24).

hRSV vaccine development has been haunted by the disastrous results obtained with the formalin-inactivated virus vaccine that was tested in the 1960s. Disease severity and hospital admission rates were increased in vaccinated children, who were naturally infected with RSV later, and several deaths occurred. The mechanism of this vaccine-induced disease enhancement remains incompletely understood, but appears associated with low induction of neutralizing antibodies and recruitment of eosinophils. Next to this effort, a large number of RSV vaccine strategies has been explored with varying success, including live attenuated RSV strains, subunit vaccines and viral vectored vaccines (Groothuis J R et al. 2011. Prevention of serious respiratory syncytial virus-related illness. I: Disease pathogenesis and early attempts at prevention. *Adv Ther* 28:91-109; Hurwitz J L. 2011. Respiratory syncytial virus vaccine development. *Expert Rev Vaccines* 10:1415-33). Obviously, successful RSV vaccines should induce protective immunity, but no immunopathology.

REPORTED DEVELOPMENTS

Currently, the only available option to prevent RSV-mediated disease is the passive administration of the commercially available RSV neutralizing monoclonal antibody Palivizumab. This product is used as prophylaxis for RSV infection and recognizes a highly conserved epitope in the F protein (Beeler J A and K van Wyke Coelingh. 1989. Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. *J Virol* 63:2941-50; Groothuis J R et al. 2011. Prevention of serious respiratory syncytial virus-related illness. II: Immunoprophylaxis. *Adv Ther* 28:110-25). However, due to its high cost the use of Palivizumab is restricted to infants considered at high risk of developing severe respiratory disease.

Although there is a need for a vaccine for protection of the general population, there is currently no approved vaccine against RSV available. Many vaccine candidates based on the main RSV neutralizing antigen, which is the F protein, failed due to problems with stability, reproducibility and potency.

Although the post-fusion form of RSV F was shown to contain neutralizing epitopes (McLellan J S et al. 2011. Structure of respiratory syncytial virus fusion glycoprotein in the postfusion conformation reveals preservation of neutralizing epitopes. *J Virol* 85:7788-96; Swanson K A et al. 2011. Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. *Proc Natl Acad Sci USA* 108:9619-24) Magro and coworkers showed that antibodies specific for the pre-fusion form of F account for most of the neutralizing activity found in human sera (Magro M et al. 2012. Neutralizing antibodies against the preactive form of respiratory syncytial virus fusion protein offer unique possibilities for clinical intervention. *Proc Natl Acad Sci USA* 109:3089-94). Furthermore, RSV neutralizing antibodies were identified that recognize F, but that do not recognize recombinant soluble ectodomains thereof that are

presumably in the post-fusion conformation (WO 2008/147196; US 2012/0070446; McLellan J S et al. 2011. *J Virol* 85:7788-96; Swanson K A et al. 2011. *Proc Natl Acad Sci USA* 108:9619-24; Gonzalez-Reyes L et al. 2001. Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion. *Proc Natl Acad Sci USA* 98:9859-64; Ruiz-Arguello M B et al. 2002. Effect of proteolytic processing at two distinct sites on shape and aggregation of an anchorless fusion protein of human respiratory syncytial virus and fate of the intervening segment. *Virology* 298:317-26; Ruiz-Arguello M B et al. 2004. Thermostability of the human respiratory syncytial virus fusion protein before and after activation: implications for the membrane fusion mechanism. *J Gen Virol* 85:3677-87), see FIG. 2. These antibodies, which presumably recognize pre-fusion F or an intermediate form between pre- and post-fusion F, and not post-fusion F, were shown to more effectively neutralize RSV than Palivizumab, which recognizes all forms of F protein.

Attempts to modify the RSV F protein for use in an immunological composition have been reported (WO2010/149743; WO2012/158613).

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the USPTO upon request and payment of the necessary fee.

FIG. 1 shows (a) a comparison of wild-type fusion (F) protein from respiratory syncytial virus (RSV) with an engineered RSV F antigen. Arrowheads indicate furin cleavage sites, and the peptide p27 is released after cleavage. The F1 and F2 fragments of the wild-type sequence, which are produced as a result of furin cleavage, are indicated. Panel (b) is a model of the engineered RSV pre-fusion F, based on data from parainfluenza virus 5 (PIV-5) pre-fusion F and engineered RSV post-fusion F crystal structures. Panel (c) displays the engineered RSV post-fusion F structure of (a). CT=cytoplasmic tail; SP=signal peptide; TM=transmembrane region. (Taken from *Nature Reviews Microbiology* 10, 807-813; December 2012)

FIG. 2 (Taken from *J. Virol.* August 2011 vol. 85 no. 15 7788-7796) shows in panel (A) a three-dimensional model of the proposed pre-fusion conformation of the hRSV F trimer, built using the SWISS-MODEL server facilities (see the website found at swissmodel.expasy.org) and the atomic coordinates of the pre-fusion structure of the PIVS F protein (Protein Data Bank code, 2B9B) as a template. The backbone structure of the three monomers is shown in gray. Fusion peptide sequences of one monomer are shown in pink, and those of HRA are shown in black. Residues that are changed in virus isolates or in escape mutants selected with monoclonal antibodies, whose epitopes map in different antigenic sites of the F protein, are shown as colored spheres (antigenic site I, amino acid 389; antigenic site II, amino acids 262, 268, 272, and 275; and antigenic site IV, amino acids 429, 432, 433, 436, and 447). The two proteolytic cleavage sites are indicated with arrows in one of the monomers. Panel (B) shows the neutralizing epitopes on RSV F with a deleted FP. Epitopes for motavizumab and palivizumab (antigenic site II), 101F (antigenic site IV), and 131-2a (antigenic site I) are solvent exposed and in conformations compatible with antibody binding. Residues 254 to 277 are colored red (antigenic site II), residues 429 to 437 are colored blue (antigenic site IV), and atoms in Pro389 are

shown as spheres (antigenic site I) (residue numbering according to *J. Virol.* August 2011 vol. 85 no. 15 7788-7796).

FIG. 3 shows the amount of antibody needed to block 50% infection in a microneutralization assay. The lower the amount, the more effective the antibody. Synagis®=Palivizumab

FIG. 4 provides a schematic representation of the different constructs used in the present invention. TM=transmembrane region; CT=cytoplasmic tail; CD5=signal peptide; HRA=heptad repeat A; HRB=heptad repeat B; GCN=GCN4 trimerization motif.

FIG. 5 shows western blots of Fwt, Fwt-GCN, Flys and Flys-GCN proteins that were either reduced and heated (left panel), not reduced and heated (middle panel) or not reduced and not heated (right panel). Higher order proteins in the right panel indicate the post-fusion state of these proteins that almost fully disappears upon treatment with 2-mercaptoethanol (reducing) and heating prior to running the proteins on SDS-PAGE.

FIG. 6 shows a Coomassie Blue staining of gels on which Fwt, Fwt-GCN, Flys and Flys-GCN proteins were separated after heating (upper panels) or after no heating (lower panels) and increasing cleavage with trypsin (T).

FIG. 7A shows the results of an ELISA assay with which the reactivity of different soluble F proteins (Fwt, Fwt-GCN, Flys and Flys-GCN) with the AM22 antibody was checked. The treatment with trypsin is indicated for Flys and Flys-GCN.

FIG. 7B shows the results of an ELISA assay with which the reactivity of different soluble F proteins (Fwt, Fwt-GCN, Flys and Flys-GCN) with the 131-2a antibody was checked. The treatment with trypsin is indicated for Flys and Flys-GCN.

FIG. 7C shows the results of an ELISA assay with which the reactivity of different soluble F proteins (Fwt, Fwt-GCN, Flys and Flys-GCN) with the Synagis® (Palivizumab) antibody was checked. The treatment with trypsin is indicated for Flys and Flys-GCN.

FIG. 7D shows the results of an ELISA assay with which the reactivity of different soluble F proteins (Fwt, Fwt-GCN, Flys and Flys-GCN) with the anti-strep antibody was checked. The treatment with trypsin is indicated for Flys and Flys-GCN.

FIG. 8 shows a western blot of Fwt-GCN, Fwt, Fwt-cys-GCN, Fwt-cys, Flys-GCN, Flys, Flys-cys-GCN and Flys-cys proteins that were either heated but not reduced (left panel) or that were heated and reduced (right panel). The introduction of cysteines in the HRB domain as outlined in example 2 results in higher order structures that are resistant to heat and only partially sensitive to reduction.

FIG. 9 shows a Coomassie Blue staining of gels on which Fwt-cys, Fwt-cys-GCN, Flys-cys and Flys-cys-GCN proteins were separated after heating (upper panels) or after no heating (lower panels) and increasing digestion with trypsin (T).

FIG. 10A shows the results of an ELISA assay with which the reactivity of different soluble F proteins with cysteine and alanine mutations in their HRB domain (Fwt-cys, Fwt-cys-GCN, Flys-cys, Flys-cys-GCN, Fwt-ala, Fwt-ala-GCN, Flys-ala, Flys-ala-GCN) with the AM22 conformational antibody was checked.

FIG. 10B shows the results of an ELISA assay with which the reactivity of different soluble F proteins with cysteine and alanine mutations in their HRB domain (Fwt-cys, Fwt-

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cys-GCN, Flys-cys, Flys-cys-GCN, Fwt-ala, Fwt-ala-GCN, Flys-ala, Flys-ala-GCN) with the 131-2a conformational antibody was checked.

FIG. 10C shows the results of an ELISA assay with which the reactivity of different soluble F proteins with cysteine and alanine mutations in their HRB domain (Fwt-cys, Fwt-cys-GCN, Flys-cys, Flys-cys-GCN, Fwt-ala, Fwt-ala-GCN, Flys-ala, Flys-ala-GCN) with the Synagis® (Palivizumab) conformational antibody was checked.

FIG. 10D shows the results of an ELISA assay with which the reactivity of different soluble F proteins with cysteine and alanine mutations in their HRB domain (Fwt-cys, Fwt-cys-GCN, Flys-cys, Flys-cys-GCN, Fwt-ala, Fwt-ala-GCN, Flys-ala, Flys-ala-GCN) with the anti-strep conformational antibody was checked.

FIG. 11 displays western blots of Fwt-ala-GCN, Fwt-ala, Flys-ala-GCN and Flys-ala proteins that were either not reduced and heated (left panel) or both not reduced and not heated (right panel).

FIGS. 12A, B and C show the pre-fusion and post-fusion states of the F protein with the different epitopes of antibodies that are and are not available.

FIG. 12A shows that, in the pre-fusion state where 6HB is not present, the epitope for AM22 is available, while the epitope for 131-2a is shielded.

FIG. 12B shows that, in the post-fusion state of F protein where 6HB is present, the epitope for AM22 is shielded while the epitope for 131-2a is available.

FIG. 12C shows the pre-fusion state of the F protein, absent the 6HB, transitioning through two intermediate states and ending in the post-fusion state exhibiting the 6HB, and also depicting the presence, appearance and disappearance of the various antigenic sites.

FIG. 13 shows the expression of Flys-ΔHRB and Flys-ΔHRB-GCN proteins on reducing (left panel) and non-reducing conditions (middle and right panel). The heat stability was checked as well (middle panel versus right panel).

FIG. 14 shows a western blot with Flys-GCN, Flys-ΔHRB and Flys-ΔHRB-GCN under non-reducing conditions and after heat treatment (left panel) and no heat treatment (right panel). Increasing trypsin digestion is indicated (T). No trypsin treatment is indicated with a minus (-). Flys-ΔHRB-GCN forms higher order structures regardless of cleavage and regardless of heating of the purified product. These higher order structures do not relate to 6HB as a functional HRB domain is absent.

FIG. 15A shows the results of an ELISA assay with which the reactivity of two different soluble F proteins (Flys-ΔHRB and Flys-ΔHRB-GCN) with the AM22 conformational antibody was checked. The treatment with trypsin is indicated.

FIG. 15B shows the results of an ELISA assay with which the reactivity of two different soluble F proteins (Flys-ΔHRB and Flys-ΔHRB-GCN) with the 131-2a conformational antibody was checked. The treatment with trypsin is indicated.

FIG. 15C shows the results of an ELISA assay with which the reactivity of two different soluble F proteins (Flys-ΔHRB and Flys-ΔHRB-GCN) with the Synagis® (Palivizumab) conformational antibody was checked. The treatment with trypsin is indicated.

FIG. 15D shows the results of an ELISA assay with which the reactivity of two different soluble F proteins (Flys-ΔHRB and Flys-ΔHRB-GCN) with the anti-strep conformational antibody was checked. The treatment with trypsin is indicated.

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FIG. 16 is a schematic representation of the RSV F protein lacking a functional HRB domain.

SUMMARY OF THE INVENTION

The present invention relates to a heat-stable oligomeric complex of a recombinant polypeptide presenting at least one antigenic epitope of the pre-fusion Respiratory Syncytial Virus (RSV) F protein, said polypeptide comprising the RSV F protein ectodomain from which the HRB region is functionally deleted and from which the transmembrane and cytoplasmic domains are deleted and replaced with a heterologous trimerization domain, and wherein the two multi-basic furin cleavage sites in said ectodomain are deleted or mutated, thereby rendering said sites defective. The invention further relates to an immunogenic composition comprising the aforesaid heat-stable oligomeric recombinant polypeptide and a method of inducing an immune response in a subject to RSV F comprising administering to said subject said immunogenic composition.

DETAILED DESCRIPTION

The design and evaluation of recombinant soluble F proteins of RSV has suffered considerably by a lack of tools to demonstrate the conformational status of a recombinant F protein. Based on the available literature the inventors of the present invention hypothesized that the post-fusion form of F should comprise a stable 6HB and that it should be recognized by post-fusion specific antibodies, but not by pre-fusion specific antibodies. In contrast, the pre-fusion form of the F protein should not carry the 6HB, will be recognized by pre-fusion specific antibodies, but not by post-fusion specific antibodies. Using this knowledge, it was reasoned that such pre-fusion specific antibodies would enable the identification of RSV F protein mutants that are stable (resistant to for instance heat) and remain in their pre-fusion conformation also under stressful conditions. Such stable pre-fusion mutants could in a next step be used in vaccines against RSV because they would—in their stable—conformation give an immune response (in vivo) and give rise to neutralizing antibodies that would be able to neutralize the virus carrying pre-fusion state F proteins. The present invention discloses that the inventors were indeed able to identify a recombinant RSV F protein mutant that is stably maintained in its pre-fusion conformation and is therefore useful in RSV vaccines.

The present invention may be understood with reference to the following definitions.

‘Functionally deleted’ means a deletion of a sequence of amino acids (which may be referred to as a ‘domain’) from a natural protein sequence such that the function of the deleted domain is lost, and the properties of the protein are thereby altered.

‘Heat-stable’ means that a polypeptide retains its three dimensional conformation in aqueous solution over a range of temperatures, and thereby retains its properties, including for example such polypeptide’s antigenic properties. Preferred temperature ranges are from about 5 degrees C. to about 60 degrees C. A more preferred range is from about 10 degrees C. to about 80 degrees C. A most preferred range is from about 20 degrees C. to about 100 degrees C. Exemplary conformations are those characterized as a pre-fusion state.

“Oligomer” or ‘oligomeric complex’ means polypeptide that consists of two, three, or four polypeptide monomers, such as a dimeric, trimeric or tetrameric complexes of

essentially the same polypeptide monomers. Most preferred oligomeric polypeptides according to the present invention are trimeric polypeptides.

'Post-fusion conformation' or 'post-fusion state' means a three-dimensional protein configuration that differs from that configuration taken by the polypeptide or polypeptide oligomer upon initial expression or oligomeric assembly, and results from the interaction of such polypeptide or oligomer from enzymatic action and/or physical contact with other proteins or proteinaceous assemblies, such as a cell membrane. The RSV F proteins that form a post-fusion state are RSV F proteins that include an HRA-HRB 6HB.

'Pre-fusion conformation' or 'pre-fusion state' means a three-dimensional protein configuration taken by the polypeptide or polypeptide oligomer upon initial expression or oligomeric assembly. RSV F proteins form RSV F protein oligomers that exhibit a pre-fusion configuration prior to fusing with the cell membrane. Pre-fusion RSV F proteins include the following characteristics: the HRA region is packed against domain III in the RSV F head region and/or the HRB region forms a trimer coil-coil stalk in proximity to domains I and II rather than associating with the HRA region in the context of the 6-helix bundle (6HB).

'Purified' protein or polypeptide means a protein or polypeptide isolated from other components of the polypeptide production system such that the amount of protein relative to other macromolecular components present in a composition is substantially higher than that present in a crude preparation. In general, a purified protein or polypeptide will be at least about 50% homogeneous and more preferably at least about 75%, at least about 80%, at least about 90%, at least about 95% or substantially homogeneous.

The RSV F ectodomain protein sequence is exemplified by the sequence of SEQ ID NO: 18. References to the sequence numbering and identifiable domains of the RSV F protein will herein be made to SEQ ID NO: 18. However, other strains of RSV may also be used to generate equivalent recombinant polypeptides.

The present invention relates to recombinant soluble proteins that mimic the pre-fusion state of human RSV F protein. See FIG. 1 for an image depicting the pre-fusion and the post-fusion conformation of the RSV F protein. The higher order modified RSV F protein structures found to be heat-stable, as disclosed herein, are oligomers of the polypeptide, and most likely, the polypeptide trimers of the modified RSV F polypeptide. The present polypeptide is likely formed into a trimer configuration based on size as detected in the experiments disclosed herein and because a trimerization motif is present in the modified polypeptide construct.

Preferably, the heat-stable oligomeric polypeptide according to the present invention is stable at room temperature. More preferably, the polypeptide is stable at temperatures up to 40° C., and even more preferably, the polypeptide is stable at temperatures up to 60° C., and yet in an even more preferred embodiment, the heat-stable recombinant polypeptide of the invention is stable at temperatures up to 70° C. In a most preferred aspect, the heat-stable recombinant polypeptide according to the present invention remains stable at temperatures of about 96° C. for at least about 5 to about 15 minutes.

The heat-stable recombinant polypeptide according to the invention comprises a functional deletion of the HRB region. This is different from what has been performed in the art and as shown herein, for instance by introducing (cysteine or alanine) mutations in the HRB region (WO 2012/158613). Such mutants cannot—similar to what has been

shown intra—form 6HB structures, but still result in labile conformations. Such labile conformations are prevented by removing the HRB region from the RSV F protein as disclosed herein. The polypeptide of the present invention has a functional deletion of the HRB region of the RSV F protein such that the HRB region can no longer perform its natural function, for instance in building the 6-helix bundle (6HB) rendering the protein unable to form a post-fusion conformation. The deletion of the HRB region preferably comprises the amino acids of SEQ ID NO: 10. It will be appreciated by the person skilled in the art that such deletion may be slightly smaller and/or slightly bigger on either side of the HRB region, and/or may be shifted by a small number of amino acids. Nonetheless, such deletions will still render a functional deletion of the HRB region and, in combination with the mutations of the furin cleavage sites and the addition of a heterologous trimerization motif, provide a heat-stable polypeptide as shown herein.

The furin cleavage sites of the RSV protein may be mutated by different methods known in the art, for instance by replacement of the arginine residues by any other type of amino acid, or by deletions of the crucial residues. In a preferred embodiment, the mutation of the furin cleavage sites comprises the replacement of all arginine residues with lysine residues.

The heat-stable oligomeric polypeptide of the invention comprises a heterologous trimerization domain selected from the group consisting of: GCN4 leucine zipper trimerization motif, the trimerization motif from influenza virus HA protein, SARS spike, HIV gp41, NadA, ATCase and foldon sequence. In a preferred embodiment, said heterologous trimerization domain is a GCN4 leucine zipper trimerization motif. Leucine zipper motifs such as GCN4, as well as other trimerization motifs, induce the formation of trimeric-coiled coils, in which three alpha-helices are coiled together like the strands of a rope. Such trimerization motifs have been used in the art to generate RSV vaccines based on RSV F proteins (WO 2010/149743 and WO 2012/158613).

The heat-stable oligomeric polypeptide according to the invention is preferably recognized by the pre-fusion specific monoclonal antibodies AM22 and D25, the preparation and characteristics of which are both described in WO2008/147196, the recognition indicating that pre-fusion specific antigenic epitopes are available.

The polypeptides of the present invention are suitable as antigenic component(s) of a vaccine that protects against infection and disease caused by human RSV (serotype A and B). By employing the present F protein mutation/deletion strategy to F proteins of other viruses belonging to the sub-family Pneumovirinae, immunogenic polypeptides protective against infection caused by these similar viruses, such as bovine RSV or human metapneumovirus, may be prepared.

The invention further relates to an immunogenic composition that is an effective vaccine to immunize against RSV infections. In a preferred embodiment, said immunogenic composition comprises an adjuvant to boost immunogenicity. In yet another aspect, the invention relates to a recombinant expression vector comprising a nucleotide sequence encoding the heat-stable polypeptide according to the invention.

The recombinant proteins of the present invention may also be used in diagnostics assays, with which one may measure the antibody response specifically targeted against the pre-fusion form of the F protein of RSV (or relatives thereof). This response may be of predictive value with respect to disease. Furthermore, the recombinant proteins of

the present invention may be used to test the quality of the antibody response induced by a candidate vaccine, and may be used to generate conformation-specific antibodies which may be used as therapeutics, to study the epitopes present on a candidate vaccine, or to control the antigenicity of a (candidate) vaccine.

In another preferred embodiment, the invention relates to a heat-stable oligomeric complex of a recombinant polypeptide according to the invention that further comprises a LysM peptidoglycan binding domain as a tag. For easy purification and detection of the recombinant polypeptide, the polypeptide preferably comprises a triple Strep-tag. In yet another preferred aspect, the ectodomain within the polypeptide is a soluble ectodomain.

The heat-stable oligomeric complex of a recombinant polypeptide of the present invention is in a pre-fusion conformation that is antigenic, and can be confirmed by the use of antibodies that neutralize and recognize certain epitopes in the RSV F protein. In a preferred aspect, the heat-stable recombinant polypeptide according to the invention comprises an available epitope that is recognized by monoclonal antibody AM22. Whether AM22 recognizes the polypeptide can be easily checked by common methods used in the art, and as disclosed herein.

The present invention is useful in the field of medicine, and in particular in the field of vaccines against RSV infections. The heat-stable polypeptides of the present invention can be used in immunogenic compositions that may be applied in vaccination programs, in regions that present storage and handling challenges such as the third world, to protect (human) subjects who are at risk of developing disease caused by RSV.

Because the polypeptides of the present invention are stable in their pre-fusion conformation, presenting pre-fusion specific epitopes recognized by potent neutralizing antibodies, epitopes absent in polypeptides folded in the post-fusion conformation, the polypeptides are capable of inducing superior pre-fusion specific neutralizing antibodies that protect against RSV infection. The importance of pre-fusion-specific VN antibodies in naturally RSV infected humans has been demonstrated by Magro Metal. (2012. Neutralizing antibodies against the preactive form of respiratory syncytial virus fusion protein offer unique possibilities for clinical intervention. Proc Natl Acad Sci USA 109:3089-94). Hence, the present invention also relates to an immunogenic composition comprising the purified heat-stable oligomeric polypeptide of the present invention, and optionally further comprises excipients commonly used in vaccine preparations.

It should be understood that the present polypeptides used in the immunogenic compositions of the present invention do not include the signal peptide, which is co-expressed with the heat-stable polypeptide, but which is cleaved from the polypeptide before leaving the production cell. In a preferred aspect, although not strictly necessary, the additional (non-RSV F protein) tag sequences are removed by enzymatic digestion during or after the purification process. Preferably, an immunogenic composition according to the present invention further comprises an adjuvant to further boost the immune response.

The present invention also relates to recombinant expression vectors comprising the nucleotide sequences encoding the heat-stable polypeptides of the present invention. Moreover, the invention relates to a method of inducing an immune response in a subject to RSV F comprising administering to said subject an immunogenic composition according to the invention, and to methods of vaccinating human

subjects against RSV infections by applying the immunogenic compositions as disclosed herein. The invention also relates to the use of a heat-stable polypeptide according to the invention for the manufacture of a medicament for the prophylaxis or treatments of RSV infections or diseases that follow an RSV infection. The invention further relates to heat-stable recombinant polypeptides and/or recombinant expression vectors according to the invention for use in vaccines against RSV infections.

Immunogenic compositions according to the present invention may be prepared, tested for immunogenicity, efficacy and safety employing the technology disclosed in published PCT application WO2012/128628, hereby incorporated by reference. Vaccine formulations may be based on particles derived from inactivated *Lactococcus lactis* bacteria, a safe bacterium traditionally used in the food industry, such as for the production of cheese (described elsewhere as Gram-positive Enhancer Matrix or Bacterium-Like Particles and herein referred to as "BLPs"). BLPs are obtained by the acidic heat treatment of *L. lactis* bacteria, resulting in non-living spherical particles that predominantly consist of a peptidoglycan surface, the preparation of BLPs is disclosed in WO 02/101026. The antigenic polypeptides of the present invention may be loaded onto the BLPs, which employs the non-covalent coupling technology referred to as, Protan technology, disclosed in U.S. Pat. No. 7,312,311, which is hereby incorporated by reference. The resulting antigen-associated BLPs constitute the final vaccine that may be delivered to humans via the mucosal layers of the nose (e.g. drops or spray) or mouth (e.g. capsule, tablet or liquid), without the need for an injection.

EXAMPLES

Example 1

RSV F Protein Preparations

This example describes the generation of different F protein constructs and their characterization with respect to antibody binding and gel electrophoretic mobility. This analysis demonstrates that the tools and assays are suitable to demonstrate the conformational state of a RSV F protein. Also, a method is disclosed that shows the production of recombinant soluble proteins that mimic the pre-fusion state of human RSV F.

In an initial step, a comparative analysis of the virus neutralizing capacity was performed of the MAbs that were used herein (FIG. 3). In agreement with previous studies, antibody 131-2a hardly demonstrated any neutralizing capacity compared to Palivizumab or AM22 (McLellan J S et al. 2011. J Virol 85:7788-96). In contrast, AM22 efficiently neutralized infection with hRSV and was even more potent than Palivizumab, in agreement with what was shown in US 2012/0070446. Palivizumab was shown to recognize the post-fusion form of hRSV F, but it likely also recognizes the pre-fusion form of F, because its epitope appears also to be present in the pre-fusion form based on the X-ray structure solved for parainfluenza virus 5 F protein (Yin H S et al. 2006. Structure of the parainfluenza virus 5 F protein in its metastable, pre-fusion conformation. Nature 439:38-44). Antibody 131-2a recognizes an epitope that is probably not accessible for antibody binding in the pre-fusion form of F. The presumed inability of 131-2a to bind the pre-fusion form of RSV F corresponds with its lack of neutralizing activity. Finally, the epitope recognized by AM22 has not been identified yet.

The inventors of the present invention and others previously demonstrated that recombinant soluble class I fusion proteins can be stably maintained in their pre-fusion conformation by the addition of artificial trimerization domains (de Vries R P et al. 2010. The influenza A virus hemagglutinin glycosylation state affects receptor-binding specificity. *Virology* 403:17-25; Wei C J et al. 2008. Comparative efficacy of neutralizing antibodies elicited by recombinant hemagglutinin proteins from avian H5N1 influenza virus. *J Virol* 82:6200-8; Yang X et al. 2000. Characterization of stable, soluble trimers containing complete ectodomains of human immunodeficiency virus type 1 envelope glycoproteins. *J Virol* 74:5716-25; Yin H S et al. 2006. Nature 439:38-44). Use was made of a similar construct that was used previously to express recombinant soluble bioactive influenza A virus HA protein to express soluble F proteins (Cornelissen L A et al. 2010. A single immunization with soluble recombinant trimeric hemagglutinin protects chickens against highly pathogenic avian influenza virus H5N1. *PLoS One* 5:e10645; de Vries R P et al. 2011. Only two residues are responsible for the dramatic difference in receptor binding between swine and new pandemic H1 hemagglutinin. *J Biol Chem* 286:5868-75; de Vries R P et al. 2010. *Virology* 403:17-25).

In the expression constructs used in the present invention (overview provided in FIG. 4), the human codon-optimized RSV F ectodomain sequence of a European clinical isolate (Tan L et al. 2012. Genetic Variability among Complete Human Respiratory Syncytial Virus Subgroup A Genomes: Bridging Molecular Evolutionary Dynamics and Epidemiology. *PLoS One* 7:e51439) was preceded by a signal peptide-encoding sequence. The ectodomain sequence was optionally followed by a sequence coding for the GCN4 leucine zipper trimerization motif (Harbury P B et al. 1993. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* 262:1401-7). The protein with the codon-optimized ectodomain sequence (which encodes the wild type version of this part of the protein), in which the wild type furin cleavage sites and the HRB domain are present, and wherein the ectodomain is followed by the GCN4 motif was referred to as Fwt-GCN. For comparison, the wild-type F protein-encoding gene was also cloned into vectors lacking the trimerization motif (referred to as Fwt). The tags consisted either of a triple Strep-tag for easy purification, or of a LysM peptidoglycan binding domain followed by the triple Strep tag as it turned out that, for yet unknown reasons, GCN-containing F constructs were expressed to higher levels when also the LysM domain was present. In addition to the wild-type constructs (Fwt and Fwt-GCN), expression vectors encoding F proteins with modified furin-cleavage sites were also constructed. These mutations are specified in the protein sequence of SEQ ID NO: 20, shown as R to K mutations in the 1st furin cleavage site (positions 81, 83 and 84) and in the 2nd furin cleavage site (positions 108, 110 and 111). The nucleotide sequences of these mutants are given in SEQ ID NO: 19. These cleavage site (arg->lys) mutant constructs are referred to as 'Flys'. Others showed that in the absence of furin cleavage the F protein is prevented to adopt the post-fusion conformation (Ruiz-Arguello M B et al. 2002. *Virology* 298:317-26; Ruiz-Arguello M B et al. 2004. *J Gen Virol* 85:3677-87). This is in line with the idea that cleavage of F is required for activation of membrane fusion.

Gene construction and cloning was performed as follows: Two variants of a cDNA clone corresponding to residues 26 to 515 of the F protein of a European isolate of RSV serotype A (Genbank accession number JX015498.1) were synthe-

sized using human-preferred codons by GenScript USA Inc. While one cDNA clone encoded the wild type F protein ectodomain, the other clone encoded a F protein ectodomain in which the arginine residues in the two multibasic furin cleavage sites are mutated into lysines (RARR to KAKK and RKRR to KKKK). Each cDNA was cloned into the pCD5 expression vector for efficient expression in mammalian cells (de Vries R P et al. 2010. *Virology* 403:17-25). The pCD5 vector had been modified such that the F protein-encoding sequences were cloned in frame downstream of a DNA sequence coding for a CD5 signal peptide and when indicated upstream of sequences encoding the heterologous GCN4 isoleucine zipper trimerization motif and the specified tag. The tag either consisted of a triple Strep-tagII (IBA, Germany) or of a LysM peptidoglycan binding domain (van Roosmalen M L et al. 2006. Mucosal vaccine delivery of antigens tightly bound to an adjuvant particle made from food-grade bacteria. *Methods* 38:144-9; WO2012/128628) followed by a triple StreptagII. Two codon-optimized DNA fragments encoding the variable heavy and light chains of antibody AM22 (US 2012/0070446 A1) were synthesized by GenScript USA, Inc. and cloned in-frame into pCAGGS mammalian expression vectors containing human IgG1 heavy and light constant domains, respectively.

Expression of the F protein ectodomains was achieved by transient transfection as follows: pCD5 expression vectors containing RSV F ectodomain-encoding sequences were transfected into HEK293T cells using polyethyleneimine I (PEI) in a 1:5 w/w ratio ($\mu\text{g DNA} : \mu\text{g PEI}$). At 6 h post transfection, the transfection mixture was replaced by 293 SFMII expression medium (Invitrogen), supplemented with sodium bicarbonate (3.7 g/L), glucose (2.0 g/L), Primatone RL-UF (3.0 g/L), penicillin (100 units/ml), Streptomycin (100 $\mu\text{g/ml}$), glutaMAX (Gibco), and 1,5% dimethylsulfoxide. Tissue culture supernatants were harvested 5-6 days post transfection. F proteins were either purified using Strep-tactin Sepharose beads according to the manufacturer's instructions (IBA, Germany) for further analysis of the protein. The AM22 expression vectors were co-transfected at a 1:1 ratio into HEK293T cells similarly as described above. The cell culture media were clarified by centrifugation and the AM22 antibody was purified with protein A sepharose beads using standard conditions. The concentration of purified protein was determined by using a Nanodrop 1000 spectrophotometer (Isogen Life Sciences) according to the manufacturer's instructions.

Expression and secretion of recombinant proteins were confirmed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE; 10% NuPAGE BisTris, Invitrogen) followed by western blotting using anti-Strep-tag antibody conjugated with horse radish peroxidase (HRP) (StrepMAB-classic-HRP, IBA), Palivizumab (Synagis®, Abbott Laboratories) followed by HRP-conjugated anti-human IgG antibody (ITK Southern Biotech). This latter antibody was also used to confirm expression of recombinant antibody AM22. Prior to SDS-PAGE analysis, the samples were resuspended in Laemmli sample buffer (LSB) that either did or did not contain 5% 2-mercaptoethanol (Sigma), and when indicated heated at 96° C. for 5-15 minutes.

The results are given in FIG. 5. The epitope recognized by Palivizumab is located in the F1 part of the F protein. The results show that when the F ectodomains are subjected to SDS-PAGE under reducing conditions (i.e. in the presence of 2-mercaptoethanol), which results in separation of the otherwise disulfide-linked F1 and F2, they migrated with different electrophoretic mobilities corresponding to the

absence or presence of the GCN-LysM sequences. Furthermore, the F proteins migrated at a higher position in the gel, when the furin-cleavage sites were mutated (compare Fwt with Flys, and Fwt-GCN with Flys-GCN) in agreement with these proteins not being cleaved. When the same F protein preparations were subjected to SDS-PAGE in the absence of reducing agents, the migration of the non-cleaved F proteins did not appear to be much affected.

In contrast, while the Fwt and Fwt-GCN proteins clearly ran at a lower position in the gel than the Flys and Flys-GCN under reducing conditions, the difference in the electrophoretic mobility appeared much smaller in the absence of reducing agents, in agreement with the F2 part still being attached to the F1 part via disulfide bridges also in the furin-cleaved proteins. The small difference in electrophoretic mobility between the cleaved and non-cleaved F proteins that was still noticeable is most likely explained by the dissociation of the glycosylated p27 sequence from the cleaved proteins. Interestingly, the electrophoretic mobility of the cleaved F proteins was dramatically changed when the preparations were not heated prior to electrophoresis under non-reducing conditions. In contrast to the non-cleaved proteins (Flys and Flys-GCN, the electrophoretic mobility of which was not much affected) the majority of the Fwt and Fwt-GCN proteins migrated at a much higher position in the gel. The migration of these latter proteins is explained by the cleaved F proteins adopting a stable post-fusion conformation, characterized by the presence of an extremely stable 6HB, resistant to SDS unless the protein preparations are heated. These results indicate that the large majority of the soluble, cleaved F ectodomains adopts a post-fusion conformation. The post-fusion conformation is not prevented when the ectodomain is extended with an artificial trimerization domain. However, the stable post-fusion conformation is not formed when the F proteins are not cleaved.

To confirm and extend these observations, a subsequent experiment was performed in which the purified F proteins were subjected to limiting proteolysis followed by SDS-PAGE under non-reducing conditions. Despite the fact that the furin-cleavage sites in Flys and Flys-GCN had been mutated by substitution of the arginines by lysines, these positions are still sensitive to trypsin digestion. Treatment of the Flys and Flys-GCN proteins with trypsin will thus result in cleavage of these proteins and possibly in formation of the SDS-resistant higher-order structure corresponding to the post-fusion conformation of the F protein.

Purified F proteins were (mock-) treated with varying amounts of TPCK treated trypsin from bovine pancreas (Sigma) for 30 min at 23° C. The samples were next put on ice and trypsin inhibitor (Sigma) was added, after which they were analyzed by SDS-PAGE as described above. Protein bands were visualized by general staining using the Colloidal Blue Staining kit (Invitrogen). The results are shown in FIG. 6. Clearly, digestion with trypsin indeed results in the appearance of the SDS-resistant higher-order structure corresponding to the post-fusion conformation of the F protein. Fwt and Fwt-GCN proteins were taken along as controls. The purified proteins were detected using Colloidal Coomassie Blue staining. Again Fwt and Fwt-GCN ran at their expected positions in the gel, when the samples were heated prior to electrophoresis (upper panels). When the samples were not heated, proteins ran at a much higher position in the gel (lower panels). As expected, treatment of these samples with trypsin did not affect the migration of the higher order structures much. However, trypsin treatment resulted to some extent in the removal of the F protein tags as demonstrated by the appearance of lower migrating F

protein species when the samples were heated prior to electrophoresis. Treatment of the non-cleaved F proteins with trypsin resulted in the appearance of F proteins migrating at a much higher position in the gel under non-reducing conditions, similarly as observed for their Fwt and Fwt-GCN counterparts. The formation of the SDS-resistant higher-order structures was more apparent for Flys when compared to Flys-GCN, suggesting that the formation of the post-fusion conformation is prevented to some extent by the GCN trimerization motif.

The reactivity of the F protein preparations with the RSV F specific MAb Palivizumab (Synagis®), AM22 and 131-2a were probed using an ELISA format. For this, 96-well Nunc maxisorp plates were overnight coated with different F protein preparations (50 ng per well) at 4° C. After blocking and extensive washing, the plates were incubated with limiting dilutions of Palivizumab (Synagis®, starting with 1 in 500 dilution of a 3 mg/ml stock), AM22 (starting with a 200 fold dilution of a 0.7 mg/ml stock), 131-2a (Millipore, starting with a 500 fold dilution of a 1 mg/ml stock), or anti-strep (StrepMAb classic from IBA, starting with a 500 fold dilution of the stock). After extensive washing, the plates were incubated with HRP conjugated goat-anti-human IgG antibodies (ITK Southern Biotech) or HRP conjugated rabbit-antimouse IgG antibodies (DAKO) at a 1:500 dilution for 1 h at RT. Detection of HRP reactivity was performed using tetramethylbenzidine substrate (BioFX) and a ELISA plate reader (EL-808 from Biotek). The results are given in FIG. 7.

All F proteins were coated with similar efficiencies as demonstrated by the binding of MAb specific for the Strep tag (Anti-Strep panel). Palivizumab displayed a concentration-dependent binding to all F protein preparations in agreement with the assumption that this antibody recognizes the F protein regardless of its conformational state. In contrast, AM22 was not able to bind Fwt, in agreement with the assumption that this antibody is not able to bind a protein in post-fusion conformation. However, intermediate binding was observed when the cleaved protein was extended with the trimerization motif (Fwt-GCN) or when cleavage was prevented (Flys). The highest reactivity was observed when these two features were combined (Flys-GCN). Trypsin treatment of Flys and Flys-GCN prior to coating of the wells resulted in reduced AM22 reactivity, which subsequently was comparable to the reactivity observed with Fwt and Fwt-GCN. Reactivity of Palivizumab with the F proteins was not affected by the trypsin treatment. MAb 131-2a efficiently bound to all F protein preparations.

These results show that binding of neutralizing antibody AM22 differs between different F protein preparations: Fwt, which adopts the post-fusion conformation, is hardly detected, while the highest reactivity was observed for Flys-GCN. From these results it was concluded that the majority of Fwt is in the post-fusion conformation (6HB+, 131-2a+, AM22-). Fwt-GCN is probably present in a mixture of conformations (6HB+, 131-2a+, AM22+/-). In the absence of cleavage, the F proteins do not adopt the post-fusion conformation (no 6HB). Their reactivity with both 131-2a and AM22 indicates that these non-cleaved proteins are in some form of intermediate state.

Example 2

Introduction of Cysteine Residues in the HRB Region

Next, the characteristics of F proteins, in which 4 cysteine residues were introduced into their HRB domain (designated

Fwt-cys; Fwt-cys-GCN; Flys-cys; Flys-cys-GCN; FIG. 4) were investigated. These cys substitutions, when introduced in a full length F protein (containing a transmembrane domain and cytoplasmic tail), were previously shown to result in intermolecular disulfide bridges which appeared to stabilize the F protein in its pre-fusion conformation (Magro M et al. 2012. Proc Natl Acad Sci USA 109:3089-94). Purified proteins were analyzed by SDS-PAGE in the absence or presence of reducing agents. As shown in FIG. 8, introducing the cysteine residues in HRB resulted in the formation of higher order structures, which could be observed on the non-reducing gel, even when the samples were heated. These higher order structures were most apparent for the F proteins with the GCN4 trimerization motif (Fwt-cys-GCN and Flys-cys-GCN), but also to some extent for the F proteins that lacked the GCN4 motif (Fwt-cys and Flys-cys). These higher order structures were not observed for the F proteins that contained a wild type HRB under these conditions, in agreement with the results shown in FIGS. 5 and 6. While the higher order structures of Fwt-cys and Flys-cys were sensitive to the presence of reducing agents (FIG. 8; right panel), this was much less so the case for the proteins that additionally contained the GCN4 motif. These data indicate the extreme stability of the higher order structures by introduction of the cysteine residues in HRB when also the GCN4 motif is present.

To confirm and extend these observations, a subsequent experiment was performed in which the purified F proteins were subjected to limiting proteolysis followed by SDS-PAGE under non-reducing conditions. The results are shown in FIG. 9. The purified proteins were detected using Colloidal Coomassie Blue staining. Again, the higher order structures observed after introduction of the cysteine residues were shown not to be sensitive to heating of the sample prior to gel electrophoresis. Furthermore, the results indicate that trypsin-treated Fwt-cys and Flys-cys, which lack the GCN4 motif and are only present in a limited amount in the heat-resistant higher order structure, do not form higher structures when the samples were not heated prior to electrophoresis, as was observed for trypsin-treated Fwt and Flys (FIG. 6). From these results it was concluded that the higher structures observed after introduction of cysteine residues in HRB differ from the higher order structures that are observed for the cleaved F proteins with a wild type HRB, because, in contrast to the latter, the former are resistant to heating and are also formed when the F protein is not cleaved. Furthermore, the data show that introduction of cysteine residues in HRB prevents the formation of the heat-sensitive higher order structure. This indicates that these proteins do not assemble the 6HB that is present in the post-fusion conformation.

The reactivity of the cysteine mutant F proteins preparation was investigated with the RSV F specific MAbs Palivizumab, AM22 and 131-2a using the ELISA format (number 1-4 in FIG. 10), as outlined above. All cysteine mutant F proteins were coated with similar efficiencies as demonstrated by the binding of MAb specific for the Strep tag. Palivizumab displayed a concentration dependent binding to all F protein preparations, which differed only slightly between the different F preparations. MAbs 131-2a and AM22 displayed differential binding to the different F proteins. Fwt-cys-GCN and Flys-cys-GCN were not bound by 131-2a, but were clearly bound by AM22. These results indicate that the higher order structures that are observed for these two proteins correspond with stabilized pre-fusion F proteins (6HB-, 131-2a-, AM22+). The results also indicate that the epitope recognized by AM22 is affected to some

extent by cleavage of F. Fwt-cys was efficiently recognized by 131-2a (Flys-cys somewhat less), in agreement that only part of these proteins form the pre-fusion higher order structure that is not reactive with this antibody. In agreement herewith, these preparations also reacted with AM22. The reactivity observed with AM22 may also be explained in part by Fwt-cys and Flys-cys being in some form of intermediate state (6HB-, 131-2a+, AM22+). While this may be expected for Flys-cys in view of the results with Flys, an alternative explanation must account for the intermediate phenotype of Fwt-cys. The introduction of the cysteines might also promote AM22 reactivity because of HRB not being able to bind HRA, as the inability to form the 6HB may be directly coupled to preservation of the AM22 epitope.

Example 3

Introduction of Alanine Residues in the HRB Region

To study the effect of mutations in the HRB domain in more detail, mutant F proteins were produced in which the same amino acids as outlined in example 2 (see also Magro M et al. 2012. Proc Natl Acad Sci USA 109:3089-94) were substituted by alanines rather than cysteines (FIG. 4), to prevent the formation of a disulfide stabilized pre-fusion trimer. Also these proteins were subjected to SDS-PAGE analysis for the absence or presence of higher order structures. The results are shown in FIG. 11. Introduction of the alanine substitutions in the absence of GCN4 (construct referred to as Fwt-ala) prevented the formation of heat-sensitive higher order structures (6HB) under non-reducing conditions (compare FIG. 11 and FIG. 5), similarly as observed for Fwt-cys. In the presence of GCN4, some higher order structures were observed, which were not sensitive to heating of the sample prior to electrophoresis. These minor higher order structures may correspond with pre-fusion structures. Fwt-ala and Flys-ala, neither of which are able to assemble the 6HB, also do not react with AM22 (right top panel, line 5 and 6, FIG. 10), indicating that preventing the formation of the 6HB does not preserve the epitope for AM22. These proteins are probably in an intermediate state in which the stem formed by HRB in the prefusion proteins is dissociated, thereby making the epitope for 131-2a accessible. At the same time, the head domain is refolded resulting in loss of the epitope for AM22. Even so, the 6HB cannot be formed as a result of the alanine mutations in HRB, which probably prevent interaction of HRB with HRA. Introducing the GCN4 motif in these proteins (Fwt-ala-GCN and Flys-ala-GCN), probably results in a mixed population with some pre-fusion F proteins that are positive for AM22 but not 131-2a. As a result the reactivity of these proteins with AM22 increased, while their reactivity with 131-2a decreased. Strikingly, all F protein with alanine mutations in their HRB display decreased reactivity with AM22 when compared to their wild type HRB counterparts. Apparently, mutation of HRB may either help to preserve pre-fusion epitopes (F proteins with cysteine mutations in HRB) but may also decrease the reactivity with pre-fusion specific epitopes (F proteins with alanine mutations in HRB). Strikingly, these mutations are introduced at the same positions in the protein and both prevent 6HB formation to the same extent, indicating that the ability of the protein to adopt the post-fusion structure is not the driving force for the conformational change of the pre-fusion structure.

The disclosed data show that a set of assays was developed with which the conformation of recombinant soluble F proteins can be determined. With these assays four conformational states of the F protein can be discriminated that are schematically shown in FIGS. 12A and 12B:

1) Pre-fusion F (6HB-, 131-2a-, AM22+), see FIG. 12A left panel. Stable forms are Fwt-cys-GCN and Flys-cys-GCN;

2) Intermediate 1 (6HB-, 131-2a+, AM22+), see FIG. 12B left panel. The stem formed by HRB is dissociated, but the head domain with HRA is not yet refolded. Examples are Flys and Flys-GCN;

3) Intermediate 2 (6HB-, 131-2a+, AM22-), see FIG. 12B right panel. The stem formed by HRB is refolded, but also the head domain that probably contained the epitope recognized by AM22. The 6HB is however not yet assembled. Examples are Fwt-ala and Flys-ala;

4) Post-fusion F (6HB+, 131-2a+, AM22-), see FIG. 12A right panel. The best example is Fwt.

Example 4

Synthesis of Recombinant Soluble Proteins that Mimic the Pre-Fusion State of Human RSV F Protein

In the examples above it is demonstrated that F proteins in the pre-fusion conformation are antigenically different from F proteins that have other conformations. The immune response specifically targeted against pre-fusion F epitopes, may be hampered by the presence of non-pre-fusion specific epitopes, the corresponding antibodies of which do not neutralize the virus (e.g. 131-2a). Although Fwt-cys-GCN and Flys-cys-GCN are in the pre-fusion conformation, these proteins are not considered suitable for the production of pre-fusion F proteins at a larger scale, primarily because these proteins are expressed at very low levels. To solve this, the inventors set out to devise an alternative strategy to express high levels of recombinant F proteins that contain pre-fusion specific epitopes and lack post-fusion specific epitopes. The data show that GCN4 is able to confer the pre-fusion state onto recombinant proteins, but this only efficiently occurs when the HRB stem in the pre-fusion state is stabilized (e.g. in Fwt-cys-GCN and Flys-cys-GCN). This instability may even be increased by other mutations in HRB (such as the alanine mutations described herein).

The inventors hypothesized that there might be some form of inherent instability in HRB, which makes it possible for HRB to dissociate and subsequently to interact with the extended HRA. Rather than to try to stabilize HRB via the introduction of intermolecular disulfide bonds, which severely reduces protein expression levels, it was decided to remove the HRB region (and its inherent instability) altogether. However, in the complete absence of HRB it was expected that epitopes that are normally not available in the pre-fusion state would be exposed, either directly by removal of HRB (e.g. epitope of 131-2a) or because the resulting stem-less protein may behave as a monomer rather than a trimer. Furthermore, the data indicate that in the absence of cleavage the AM22 epitope and probably other pre-fusion specific epitopes are better maintained in the

recombinant F protein (compare Flys-cys-GCN and Fwt-cys-GCN). In view of these considerations, a recombinant protein was generated, in which HRB was removed, the GCN4 trimerization motif was added and the furin cleavage site was mutated (FIG. 4). The HRB part of the ectodomain that was deleted is given in SEQ ID NO: 10. The full nucleotide sequence coding for the expressed polypeptide Flys-ΔHRB-GCN is provided in SEQ ID NO: 5, whereas the amino acid sequence is provided in SEQ ID NO: 6. As a control, a variant was synthesized that lacked GCN4 (SEQ ID NO: 3 and 4). The deletion in the constructs of the present invention is somewhat larger (namely position 478-512 in SEQ ID NO: 2) than is regarded as the HRB region in literature (Swanson et al. 2011, Proc Natl Acad Sci USA 10.1073. PNAS. 1106536108), which holds HRB to run from position 482-510 (in SEQ ID NO:2).

These recombinant proteins were analyzed by SDS-PAGE using reducing and non-reducing conditions (FIG. 13). Both proteins migrated at the expected position in the reducing gel. However, in the absence of reducing agents the majority of Flys-ΔHRB-GCN ran at a much higher position in the gel. This was not the case for Flys-ΔHRB. Heating the samples did not affect the electrophoretic mobilities of the proteins much (FIG. 13, middle panel). This result indicates that the higher order structure observed for Flys-ΔHRB-GCN does not correspond to the heat-sensitive 6HB. This is expected because the 6HB cannot be formed in the absence of a functional HRB.

Next, cleavage of these recombinant proteins was induced by trypsin treatment to study the effect of protein digestion on the protein conformation. As a control Flys-GCN was taken and proteins were run on non-reducing gels. The results are shown in FIG. 14. Upon trypsin treatment Flys-GCN, and in the absence of heating, the cleaved Flys-GCN protein ran as the (heat-sensitive) higher order structure indicative of 6HB formation. In contrast, the majority of Flys-ΔHRB ran at its expected (calculated) position in the gel, and trypsin cleavage did not induce the formation of higher order structures. This is expected as in the absence of HRB it is not possible to form the 6HB. The majority of Flys-ΔHRB-GCN4 again ran as a higher order structure, which was resistant to heat. This migration was not significantly affected by the trypsin treatment. The results show that replacing the HRB domain with GCN4 results in an extremely stable higher order structure that obviously does not correspond to the 6HB.

The reactivity of the Flys-ΔHRB and Flys-ΔHRB-GCN proteins with the monoclonal antibodies was tested in an ELISA as outlined above (FIG. 15). Flys-ΔHRB was recognized both by 131-2a and AM22. In the additional presence of GCN4 (Flys-ΔHRB-GCN), the reactivity for 131-2a was lost, while the reactivity with AM22 increased. Apparently, the GCN4 domain shields the 131-2a epitope (similarly as a stabilized HRB) and stimulates the presence of the epitope of AM22. Trypsin treatment of the samples did not affect the 131-2a reactivity, but clearly decreased the AM22 reactivity. The reactivity with Palivizumab or the monoclonal against the strep tag was not affected by the trypsin treatment. From these results, it was concluded that Flys-ΔHRB-GCN is representative of the pre-fusion conformation of the RSV F protein as this protein does not form the 6HB, does react with AM22, but not with 131-2a (see for a schematic representation of this mutant, FIG. 16).

SEQUENCE LISTING

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Asn	Pro	Lys	Tyr	Asp	Cys	Lys	Ile	Met	Thr	Ser	Lys	Thr	Asp	Val	Ser
385					390					395					400
Ser	Ser	Val	Ile	Thr	Ser	Leu	Gly	Ala	Ile	Val	Ser	Cys	Tyr	Gly	Lys
			405						410					415	
Thr	Lys	Cys	Thr	Ala	Ser	Asn	Lys	Asn	Arg	Gly	Ile	Ile	Lys	Thr	Phe
			420					425					430		
Ser	Asn	Gly	Cys	Asp	Tyr	Val	Ser	Asn	Lys	Gly	Val	Asp	Thr	Val	Ser
		435						440				445			
Val	Gly	Asn	Thr	Leu	Tyr	Tyr	Val	Asn	Lys	Gln	Glu	Gly	Lys	Ser	Leu
	450					455					460				
Tyr	Val	Lys	Gly	Glu	Pro	Ile	Ile	Asn	Phe	Tyr	Asp	Pro	Leu	Ile	Asn
465					470					475					480
Asp	Tyr	Lys	Asp	Asp	Asp	Asp	Lys	Ala	Gly	Pro	Gly	Trp	Ser	His	Pro
			485						490					495	
Gln	Phe	Glu	Lys	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser
			500					505					510		
Trp	Ser	His	Pro	Gln	Phe	Glu	Lys	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser
		515					520					525			
Gly	Gly	Gly	Ser	Trp	Ser	His	Pro	Gln	Phe	Glu	Lys				
	530					535					540				

<210> SEQ ID NO 5

<211> LENGTH: 2010

<212> TYPE: DNA

<213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 5

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atgggggtctc tgcaaccgct ggccaccttg tacctgctgg ggatgctggt cgcttccgtg    60
ctagcacaga atatcaccga ggagttttat cagtccacct gttccgcagt gtccaaaggc    120
tatctgtccg ccctgagaac cggctggtat acaagtgtga tcactattga gctgtcaaac    180
atcaaggaaa acaaatgcaa tggcaccgac gctaagggtga aactgattaa gcaggagctg    240
gataagtaca aaaatgcagt gaccgaactg cagctgctga tgcagtcaac accagccgct    300
aacagcaaag ccaagaaaga gctgccccgg ttcattgaatt atacactgaa caataccaag    360
aacacaaatg tgactctgag caagaaaaag aaaaagaaat tcctgggggt tctgctggga    420
gtgggatcag caatgccag cggcattgcc gtgagcaaag tcctgcacct ggagggggaa    480
gtgaacaaga tcaaatccgc tctgctgtct acaacaagg cagtggtcag tctgtcaaat    540
ggcgtgagtg tcctgacttc aaagggtctg gacctgaaaa attacatcga taagcagctg    600
ctgcctattg tcaacaaca gagctgttcc atctctaata ttgagaccgt gatcgaattc    660
cagcagaaga acaataggct gctggagatt acacgcgaat tttctgtgaa cgcaggcgtc    720
accacaccg tgagtacata catgctgact aatagcgagc tgctgtccct gatcaacgac    780
atgcctatta ccaatgatca gaagaaactg atgtccaaca atgtgcagat cgtccggcag    840
cagagttact caatcatgtc tatcattaag gaggaagtcc tggcttacgt ggtccagctg    900

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ccactgtatg gagtgatcga cacaccctgc tggaaactgc atactagccc cctgtgcact    960
accaacacaaa aggaaggcag caatatttgc ctgacacgga ctgacagagg atggtactgt    1020
gataacgccg gcagcgtgtc cttctttcca caggctgaga cctgcaaggt ccagagcaac    1080
cgagtgttct gtgacaccat gaattctctg aactgccta gtgaagtгаа cctgtgcaat    1140
atcgacatct tcaacccaaa gtacgattgt aagatcatga cctctaagac agatgtcagc    1200
tcctctgtga tcacttcctt gggggcaatc gtgagctgct acggaaagac taaatgtacc    1260
gcctccaaca aaaatagagg gatcattaag accttcagca acggatgсga ctatgtctcc    1320
aacaagggcg tggatactgt gagtgctggg aacaccctgt actatgtcaa taagcaggag    1380
ggaaaaagcc tgtacgtгаа gggcgaacct atcattaact tttatgatcc cttaattaag    1440
aggatгааac agattgaggа taaaatcgag gaaattгаа gcaagcagaa gaaaattgag    1500
aacгааatcg cccgcattaa gaaagggaat accaactccg gcgggtctac aactaccatc    1560
acaacaata acagtггaac taacagttca agcacaactt acaccgtгаа gtctggcgat    1620
aactgtggg ggatctcaca gcgatatggc atcagcgtgg ctcagattca gtccgcaaat    1680
aacctгааat ctaccatcat ctacatcggg cagaagctgg tgctgactgg aagcgcctcc    1740
tctaccaaca gtggaggctc aaataactca gttccacca caccaactac cagcgtgacc    1800
cccгaaagc ctacatcaca gactaccgac tacaaggatg acgacгaaа ggctggaccc    1860
ggttggtccc atccacagtt cgagaagggc ggagгааagcg gaggcggctc cggaggagga    1920
tcctgggtccc acccгcagtt tgagaagggc ggcгcagcg gcggaggctc cggcгgaggc    1980
tcctggagcc acccccagtt cgagaagtaa    2010

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<210> SEQ ID NO 6
<211> LENGTH: 669
<212> TYPE: PRT
<213> ORGANISM: Respiratory Syncytial Virus

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<400> SEQUENCE: 6

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

Val Ala Ser Val Leu Ala Gln Asn Ile Thr Glu Glu Phe Tyr Gln Ser
20          25          30

Thr Cys Ser Ala Val Ser Lys Gly Tyr Leu Ser Ala Leu Arg Thr Gly
35          40          45

Trp Tyr Thr Ser Val Ile Thr Ile Glu Leu Ser Asn Ile Lys Glu Asn
50          55          60

Lys Cys Asn Gly Thr Asp Ala Lys Val Lys Leu Ile Lys Gln Glu Leu
65          70          75          80

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

Thr Pro Ala Ala Asn Ser Lys Ala Lys Lys Glu Leu Pro Arg Phe Met
100         105         110

Asn Tyr Thr Leu Asn Asn Thr Lys Asn Thr Asn Val Thr Leu Ser Lys
115        120        125

Lys Lys Lys Lys Lys Phe Leu Gly Phe Leu Leu Gly Val Gly Ser Ala
130        135        140

Ile Ala Ser Gly Ile Ala Val Ser Lys Val Leu His Leu Glu Gly Glu
145        150        155        160

Val Asn Lys Ile Lys Ser Ala Leu Leu Ser Thr Asn Lys Ala Val Val
165        170        175

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Ser Leu Ser Asn Gly Val Ser Val Leu Thr Ser Lys Val Leu Asp Leu
 180 185 190

Lys Asn Tyr Ile Asp Lys Gln Leu Leu Pro Ile Val Asn Lys Gln Ser
 195 200 205

Cys Ser Ile Ser Asn Ile Glu Thr Val Ile Glu Phe Gln Gln Lys Asn
 210 215 220

Asn Arg Leu Leu Glu Ile Thr Arg Glu Phe Ser Val Asn Ala Gly Val
 225 230 235 240

Thr Thr Pro Val Ser Thr Tyr Met Leu Thr Asn Ser Glu Leu Leu Ser
 245 250 255

Leu Ile Asn Asp Met Pro Ile Thr Asn Asp Gln Lys Lys Leu Met Ser
 260 265 270

Asn Asn Val Gln Ile Val Arg Gln Gln Ser Tyr Ser Ile Met Ser Ile
 275 280 285

Ile Lys Glu Glu Val Leu Ala Tyr Val Val Gln Leu Pro Leu Tyr Gly
 290 295 300

Val Ile Asp Thr Pro Cys Trp Lys Leu His Thr Ser Pro Leu Cys Thr
 305 310 315 320

Thr Asn Thr Lys Glu Gly Ser Asn Ile Cys Leu Thr Arg Thr Asp Arg
 325 330 335

Gly Trp Tyr Cys Asp Asn Ala Gly Ser Val Ser Phe Phe Pro Gln Ala
 340 345 350

Glu Thr Cys Lys Val Gln Ser Asn Arg Val Phe Cys Asp Thr Met Asn
 355 360 365

Ser Leu Thr Leu Pro Ser Glu Val Asn Leu Cys Asn Ile Asp Ile Phe
 370 375 380

Asn Pro Lys Tyr Asp Cys Lys Ile Met Thr Ser Lys Thr Asp Val Ser
 385 390 395 400

Ser Ser Val Ile Thr Ser Leu Gly Ala Ile Val Ser Cys Tyr Gly Lys
 405 410 415

Thr Lys Cys Thr Ala Ser Asn Lys Asn Arg Gly Ile Ile Lys Thr Phe
 420 425 430

Ser Asn Gly Cys Asp Tyr Val Ser Asn Lys Gly Val Asp Thr Val Ser
 435 440 445

Val Gly Asn Thr Leu Tyr Tyr Val Asn Lys Gln Glu Gly Lys Ser Leu
 450 455 460

Tyr Val Lys Gly Glu Pro Ile Ile Asn Phe Tyr Asp Pro Leu Ile Lys
 465 470 475 480

Arg Met Lys Gln Ile Glu Asp Lys Ile Glu Glu Ile Glu Ser Lys Gln
 485 490 495

Lys Lys Ile Glu Asn Glu Ile Ala Arg Ile Lys Lys Gly Asn Thr Asn
 500 505 510

Ser Gly Gly Ser Thr Thr Thr Ile Thr Asn Asn Asn Ser Gly Thr Asn
 515 520 525

Ser Ser Ser Thr Thr Tyr Thr Val Lys Ser Gly Asp Thr Leu Trp Gly
 530 535 540

Ile Ser Gln Arg Tyr Gly Ile Ser Val Ala Gln Ile Gln Ser Ala Asn
 545 550 555 560

Asn Leu Lys Ser Thr Ile Ile Tyr Ile Gly Gln Lys Leu Val Leu Thr
 565 570 575

Gly Ser Ala Ser Ser Thr Asn Ser Gly Gly Ser Asn Asn Ser Ala Ser
 580 585 590

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Thr Thr Pro Thr Thr Ser Val Thr Pro Ala Lys Pro Thr Ser Gln Thr
 595 600 605

Thr Asp Tyr Lys Asp Asp Asp Asp Lys Ala Gly Pro Gly Trp Ser His
 610 615 620

Pro Gln Phe Glu Lys Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly
 625 630 635 640

Ser Trp Ser His Pro Gln Phe Glu Lys Gly Gly Gly Ser Gly Gly Gly
 645 650 655

Ser Gly Gly Gly Ser Trp Ser His Pro Gln Phe Glu Lys
 660 665

<210> SEQ ID NO 7
 <211> LENGTH: 65
 <212> TYPE: DNA
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 7

atgggggtctc tgcaaccgct ggccaccttg tacctgctgg ggatgctggt cgcttccgtg 60
 ctagc 65

<210> SEQ ID NO 8
 <211> LENGTH: 22
 <212> TYPE: PRT
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 8

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

Val Ala Ser Val Leu Ala
 20

<210> SEQ ID NO 9
 <211> LENGTH: 105
 <212> TYPE: DNA
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 9

ctggtcttcc ctagecgacga gtttgatgcc tctatcagtc aggtgaacga aaaaatcaat 60
 cagagcctgg cattcatccg aaagagcgac gaactgctgc acaac 105

<210> SEQ ID NO 10
 <211> LENGTH: 35
 <212> TYPE: PRT
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 10

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

Glu Lys Ile Asn Gln Ser Leu Ala Phe Ile Arg Lys Ser Asp Glu Leu
 20 25 30

Leu His Asn
 35

<210> SEQ ID NO 11
 <211> LENGTH: 192
 <212> TYPE: DNA
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 11

ttaattaatg actacaagga tgacgacgac aaggctggac ccggttggtc ccatccacag 60

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ttcgagaagg gcgagggaag cggaggcggc tccggaggag gatcctggtc ccaccgcag 120
tttgagaagg gcgggcggcag cggcggaggc tccggcggag gctcctggag ccacccccag 180
ttcgagaagt aa 192

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<210> SEQ ID NO 12
<211> LENGTH: 63
<212> TYPE: PRT
<213> ORGANISM: Respiratory Syncytial Virus

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<400> SEQUENCE: 12

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Leu Ile Asn Asp Tyr Lys Asp Asp Asp Lys Ala Gly Pro Gly Trp
1           5           10           15
Ser His Pro Gln Phe Glu Lys Gly Gly Gly Ser Gly Gly Gly Ser Gly
                20           25           30
Gly Gly Ser Trp Ser His Pro Gln Phe Glu Lys Gly Gly Gly Ser Gly
            35           40           45
Gly Gly Ser Gly Gly Gly Ser Trp Ser His Pro Gln Phe Glu Lys
        50           55           60

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<210> SEQ ID NO 13
<211> LENGTH: 314
<212> TYPE: DNA
<213> ORGANISM: Respiratory Syncytial Virus

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<400> SEQUENCE: 13

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gggaatacca actccggcgg gtctacaact accatcacia acaataacag tggaactaac 60
agttcaagca caacttacac cgtgaagtct ggcgatacac tgtgggggat ctcacagcga 120
tatggcatca gcgtggctca gattcagtc gaaataaacc tgaaatctac catcatctac 180
atcgggcaga agctggtgct gactggaagc gctcctcta ccaacagtgg aggctcaaat 240
aactcagctt ccaccacacc aactaccagc gtgacccccg caaagcctac atcacagact 300
accgactaca agga 314

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<210> SEQ ID NO 14
<211> LENGTH: 101
<212> TYPE: PRT
<213> ORGANISM: Respiratory Syncytial Virus

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<400> SEQUENCE: 14

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Gly Asn Thr Asn Ser Gly Gly Ser Thr Thr Thr Ile Thr Asn Asn Asn
1           5           10           15
Ser Gly Thr Asn Ser Ser Ser Thr Thr Tyr Thr Val Lys Ser Gly Asp
                20           25           30
Thr Leu Trp Gly Ile Ser Gln Arg Tyr Gly Ile Ser Val Ala Gln Ile
            35           40           45
Gln Ser Ala Asn Asn Leu Lys Ser Thr Ile Ile Tyr Ile Gly Gln Lys
        50           55           60
Leu Val Leu Thr Gly Ser Ala Ser Ser Thr Asn Ser Gly Gly Ser Asn
        65           70           75           80
Asn Ser Ala Ser Thr Thr Pro Thr Thr Ser Val Thr Pro Ala Lys Pro
            85           90           95
Thr Ser Gln Thr Thr
        100

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<210> SEQ ID NO 15
<211> LENGTH: 93

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<212> TYPE: DNA
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 15

ttaattaaga g gatgaaaca gattgaggat aaaatcgagg aaattgaaag caagcagaag 60
 aaaattgaga acgaaatcgc ccgattaag aaa 93

<210> SEQ ID NO 16
 <211> LENGTH: 31
 <212> TYPE: PRT
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 16

Leu Ile Lys Arg Met Lys Gln Ile Glu Asp Lys Ile Glu Glu Ile Glu
 1 5 10 15
 Ser Lys Gln Lys Lys Ile Glu Asn Glu Ile Ala Arg Ile Lys Lys
 20 25 30

<210> SEQ ID NO 17
 <211> LENGTH: 1471
 <212> TYPE: DNA
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 17

acagaatatt accgaggagt tttatcagag cacctgttca gccgtgagta agggctacct 60
 gtcagcactg cggactggat ggtacaccag tgtgatcact attgagctgt caaacatcaa 120
 ggaaaacaaa tgcaatggca ccgacgctaa ggtgaaactg attaagcagg agctggataa 180
 gtacaaaaat gcagtgaccg aactgcagct gctgatgcag tcaacaccag cagctaacag 240
 ccgagcagga agagagctgc cccgattcat gaactacaca ctgaacaaca ccaagaacac 300
 aatgtgact ctgagcaaga aacggaagag gcgcttctctg gggtttctgc tgggagtggg 360
 atcagcaatc gccagcggca ttgccgtgag caaagtctctg cacctggagg gggaagtgaa 420
 caagatcaaa tccgctctgc tgtctacaaa caaggcagtg gtcagtctgt caaatggcgt 480
 gagtgtcctg acttcaaagg tgctggacct gaaaaattac atcgataagc agctgctgcc 540
 tattgtcaac aacagagct gttccatctc taatattgag accgtgatcg aattccagca 600
 gaagaacaat agactgctgg agattacaag ggaattttct gtgaacgcag gcgtcaccac 660
 acccgtgagt acatacatgc tgactaatag cgagctgctg tccctgatca acgacatgcc 720
 tattaccaat gatcagaaga aactgatgtc caacaatgtg cagatcgtca gacagcagag 780
 ttactcaatc atgtctatca ttaaggagga agtcctggct tacgtggtcc agctgccact 840
 gtatggagtg atcgacacac cctgctggaa actgcatact tcacctctgt gcaactacaa 900
 caciaaggaa ggcagcaata tttgctgac acgaaactgac cggggatggt actgtgataa 960
 cgccggcagc gtgtccttct tccacaggc tgagacctgc aaggccaga gcaacagggt 1020
 gttctgtgac accatgaatt ctctgacact gcctagttaa gtgaacctgt gcaatatcga 1080
 catcttcaac ccaaagtacg attgtaagat catgacctct aagacagatg tcagctctctc 1140
 tgtgatcact tccctggggg caatcgtgag ctgctacgga aagactaaat gtaccgctctc 1200
 caacaaaaat cgcgggatca ttaagacctt cagcaacgga tgcgactatg tctccaacaa 1260
 gggcgtggat actgtgagtg togggaacac cctgtactat gtcaataagc aggagggaaa 1320
 aagcctgtac gtgaagggcg aacctatcat taacttttat gatccctgg tcttccctag 1380
 cgacgagttt gatgcctcta tcagtcaggt gaacgaaaa atcaatcaga gcctggcatt 1440

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catccgaaag agcgacgaac tgctgcacaa c

1471

<210> SEQ ID NO 18

<211> LENGTH: 490

<212> TYPE: PRT

<213> ORGANISM: Respiratory Syncytial Viru

<400> SEQUENCE: 18

Gln Asn Ile Thr Glu Glu Phe Tyr Gln Ser Thr Cys Ser Ala Val Ser
 1 5 10 15
 Lys Gly Tyr Leu Ser Ala Leu Arg Thr Gly Trp Tyr Thr Ser Val Ile
 20 25 30
 Thr Ile Glu Leu Ser Asn Ile Lys Glu Asn Lys Cys Asn Gly Thr Asp
 35 40 45
 Ala Lys Val Lys Leu Ile Lys Gln Glu Leu Asp Lys Tyr Lys Asn Ala
 50 55 60
 Val Thr Glu Leu Gln Leu Leu Met Gln Ser Thr Pro Ala Ala Asn Ser
 65 70 75 80
 Arg Ala Arg Arg Glu Leu Pro Arg Phe Met Asn Tyr Thr Leu Asn Asn
 85 90 95
 Thr Lys Asn Thr Asn Val Thr Leu Ser Lys Lys Arg Lys Arg Arg Phe
 100 105 110
 Leu Gly Phe Leu Leu Gly Val Gly Ser Ala Ile Ala Ser Gly Ile Ala
 115 120 125
 Val Ser Lys Val Leu His Leu Glu Gly Glu Val Asn Lys Ile Lys Ser
 130 135 140
 Ala Leu Leu Ser Thr Asn Lys Ala Val Val Ser Leu Ser Asn Gly Val
 145 150 155 160
 Ser Val Leu Thr Ser Lys Val Leu Asp Leu Lys Asn Tyr Ile Asp Lys
 165 170 175
 Gln Leu Leu Pro Ile Val Asn Lys Gln Ser Cys Ser Ile Ser Asn Ile
 180 185 190
 Glu Thr Val Ile Glu Phe Gln Gln Lys Asn Asn Arg Leu Leu Glu Ile
 195 200 205
 Thr Arg Glu Phe Ser Val Asn Ala Gly Val Thr Thr Pro Val Ser Thr
 210 215 220
 Tyr Met Leu Thr Asn Ser Glu Leu Leu Ser Leu Ile Asn Asp Met Pro
 225 230 235 240
 Ile Thr Asn Asp Gln Lys Lys Leu Met Ser Asn Asn Val Gln Ile Val
 245 250 255
 Arg Gln Gln Ser Tyr Ser Ile Met Ser Ile Ile Lys Glu Glu Val Leu
 260 265 270
 Ala Tyr Val Val Gln Leu Pro Leu Tyr Gly Val Ile Asp Thr Pro Cys
 275 280 285
 Trp Lys Leu His Thr Ser Pro Leu Cys Thr Thr Asn Thr Lys Glu Gly
 290 295 300
 Ser Asn Ile Cys Leu Thr Arg Thr Asp Arg Gly Trp Tyr Cys Asp Asn
 305 310 315 320
 Ala Gly Ser Val Ser Phe Phe Pro Gln Ala Glu Thr Cys Lys Val Gln
 325 330 335
 Ser Asn Arg Val Phe Cys Asp Thr Met Asn Ser Leu Thr Leu Pro Ser
 340 345 350
 Glu Val Asn Leu Cys Asn Ile Asp Ile Phe Asn Pro Lys Tyr Asp Cys
 355 360 365

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Lys	Ile	Met	Thr	Ser	Lys	Thr	Asp	Val	Ser	Ser	Ser	Val	Ile	Thr	Ser
	370					375						380			
Leu	Gly	Ala	Ile	Val	Ser	Cys	Tyr	Gly	Lys	Thr	Lys	Cys	Thr	Ala	Ser
385					390					395					400
Asn	Lys	Asn	Arg	Gly	Ile	Ile	Lys	Thr	Phe	Ser	Asn	Gly	Cys	Asp	Tyr
				405					410					415	
Val	Ser	Asn	Lys	Gly	Val	Asp	Thr	Val	Ser	Val	Gly	Asn	Thr	Leu	Tyr
			420					425					430		
Tyr	Val	Asn	Lys	Gln	Glu	Gly	Lys	Ser	Leu	Tyr	Val	Lys	Gly	Glu	Pro
		435					440					445			
Ile	Ile	Asn	Phe	Tyr	Asp	Pro	Leu	Val	Phe	Pro	Ser	Asp	Glu	Phe	Asp
	450					455					460				
Ala	Ser	Ile	Ser	Gln	Val	Asn	Glu	Lys	Ile	Asn	Gln	Ser	Leu	Ala	Phe
465					470					475					480
Ile	Arg	Lys	Ser	Asp	Glu	Leu	Leu	His	Asn						
				485					490						

<210> SEQ ID NO 19
 <211> LENGTH: 1365
 <212> TYPE: DNA
 <213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 19

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cagaatatca ccgaggagt ttatcagtc acctgttccg cagtgtcca aggctatctg    60
tccgcctga gaaccggctg gtatacaagt gtgatcacta ttgagctgtc aaacatcaag   120
gaaaacaaat gcaatggcac cgacgctaag gtgaaactga ttaagcagga gctggataag   180
tacaanaatg cagtgaccga actgcagctg ctgatgcagt caacaccagc cgctaacagc   240
aaagccaaga aagagctgcc cgggttcctg aattatacac tgaacaatac caagaacaca   300
aatgtgactc tgagcaagaa aaagaaaaag aaattcctgg ggtttctgct gggagtggga   360
tcagcaatcg ccagcggcat tgccgtgagc aaagtcctgc acctggaggg ggaagtgaac   420
aagatcaaat ccgctctgct gtctacaaac aaggcagtg tcaagtctgc aaatggcgtg   480
agtgtcctga cttcaaaggt gctggacctg aaaaattaca tcgataagca gctgctgcct   540
attgtcaaca aacagagctg ttccatctct aatattgaga ccgtgatcga attccagcag   600
aagaacaata ggctgctgga gattacacgc gaattttctg tgaacgcagg cgtcaccaca   660
cccgtgagta catacatgct gactaatagc gagctgctgt ccctgatcaa cgacatgcct   720
attaccaatg atcagaagaa actgatgtcc aacaatgtgc agatcgtccg gcagcagagt   780
tactcaatca tgtctatcat taaggaggaa gtcttgctt acgtggcca gctgccactg   840
tatggagtga tcgacacacc ctgctgaaa ctgcatacta gccccctgtg cactaccaac   900
acaaaggaag gcagcaatat ttgctgaca cggactgaca gaggatggta ctgtgataac   960
gccggcagcg tgtccttctt tccacaggct gagacctgca aggtccagag caaccgagtg  1020
ttctgtgaca ccatgaatc tctgacactg cctagtgaag tgaacctgtg caatatcgac  1080
atcttcaacc caaagtacga ttgtaagatc atgacctcta agacagatgt cagctcctct  1140
gtgatcactt ccctgggggc aatcgtgagc tgctacggaa agactaaatg taccgcctcc  1200
aacaaaaata gagggatcat taagacctc agcaacggat gcgactatgt ctccaacaag  1260
ggcgtggata ctgtgagtgt cgggaacacc ctgtactatg tcaataagca ggagggaaaa  1320
agcctgtacg tgaagggcga acccatcatt aacttttatg atccc                    1365

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<210> SEQ ID NO 20
<211> LENGTH: 455
<212> TYPE: PRT
<213> ORGANISM: Respiratory Syncytial Virus

<400> SEQUENCE: 20

Gln Asn Ile Thr Glu Glu Phe Tyr Gln Ser Thr Cys Ser Ala Val Ser
1          5          10          15
Lys Gly Tyr Leu Ser Ala Leu Arg Thr Gly Trp Tyr Thr Ser Val Ile
20          25          30
Thr Ile Glu Leu Ser Asn Ile Lys Glu Asn Lys Cys Asn Gly Thr Asp
35          40          45
Ala Lys Val Lys Leu Ile Lys Gln Glu Leu Asp Lys Tyr Lys Asn Ala
50          55          60
Val Thr Glu Leu Gln Leu Leu Met Gln Ser Thr Pro Ala Ala Asn Ser
65          70          75          80
Lys Ala Lys Lys Glu Leu Pro Arg Phe Met Asn Tyr Thr Leu Asn Asn
85          90          95
Thr Lys Asn Thr Asn Val Thr Leu Ser Lys Lys Lys Lys Lys Lys Phe
100         105         110
Leu Gly Phe Leu Leu Gly Val Gly Ser Ala Ile Ala Ser Gly Ile Ala
115         120         125
Val Ser Lys Val Leu His Leu Glu Gly Glu Val Asn Lys Ile Lys Ser
130         135         140
Ala Leu Leu Ser Thr Asn Lys Ala Val Val Ser Leu Ser Asn Gly Val
145         150         155         160
Ser Val Leu Thr Ser Lys Val Leu Asp Leu Lys Asn Tyr Ile Asp Lys
165         170         175
Gln Leu Leu Pro Ile Val Asn Lys Gln Ser Cys Ser Ile Ser Asn Ile
180         185         190
Glu Thr Val Ile Glu Phe Gln Gln Lys Asn Asn Arg Leu Leu Glu Ile
195         200         205
Thr Arg Glu Phe Ser Val Asn Ala Gly Val Thr Thr Pro Val Ser Thr
210         215         220
Tyr Met Leu Thr Asn Ser Glu Leu Leu Ser Leu Ile Asn Asp Met Pro
225         230         235         240
Ile Thr Asn Asp Gln Lys Lys Leu Met Ser Asn Asn Val Gln Ile Val
245         250         255
Arg Gln Gln Ser Tyr Ser Ile Met Ser Ile Ile Lys Glu Glu Val Leu
260         265         270
Ala Tyr Val Val Gln Leu Pro Leu Tyr Gly Val Ile Asp Thr Pro Cys
275         280         285
Trp Lys Leu His Thr Ser Pro Leu Cys Thr Thr Asn Thr Lys Glu Gly
290         295         300
Ser Asn Ile Cys Leu Thr Arg Thr Asp Arg Gly Trp Tyr Cys Asp Asn
305         310         315         320
Ala Gly Ser Val Ser Phe Phe Pro Gln Ala Glu Thr Cys Lys Val Gln
325         330         335
Ser Asn Arg Val Phe Cys Asp Thr Met Asn Ser Leu Thr Leu Pro Ser
340         345         350
Glu Val Asn Leu Cys Asn Ile Asp Ile Phe Asn Pro Lys Tyr Asp Cys
355         360         365
Lys Ile Met Thr Ser Lys Thr Asp Val Ser Ser Ser Val Ile Thr Ser
370         375         380

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-continued

Leu	Gly	Ala	Ile	Val	Ser	Cys	Tyr	Gly	Lys	Thr	Lys	Cys	Thr	Ala	Ser
385					390					395					400
Asn	Lys	Asn	Arg	Gly	Ile	Ile	Lys	Thr	Phe	Ser	Asn	Gly	Cys	Asp	Tyr
				405					410					415	
Val	Ser	Asn	Lys	Gly	Val	Asp	Thr	Val	Ser	Val	Gly	Asn	Thr	Leu	Tyr
			420					425					430		
Tyr	Val	Asn	Lys	Gln	Glu	Gly	Lys	Ser	Leu	Tyr	Val	Lys	Gly	Glu	Pro
		435					440					445			
Ile	Ile	Asn	Phe	Tyr	Asp	Pro									
	450					455									

The invention claimed is:

1. A heat-stable oligomeric recombinant polypeptide presenting at least one antigenic epitope of the pre-fusion Respiratory Syncytial Virus F protein, said polypeptide comprising

the Respiratory Syncytial Virus F protein ectodomain, from which the HRB region is deleted, and [from which the transmembrane and cytoplasmic domains are deleted and replaced with] *which Respiratory Syncytial Virus F protein ectodomain is extended with a heterologous trimerization domain[,] that replaces the deleted Respiratory Syncytial Virus F protein transmembrane and cytoplasmic domains;*

and

wherein the two multibasic furin cleavage sites in said Respiratory Syncytial Virus F protein ectodomain are mutated by the substitution of all [lysine] *arginine* residues in said sites with [arginine] *lysine* residues, thereby rendering said furin cleavage sites defective.

2. The heat-stable oligomeric recombinant polypeptide according to claim 1, wherein said heterologous trimerization domain is a GCN4 leucine zipper trimerization motif.

3. The heat-stable oligomeric recombinant polypeptide according to claim 1, wherein said deletion of the HRB region comprises a deletion of the HRB wherein the [HBR] *HRB* comprises the sequence of SEQ ID NO: 10.

4. The heat-stable oligomeric recombinant polypeptide according to claim 1, further comprising a LysM peptidoglycan binding domain linked to the carboxy-terminal end of said trimerization domain.

5. The heat-stable oligomeric recombinant polypeptide according to claim 1, further comprising a triple Strep-tag.

6. The heat-stable oligomeric recombinant polypeptide according to claim 1, wherein said ectodomain is a soluble ectodomain.

7. The heat-stable oligomeric recombinant polypeptide according to claim 1, wherein said antigenic epitope is recognized by a pre-fusion specific monoclonal antibody AM22 or D25, or AM22 and D25.

8. An immunogenic composition comprising the oligomeric recombinant polypeptide of claim 1.

9. An immunogenic composition according to claim 8, further comprising an adjuvant.

10. An immunogenic composition according to claim 8, in wherein said oligomeric recombinant polypeptide is bound, covalently or non-covalently, to a carrier particle.

11. An immunogenic composition according to claim 10, wherein said carrier particle is a bacterium-like particle.

12. A recombinant expression vector comprising a nucleotide sequence encoding the polypeptide forming the heat-stable oligomer of claim 1.

13. A method of inducing an immune response in a subject to RSV comprising administering to said subject an immunogenic composition according to claim 8.

14. *A heat-stable oligomeric recombinant polypeptide presenting at least one antigenic epitope of the pre-fusion Respiratory Syncytial Virus F protein, said polypeptide comprising*

the Respiratory Syncytial Virus F protein ectodomain, which is extended with a heterologous trimerization domain that replaces the deleted Respiratory Syncytial Virus F protein transmembrane and cytoplasmic domains; and

wherein the two multibasic furin cleavage sites in said Respiratory Syncytial Virus F protein ectodomain are mutated by the substitution of all arginine residues in said sites with lysine residues, thereby rendering said furin cleavage sites defective.

15. *The heat-stable oligomeric recombinant polypeptide according to claim 14, wherein said heterologous trimerization domain is a GCN4 leucine zipper trimerization motif.*

16. *The heat-stable oligomeric recombinant polypeptide according to claim 14, further comprising a LysM peptidoglycan binding domain linked to the carboxy-terminal end of said trimerization domain.*

17. *The heat-stable oligomeric recombinant polypeptide according to claim 14, further comprising a triple Strep-tag.*

18. *The heat-stable oligomeric recombinant polypeptide according to claim 14, wherein said ectodomain is a soluble ectodomain.*

19. *The heat-stable oligomeric recombinant polypeptide according to claim 14, wherein said antigenic epitope is recognized by a pre-fusion specific monoclonal antibody AM22 or D25, or AM22 and D25.*

20. *An immunogenic composition comprising the oligomeric recombinant polypeptide of claim 14.*

21. *An immunogenic composition according to claim 20, further comprising an adjuvant.*

22. *An immunogenic composition according to claim 20, in wherein said oligomeric recombinant polypeptide is bound, covalently or non-covalently, to a carrier particle.*

23. *An immunogenic composition according to claim 22, wherein said carrier particle is a bacterium-like particle.*

24. *A recombinant expression vector comprising a nucleotide sequence encoding the polypeptide forming the heat-stable oligomer of claim 14.*

25. *A method of inducing an immune response in a subject to RSV comprising administering to said subject an immunogenic composition according to claim 20.*

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