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(57) **Abrégé/Abstract:**

Methods and composition for the screening and isolation of aglycosylated antibody Fc domain polypeptides. For example, in certain aspects methods for identifying aglycosylated Fc domains that bind to Fc receptors or preferentially bind to particular Fc receptors are described. Furthermore, the invention provides aglycosylated Fc domains that bind to Fc receptors with high affinity. Enhanced methods and media for prokaryotic based interaction screening are also provided.

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(57) Abstract: Methods and composition for the screening and isolation of aglycosylated antibody Fc domain polypeptides. For example, in certain aspects methods for identifying aglycosylated Fc domains that bind to Fc receptors or preferentially bind to particular Fc receptors are described. Furthermore, the invention provides aglycosylated Fc domains that bind to Fc receptors with high affinity. Enhanced methods and media for prokaryotic based interaction screening are also provided.



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DESCRIPTION**IMMUNOGLOBULIN FC LIBRARIES****BACKGROUND OF THE INVENTION**

[0001]

1. Field of the Invention

[0002] The present invention relates generally to the field of protein engineering. More particularly, it concerns improved methods and compositions for the screening of combinatorial antibody Fc libraries expressed in bacteria.

2. Description of Related Art

[0003] Currently recombinant therapeutic antibodies have sales of well over \$10 bn/yr and with a forecast of annual growth rate of 20.9%, they are projected to increase to \$25 bn/yr by 2010. Monoclonal antibodies (mAbs) comprise the majority of recombinant proteins currently in the clinic, with more than 150 products in studies sponsored by companies located worldwide (Pavlou and Belsey, 2005). In terms of therapeutic focus, the mAb market is heavily focused on oncology and arthritis, immune and inflammatory disorders, and products within these therapeutic areas are set to continue to be the key growth drivers over the forecast period. As a group, genetically engineered mAbs generally have higher probability of FDA approval success than small-molecule drugs. At least 50 biotechnology companies and all the major pharmaceutical companies have active antibody discovery programs in place.

[0004] The original method for isolation and production of mAbs was first reported at 1975 by Milstein and Kohler (Kohler and Milstein, 1975), and it involved the fusion of mouse lymphocyte and myeloma cells, yielding mouse hybridomas. Therapeutic murine mAbs entered clinical study in the early 1980s; however, problems with lack of efficacy and rapid clearance due to patients' production of human anti-mouse antibodies (HAMA) became apparent. These issues, as well as the time and cost consuming related to the technology became driving

forces for the evolution of mAb production technology. Polymerase Chain Reaction (PCR) facilitated the cloning of monoclonal antibodies genes directly from lymphocytes of immunized animals and the expression of combinatorial library of fragments antibodies in bacteria (Orlandi *et al.*, 1989). Later libraries were created entirely by *in vitro* cloning techniques using naïve genes with rearranged complementarity determining region 3 (CDR3) (Griffiths and Duncan, 1998; Hoogenboom *et al.*, 1998). As a result, the isolation of antibody fragments with the desired specificity was no longer dependent on the immunogenicity of the corresponding antigen. Moreover, the range of antigen specificities in synthetic combinatorial libraries was greater than that found in a panel of hybridomas generated from an immunized mouse. These advantages have facilitated the development of antibody fragments to a number of unique antigens including small molecular compounds (haptens) (Hoogenboom and Winter, 1992), molecular complexes (Chames *et al.*, 2000), unstable compounds (Kjaer *et al.*, 1998) and cell surface proteins (Desai *et al.*, 1998).

[0005] In microbial cells, display screening may be carried out by flow cytometry. In particular, Anchored Periplasmic Expression (APEx) is based on anchoring the antibody fragment on the periplasmic face of the inner membrane of *E.coli* followed by disruption of the outer membrane, incubation with fluorescently labeled target and sorting of the spheroplasts (U.S. Patent 7,094,571). APEx was used for the affinity maturation of antibody fragments (Harvey *et al.*, 2004; Harvey *et al.*, 2006). In one study over 200-fold affinity improvement was obtained after only two rounds of screening.

[0006] One important mechanism underlying the potency of antibody therapeutics is the ability of antibody to recruit immune cells to a target antigen (or cell). Thus, the Fc region of an antibody is crucial for recruitment of immunological cells and antibody dependent cytotoxicity (ADCC). In particular, the nature of the ADCC response elicited by antibodies depends on the interaction of the Fc region with receptors (FcRs) located on the surface of many cell types. Humans contain five different classes of Fc receptors. In addition haplotypes, or genetic variants of different FcRs belonging to a particular class are known. The binding of an antibody to FcRs determines its ability to recruit other immunological cells and the type of cell

recruited. Hence, the ability to engineer antibodies that can recruit only certain kinds of cells can be critically important for therapy.

[0007] However, to the inventors' knowledge, previous attempts to engineer Fc domains have been performed using mammalian-expressed IgG molecules. Mammalian antibodies are glycosylated. The carbohydrate chain is attached to the Fc region and alters the conformation of the protein and enables the antibody to bind to FcRs. In contrast, aglycosylated antibodies produced in bacteria cannot bind to FcRs and therefore are unable to elicit ADCC. It is desirable to engineer aglycosylated antibodies that are capable of eliciting ADCC and thus benefit from the lower production costs that are derived from bacterial expression.

[0008] Second, and most importantly, mammalian antibodies with engineered Fc regions display increased binding to a particular FcR of interest but in addition they are still capable of binding to other FcRs with normal affinity. Thus, while such antibodies are more selective than the molecules naturally produced by the immune system they can nonetheless still mediate undesirable immunological responses.

[0009] Nonetheless, all high throughput antibody screening technologies available to-date rely on microbial expression of antibody fragments. The use of antibody fragments rather than intact or full length IgGs, in the construction and screening of libraries has been dictated by limitations related to the expression of the much larger IgGs in microorganisms. IgG libraries have never before been expressed or screened using microorganisms such as bacteria or yeasts. As a result the isolation of antigen binding proteins has been carried out exclusively using antibody fragments that are smaller and much easier to produce. Once isolated, such antibody fragments have to then be fused to vectors that express full length immunoglobulins which in turn are expressed preferentially in mammalian cells such as CHO cells.

[0010] *E.coli* possesses a reducing cytoplasm that is unsuitable for the folding of proteins with disulfide bonds which accumulate in an unfolded or incorrectly folded state (Baneyx and Mujacic, 2004). In contrast to the cytoplasm, the periplasm of *E. coli* is maintained in an oxidized state that allows the formation of

protein disulfide bonds. Notably, periplasmic expression has been employed successfully for the expression of antibody fragments such as Fvs, scFvs, Fabs or F(ab')₂s (Kipriyanov and Little, 1999). These fragments can be made relatively quickly in large quantities with the retention of antigen binding activity. However, because antibody fragments lack the Fc domain, they do not bind the FcRn receptor and are cleared quickly; thus, they are only occasionally suitable as therapeutic proteins (Knight *et al.*, 1995). Until recently, full-length antibodies could only be expressed in *E. coli* as insoluble aggregates and then refolded *in vitro* (Boss *et al.*, 1984; Cabilly *et al.*, 1984). Clearly this approach is not amenable to the high throughput screening of antibody libraries since with the current technology it is not possible to refold millions or tens of millions of antibodies individually. A further problem is that since *E. coli* expressed antibodies are not glycosylated, they fail to bind to complement factor 1q (C1q) or Fc and many other Fc receptors. However, aglycosylated Fc domains can bind to the neonatal Fc receptor efficiently (FcRn). Consequently bacterially expressed aglycosylated antibodies do exhibit serum persistence and pharmacokinetics similar to those of fully glycosylated IgGs produced in human cells. Nonetheless, since the aglycosylated antibodies fail to elicit complement activation and can not mediate the recruitment of immune cells such as macrophages, they have previously been ineffective for many therapeutic applications.

SUMMARY OF THE INVENTION

[0011] The present invention overcomes a major deficiency in the art in providing aglycosylated antibody Fc domains that bind to Fc receptors and providing methods for the screening and production thereof. In a first embodiment there is provided a method of selecting a bacterial cell comprising an aglycosylated antibody Fc domain having specific affinity for an Fc receptor (FcR) polypeptide comprising the steps of: (a) obtaining a population of Gram negative bacterial cells, cells of which population express an aglycosylated antibody Fc domain in their periplasm, wherein the population expresses a plurality of different Fc domains; (b) contacting the bacterial cells with an FcR polypeptide under conditions wherein the FcR polypeptide contacts the aglycosylated Fc domains; and (c) selecting at least one bacterial cell based on binding of the aglycosylated Fc domain to the FcR polypeptide. Method for expressing polypeptides and in particular antibodies in the

periplasmic space are known in the art for example see U.S. Patent 7,094,571 and U.S. Patent Publ. 20030180937 and 20030219870. In some cases, a gram negative bacterial cell of the invention may be defined as an *E. coli* cell. Furthermore, in some preferred aspects a Gram negative bacterial cell of the invention may be defined as a genetically engineered bacterial cell such as a Jude-1 strain of *E. coli*. Preferably, Gram negative bacterial cells of the invention are viable bacterial cells.

[0012] In certain further embodiments, the invention involves disrupting, permeablizing or removing the outer membrane of bacteria are well known in the art, for example, see U.S. Patent 7,094,571. For instance, prior to contacting the bacterial cells with an FcR polypeptide the outer membrane of the bacterial cell may be treated with hyperosmotic conditions, physical stress, lysozyme, EDTA, a digestive enzyme, a chemical that disrupts the outer membrane, or by infecting the bacterium with a phage or a combination of the foregoing methods. Thus, in some cases, the outer membrane may be disrupted by lysozyme and EDTA treatment. Furthermore, in certain aspects of the invention the bacterial outer membrane may be removed entirely.

[0013] In still further aspects of the invention, an antibody Fc domain that is comprised in the bacterial periplasm may be defined as comprising a hinge, CH2 and CH3 region. However, in some aspects, Fc domains of the invention comprise a functional domain fragment. As used herein the term functional domain fragment means that antibody Fc domain that comprises amino acid deletions relative to wild-type sequence but nonetheless is able to bind to an FcR polypeptide. A skilled artisan will recognize that an antibody Fc domain for use in the invention may be an IgA, IgM, IgE, IgD or IgG antibody Fc domain or a variant thereof. Preferably, an antibody of the invention is an IgG antibody Fc domain such as an IgG1, IgG2a, IgG2b, IgG3 or IgG4 antibody Fc domain. Furthermore, the antibody Fc domain may be defined as a human Fc domain. In certain aspects, the Fc domain may be an IgG1 Fc domain, specifically, the Fc domain of an anti-HER2 antibody, more specifically, the Fc domain of trastuzumab.

[0014] In some further aspects, a Gram negative bacterial cell of the invention further comprises a nucleic acid sequence encoding an antibody Fc domain.

The encoded antibody may be any of the antibody Fc domains defined herein. In further aspects, a nucleic acid of the invention comprises sequences that facilitate Fc export into the periplasmic space. Such sequences are well known in the art and may comprise a secretion signal fused to the Ig chain (U.S. Patent Publ. 20030180937 and 20030219870). Furthermore, an antibody Fc domain encoding nucleic acid may comprise additional elements such as an origin of replication or a selectable marker gene. In some preferred aspects the Fc domain encoding sequences are flanked by known sequences such that the Ig sequence may be amplified by PCR using primers that anneal to the known sequence. Furthermore, the skilled artisan will recognize that a nucleic acid sequence encoding an Fc domain of the invention will comprise sequences that mediate periplasmic expression, such as a secretion signal. For example, in some cases a dual arginine secretion signal may be used. In some highly preferred embodiments the secretion signal is from PelB. In other embodiments, the dsbA secretion signal or any other signal peptide capable of co-translational secretion may be used in order to achieve higher expression.

[0015] Furthermore, in highly preferred aspects of the invention Gram negative bacterial cells for use in the invention comprise a plurality of distinct Fc domain sequences. As used herein a “distinct Fc domain” may be defined as a domain that differs from another Fc by as little as one amino acid. Methods for making a library of distinct antibody Fc domains or nucleic acids that encode antibodies are well known in the art and exemplified herein. For example, in some cases Fc domains may be amplified by error prone PCR as exemplified herein. Furthermore, in certain cases a plurality of antibody Fc domains may comprise a stretch (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) amino acids that have been randomized. In certain cases specific mutations may be engineered into Fc domains. For example, in some aspects, residues that are normally glycosylated in an antibody Fc domain may be mutated. Furthermore, in certain aspects, residues that are normally glycosylated (or adjacent residues) may be used as a site for an insertion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids. In still further embodiments, an amino acid insertion may be made at, or adjacent to, a residue corresponding to amino acid 384 of the IgG1 Fc (SEQ ID NO:1). In still further cases, a population of gram negative bacteria according to the invention may be defined as comprising at least about 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 , or more distinct antibodies Fc domains. In some specific cases, a

population of Gram negative bacterial cells may be produced by a method comprising the steps of: (a) preparing a plurality of nucleic acid sequences encoding a plurality of distinct antibody Fc domains; and (b) transforming a population of Gram negative bacteria with said nucleic acids wherein the Gram negative bacteria comprise a plurality of antibody Fc domains expressed in the periplasm.

[0016] A variety of antibody-binding domains (*e.g.*, FcR polypeptides) are known in the art and may be used in the methods and compositions of the invention. For example, in some aspects, an FcR may have specificity for a particular type or subtype of Ig, such as IgA, IgM, IgE or IgG (*e.g.*, IgG1, IgG2a, IgG2b, IgG3 or IgG4). Thus, in some preferred cases the antibody-binding domain may be defined as an IgG binding domain. The FcR polypeptide may comprises an eukaryotic, prokaryotic, or synthetic FcR domain. For instance, an antibody Fc-binding domain may be defined as a mammalian, bacterial or synthetic binding domain. Some Fc-binding domains for use in the invention include but are not limited to a binding domain from one of the polypeptides of Table 1. For example, an Fc-binding polypeptide may be encoded by an FCGR2A, FCGR2B, FCGR2C, FCGR3A, FCGR3B, FCGR1A, Fcgr1, FCGR2, FCGR2, Fcgr2, Fcgr2, FCGR3, FCGR3, Fcgr3, FCGR3, Fcgr3, FCGRT, mrp4, spa or spg gene. Preferably, an FcR polypeptide for use according to the invention may be an Fc binding region from human Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, Fc α RI or C1q.

[0017] In still further embodiments of the invention an Fc polypeptide may be anchored to the inner membrane of a Gram negative bacteria. Methods and compositions for the anchoring of polypeptides to the inner membrane of Gram negative bacterial have previously been described (U.S. Patent 7,094,571 and U.S. Patent Publ. 20050260736). Thus, in some aspects, an Fc domain may be fused to a polypeptide that is associated with or integrated in a bacterial inner membrane. Such a fusion protein may comprise an N terminal or C terminal fusion with an Fc domain and in some case may comprise additional linker amino acids between the membrane anchoring polypeptide and the Fc domain. In certain specific cases, a membrane anchoring polypeptide may be the first six amino acids encoded by the *E. coli* NlpA gene, one or more transmembrane α -helices from an *E. coli* inner membrane protein, a gene III protein of filamentous phage or a fragment thereof, or an inner membrane

lipoprotein or fragment thereof. Thus, as an example, a membrane anchoring polypeptide may be an inner membrane lipoprotein or fragment thereof such as from AraH, MglC, MalF, MalG, MalC, MalD, RbsC, RbsC, ArtM, ArtQ, GlnP, ProW, HisM, HisQ, LivH, LivM, LivA, LivE, DppB, DppC, OppB, AmiC, AmiD, BtuC, ThuD, FecC, FecD, FecR, FepD, NikB, NikC, CysT, CysW, UgpA, UgpE, PstA, PstC, PotB, PotC, PotH, Pod, ModB, NosY, PhnM, LacY, SecY, TolC, Dsb, B, DsbD, TouB, TatC, CheY, TraB, ExbD, ExbB or Aas.

[0018] The skilled artisan will understand that methods for selecting cells based upon their interaction (binding) with an FcR are well known in the art. For example, an FcR may be immobilized on a column or bead (*e.g.*, a magnetic bead) and the bacterial cell binding to the FcR separated by repeated washing of the bead (*e.g.*, magnetic separation) or column. Furthermore, in some aspects a target ligand may be labeled such as with a fluorophor, a radioisotope or an enzyme. Thus, bacterial cells may, in some cases, be selected by detecting a label on a bound FcR. For example, a fluorophore may be used to select cells using fluorescence activated cell sorting (FACS). Furthermore, in some aspects, bacterial cells may be selected based on binding or lack of binding two or more FcR polypeptides. For instance, bacteria may be selected that display antibodies that bind to two FcR polypeptides, wherein each FcR is used to select the bacterial sequentially. Conversely, in certain aspects, bacteria may be selected that display antibody Fc domains that bind to one FcR (such as an FcR comprising a first label) but not to a second FcR (*e.g.*, comprising a second label). The foregoing method maybe used, for example, to identify antibody Fc domains that bind to a specific FcR but not a second specific FcR.

[0019] In further embodiments, methods for producing bacteria of the invention, may comprise at least two rounds of selection (step c) wherein the sub-population of bacterial cells obtained in the first round of selection is subjected to at least a second round of selection based on the binding of the candidate antibody Fc domain to an FcR. Furthermore in some aspects the sub-population of bacterial cells obtained in the first round of selection may be grown under permissive conditions prior to a second selection (to expand the total number of cells). Thus, in some aspects, methods of the invention may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more

rounds of selection. Furthermore, in some aspects, a sub-population of bacterial cells obtained from each round of selection will be grown under permissive conditions before a subsequent round of selection. Cells isolated following one or more such rounds of selection may be subjected to additional rounds of mutagenesis. In some cases, selection will be performed after removing FcR polypeptide that is not bound to the antibody. Furthermore, in some cases the stringency of selection may be modified by adjusting the pH, salt concentration, or temperature of a solution comprising bacteria that display antibodies. Thus, in some aspects, it may be preferred that a bacterial cell of the invention is grown at a sub-physiological temperature such as at about 25 °C.

[0020] In still further aspects, a method of producing a bacterial cell according to the invention may be further defined as a method of producing a nucleic acid sequence encoding an Fc domain that binds to at least a first FcR. Thus, a bacterial cell produced by the methods herein may be used to clone a nucleic acid sequence encoding the Fc domain having a specific affinity for an FcR polypeptide. Methods for isolating and amplifying such a nucleic acid from a cell for example by PCR are well known in the art and further described below. Thus, a nucleic acid sequence produced by the forgoing methods is included as part of the instant invention. Furthermore, such a sequence maybe expressed in a cell to produce an Fc domain having a specific affinity for an FcR. Thus, in some aspects, the invention provides a method for producing an Fc domain having a specific affinity for an FcR. Furthermore, the invention includes antibody Fc domains produced by the methods of the invention. It will be understood however that the antibody Fc domains produced by such a screen may be combine with antibody variable regions that have an affinity for a particular target ligand and these antibodies are also included as part of the invention.

[0021] In yet a further embodiment the invention provides a polypeptide comprising an aglycosylated antibody Fc domain capable of binding an FcR polypeptide. In some aspects, the aglycosylated Fc domain may be further defined as having a specific affinity for an FcR polypeptide under physiological conditions. For instance an Fc domain may have an equilibrium dissociation constant between about 10^{-6} M to about 10^{-9} M under physiological conditions. Furthermore in

some aspects an aglycosylated Fc domain may be defined as comprising one or more amino acid substitution or insertion relative to a wild type human sequence.

[0022] Of course, it is contemplated that a preferred means of preparing such a polypeptide is through the practice of the methods discussed above. However, one can alternatively prepare such polypeptides directly by genetic engineering techniques such as, for example, by introducing selected amino acid substitutions or insertions into a known Fc background, wherein the insertion or substitution provides an improved FcR binding capability to aglycosylated Fc regions. The inventors have identified as particularly preferred substitutions for achieving such improved FcR binding as those at positions 331, 382 and/or 428 of the Fc domain (for example, see Nagaoka and Akaike 2003; such as P331, E382 and/or M428 of the human IgG Fc domain sequence as shown in **FIG. 46** and also in, *e.g.*, U.S. Patent Publ. US20060173170), and still more preferred are one or more substations defined by P331L, E382V, M428I or M428L.

[0023] Preferred substitutions may further include one or more of 426, 229, 322, 350, 361, 372, 442, 402, 224, 430, 238, 436, 310, 313, 384, 372, 380 or 331 of the Fc domain, such as S426, C229, K322, T350, N361, F372, S442, G402, H224, E430, P238, Y436, H310, W313, N384, F372, E380 or P331 of the human IgG Fc domain, with the specific preferred examples being a) E382 and M428; b) N361, E382 and M428; c) N361, F372, E382 and M428; d) H310, K322, T350, E382, S426 and S442; e) C229R, E382 and M428; f) W313 and M428; g) E382, N384 and M428; h) E380, E382 and N384; i) N361, E382 and M428; j) E382, M428 and Y436; k) P238, E382, S426, M428 and E430; l) E380, E382, N384, S426, M428 and E430; m) E382, S426, M428 and E430; n) H224, E382, S426, M428 and E430; o) P331; p) S239, I253, Q347, E382; q) E382, G402 and M428; and r) E382, P331 and M428. Of these, the most preferred include a) E382V and M428I; b) E382V; c) N361D, E382V and M428I; d) N361D, F372L, E382V and M428I; e) H310Y, K322R, T350A, E382V, S426T and S442P; f) C229R, E382V and M428I; g) W313R and M428I; h) E382T, N384D and M428I; i) E380R, E382M and N384E; j) N361S, E382V and M428I; k) E382V, M428I and Y436A; l) P238S, E382V, S426V, M428L and E430H; m) E380D, E382V, N384R, S426V, M428L and E430D; n) E382V, S426I, M428L and E430S; o) H224R, E382V, S426T, M428S and E430P; p) P331L; q) S239L,

I253T, Q347L, E382V; r) E382V, G402D and M428I; and s) E382V, P331L and M428I.

[0024] The inventors have also identified various insertion points that upon insertion of additional amino acids, provide improved FcR binding capability. Most preferred in this regard are insertions of 5 to 15 amino acids, and preferably 10 amino acids, between amino acids N297 and S298 of an Fc domain, such as a human IgG Fc domain. Particularly preferred insertions at this position (as well as substitutions) include a) RTETPVYVMVM (SEQ ID NO:60); b) WQVFNKYTKP (SEQ ID NO:61); c) LGDGSPCKAN (SEQ ID NO:62); d) EVPLVWMWVS (SEQ ID NO:63) together with F241L and K326E; and e) EQWGSQFGCG (SEQ ID NO:64) together with V282A.

[0025] The Fc domain of the invention may be a human IgG Fc that comprises an amino acid substitution at an amino acid residue corresponding to E382 of the IgG Fc domain. Furthermore, an aglycosylated Fc domain may comprise an amino acid sequence insertion (*e.g.*, about 1 to 5 amino acids) adjacent to an amino acid residue corresponding to E382 of the IgG Fc domain. Thus, in some specific aspects an Fc domain may comprise a hydrophobic amino acid substitution at E382 such as an E to V substitution. Furthermore, in some aspects an Fc domain of the invention may comprise an amino acid substitution at a residue corresponding to M428 (*e.g.*, M428 to I), S426, C229, H310, K322, T350, N361, F372 or S442 of the human IgG Fc. In certain specific embodiments, an aglycosylated Fc domain may comprise an amino acid substitution corresponding to those found in the Fc11 (SEQ ID NO:2), Fc5 (SEQ ID NO:3), Fc12 (SEQ ID NO:4), Fc 20 (SEQ ID NO:5), Fc49 (SEQ ID NO:6) or Fc23 Fc (SEQ ID NO:7) domains described herein (see **FIG. 14**). Hence in a very specific case an aglycosylated Fc domain may comprise the amino acid sequence of Fc11 (SEQ ID NO:2), Fc5 (SEQ ID NO:3), Fc12 (SEQ ID NO:4), Fc 20 (SEQ ID NO:5), Fc49 (SEQ ID NO:6), Fc23 (SEQ ID NO:7), Fc104 (SEQ ID NO:65), Fc106 (SEQ ID NO:66), Fc110 (SEQ ID NO:67), Fc114 (SEQ ID NO:68), Fc117 (SEQ ID NO:69), Fc143 (SEQ ID NO:70), Fc149 (SEQ ID NO:71), Fc151 (SEQ ID NO:72), Fc152 (SEQ ID NO:73), Fc207 (SEQ ID NO:74), Fc209 (SEQ ID NO:75), Fc216 (SEQ ID NO:76), Fc217 (SEQ ID NO:77), Fc236 (SEQ ID NO:78), Fc331 (SEQ ID NO:79), Fc336 (SEQ ID NO:80), Fc 401 (SEQ ID NO:122); Fc402

(SEQ ID NO:81), or Fc403 (SEQ ID NO:82). As described supra the instant invention also contemplates antibodies or antibody fragments that comprise an aglycosylated Fc domain of the invention. Thus, in some cases, polypeptides described herein (Fc domains) may comprise an Ig variable domain and may be further defined as a full length antibody.

[0026] Preferably, an aglycosylated Fc domain of the invention comprises a specific binding affinity for an FcR such as human Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, Fc α RI or C1q. Thus, in some aspects an aglycosylated Fc domain of the invention is defined as an Fc domain with a specific affinity for Fc γ RIa. Furthermore, such an Fc domain may be defined as having an equilibrium dissociation constant, with respect to Fc γ RIa binding, of about 10^{-6} M to about 10^{-9} M under physiological conditions.

[0027] Of course, a still further aspect of the invention includes isolated DNA segments encoding a polypeptide in accordance with any one of the foregoing modified Fc regions as well as antibodies, etc., incorporating such a polypeptide. Such DNA segments may preferably be positioned in an expression vector, which is preferably a bacterial expression vector.

[0028] In still a further aspect of the invention there is provided a bacterial growth media that comprises trehalose. In certain aspects such a media may be used in a method A method of identifying a bacteria cell comprising a first binding partner associated with an inner membrane comprised in the bacteria cell, wherein the binding partner having specific affinity for a second binding partner, comprising the steps of: a) obtaining a population of bacteria cells, cells of which population comprise the first binding partner associated with the inner membrane in the periplasm of the bacteria cells, wherein the population comprises a plurality of different such first binding partners; b) contacting the bacteria cells with the second binding partner, wherein the first binding partner or the second binding partner comprises a label, wherein a signal is elicited when the first binding partner binds to the second binding partner; and c) selecting at least one bacterial cell by detecting such a signal from at least such a first binding partner binding to at least such second binding partner. Preferably, the signal may be a fluorescent signal. In this respect a media comprising trehalose, as demonstrated herein, provides enhanced fluorescence

signal and greatly improves the screening process. Thus, methods for the use of the trehalose bacterial media in screening such binding partners are included as part of the instant invention. Any of the fluorescence screening methods known in the art or described herein may be used in combination with a trehalose bacterial media of the invention. For example, a fluorescence signal may be detected by flow cytometry. Furthermore, bacteria comprising binding partners for detection may have their outer membrane disrupted or partially disrupted. Furthermore, in certain preferred aspects of the one of the binding partners for use in the instant methods may be defined as an antibody or an antibody domain. In some very aspects a bacterial growth media comprising trehalose may be further defined based upon the trehalose concentration in the media. For example a media comprising about between about 0.05 and 1.5M trehalose or preferably between about 0.1 and 1.0 M trehalose is specifically contemplated herein. Thus, in a very specific aspect, bacterial media comprising about 0.5 M trehalose is provided.

[0029] Embodiments discussed in the context of a methods and/or composition of the invention may be employed with respect to any other method or composition described herein. Thus, an embodiment pertaining to one method or composition may be applied to other methods and compositions of the invention as well.

[0030] As used herein the terms “encode” or “encoding” with reference to a nucleic acid are used to make the invention readily understandable by the skilled artisan however these terms may be used interchangeably with “comprise” or “comprising” respectively.

[0031] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one.

[0032] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

[0033] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0034] The scope of the claims should not be limited by the preferred embodiments and examples, but should be given the broadest interpretation consistent with the description as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0036] **FIG. 1:** Two plasmids system for the periplasmic display of Fc using cJun-cFos or cJun(Cys)-cFos(Cys) interaction.

[0037] **FIG. 2a-b:** FACS analysis results of periplasmic displayed Fc homodimer using cJun-cFos and cJun(Cys)-cFos(Cys) interaction pairs. FIG. 2a, FACS signals of periplasmic displayed Fc using cJun-cFos and cJun(Cys)-cFos(Cys) were compared with a positive and a negative controls. FIG. 2b, FACS signals of periplasmic displayed Fc using cJun-cFos and cJun(Cys)-cFos(Cys) were compared with one plasmid systems not co-expressing NlpA and 6 amino acid residues (CDQSSS (SEQ ID N:84)) fused cJun or cJun(Cys). Spheroplasts were incubated with Protein A-FITC probe for detection. Mn: Mean fluorescence intensity.

[0038] **FIG. 3:** Two plasmids system for the periplasmic display of Fc using ColE2-Im2 interaction.

[0039] **FIG. 4a-b:** FACS analysis results for the periplasmic display of Fc homodimer using ColE2-Im2 interaction pairs. FIG. 4a, Display of Im2 fused

M18 scFv or 26-10 scFv co-expressed with APEx displayed ColE2(H578A) and incubated with PA-FITC. FIG. 4b, Display of Im2 fused M18 scFv or 26-10 scFv co-expressed with APEx displayed ColE2(H578A) and incubated with digoxin-BODIPY. Mn: Mean fluorescence intensity.

[0040] FIG. 5: Effect of ColE2 for the expression of target proteins, M18 scFv (Lane 1-3), 26-10 scFv (Lane 4-6), and Fc (Lane 7-9). In lane 1, 4 and 7, Im2 fused proteins were co-expressed with APEx displayed ColE2(H578A). In lane 2, 5 and 7, Im2 fused proteins were expressed without APEx displayed ColE2(H578A). In lane 3, 6 and 9, proteins without Im2 fusions were co-expressed APEx displayed ColE2(H578A). Anti-ECS antibody peroxidase conjugated was used as a detection antibody for Western blot.

[0041] FIG. 6: Effect of sugars (sorbitol and trehalose) on the FACS analysis for periplasmic displayed Fc or APEx displayed Fc. Spheroplasts were incubated with Protein A-FITC probe for detection. Mn: Mean fluorescence intensity.

[0042] FIG. 7: Effect of trehalose on the periplasmic display of Fc. As a negative control, M18 scFv was used. Spheroplasts were incubated with Protein A-FITC probe for detection. Mn: Mean fluorescence intensity.

[0043] FIG. 8: One plasmid system for the periplasmic display of trapped Fc with trehalose.

[0044] FIG. 9a-b: Effect of trehalose on the expression level and the retention after spheroplasting for homodimeric Fc. FIG.9a, Western blot result from reduced gel for the periplasmic expressed Fc and M18 scFv cultured in the media with or without trehalose. FIG.9b, Western blot result from reduced or non-reduced gel for the periplasmic expressed Fc cultured in the media with or without trehalose. Anti-ECS antibody peroxidase conjugated was used as a detection antibody for Western blot.

[0045] FIG. 10a-b: Effect of signal leader peptides (PelB and dsbA) on the periplasmic display of Fc. FIG. 10a, Comparison of FACS signals between PelB and dsbA fused proteins. PelB or dsbA signal peptide fused proteins were cultured with 0.5M trehalose. FIG. 10b, Comparison of FACS signals between with

and without trehalose in the media. DsbA signal peptide fused proteins were cultured with or without 0.5M trehalose. Mn: Mean fluorescence intensity. Spheroplasts were incubated with Protein A-FITC probe for detection.

[0046] **FIG. 11:** FACS analysis for the periplasmic displayed antibodies. M18.1 humanized antibodies and 26-10 antibodies with various formats, scFv, scAb, and IgG., were periplasmic displayed and detected by PA-FITC. Mn: Mean fluorescence intensity.

[0047] **FIG. 12a-b:** Fluorescence ELISA to detect affinity of FITC labeled Fc γ RIa for IgG-Fc. FIG. 12a, IgG-Fc was coated onto fluorescence ELISA plate. The fluorescence of serially diluted and bound Fc γ RIa-FITC was detected at excitation 485 nm and emission 528 nm. FIG. 12b, Fluorescence signals of serially diluted Fc γ RIa-FITC in the IgG-Fc coated wells compared to the signals in the BSA coated wells.

[0048] **FIG. 13a-b:** Fc library screening using FACS sorting. FIG. 13a, Histogram showing enrichment of high affinity clones sorted by Fc γ RIa-FITC. FIG. 13b, Histogram showing fluorescence signals of Fc mutants comparing with wild type Fc. Spheroplasts were incubated with Fc γ RIa-FITC for detection. Mn: Mean fluorescence intensity.

[0049] **FIG. 14:** Sequences of isolated Fc mutant clones exhibiting high affinity to Fc γ RIa. Depicted sequences are as follows used in the experiment with a FLAG tag attached to the C-terminal end, wt-IgG1 Fc, SEQ ID NO:1, Fc11, SEQ ID NO:2; Fc5, SEQ ID NO:3; Fc12, SEQ ID NO:4; Fc20, SEQ ID NO:5; Fc49, SEQ ID NO:6; and Fc23, SEQ ID NO:7;

[0050] **FIG. 15a-b:** Mutation points of isolated aglycosylated Fcs in 3D structure of glycosylated IgG (PBD Code: 1FC1). FIG. 15a, Major mutation points in full glycosylated IgG. FIG. 15b, Interaction of two beta sheets including 382E and 428M in the CH3 region.

[0051] **FIG. 16:** Fc library comprising 3 kinds of sub-libraries randomized and inserted around 382E and 428M.

[0052] **FIG. 17:** Histogram showing enrichment of clones showing high affinity to Fc γ RIa by FACS sorting from the library randomized around 382E and 428M in **FIG. 16**.

[0053] **FIG. 18:** Sequence of isolated Fc mutant clones exhibiting high affinity to Fc γ RIa. Spheroplasts were incubated with Fc γ RIa-FITC for detection. FACS mean values are indicated in the parenthesis.

[0054] **FIG. 19:** SDS-PAGE of purified and refoled Fc γ RIIIa from *E. coli* inclusion bodies.

[0055] **FIG. 20:** Histogram showing enrichment of high affinity clones sorted by Fc γ RIIIa-FITC.

[0056] **FIG. 21:** Histogram showing fluorescence signals of Fc mutants comparing with wild type Fc. Spheroplasts were incubated with Fc γ RIIIa-FITC for detection. M: Mean fluorescence intensity.

[0057] **FIG. 22:** Sequences of isolated Fc mutant clones exhibiting high affinity to Fc γ RIIIa.

[0058] **FIG. 23:** Histogram showing enrichment of high affinity clones sorted by Fc γ RIIa-FITC

[0059] **FIG. 24:** Histogram showing fluorescence signals of Fc mutants comparing with wild type Fc. Spheroplasts were incubated with Fc γ RIIa-FITC for detection. M: Mean fluorescence intensity.

[0060] **FIG. 25:** Sequences of isolated Fc mutant clones exhibiting high affinity to Fc γ RIIIa.

[0061] **FIG. 26.** SDS-PAGE showing the expression of wild type Fc γ RIIa and codon optimized Fc γ RIIa, Lane 1: Wild type Fc γ RIIa; Lane 2: codon optimized Fc γ RIIa.

[0062] **FIG. 27.** SDS-PAGE showing the localization of codon optimized Fc γ RIIa, Lane 1: Total fraction; Lane 2: soluble fraction; Lane 3: insoluble fraction.

[0062] **FIG. 27.** SDS-PAGE showing the localization of codon optimized FcγRIIa, Lane 1: Total fraction; Lane 2: soluble fraction; Lane 3: insoluble fraction.

[0063] **FIG. 28.** SDS-PAGE showing the purified FcγRIIa. Lane 1: purified FcγRIIa.

[0064] **FIG. 29.** ELISA result of Fc mutants to FcγRIIa from the media fraction of cultured Jude-1 cells harboring pDsbAFLAG-Fc mutant plasmids.

[0065] **FIG. 30:** Soluble expression of homodimeric wild type Fc and Fc mutants (5 ml tube culture). Wild type Fc with two different signal peptides (PelB and DsbA) was expressed at different culture temperatures after induction and was harvested at different times. The localization of the protein was also analyzed.

[0066] **FIG. 31:** Soluble expression of homodimeric wild type Fc and Fc mutants (500 ml flask culture). DsbA leader peptide fused wild type Fc was expressed at different culture temperatures and culture time after induction. The localization of the protein was also analyzed.

[0067] **FIG. 32:** SDS-PAGE of wild type Fc and Fc mutants purified with Protein A affinity chromatography.

[0068] **FIG. 33a-d:** Chromatogram of wild type Fc (FIG. 33a) and Fc mutants using SuperdexTM 200 gel filtration chromatography, including Fc5 (FIG. 33b), Fc11 (FIG. 33c) and Fc49 (FIG. 33d).

[0069] **FIG. 34:** SDS-PAGE of wild type Fc and Fc mutants purified with Superdex 200 gel filtration chromatography.

[0070] **FIG. 35:** Direct coating ELISA for the detection of affinity of Fc mutants to FcγRs

[0071] **FIG. 36.** ELISA result of Fc mutants to FcγRI.

[0072] **FIG. 37.** SPR Sensorgrams of Fc protein binding onto immobilized FcγRI.

working volume. The OD₆₀₀ is shown as a function of time after inoculation during the expression of trastuzumab in *E. coli*

[0075] **FIG. 40.** Fully assembled IgG as detected by non-denaturing gel electrophoresis and Western blotting with goat anti-human IgG (H+L) antibodies. Results are shown for cells expressing wild type trasuzumab; similar results were obtained for cells expressing trastuzumab-Fc5.

[0076] **FIG. 41.** Expression of aglycosylated trastuzumab and trastuzumab-Fc5, Lane 1: IgG1 standard; Lane 2: Before induction; Lane 3: aglycosylated trastuzumab; Lane 4: trastuzumab-Fc5.

[0077] **FIG. 42.** SDS-PAGE showing the purified aglycosylated trastuzumab and trastuzumab-Fc5, Lane 1, 3: Wild type Fc aglycosylated trastuzumab; Lane 2, 4: trastuzumab-Fc5.

[0078] **FIG. 43.** ELISA assays for binding to Fc γ RIIa. Plates were coated with purified trastuzumab or trastuzumab-Fc5 and the binding of Fc γ R was detected using anti-GST-HRP.

[0079] **FIG. 44.** ELISA assays for binding to Fc γ RIIb. Plates were coated with purified trastuzumab or trastuzumab-Fc5 and the binding of Fc γ R was detected using either anti-polyhistidine-HRP or anti-GST-HRP.

[0080] **FIG. 45.** ELISA assays for binding to FcRn at pH 7.4 and 5.5. Plates were coated with purified trastuzumab or trastuzumab-Fc5 and the binding of Fc γ R was detected using anti-GST-HRP.

[0081] **FIG. 46.** Alignment of sequences for human IgG subclasses (SEQ ID NOS: 110, 111, 112 and 113).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0082] The instant invention overcomes several major problems with current immunotherapeutic technologies in providing aglycosylated antibody Fc domains that are able to bind to Fc receptor polypeptides. Furthermore, now methods for identifying aglycosylated Fc domains capable of binding to Fc receptors are described. These methods enable isolation of antibody Fc domains that preferentially

or selectively bind to specific Fc receptors. Thus, the new compositions and methods will enable manufacture of antibody therapeutics that may be produced in bacteria while retaining their ability to interact with FcR polypeptides and thereby recruit immune affecter cells. Furthermore, Fc receptors may be selected for a particular FcR binding affinity thereby allowing therapeutics to be tailored for recruitment or targeting of specific cell types. Finally, the instant invention provided new media and methods that may be used to enhance prokaryotic interaction screening techniques. Further embodiments and advantages of the invention are described below.

I. Periplasmic Expression

[0083] In some aspects of the invention a polypeptide comprising an antibody Fc domain is expressed in the periplasmic space of a gram negative bacteria. Furthermore, in some aspects an antibody Fc domain may be anchored to the periplasmic face of the inner membrane. For example, an Fc domain may be directly fused to a membrane spanning or membrane bound polypeptide or may interact (*e.g.*, via protein-protein interactions) with a membrane spanning or membrane bound polypeptide. Such a technique may be termed “Anchored Periplasmic Expression” or “APEX”.

[0084] The periplasmic compartment is contained between the inner and outer membranes of Gram negative cells (see, *e.g.*, Oliver, 1996). As a sub-cellular compartment, it is subject to variations in size, shape and content that accompany the growth and division of the cell. Within a framework of peptidoglycan heteropolymer is a dense milieu of periplasmic proteins and little water, lending a gel-like consistency to the compartment (Hobot *et al.*, 1984; van Wielink and Duine, 1990). The peptidoglycan is polymerized to different extents depending on the proximity to the outer membrane, close-up it forms the murein sacculus that affords cell shape and resistance to osmotic lysis.

[0085] The outer membrane (see Nikaido, 1996) is composed of phospholipids, porin proteins and, extending into the medium, lipopolysaccharide (LPS). The molecular basis of outer membrane integrity resides with LPS ability to bind divalent cations (Mg^{2+} and Ca^{2+}) and link each other electrostatically to form a highly ordered quasi-crystalline ordered “tiled roof” on the surface (Labischinski *et al.*, 1985). The membrane forms a very strict permeability barrier allowing passage of molecules no greater than around 650 Da (Burman *et al.*, 1972; Decad and Nikaido, 1976) via the porins. The large water filled porin channels are primarily responsible for allowing free passage of mono and disaccharides, ions and amino acids in to the periplasm compartment (Nikaido and Nakae, 1979; Nikaido and Vaara, 1985). With such strict physiological regulation of access by molecules to the periplasm it may appear, at first glance, inconceivable that large ligands (*i.e.*, larger than the 650 Da exclusion limit) could be employed in screening methods. However, the inventors have shown that ligands greater than 2000 Da in size can diffuse into the periplasm

without disruption of the periplasmic membrane. Such diffusion can be aided by one or more treatments of a bacterial cell, thereby rendering the outer membrane more permeable, as is described herein below.

II. Permeabilization of the Outer Membrane

[0086] In one embodiment of the invention, methods are employed for increasing the permeability of the outer membrane to one or more labeled ligand. This can allow screening access of labeled ligands otherwise unable to cross the outer membrane. However, certain classes of molecules, for example, hydrophobic antibiotics larger than the 650 Da exclusion limit, can diffuse through the bacterial outer membrane itself, independent of membrane porins (Farmer *et al.*, 1999). The process may actually permeabilize the membrane on so doing (Jouenne and Junter, 1990). Such a mechanism has been adopted to selectively label the periplasmic loops of a cytoplasmic membrane protein *in vivo* with a polymyxin B nonapeptide (Wada *et al.*, 1999). Also, certain long chain phosphate polymers (100 Pi) appear to bypass the normal molecular sieving activity of the outer membrane altogether (Rao and Torriani, 1988).

[0087] Conditions have been identified that lead to the permeation of ligands into the periplasm without loss of viability or release of the expressed proteins from the cells, but the invention may be carried out without maintenance of the outer membrane. As demonstrated herein Fc domains expressed or anchored candidate binding polypeptides in the periplasmic space the need for maintenance of the outer membrane (as a barrier to prevent the leakage of the binding protein from the cell) to detect bound labeled ligand is removed. As a result, cells expressing binding proteins anchored to the outer (periplasmic) face of the cytoplasmic membrane can be fluorescently labeled simply by incubating with a solution of fluorescently labeled ligand in cells that either have a partially permeabilized membrane or a nearly completely removed outer membrane.

[0088] The permeability of the outer membrane of different strains of bacterial hosts can vary widely. It has been shown previously that increased permeability due to OmpF overexpression was caused by the absence of a histone like protein resulting in a decrease in the amount of a negative regulatory mRNA for OmpF translation (Painbeni *et al.*, 1997). Also, DNA replication and chromosomal

segregation is known to rely on intimate contact of the replisome with the inner membrane, which itself contacts the outer membrane at numerous points. A preferred host for library screening applications is *E. coli* ABLEC strain, which additionally has mutations that reduce plasmid copy number.

[0089] Treatments such as hyperosmotic shock can improve labeling significantly. It is known that many agents including, calcium ions (Bukau *et al.*, 1985) and even Tris buffer (Irvin *et al.*, 1981) alter the permeability of the outer-membrane. Further, phage infection stimulates the labeling process. Both the filamentous phage inner membrane protein pIII and the large multimeric outer membrane protein pIV can alter membrane permeability (Boeke *et al.*, 1982) with mutants in pIV known to improve access to maltodextrins normally excluded (Marciano *et al.*, 1999). Using the techniques of the invention, comprising a judicious combination of strain, salt and phage, a high degree of permeability may be achieved (Daugherty *et al.*, 1999). Cells comprising anchored or periplasm-associated polypeptides bound to fluorescently labeled ligands can then be easily isolated from cells that express binding proteins without affinity for the labeled ligand using flow cytometry or other related techniques. However, in some cases, it will be desired to use less disruptive techniques in order to maintain the viability of cells. EDTA and Lysozyme treatments may also be useful in this regard.

III. Antibody-binding polypeptides

[0090] In certain aspects the invention concerns methods for identifying antibody Fc domains with a specific affinity for antibody-binding polypeptide such as an Fc receptor. A variety of Fc receptors are well known in the art and some examples of receptors are listed below in Table 1.

Table 1: Selected FcR Polypeptides

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
Fc-gamma RII-a (CD32)	FCGR2A	Low affinity immunoglobulin gamma Fc region receptor II-a precursor	<i>Homo sapiens</i> (Human)	317	(Stuart <i>et al.</i> , 1987)
Fc-gamma RII-a	FCGR2A	Low affinity immunoglobulin gamma Fc region receptor II-a precursor	<i>Pan troglodytes</i> (Chimpanzee)	316	
Fc-gamma RII-b	FCGR2B	Low affinity immunoglobulin gamma Fc region receptor II-b precursor	<i>Homo sapiens</i> (Human)	310	(Stuart <i>et al.</i> , 1989)
Fc-gamma RII-c	FCGR2C	Low affinity immunoglobulin gamma Fc region receptor II-c precursor	<i>Homo sapiens</i> (Human)	323	(Stuart <i>et al.</i> , 1989)
Fc-gamma RIIIa	FCGR3A	Low affinity immunoglobulin gamma Fc region receptor III-A precursor	<i>Homo sapiens</i> (Human)	254	(Ravetch and Perussia, 1989)
Fc-gamma RIIIb	FCGR3B	Low affinity immunoglobulin gamma Fc region receptor III-B precursor	<i>Homo sapiens</i> (Human)	233	(Ravetch and Perussia, 1989)
Fc-gamma RI (CD64)	FCGR1A	High affinity immunoglobulin gamma Fc receptor I precursor	<i>Homo sapiens</i> (Human)	374	(Allen and Seed, 1988)
Fc-gamma RI	Fcgr1	High affinity immunoglobulin gamma Fc receptor I precursor	<i>Mus musculus</i> (Mouse)	404	(Sears <i>et al.</i> , 1990)
Fc-gamma RII	FCGR2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Bos taurus</i> (Bovine)	296	(Zhang <i>et al.</i> , 1994)

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
Fc-gamma RII	FCGR2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Cavia porcellus</i> (Guinea pig)	341	(Tominaga <i>et al.</i> , 1990)
Fc-gamma RII	Fcgr2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Mus musculus</i> (Mouse)	330	(Ravetch <i>et al.</i> , 1986)
Fc-gamma RII	Fcgr2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Rattus norvegicus</i> (Rat)	285	(Bocek and Pecht, 1993)
Fc-gamma RIII	FCGR3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Bos taurus</i> (Bovine)	250	(Collins <i>et al.</i> , 1997)
Fc-gamma RIII	FCGR3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Macaca fascicularis</i> (Crab eating macaque) (<i>Cynomolgus</i> monkey)	254	
Fc-gamma RIII	Fcgr3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Mus musculus</i> (Mouse)	261	(Ravetch <i>et al.</i> , 1986)
Fc-gamma RIII	FCGR3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Sus scrofa</i> (Pig)	257	(Halloran <i>et al.</i> , 1994)
Fc-gamma RIII	Fcgr3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Rattus norvegicus</i> (Rat)	267	(Zeger <i>et al.</i> , 1990)
FcRn	FCGRT	IgG receptor transporter FcRn large subunit p51 precursor	<i>Homo sapiens</i> (Human)	365	

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
FcRn	FCGRT	IgG receptor transporter FcRn large subunit p51 precursor	<i>Macaca fascicularis</i> (Crab eating macaque) (Cynomolgus monkey)	365	
FcRn	Fcgrt	IgG receptor transporter FcRn large subunit p51 precursor	<i>Mus musculus</i> (Mouse)	365	(Ahouse <i>et al.</i> , 1993)
FcRn	Fcgrt	IgG receptor transporter FcRn large subunit p51 precursor	<i>Rattus norvegicus</i> (Rat)	366	(Simister and Mostov, 1989)
MRP protein	mrp4	Fibrinogen- and Ig-binding protein precursor	<i>Streptococcus pyogenes</i>	388	(Stenberg <i>et al.</i> , 1992)
Protein B		cAMP factor	<i>Streptococcus agalactiae</i>	226	(Ruhlmann <i>et al.</i> , 1988)
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i> (strain NCTC 8325)	516	(Uhlen <i>et al.</i> , 1984)
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i>	508	(Shuttleworth <i>et al.</i> , 1987)
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i> (strain Mu50 / ATCC 700699)	450	(Kuroda <i>et al.</i> , 2001)
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i> (strain N315)	450	(Kuroda <i>et al.</i> , 2001)
protein G	spg	Immunoglobulin G-binding protein G precursor	<i>Streptococcus</i> sp. group G	448	(Fahnestock <i>et al.</i> , 1986)
protein G	spg	Immunoglobulin G-binding protein G precursor	<i>Streptococcus</i> sp. group G	593	(Olsson <i>et al.</i> , 1987)

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
protein H		Immunoglobulin G-binding protein H precursor	<i>Streptococcus pyogenes</i> serotype M1	376	(Gomi <i>et al.</i> , 1990)
Protein sbi	sbi	Immunoglobulin G-binding protein sbi precursor	<i>Staphylococcus aureus</i> (strain NCTC 8325-4)	436	(Zhang <i>et al.</i> , 1998)
Allergen Asp fl 1		Allergen Asp fl 1 causes an allergic reaction in human. Binds to IgE and IgG	<i>Aspergillus flavus</i>	32	
Allergen Asp fl 2		Allergen Asp fl 2 causes an allergic reaction in human. Binds to IgE and IgG	<i>Aspergillus flavus</i>	20	
Allergen Asp fl 3		Allergen Asp fl 3 causes an allergic reaction in human. Binds to IgE and IgG	<i>Aspergillus flavus</i>	32	
Fc-epsilon RI		IgE receptor displayed on Mast cells, Eosinophils and Basophils	<i>Homo sapiens</i> (Human)		
Fc-alpha RI (CD86)		IgA (IgA1, IgA2) receptor displayed on Macrophages	<i>Homo sapiens</i> (Human)		
C1q	C1QA NP_057075.1, C1QB NP_000482.3, C1QC NP_758957.1	C1q is multimeric complex that binds to antibody Fc composed of 6 A chains, 6 B chains and 6 C chains	<i>Homo sapiens</i> (Human)		

IV. Antibody Fc Libraries

[0091] Examples of techniques that could be employed in conjunction with the invention for creation of diverse antibody Fc domains and/or antibodies

comprising such domains may employ techniques similar to those for expression of immunoglobulin heavy chain libraries described in U.S. Patent 5,824,520.

V. Screening Antibody Fc Domains

[0092] The present invention provides methods for identifying molecules capable of binding to a particular FcR. The binding polypeptides screened may comprise a large library of diverse candidate Fc domains, or, alternatively, may comprise particular classes of Fc domains (*e.g.*, engineered point mutations or amino acid insertions) selected with an eye towards structural attributes that are believed to make them more likely to bind the target ligand. In one embodiment of the invention, the candidate binding protein is an intact antibody, or a fragment or portion thereof comprising an Fc domain.

[0093] To identify a candidate Fc domain capable of binding a target ligand in accordance with the invention, one may carry out the steps of: providing a population of Gram negative bacterial cells that express a distinct antibody Fc domain; admixing the bacteria or phages and at least a first labeled or immobilized target ligand (FcR polypeptide) capable of contacting the antibody and identifying at least a first bacterium expressing a molecule capable of binding the target ligand.

[0094] In some aspects of the aforementioned method, the binding between antibody Fc domain and a labeled FcR polypeptide will prevent diffusing out of a bacterial cell. In this way, molecules of the labeled ligand can be retained in the periplasm of the bacterium comprising a permeabilized outer membrane. Alternatively, the periplasm can be removed, whereby the Fc domain will cause retention of the bound candidate molecule since Fc domains are shown to associate with the inner membrane. The labeling may then be used to isolate the cell expressing a binding polypeptide capable of binding the FcR polypeptide, and in this way, the gene encoding the Fc domain polypeptide isolated. The molecule capable of binding the target ligand may then be produced in large quantities using *in vivo* or *ex vivo* expression methods, and then used for any desired application, for example, for diagnostic or therapeutic applications, as described below. Furthermore, it will be understood that isolated antibody Fc domains identified may be used to construct an antibody fragment or full-length antibody comprising an antigen binding domain.

A. Cloning of Fc domain Coding Sequences

[0095] The binding affinity of an antibody Fc or other binding protein can, for example, be determined by the Scatchard analysis of Munson & Pollard (1980). Alternatively, binding affinity can be determined by surface plasmon resonance or any other well known method for determining the kinetics and equilibrium constants for protein:protein interactions. After a bacterial cell is identified that produces molecules of the desired specificity, affinity, and/or activity, the corresponding coding sequence may be cloned. In this manner, DNA encoding the molecule can be isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the antibody or binding protein).

[0096] Once isolated, the antibody Fc domain DNA may be placed into expression vectors, which can then transfected into host cells such as bacteria. The DNA also may be modified, for example, by the addition of sequence for human heavy and light chain variable domains, or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" binding proteins are prepared to have the desired binding specificity. For instance, an identified antibody Fc domain may be fused to a therapeutic polypeptide or a toxin and used to target cells (*in vitro* or *in vivo*) that express a particular FcR.

[0097] Chimeric or hybrid Fc domains also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, targeted-toxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

[0098] It will be understood by those of skill in the art that nucleic acids may be cloned from viable or inviable cells. In the case of inviable cells, for example, it may be desired to use amplification of the cloned DNA, for example, using PCR. This may also be carried out using viable cells either with or without further growth of cells.

B. Labeled Ligands

[0099] In one embodiment of the invention, an Fc domain is isolated which has affinity for a labeled FcR polypeptide. By permeabilization and/or removal of the periplasmic membrane of a Gram negative bacterium in accordance with the invention, labeled ligands of potentially any size may be screened. In the absence of removal of the periplasmic membrane, it will typically be preferable that the labeled ligand is less than 50,000 Da in size in order to allow efficient diffusion of the ligand across the bacterial periplasmic membrane.

[00100] As indicated above, it will typically be desired in accordance with the invention to provide an FcR polypeptide which has been labeled with one or more detectable agent(s). This can be carried out, for example, by linking the ligand to at least one detectable agent to form a conjugate. For example, it is conventional to link or covalently bind or complex at least one detectable molecule or moiety. A "label" or "detectable label" is a compound and/or element that can be detected due to specific functional properties, and/or chemical characteristics, the use of which allows the ligand to which it is attached to be detected, and/or further quantified if desired. Examples of labels which could be used with the invention include, but are not limited to, enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, luminescent molecules, photoaffinity molecules, colored particles or ligands, such as biotin.

[00101] In one embodiment of the invention, a visually-detectable marker is used such that automated screening of cells for the label can be carried out. In particular, fluorescent labels are beneficial in that they allow use of flow cytometry for isolation of cells expressing a desired binding protein or antibody. Examples of agents that may be detected by visualization with an appropriate instrument are known in the art, as are methods for their attachment to a desired ligand (see, *e.g.*, U.S. Patents 5,021,236; 4,938,948; and 4,472,509). Such agents can include paramagnetic ions; radioactive isotopes; fluorochromes; NMR-detectable substances and substances for X-ray imaging. Types of fluorescent labels that may be used with the invention will be well known to those of skill in the art and include, for example, Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-

TRX, Cascade Blue, Cy3, Cy5,6-FAM, Fluorescein Isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, TAMRA, TET, Tetramethylrhodamine, and/or Texas Red.

[00102] Magnetic screening techniques are well known to those of skill in the art (see, for example, U.S. Pat. No. 4,988,618, U.S. Pat. No. 5,567,326 and U.S. Pat. No. 5,779,907). Examples of paramagnetic ions that could be used as labels in accordance with such techniques include ions such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and/or erbium (III). Ions useful in other contexts include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III).

[00103] Another type of FcR conjugate contemplated in the present invention are those where the ligand is linked to a secondary binding molecule and/or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of such enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase or glucose oxidase. In such instances, it will be desired that cells selected remain viable. Preferred secondary binding ligands are biotin and/or avidin and streptavidin compounds. The use of such labels is well known to those of skill in the art and are described, for example, in U.S. Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241.

[00104] Molecules containing azido groups also may be used to form covalent bonds to proteins through reactive nitrene intermediates that are generated by low intensity ultraviolet light (Potter & Haley, 1983). In particular, 2- and 8-azido analogues of purine nucleotides have been used as site-directed photoprobes to identify nucleotide-binding proteins in crude cell extracts (Owens & Haley, 1987; Atherton *et al.*, 1985). The 2- and 8-azido nucleotides have also been used to map nucleotide-binding domains of purified proteins (Khatoon *et al.*, 1989; King *et al.*, 1989; and Dholakia *et al.*, 1989) and may be used as ligand binding agents.

[00105] Labeling can be carried out by any of the techniques well known to those of skill in the art. For instance, FcR polypeptides can be labeled by contacting the ligand with the desired label and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Similarly, a ligand exchange process could be used. Alternatively, direct labeling techniques may be used, *e.g.*, by incubating the label, a reducing agent such as SnCl_2 , a buffer solution such as sodium-potassium phthalate solution, and the ligand. Intermediary functional groups on the ligand could also be used, for example, to bind labels to a ligand in the presence of diethylenetriaminepentaacetic acid (DTPA) or ethylene diaminetetracetic acid (EDTA).

[00106] Other methods are also known in the art for the attachment or conjugation of a ligand to its conjugate moiety. Some attachment methods involve the use of an organic chelating agent such as diethylenetriaminepentaacetic acid anhydride (DTPA); ethylenetriaminetetraacetic acid; N-chloro-p-toluenesulfonamide; and/or tetrachloro-3 α -6 α -diphenylglycouril-3 attached to the ligand (U.S. Patents 4,472,509 and 4,938,948). FcR polypeptides also may be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers can be prepared in the presence of these coupling agents or by reaction with an isothiocyanate. In U.S. Patent 4,938,948, imaging of breast tumors is achieved using monoclonal antibodies and the detectable imaging moieties are bound to the antibody using linkers such as methyl-p-hydroxybenzimidate or N-succinimidyl-3-(4-hydroxyphenyl)propionate. In still further aspects an FcR polypeptide may be fused to a reporter protein such as an enzyme as described *supra* or a fluorescence protein.

[00107] The ability to specifically label periplasmic expressed proteins with appropriate fluorescent ligands also has applications other than library screening. Specifically labeling with fluorescent ligands and flow cytometry can be used for monitoring production of Fc domains during protein manufacturing.

[00108] Once an Fc domain has been isolated in accordance with the invention, it may be desired to link the molecule to at least one agent to form a conjugate to enhance the utility of that molecule. For example, in order to increase the efficacy of Fc domains or antibody molecules as diagnostic or therapeutic agents, it is

conventional to link or covalently bind or complex at least one desired molecule or moiety. Such a molecule or moiety may be, but is not limited to, at least one effector or reporter molecule. Effector molecules comprise molecules having a desired activity, *e.g.*, cytotoxic activity. Non-limiting examples of effector molecules which have been attached to antibodies include toxins, anti-tumor agents, therapeutic enzymes, radio-labeled nucleotides, antiviral agents, chelating agents, cytokines, growth factors, and oligo- or poly-nucleotides. By contrast, a reporter molecule is defined as any moiety which may be detected using an assay. Techniques for labeling such a molecule are known to those of skill in the art and have been described herein above.

[00109] Labeled binding proteins such as Fc domains which have been prepared in accordance with the invention may also then be employed, for example, in immunodetection methods for binding, purifying, removing, quantifying and/or otherwise generally detecting biological components such as protein(s), polypeptide(s) or peptide(s). Some immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay, and Western blot to mention a few. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle and Ben-Zeev, 1999; Gulbis and Galand, 1993; and De Jager *et al.*, 1993. Such techniques include binding assays such as the various types of enzyme linked immunosorbent assays (ELISAs) and/or radioimmunoassays (RIA) known in the art.

[00110] The Fc domain molecules, including antibodies, prepared in accordance with the present invention may also, for example, in conjunction with both fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and/or is well known to those of skill in the art (Abbondanzo *et al.*, 1990).

VI. Automated Screening with Flow Cytometry

[00111] In one embodiment of the invention, fluorescence activated cell sorting (FACS) screening or other automated flow cytometric techniques may be used for the efficient isolation of a bacterial cell comprising a labeled ligand bound to an Fc domain. Instruments for carrying out flow cytometry are known to those of skill in the art and are commercially available to the public. Examples of such instruments include FACS Star PlusTM, FACScanTM and FACSsortTM instruments from Becton Dickinson (Foster City, Calif.) Epics C from Coulter Epics Division (Hialeah, Fla.) and MOFLOTM from Cytomation (Colorado Springs, Co).

[00112] Flow cytometric techniques in general involve the separation of cells or other particles in a liquid sample. Typically, the purpose of flow cytometry is to analyze the separated particles for one or more characteristics thereof, for example, presence of a labeled ligand or other molecule. The basic steps of flow cytometry involve the direction of a fluid sample through an apparatus such that a liquid stream passes through a sensing region. The particles should pass one at a time by the sensor and are categorized base on size, refraction, light scattering, opacity, roughness, shape, fluorescence, *etc.*

[00113] Rapid quantitative analysis of cells proves useful in biomedical research and medicine. Apparati permit quantitative multiparameter analysis of cellular properties at rates of several thousand cells per second. These instruments provide the ability to differentiate among cell types. Data are often displayed in one-dimensional (histogram) or two-dimensional (contour plot, scatter plot) frequency distributions of measured variables. The partitioning of multiparameter data files involves consecutive use of the interactive one- or two-dimensional graphics programs.

[00114] Quantitative analysis of multiparameter flow cytometric data for rapid cell detection consists of two stages: cell class characterization and sample processing. In general, the process of cell class characterization partitions the cell feature into cells of interest and not of interest. Then, in sample processing, each cell is classified in one of the two categories according to the region in which it falls. Analysis of the class of cells is very important, as high detection performance may be expected only if an appropriate characteristic of the cells is obtained.

[00115] Not only is cell analysis performed by flow cytometry, but so too is sorting of cells. In U.S. Patent 3,826,364, an apparatus is disclosed which physically separates particles, such as functionally different cell types. In this machine, a laser provides illumination which is focused on the stream of particles by a suitable lens or lens system so that there is highly localized scatter from the particles therein. In addition, high intensity source illumination is directed onto the stream of particles for the excitation of fluorescent particles in the stream. Certain particles in the stream may be selectively charged and then separated by deflecting them into designated receptacles. A classic form of this separation is *via* fluorescent-tagged antibodies, which are used to mark one or more cell types for separation.

[00116] Other examples of methods for flow cytometry that could include, but are not limited to, those described in U.S. Patent Nos. 4,284,412; 4,989,977; 4,498,766; 5,478,722; 4,857,451; 4,774,189; 4,767,206; 4,714,682; 5,160,974; and 4,661,913.

[00117] For the present invention, an important aspect of flow cytometry is that multiple rounds of screening can be carried out sequentially. Cells may be isolated from an initial round of sorting and immediately reintroduced into the flow cytometer and screened again to improve the stringency of the screen. Another advantage known to those of skill in the art is that nonviable cells can be recovered using flow cytometry. Since flow cytometry is essentially a particle sorting technology, the ability of a cell to grow or propagate is not necessary. Techniques for the recovery of nucleic acids from such non-viable cells are well known in the art and may include, for example, use of template-dependent amplification techniques including PCR.

VII. Nucleic Acid-Based Expression Systems

[00118] Nucleic acid-based expression systems may find use, in certain embodiments of the invention, for the expression of recombinant proteins. For example, one embodiment of the invention involves transformation of Gram negative bacteria with the coding sequences for an antibody Fc domain, or preferably a plurality of distinct Fc domains.

A. Methods of Nucleic Acid Delivery

[00119] Certain aspects of the invention may comprise delivery of nucleic acids to target cells (*e.g.*, gram negative bacteria). For example, bacterial host cells may be transformed with nucleic acids encoding candidate Fc domains potentially capable binding an FcR. In particular embodiments of the invention, it may be desired to target the expression to the periplasm of the bacteria. Transformation of eukaryotic host cells may similarly find use in the expression of various candidate molecules identified as capable of binding a target ligand.

[00120] Suitable methods for nucleic acid delivery for transformation of a cell are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA) can be introduced into such a cell, or even an organelle thereof. Such methods include, but are not limited to, direct delivery of DNA such as by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859), including microinjection (Harland and Weintraub, 1985; U.S. Patent 5,789,215); by electroporation (U.S. Patent 5,384,253); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patents 5,610,042; 5,322,783, 5,563,055, 5,550,318, 5,538,877 and 5,538,880); or by agitation with silicon carbide fibers (Kaeppeler *et al.*, 1990; U.S. Patents 5,302,523 and 5,464,765); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985). Through the application of techniques such as these, cells may be stably or transiently transformed.

1. Electroporation

[00121] In certain embodiments of the present invention, a nucleic acid is introduced into a cell *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants

of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent 5,384,253). Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding.

2. Calcium Phosphate

[00122] In other embodiments of the present invention, a nucleic acid is introduced to the cells using calcium phosphate precipitation.

B. Vectors

[00123] Vectors may find use with the current invention, for example, in the transformation of a Gram negative bacterium with a nucleic acid sequence encoding a candidate Fc domain which one wishes to screen for ability to bind a target FcR. In one embodiment of the invention, an entire heterogeneous “library” of nucleic acid sequences encoding target polypeptides may be introduced into a population of bacteria, thereby allowing screening of the entire library. The term “vector” is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be “exogenous,” or “heterologous”, which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids and viruses (*e.g.*, bacteriophage). One of skill in the art may construct a vector through standard recombinant techniques, which are described in Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, both of which references are incorporated herein by reference.

[00124] The term “expression vector” refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of “control sequences,” which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation,

vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

1. Promoters and Enhancers

[00125] A “promoter” is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases “operatively positioned,” “operatively linked,” “under control,” and “under transcriptional control” mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an “enhancer,” which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[00126] A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as “endogenous.” Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic cell, and promoters or enhancers not “naturally occurring,” *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906).

[00127] Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type chosen for expression. One example of such promoter that may be used with the invention is the *E. coli* arabinose or T7 promoter. Those of skill in the art of molecular biology generally are familiar with the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

2. Initiation Signals and Internal Ribosome Binding Sites

[00128] A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be “in-frame” with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

3. Multiple Cloning Sites

[00129] Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997). “Restriction enzyme digestion” refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to

enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

4. Termination Signals

[00130] The vectors or constructs prepared in accordance with the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments, a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

[00131] Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, rhp dependent or rho independent terminators. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

5. Origins of Replication

[00132] In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated.

6. Selectable and Screenable Markers

[00133] In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its

presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

[00134] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

C. Host Cells

[00135] In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic cell, and it includes any transformable organism that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

[00136] In particular embodiments of the invention, a host cell is a Gram negative bacterial cell. These bacteria are suited for use with the invention in that they possess a periplasmic space between the inner and outer membrane and, particularly, the aforementioned inner membrane between the periplasm and cytoplasm, which is also known as the cytoplasmic membrane. As such, any other cell with such a periplasmic space could be used in accordance with the invention. Examples of Gram negative bacteria that may find use with the invention may include, but are not limited to, *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Salmonella typhimurium*, *Shigella flexneri*, *Haemophilus influenza*, *Bordetella*

pertussi, *Erwinia amylovora*, *Rhizobium sp.* The Gram negative bacterial cell may be still further defined as bacterial cell which has been transformed with the coding sequence of a fusion polypeptide comprising a candidate binding polypeptide capable of binding a selected ligand. The polypeptide is anchored to the outer face of the cytoplasmic membrane, facing the periplasmic space, and may comprise an antibody coding sequence or another sequence. One means for expression of the polypeptide is by attaching a leader sequence to the polypeptide capable of causing such directing.

[00137] Numerous prokaryotic cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials (www.atcc.org). An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote host cell for replication of many vectors. Bacterial cells used as host cells for vector replication and/or expression include DH5 α , JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE[®] Competent Cells and SOLOPACK[™] Gold Cells (STRATAGENE[®], La Jolla). Alternatively, bacterial cells such as *E. coli* LE392 could be used as host cells for bacteriophage.

[00138] Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with a prokaryotic host cell, particularly one that is permissive for replication or expression of the vector. Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

D. Expression Systems

[00139] Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Such systems could be used, for example, for the production of a polypeptide product identified in accordance with the

invention as capable of binding a particular ligand. Prokaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available. Other examples of expression systems comprise of vectors containing a strong prokaryotic promoter such as T7, Tac, Trc, BAD, lambda pL, Tetracycline or Lac promoters, the pET Expression System and an *E. coli* expression system.

E. Candidate Binding Proteins and Antibodies

[00140] In certain aspects of the invention, antibody Fc domains are expressed on the cytoplasmic or in the periplasmic space membrane of a host bacterial cell. By expression of a heterogeneous population of such Fc domains, those polypeptides having a high affinity for a target ligand (FcR) may be identified. The identified Fc domains may then be used in various diagnostic or therapeutic applications, as described herein.

[00141] As used herein, the term "Fc domain" is intended to refer broadly to any immunoglobulin Fc region such as an IgG, IgM, IgA, IgD or IgE Fc. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

[00142] Once an antibody having affinity for a target ligand is identified, the Fc domain may be purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography. Alternatively, Fc domains encompassed by the present invention can be synthesized using an automated peptide synthesizer.

VIII. Manipulation and Detection of Nucleic Acids

[00143] In certain embodiments of the invention, it may be desired to employ one or more techniques for the manipulation, isolation and/or detection of nucleic acids. Such techniques may include, for example, the preparation of vectors for transformation of host cells as well as methods for cloning selected nucleic acid

segments from a transgenic cell. Methodology for carrying out such manipulations will be well known to those of skill in the art in light of the instant disclosure.

[00144] Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 1989). In certain embodiments, analysis may be performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

[00145] The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

[00146] Pairs of primers designed to selectively hybridize to nucleic acids corresponding to a selected nucleic acid sequence are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids comprising one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

[00147] The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent

label or even via a system using electrical and/or thermal impulse signals (AffymaxTM technology; Bellus, 1994).

[00148] A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCRTM) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1988.

[00149] A reverse transcriptase PCRTM amplification procedure may be performed to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known (see Sambrook *et al.*, 1989). Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art. Representative methods of RT-PCR are described in U.S. Patent 5,882,864.

[00150] Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application 320 308. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence. A method based on PCRTM and oligonucleotide ligase assay (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

[00151] Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patents 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Publication No. WO 89/09284.

[00152] Qbeta Replicase, described in PCT Publication No. WO 87/06270, may also be used as an amplification method in the present

complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which may then be detected.

[00153] An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement Amplification (SDA), disclosed in U.S. Patent 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

[00154] Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

[00155] PCT Application WO 89/06700 (incorporated herein by reference in its entirety) discloses a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

IX. Examples

[00156] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus

discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. The scope of the claims should not be limited by the preferred embodiments and examples, but should be given the broadest interpretation consistent with the description as a whole.

Example 1

Reagents for studies

[00157] Oligonucleotides primers (Table 2) and restriction endonucleases used to construct plasmids to display homodimeric protein IgG-Fc were obtained from Integrated DNA Technologies (Coralville, IA) and New England Biolabs (Ipswich, MA), respectively. TaqTM Polymerase and FITC protein labeling kit were from Invitrogen (Carlsbad, CA). Recombinant human FcγRI/CD64 was purchased from R&D Systems (Minneapolis, MN). Trehalose was obtained from Fisher Scientific (Fair Lawn, NJ). Human IgG-Fc and Rabbit anti-ECS antibody peroxidase conjugated were from Bethyl Laboratories (Montgomery, TX). Digoxigenin-BODIPY (Digoxigenin-4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl ethylenediamine) was synthesized as described previously (Harvey *et al.*, 2004). PA-FITC was obtained from List Biological Laboratories (Campbell, CA). Protein A-FITC and analytical grades of all other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Table 2: Oligonucleotide sequences (Underlining indicates the restriction enzyme sites)

Primer Name	Primer nucleotide sequence (5' → 3')
STJ#16	TTGTGAGCGGATAACAATTTC (SEQ ID NO:8)
STJ#58	CGAACTGGCCAGCCGGCCATCGCCGGCTAGAGGAAAAAG (SEQ ID NO:9)
STJ#59	CGAACTGGCCCCCGAGGCCCGGTTCATGACTTCTGTTTAAG (SEQ ID NO:10)
STJ#68	GATATCGCGGCCGCACTGACCCGACACCCCTGCAGG (SEQ ID NO:11)
STJ#69	TTTAGGGTCTGACTGCGGCGTGTGCCGCCAGGATGAAC (SEQ ID NO:12)
STJ#74	CGCAGCGAGGCCAGCCGGCCATGGCGCAAGCTGCTCCCCCAAAGGC (SEQ ID NO:13)
STJ#78	CGCAGCGAGGCCAGCCGGCCATGGCGATCCAGCGTACTCCAAAGATTTC (SEQ ID NO:14)
STJ#80	CGCAATTTCGGCCCCCGAGGCCCAATGACCCCCCATTTGGTGAAGAG (SEQ ID NO:15)
STJ#84	CGCAATTTCGGCCCCCGAGGCCCATGTCTCGATCCCACCTAAC (SEQ ID NO:16)
STJ#86	CAGCGTACTCCAAAGATTCAAGTTTACTCACGTCCAGCAGAGAATGGAAAG (SEQ ID NO:17)
STJ#87	CAGCAGAGAAATGGAAGTCAAAATTCCTGAATTGCTATGTGCTGGTTTCATC (SEQ ID NO:18)
STJ#88	CTATGTGCTGGTTTCATCCATCCGACATTGAAGTTGACTTACTGAAGAATGG (SEQ ID NO:19)
STJ#89	GTTGACTTACTGAAGAAATGGAGAGAGAAATTGAAAGAGTGAGCATTCAGACTTG (SEQ ID NO:20)
STJ#90	GTACAAAGAGATAGAAAGACCAGTCCTTGCTGAAAGACAAGTCTGAATGCTCCAC (SEQ ID NO:21)
STJ#91	ACTCATCTTTTTCAGTGGGGTGAATTCAGTGTAGTACAAGAGATAGAAAGACC (SEQ ID NO:22)
STJ#92	CTGTGACAAAGTCACATGGTTCACACGGCAGGCATACATCTTTTTCAGTGGG (SEQ ID NO:23)
STJ#93	CATGTCTCGATCCCACCTTAACTATCTTGGGCTGTGACAAAGTCACATGG (SEQ ID NO:24)
STJ#94	CGAACTGGCCAGCCGGCCATGGCGTGCGGCGCATCGCCGGCTAGAGGAAAA (SEQ ID NO:25)
STJ#95	CGAACTGGCCCGGAGGCCCGGCGAGCCGCGTGGTTCATGACTTCTGTTTAAG (SEQ ID NO:26)
STJ#96	GATATCGCGGCCGCACTGCGGGCGGCTGACCCGACACCCTGCAGG (SEQ ID NO:27)
STJ#97	TTTTAGGGGTCTGACTGCGGGCGGCGCAGCGCCGTGTGCCGCCAGGATGAAC (SEQ ID NO:28)
STJ#114	GACGAACCTGGCCAGCCGGCCATGGCGGAGAGTAAACGGAATAAGCCAGGGAAG (SEQ ID NO:29)
STJ#115	GCGAACTGGCCCCCGAGGCCCCCTTACCCCGATGAATATCAATATGTCGCTTAG (SEQ ID NO:30)
STJ#116	CGAGATATCGCGGCCGCAATGGAACTGAACATAGTATTAGTGATTATACCGAG (SEQ ID NO:31)
STJ#117	GTTTTAGGGGTCGACTGCGGGCCCTGTTTAAATCCTGACTTACCGTTAGC (SEQ ID NO:32)
STJ#118	CTTACCCCGATGAATATCAATCGCTCGCTTAGGTGTGGTCACTCTGATATTATT (SEQ ID NO:33)

STJ#119	GCGAACTGGCCCCCGAGGCCCCCTTACCCCGATGAATATCAATCGCTCGCTTAG (SEQ ID NO:34)
STJ#120	CTTACCCCGCGCAATATCAATATGTCGCTTAGGTGGTCACTC (SEQ ID NO:35)
STJ#121	GCGAACTGGCCCCCGAGGCCCCCTTACCCCGCAATATCAATATGTCGCTTAG (SEQ ID NO:36)
STJ#122	CTTACCCCGCGCAATATCAATCGCTCGCTTAGGTGGTCACTCTGATATTATT (SEQ ID NO:37)
STJ#123	GCGAACTGGCCCCCGAGGCCCCCTTACCCCGCAATATCAATCGCTCGCTTAG (SEQ ID NO:38)
STJ#136	TTTAGGGGTCGACCAAGCTGCTCCCCCAAAGGCTG (SEQ ID NO:39)
STJ#139	TTTAAGGGAAGCTTCTATCAATGGTGGTGGTGGTGATG (SEQ ID NO:40)
STJ#144	TTTAGGGGTCGACGACAAAACTCACACATGCCACCGTG(SEQ ID NO:41)
STJ#145	TTTAAGGGAAGCTTCTATTAGGCGGCCCTTTGTCAATCG (SEQ ID NO:42)
STJ#194	CTAGGGAGCCGCGGAGGAGCAGTACAACNNNSNNSNNSNNSNNSNNSNNSNNSNNSAGCACGTA CCGTGTGTCAGCG (SEQ ID NO:43)
STJ#195	CTAGAGGAAATTCGGCCCCCGAGGCCCTTTAC (SEQ ID NO:44)
STJ#196	CGCAGCGAGGCCCGAGCCGGCCATGGCG (SEQ ID NO: 45)
STJ#197	CGCAATTCGAATTCGGCCCCCGAGGGCCCC (SEQ ID NO:46)
STJ#220	CAATTTTGTCAAGCCGCTGAGCAGAAAG (SEQ ID NO:47)
STJ#283	CTTCTATCCCAGCGACATCGCCGTGNNSTGGNNSAGCNNNSGGGCAAGGCTTCTATCCAGCGACATCGCCGTG (SEQ ID NO:48)
STJ#284	GACCAAGAACCCAGGTCAGCCTGACCTGCCCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTG (SEQ ID NO:49)
STJ#285	AGGGAGAGGGCTCTTCTGCGTGTAGTGGTTGTGCAGAGCWNNAATGWNNCACWNNGCATGAGAAGAC GTTCCCTGCTG (SEQ ID NO:50)
STJ#286	AGGGAGAGGGCTCTTCTGCGTGTAGTGGTTGTGCAGAGCCTCATGWNNWNNCACGGAGCATGAGAA GACGTTCCCTGCTG (SEQ ID NO:51)
STJ#287	AGGGAGAGGGCTCTTCTGCGTGTAGTGGTTGTGCAGAGCCTCATGWNNWNNWNNCACGGAGCATGA GAAGACGTTCCCTGCTG (SEQ ID NO:52)
STJ#302	GCGGAATTCCCATGGCGGATATTCAAATGACCC (SEQ ID NO:53)
STJ#303	CAGACGCGCTTAAAGAAGACGGGCTTTGGGTCAATTGAATATCCGCCATG (SEQ ID NO:54)
STJ#304	CGTCTTCTTTAAGCGCGTCTGTCCGTGATCGCGTGACCATCACGTGTCGT (SEQ ID NO:55)
STJ#305	AGGCCACCGCCGTATTAAACATCTTGGCTCGCACGACGTCATGATGGTCAAG (SEQ ID NO:56)

STJ#306	GTTAATACGGCGGTGGCCTGGTATCAACAAAAACCGGGTAAAGCCCCGAA (SEQ ID NO:57)
STJ#307	GAGTACAGAAAGCTGGCGCTGTAGATTACAGCTTCGGGGCTTTACCCGG (SEQ ID NO:58)
STJ#308	CAGCGCCAGCTTTCGTACTCTGGCGTCCCGAGCCGCTTTCTGGCAGCC (SEQ ID NO:59)

Example 2

Construction of plasmids to display homodimeric protein IgG-Fc

[00158] All plasmids and primers used in the present examples are described in Table 3 and Table 2. Plasmid pPelBHis was generated by ligating BamHI-HindIII digested *skp* gene from pMopac12 into pMopac1 digested with the same restriction endonucleases. pPelBFLAG was derived from pPelBHis in which polyhistidine tag and c-myc tag were replaced by FLAG tag (DYKDDDDK; SEQ ID NO:114). Subcloning of PCR amplified and SfiI digested Fc gene encoding human IgG1-Fc fragment, hinge, CH2 and CH3 region of human IgG1 heavy chain (GeneBank Accession No. AF237583; SEQ ID NO:83) into *Sfi*I digested pPelBHis and pPelBFLAG generated pPelBHis-Fc and pPelBFLAG-Fc, respectively. pPelBHis-beta 2 microglobulin was constructed by subcloning soluble mature human beta 2 microglobulin gene synthesized from overlap PCR amplification using 10 primers including 2 external primers (STJ#78 and STJ#84; SEQ ID NOS:14 and 16) and 8 internal primers (STJ#86-93; SEQ ID NOS:17-24) into pPelBHis using *Sfi*I restriction endonuclease site. pPelBHis-FcγRIIa was generated by introducing *Sfi*I digested soluble mature human FcγRIIa gene (GeneBank Accession No. P12318) (Stengelin *et al.*, 1988) amplified from pDNR-LIB-FcγRIIa (ATCC: MGC-23887) using primers STJ#74 and STJ#80 (SEQ ID NOS:13 and 15) into pPelBHis. *Sfi*I digested genes from pMoPac1-FLAG-M18 and pMoPac1-FLAG-2610 for M18 scFv (Harvey *et al.*, 2006) and 26-10 scFv (Francisco *et al.*, 1993) specific for the PA antigen of *Bacillus anthracis* and cardiac glycoside digoxin, respectively, were introduced to pPelBFLAG to generate pPelBFLAG-M18 scFv and pPelB-2610 scFv.

Table 3. Plasmids Used in The Present Examples

Plasmids	Relevant characteristics	Reference or source
pMoPac1	Cm ^r , <i>lac</i> promoter, <i>tetA</i> gene, C-terminal polyhistidine tag and c-myc tag	Hayhurst <i>et al.</i> , 2003
pMoPac12	Ap ^r , <i>lac</i> promoter, <i>tetA</i> gene, <i>skp</i> gene, C-terminal polyhistidine tag and c-myc tag	Hayhurst <i>et al.</i> , 2003

pMoPac16	Ap ^r , <i>lac</i> promoter, <i>tetA</i> gene, <i>HuCk</i> gene, <i>skp</i> gene, C-terminal polyhistidine tag and c-myc tag	Hayhurst <i>et al.</i> , 2003
pMoPac1-FLAG-M18	NlpA fused <i>M18 scFv</i> gene, C-terminal FLAG tag in pMoPac1	Jung <i>et al.</i> , 2007
pMoPac1-FLAG-2610	NlpA fused <i>26-10 scFv</i> gene, C-terminal FLAG in pMoPac1	Jung <i>et al.</i> , 2007
pPelBHis	Cm ^r , <i>lac</i> promoter, <i>tetA</i> gene, <i>skp</i> gene, C-terminal polyhistidine tag and c-myc tag	The Present Examples
pPelBHis-Fc	<i>IgG1-Fc</i> gene in pPelBHis	The Present Examples
pPelBHis-beta 2 microglobulin	<i>Human beta 2 microglobulin</i> gene in pPelBHis	The Present Examples
pPelBHis-FcγRIIa	<i>FcγRIIa</i> gene in pPelBHis	The Present Examples
pPelBHis-Fc-cFos	<i>IgG1-Fc</i> gene fused to C-terminal <i>cFos</i> gene in pPelBHis	The Present Examples
pPelBHis-Fc-cFos(Cys)	<i>IgG1-Fc</i> gene fused to C-terminal <i>cFos(Cys)</i> gene in pPelBHis	The Present Examples
pPelBFLAG	Cm ^r , <i>lac</i> promoter, <i>tetA</i> gene, <i>skp</i> gene, C-terminal FLAG tag	The Present Examples
pPelBFLAG-Fc	<i>IgG1-Fc</i> gene in pPelBFLAG	The Present Examples
pPelBFLAG-M18 scFv	<i>M18 scFv</i> gene in pPelBFLAG	The Present Examples
pPelBFLAG-2610 scFv	<i>26-10 scFv</i> gene in pPelBFLAG	The Present Examples
pPelBFLAG-Fc-Im2	<i>IgG1-Fc</i> gene fused to C-terminal <i>Im2</i> gene in pPelBFLAG	The Present Examples
pPelBFLAG-M18 scFv-Im2	<i>M18 scFv</i> gene fused to C-terminal <i>Im2</i> gene in pPelBFLAG	The Present Examples
pPelBFLAG-2610 scFv-Im2	<i>26-10 scFv</i> gene fused to C-terminal <i>Im2</i> gene in pPelBFLAG	The Present Examples

pMopac12-M18.1 hum scFv	<i>M18.1 humanized scFv</i> gene in pMoPac12	The Present Examples
pMopac12-2610 scFv	<i>26-10 scFv</i> gene in pMoPac12	The Present Examples
pMopac16-M18.1 hum scAb	<i>M18.1 humanized scAb</i> gene in pMoPac16	The Present Examples
pMopac16-2610 scAb	<i>26-10 scAb</i> gene in pMoPac16	The Present Examples
pMAZ360-M18.1-Hum-IgG	<i>M18.1 humanized IgG1</i> gene in pMAZ360	Mazor <i>et al.</i> , 2007
pMAZ360-26.10 IgG	<i>26-10 IgG1</i> gene in pMAZ360	Mazor <i>et al.</i> , 2007
pNlpAFLAG-M18	NlpA fused <i>M18 scFv</i> gene in pPelBFLAG	The Present Examples
pNlpAHis-Fc	NlpA fused <i>IgG-Fc</i> gene in pPelBHis	The Present Examples
pBAD30	Ap ^r , BAD promoter	Guzman <i>et al.</i> , 1995
pBADNlpAFLAG-M18	NlpA fused <i>M18 scFv</i> gene, C-terminal FLAG tag in pBAD30	The Present Examples
pBADNlpAFLAG-cJun	NlpA fused <i>cJun</i> gene, C-terminal FLAG tag in pBAD30	The Present Examples
pBADNlpAFLAG-cJun(Cys)	NlpA fused <i>cJun(Cys)</i> gene, C-terminal FLAG in pBAD30	The Present Examples
pBADNlpAHis-Fc	NlpA fused <i>IgG-Fc</i> gene, C-terminal polyhistidine tag in pBAD30	The Present Examples
pBADNlpAHis-ColE2(H574A)	NlpA fused <i>ColE2(H574A)</i> gene, C-terminal polyhistidine tag in pBAD30	The Present Examples
pBADNlpAHis-ColE2(H578A)	NlpA fused <i>ColE2(H578A)</i> gene, C-terminal polyhistidine tag in pBAD30	The Present Examples
pBADNlpAHis-ColE2(H574A/H578A)	NlpA fused <i>ColE2(H574A/H578A)</i> gene, C-terminal polyhistidine tag in pBAD30	The Present Examples
pTrc99A	Ap ^r , trc promoter, lacI ^q	Amersham Biosci., (Piscataway, NJ)

pTrcdsbAHis-Fc	dsbA fused <i>IgG-Fc</i> gene, C-terminal FLAG tag in pTrc99A	The Present Examples
pTrcdsbAHis-Fc γ RIIa	dsbA fused <i>FcγRIIa</i> gene, C-terminal polyhistidine tag in pTrc99A	The Present Examples
pSTJ4-Herceptin IgG1	<i>Herceptin IgG1</i> gene in pMAZ360-M18.1-Hum-IgG1	The Present Examples

[00159] pNlpAFLAG-M18 was constructed by ligating XbaI–HindIII digested fragments for NlpA and 6 amino acid residues (CDQSSS; SEQ ID NO:84) fused M18 scFv gene from pMopac1-FLAG-M18 into pPelBFLAG-M18. pNlpAHis-Fc was generated by subcloning SfiI digested Fc gene into pNlpAFLAG-M18 and by replacing FLAG with polyhistidine tag and c-myc tag. pBADNlpAFLAG-M18 and pBADNlpAHis-Fc were generated by ligating XbaI–HindIII digested M18 scFv gene and Fc gene from pNlpAFLAG-M18 and pNlpAHis-Fc, respectively, into pBAD30 digested with same restriction endonucleases.

[00160] To display Fc domain using leucine zipper pair of cJun-cFos interaction, NotI–SalI digested cFos fragments amplified using two primers (STJ#68 and STJ#69; SEQs ID NO:11 and 12) and the cFos(Cys) fragments encoding additional three amino acids including internal two Gly residues and external Cys residue at both ends of C-terminus and N-terminus amplified using two primers (STJ#96 and STJ#97; SEQ ID NOS:27 and 28) were cloned into pPelBHis-Fc to make pPelBHis-Fc-cFos for non-covalent bonding of cJun-cFos interaction pair and pPelBHis-Fc-cFos(Cys) for covalent disulfide bonding of both N terminal and C terminal ends of cJun-cFos pair in *E. coli* periplasmic space. For anchoring periplasmic expressed Fc domain fused to cFos or cFos(Cys), pBADNlpAFLAG-cJun and pBADNlpAFLAG-cJun(Cys) were generated by subcloning SfiI digested cJun fragments amplified with primers (STJ#58 and STJ#59; SEQ ID NOS:11 and 12) and cJun(Cys) fragments amplified with primers (STJ#94 and STJ#95; SEQ ID NOS:25 and 26) into SfiI digested pBADNlpAFLAG-M18.

[00161] For the display of Fc using tight Cole2–Im2 interaction, Im2 gene that is PCR amplified using two primers (ST#116 and STJ#117; SEQ ID NOS:31 and 32) and template *E. coli* WTZ1011 Cole2 harboring plasmid Cole2-P9

(The Coli Genetic Stock Center, Yale Univ. CGSC No. 8203) (Masaki *et al.*, 1985) was NotI-SalI digested and ligated into pPelBFLAG-Fc, pPelBFLAG-M18, and pPelBFLAG-2610 to generate pPelBFLAG-Fc-Im2, pPelBFLAG-M18-Im2, and pPelbFLAG-2610-Im2. To construct plasmids encoding NlpA fused Cole2 mutants binding to Im2 with strong protein protein interaction, the catalytic domains of three Cole2 mutants were amplified by overlap PCR with the template plasmid used for Im2 gene amplification and with four primers including two common external primers (STJ#114 and STJ#115; SEQ ID NOS:29 and 30) and two internal reverse primers (STJ#120 and STJ#121; SEQ ID NOS:35 and 36) for Cole2(H574A), internal primers (STJ#118 and STJ#119; SEQ ID NOS:33 and 34) for Cole2(H578A), and internal primers (STJ#122 and STJ#123; SEQ ID NOS:37 and 38) for Cole2(H574A/H578A), respectively. The amplified PCR products were SfiI digested and introduced into pBADNlpAHis to generate pBADNlpAHis-Cole2(H574A), pBADNlpAHis-Cole2(H578A), and pBADNlpAHis-Cole2(H574A/H578A).

[00162] Subcloning of SfiI digested M18.1 hum scFv (1) and 26-10 scFv gene into pMopac12 generated pMopac12-M18.1 hum scFv and pMopac12-2610 scFv. Also, subcloning of the SfiI digested M18.1 hum scFv and 26-10 scFv into pMopac16 generated pMopac16-M18 scAb and pMopac16-2610 scAb. For pTrcdsabAHis-Fc and pTrcdsbAHis-Fc γ RIIa, Fc and Fc γ RIIa gene fragments were PCR amplified using primers (STJ#144 and STJ#139; SEQ ID NOS:41 and 40) with the templates pPelBHis-Fc for Fc gene and primers (STJ# 136 and STJ#139; SEQ ID NOS:39 and 40) with the template pPelB-Fc γ RIIa for Fc γ RIIa gene, respectively, SalI-HindIII digested, and ligated into dsbA signal sequence (Schierle *et al.*, 2003) inserted pTrc99A.

[00163] All plasmids were transformed into E. coli Jude-1 (F' [Tn10(Tetr) proAB⁺ lacIq Δ (lacZ)M15] mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 Δ lacX74 deoR recA1 araD139 Δ (ara leu)7697 galU galK rpsL endA1 nupG) (Kawarasaki *et al.*, 2003).

Example 3

Culture conditions

Culture conditions for two plasmids system

[00164] For the periplasmic display using leucine zippers, cJun-cFos, pPelBHis-Fc-cFos and pPelBHis-Fc-cFos(Cys) were co-transformed with pBADNlpAFLAG-cJun or pBADNlpAFLAG-cJun(Cys) into *E. coli* Jude-1. To display Fc using the interaction of Cole2-Im2, pPelBFLAG-Fc-Im2, pPelB-M18 scFv-Im2, and pPelBFLAG-2610 scFv-Im2 were co-transformed with pBADNlpAHis-Cole2(H574A), pBADNlpAHis-Cole2(H578A), or pBADNlpAHis-Cole2(H574/578) containing single or double mutations at C-terminal Cole2 DNase catalytic domain. The transformants harboring two plasmids were grown overnight at 37°C with 250 rpm shaking in Terrific Broth (TB) (Becton Dickinson Diagnostic Systems DIFCO™, Sparks, MD) supplemented with 2% (wt/vol) glucose, chloramphenicol (40 µg/ml) and ampicillin (50 µg/ml). After overnight culture, the cells were diluted 1:100 in fresh TB medium without glucose, incubated at 37°C for 2 h and then cooled at 25°C for 20 min. Firstly, PelB signal sequence fused proteins were induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to allow time for correct folding in periplasmic space prior to binding to inner membrane anchored Cole2 mutants. And 2h after IPTG induction, 0.2% (wt/vol) arabinose was added to induce expression of inner membrane anchored cJun, cJun(Cys), Cole2(H574A), Cole2(H578A), or Cole2(H574A/H578A).

Culture Conditions for One Plasmid System

[00165] *E. coli* transformed with various plasmids, pPelBHis-Fc, pPelBHis-beta 2 microglobulin, pPelBFLAG-M18 scFv, pMopac12-M18.1 hum scFv, pMopac12-2610 scFv, pMopac16-M18.1 hum scAb, pMopac16-2610 scAb, pMAZ360-M18.1-Hum-IgG, pMAZ360-26.10 IgG, pNlpAHis-Fc, pTrcdsbAHis-Fc, and pTrcdsbAHis-FcγRIIa were cultured overnight at 37 °C with 250 rpm shaking in Terrific Broth (TB) with 2% (wt/vol) glucose. Antibiotics, chloramphenicol (40 µg/ml) or ampicillin (50 µg/ml) appropriate for antibiotic resistance gene of each plasmid, were added for overnight culture. The overnight cultured cells were diluted 1:50 in fresh TB medium with 0.5M trehalose and the supplement of appropriate antibiotics, chloramphenicol (40 µg/ml) or ampicillin (50 µg/ml). After incubation at

37°C for 3 h and cooling at 25°C for 20 min with 250 rpm shaking, the protein expression was induced with 1 mM of isopropyl-1-thio- β -D-galactopyranoside (IPTG).

[00166] For the culture of various *E. coli* transformants in the media without trehalose was performed as same as above culture condition for one plasmid cultured in the media with trehalose except 1:100 dilution after overnight culture and incubation for 2 h instead of 3 h before IPTG induction.

Example 4

Flow cytometry analysis for screening of Fc libraries

Spheroplasts preparation and flow cytometry analysis

[00167] 5 h after IPTG induction, an aliquot of the culture broth equivalent to 8 ml/OD600 was harvested by centrifugation and washed two times in 1 ml of cold 10 mM Tris-HCl (pH 8.0). After resuspension in 1 ml of cold STE solution (0.5 M Sucrose, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0), the cells were incubated with rotating mixing at 37°C for 30 min, pelleted by centrifugation at 12,000 x g for 1 min and washed in 1 ml of cold Solution A (0.5 M Sucrose, 20 mM MgCl₂, 10 mM MOPS, pH 6.8). The washed cells were incubated in 1 ml of Solution A with 1 mg/ml of hen egg lysozyme at 37°C for 15 min. After centrifugation at 12,000 x g for 1 min and the resulting spheroplasts pellet were resuspended in 1 ml of cold PBS. 200 μ l of the spheroplasts further diluted in 800 μ l of PBS was mixed with each fluorescent labeled probes, 0.5 μ l of Protein A-FITC (5 mg/ml), 2 μ l of PA-FITC (0.25 mg/ml), 2.5 μ l of Fc γ RIa-FITC (0.6 mg/ml), or 200 nM Digoxin-BODIPY. After incubation for 1 h with vigorous shaking at 25°C in dark condition, the mixture was pelleted by centrifugation at centrifuged at 12,000 x g for 1 min and resuspended in 1 ml of PBS. The 100 μ l of the resuspension was diluted in 1 ml of PBS and analyzed on BD FACSort (BD Bioscience, San Jose, CA).

Screening of Fc Libraries Using Flow Cytometry

[00168] To construct random peptide loop inserted Fc library, 10 degenerate codons (NNS: N = A, T, G, or C; S = G or C) encoding 10 random amino acid residues were introduced between 297Asn and 298Ser. Fc partial gene fragments amplified using primers (STJ#194 and STJ195 (SEQ ID NOS:43 and 44) were

digested with SacII and EcoRI restriction endonucleases and subcloned into SacII-EcoRI digested pPelBFLAG-Fc to generate random peptide loop inserted Fc library. For the error prone PCR library for full Fc region, standard error prone PCR methods (Fromant *et al.*, 1995) were employed using primers STJ#196 and STJ#197 (SEQ ID NOS:45 and 46). The amplified PCR fragments were digested with SfiI and cloned into SfiI digested pPelBFLAG-Fc to generate error prone PCR Fc library. Two kinds of libraries were mixed at a 1:1 volume ratio and cultured in TB with 0.5M trehalose and chloramphenicol (40 µg/ml). After 2 h incubation at 37°C and cooling at 25°C for 20 min, the protein expression was induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG). After spheroplasting and incubation with glycosylated human FcγRIa-FITC, spheroplasts were sorted on a MoFlo™ droplet deflection flow cytometry (Dako Cytomation, Fort Collins, CO) equipped with a 488 nm Argon laser for excitation. By gating spheroplasts exhibiting the approximately high 3% of FL2 signal, high fluorescent spheroplasts were sorted and resorted immediately after the initial sorting. The Fc genes in the spheroplasts were rescued by PCR amplification using two specific primers (STJ#16 and STJ#220; SEQ ID NOS:8 and 47), ligated into pPelBFLAG-Fc using SfiI restriction enzyme site, and transformed in electrocompetent *E. coli* Jude-1 cells. The resulting transformants were employed for the next round sorting.

Example 5

Bacterial display system for homo-multimeric proteins

[00169] In the bacterial display of homomultimeric protein including dimeric protein Fc, three factors should be made a consideration. Firstly, for efficient construction of libraries encompassing random peptide loop library or error prone PCR library, the multimeric protein should be encoded by single gene to make the homomultimer. Fc encoded by two separate genes generates heterodimeric Fc. Secondly, the homomultimeric proteins should be expressed in the space enabling correct folding and assembly. In bacterial expression system, *E. coli* periplasmic space provides oxidative environment for disulfide bonds and is suitable for the production of correct folded heterologous protein with the use of cellular folding machinery (Georgiou and Segatori, 2005). Finally, the expressed and folded multimeric proteins should be tightly anchored to bacterial cells during high throughput Fluorescent Activated Cell Sorting (FACS).

[00170] The first attempted system was leucine zippers cJun-cFos interaction pair. The repetitive leucine residues at every seven amino acid of cJun and cFos allow strong non-covalent interaction (Landschulz *et al.*, 1988; Kouzarides and Ziff, 1988). Expression of cFos fused Fc from pPelBHis-Fc-cFos was firstly induced for periplasmic expression and then pBADNlpAFLAG-cJun for cJun fused to NlpA leader sequence and six amino acid residues (CDQSSS; SEQ ID NO:84) for inner membrane anchoring was induced for binding to the periplasmic assembled Fc homodimer (**FIG. 1**). Also, for another Fc display system, three amino acid residues including one external Cys and two internal Gly were introduced to N and C terminal ends of both cJun and cFos for more tight anchoring of periplasmic expressed Fc for the purpose of inhibiting the dissociation of cJun-cFos non-covalent bond. The resulting cJun(Cys)-cFos(Cys) enables disulfide covalent bond between the two leucine zippers in the periplasmic space (de Kruif and Logtenberg, 1996). With the APEX displayed pNlpAHis-Fc as a positive control and the pNlpAFLAG-cJun(Cys) not anchoring Fc domains as a negative control, the two Fc display systems were analyzed on flow cytometry after spheroplasting and incubation with Protein A-FITC (**FIG. 2a**). As expected, the periplasmic Fc display system employing engineered cJun(Cys)-cFos(Cys) showed higher fluorescence signal compared with native cJun-cFos, suggesting improved anchoring of Fc domains to inner membrane. However, when the system was compared with other negative controls that express only periplasmic Fc domains without co-expression of anchoring partner cJun or cJun(Cys), it did not show selective high signals. The spheroplasts harboring pPelBFLAG-Fc for PelB leader peptide fused Fc without additional inner membrane anchoring motif showed very high fluorescence signal suggesting that most of the periplasmic expressed Fc proteins are remained binding to the cells without additional inner membrane anchoring motif even after spheroplasting (**FIG. 2b**).

[00171] As an alternative display system, stronger protein-protein interaction pair than leucine zippers can be considered. For example, the ColE2-Im2 interaction pair, one of the tightest protein-protein interaction pair in nature ($K_d = 10^{-15}$) may be used (Li *et al.*, 2004). To display homodimeric Fc using the tight ColE2-Im2 interaction, Im2 fused Fc was firstly induced for periplasmic Fc assembly and then the expression of ColE2 mutants fused to NlpA leader sequence and six amino acids (CDQSSS; SEQ ID NO:84) was induced for inner membrane anchoring (**FIG.**

3). To prevent auto degradation of host DNA, zinc binding histidines (H574, H578) were substituted to Ala by site directed mutagenesis (Garinot-Schneider *et al.*, 1996). The resulting three mutants (H574A, H578A, or H574A/H578A) could inhibit host DNase activity with retaining Im2 binding property. Of the three ColE2 mutants, the single mutant, ColE2(H578A) showed the best result for the display of Im2 fused 26-10 scFv on the FACS analysis detected by digoxin BODIPY. The feasibility of the display system using ColE2(H578A)-Im2 interaction was further investigated with M18 scFv, 26-10 scFv, and homodimeric Fc. Although Im2 fused antibodies, M18 scFv-Im2 and 26-10 scFv-Im2 showed selectively higher fluorescence signal comparing negative controls, M18 scFv and 26-10 scFv not fused to Im2, respectively (FIG. 4), this selective high signals were derived from the deviation in expression levels. When ColE2 was not expressed, M18 scFv, 26.10 scFv, and Fc were well expressed. However, the expression of ColE2 with Im2 or without Im2 inhibited the expression of M18 scFv, 26-10 scFv and Fc partially or completely, respectively (FIG. 5).

[00172] In cJun-cFos or cJun(Cys)-cFos(Cys) system, it was found that periplasmic expressed Fc proteins are not clearly removed even in harsh spheroplasting conditions and keep bound strongly to the spheroplasts with enabling access of fluorescent dye labeled ligands. PelB fused small globular protein such as human beta 2 microglobulin was well removed after spheroplasting. On the contrary, PelB fused larger proteins including antibody domains or full antibody including Fc, scFv, scAb, and full IgG remained bound to the cells after spheroplasting.

Example 6

Trehalose effect in periplasmic display

[00173] For affinity maturation using FACS sorting method based on gating selective fluorescence and scattering regions, it is required to get distinguishable high or low fluorescence signal comparing a negative control with low coefficient of variation ($CV = [\text{Standard Deviation}/\text{Mean Value}] \times 100$). Some carbohydrates such as sucrose, sorbitol, mannitol, and trehalose are widely used sugars for protein stabilization at protein drug formulation or long term storage (Jung *et al.*, 2003; Elbein *et al.*, 2003; Purvis *et al.*, 2005). Sugars have been used to enhance periplasmic folding and stabilize protein (Bowden and Georgiou, 1990). The

fluorescence signal for the PelB fused Fc was tested when cultured in media comprising sorbitol or trehalose. Surprisingly, 0.5M trehalose greatly increased fluorescence signal intensity in the FACS analysis for both the APEx displayed Fc and the PelB leader peptide fused Fc (**FIG. 6**). Also, in comparison with other negative controls, PelB fused M18 scFv cultured in the media with or without 0.5M trehalose, the PelB fused Fc clearly exhibited dramatically improved signal intensity and CV value (**FIG. 7**) providing a selective display system for real affinity maturation of homodimeric Fc (**FIG. 8**).

[00174] Culture with trehalose did not significantly change the expression levels in total cell lysate and spheroplasts fraction (**FIG. 9a**). However, on the Western blot result of non-reduced samples, it clearly shows that trehalose increases the retention of dimeric Fc after spheroplasting (**FIG. 9b**). When PelB signal peptide was replaced by dsbA signal sequence depending on SRP (Signal recognition pathway), culture with trehalose did not significantly change fluorescence signal (**FIG. 10**). The display system using periplasmic expression with trehalose and spheroplasting has been tested for various formats of antibodies, full IgG1, scAb, and scFv. In the FACS analysis with PA-FITC probe, periplasmic expressed M18.1 antibodies showed significantly higher fluorescence signal than negative control, periplasmic expressed 26.10 antibodies (**FIG. 11**).

Example 7

Fc library construction and screening using high throughput flow cytometry

[00175] The native human IgG has two N-linked biantennary complex type oligosaccharide chains at the Asn297 amino acid residue of each CH2 domain. The two chains are located between the CH2 domains and interact with hydrophobic parts of the domains. Effector functions are largely dependent on the presence of the oligosaccharide chains (Wright and Morrison, 1997; Jefferis, 2005) to keep open structure of heavy chains for immune ligands binding (Sondermann *et al.*, 2001). Aglycosylation causes great reduction or complete loss of effector functions (Jefferis, 2005). In the first library, 10 random amino acids were introduced between N-linked glycosylation site 297Asn and 298Ser using random degenerate codons (NNS) to find random peptide loop showing similar function with the oligosaccharide chains of mammalian IgG molecules. The gene encoding the Fc domain was used as a template

for random mutagenesis by error-prone PCR (Fromant *et al.*, 1995) with primers STJ#196 and STJ#197; SEQ ID NOS:45 and 46. The random 10 a.a. insertion library was constructed by PCR amplification using forward primer STJ#194 (SEQ ID NO:43) containing 10 degenerate codons encoded by the NNS randomization scheme and reverse primer STJ#195 (SEQ ID NO:44) with the same template. The amplified PCR fragments were ligated into pPelBFLAG cut with *Sfi*I restriction sites for the error prone PCR library and with *Sac*II / *Eco*RI for random 10 a.a. insertion library, respectively. The transformation of the resulting library generated 2.8×10^7 transformants. In the second library, error prone PCR was used to generate random mutation for full Fc region. The resulting library was 9.2×10^8 individual transformants with 0.49% error rate per gene based on the sequence of 20 library clones randomly selected.

[00176] For library screening, extracellular domain of glycosylated Fc γ RIa was labeled with FITC as manufacturer's instruction. After the labeling reaction, the affinity of FITC labeled Fc γ RIa for human IgG Fc was confirmed by fluorescent ELISA displaying high fluorescence in the Fc glycosylated human IgG-Fc coated well comparing in the BSA coated well (**FIG. 12**). Total 1×10^8 spheroplasts were sorted and high fluorescent clones were enriched as sorting rounds go on (**FIG. 13a**). After the 4th round sorting, six individual clones showing high affinity to Fc γ RIa were isolated (**FIG. 13b**). All the six clones were from error prone PCR Fc library. The Fc5 showing the highest affinity to Fc γ RIa had two mutations E382V and M428I in CH3 region. The other five clones contained consensus mutations in E382V as well as M428I or S426T (**FIG. 14**) suggesting a critical role of two interacting beta sheets including the major mutation points in CH3 region for the binding of aglycosylated Fc to Fc γ RIa (**FIGs. 15a and 15b**).

Example 8

Randomization of residues around the amino acid substitutions 382E and 428M

[00177] For the screening of Fc exhibiting high affinity to Fc γ RIa by randomization around the two critical mutation points 382E and 428M, a new library comprising three kinds of sub-libraries was constructed from the PelB leader peptide fused Fc (**FIG. 16**). In the first sub-library, three amino acids (380E, 382E, and 384N) around 382E interacting with the beta sheet containing 482M, were replaced by three

random amino acid residues using random degenerate codons (NNS) (Kabat *et al.*, 1991). Also, for the combinatorial library of beta sheet around 428M, three amino acids (426S, 428M, and 420E) interacting with the beta sheet including of 382E, were replaced by three random amino acids. In the second and third sub-libraries, to increase the interaction of the two beta sheets containing 382E and 428M in CH3 region with the alpha helix of CH2 region and possibly increase accessibility of FcγRIa (**FIG. 15b**), one or two random amino acids were inserted between 428M and 429H with the randomization of 428M and three amino acid residues (380E, 382E, and 384N) around 382E. The three sublibraries randomized around E382V and M428I, were generated using PCR products amplified using forward primers STJ#283 and STJ#284 (SEQ ID NOS:48 and 49) and reverse primers STJ#285, STJ#286, or STJ#287 (SEQ ID NOS: 50, 51 or 52). Each of the three sublibraries was subcloned into *SexAI* / *SapI* digested pPelBFLAG-Fc. The resulting plasmids were transformed into *E. coli* Jude-1(F' [*Tn10*(Tet^r) *proAB*⁺ *lacI*^q Δ(*lacZ*)M15] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74* *deoR* *recA1* *araD139* Δ(*ara leu*)7697 *galU* *galK* *rpsL* *endA1* *nupG*) (Kawarasaki *et al.*, 2003).

[00178] The transformation of the resulting library, mixture of three sub-libraries, generated over 10⁷ transformants. Table 4 shows the sequencing results of 10 randomly picked clones indicating that the expected sequence diversity had been obtained. The library cells were cultured in media containing trehalose, protein synthesis was induced with 1 mM IPTG and after 5 hours the cells were harvested and converted into spheroplasts as described in Example 4. Following labeling, spheroplasts were sorted by FACS. In each round the top 3% of the population showing the highest fluorescence due to FcγRIa-FITC binding labeling was isolated (-1×10⁸ spheroplasts were sorted in each round of sorting). The Fc encoding genes were recovered by PCR ligated into vector and the ligation mix was transformed into *E. coli* Jude-1. Transformants were selected on chloramphenicol containing media and then grown, spheroplasted as above in preparation for the next round of sorting (**FIG. 17**). After the 4th round of sorting, 14 individual clones exhibiting high fluorescence were isolated (**FIG. 18**). However, the parental Fc5 clone (E382V/M428I) showed the highest fluorescence; importantly most of the selected mutants contained the mutations E382V and/or M428I or M428L, again suggesting the importance of these two amino acid substitutions.

Table 4: Sequence of randomly picked up 10 clones from library randomized around 382E and 428M

Underlining indicates mutated or inserted amino acids; * : Stop codon; ~ : Blank

Wild type	378-AVEWESNG-385	425-CSVMHE~AL-432
1	AVA <u>W</u> <u>D</u> S <u>R</u> G (SEQ ID NO:85)	CSV <u>A</u> LHE~AL (SEQ ID NO:95)
2	AVY <u>W</u> <u>S</u> S <u>L</u> G (SEQ ID NO:86)	CLV <u>C</u> H <u>S</u> ~AL (SEQ ID NO:96)
3	AV <u>L</u> W <u>G</u> S <u>L</u> G (SEQ ID NO:87)	CLV <u>L</u> H <u>G</u> ~AL (SEQ ID NO:97)
4	AVV <u>C</u> <u>Y</u> S <u>Y</u> G (SEQ ID NO:88)	CRV* <u>H</u> <u>P</u> ~AL (SEQ ID NO:98)
5	AVS <u>W</u> I <u>S</u> Q <u>G</u> (SEQ ID NO:89)	CSV <u>G</u> <u>G</u> H <u>E</u> ~AL (SEQ ID NO:99)
6	AVN <u>W</u> E <u>S</u> K <u>G</u> (SEQ ID NO:90)	CSV <u>L</u> <u>L</u> S <u>H</u> EAL (SEQ ID NO:100)
7	AVT <u>W</u> <u>R</u> S <u>W</u> G (SEQ ID NO:91)	CSV <u>P</u> <u>V</u> H <u>E</u> ~AL (SEQ ID NO:101)
8	AV* <u>W</u> <u>S</u> S <u>Q</u> G (SEQ ID NO:92)	CSV <u>H</u> <u>L</u> H <u>E</u> ~AL (SEQ ID NO:102)
9	AVN <u>W</u> N <u>S</u> <u>W</u> G (SEQ ID NO:93)	CSV <u>R</u> <u>D</u> H <u>E</u> ~AL (SEQ ID NO:103)
10	AVD <u>W</u> <u>R</u> S <u>V</u> G (SEQ ID NO:94)	CTV <u>C</u> H <u>I</u> ~AL (SEQ ID NO:104)

Example 9

Preparation and labeling of the extracellular domain of the Fc γ RIIIa protein

[00179] For library screening, the extracellular domain of aglycosylated Fc γ RIIIa was first purified from *E. coli* inclusion bodies. First an *E. coli* codon optimized Fc γ RIIIa synthetic gene (Nucleotide Sequence #1 (SEQ ID NO:105)) was subcloned into pET21a (Novagen) and transformed into *E. coli* BL21(DE3). After 5 hr induction with 1 mM IPTG induction, Western blot analysis revealed that the majority of the Fc γ RIIIa protein was present as inclusion bodies. Inclusion bodies were harvested by centrifugation of cell lysates, washed with U2KP buffer (2M urea in 10 mM potassium phosphate buffer, pH 8.2) and solubilized in U8KP buffer (8M urea, 10mM potassium phosphate buffer, pH 8.2). The solubilized and denatured Fc γ RIIIa protein was purified using Ni-NTA affinity chromatography and refolded by consecutive dialysis (FIG. 19) (Jung *et al.*, 2003). The purified Fc γ RIIIa was labeled with FITC using a commercial FITC labeling kit (Molecular Probes) as described in

the manufacturer's instructions. 1.5 μ l of FITC labeled Fc γ RIIIa (0.8 mg/ml) per 1 ml reaction was used for the labeling of spheroplasts.

Example 10

Selection of Fc γ RIIIa binders

[00180] In order to select candidate Fc mutants having the ability to bind Fc γ RIIIa polypeptides, a technique similar to that described above for Fc γ RIa binders was employed, with the exception Fc γ RIIIa polypeptides were employed in place of the Fc γ RIa polypeptides. In the context of Fc γ RIIIa binders, however, the approach has not been uniformly reproducible. That is, mutants so identified demonstrate Fc γ RIIIa binding capability in some studies and fail to demonstrate binding capability in other studies. At the time of the present filing, the inventors are confirming that this technique can indeed be used to identify reproducible Fc γ RIIIa binders. In this technique, to isolate binders to Fc γ RIIIa two libraries were constructed: First, the Fc gene was subjected to random mutagenesis by error prone PCR. Second, a 10 random amino acids insertion library was employed. The library cells were cultured in media containing trehalose, protein synthesis was induced with 1 mM IPTG and after 5 hours the cells were harvested and converted into spheroplasts as described in Example 4. Following labeling, spheroplasts were sorted by FACS. In each round the top 3% of the population showing the highest fluorescence due to Fc γ RIIIa-FITC binding labeling was isolated (1×10^8 spheroplasts were sorted in each round of sorting). The Fc encoding genes were recovered by PCR ligated into vector and the ligation mix was transformed into *E. coli* Jude-1. Transformants were selected on chloramphenicol containing media and then grown, spheroplasted as above in preparation for the next round of sorting (**FIG. 20**). After the 4th round sorting, five individual clones exhibiting high affinity to Fc γ RIIIa were isolated (**FIG. 21**). All five clones contained 10 random amino acid insertions. Two of these clones had additional mutations that presumably resulted from PCR amplification (**FIG. 22**). Although these mutant clones are generally considered to be high affinity binders, as noted above they have been found to exhibit certain variability in different tests.

Example 11

Selection of Fc γ RIIa binders from a library of random mutants of Fc5

[00181] For the selection of Fc γ RIIa binders, either the same mixture of library including 10 a.a. insertion library and an error prone PCR library described in Example 7 or the Fc5 error prone PCR library using the template Fc5 (Fc: E382V, M428I) are used. The library size of Fc5 error prone PCR library was 1.1×10^7 and the error rate was 0.131% as determined by the sequencing of 20 randomly selected clones. For library screening, the extracellular domain of glycosylated Fc γ RIIa (R&D systems) was conjugated to FITC using a FITC labeling kit (Molecular Probes) as described in the manufacturer's instruction. For the labeling of spheroplasts, 2 μ l of FITC labeled Fc γ RIIa (0.975 mg/ml) per 1 ml reaction was used. The library cells were cultured in media containing trehalose, protein synthesis was induced with 1 mM IPTG and after 5 hours the cells were harvested and converted into spheroplasts as described in Example 4. Following labeling spheroplasts were sorted by FACS. In each round the top 3% of the population showing the highest fluorescence due to Fc γ RIIa-FITC binding labeling was isolated (1×10^8 spheroplasts were sorted in each round of sorting) (**FIG. 23**). The Fc encoding genes were recovered by PCR ligated into vector and the ligation mix was transformed into *E. coli* Jude-1. Transformants were selected on chloramphenicol containing media and then grown, spheroplasted as above in preparation for the next round of sorting. After the 6th round sorting from the Fc5 error prone PCR library, two individual clones showing high affinity to Fc γ RIIa were isolated (**FIG. 24**). In addition to the two mutations encoded by the Fc5 parental gene (E382V/M428I), the two isolated clones Fc331 and Fc336 had the mutations G402D and P331L, respectively (**FIG. 25**). Although these mutant clones are generally considered to be high affinity binders, they may exhibit certain variability in different tests.

[00182] Alternatively, after the 5th round sorting from the same mixture of library including 10 a.a. insertion library and error prone PCR library described in Example 7, Fc fragment genes were subcloned into *Sa*II / *Hind*III digested pDsbAFLAG plasmid and 192 individual colony harboring pDsbAFLAG-Fc mutant genes were cultured in 96 well plates with 200 μ l working volume. The culture supernatant from the induced cells was separated by centrifugation at 4000 rpm for 30 min. For ELISA analysis, the extracellular domain of aglycosylated Fc γ RIIa was

purified from *E. coli* inclusion bodies. First an *E. coli* codon optimized FcγRIIa synthetic gene (Nucleotide Sequence #2 SEQ ID NO:106)) was subcloned into pET21a (Novagen) and transformed into *E. coli* BL21(DE3). After 5 hr induction with 1 mM IPTG induction, SDS-PAGE analysis revealed that codon optimized FcγRIIa synthetic gene shows dramatically increased expression level comparing with wild type FcγRIIa gene (**FIG. 26**) and the majority of the FcγRIIa protein was present as inclusion bodies (**FIG. 27**). Inclusion bodies were harvested by centrifugation of cell lysates, washed with U2KP buffer (2M urea in 10mM potassium phosphate buffer, pH 8.2) and solubilized in U8KP buffer (8M urea, 10mM potassium phosphate buffer, pH 8.2). The solubilized and denatured FcγRIIa protein was purified using Ni-NTA affinity chromatography and refolded by consecutive dialysis (**FIG. 28**) (Jung *et al.*, 2003). 100 μl of the culture supernatants were transferred to 96 well ELISA plates and incubated at 4 °C for overnight. After coating with PBS, 0.5% BSA for 2 h at room temperature, the plate was washed 4 times with PBS, 0.05% Tween20 and then added with 12.5 μg/ml of aglycosylated FcγRIIa purified from *E. coli*. After 1 h incubation at room temperature and washing with PBS, 0.05% Tween20, 1:10000 diluted Anti-His antibody HRP conjugate (Sigma-Aldrich) was added. After additional 1 h incubation at room temperature and washing, TMB was added for detection and 2M H₂SO₄ was added to quench the reaction. The plate was read at 450 nm with 96 well plate reader (Bio-Tek). Three Fc mutants, (Fc401, 402, and Fc403) showed higher ELISA signal comparing with wild type Fc (**FIG. 29**).

Example 12

Sequences of selected clones

[00183] Isolated Fc mutants have substitution or insertion mutations in the sequence of wild type Fc (Nucleotide Sequence #3 (SEQ ID NO: 107) and Protein Sequence #1 (SEQ ID NO:1)). Mutation points of the isolated clones showing high affinity to FcγRs are summarized in Table 5. Fc mutants (Protein Sequence #2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 17; SEQ ID NOS:3, 2, 4, 5, 7, 6 and SEQ ID NOS:65-73) show high affinity to FcγRIa. Fc mutants (Protein Sequences #18, 19, 20, 21, and 22; SEQ ID NOS:74-78) show high affinity to FcγRIIIa. Fc mutants (Protein Sequence #23, 24, 25, 26, 27; SEQ ID NOS:79, 80, 122, 81 and 82) show high affinity to FcγRIIa. Although these mutant clones binding to FcγRIIIa and FcγRIIa

are generally considered to be high affinity binders, some of them have been found to exhibit certain variability in different tests.

Table 5
Mutations in Fc showing high affinity to Fc γ Rs

Fc mutants	Binding FcγR	Mutations
Fc5	Fc γ RIa	E382V, M428I
Fc11		E382V
Fc12		N361D, E382V, M428I
Fc20		N361D, F372L, E382V, M428I
Fc23		H310Y, K322R, T350A, E382V, S426T, S442P
Fc49		C229R, E382V, M428I
Fc104		W313R, M428I
Fc106		E382T, N384D, M428I
Fc110		E380R, E382M, N384E
Fc114		N361S, E382V, M428I
Fc117		E382V, M428I, Y436A
Fc143		P238S, E382V, S426V, M428L, E430H
Fc149		E380D, E382V, N384R, S426V, M428L, E430D
Fc151		E382V, S426I, M428L, E430S
Fc152		H224R, E382V, S426T, M428S, E430P
Fc207	Fc γ RIIIa	QLISHYRHLT (SEQ ID NO:108) insertion between N297 and S298
Fc209		F241L, K326E , EVPLVWMWVS (SEQ ID NO:63)

		insertion between N297 and S298
Fc216		WQVFNKYTKP (SEQ ID NO:61) insertion between N297 and S298
Fc217		LGDGSPCKAN (SEQ ID NO:62) insertion between N297 and S298
Fc236		V282A, EQWGSQFGCG (SEQ ID NO:64) insertion between N297 and S298
Fc331		E382V, G402D, M428I
Fc336	Fc γ RIIa	E382V, P331L, M428I
Fc401		RTETPVYMM (SEQ ID NO:60), 10 a.a. insertion between N297 and S298
Fc402		P331L
Fc403		S239L, I253T, Q347L, E382V

Example 13

Soluble expression and purification of wild type Fc and Fc mutants

[00184] For the expression of correctly assembled, homodimeric Fc in the periplasmic space of *E.coli*, two different signal peptides were examined: The PelB signal peptide which is directed to the general secretory pathway post-translationally (Lei *et al.*, 1987; Better *et al.*, 1988) and the DsbA signal peptide which is exported co-translationally in an SRP (signal recognition particle)-dependent fashion (Schierle *et al.*, 2003). For the former, the Fc gene was cloned into the pPelBFLAG-Fc plasmid described in Example 2. The plasmid pDsbAFLAG-Fc was constructed for the export of Fc via the DsbA signal peptide. To construct pDsbAFLAG-Fc first, a synthetic DNA fragment encoding the 53 bp DsbA signal peptide gene (ATGAAAAAGATTTGGCTGGCGCTGGCTGGTTTAGTTTTAGCGTTTAGCGCATCGGCG (SEQ ID NO:109)) was introduced into pTrc99A following cleavage with *FatI* which is compatible with the *NcoI* in pTrc99A (Amersham Pharmacia) and also with *SalI*. The resulting plasmid was named pDsbA. The parental Fc or Fc mutant genes were amplified using the primers STJ#144 (TTTTAGGGGTCGACGACAAAACACACATGCCCACCGTG (SEQ ID NO:41)) and STJ#145 (TTTAAGGGAAGCTTCTATTAGGCGCGCCCTTTGTCATCG (SEQ ID NO:42)), ligated into pDsbA plasmid using *SalI* and *HindIII* restriction enzyme sites giving rise to pDsbAFLAG-Fc.

[00185] The effect of growth temperature, following induction of protein synthesis on the localization of Fc proteins was examined in *E.coli* Jude-1 cells harboring pPelBFLAG-Fc and on pDsbAFLAG-Fc was examined as follows: Cells were grown at 37 °C and then growth temperature was either changed to 25 °C or 30 °C or kept 37 °C 15 minutes before induction. The cells were induced with 1 mM IPTG at mid-exponential growth phase ($OD_{600} = 0.6$) and harvested either 5 h or 20 h after induction. Cells were fractionated by the periplasmic osmotic shock procedure (Osborn *et al.*, 1972) and the level of Fc protein in the extracellular fluid (growth medium) periplasmic (osmotic shock) and cytoplasmic fractions was determined by SDS-PAGE and Western blotting (O'Brien *et al.*, 2002) (FIG. 30).

Export via the DsbA leader peptide showed a substantially higher amount of correctly assembled Fc both in the periplasmic fraction and in the growth media.

[00186] The effect of growth temperature and harvest time after induction, were tested in 500 ml shake flask cultures. Optimal expression of Fc in the media (thus alleviating further purification) was obtained in cultured incubated for 8 h after induction at 30 °C with 1 mM IPTG (**FIG. 31**).

[00187] For purification, *E. coli* Jude-1 cells harboring pDsbAFLAG-Fc or Fc mutants were cultured in 2L flask with 500 ml working volume. The culture supernatant from the induced cells was separated by centrifugation at 7000 rpm for 30 min. The supernatant was filtered using 0.22 µm bottle top filters (Corning) and loaded onto a column packed with 1 ml of Immobilized Protein A agarose (Pierce). After loading of 400 ml of supernatants by gravity flow, the columns were washed with 75 ml of 20 mM sodium phosphate buffer (pH 7.0) and with 50 ml of 40 mM sodium citrate (pH 5.0). Wild type Fc and Fc mutants were eluted using 0.1M glycine (pH 2.5) and neutralized immediately with 1M Tris (pH 8.0) solution. The eluted wild type Fc and Fc mutants were analyzed by SDS-PAGE (**FIG. 32**). To collect dimeric Fc, the eluted samples from Protein A affinity chromatography column were concentrated with an ultrafiltration unit (10 kDa Mw cutoff: Millipore) and purified using Superdex 200 (Amersham Pharmacia) gel filtration chromatography (**FIG. 33**). Most of the purified wild type Fc and Fc mutants were dimeric forms (**FIG. 34**). The final yield of purified dimeric Fc and Fc mutants was approximately 800 µg/ml.

Example 14

Quantification of Fc binding to FcγRs by ELISA

[00188] The affinity of purified Fc or Fc mutant proteins for FcγRIa, FcγRIIa or FcγRIIIa was analyzed by ELISA (**FIG. 35**). 50 µl of 5 µg/ml purified wild type Fc, Fc mutants (Fc5, Fc11, Fc49), or glycosylated IgG-Fc (Bethyl laboratories) diluted in 0.05 M Na₂CO₃ (pH 9.6) buffer were coated on 96 well polystyrene ELISA plate (Corning) by overnight incubation at 4 °C. After coating with PBS, 0.5% BSA for 3 h at room temperature, the plate was washed 4 times with PBS, 0.05% Tween20TM and then added with 2 fold-diluted FcγRIa from 4 µg/ml of initial concentration. After 1 h incubation at room temperature and washing with PBS, 0.05% Tween20, 1:10000 diluted Anti-His antibody HRP conjugate (Sigma-Aldrich)

was added. After additional 1 h incubation at room temperature and washing, TMB was added for detection and 2M H₂SO₄ was added to quench the reaction. The plate was read at 450 nm with 96 well plate reader (Bio-TekTM). Soluble Fc mutants, (Fc5 and Fc49) showed higher affinity comparing with glycosylated IgG-Fc (**FIG. 36**).

Example 15

Quantification of Fc binding to FcγRs by BIAcore

[00189] Binding of IgG1-Fc domains to the human FcγRI was analyzed by surface plasmon resonance using a BIAcoreTM 3000 biosensor (BIAcore). The soluble monomeric FcγRIa was immobilized on the CM-5 sensor chip by the amine coupling kit as recommended by the manufacturer. Binding experiments were performed in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20 surfactant). Aglycosylated IgG1-Fc fragments, aglycosylated Fc mutants (Fc5, Fc49), or glycosylated IgG1 were injected at flow rate of 100 ul/min for 30 s with dissociation time 300 s. Regeneration was performed by a single injection of 100 mM citric acid, pH 3.0. Fc5 and Fc49 were injected in duplicate at concentrations 0, 80, 100, 200, 400, 600 nM and 0, 200, 400, 600, 800, and 1,000 nM. BIAcore analysis revealed that wt Fc does not bind to FcγRI ($K_D > 50 \mu\text{M}$). In contrast, Fc5 and Fc49 exhibited K_D values of 31 and 92 nM respectively. For comparison, the equilibrium dissociation constant of commercially available, glycosylated IgG1 was 18 nM. Notably, the aglycosylated Fc5 mutant and the glycosylated human Fc exhibited experimentally indistinguishable dissociation rate constants, k_{off} and a 2-fold lower association rate constant, k_{on} (Table 6) (**FIG. 37**).

Table 6: Kinetic rates and equilibrium dissociation constants of isolated Fc mutants determined by BIAcore.

	$k_{\text{on}} (\text{M}^{-1} \text{sec}^{-1})$	$k_{\text{off}} (\text{sec}^{-1})$	$K_D (\text{nM})$
Glycosylated-hIgG1	8.0×10^4	1.4×10^{-3}	18
aglycosylated-Fc	Undetectable	Undetectable	(a)
aglycosylated-Fc49	2.5×10^4	2.3×10^{-3}	92

aglycosylated-Fc5	4.5×10^4	1.4×10^{-3}	31
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^(a)K_D > 50 μM

Example 16

Plasmid construction for the expression of aglycosylated wild type and Fc5

trastuzumab

[00190] For the construction of pSTJ4-Herceptin™ IgG1, *E. coli* codon-optimized (Hoover and Lubkowski, 2002) V_L and V_H domains of humanized 4D5-8 (anti-p185HER2) (Eigenbrot *et al.*, 1993) were synthesized by total gene synthesis with overlap extension PCR using 12 oligonucleotides that included 2 external primers (STJ#302 and STJ#313) and 10 internal primers (STJ#303-312) for V_L and 14 primers total 2 external primers (STJ#314 and STJ#327) and 12 internal primers (STJ#315-326) for V_H, respectively. The ligation of the amplified V_L and V_H into pMAZ360-M18.1-Hum-IgG1 using *Nco*I / *Not*I for V_L and *Nhe*I / *Hind*III restriction endonuclease sites generated pSTJ4-Herceptin™ IgG1.

Example 17

Expression of aglycosylated wild type and Fc5 trastuzumab in *E. coli*

[00191] Trastuzumab (Herceptin™) recognizes HER2/neu (Erb2) which is overexpressed in about 30% of breast carcinomas (Sergina and Moasser, 2007). Extensive evidence indicates that recruitment of innate immune cells via interactions with Fcγ receptors plays an important role in the therapeutic action of trastuzumab (Sergina and Moasser, 2007; Lazar *et al.*, 2006). For preparative production of aglycosylated trastuzumab and trastuzumab-Fc5 in *E. coli* the heavy and light chains were fused to the PelB signal peptide and placed downstream from the *lac* promoter in a dicistronic operon (FIG. 38). Preparative expression was performed by fed-batch fermentation using a 3.3 L jar fermentor (New Brunswick Scientific Co., Edison, NJ) with 1.2 L working volume. BL21(DE3) cells were grown at 30°C in R/2 medium (Jeong and Lee, 2003) consisting of: 2 g of (NH₄)₂HPO₄, 6.75 g of KH₂PO₄, 0.93 g of citric acid H₂O, 0.34 g of MgSO₄, 20 g of glucose, 0.05 g of ampicillin and 5 ml of

trace metal solution dissolved in 2 N HCl (10 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.25 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.35 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.23 g of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 1.5 g of CaCl_2 , and 0.1 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$) per L). *E. coli* BL21(DE3) (EMD Chemicals, Gibbstown, NJ) harboring pSTJ4-Herceptin™ IgG1 or pSTJ4-Herceptin™ IgG1-Fc5 were cultured in 500 mL baffled-flask with 120 ml R/2 media at 30°C at 250 rpm for 8 h and used to inoculate the fermenter. The dissolved oxygen (DO) concentration was maintained at 40% of air saturation using automatic cascade control by increasing agitation speed from 100 rpm to 1000 rpm, air flow rate from 1 to 3 SLPM (Standard liquid per minute) and pure oxygen flow rate from 0 to 1.5 SLPM when required. Fed-batch fermentation were performed using pH-stat glucose feed control (**FIG. 39**). The initial pH was adjusted to 6.8 and controlled by the addition of 30% (v/v) ammonium hydroxide when it decreased to less than 6.75 and by the supply of feeding solutions, (700 g/L of glucose and 10 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; before induction) and (500 g/L glucose, 10 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 100 g/L of yeast extract; after induction), when it increased to more than 6.9. When OD_{600} reached 100, the culture temperature was reduced to 25°C and 30 min later, protein expression was induced with 1 mM of isopropyl-1-thio- β -D-galactopyranoside (IPTG). The culture broth was harvested 7 h after induction. The yield of aglycosylated tertameric IgG was 40 mg/L; a significant amount of light chain and minor amounts of incompletely assembled antibody molecules were also observed (**FIG. 40** and **FIG. 41**).

[00192] Cells were pelleted by centrifugation at $11,000 \times g$ for 30 min, suspended in 1.2 L 100 mM Tris, 10 mM EDTA (pH 7.4), 4 mg of lysozyme (per g of dry cell weight), and 1 mM PMSF and were incubated with shaking at 250 rpm at 30°C for 16 h to release periplasmic proteins. After centrifugation at $14,000 \times g$ for 30 min, the supernatant was mixed with polyethyleneimine (MP Biomedical, Solon, OH) to a final concentration of 0.2% (w/v) recentrifuged at $14,000 \times g$ for 30 min, and filtered through 0.2 μm filter. Immobilized Protein A agarose resin pre-equilibrated in 20 mM sodium phosphate buffer (pH 7.0) was added to the supernatant and incubated at 4°C for 16 h. After washing with 200 ml of 20 mM sodium phosphate buffer (pH 7.0) and 200 ml of 40 mM sodium citrate (pH 5.0), IgG1 was eluted from the resin using 15 ml of 0.1 M glycine (pH 3.0) and neutralized immediately with 1M Tris (pH 8.0) solution. The eluted samples were concentrated by ultrafiltration through a 10

kDa Mw cutoff membrane and the retentate was applied to a Superdex 200 gel filtration column developed with PBS (pH 7.4) (**FIG. 42**).

Example 18

ELISA and BIAcore analysis of aglycosylated wild type and Fc5 trastuzumab for FcγRs

[00193] The affinity of the purified IgGs for the extracellular domain of FcγRIIa and FcγRIIb expressed as an N-terminal fusion to GST in 293E cells (Berntzen *et al.*, 2005) was determined by ELISA. 50 μl of 4 μg/ml of wild type Fc, Fc mutants, aglycosylated trastuzumab, or trastuzumab-Fc5 purified from *E. coli*, glycosylated IgG trastuzumab (Clinical grade, Fox Chase Cancer Center Pharmacy) or glycosylated IgG1 (Sigma-Aldrich, St. Louis, MO), or were diluted in 0.05 M Na₂CO₃ (pH 9.6) buffer and used to coat 96 well polystyrene ELISA wells (Corning, Corning, NY) overnight at 4 °C. After blocking with 1x PBS (pH 7.4), 0.5% BSA for 2 hr at room temperature, the plate was washed 4 times with PBS containing 0.05% Tween20, and incubated with serially diluted FcγRIIa, FcγRIIb C-terminal fused to GST (Berntzen *et al.*, 2005), FcγRIa or FcγRIIb (R&D Systems, Minneapolis, MN) at room temperature for 1 h. After washing 4 times with the same buffer, 1:10,000 diluted anti-polyhistidine antibody HRP conjugate (Sigma-Aldrich, St. Louis, MO) for FcγRIIb or 1:5,000 diluted anti-GST antibody HRP conjugate (Amersham Pharmacia, Piscataway, NJ) for FcγRIIa and FcγRIIb was added and plates were washed and developed as described previously (Mazor *et al.*, 2007). To determine the binding of IgG to FcRn at pH 7.4, 2 μg/ml FcRn preincubated with 1:5,000 diluted anti-GST-HRP for 1 h as previously described (Andersen *et al.*, 2006) was added to plates coated with IgG.. To evaluate binding at pH 5.5, ELISAs were carried out as above except that the washing buffer and sample dilution buffers were adjusted to pH 5.5. As expected, the aglycosylated tratuzumab exhibited low affinity to FcγRIIa or FcγRIIb (**FIG. 43** and **FIG. 44**). Trastuzumab-Fc5 antibody exhibited only slightly higher affinity for FcγRIIb. The neonatal FcγRn receptor binds to the CH3 domain and is responsible for the endosomal recycling of IgG in plasma (Ghetie and Ward, 2000). Glycosylated, aglycosylated and trastuzumab-Fc5 exhibited near identical binding to FcRn at pH 5.5 and low binding at pH 7.5 suggesting that the E382V and

M42I substitutions are not likely to affect the circulation half-life of this antibody (FIG. 45).

[00194] Binding of FcγRI to the full assembled IgG trastuzumab was also analyzed by immobilizing glycosylated trastuzumab, aglycosylated trastuzumab, and aglycosylated trastuzumab-Fc5 individually on the CM-5 sensor chip. Binding experiments were done in the same HBS-EP buffer. For trastuzumab or trastuzumab-Fc5 FcγRIa was injected in duplicate at concentrations 0, 10, 20, 30, 50, and 100 nM for 60 s at a flow rate of 10 μl/min. For aglycosylated trastuzumab FcγRIa was injected at concentrations 0, 100, 200, 300, 500, and 1,000. Regeneration was performed by single injection of 100 mM H₃PO₄. Data were analyzed using the BIAevaluation 3.0 software. On the other hand consistent with the results shown in Table 6 for the Fc domains alone, trastuzumab-Fc5 bound strongly to FcγRIa. Specifically, the equilibrium dissociation constants for glycosylated trastuzumab from CHO cells, the *E. coli* expressed trastuzumab and trastuzumab-Fc5 were 1.7 nM, 0.8 μM and 3.6 nM respectively. (glycosylated trastuzumab: $k_{on} = 2.1 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ $k_{off} = 3.5 \times 10^{-4} \text{ sec}^{-1}$; *E. coli*, aglycosylated trastuzumab $k_{on} = 4.6 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ $k_{off} = 3.7 \times 10^{-2} \text{ sec}^{-1}$; trastuzumab Fc5: $k_{on} = 1.4 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, $k_{off} = 5 \times 10^{-5} \text{ sec}^{-1}$). Thus, trastuzumab-Fc5 exhibits selective binding only to the FcγRIa receptor.

* * *

[00195] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. The scope of the claims should not be limited by the preferred embodiments and examples, but should be given the broadest interpretation consistent with the description as a whole.

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CLAIMS

1. A method of selecting a bacterial cell comprising an aglycosylated antibody Fc domain having specific affinity for an Fc receptor (FcR) polypeptide comprising
5 the steps of:
- a) obtaining a population of Gram negative bacterial cells, cells of which population express an aglycosylated antibody Fc domain in their periplasm, wherein the population expresses a plurality of different Fc domains;
 - b) contacting the bacterial cells with an FcR polypeptide under conditions
10 wherein the FcR polypeptide contacts the aglycosylated Fc domains; and
 - c) selecting at least one bacterial cell based on binding of the aglycosylated Fc domain to the FcR polypeptide.
2. The method of claim 1, wherein the bacterial cells are *E. coli* cells.
15
3. The method of claim 1, wherein the Fc domain is an IgG, IgA or IgE Fc domain.
4. The method of claim 3, wherein the IgG Fc domain is an IgG1 Fc domain.
20
- 5 The method of claim 4, wherein the IgG1 Fc domain is the Fc domain of an anti-HER2 antibody.
- 6 The method of claim 5, wherein the IgG1 Fc domain is the Fc domain of the
25 Fc domain of trastuzumab.
7. The method of claim 1, wherein the population of Gram negative bacterial cells comprise a plurality of nucleic acids encoding said plurality of aglycosylated Fc domains.
30
8. The method of claim 7, wherein the plurality of nucleic acids further encodes a membrane secretion signal fused to said plurality of aglycosylated Fc domains.

9. The method of claim 8, wherein the membrane secretion signal is PelB.
10. The method of claim 8, wherein the membrane secretion signal is DsbA.
- 5 11. The method of claim 1, wherein the aglycosylated Fc domain comprises a hinge, CH2 and CH3 region.
12. The method of claim 1, wherein the FcR polypeptide comprises an eukaryotic
10 FcR domain.
- 13 The method of claim 1, wherein the FcR polypeptide comprises a prokaryotic or synthetic FcR domain.
- 15 14. The method of claim 12, wherein the FcR polypeptide comprises an antibody-binding domain from one of the polypeptides of Table 1.
15. The method of claim 14, wherein the FcR polypeptide comprises an antibody-binding domain from human FcγRIa, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, FcγRIIIb,
20 FcαRI or C1q.
16. The method of claim 15, wherein the FcR polypeptide comprises an antibody-binding domain from human FcγRIa.
- 25 17. The method of claim 1, wherein the FcR polypeptide is labeled.
18. The method of claim 17, wherein the FcR polypeptide is labeled with a fluorophore, a radioisotope or an enzyme.
- 30 19. The method of claim 18, wherein the FcR polypeptide is labeled with a fluorophore.

20. The method of claim 17, wherein the FcR polypeptide is fused to a fluorescence protein or an enzyme.
21. The method of claim 20, wherein the FcR polypeptide is fused to a green
5 fluorescence protein (GFP).
22. The method of claim 1, wherein the FcR polypeptide is immobilized.
23. The method of claim 1, wherein the selecting of step (c) is further defined as
10 comprising at least two rounds of selection wherein the sub-population of bacterial cells obtained in the first round of selection is subjected to at least a second round of selection based on the binding of the Fc domain to FcR polypeptide.
24. The method of claim 23, comprising two to ten rounds of selection.
15
25. The method of claim 24, wherein the selecting is carried out by FACS or magnetic separation.
26. The method of claim 1, further comprising contacting the bacterial cells with
20 at least two FcR polypeptides.
27. The method of claim 26, wherein the at least two FcR polypeptides comprise distinct labels.
- 25 28. The method of claim 27, further comprising selecting bacterial cells based on binding of the aglycosylated Fc domain to the at least two FcR polypeptides.
29. The method of claim 27, further comprising selecting bacterial cells based on
binding of the aglycosylated Fc domain to at least one FcR polypeptide and based on
30 the aglycosylated Fc domain not binding to at least one other FcR polypeptide.

30. The method of claim 1, further comprising disrupting the outer membrane of the bacterial cells before contacting the bacterial cells with an FcR polypeptide.

31. The method of claim 30, wherein disrupting the outer membrane of the bacterial cell comprises treatment with hyperosmotic conditions, treatment with physical stress, infecting the bacterium with a phage, treatment with lysozyme, treatment with EDTA, treatment with a digestive enzyme or treatment with a chemical that disrupts the outer membrane.

32. The method of claim 31, wherein disrupting the outer membrane of the bacterial cell comprises a combination of said methods.

33. The method of claim 30, wherein disrupting the outer membrane of the bacterial cell comprises heating the bacterial cell with a combination of physical, chemical and enzyme disruption of the outer membrane.

34. The method of claim 30, wherein disrupting the outer membrane of the bacterial cell further comprises removing the outer membrane of said bacterium.

35. The method of claim 1, further comprising removing FcR polypeptide not bound to the aglycosylated Fc domain.

36. The method of claim 1, wherein the bacteria are grown in a media comprising sucrose, sorbitol, mannitol or trehalose.

37. The method of claim 1, wherein the bacteria are grown in a media comprising trehalose.

38. The method of claim 1, further defined as a method of producing a nucleic acid sequence encoding an antibody Fc polypeptide having a specific affinity for an FcR polypeptide and further comprising the step of cloning a nucleic acid sequence encoding the Fc polypeptide from the bacterial cell to produce a nucleic acid sequence

encoding an antibody Fc polypeptide having a specific affinity for an FcR polypeptide.

39. The method of claim 38, wherein cloning comprises amplification of the
5 nucleic acid sequence.

40. The method of claim 38, further defined as a method of producing an antibody Fc polypeptide having a specific affinity for an FcR polypeptide further comprising the step of expressing a nucleic acid sequence encoding the antibody Fc polypeptide
10 to produce an antibody Fc polypeptide having a specific affinity for an FcR polypeptide.

41. A polypeptide comprising an aglycosylated antibody Fc domain capable of binding an FcR polypeptide, wherein the Fc domain is a human IgG Fc domain which
15 comprises an amino acid substitution at P331, E382 or M428 of the human IgG Fc domain.

42. The polypeptide of claim 41, wherein the amino acid substitution is one or more of P331L, E382V, M428I or M428L.
20

43. The polypeptide of claim 41 or 42, further comprising an amino acid substitution at a residue at one or more of S426, C229, K322, T350, N361, F372, S442, G402, H224, E430, P238, Y436, H310, W313, N384, F372, E380 or P331 of the human IgG Fc domain.
25

44. The polypeptide of claim 41 or 42, wherein the Fc domain comprises a substitution at an amino acid position selected from the group consisting of a) E382 and M428; b) N361, E382 and M428; c) N361, F372, E382 and M428; d) H310, K322, T350, E382, S426 and S442; e) C229R, E382 and M428; f) W313 and M428;
30 g) E382, N384 and M428; h) E380, E382 and N384; i) N361, E382 and M428; j) E382, M428 and Y436; k) P238, E382, S426, M428 and E430; l) E380, E382, N384, S426, M428 and E430; m) E382, S426, M428 and E430; n) H224, E382, S426, M428

and E430; o) P331; p) S239, I253, Q347, E382; q) E382, G402 and M428; and r) E382, P331 and M428.

45. The polypeptide of claim 41 or 42, wherein the Fc domain comprises a substitution selected from the group consisting of a) E382V and M428I; b) E382V; c) N361D, E382V and M428I; d) N361D, F372L, E382V and M428I; e) H310Y, K322R, T350A, E382V, S426T and S442P; f) C229R, E382V and M428I; g) W313R and M428I; h) E382T, N384D and M428I; i) E380R, E382M and N384E; j) N361S, E382V and M428I; k) E382V, M428I and Y436A; l) P238S, E382V, S426V, M428L and E430H; m) E380D, E382V, N384R, S426V, M428L and E430D; n) E382V, S426I, M428L and E430S; o) H224R, E382V, S426T, M428S and E430P; p) P331L; q) S239L, I253T, Q347L, E382V; r) E382V, G402D and M428I; and s) E382V, P331L and M428I.

46. The polypeptide of claim 41, wherein the aglycosylated antibody Fc domain is Fc11, Fc5, Fc12, Fc 20, Fc49, Fc23, Fc104, Fc106, Fc110, Fc114, Fc117, Fc143, Fc149, Fc151, Fc152, Fc331, Fc336, Fc 402 or Fc403.

47. The polypeptide of claim 41, further comprising an Ig variable domain.

20

48. The polypeptide of claim 47, further defined as a full length antibody.

49. The polypeptide of claim 41, wherein the Fc domain binds to FcγRIa with an equilibrium dissociation constant of about 10^{-6} M to about 10^{-9} M under physiological conditions.

25

50. A polypeptide comprising an aglycosylated antibody Fc domain capable of binding an FcR polypeptide, wherein the Fc domain is a human IgG Fc domain and comprises an amino acid insertion between amino acids N297 and S298.

30

51. The polypeptide of claim 50 wherein the amino acid insertion is 10 amino acids or less.

52. The polypeptide of claim 51, wherein the insertion is selected from the group consisting of a) RTETPVYVM (SEQ ID NO:60); b) WQVFNKYTKP (SEQ ID NO:61); c) LGDGSPCKAN (SEQ ID NO:62); d) EVPLVWMWVS (SEQ ID NO:63)
5 together with F241L and K326E; and e) EQWGSQFGCG (SEQ ID NO:64) together with V282A; f) QLISHYRHLT (SEQ ID NO:108).

53. An isolated DNA segment encoding a polypeptide in accordance with any one of claims 42 through 52.

10

54. The DNA segment of claim 53, positioned in an expression vector.

55. The DNA segment of claim 54, wherein the expression vector is a bacterial expression vector.

SUBSTITUTE SHEET (RULE 26)

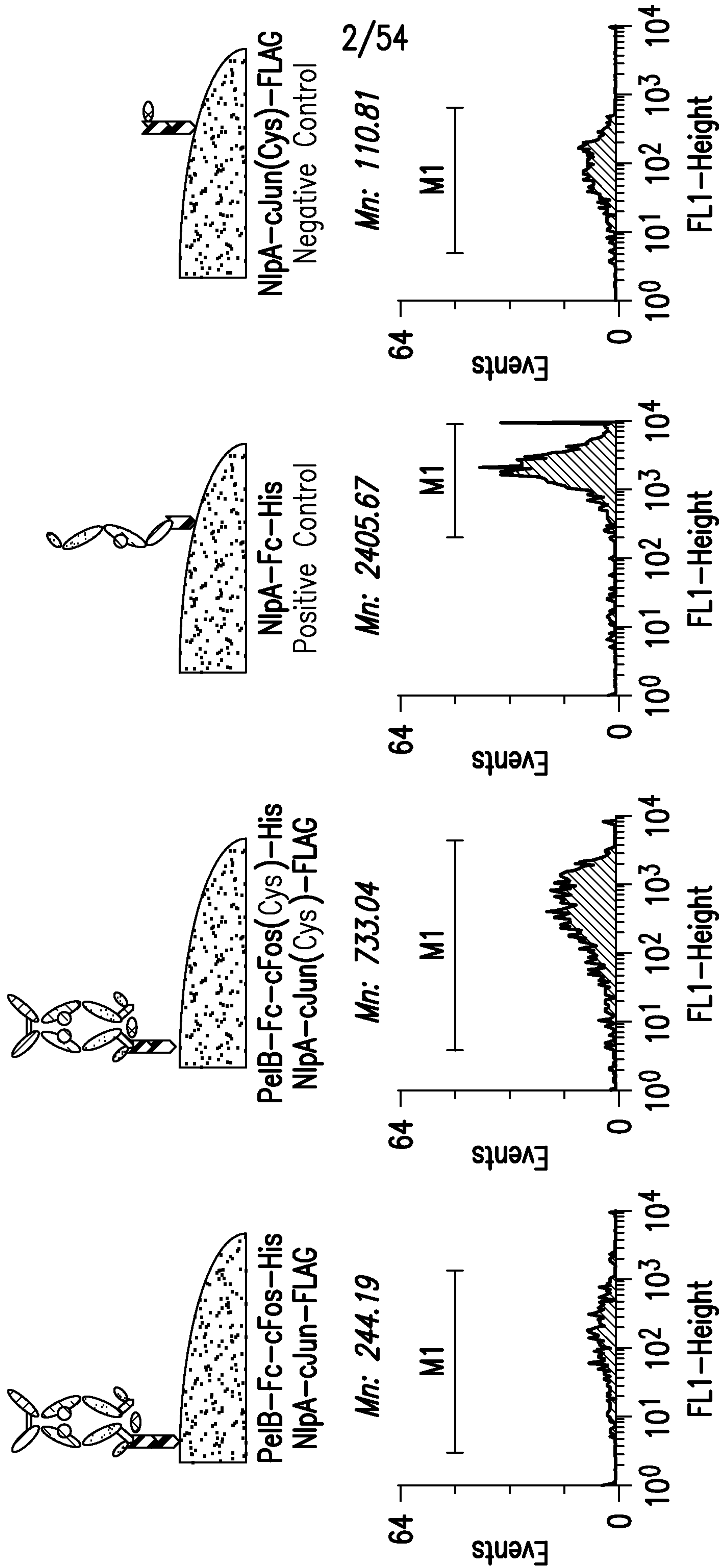


FIG. 2A

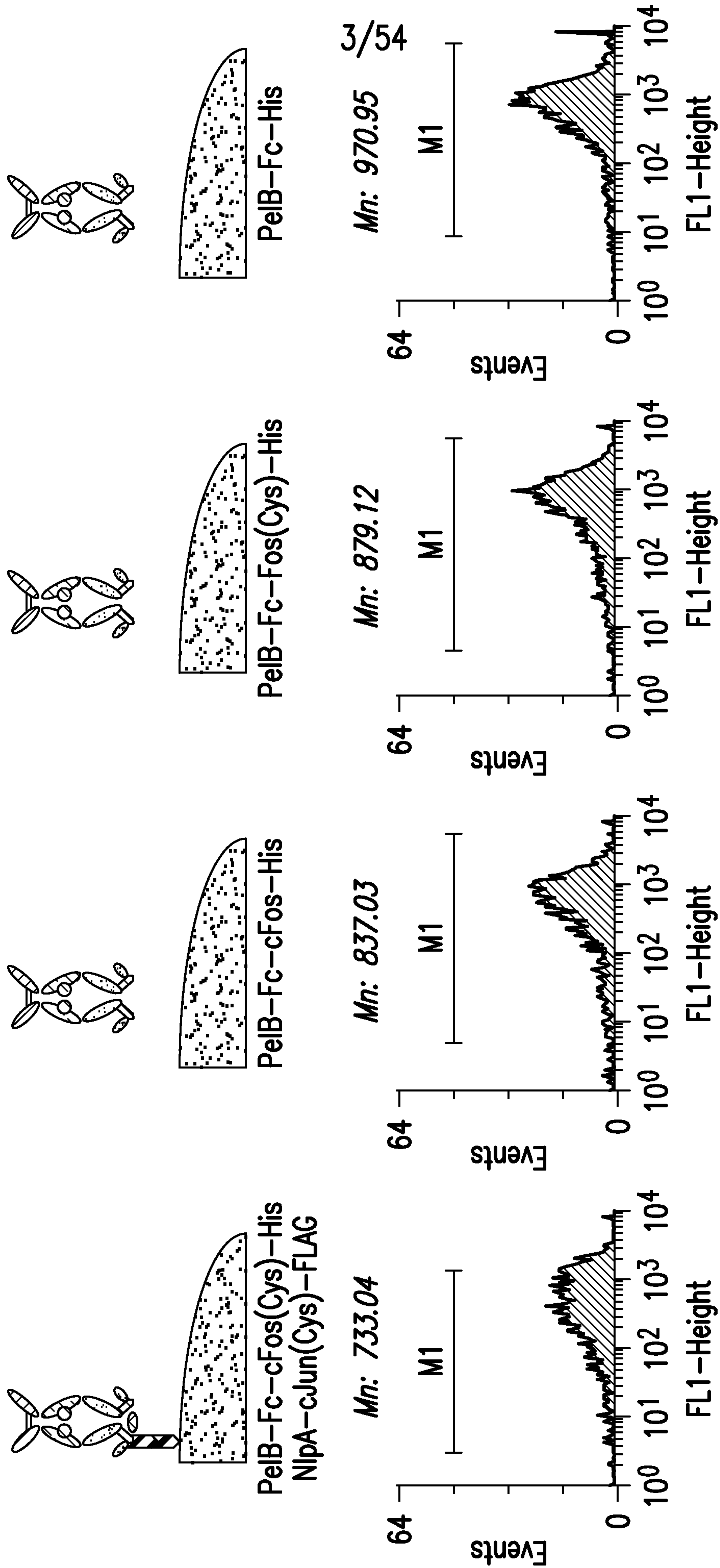
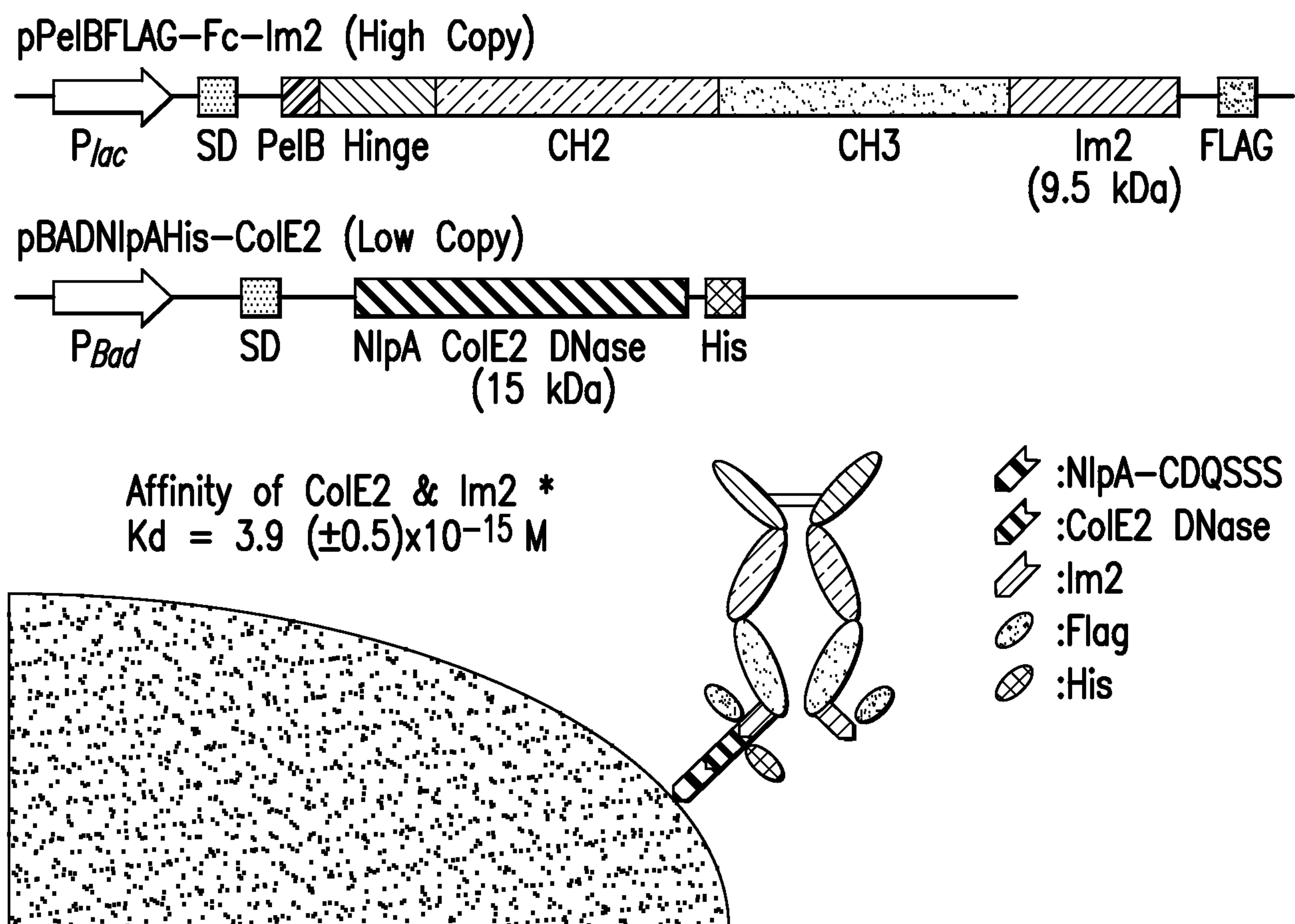


FIG.2B

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* Wei Li et al. (2004) Journal of Molecular Biology 337, 743-759

FIG.3

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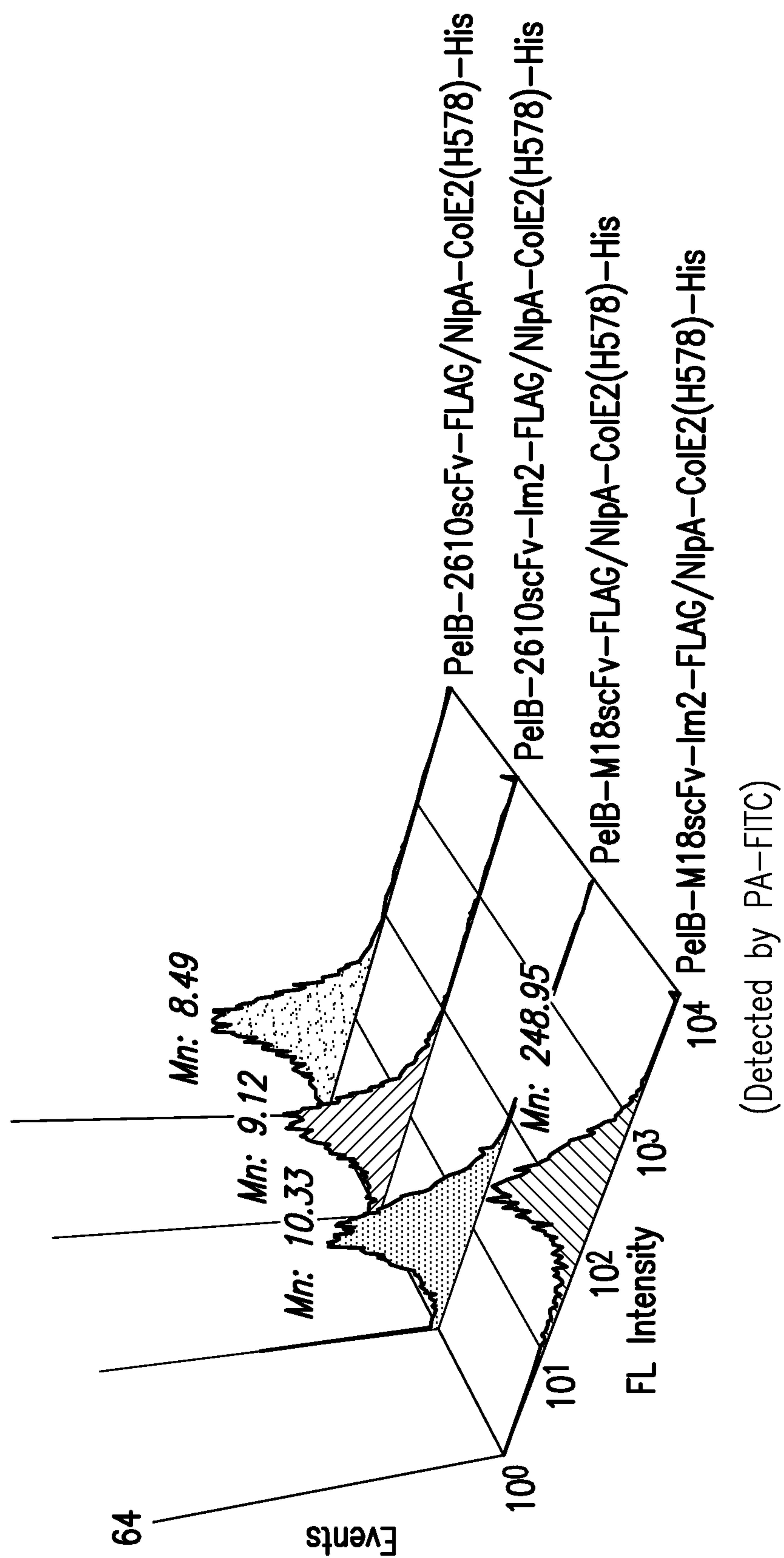


FIG. 4A

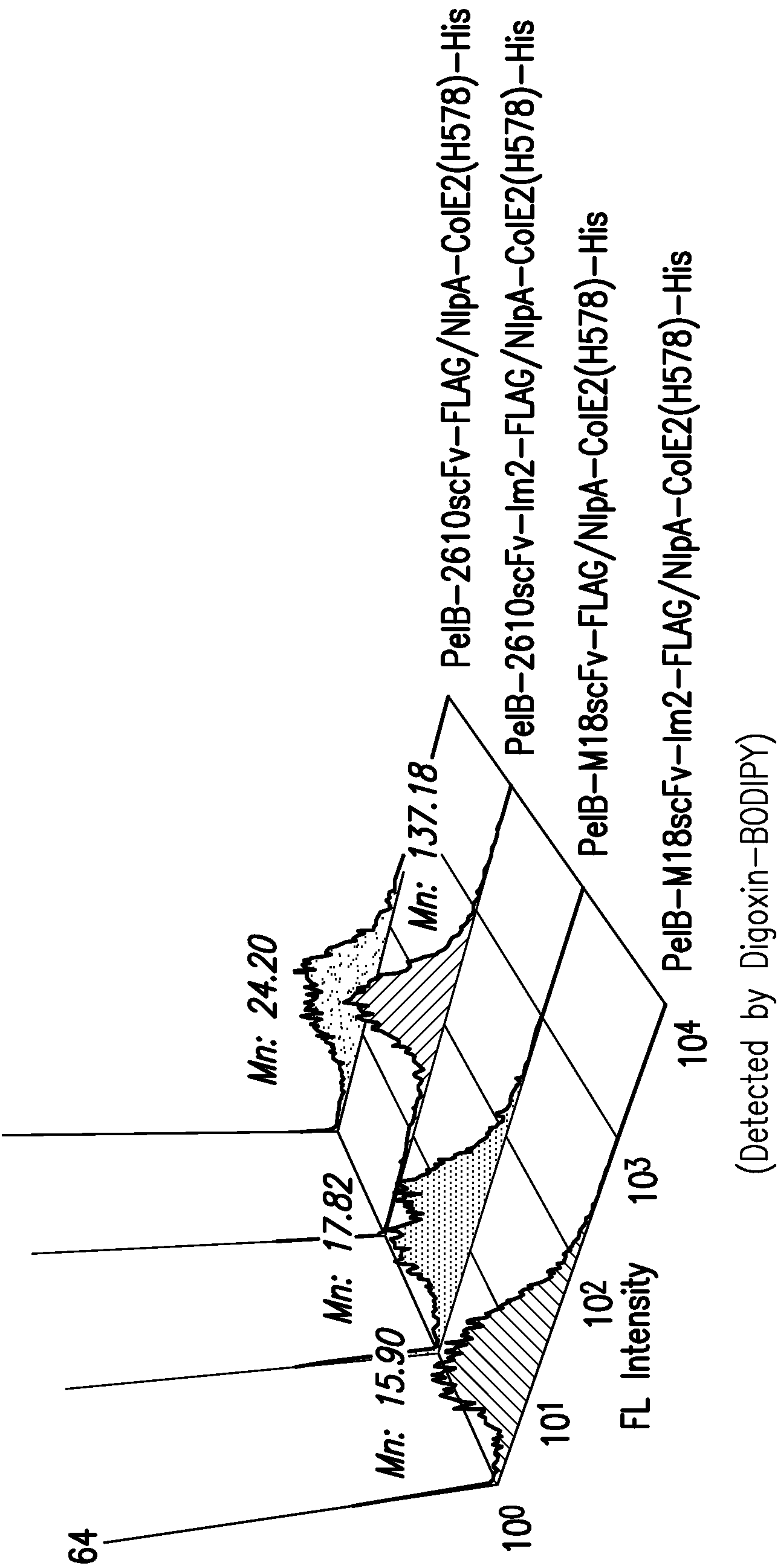


FIG.4B

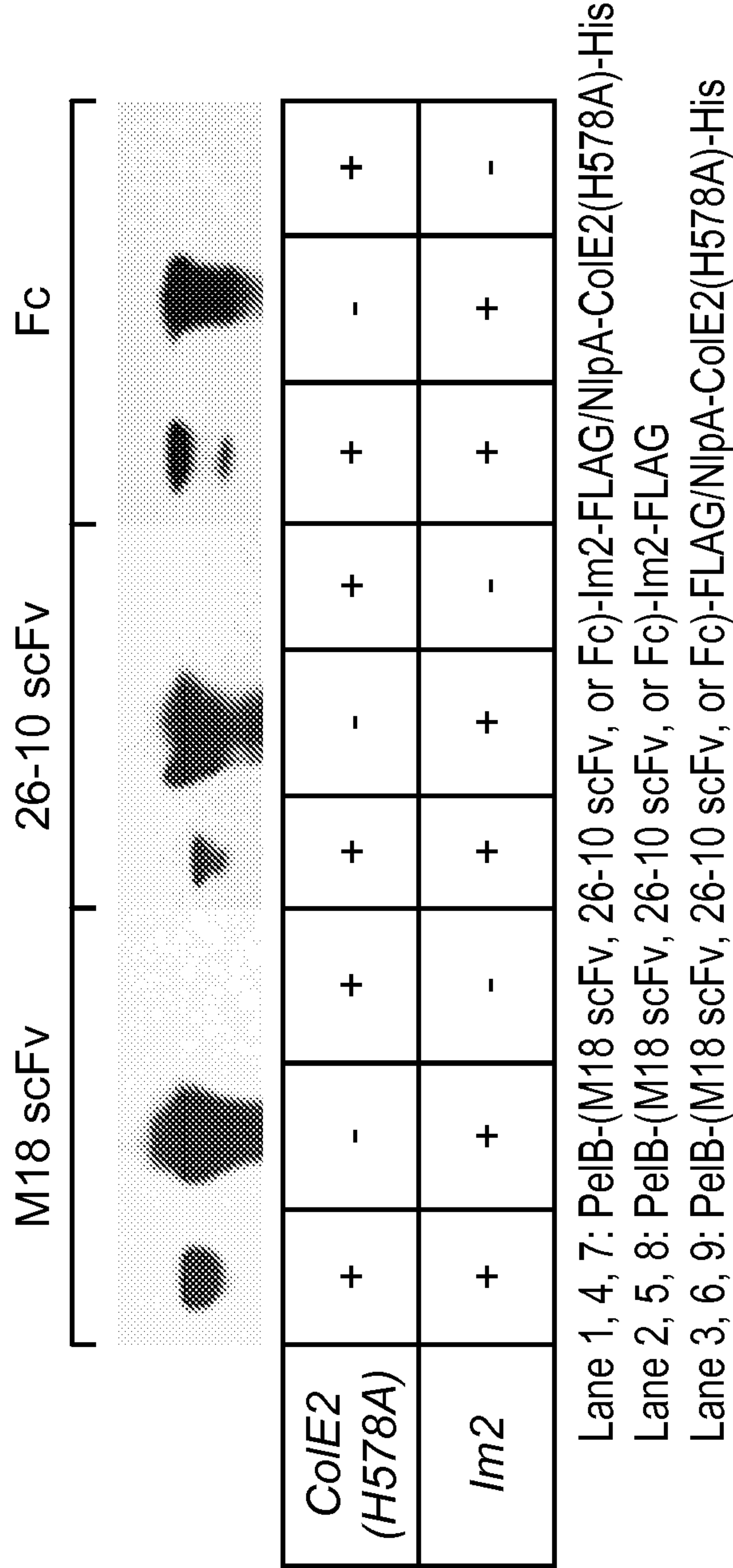


FIG.5

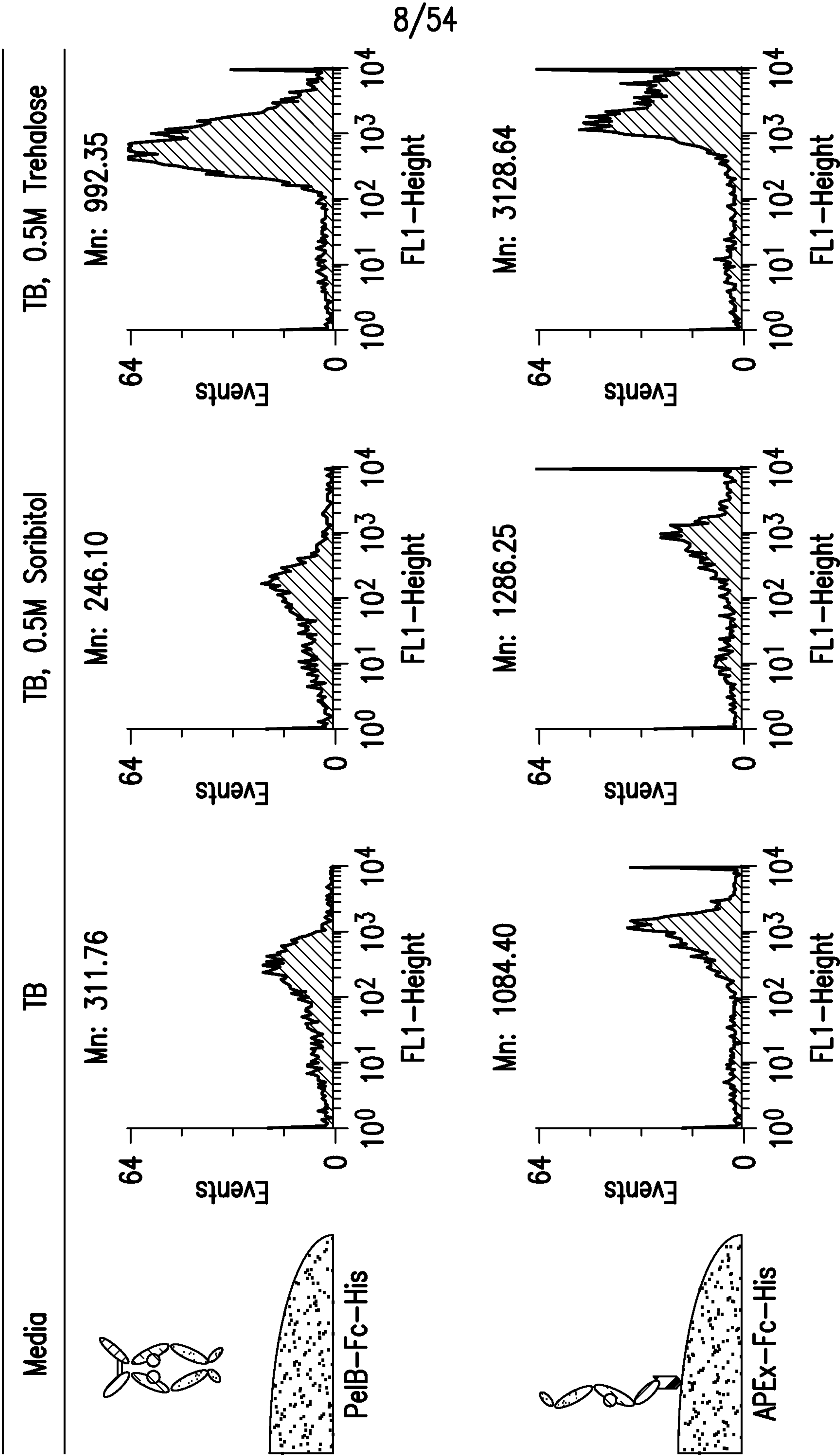
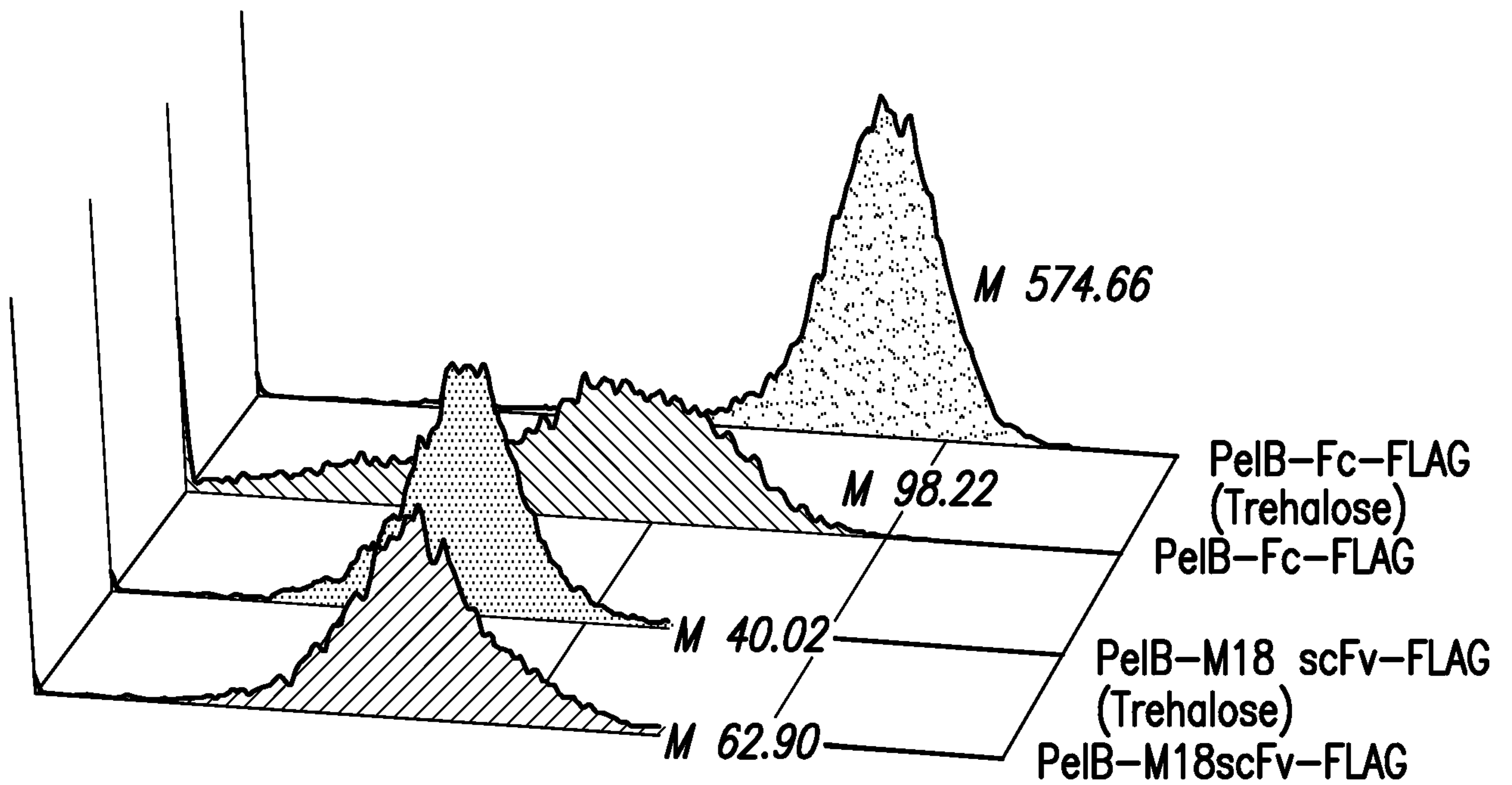


FIG.6

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PMT: 700V, Cultured in TB or TB w/0.5M Trehalose,
Labeling with Protein A-FITC

FIG.7

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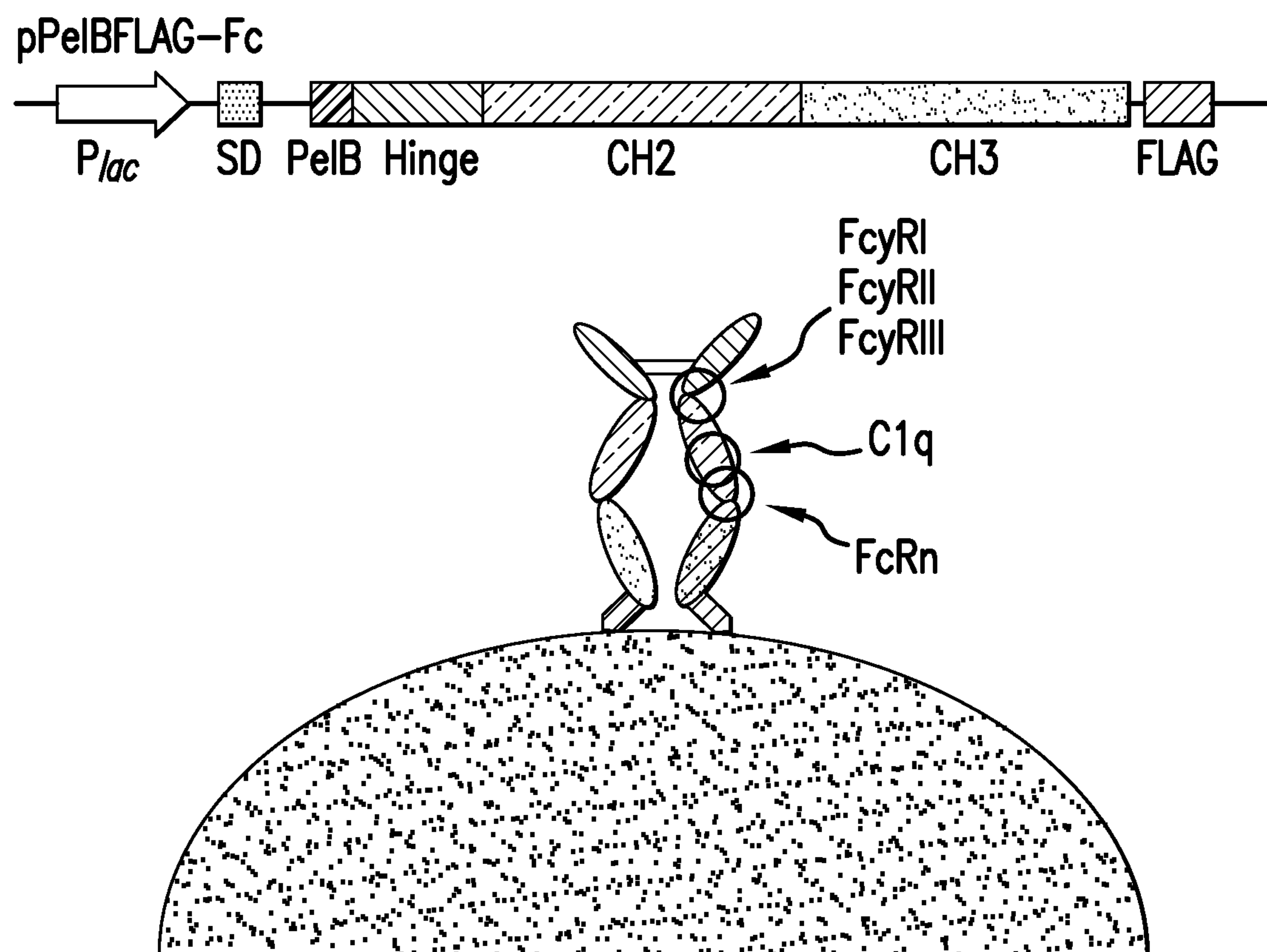
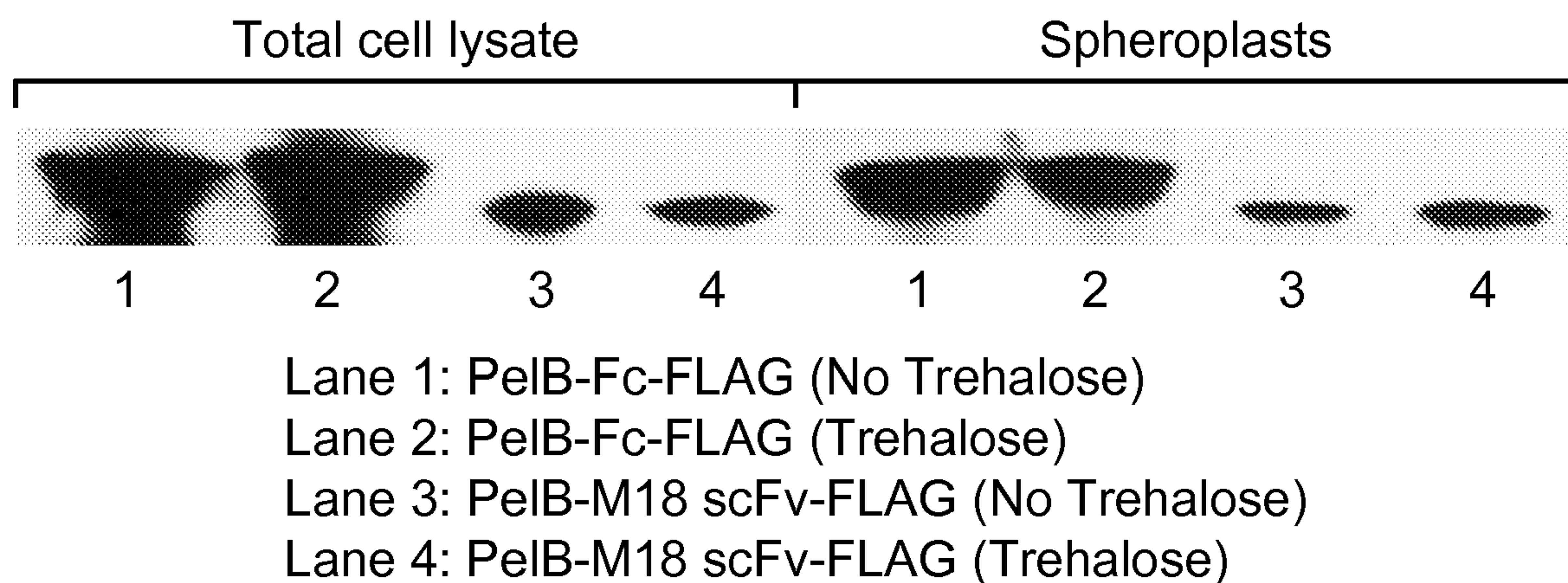
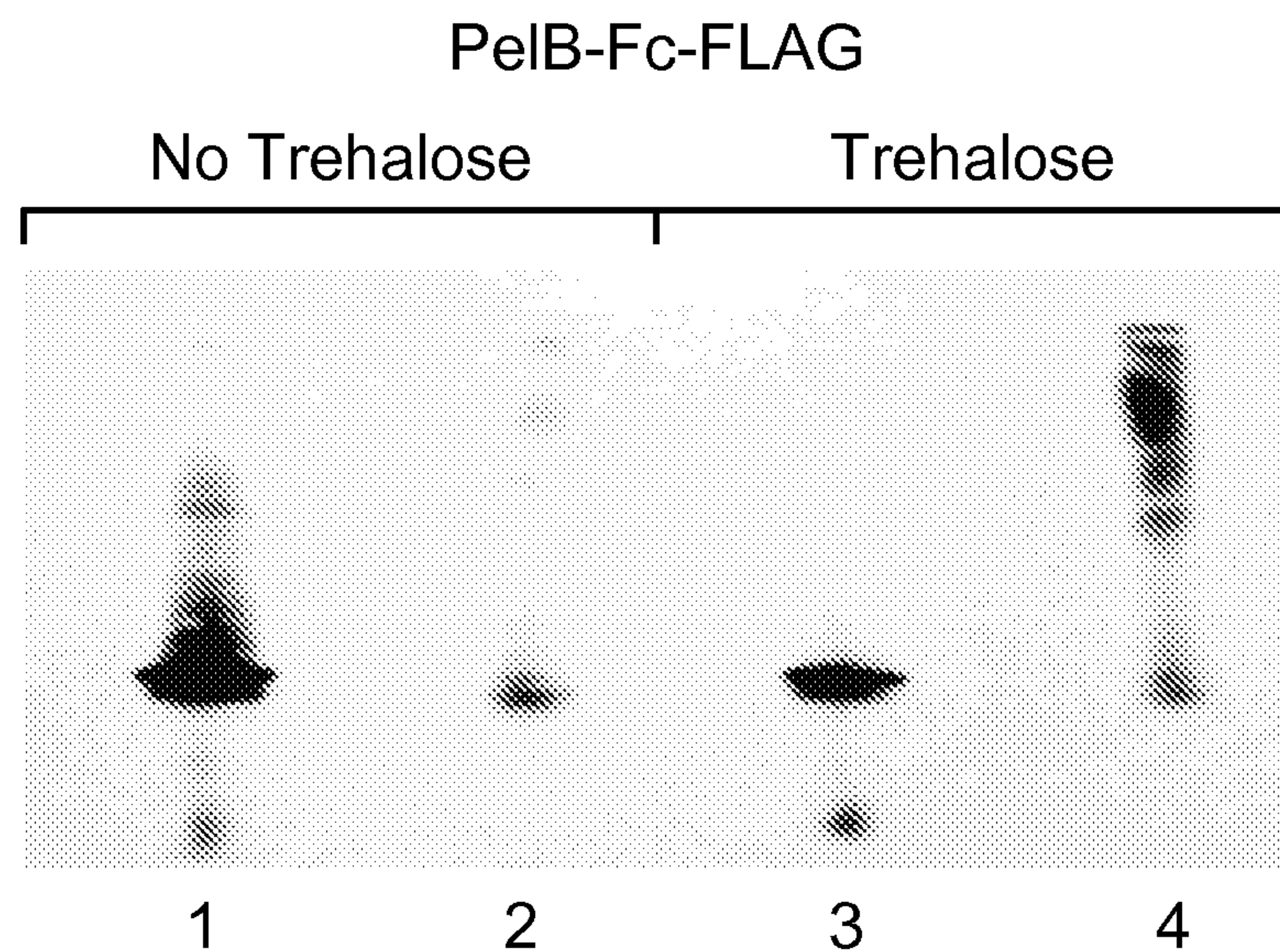


FIG.8

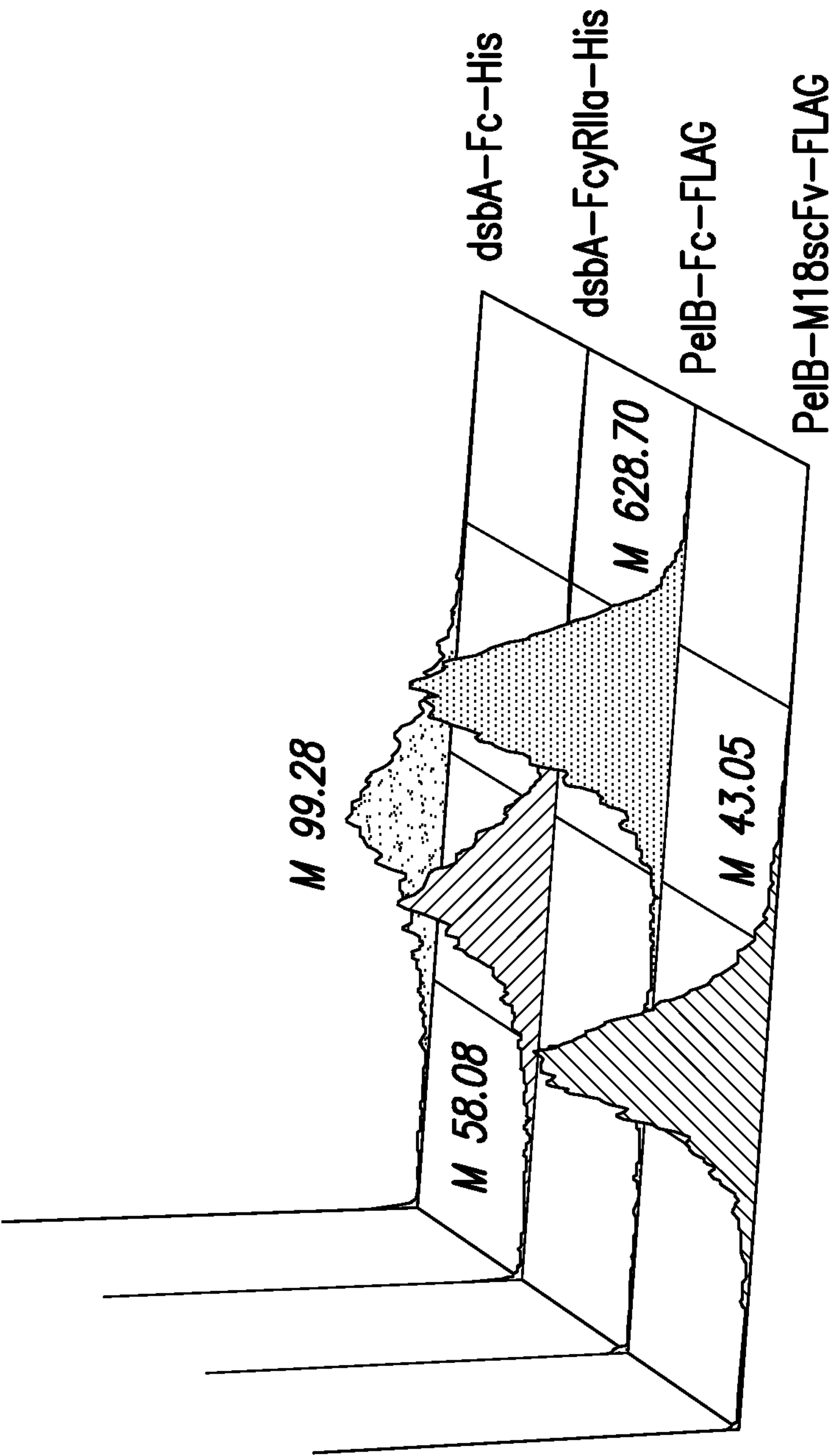
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**FIG.9A**

Lane 1,3: Reduced sample after spheroplasting
Lane 2,4: Not reduced sample after spheroplasting

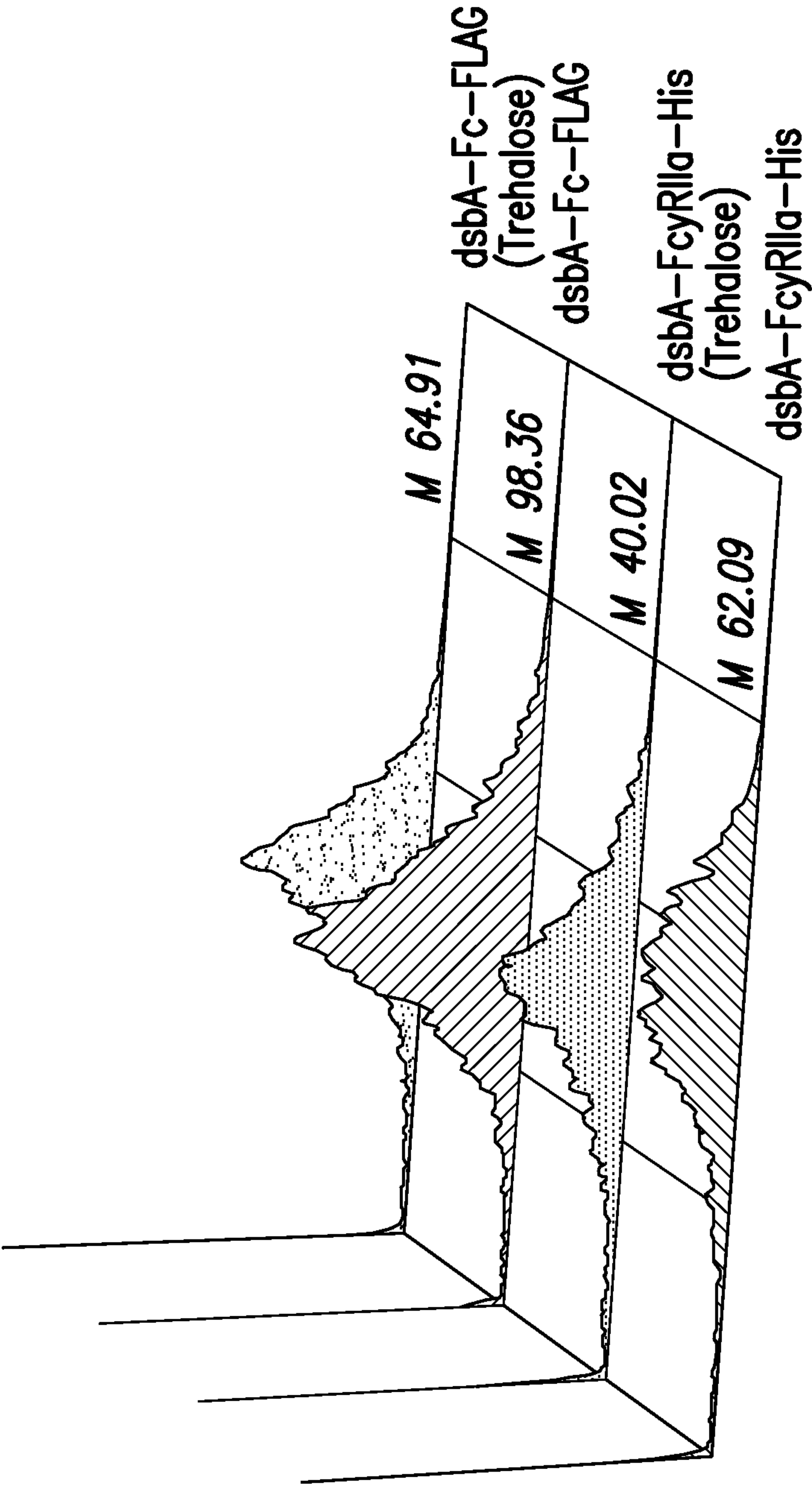
12.5 μ l/OD600 equivalent culture volume loading for each well

FIG.9B



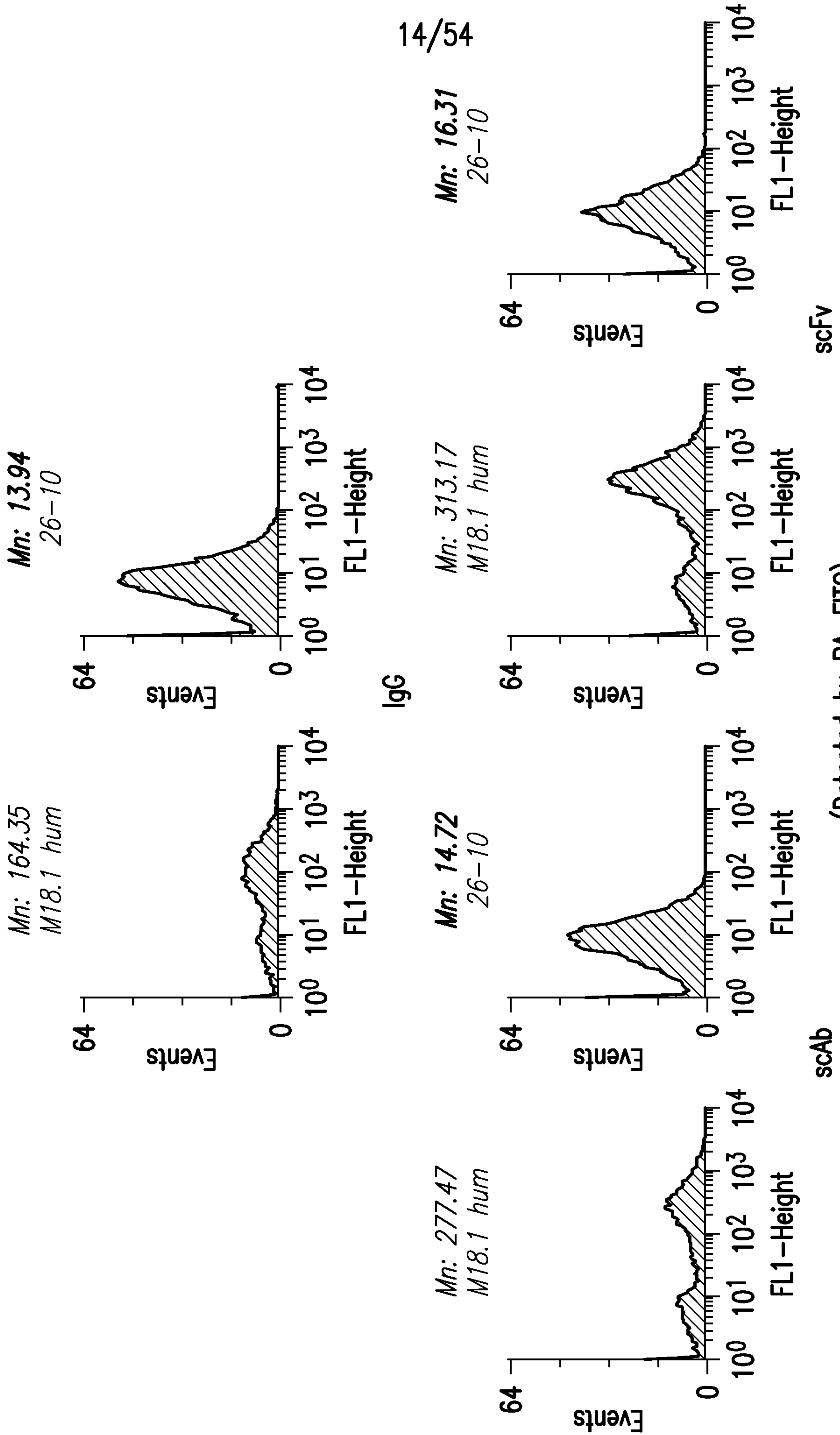
PMT: 700V, Cultured in TB w/0.5M Trehalose
Labeling with Protein A-FITC

FIG.10A



PMT: 700V, Cultured in TB or TB w/0.5M Trehalose,
Labeling with Protein A-FITC

FIG.10B



(Detected by PA-FITC)

FIG. 11

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Capture	Binding Protein
Human IgG Fc Fragments	FITC labeled FcR1a

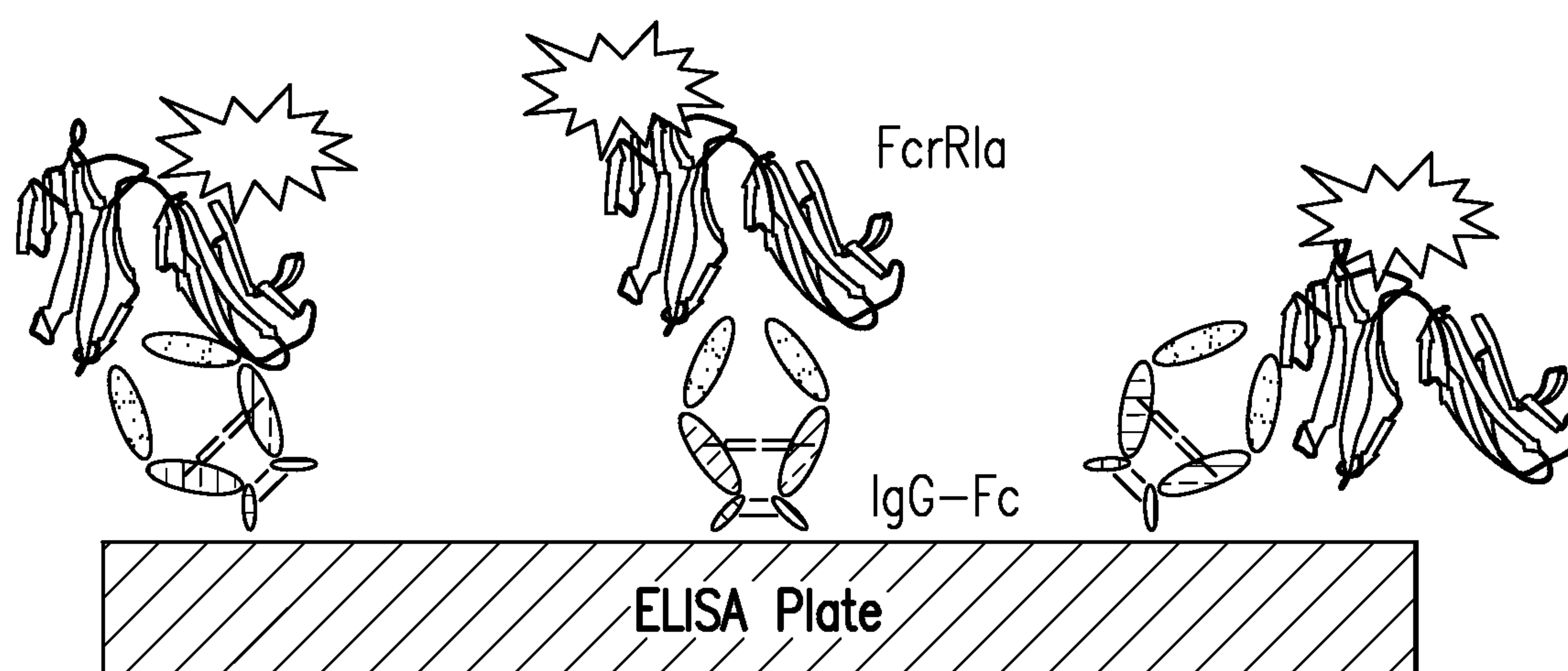


FIG.12A

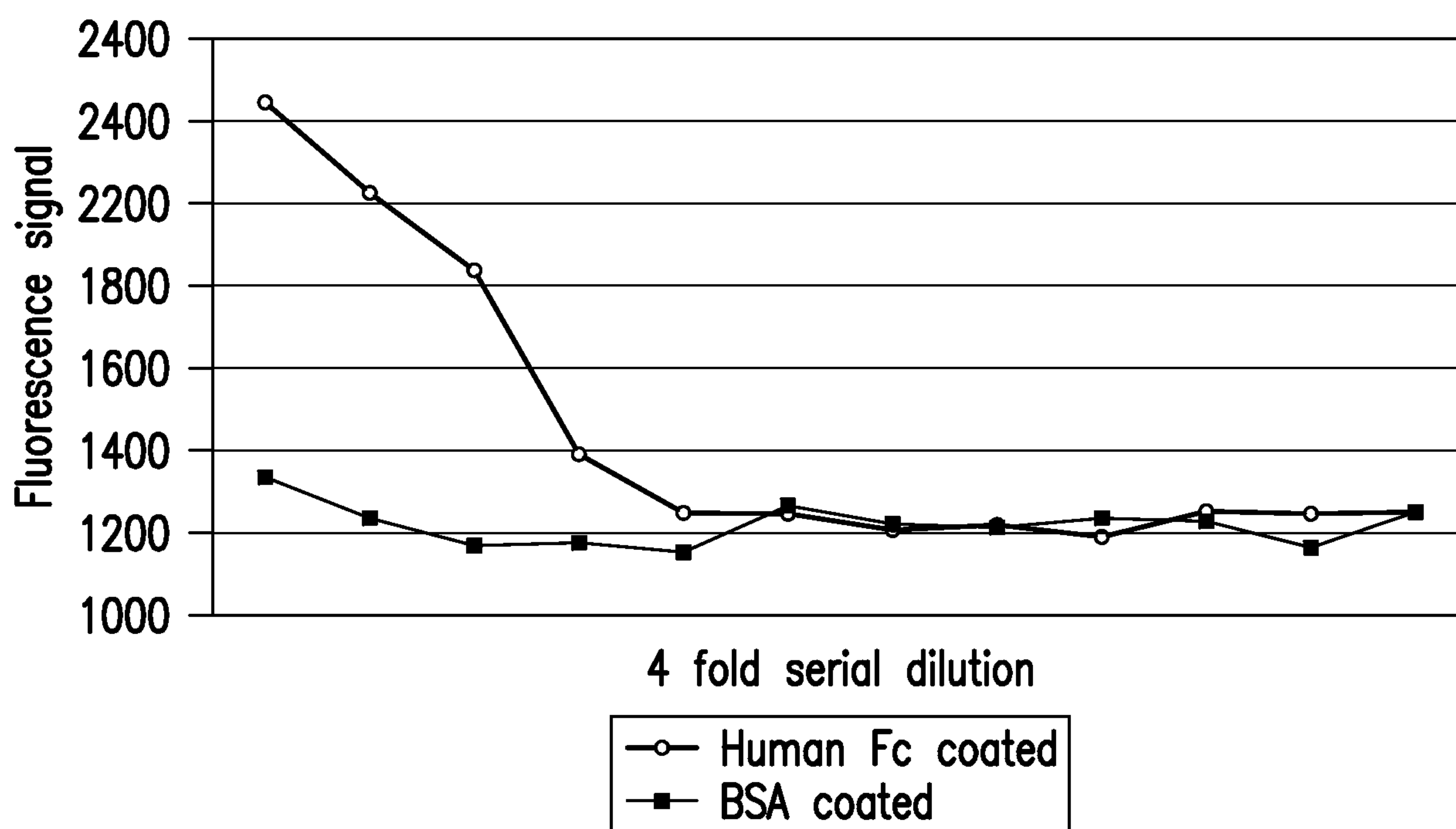
5.5 μ l FcR1a-FITC loading for the first wells

FIG.12B

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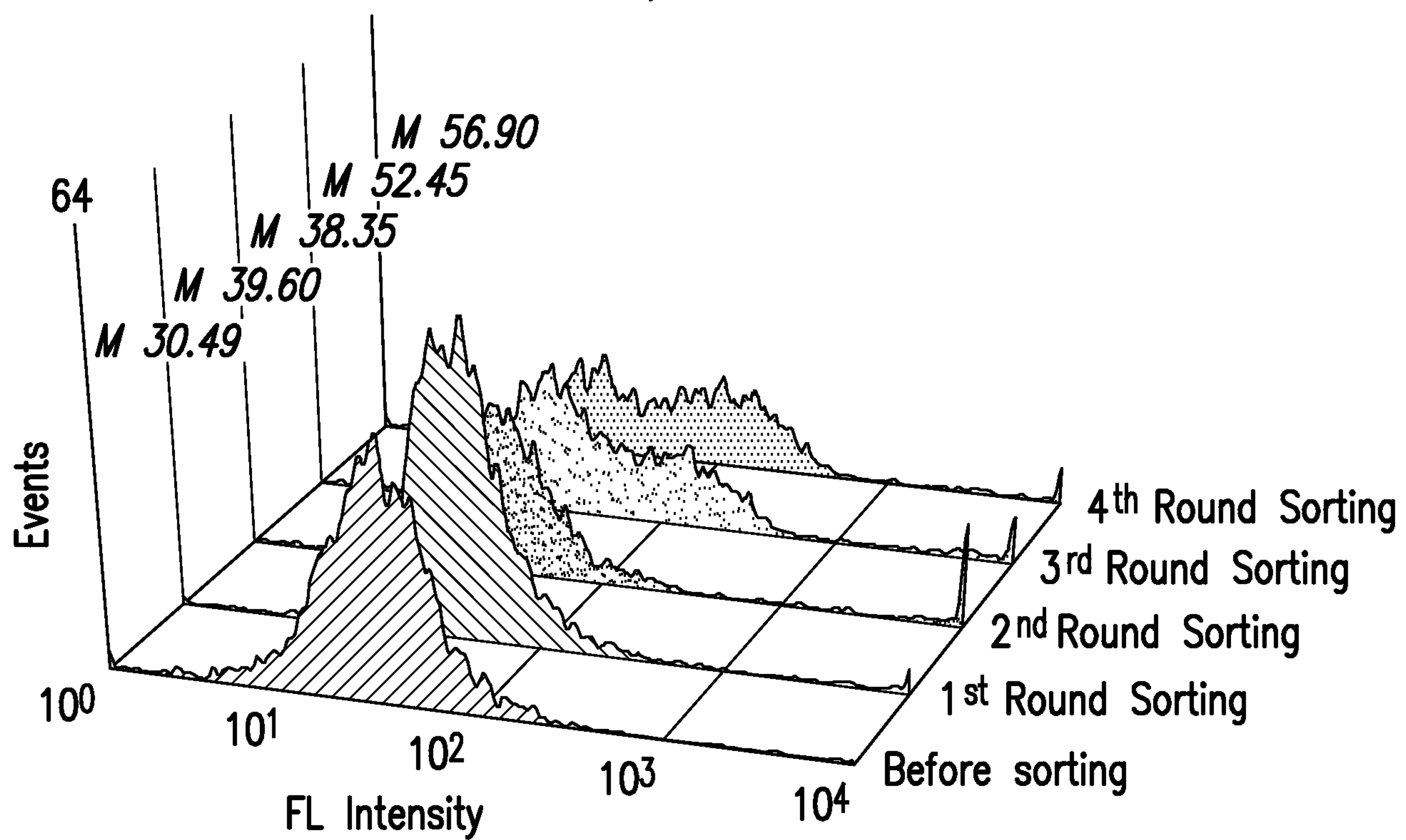


FIG.13A

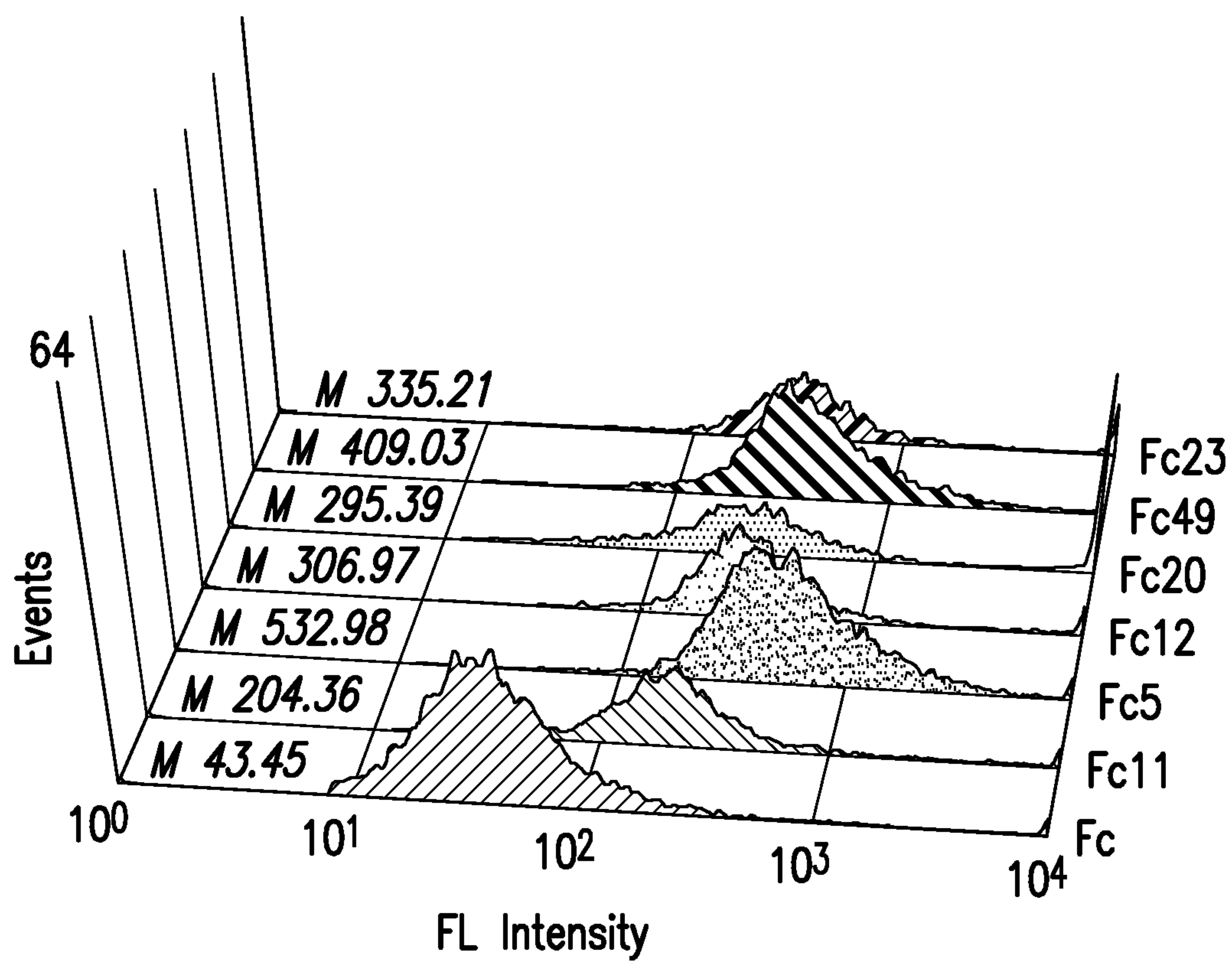


FIG.13B

	380	390	400	410	420	430	440	FLAG
IgG-Fc								
Fc11	E	W	S	N	G	Q	P	E
Fc5	E	W	S	N	G	Q	P	E
Fc12	E	W	S	N	G	Q	P	E
Fc20	E	W	S	N	G	Q	P	E
Fc49	E	W	S	N	G	Q	P	E
Fc23	E	W	S	N	G	Q	P	E

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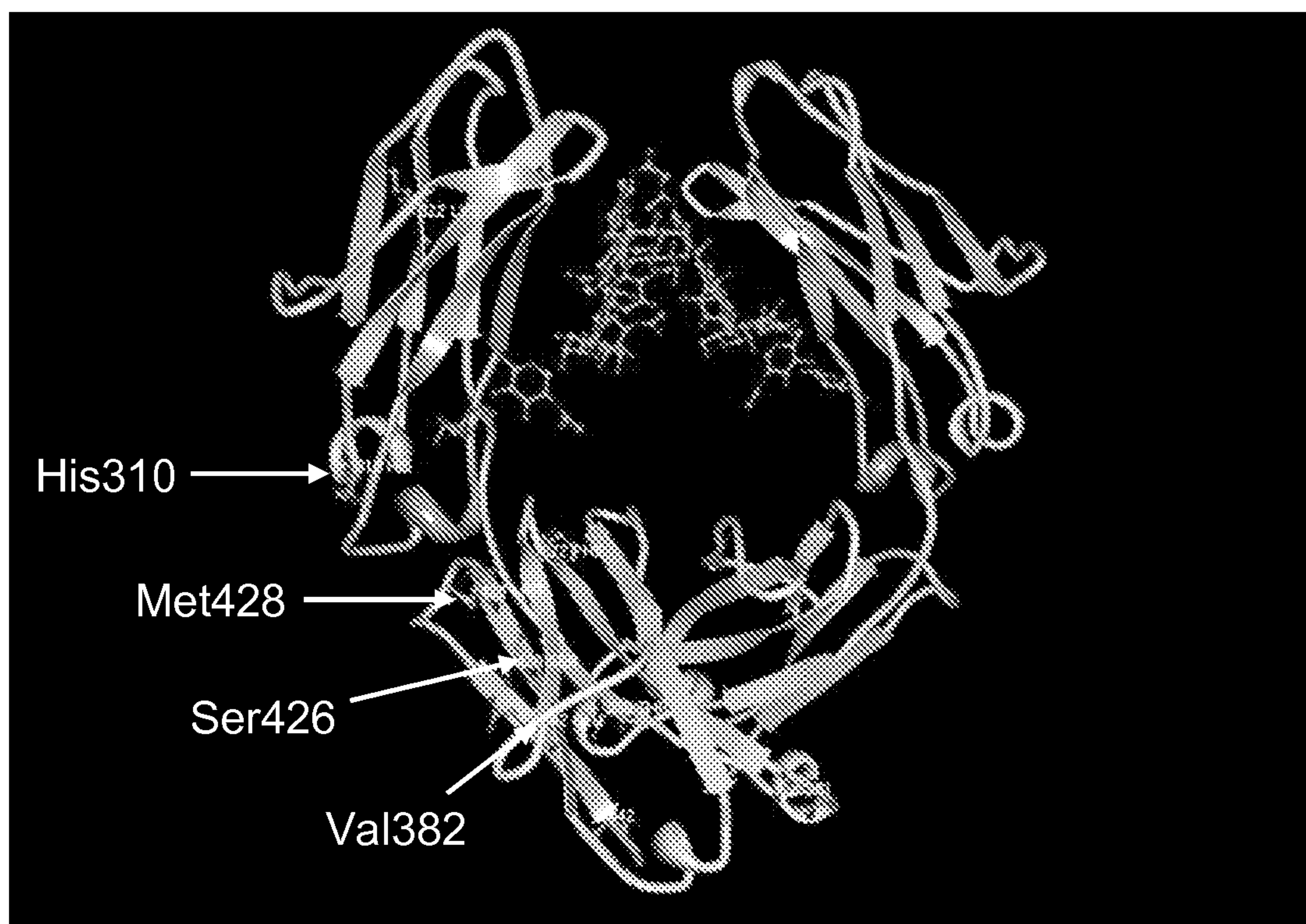


FIG.15A

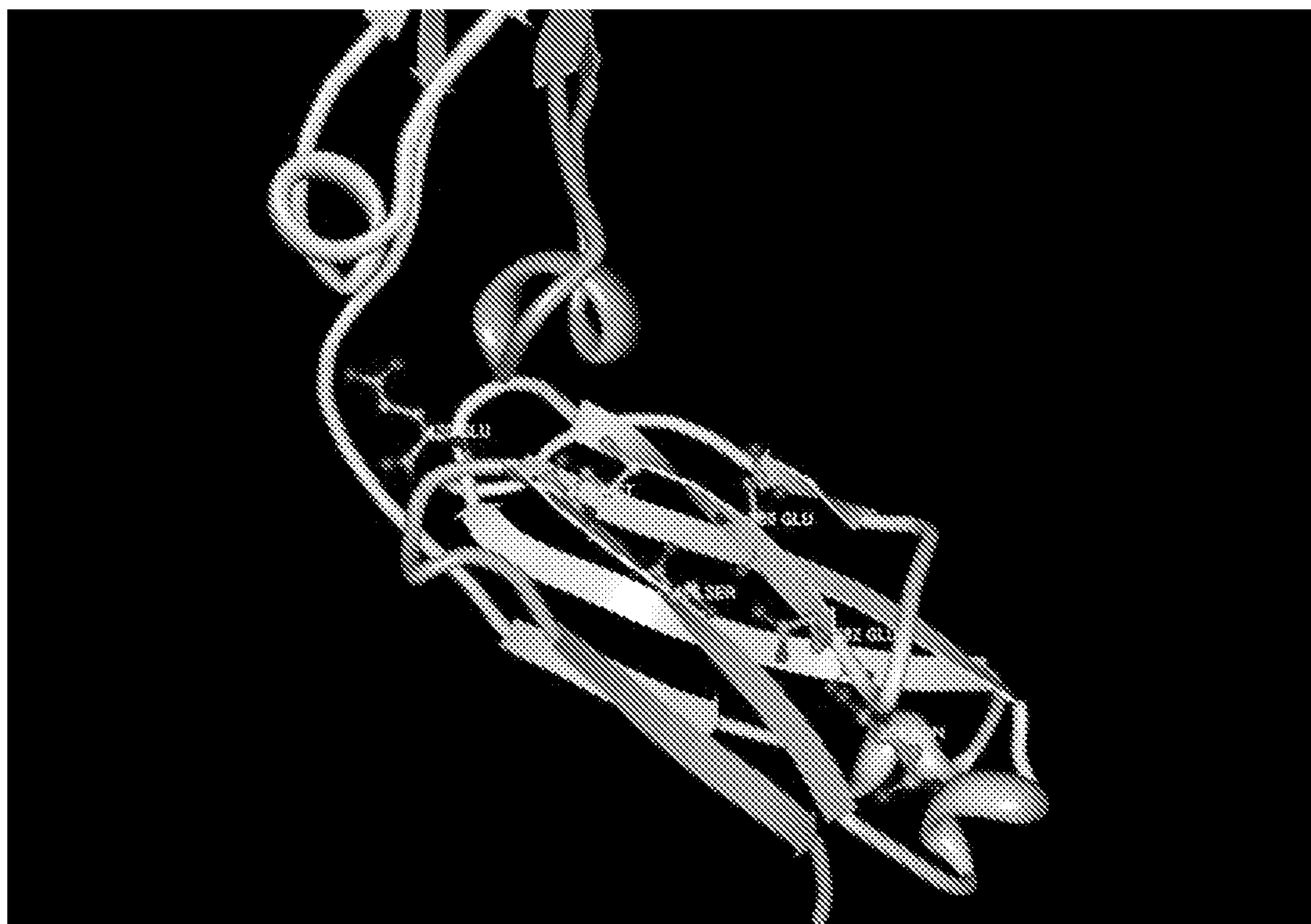


FIG.15B

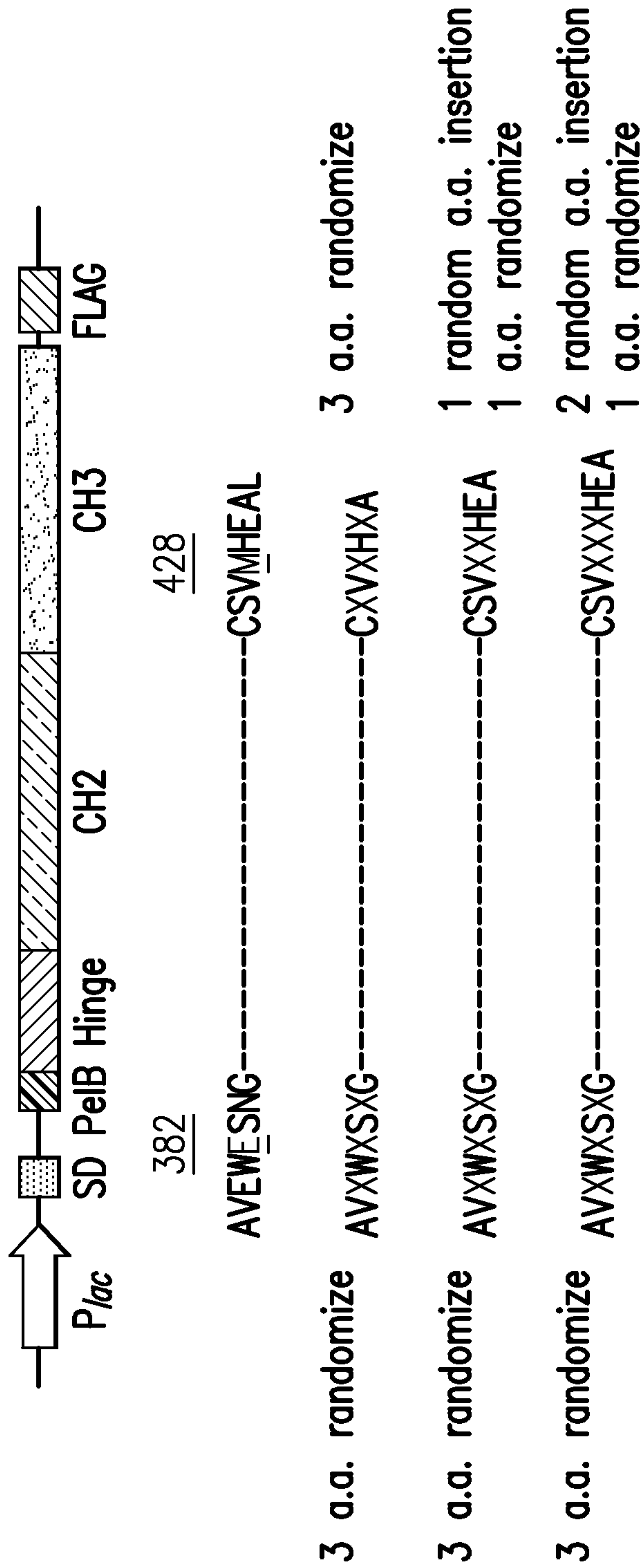


FIG.16

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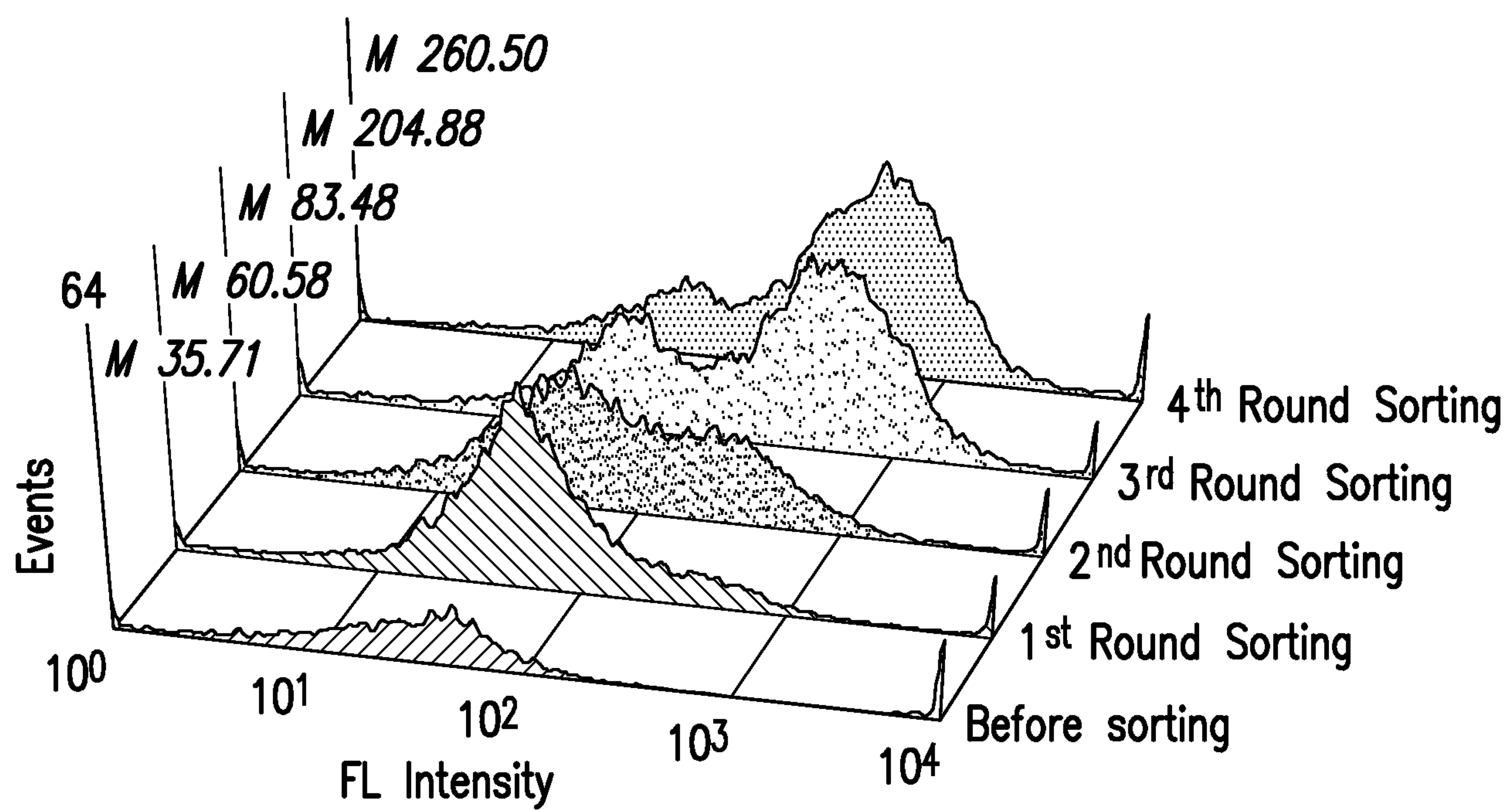


FIG.17

Hinge		230	240	250	260	270	280	290	300
FcWT	(16.39)	[DKTHTCPPC]	PAPELLGGPSVFLFPPKPKDILMISRTPEVTCWVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV						
Fc104	(87.17)	[-----]							
Fc107	(316.58)	[-----]							
Fc101	(521.92)	[-----]							
Fc147	(464.35)	[-----R]							
Fc102	(373.63)	[-----]							
Fc114	(370.26)	[-----]							
Fc117	(390.62)	[-----]							
Fc151	(325.53)	[-----]							
Fc143	(211.23)	[-----]	S						
Fc152	(172.61)	[-----R]							
Fc149	(139.57)	[-----]							
Fc106	(268.78)	[-----]							
Fc100	(137.93)	[-----]							

1) Fc101 = Fc103, 111, 115, 116, 125, 136, 141, 146, 148; FACS mean values are indicated in the parenthesis

FIG.18A

		310	320	330	340	350	360	370	380
		CH3							
FcWT	(16.39)	SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK	[GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP						
Fc104	(87.17)	[-----R]	-----	-----	-----	-----	-----	-----	-----
Fc107	(316.58)	[-----]	-----	-----	-----	-----	-----	-----	V-----
Fc101	(521.92)	[-----]	-----	-----	-----	-----	-----	-----	V-----
Fc147	(464.35)	[-----]	-----	-----	-----	-----	-----	-----	V-----
Fc102	(373.63)	[-----]	-----	-----	-----	-----	D-----	-----	V-----
Fc114	(370.26)	[-----]	-----	-----	-----	-----	S-----	-----	V-----
Fc117	(390.62)	[-----]	-----	-----	-----	-----	-----	-----	V-----
Fc151	(325.53)	[-----]	-----	-----	-----	-----	-----	-----	V-----
Fc143	(211.23)	[-----]	-----	-----	-----	-----	-----	-----	V-----
Fc152	(172.61)	[-----]	-----	-----	-----	-----	-----	-----	V-----
Fc149	(139.57)	[-----]	-----	-----	-----	-----	-----	-----	D-V-R-----
Fc106	(268.78)	[-----]	-----	-----	-----	-----	-----	-----	T-D-----
Fc100	(137.93)	[-----]	-----	-----	-----	-----	-----	-----	T-M-E-----

1) Fc101 = Fc103, 111, 115, 116, 125, 136, 141, 146, 148; FACS mean values are indicated in the parenthesis

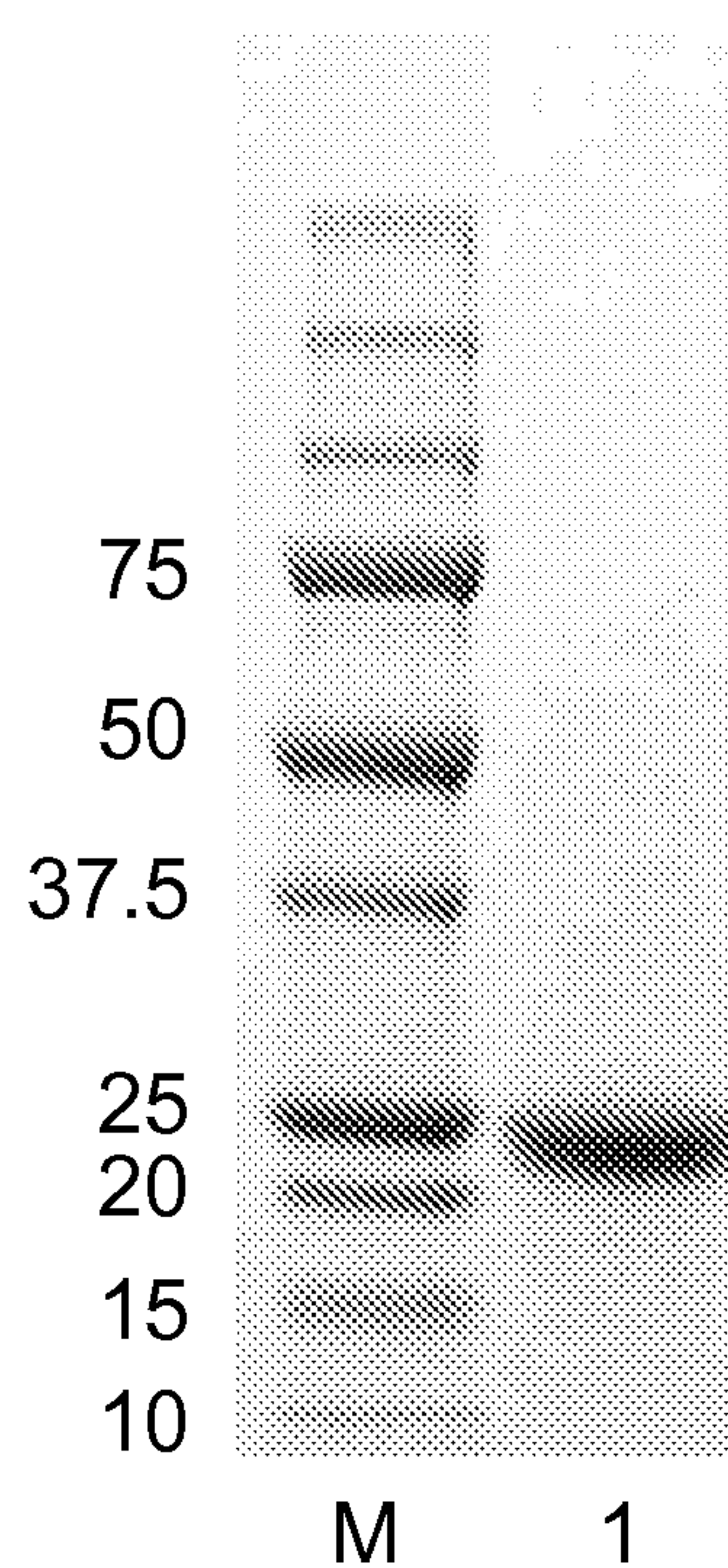
FIG.18B

FcWT	(16.39)	390	400	410	420	430	440
Fc104	(87.17)	ENNYKTTTPPVLDSDGSFFLYSKLTVD	SKRWQQGNVFS	SCSVMHEALHNHYTQKSLSPGK]	I-----		
Fc107	(316.58)						
Fc101	(521.92)				I-----		
Fc147	(464.35)				I-----		
Fc102	(373.63)				I-----		
Fc114	(370.26)				I-----		
Fc117	(390.62)				I-----	A-----	
Fc151	(325.53)				I-L-S-----		
Fc143	(211.23)				V-L-H-----		
Fc152	(172.61)				T-S-P-----		
Fc149	(139.57)				V-L-D-----		
Fc106	(268.78)				I-----		
Fc100	(137.93)						

1) Fc101 = Fc103, 111, 115, 116, 125, 136, 141, 146, 148; FACS mean values are indicated in the parenthesis

FIG.18C

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Lane M: Molecular weight marker
Lane 1: Purified FcrRIIIa (23.45 kDa)

FIG.19

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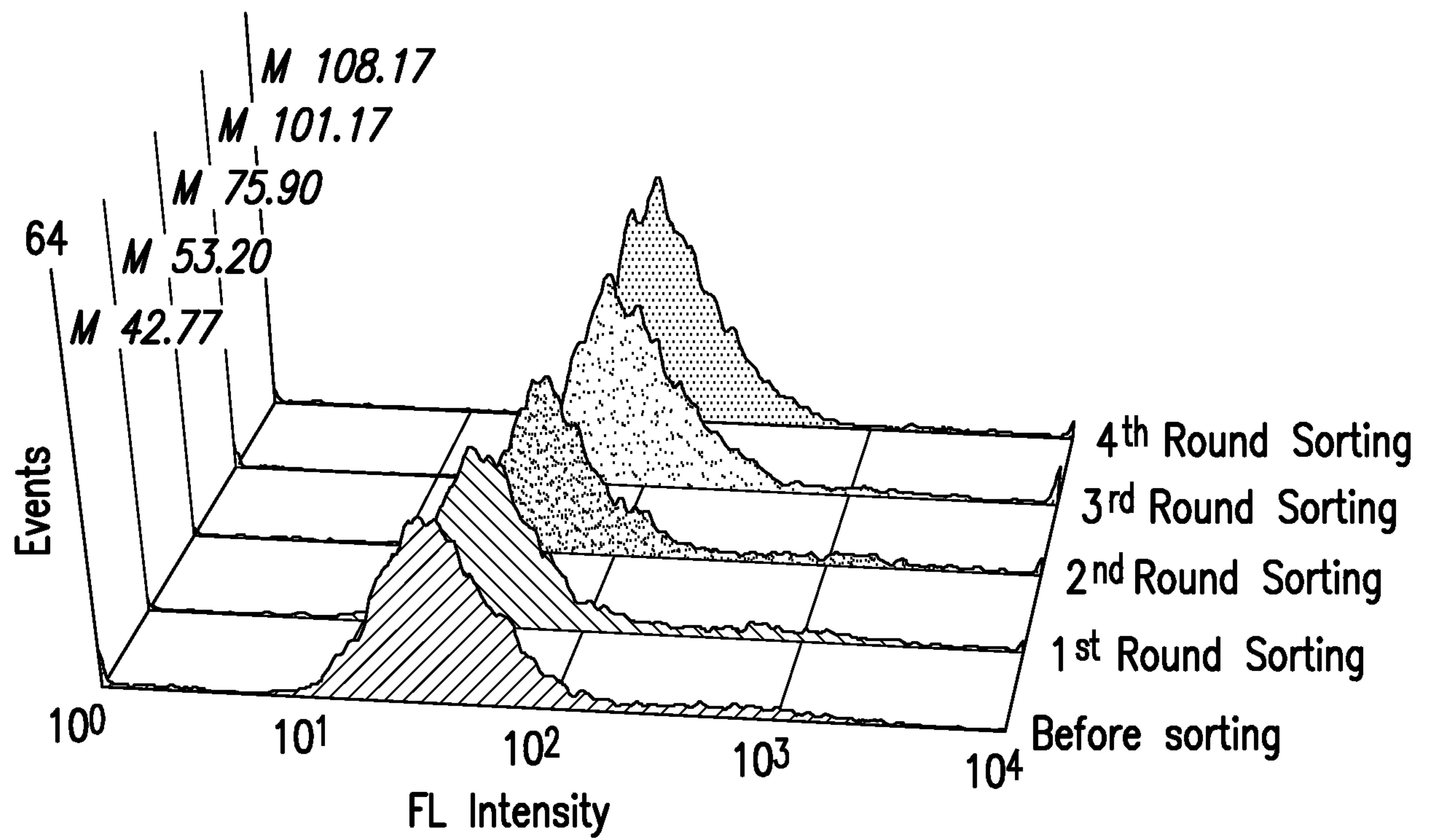


FIG.20

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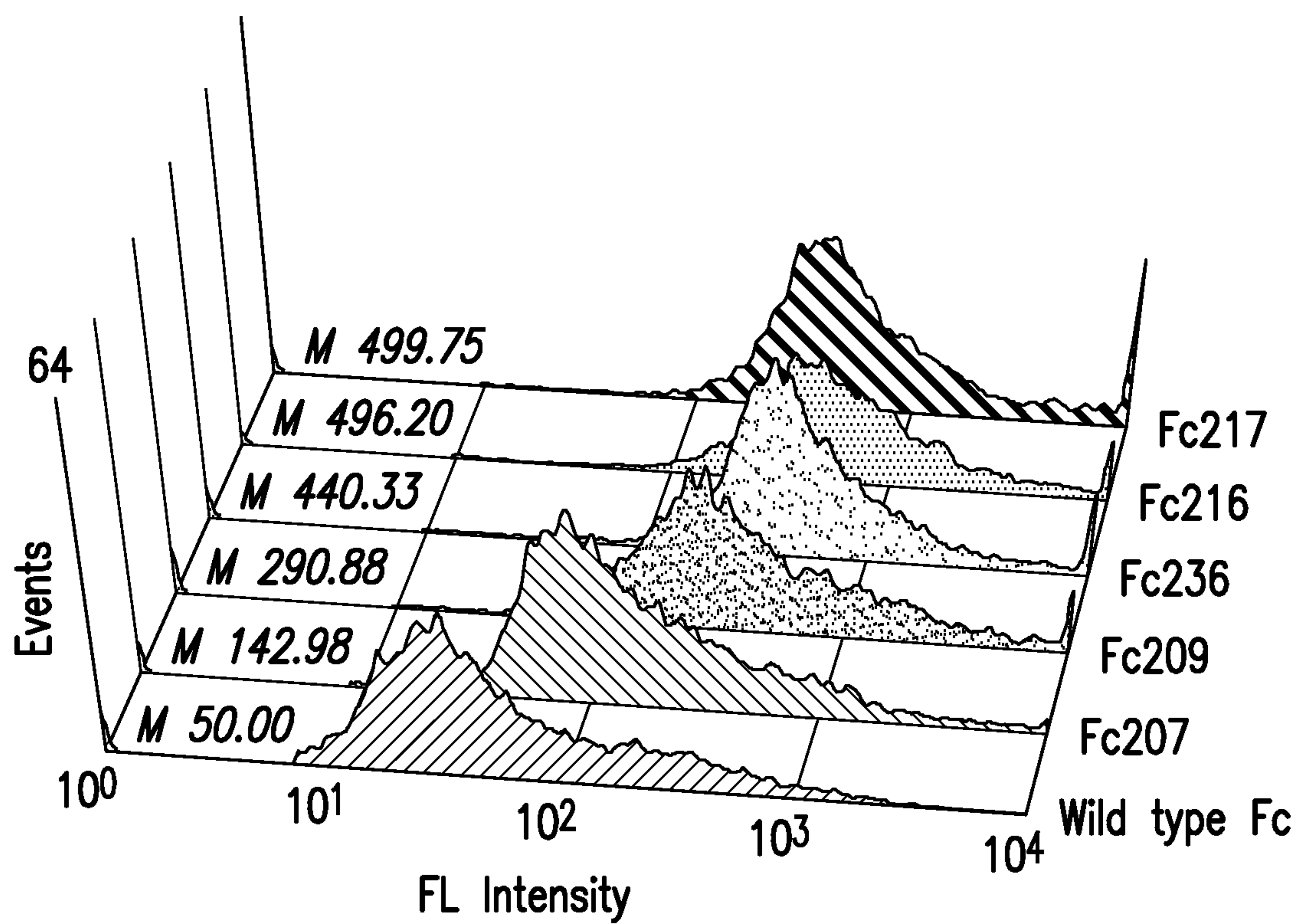


FIG.21

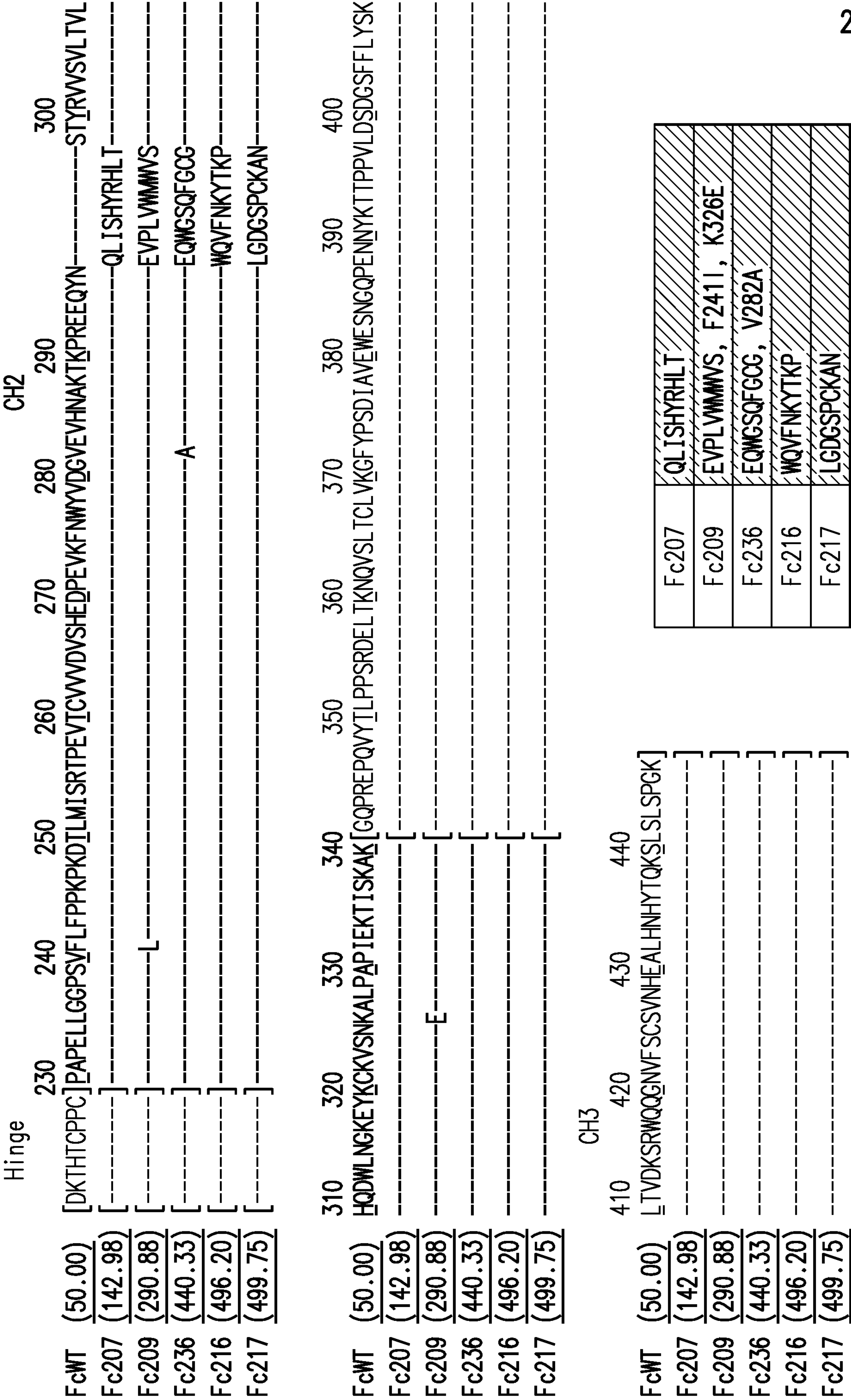


FIG.22

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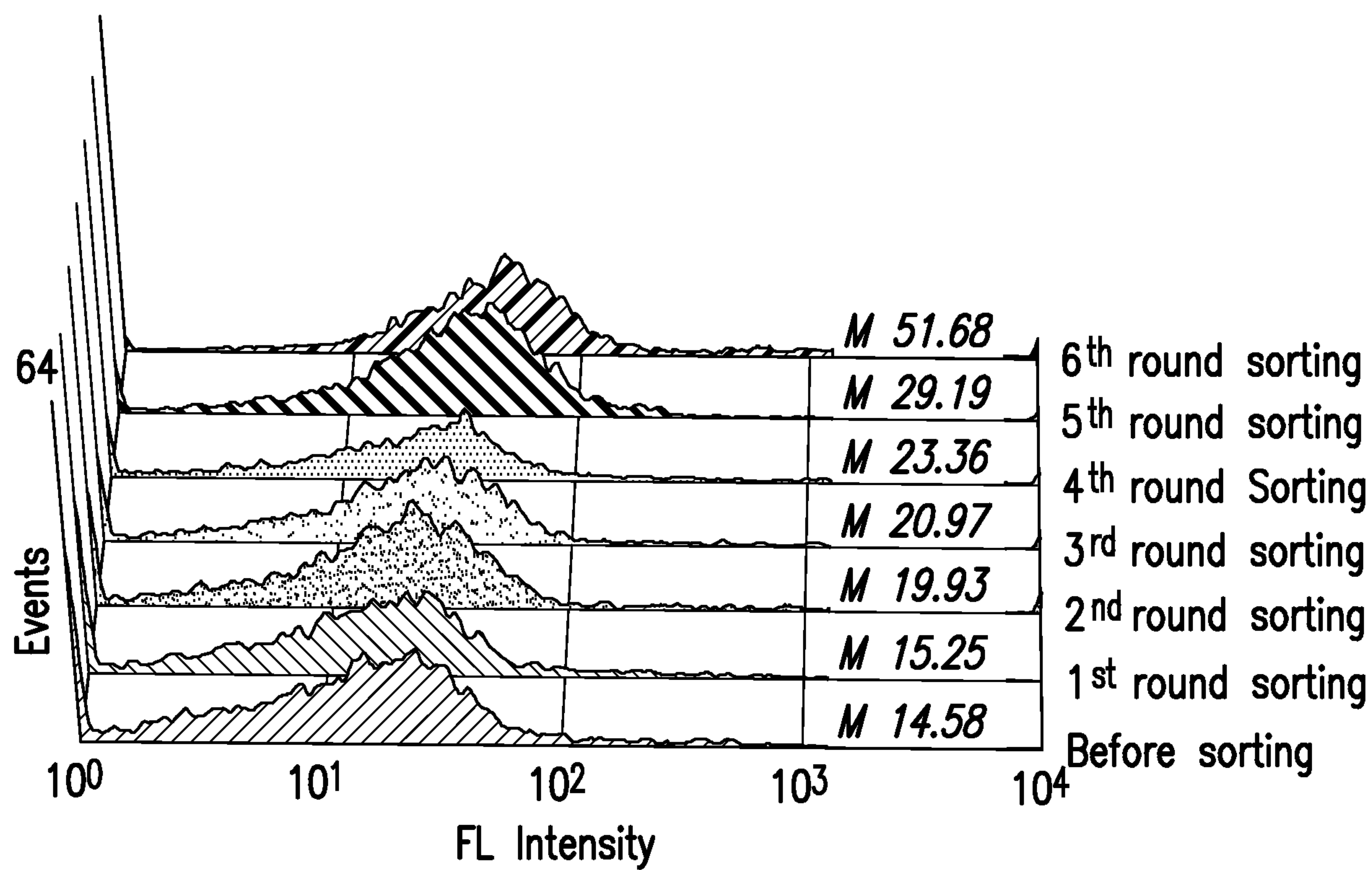


FIG.23

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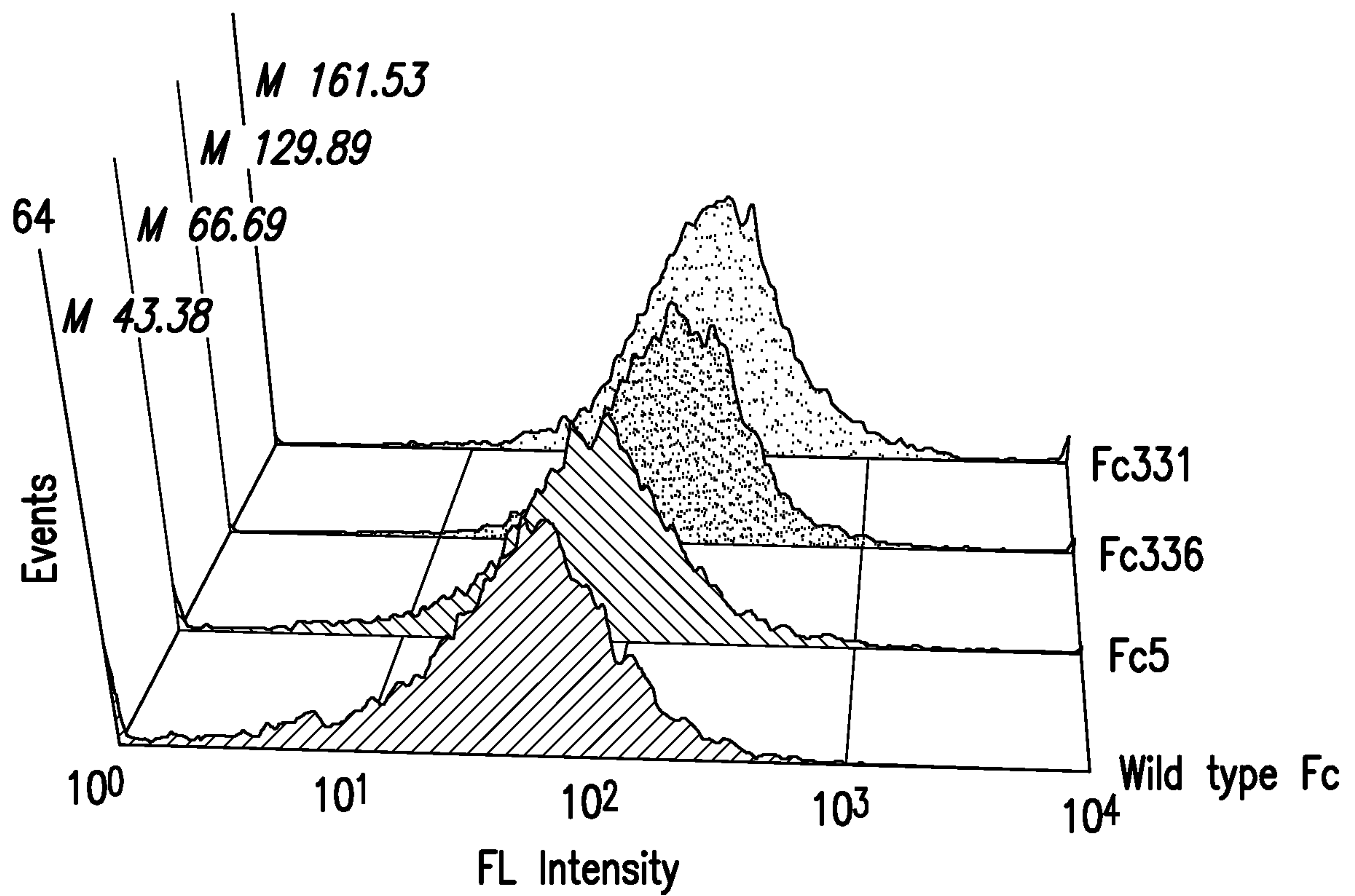
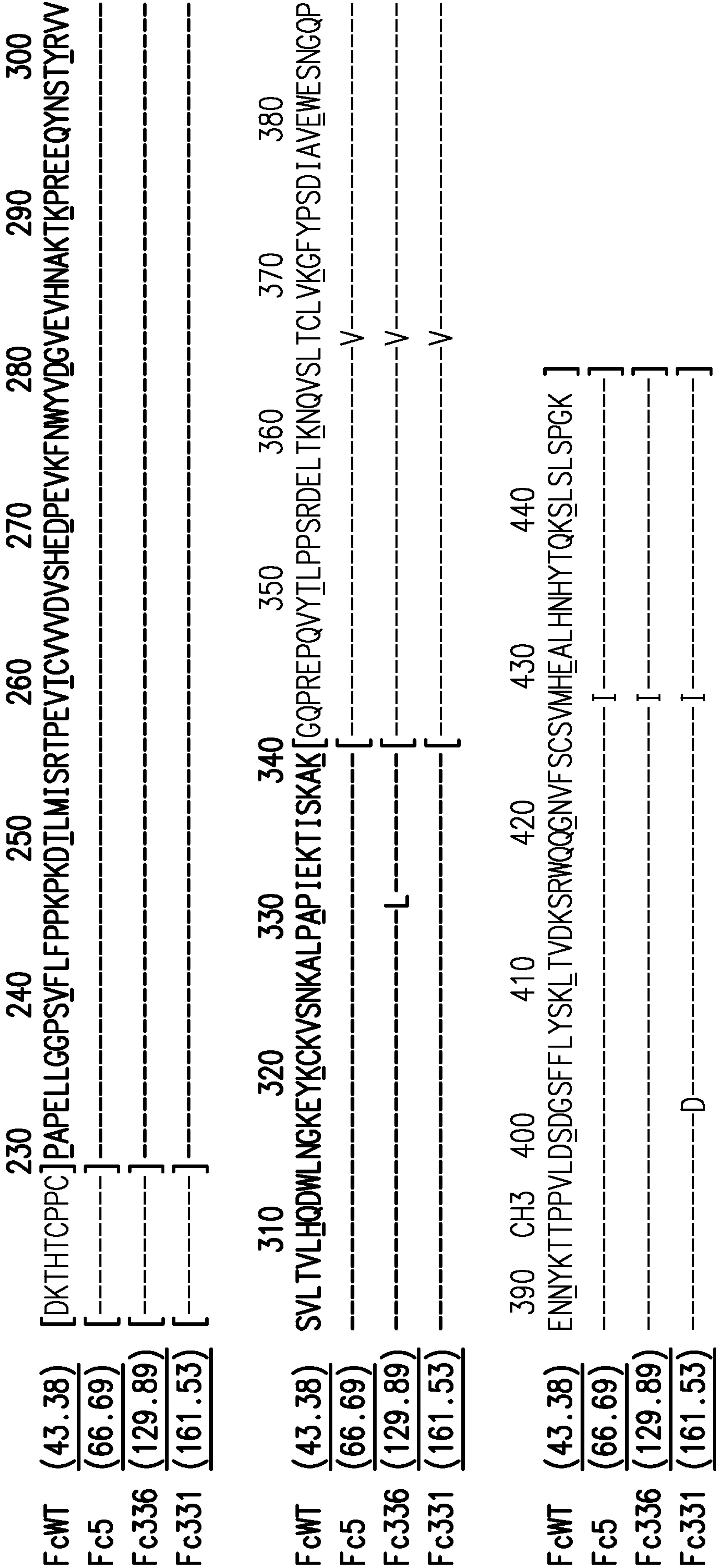


FIG.24



Fc5	E382V, M428I
Fc336	E382V, P331L, M428I
Fc331	E382V, G402D, M428I

FIG.25

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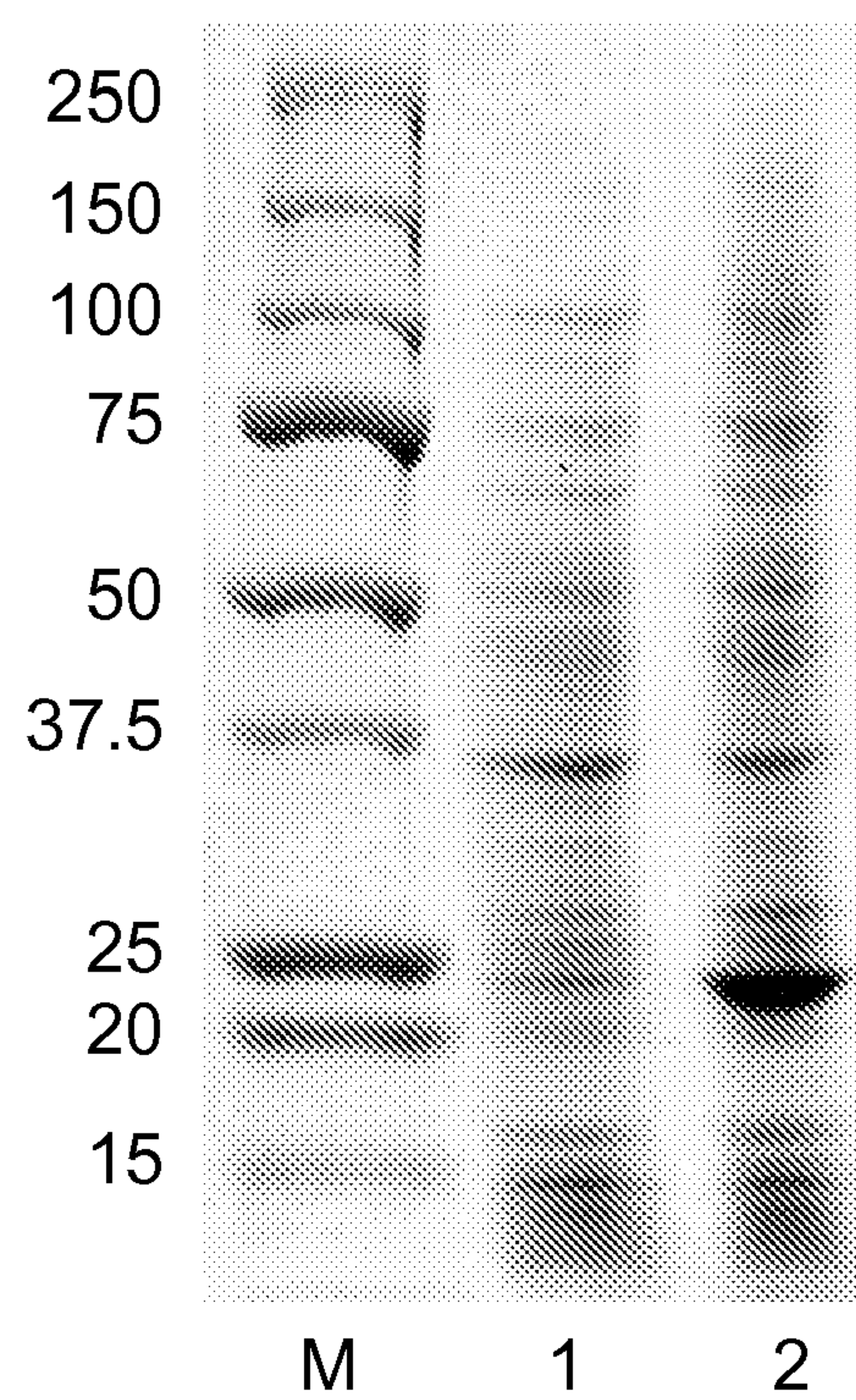


FIG.26

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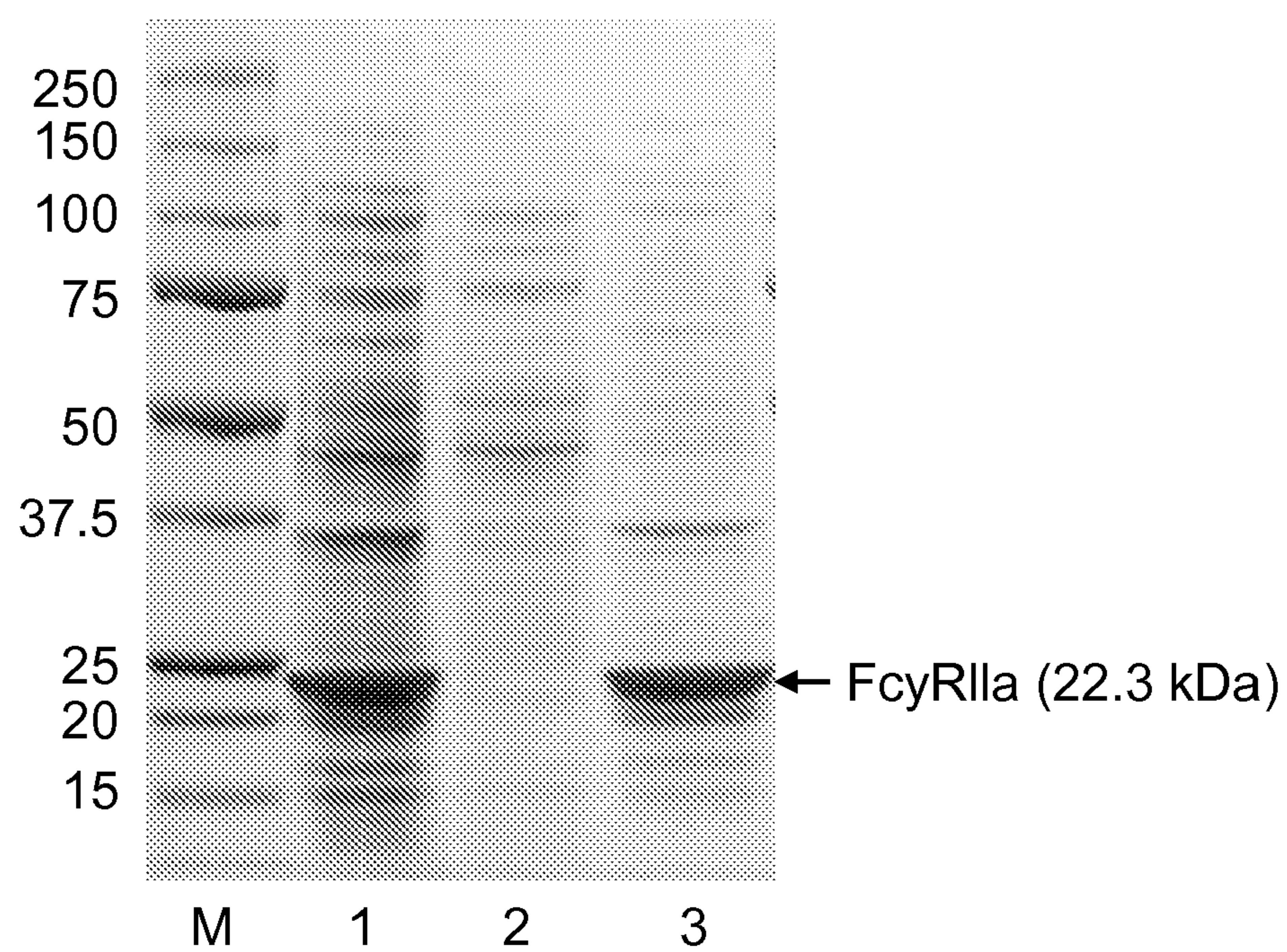


FIG.27

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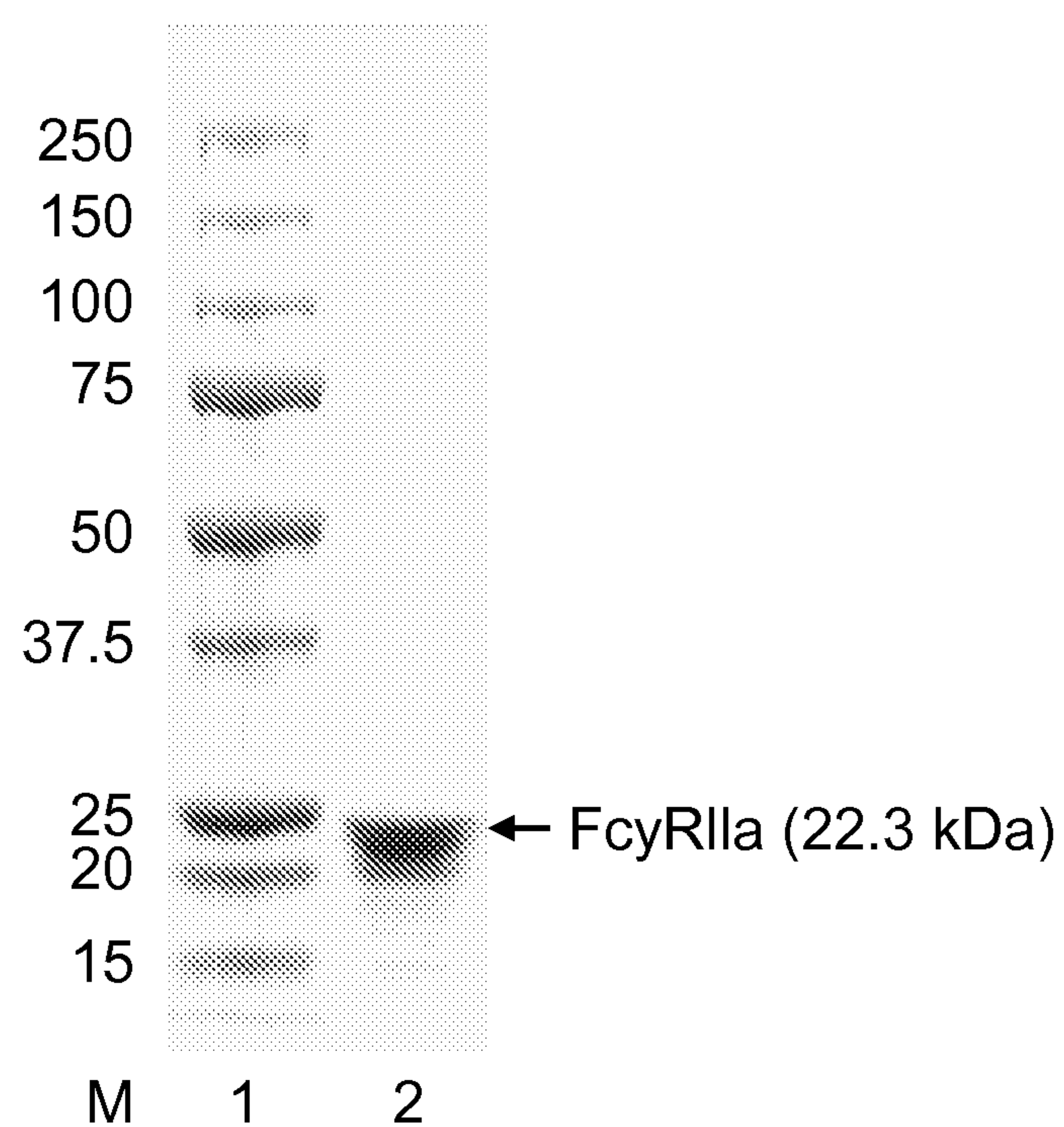


FIG.28

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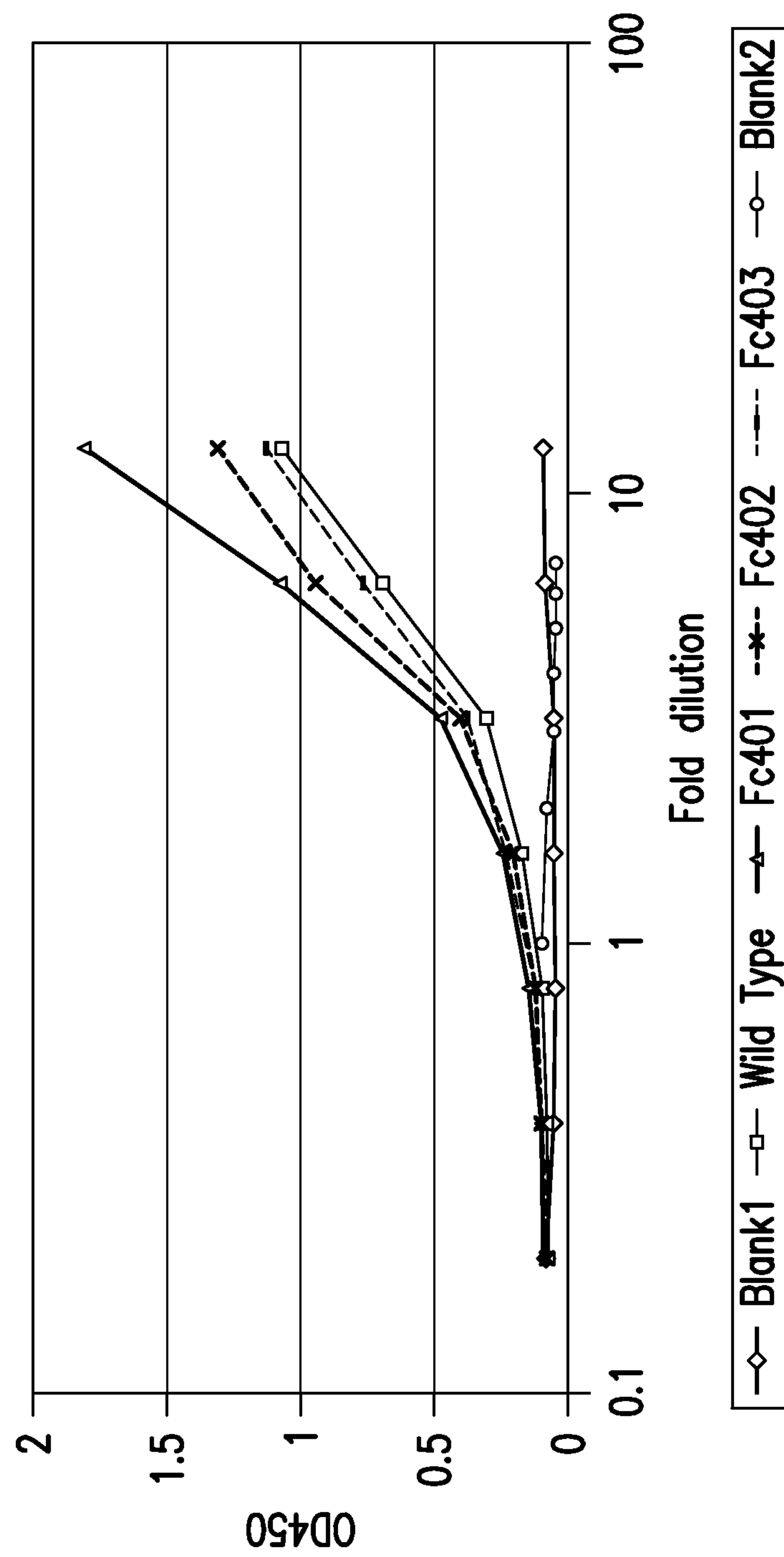


FIG. 29

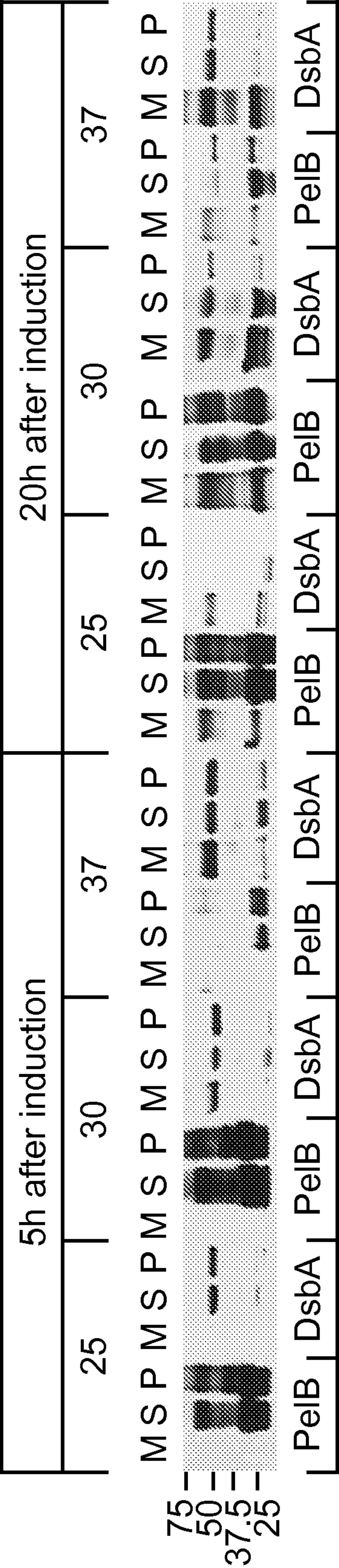
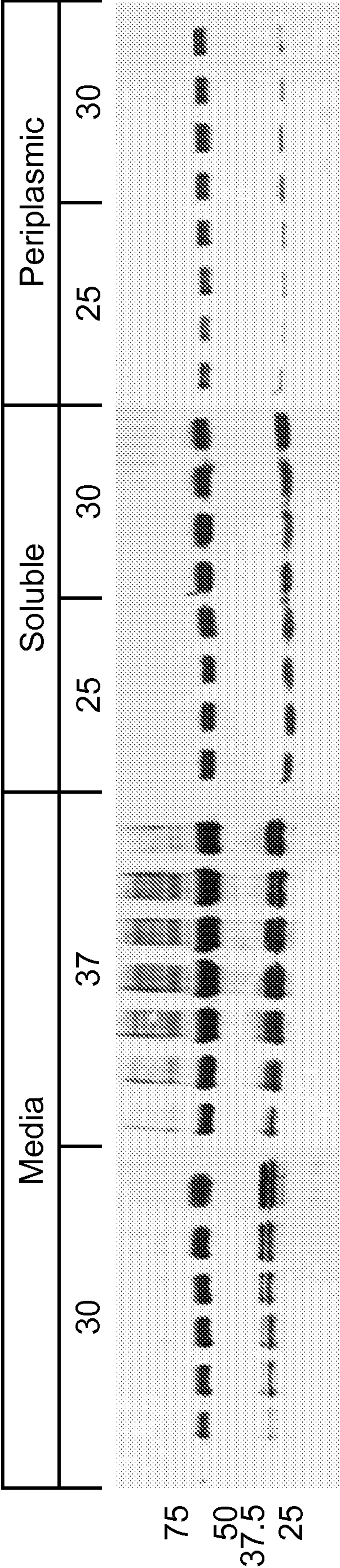


FIG.30

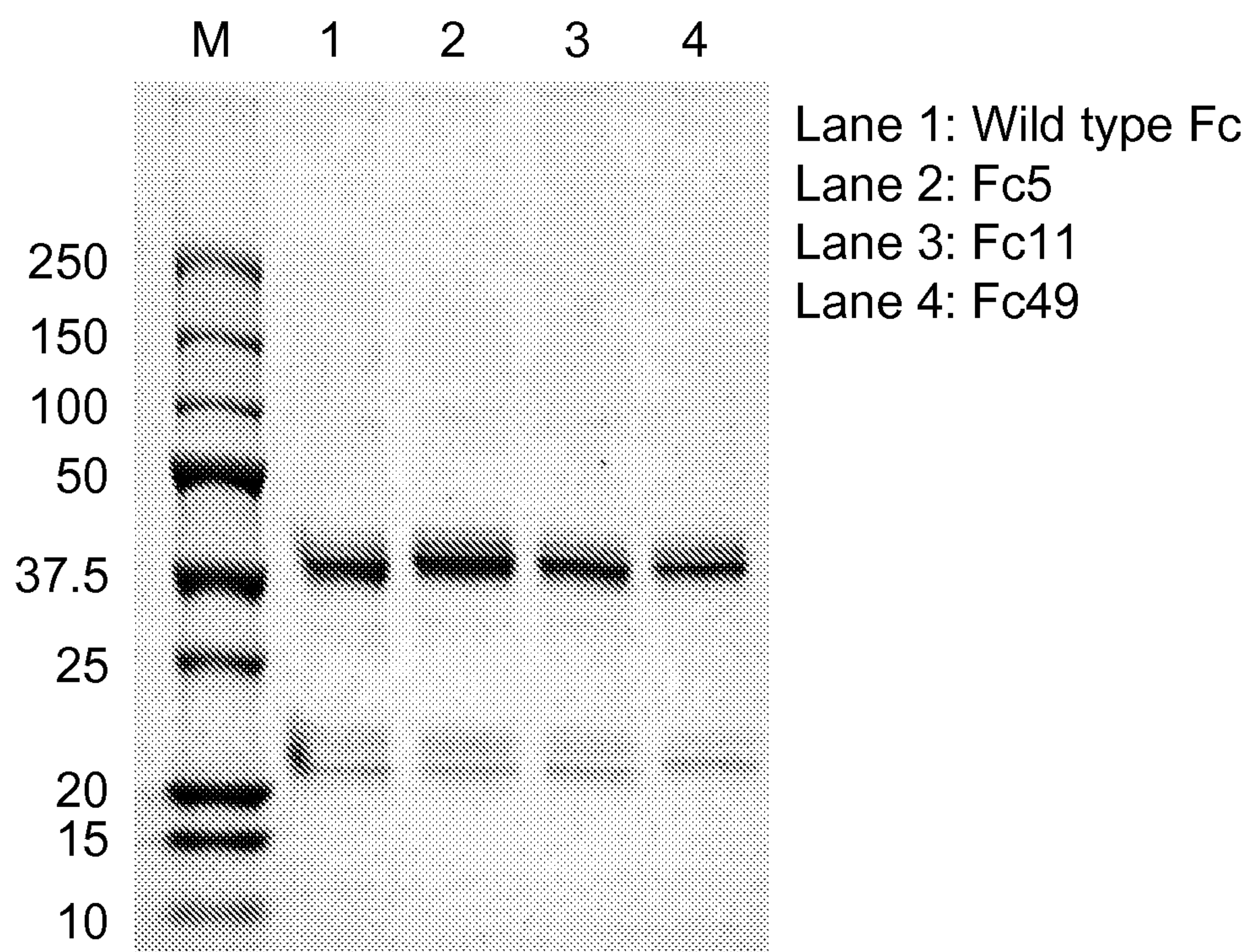
pTrc99A-DsbA-Fc in Jude1
500 ml flask culture
Localizations (Media, Soluble, Periplasmic, fraction)
Different culture temperature and time after induction



20 µl equivalent culture loading

FIG.31

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Expression from pTrc99A-dsbA-Fc mutants-FLAG
500 ml flask culture
Harvest 400 ml of culture supernatant
Purification using Protein A affinity chromatography

FIG.32

Wild type Fc

— ST093007Fcwild01:1_UV

— ST093007Fcwild01:1_Conc

— ST093007Fcwild01:1_Conc

— ST093007Fcwild01:1_Inject

— ST093007Fcwild01:1_Flow

--- ST093007Fcwild01:1_Temp

--- ST093007Fcwild01:1_Fractions

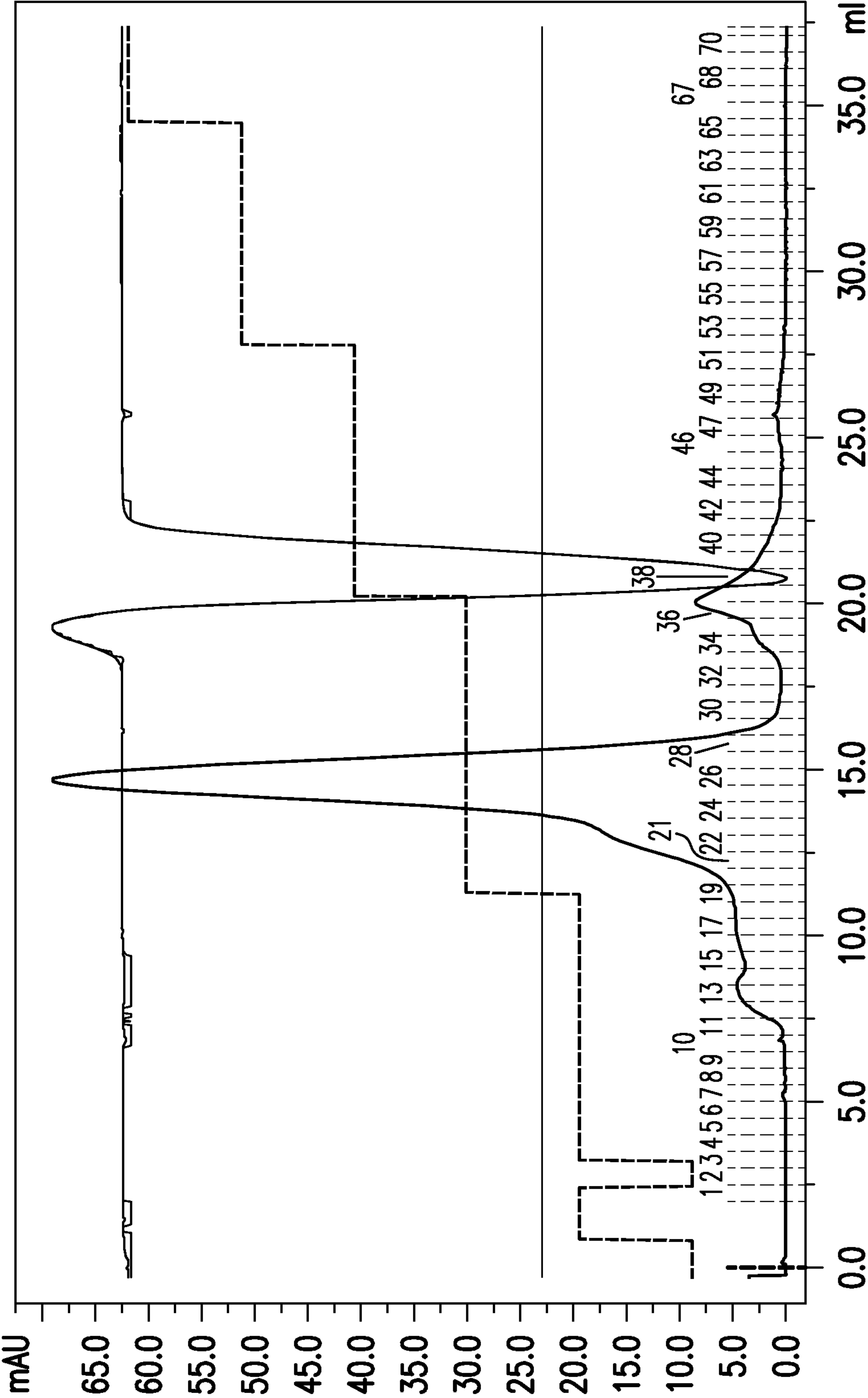


FIG.33A

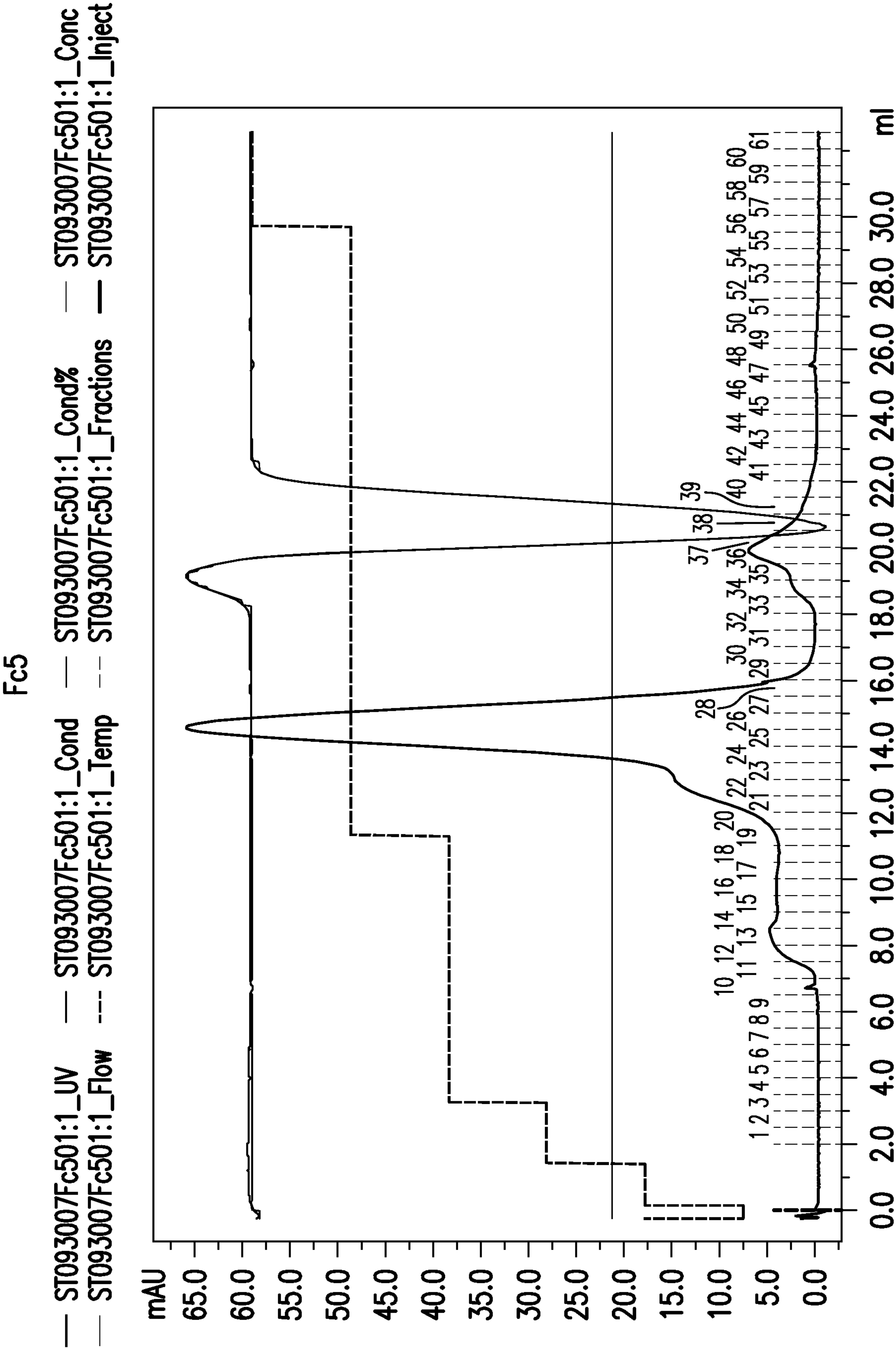


FIG.33B

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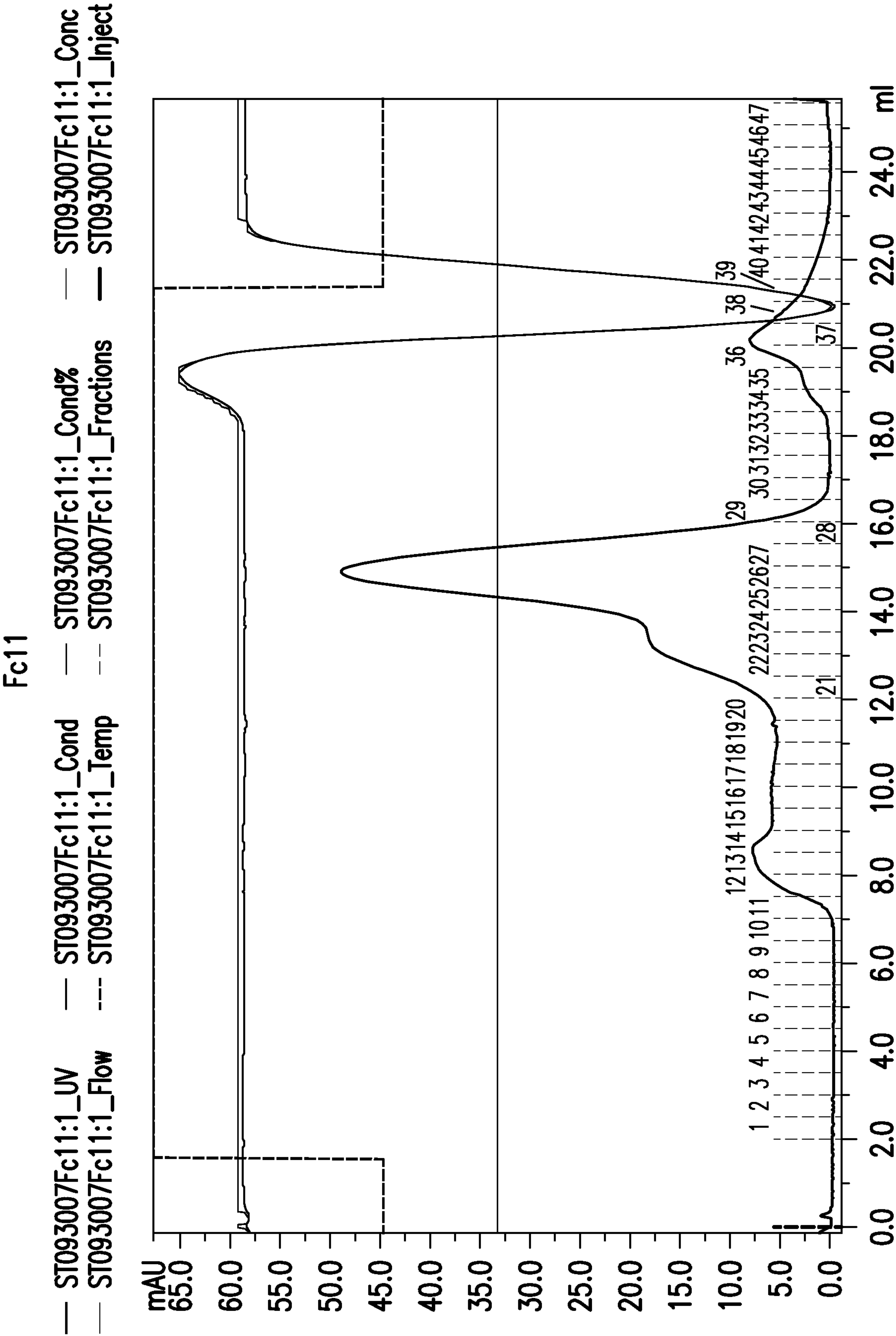


FIG.33C

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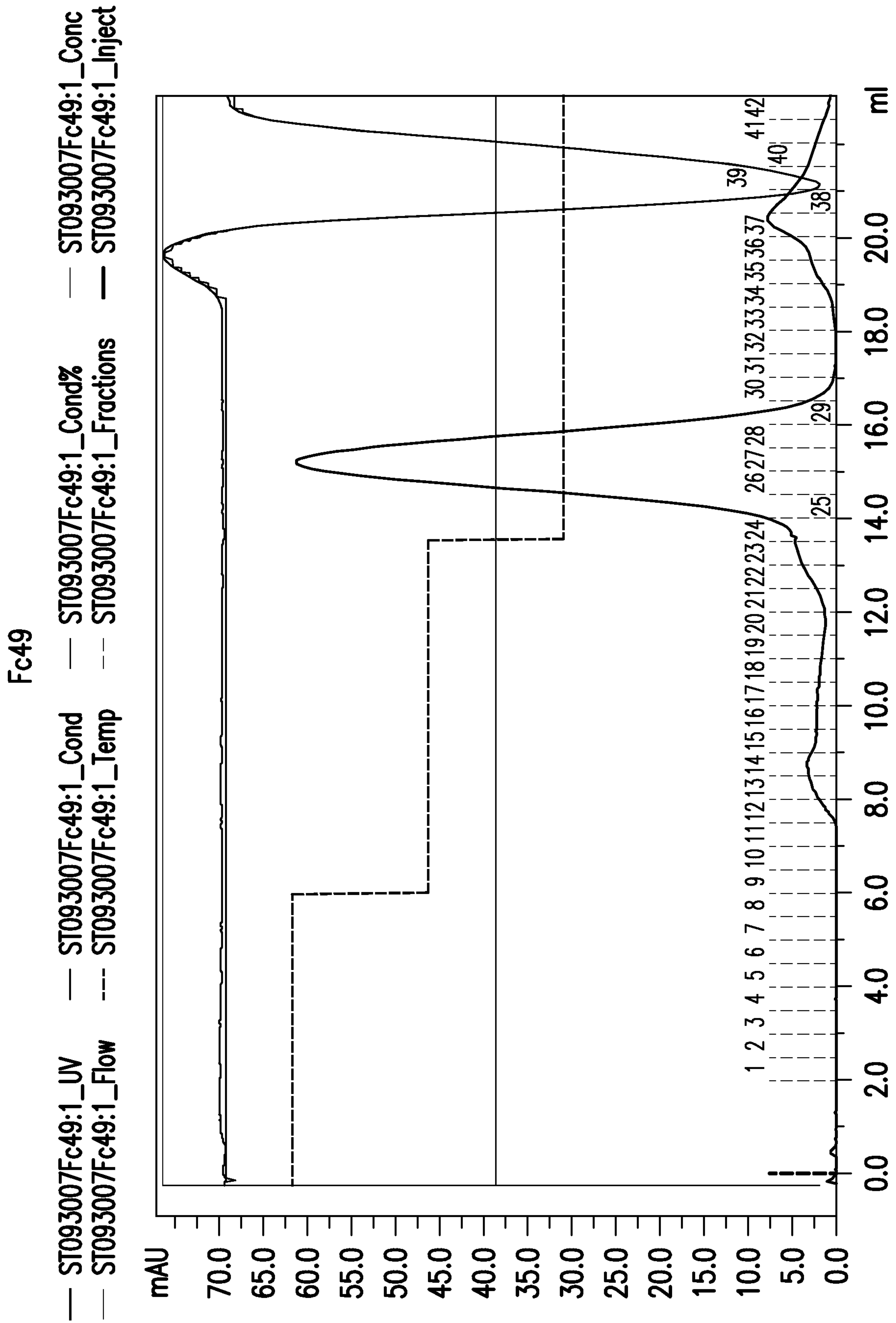


FIG.33D

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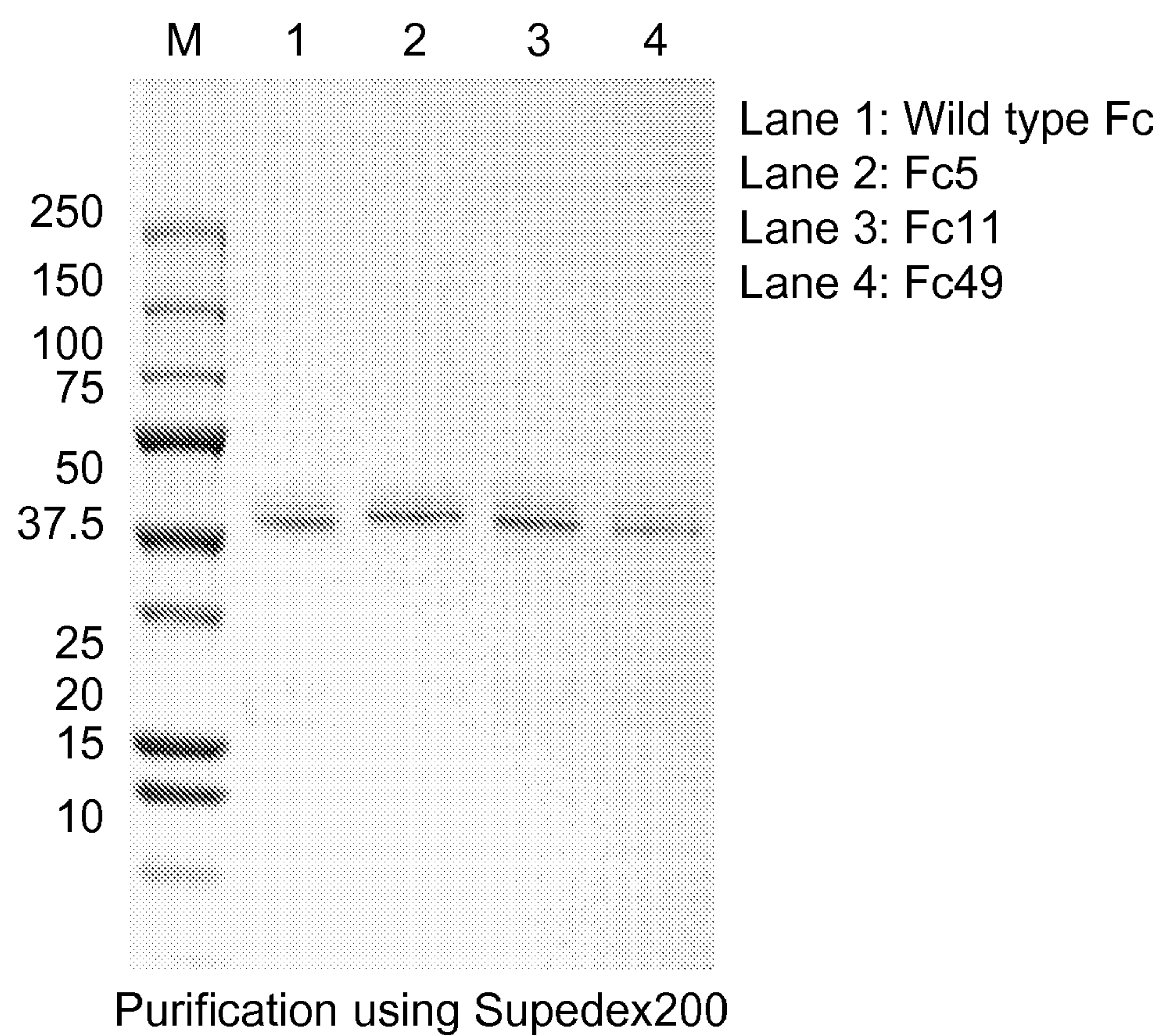


FIG.34

Capture	Binding Protein	Detection Ab
Purified aglycosylated Fc or glycosylated Fc	Diluted Fc receptors	Anti-His-HRP

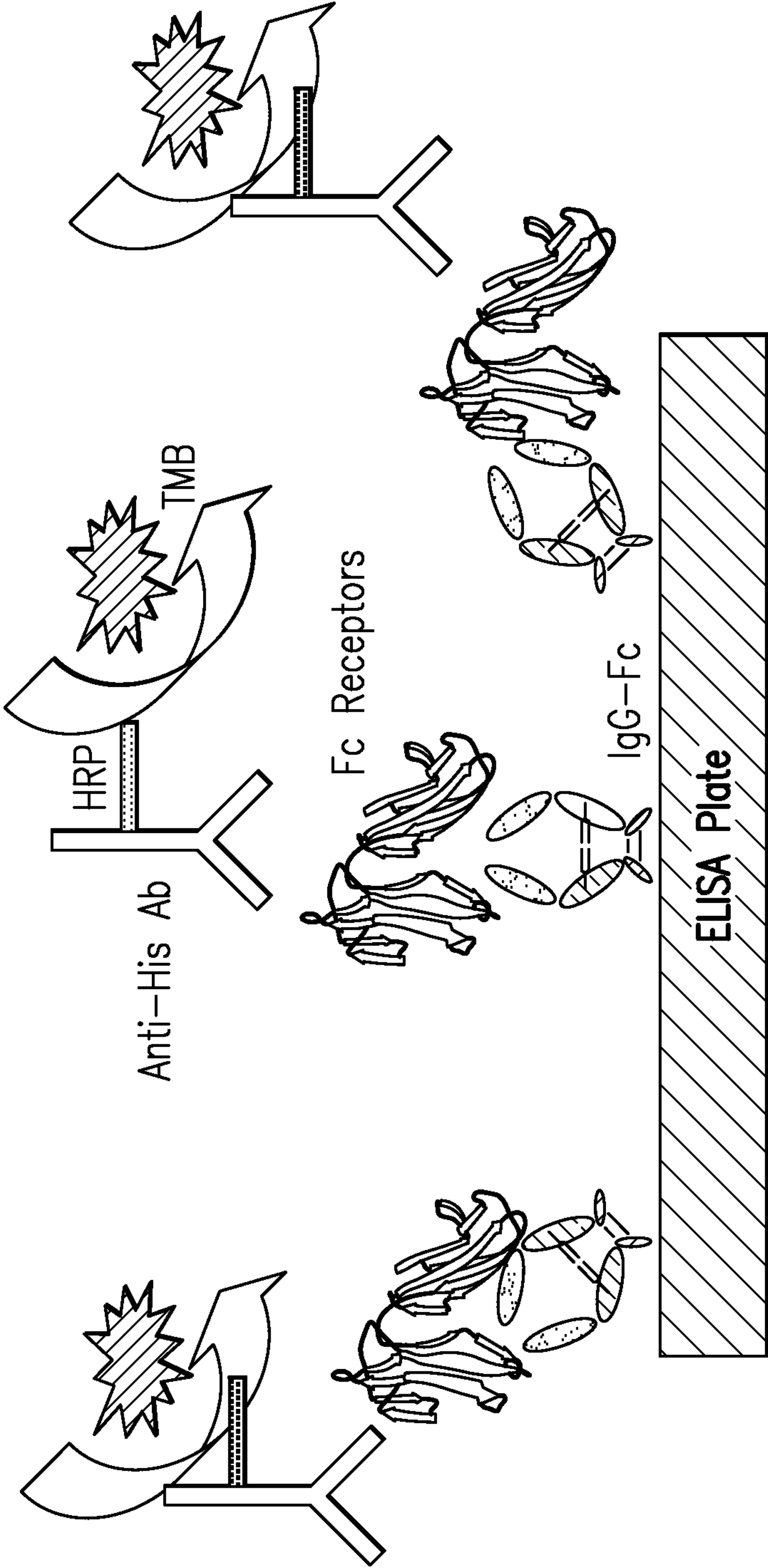


FIG.35

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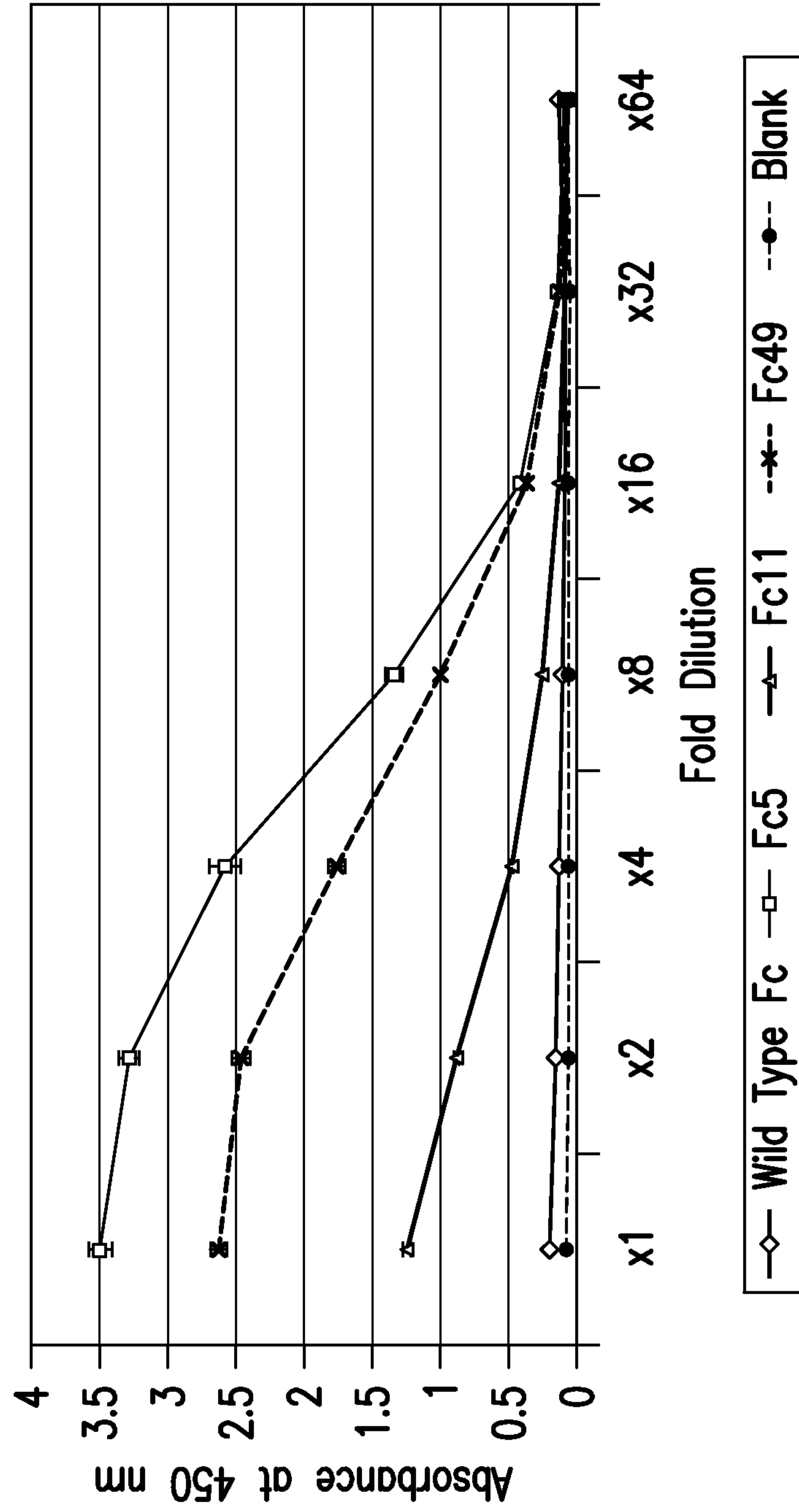


FIG.36

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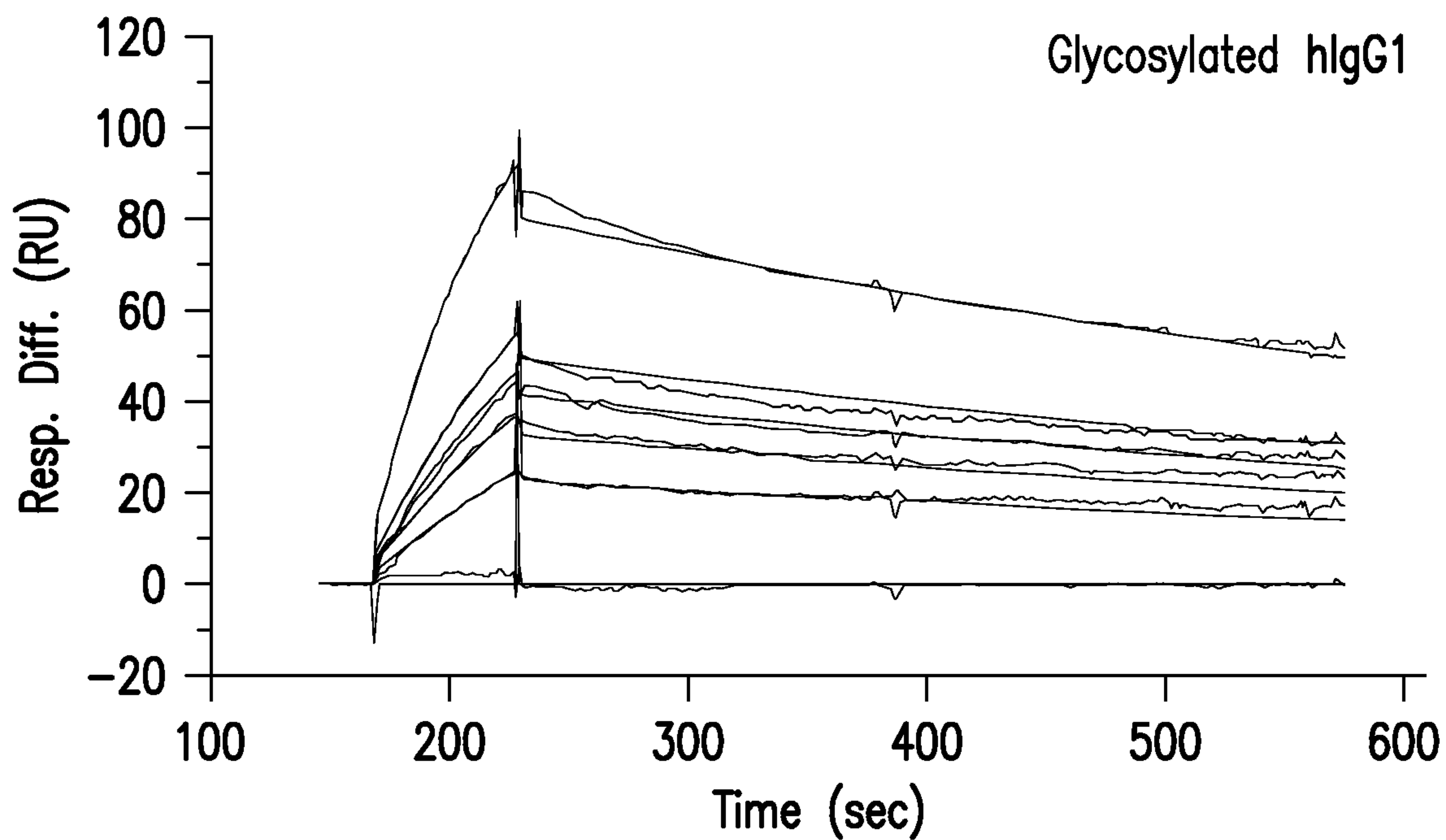


FIG.37A

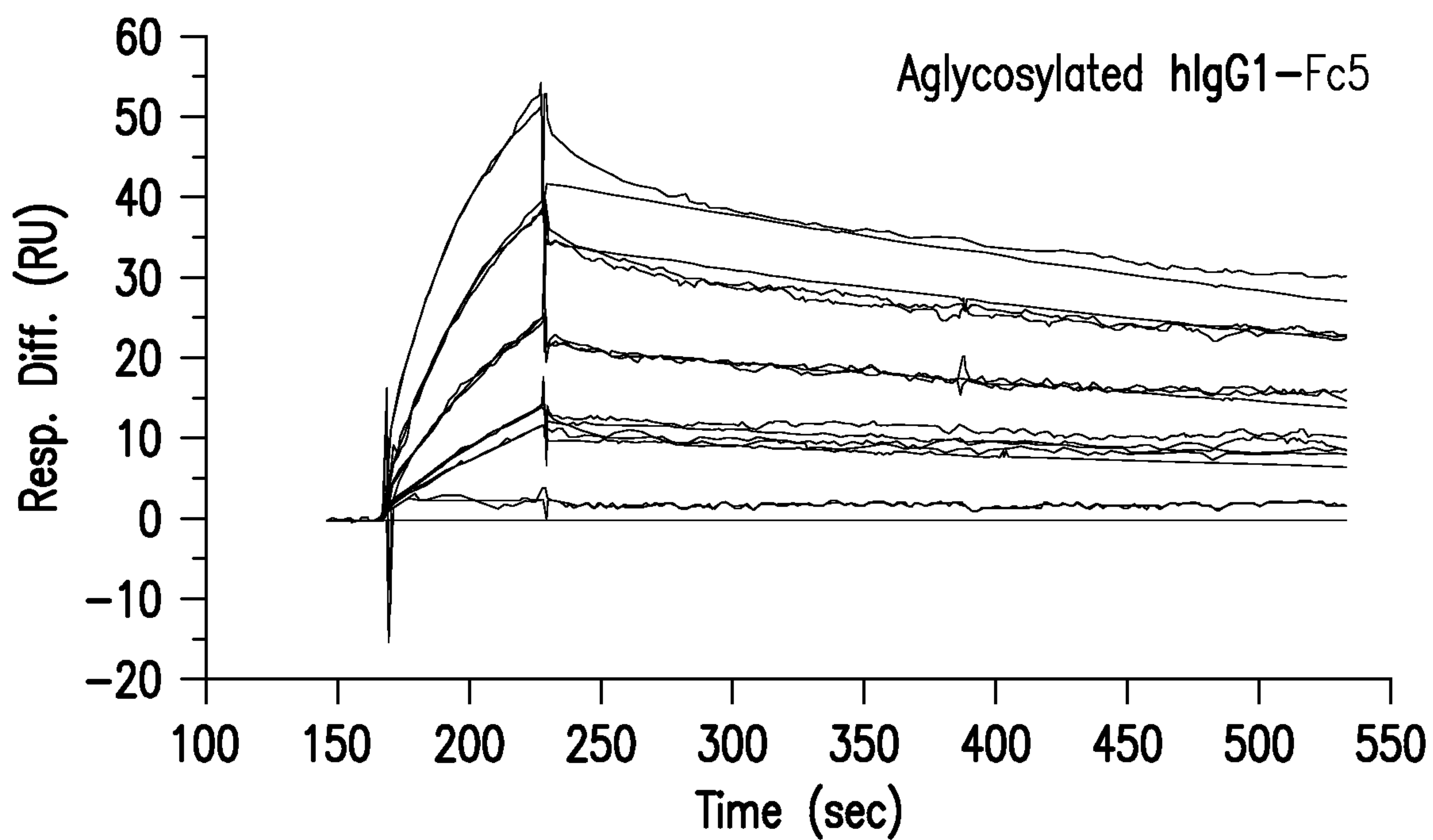


FIG.37B

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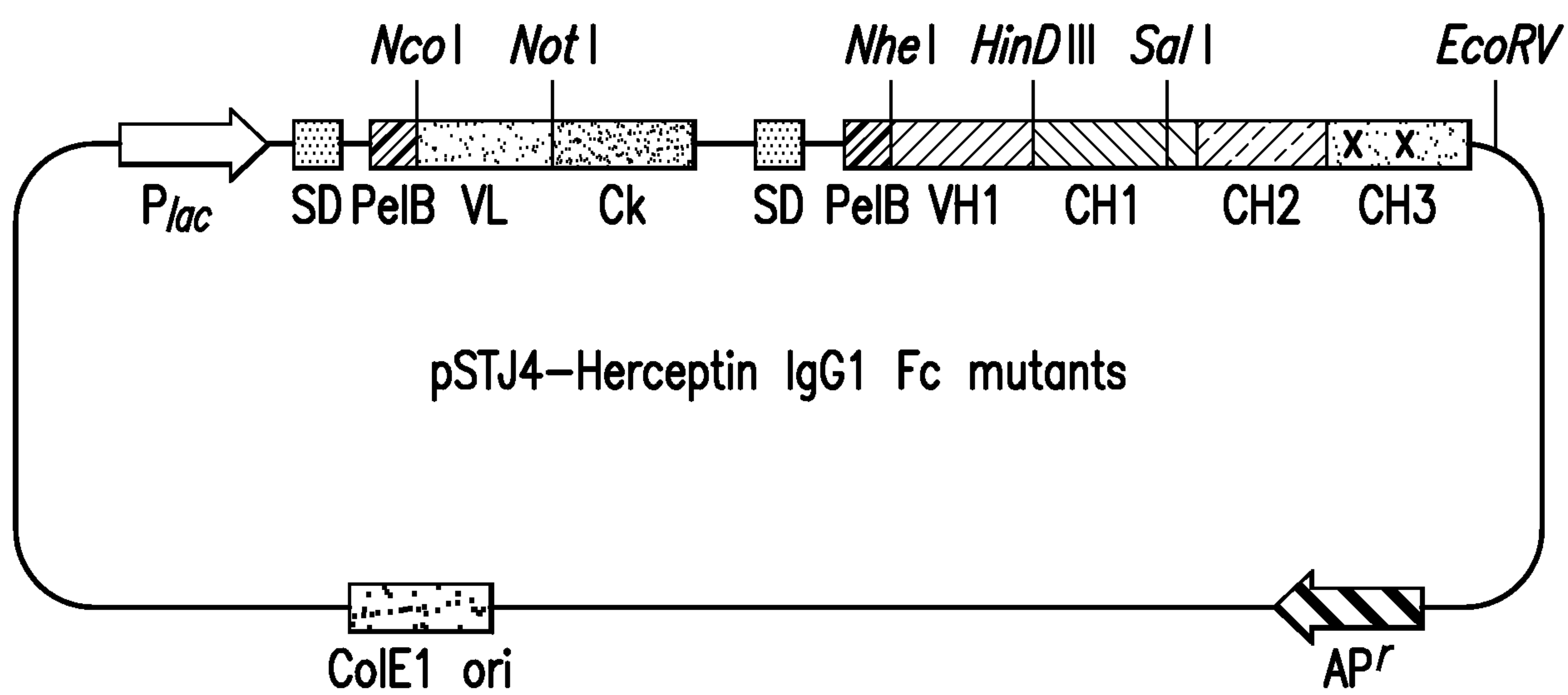


FIG.38

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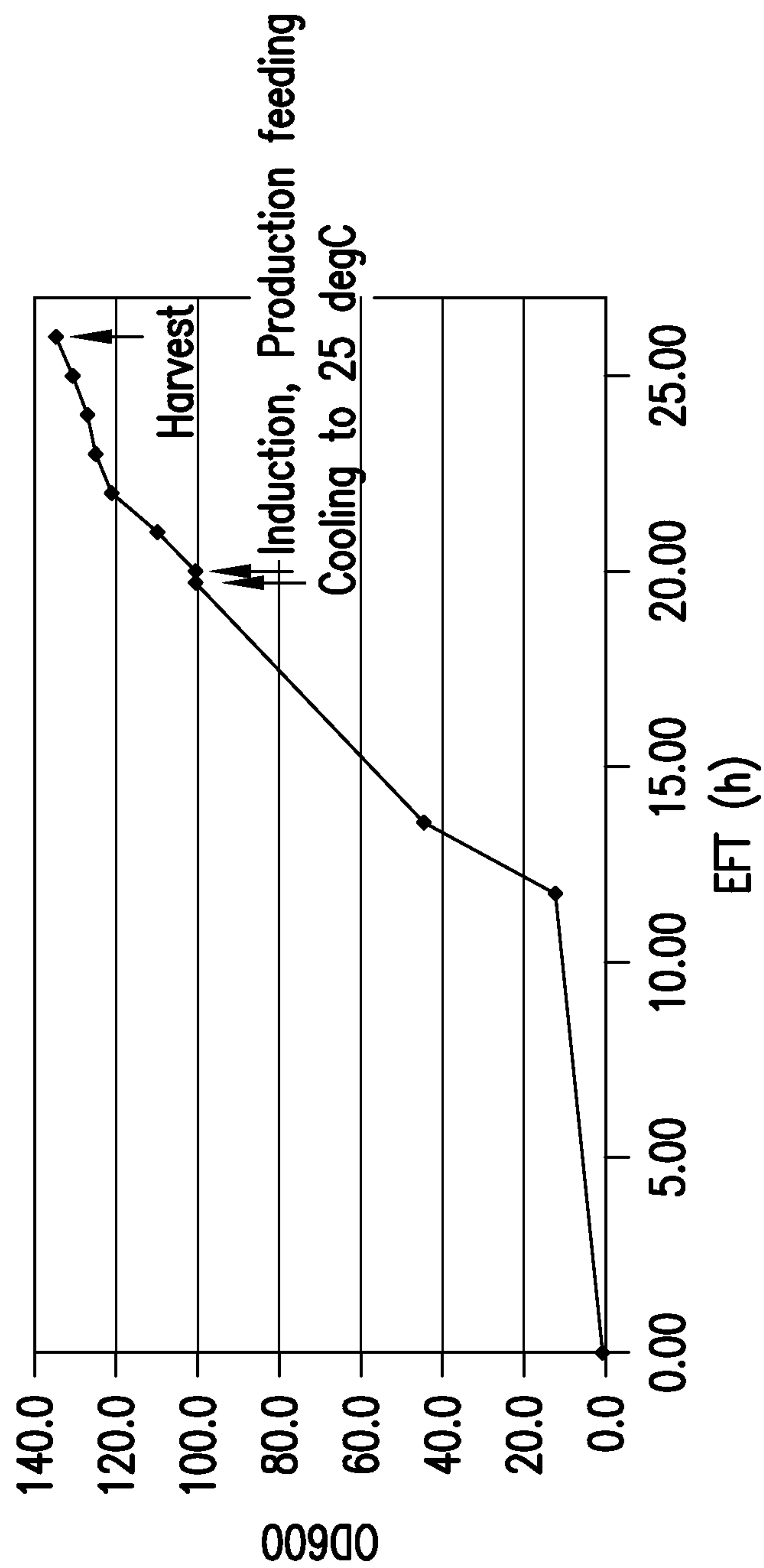


FIG. 39

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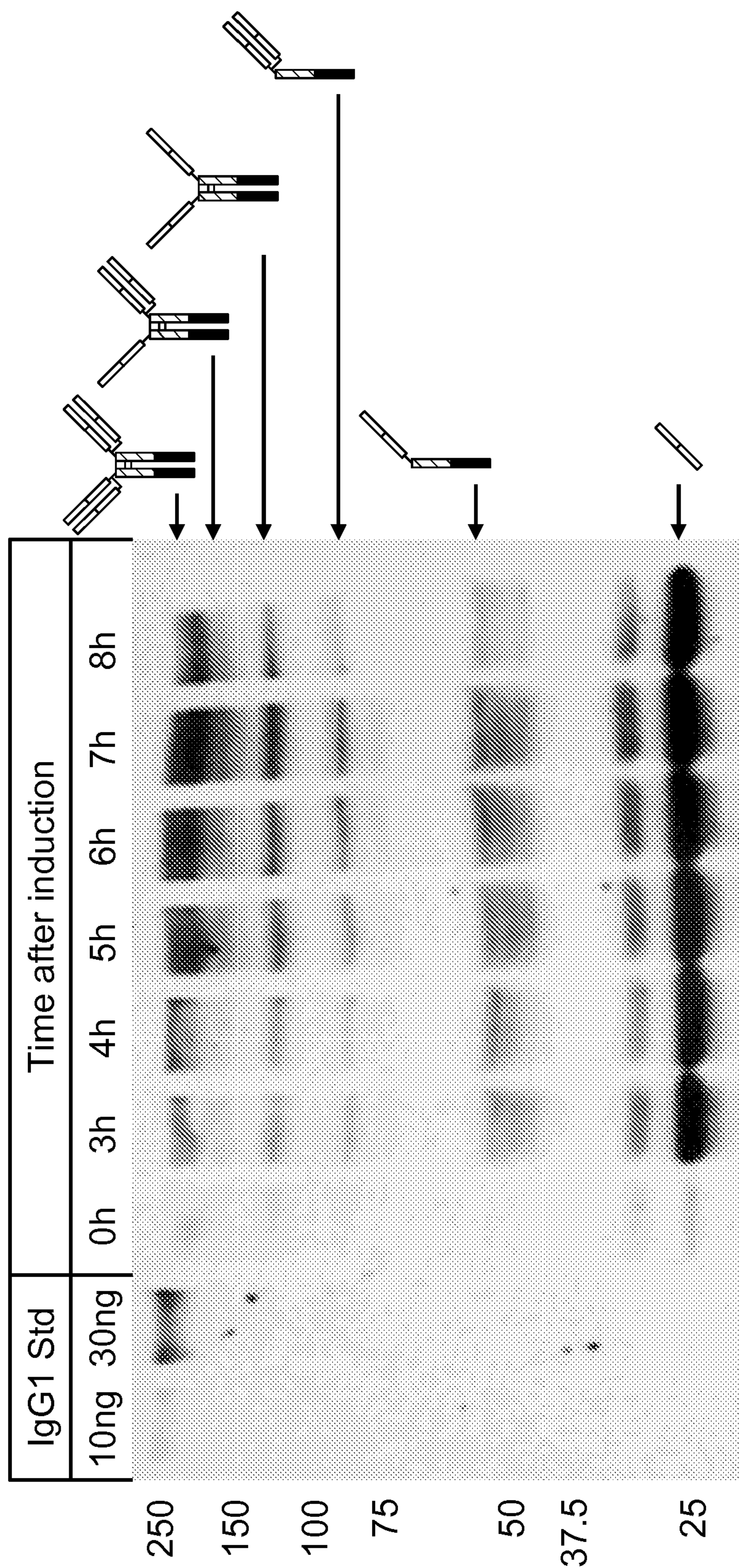


FIG. 40

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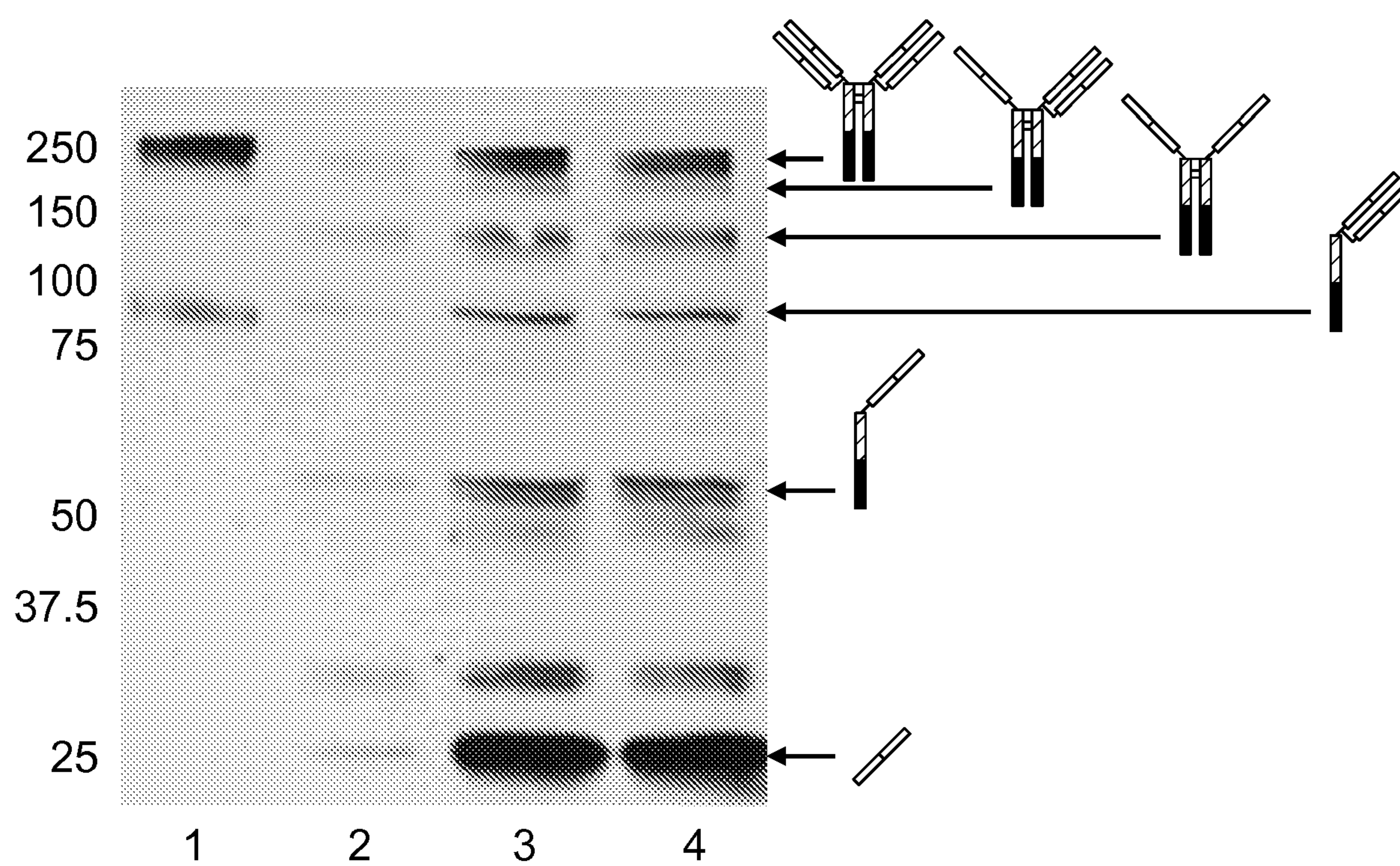
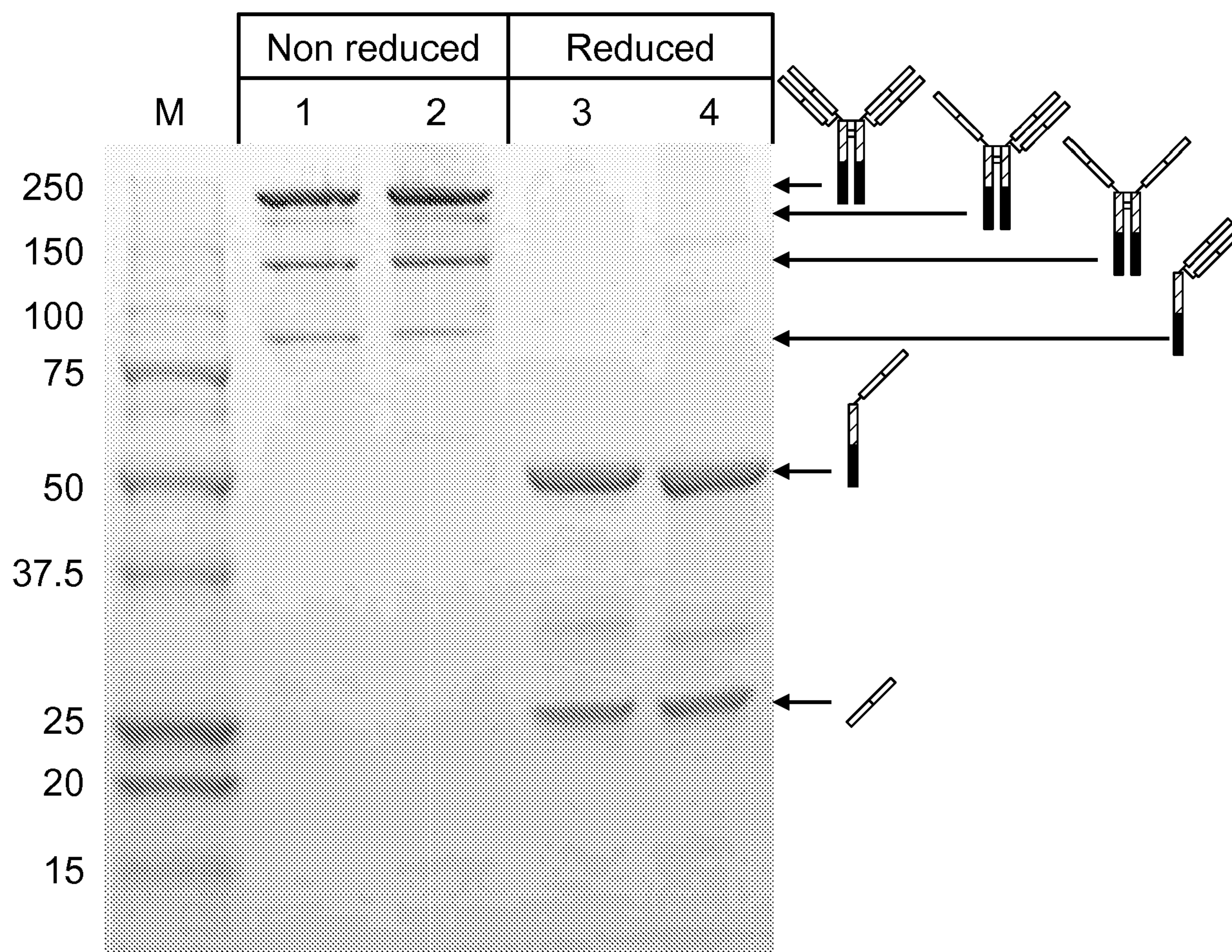


FIG.41

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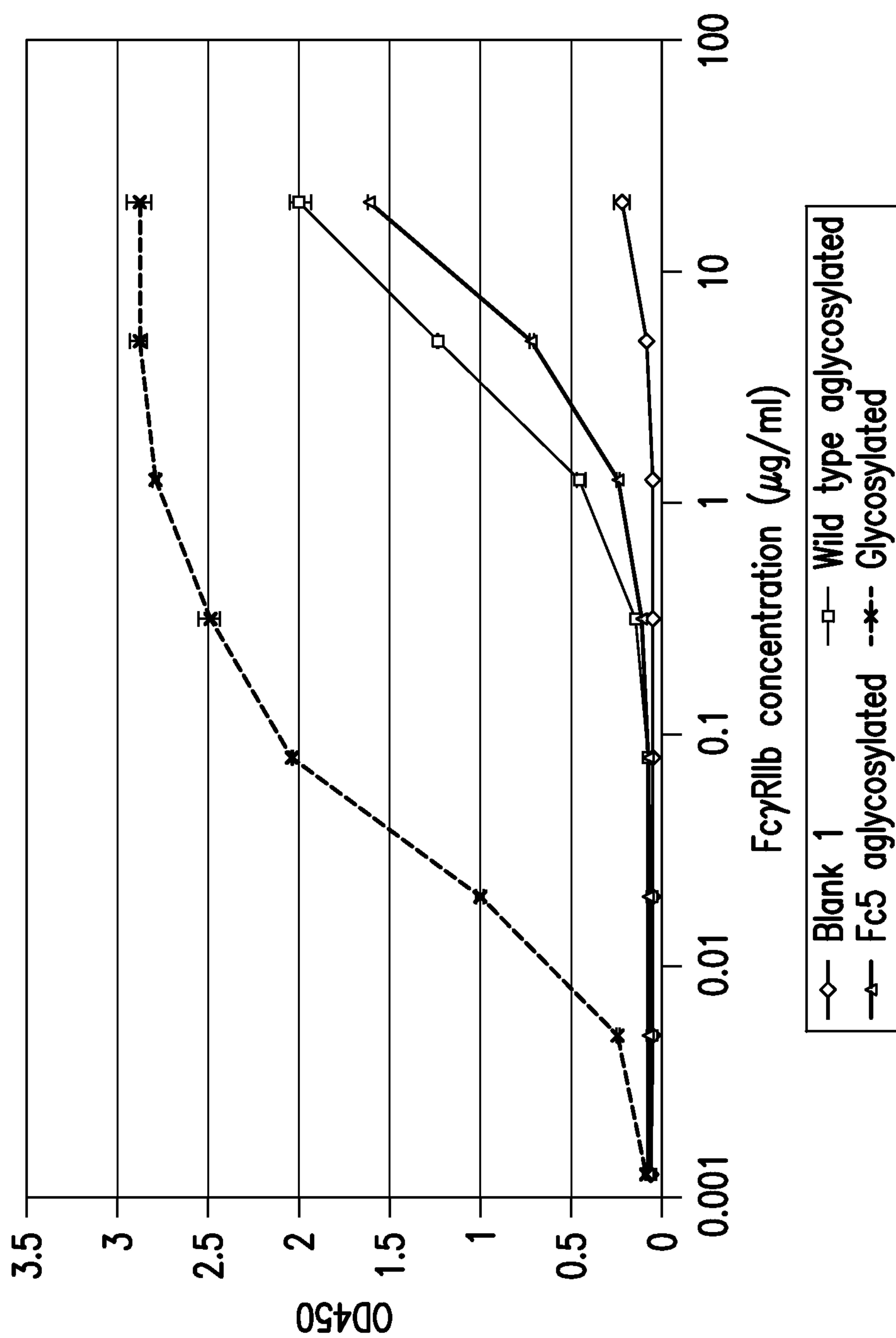


FIG.43

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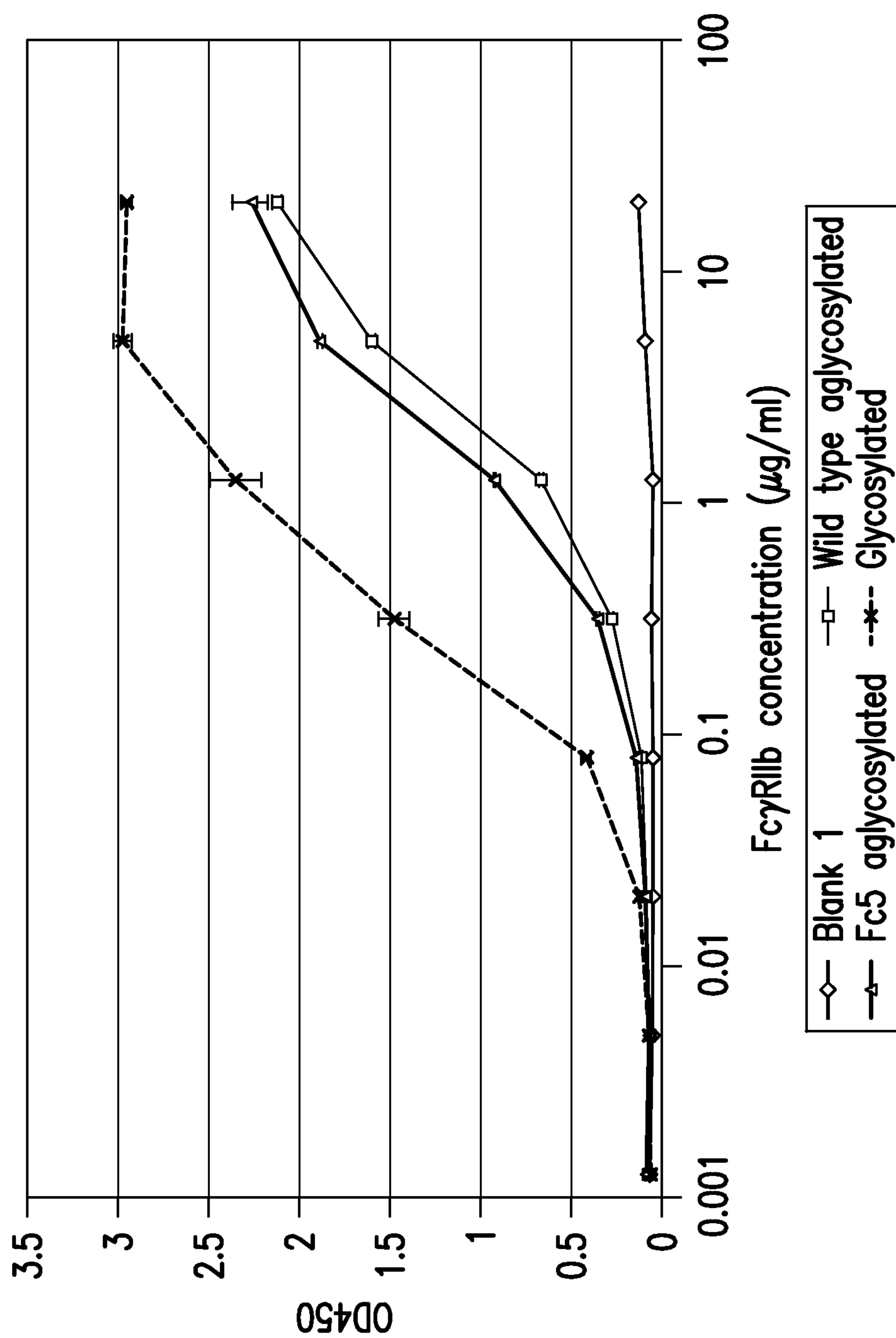


FIG.44

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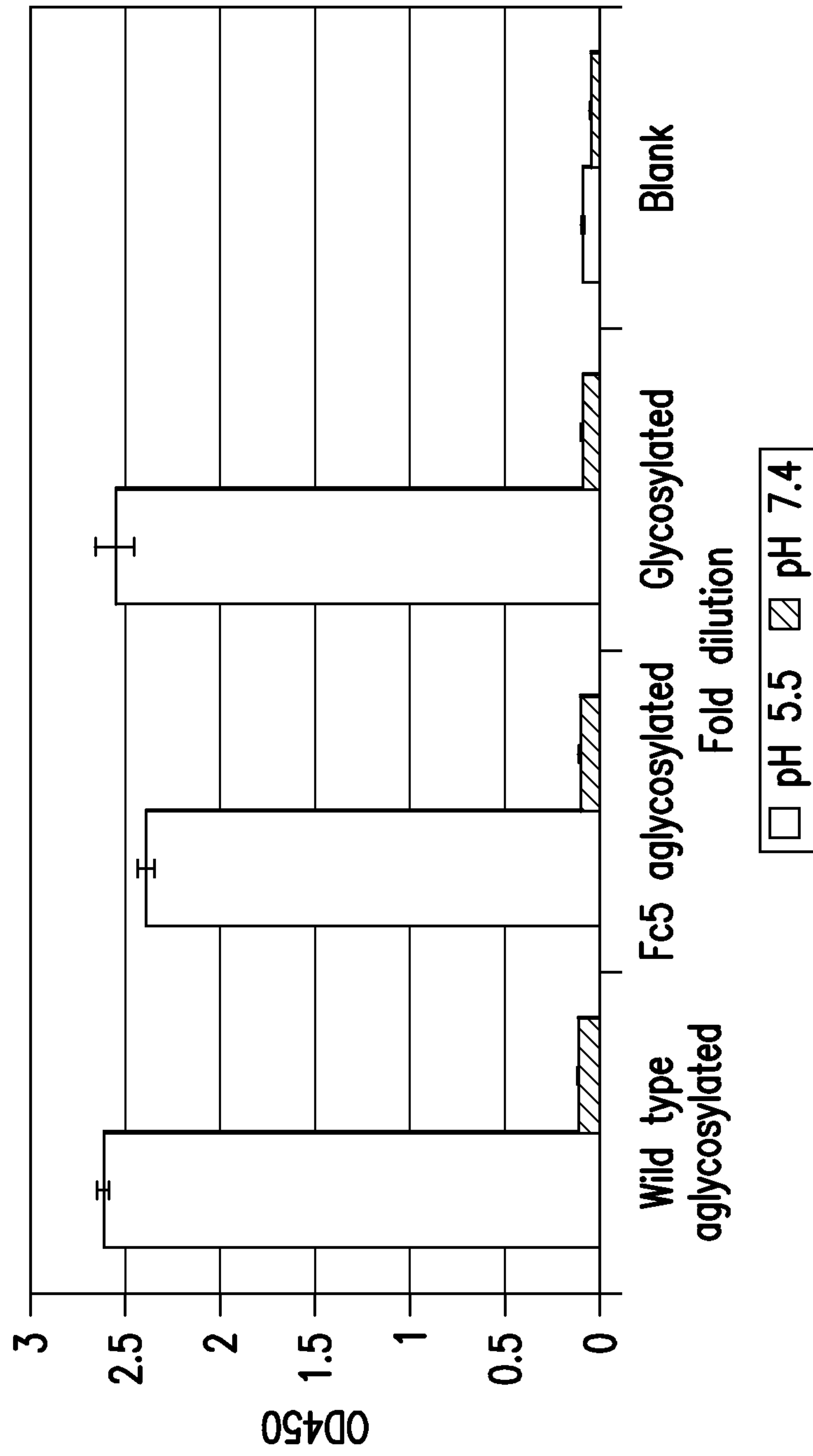


FIG.45

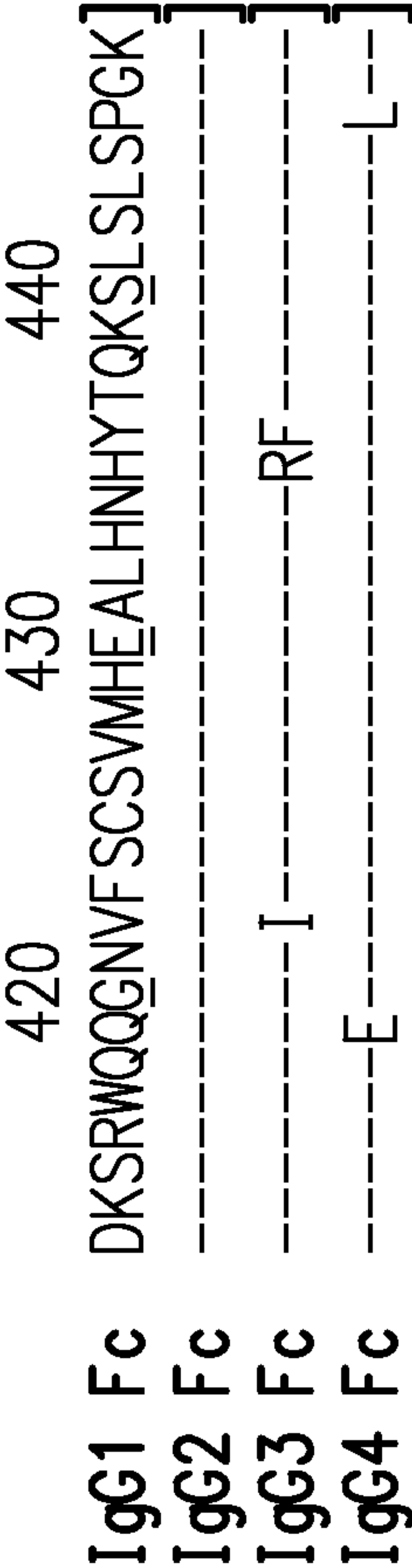
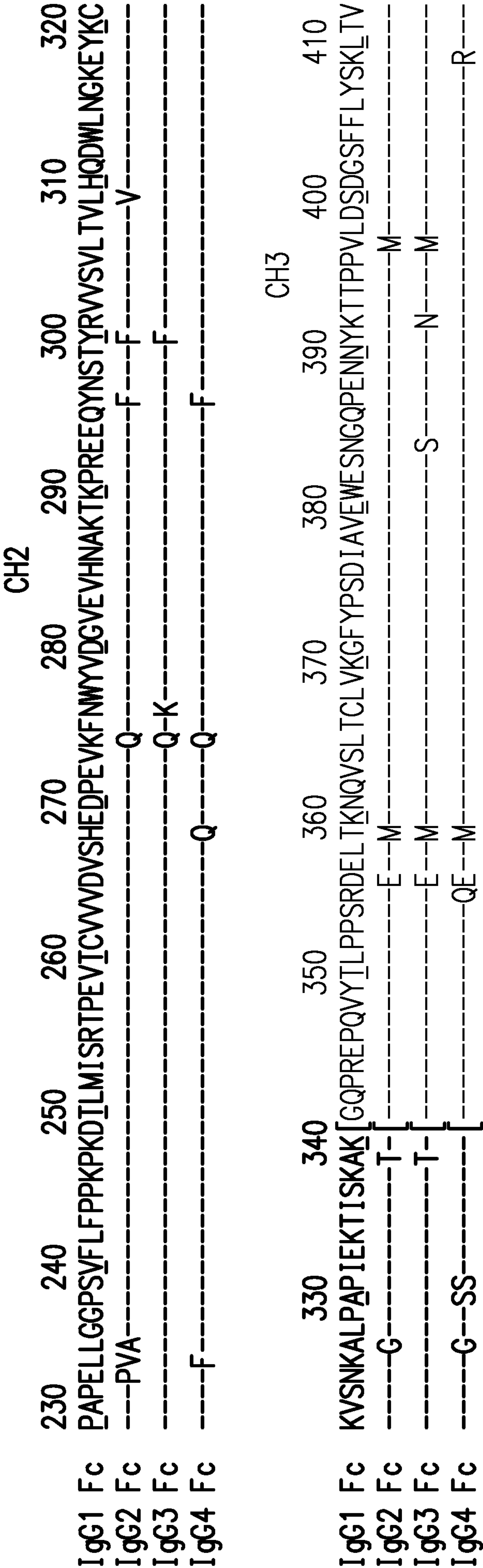


FIG.46