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(54) **Titre : ANTICORPS SPECIFIQUES DE PD-1 ET LEURS UTILISATIONS**
(54) **Title: PD-1 SPECIFIC ANTIBODIES AND USES THEREOF**

(57) **Abrégé/Abstract:**

One aspect of the present disclosure provides antibodies that can act as agonists of PD-1, thereby modulating immune responses regulated by PD-1. Another aspect of the disclosure provides compositions comprising PD-1 specific antibodies and their use in methods of down regulating the immune response. These methods can be practiced on any subject, including humans or animals. Anti-PD-1 antibodies disclosed herein may be used, in another aspect of the invention to detect PD-1 or its fragments in a biological sample. The amount of PD-1 detected may be correlated with the expression level of PD-1, and associated with the activation status of immune cells (e.g., activated T cells, B cells, and/or monocytes) in the subject.

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(54) Title: PD-1 SPECIFIC ANTIBODIES AND USES THEREOF

(57) Abstract: One aspect of the present disclosure provides antibodies that can act as agonists of PD-1, thereby modulating immune responses regulated by PD-1. Another aspect of the disclosure provides compositions comprising PD-1 specific antibodies and their use in methods of down regulating the immune response. These methods can be practiced on any subject, including humans or animals. Anti-PD-1 antibodies disclosed herein may be used, in another aspect of the invention to detect PD-1 or its fragments in a biological sample. The amount of PD-1 detected may be correlated with the expression level of PD-1, and associated with the activation status of immune cells (e.g., activated T cells, B cells, and/or monocytes) in the subject.



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PD-1 SPECIFIC ANTIBODIES AND USES THEREOF

SUMMARY OF THE INVENTION

One aspect of the present disclosure provides antibodies that can act as agonists of PD-1, thereby modulating immune responses regulated by PD-1. In one embodiment, the anti-PD-1 antibodies can be novel antigen-binding fragments. Anti-PD-1 antibodies disclosed herein are able to bind to human PD-1 and agonize the activity of PD-1, thereby inhibiting the function of immune cells expressing PD-1. Exemplary antibodies for use in the context of this disclosure include, but are not limited to monoclonal antibody produced by clone 19.

Another aspect of the disclosure provides compositions comprising PD-1 specific antibodies and their use in methods of down regulating the immune response. These methods can be practiced on any subject, including humans or animals. In particular embodiments, anti-PD-1 antibodies are used to treat or prevent immune disorders by reducing the T cell response. Non-limiting examples of immune disorders that can be treated via the administration of PD-1 specific antibodies to a subject include, but are not limited to, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, type I diabetes, transplant rejection, graft-versus-host disease, hyperproliferative immune disorders, cancer, and infectious diseases. Some embodiments of this aspect of the invention may use two PD-1 specific antibodies that bind to distinct, non-overlapping epitopes.

Anti-PD-1 antibodies disclosed herein may be used, in another aspect of the invention to detect PD-1 or its fragments in a biological sample. The amount of PD-1 detected may be correlated with the expression level of PD-1, and associated with the activation status of immune cells (e.g., activated T cells, B cells, and/or monocytes) in the subject.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Detection of antibody binding to human PD-1 transfectants. Cells were transfected with constructs expressing the extracellular region of human PD-1 (panels A and B) or mutants that affected the binding of Clone 19 (L16R; panel C) or Clone 10 (L103E; panel D) anti-PD-1 antibodies. The cells were labelled with isotype control antibody (panel A) or with Clone 19 (panels B and C) or Clone 10 (panel D) antibody, followed by Alexa647 labelled secondary antibody. Transfected 293T cells are eGFP-positive (x-axis). Antibody binding is shown on the y-axis.

Fig. 2: PD-1 epitope screen. The geometric means of the Alex647 fluorescence levels for the GFP⁺ cells is given for each of the mutants of PD-1, expressed as full length proteins in HEK 293T cells.

Fig. 3: Anti-PD-1 antibody epitopes. The epitopes were mapped by antibody binding analysis following expression of single-residue mutated forms of PD-1 in HEK 293T cells. Mouse PD-1 residues equivalent to human PD-1 residues that when mutated partially or fully block the binding of Clones 2, 10 and 19 antibody are highlighted in black on the mouse PD-1 crystal structure (Zhang *et al. Immunity* 20, 337-47 (2004)). Mouse-equivalents of human PD-1 residues that have no effect on the binding of the antibodies when mutated are coloured grey. The mutated residue numbers for the non-binding mutants are given alongside the structure, for each antibody. Clone 2 and clone 10 antibodies appear to compete with one another for binding to PD-1 based upon the results of this analysis.

Figs. 4A-B: IL-2 secretion induced by anti-PD-1 antibodies binding to a hPD-1/mCD3 ζ WT/mCD28 chimera. (Figure 4A) A chimera consisting of the extracellular region of human PD-1 and the transmembrane and cytoplasmic regions of mouse (m) TCR ζ and CD28 was expressed in DO11.10 cells. (Figure 4B) The cells were treated with immobilized anti-CD3 (KT3) or anti-PD-1 antibodies and the amount of IL-2 released was measured.

Fig. 5: Quantification of monoclonal antibodies loaded onto tosyl-activated DYNALBEADS. Amount of anti-human CD3 OKT3 antibody used per loading (10^7 beads with 2.5 μ g total antibody) is shown on the x-axis. Remaining amount of antibody was made up with Rabbit IgG or anti-PD-1 antibodies (Clone 19, Clone 10, Clone 2) to a total of 2.5 μ g. The number of IgG1 (Rabbit IgG or anti-PD-1 antibodies; bar graphs with one asterisk (*)) or IgG2a (OKT3; bar graphs with two asterisks (**)) molecules detected per bead is shown on the y-axis. Arrows indicate beads selected for use in the experiments shown in Fig. 6. Values are averages of duplicates.

Fig. 6: Titration of anti-PD-1 antibodies coupled to tosyl-activated DYNALBEADS. Bulk preparations of PBL were incubated with beads containing anti-CD3 and increasing amounts of anti-PD-1 antibodies (Clone 19 or Clone 10). Amount of anti-PD-1 antibody loaded per 10^7 beads (in a total of $2.5 \mu\text{g}$ mAb per 10^7 beads) is shown on the x-axis. Proliferation (y-axis) was measured by CFSE dilution at day 5. Bars represent means of triplicates \pm SD.

Fig. 7: Stimulation of a PD-1/mCD28 chimera-expressing DO11.10 cell line with titrations of two anti-PD-1 antibodies. DO11.10 cells expressing a PD-1/mCD28 chimera were incubated with titrations of anti-PD-1 Clone 19 antibody (from $100 \mu\text{g/ml}$ to $0 \mu\text{g/ml}$) and anti-PD-1 Clone 10 antibody (from $100 \mu\text{g/ml}$ to $0 \mu\text{g/ml}$). Cells were then incubated in donkey anti-mouse IgG antibody coated ($500 \mu\text{g/ml}$) 96 well plates for 48 hours before tissue culture supernatant was assayed for IL-2 by ELISA.

Fig. 8: Activation of T cells with beads quantified for Ig content. PBL were depleted of monocytes by plastic adherence (bulk PBL). The amount of anti-CD3 (OKT3) and anti-PD-1 antibodies (Clone 19 or Clone 10) was quantified and is shown in the table (left) expressed as number of molecules per bead. Proliferation (y-axis) was measured by CFSE dilution at day 5. Bars are duplicates \pm SD.

Fig. 9: Explanation for differential signaling by the two antibodies. Clone 19 induces stronger signaling by a hPD-1/mCD3 ζ WT/mCD28 chimera than Clone 10. PD-1 has ITIM (inhibitory) and ITSM (activating) tyrosine-based signaling motifs. It is suggested that, *in vitro*, Clone 19 triggers the phosphorylation of both motifs whereas Clone 10 ligation results in phosphorylation of the inhibitory motif only, leading to more potent inhibitory signaling.

Figs. 10A-10B: IL-2 secretion induced by anti-PD-1 antibodies binding to a hPD-1/mCD28 chimera. (Figure 10A) A chimera consisting of the extracellular region of human PD-1 and the transmembrane and cytoplasmic regions of mouse CD28 was expressed in DO11.10 cells. (Figure 10B) The cells were treated with immobilized anti-CD3 (KT3) or anti-PD-1 antibodies and the amount of IL-2 released was measured.

Figs. 11A-11C: Strong signaling induced by a pair of antibodies binding to a monomeric signaling protein. (Figure 11A) Antibodies, which are bivalent, cause strong signaling by homodimeric receptors because they are able to generate a high local density of signaling domains. (Figure 11B) In contrast, antibodies are only able to recruit pairs of

monomeric receptors, such as PD-1, leading to much less intense signaling. (Figure 11C) By using antibodies that bind to two non-overlapping epitopes, higher densities of monomeric signaling receptors can be generated, giving much more potent signaling.

Fig. 12: Dissociation rates for Clone 2, 10 and 19 antibodies determined by surface plasmon resonance-based analysis. The three antibodies and a negative control (OX-7) were bound indirectly to the biosensor surface, *i.e. via* a covalently coupled rabbit anti-mouse Fc antibody. Monomeric soluble human PD-1 was then injected to saturating levels over the immobilized antibodies in the buffer 10 mM Hepes, 150 mM NaCl pH 7.4. Following injection of the soluble PD-1, the buffer only was injected, allowing dissociation of the bound soluble PD-1 from each of the antibodies simultaneously. Dissociation rates were fitted using Origin v.5.0 software (MicroCal Software Inc, Northampton, MA) after subtraction of the dissociation rate for OX-7 dissociating from the anti-mouse Fc antibody.

Fig. 13: Inhibition of CD4⁺ T cell proliferation by anti-PD-1 antibodies. CD4⁺ T cells were purified from human PBL by negative selection and cultured with Dynalbeads coated with anti-CD3 plus control (BSA or MOPC21) or Clone 10 antibody. Proliferation (y-axis) was measured by ³H-thymidine incorporation at day 6. Bars represent the % of maximal response (anti-CD3/BSA) and are the mean +/- S.E.M. of 4 different donor cultures.

DETAILED DESCRIPTION

The term “antibody”, as used in this disclosure, refers to an immunoglobulin or a fragment or a derivative thereof, and encompasses any polypeptide comprising an antigen-binding site, regardless of whether it is produced *in vitro* or *in vivo*. Thus, an antibody includes, but is not limited to, polyclonal, monoclonal, monospecific, polyspecific, bispecific, humanized, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, and grafted antibodies.

The term “antibody fragment” or “an antigen binding fragment” includes antibody fragments such as Fab, F(ab')₂, Fv, scFv, Ed, dab, and other antibody fragments that retain antigen-binding function, *i.e.*, the ability to bind PD-1 specifically and/or that are produced from a monoclonal antibody disclosed herein. These fragments comprise an antigen-binding domain and can also, in some embodiments, agonize the function of PD-1. Antibodies disclosed herein, and fragments thereof, include those antibodies having altered glycosylation patterns when compared to the parent antibody (*e.g.*, the antibody produced by clone 10 and/or clone 19).

As discussed above, the PD-1 antibodies disclosed herein are able to antagonize the activity and/or proliferation of lymphocytes by agonizing PD-1. The term “antagonize the activity” relates to a decrease (or reduction) in lymphocyte proliferation or activity that is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more. The term “antagonize” may be used interchangeably with the terms “inhibitory” and “inhibit”. PD-1-mediated activity can be determined quantitatively using T cell proliferation assays as described herein.

The terms “therapeutically effective”, “therapeutically effective amount”, “effective amount” or “in an amount effective” refers to a dosage or amount of the disclosed antibodies that is sufficient to agonize the activity of PD-1 and provide for the amelioration of symptoms in a subject or to achieve a desired biological response, e.g., decreased T cell activity, etc.

The term “isolated” refers to a molecule that is substantially free of its natural environment. For instance, an isolated antibody is substantially free of cellular material or other proteins from the cell (e.g., hybridoma) or other source from which it is derived. The term isolated also refers to preparations where the isolated protein is sufficiently pure to be administered as a pharmaceutical composition, or at least 70-80% (w/w) pure, at least 80-90% (w/w) pure, 90-95% pure; or at least 95%, 96%, 97%, 98%, 99%, or 100% (w/w) pure.

One aspect of the present disclosure provides antibodies that can act as agonists of PD-1, thereby modulating immune responses regulated by PD-1. In one embodiment, the anti-PD-1 antibodies can be novel antigen-binding fragments. Anti-PD-1 antibodies disclosed herein are able to bind to including human PD-1 and agonize PD-1, thereby inhibiting the function of immune cells expressing PD-1. In some embodiments, the immune cells are activated lymphocytes, such as T-cells, B-cells and/or monocytes expressing PD-1. Exemplary antibodies for use in the context of this disclosure include, but are not limited to monoclonal antibodies produced by clone 19. Some embodiments of this aspect of the invention may use two PD-1 specific antibodies that bind to distinct, non-overlapping epitopes.

Another aspect of the invention provides anti-PD-1 specific monoclonal antibodies having modified binding affinity. One embodiment provides for modifying the binding affinity such that the antibody has a low affinity for PD-1 (e.g., the antibody has a dissociation rate of between 0.1 sec^{-1} and 0.5 sec^{-1} or less than 0.90 sec^{-1}). Particular embodiments provided antibodies having off rates of 0.10 sec^{-1} , 0.15 sec^{-1} , 0.20 sec^{-1} , 0.25

sec⁻¹, 0.30 sec⁻¹, 0.35 sec⁻¹, 0.40 sec⁻¹, 0.45 sec⁻¹ or 0.50 sec⁻¹ or for antibodies having dissociation rates ranging from 0.04 sec⁻¹ to 2.0 sec⁻¹ (e.g., 0.04 sec⁻¹, 0.05 sec⁻¹, 0.06 sec⁻¹, 0.07 sec⁻¹, 0.08 sec⁻¹, 0.09 sec⁻¹, 0.10 sec⁻¹, 0.15 sec⁻¹, 0.20 sec⁻¹, 0.25 sec⁻¹, 0.30 sec⁻¹, 0.35 sec⁻¹, 0.40 sec⁻¹, 0.45 sec⁻¹, 0.50 sec⁻¹, 0.55 sec⁻¹, 0.60 sec⁻¹, 0.65 sec⁻¹, 0.70 sec⁻¹, 0.75 sec⁻¹, 0.80 sec⁻¹, 0.85 sec⁻¹, 0.90 sec⁻¹, 0.95 sec⁻¹, 1.0 sec⁻¹, 1.10 sec⁻¹, 1.20 sec⁻¹, 1.30 sec⁻¹, 1.40 sec⁻¹, 1.50 sec⁻¹, 1.60 sec⁻¹, 1.70 sec⁻¹, 1.80 sec⁻¹, 1.90 sec⁻¹, or 2.00 sec⁻¹). Antibodies having such binding affinities can be modified in any suitable process.

Thus, the binding affinity of the antibodies (such as those produced by clone 2, clone 10 or clone 19) can be increased or decreased via various methods known in the art. For example, binding characteristics can be modified by direct mutation, methods of affinity maturation, phage display, or chain shuffling within the nucleic acids encoding the antibody molecules. Individual residues or combinations of residues can be randomized so that in a population of otherwise identical antigen binding sites, all twenty amino acids are found at particular positions and binding characteristics/affinities can also be modified by methods of affinity maturation. (See, e.g., Yang *et al.* (1995) *J. Mol. Biol.* 254, 392-403; Hawkins *et al.* (1992) *J. Mol. Bio.* 226,889-896; or Low *et al.* (1996) *J. Mol. Biol.* 250, 359-368 (

particularly with respect to methods of increasing or decreasing the binding affinity of antibodies). Methods known in the art include, for example, Marks *et al.* *BioTechnology*, 10, 779-783 (1992), which describes affinity maturation by VH and VL domain shuffling; random mutagenesis of CDR and/or framework residues is described by: Barbas *et al.* *Proc Nat. Acad. Sci, USA* 91, 3809-3813 (1994); Schier *et al.* *Gene*, 169, 147-155 (1995); Yelton *et al.* *J. Immunol.*, 155, 1994-2004 (1995); Jackson *et al.* *J. Immunol.*, 154, 3310-9 (1995); and Hawkins *et al.* *J. Mol. Biol.*, 226, 889-896 (1992).

Strategies for antibody optimization are sometimes carried out using random mutagenesis. In these cases positions are chosen randomly, or amino acid changes are made using simplistic rules. For example all residues may be mutated to alanine, referred to as alanine-scanning. WO 9523813

teaches *in vitro* methods of altering antibody affinities utilizing alanine-scanning mutagenesis. Alanine-scanning mutagenesis can also be used, for example, to map the antigen binding residues of an antibody (Kelley *et al.* *Biochemistry* 32, 6828-6835 (1993); Vajdos *et al.* *J. Mol. Biol.* 320, 415-428 (2002)). Sequence-based methods of affinity maturation (see, U.S. Pat. Application No. 2003/022240 A1 and U.S. Pat. No. 2002/177170A1

) may also be used to increase or decrease the binding affinities of antibodies. Finally, the binding affinities of antibodies in which the binding affinity has been altered can be determined using methods as disclosed herein (for example, dissociation rates for modified antibodies can be determined by surface plasmon resonance-based analysis as described for Figure 12).

Anti-PD1 antibodies described herein can be linked to another molecule/moiety. Non-limiting examples include another peptide or protein (albumin, another antibody, etc.), toxins, radioisotopes, cytotoxic agents or cytostatic agents. The term "link" or "linked" relates to the chemical cross-linking or covalent attachment of another molecule/moiety by recombinant methods. Antibodies disclosed herein may also be linked to one or more nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes (see, for example, U. S. Patent Nos. 4,791,192; 4,766,106; 4,670,417; 4,640,835; 4,609,546; 4,496,689; 4,495,285; 4,301,144; and 4,179,337).

The antibodies may also be tagged with a detectable, or functional, label. Detectable labels include radiolabels such as ⁹⁹Tc, which may also be attached to antibodies using conventional chemistry. Detectable labels also include enzyme labels such as horseradish peroxidase or alkaline phosphatase. Other types of detectable labels include chemical moieties such as biotin, which may be detected via binding to a specific cognate detectable moiety, *e.g.*, labeled avidin.

Another aspect of the invention provides for the use of antibodies disclosed herein for isolating PD-1 or PD-1-expressing cells. Yet another aspect of the invention provides methods of inducing tolerance to a specific antigen. For example, tolerance can be induced by co-administration of antigen and an anti-PD-1 antibody disclosed herein. Still other aspects of the invention relate to reducing immune responses mediated by activated lymphocytes in a subject comprising the administration of anti-PD-1 antibodies disclosed herein. Another aspect of the invention provides for the use of the disclosed anti-PD-1 antibodies for agonizing PD-1 and down regulating immune responses (or in some cases inhibiting or reducing the proliferation of activated lymphocytes). In particular embodiments, the immune response is TcR/CD28-mediated. As discussed herein, allergies, rheumatoid arthritis, type I diabetes mellitus, multiple sclerosis, inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, tissue, skin and organ transplant rejection or graft-versus-host disease (GVHD) can be treated *via* the administration of anti-PD-1 antibodies.

Some embodiments of this aspect of the invention may use two PD-1 specific antibodies that bind to distinct, non-overlapping epitopes.

Another aspect of the disclosure provides compositions comprising PD-1 specific antibodies and their use in methods of down regulating the immune response (or reducing the proliferation of activated T-cells, B-cells or mononuclear cells). These methods can be practiced on any subject, including humans or animals. In particular embodiments, anti-PD-1 antibodies are used to treat or prevent immune disorders by reducing the T cell response. Non-limiting examples of immune disorders that can be treated via the administration of PD-1 specific antibodies to a subject include, but are not limited to, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, type I diabetes, transplant rejection, graft-versus-host disease, hyperproliferative immune disorders, cancer, and infectious diseases. Yet other aspects of the invention provide for inhibiting or reducing lymphocyte (T-cell, B-cell and/or monocyte) activity in inflammatory lesions. Some embodiments of this aspect of the invention may use two PD-1 specific antibodies that bind to distinct, non-overlapping epitopes (such antibodies can be affinity matched to provide a desired activity *in vivo* (e.g., Clone 19 and Clone 2)).

Anti-PD-1 antibodies disclosed herein may be used, in another aspect of the invention to detect PD-1 or its fragments in a biological sample. The amount of PD-1 detected may be correlated with the expression level of PD-1, and associated with the activation status of immune cells (e.g., activated T cells, B cells, and/or monocytes) in the subject.

T-cells can be activated by any T-cell activating compound. As discussed in the examples, one such T-cell-activating compound is an anti-CD3 antibody, which binds TcR. Activating anti-CD3 antibodies are known in the art (see, for example, U. S. Patent Nos. 6,405,696 and 5,316,763). The ratio between the activating TcR signal and negative PD-1 signal is determined experimentally using conventional procedures known in the art or as described in the Examples. Some embodiments of this aspect of the invention may use two PD-1 specific antibodies that bind to distinct, non-overlapping epitopes.

The antibodies or antibody compositions of the present invention are administered in therapeutically effective amounts. Generally, a therapeutically effective amount may vary with the subject's age, condition, and sex, as well as the severity of the medical condition of the subject. A therapeutically effective amount of antibody ranges from about 0.001 to about 25 mg/kg body weight, preferably from about 0.01 to about 25 mg/kg body weight, from

about 0.1 to about 20 mg/kg body weight, or from about 1 to about 10 mg/kg. The dosage may be adjusted, as necessary, to suit observed effects of the treatment. The appropriate dose is chosen based on clinical indications by a treating physician.

In another aspect, the antibodies of the invention can be used as a targeting agent for delivery of another therapeutic or a cytotoxic agent (*e.g.*, a toxin) to a cell expressing PD-1. The method includes administering an anti-PD-1 antibody coupled to a therapeutic or a cytotoxic agent or under conditions that allow binding of the antibody to PD-1 expressed on the cell surface.

Still other aspects of the invention provide for the use of the disclosed antibodies for detecting the presence of PD-1 in biological samples. The amount of PD-1 detected may be correlated with the expression level of PD-1, which, in turn, is correlated with the activation status of immune cells (*e.g.*, activated T cells, B cells, and monocytes) in the subject.

The subject invention also provides methods of binding an antibody to a PD-1 polypeptide comprising contacting a sample that may contain PD-1 or cells expressing PD-1 with an antibody under conditions that allow for the formation of an antibody-antigen complex. These methods can further comprise the step of detecting the formation of said antibody-antigen complex. The complex can be detected using any means known in the art (*e.g.*, fluorescence activated cell sorting, radioimmunoassays, or chromogenic assays).

Another aspect of the disclosure provides compositions comprising anti-PD-1 antibodies. These compositions can be formulated according to known methods for preparing pharmaceutically useful compositions. Formulations are described in a number of sources which are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Science* (Martin E.W., Easton Pennsylvania, Mack Publishing Company, 19th ed., 1995) describes formulations which can be used in connection with the subject invention. Formulations suitable for administration include, for example, aqueous sterile injection solutions, which may contain antioxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the condition of the sterile liquid carrier, for example, water for injections, prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powder, granules, tablets, *etc.* It should be understood that in addition

to the ingredients particularly mentioned above, the formulations of the subject invention can include other agents conventional in the art having regard to the type of formulation in question.

Another aspect of the invention provides nucleic acids encoding PD-1 specific antibodies disclosed herein. For example, the nucleic acids encoding the antibody secreted by clone 19 can be isolated according to methods known to those skilled in the art. Yet another aspect of the invention provides vectors and transformed host cells comprising a nucleic acid encoding a PD-1 specific antibody as secreted by clone 19. As would be apparent to those skilled in the art, constant regions of the murine antibodies disclosed herein can be substituted with human constant regions to form chimeric antibodies comprising murine variable regions and human constant regions. Some embodiments provide for the substitution of heavy chain constant regions on the disclosed antibodies that provide for higher Fc receptor binding by the antibodies (*e.g.*, human IgG1, IgG3, and murine IgG2a isotypes, all of which bind Fc receptors strongly, can be grafted onto variable regions of the disclosed antibodies without affecting binding specificity). Alternatively, CDRs from the murine antibodies disclosed herein can be isolated and grafted into human framework regions to form humanized antibodies. Finally, methods of producing the disclosed PD-1 specific antibodies (including methods of producing the aforementioned humanized and chimeric antibodies) are also provided by the subject invention.

The hybridomas disclosed herein were deposited on September 9, 2008 with European Collection of Cell Cultures (ECACC), Centre For Emergency Preparedness and Response, The Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG United Kingdom. The accession numbers for the hybridomas are as follows:

Clone 2: 08090903;

Clone 10: 08090902; and

Clone 19: 08090901.

As discussed above, antibodies disclosed herein can be a full-length murine, human, humanized, or chimeric antibody; or a fragment or derivative thereof. In one embodiment, the antibody binds the same, or substantially the same, epitope as clone 19 or by a monoclonal antibody comprising a VH sequence of SEQ ID NO: 14 and a Vk sequence of SEQ ID NO: 12. In another embodiment, the antibody, including a fragment or derivative thereof, comprises the same or substantially identical VH and/or Vk regions as clone 19 (SEQ ID NOs: 14 and 12).

In another embodiment, the antibody, including a fragment or derivative thereof, comprises the same or substantially identical CDR1, CDR2 and CDR3 regions as those found in the Vk and VH sequences of clone 19 (SEQ ID NOs: 27-32). In one embodiment, the antibody comprises a VH sequence of SEQ ID NO: 14, a Vk sequence of SEQ ID NO: 12, as well as the sequence for murine IgG1 constant heavy chain region (GenBank accession No. D78344) and the sequence for murine IgG1 constant light chain region (GenBank accession No. V00807

). Other aspects of the invention provide nucleotide sequences encoding the disclosed antibodies, expression vectors comprising such sequences, host cells comprising such vectors, and methods of producing such antibodies from such host cells.

Fragments and derivatives of antibodies of this invention can be produced by techniques that are known in the art. "Immunoreactive fragments" comprise a portion of the intact antibody, generally the antigen binding site or variable region. Examples of antibody fragments include Fab, Fab', Fab'-SH, F(ab')₂, and Fv fragments; diabodies; any antibody fragment that is a polypeptide having a primary structure consisting of one uninterrupted sequence of contiguous amino acid residues (referred to herein as a "single-chain antibody fragment" or "single chain polypeptide"), including without limitation (1) single-chain Fv (scFv) molecules (2) single chain polypeptides containing only one light chain variable domain, or a fragment thereof that contains the three CDRs of the light chain variable domain, without an associated heavy chain moiety and (3) single chain polypeptides containing only one heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multispecific antibodies formed from antibody fragments. For instance, Fab or F(ab')₂ fragments may be produced by protease digestion of the isolated antibodies, according to conventional techniques. Alternatively, the DNA of a hybridoma producing an antibody of this invention may be modified so as to encode for a fragment of this invention. The modified DNA is then inserted into an expression vector and used to transform or transfect an appropriate cell, which then expresses the desired fragment.

In an alternate embodiment, the DNA of a hybridoma producing an antibody of this invention can be modified prior to insertion into an expression vector, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous non-human sequences (*e.g.*, Morrison et al., Proc. Natl. Acad. Sci. U.S.A.,

81, pp. 6851 (1984)), 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" antibodies are prepared that have the binding specificity of the original antibody. Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention. Thus, the antibodies of the present invention may also be made into "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in the original antibody, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly et al., *supra*; Morrison et al., Proc. Natl. Acad. Sci. U.S.A., 81, pp. 6851 (1984)).

In an exemplary embodiment, a chimeric recombinant mAb from clone 19 VH and Vk sequences, or a derivative or variant thereof, is produced. Nucleic acid sequences encoding the clone 19 VH and Vk sequences (SEQ ID NOs: SEQ ID NOs: 14 and 12, respectively) are cloned into a commercially available or otherwise known eukaryotic expression vector containing the light and heavy chain constant regions for a human or non-human antibody, using standard techniques. One example of a commercially available vector is pASK84, available from the ATCC (American Type Culture Collection, catalog number 87094). CHO cells, or other mammalian cell lines are then transfected with the vectors by standard methods, as described for example in "Molecular Cloning", Sambrook et al. The result is transfected cell lines that stably express and secrete the antibody molecule of interest, such as a chimeric version of clone 19 comprising its original VH and Vk regions and the constant regions from a human mAb. The entire cDNA sequences encoding the constant regions of human IgG can be found in the following GenBank entries:

Human IgG1 constant heavy chain region: GenBank accession #: J00228; Human IgG2 constant heavy chain region: GenBank accession #: J00230; Human IgG3 constant heavy chain region: GenBank accession #: X04646; Human IgG4 constant heavy chain region: GenBank accession #: K01316; and Human kappa light chain constant region: GenBank accession #: J00241.

Alternatively, VH and Vk regions of clone 19, or mutants or derivatives thereof, can be cloned into vectors encoding truncated constant regions in order to express antibody fragments (*e.g.*, Fab fragments). Isotype-switching of antibody can be made according to

similar principles. For example, an antibody with the exact same specificity as clone 19 but of a different isotype can be obtained by sub-cloning the cDNA encoding Vk and VH sequences into plasmids containing cDNA encoding human kappa light chain constant regions and a human heavy constant chain region selected from IgG1 or IgG2 or IgG3 or IgG4 constant heavy chain regions. Thus, an antibody as generated can possess any isotype and the antibody can then be isotype switched using conventional techniques in the art. Such techniques include the use of direct recombinant techniques (see, *e.g.*, US Patent 4,816,397), cell-cell fusion techniques (see *e.g.*, US Patent 5,916,771), and other suitable techniques known in the art. Accordingly, the effector function of antibodies provided by the invention may be “changed” with respect to the isotype of a parent antibody by isotype switching to, *e.g.*, an IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM antibody for various therapeutic or other uses.

According to another embodiment, the antibody of this invention is humanized. “Humanized” forms of antibodies according to this invention are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from the murine immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of the original antibody (donor antibody) while maintaining the desired specificity, affinity, and capacity of the original antibody. In some instances, Fv framework residues of the human immunoglobulin may be replaced by corresponding non-human residues. Furthermore, humanized antibodies can comprise residues that are not found in either the recipient antibody or in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of the original antibody and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones et al., *Nature*, 321, pp. 522 (1986); Reichmann et al., *Nature*, 332, pp. 323 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2, pp. 593 (1992). Accordingly, humanized versions of clone 19 comprising the VH and Vk CDR regions of clone 19 and constant and

framework regions from a human mAb can be made, using known constant and framework human mAb sequences and established techniques in the art, as described herein. For any humanized antibody incorporating the clone 19 VH CDR1 domain, the domain can contain SEQ ID NO: 30 or amino acids 6-10 of SEQ ID NO: 30.

Methods for humanizing the antibodies of this invention are well known in the art. Generally, a humanized antibody according to the present invention has one or more amino acid residues introduced into it from the original antibody. These murine or other non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321, pp. 522 (1986); Riechmann et al., *Nature*, 332, pp. 323 (1988); Verhoeyen et al., *Science*, 239, pp. 1534 (1988)). Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly et al., U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from the original antibody. In practice, humanized antibodies according to this invention are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in the original antibody.

EXAMPLES

EXAMPLE 1—METHODS FOR GENERATION OF ANTI-PD-1 ANTIBODIES

1.1 Myeloma cell line

For fusion the myeloma cell line SP2/0-Ag14 from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig) was used. This cell line is a hybrid between BALB/c spleen cells and the myeloma cell line P3x63Ag8. The cells have been described as not synthesizing or secreting immunoglobulin chains, being resistant to 8-azaguanine at 20 µg/ml, and not growing in HAT (Hypoxanthine, Aminopterin, Thymidine) medium. The SP2/0 cells are routinely maintained in tissue culture flasks in standard growth medium (with 10% FCS). A new aliquot of frozen SP2/0 cells was used after a period of 2 weeks in order to avoid the implementation of HGPRT-positive revertants. The myeloma cells were shown to be negative in all mycoplasma tests.

1.2 Antigens for immunization and screening

The recombinant protein PD-1Fc was prepared using the methods described for the production of CD28Fc (Evans *et al. Nat Immunol.* 6, 271-9 (2005)) and concentrated to 5.1 mg/ml in 0.01 M HEPES, 150 mM NaCl, pH 7.4. SDS-PAGE analysis of the antigen run under reducing and non-reducing conditions established the purity of the protein to be >95%.

1.3 Immunization

Five mice (about 8 weeks old) were immunized *via* the intraperitoneal cavity using an immunization protocol over 60 days. For immunization an alum precipitate of the immunogen was prepared. The alum precipitate was freshly prepared for each boost. The mice were immunized with 50 µg protein and boosted with 25 µg protein. Three mice were used for fusion.

1.4 General handling of cells

Cells were handled under sterile conditions using a laminar air-flow system, sterile materials and sterile solutions. Cells were incubated at 37°C in a humid atmosphere containing 5% carbon dioxide. For cultivation of the hybridoma cells a complete growth medium (CGM) containing DMEM with supplements 2-mercaptoethanol, L-Glutamine, GlutaMax, HT, non essential amino acids, sodium pyruvate, antibiotics/antimycotic solution (in concentrations recommended by the supplier) and FCS at different concentrations (10%, 15% or 20%) was used.

1.5 Preparation of spleen cells and cell fusions

After asphyxiation of the three immunized mice in CO₂ spleens were aseptically removed. A single cell suspension of pooled spleens was prepared. The spleen cells and the myeloma cells were washed several times with DMEM and fused twice in the presence of 1 ml 50% (w/v) PEG 3550 (ratio spleen cells to SP2/0 2.5:1 and 2.4:1). The hybridomas produced were resuspended in CGM containing 20% FCS and aminopterin (HAT medium). The cell suspension (140 Cl/well) of each fusion was plated out into eight 96-well tissue culture flat-bottom plates (Corning-Costar) containing 140 Cl/well peritoneal exudate cells as feeder cells in CGM with 20% FCS. The plates were incubated for 10 days. During this period cells were fed two times with HAT medium. An aliquot of the spleen cell preparation (about 8x10⁶ spleen cells) was cultivated 10 days in a well of a 24-well plate and the cell culture supernatant served as positive control in ELISA.

1.6 Screening assay

An ELISA was used for screening of IgG in cell culture supernatants. 96 well flat-bottom polystyrene microtiter plates (Greiner, Cat. No 655061) were coated with 50 µl/well PD-1Fc antigen (5 µg/ml) in 0.5 M carbonate/bicarbonate buffer, pH 9.6. After incubation overnight in a moist chamber at 4°C the plates were washed with tris-buffered saline (TBS, 50 mM Tris, pH 7.8, 500 mM sodium chloride) containing 0.01% Triton™ X-100 (washing buffer) and blocked with 200 µl/well 2% FCS in TBS (blocking buffer) for 1 hour at room temperature (RT) on a shaker. The wells were washed with washing buffer and 100µl cell culture supernatant was added in the appropriate well. Cell culture supernatant from SP 2/0 myeloma cells was used as a negative control. As positive control cell culture supernatant from spleen cell culture was used. The plates were incubated on a shaker for 1 h at RT, followed by several washes. For detection of bound antibodies plates were incubated with 50 µl/well goat anti-mouse IgG (Fab specific) conjugated to alkaline phosphatase (1:5000) in blocking buffer for 1 h at RT on a shaker, followed by several washes and addition of 150 µl/well substrate buffer (2 mM 4-nitrophenyl phosphate in 5% diethanolamine + 0.5 mM MgCl₂, pH 9.8). The optical density (OD) was estimated in a 12-channel Dynex Opsys MR microplate reader at 405 nm. Wells with OD_{405nm} 2-fold higher than the OD_{405nm} of the average plate value were selected as positive.

1.7 Selection of stable antibody producers

Cells from positive IgG producing cultures were transferred into wells of a 48-well plate and cultivated for several days (depending on the growth characteristics of the cells). An ELISA on PD-1Fc and without precoated antigen in order to select the specific binders was carried out. The cells from ELISA-positive wells were frozen in freezing medium (90 % FCS, 10 % DMSO). An aliquot of the cells was further cultivated for production of cell culture supernatants for further characterization.

1.8 Limiting dilution cloning

As soon as positive wells were identified, hybridoma cells were cloned to reduce the risk of overgrowth by non-producing cells (first cloning). To ensure that the antibodies are truly monoclonal the hybridomas were cloned again (second cloning). The method of limiting dilution was used for both cloning procedures. IgG producing cells were distributed into one

96 well plate containing feeder cells at a density of 1-3 cells per well. After 8-10 days (depending on growth characteristics) all plates were visually inspected under the microscope for detection of monoclonal growth. Culture supernatants from such wells were screened for specific immunoglobulin content using the above-described screening assay. The appropriate clones concerning growth characteristic and ELISA signal were selected, transferred into wells of a 24- well plate and cultivated for some days. A screening assay was performed. This procedure was repeated two to three times. The appropriate subclone was selected respectively for the second cloning procedure or cultivation for cryopreservation. This procedure resulted in the production of three anti-PD-1 antibodies: Clone 2, Clone 10 and Clone 19. Clone 2 is characterized only with respect to its epitope and binding off-rate.

EXAMPLE 2—CHARACTERIZATION OF THE CLONE 10 AND CLONE 19 ANTIBODIES

2.1 Reagents used for characterization of the properties of the antibodies

The following directly labelled antibodies were used: donkey anti-mouse IgG Alexa647 conjugate (Molecular Probes), anti-human C4 Alexa647 conjugate (Serotec Ltd) and anti-human CD4 FITC conjugate (Serotec Ltd). OX7 (mIgG₁ culture supernatant; in-house) and MOPC21 (mIgG₁; Sigma-Aldrich Ltd) were used as isotype controls. Isotype-specific PE-labelled goat anti-mouse IgG₁ and IgG_{2a} antibodies (STAR81PE and STAR82PE respectively) were obtained from Serotec Ltd and exhibited <1% cross reactivity with other murine Ig subclasses. Propidium iodide and rabbit IgG were from Sigma Ltd. Clone19 anti-PD1 antibody produced as described above was conjugated to Alexa647 using a kit following the manufacturer's instructions (Molecular Probes). IL-2 levels in cell culture supernatants were quantified using the DuoSet Human IL-2 ELISA Kit (R&D Systems Ltd).

2.2 Preparation and isotyping of antibodies

Hybridoma supernatant was prepared and diluted into sterile, azide-free PBS. Purified stocks of monoclonal antibodies were isotyped at 1 µg/ml in PBS using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Santa Cruz; sc-24958). The isotypes of Clone 19, Clone 10 and Clone 2 were IgG_{1K}.

2.3 Epitope mapping

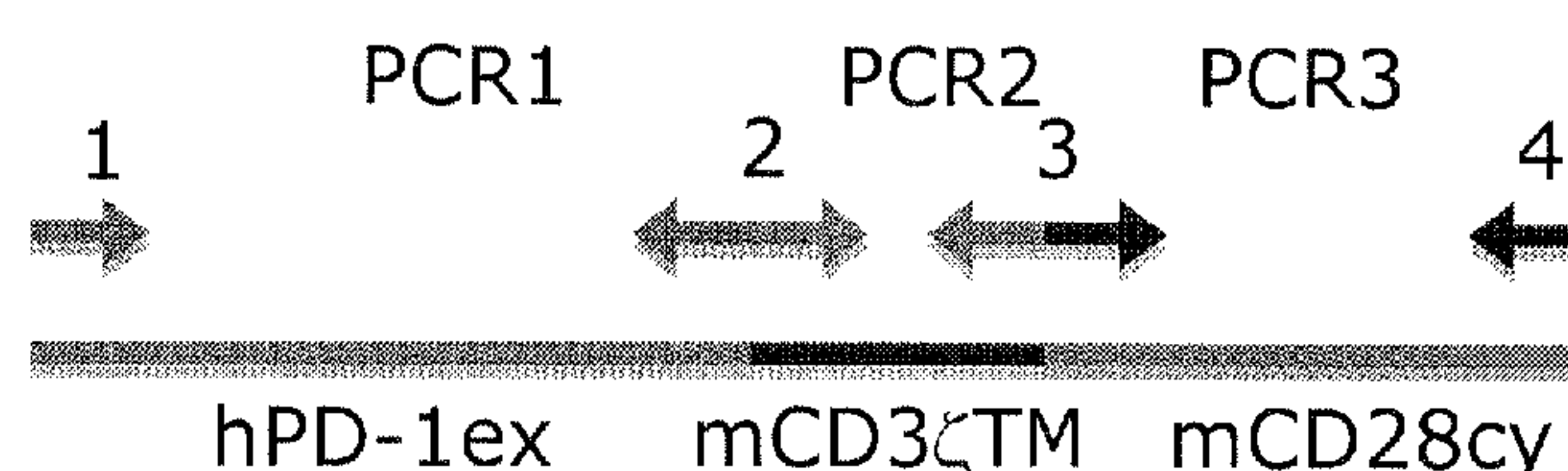
Constructs encoding the human extracellular region of PD-1 with the transmembrane and intracellular regions of murine CD28 were cloned into the bi-cistronic mammalian expression vector pGFP2-n2 from BioSignal Packard Ltd, which also encodes GFP. Mutant constructs varying by one amino acid were prepared using the “drastic” mutagenesis approach (Davis *et al. Proc Natl Acad Sci USA.* 95, 5490-4 (1998)). Plasmids (2 µg/well) were transfected into HEK-293T cells in 6 well plates using Genejuice transfection reagent (Novagen; 6 µl/well). Mock and no-transfection controls were included with each experiment. Cells were harvested at 18-24 hours and stained with anti-PD1 antibodies or isotype controls at 10 µg/ml in PBS-azide for 1h at 4°C. Cells were washed with PBS-Azide, pelleted at 1500rpm/5min and primary antibodies were labelled with Alexa647-conjugated donkey anti-mouse IgG (5 µg/ml) in PBS-Azide for 30 min at 4°C. Cells were washed as above and resuspended in 200 µl PBS-Azide before being analysed at the flow cytometer. Propidium iodide (5 µg/ml) was added immediately prior to analysis to identify dead cells. GFP-positive (transfected) viable cells were gated and analysed for binding of anti-PD1 antibodies. Mutants were defined as ‘knock-out’ (reducing the percentage of cells bound by the anti-PD1 antibody) or ‘knock-down’ (reducing the intensity of antibody staining relative to other PD-1 antibodies).

Following transfection, cells analysed at the flow cytometer were 85-90% viable by propidium iodide exclusion. An example of the binding analysis is shown in Figure 1. Transfection efficiencies ranged from 15-50% (GFP⁺). Isotype controls were negative on all transfectants. Analysis of the percentage of GFP⁺ cells that are also positive for Alexa647 (anti-PD-1 antibody binding) shows that the L16R and R118D mutations completely eliminate Clone 19 binding (Fig. 2). All R118D expressing cells bind Clone 10, indicating functional expression of PD-1, but have the lowest intensity of all mutants (Fig. 2), suggesting a low level of expression. V18R partially eliminates Clone 19 binding. Clone 10 binds all the mutants but for mutants N41K and L103E the binding intensity for this antibody versus the other PD-1 antibodies is significantly decreased (Fig. 2). The binding analyses thus define two distinct epitopes each defined in turn by at least two residues: anti-PD-1 antibody Clone 10 binds to a membrane-distal epitope that overlaps with the ligand-binding region (Zhang *et al. Immunity* 20, 337-47 (2004)); Clone 19 binds to a membrane-proximal epitope. The binding-disrupting residues are mapped onto the murine PD-1 crystal structure in Figure 3.

EXAMPLE 3—ANALYSIS OF CLONE 10- AND CLONE 19-INDUCED SIGNALING BY A DIMERIC FORM OF PD-1 WITH AN ACTIVATING CYTOPLASMIC DOMAIN

To directly compare the signal-generating activities of the antibodies, a dimeric form of PD-1 was generated that consisted of the extracellular (antibody-binding) region of human PD-1 spliced to the transmembrane region of CD3 ζ (to produce dimers) and the cytoplasmic region of CD28 (in order to have an “active” readout consisting of IL-2 secretion; Fig. 4A).

3.1 Construction of a dimeric form of PD-1 for detecting anti-PD1 antibody-induced activating signaling in a T-cell hybridoma



The hPD-1/mCD3 ζ WT/mCD28 construct was created in a series of five steps. In step 1, oligonucleotide 1 (left arrow; sequence 5'-TAGTAGAGATCTCTCAAGCAGGCCACCATGCAAATCCCACAGGCGCCGTGG-3', SEQ ID NO: 33), which encodes a BglIII restriction site and the rat ribosome binding site followed by the initiating codon and the first 21 bases of the signal peptide-encoding sequence of human PD-1, was used in a polymerase chain reaction (PCR1) with the complement of oligonucleotide 2 (5'-TCAGCCGGATCCTTCCAAACCCTGGTGCTCTGCTACTTGCTAGATGG-3', SEQ ID NO: 34). Oligonucleotide 2 encodes the last nine residues of the human PD-1 extracellular domain (up to residue 170 of the mature polypeptide) inserting a Bam H1 site, followed by 20 bases encoding the NH₂-terminal end of the mouse CD3 ζ transmembrane domain. PCR reactions were carried out under standard conditions. In step 2, oligonucleotide 2 was used in a PCR reaction (PCR2) with the complement of oligonucleotide 3 (5'-ATCACAGCCCTGTACCTGAATAGTAGAAGGAATAGACTC-3', SEQ ID NO: 35) which encodes the COOH-terminal end of the transmembrane region of mouse CD3 ζ , followed by the first 21 bases encoding the NH₂-terminal end of the mouse CD28 cytoplasmic domain. In step 3, the PCR1 and PCR2 reaction products were purified, annealed, extended and then amplified in the presence of oligonucleotide 1 and the complement of oligonucleotide 3, to generate a cDNA encoding the extracellular region of PD-1 fused with the transmembrane region of CD3 ζ . In step 4,

oligonucleotide 3 was used in a PCR reaction (PCR3) with oligonucleotide 4 (5'-CTTCGAGCTACTAGGGGCGGTACGCTGCAAA-3', SEQ ID NO: 36), which encodes the COOH-terminal end of the cytoplasmic domain of mouse CD28 followed by a stop codon and a Xho I restriction site. In step 5, the purified PCR3 product was fused with the purified PCR product from step 3 by annealing the two products, extending the annealed hybrid, and then amplifying it with oligonucleotides 1 and 4.

Human PD-1 and mouse CD28 cDNA was amplified using pENTRhPD-1/mCD28 as template, which was originally constructed from IMAGE clones obtained from Geneservices Ltd (Cambridge UK). Mouse CD3 ζ was amplified from DO11.10 mouse T cell hybridoma cDNA. The fusion PCR products were cloned into pCR4 \oplus -TOPO \oplus (Invitrogen) and the final products sequenced by the dideoxy method. The constructs were cut with BglII and XhoI and inserted into the lentiviral vector pHR-SIN-BX-IRES-Em.

3.2 Detection of activating signaling by the hPD-1/mCD3 ζ WT/mCD28 chimera

HEK 293T cells were transfected with pHR-SIN-BX-IRES-Em encoding hPD-1/mCD3 ζ WT/mCD28, and the supernatant used to infect DO11.10 T-cell hybridomas. Infected DO11.10 cells were propagated and FACS sorted for mouse PD-1 and EGFP expression, and then tested for agonistic signaling by the anti-PD-1 antibodies using IL-2 release as a stimulation assay readout. The IL-2 secretion results indicate that both antibodies are capable of inducing signaling *via* the hPD-1/mCD3 ζ WT/mCD28 chimera; however Clone 19, which binds PD-1 closest to the membrane induces the largest amount of IL-2 release (representative data is shown in Fig. 4b). This supports the notion that the topology of the complex formed by the antibodies is what determines the relative levels of signaling induced by agonists. The data also suggest that the degree of agonistic signaling can be varied with choice of antibody.

EXAMPLE 4—ANALYSIS OF INHIBITORY SIGNALING BY CLONE 10 AND CLONE 19 ANTIBODIES IN HUMAN PERIPHERAL LYMPHOCYTES (PBL)

The antibodies were tested for their ability to inhibit TCR-derived activating signals by covalently coupling the antibodies, along with anti-CD3 antibodies, to tosyl-activated DYNALBEADS. The beads were then added to cultures of PBL labelled with carboxyfluorescein succinimidyl ester (CFSE). Proliferation levels were indicated by the fraction of cells with diluted CFSE determined by flow cytometric analysis.

4.1 Loading and quantification of antibody on DYNALBEADS

Tosyl-activated 4.5 μm DYNALBEADS (M450; Invitrogen) were washed in 0.1M sterile phosphate buffer (pH 8) and loaded with 2.5 μg total antibody per 3×10^7 beads at 37°C for 18-24 h with continuous inversion mixing. Rabbit IgG (Sigma) was used to equalise the amount of total antibody per bead-loading reaction. Beads were blocked for at least 30 min in RPMI with 10% FCS at room temperature and washed three times in serum-free RPMI. For some experiments, bead-bound antibody was quantified in duplicate with saturating amounts of isotype-specific PE-labelled goat antibodies and compared with Quantibrite™ prelabelled quantification kit (BD Biosciences Ltd.). The geometric mean fluorescence PE intensities of bead singlets (minus background of unloaded beads as a control) were used to calculate the absolute amount of antibody loaded per bead from the standard curve. An example of such a titration is given in Fig. 5. Loaded beads were stored at 4°C. During bead loading the amounts of anti-CD3 antibody added were varied so that, at the time of the experiments, the effects of matched sets of beads with near-equivalent levels of anti-CD3 antibody could be compared. The level of stimulation provided by anti-CD3 loaded beads was defined as low (resulting in 15% proliferation of bulk lymphocytes at day 5), medium-low (30% proliferation), medium-high (60% proliferation) and high (80% proliferation).

4.2 Proliferation studies

Fresh heparinized blood was diluted 1:1 with PBS and the lymphocytes isolated by density gradient separation (Ficoll Hypaque). In some experiments, accessory cells were depleted by plastic adherence for 2 h at 37°C or with specific antibody-labelled DYNALBEADS (against CD14/19/8/56). Cells were washed in PBS and RPMI and resuspended at 10^7 cells/ml in serum-free RPMI. Cells were labeled with 25 μM CFSE in PBS for 10 min in the dark at RT. CFSE was quenched with an equal volume of FCS at RT for 5 min. Cells were washed 3-5 times with RPMI and resuspended at 10^6 cells/ml in RPMI +10% FCS + PSG + 2-ME (final concentration 5×10^{-5} M). Antibodies (beads), mitogen or media was added to relevant wells in 96-well round-bottomed plates and 10^5 cells/well were distributed and incubated at 37°C for 3-5 days. For proliferation studies, cells were stained with directly-labelled cell-surface antibodies for 1 h at 4°C. Cells were washed with PBS-Azide, pelleted at 1500rpm/5min and resuspended in 200 μl PBS-Azide. Cells were analysed

for CFSE and antibody labelling at the flow cytometer using FlowJo Flow Cytometry Analysis Software.

4.3 Effects of the antibodies

In the experimental results described in Figure 6, the tosyl-activated beads used had been incubated with 2.5µg of total antibody containing up to 2375 ng of anti-PD-1 Clone 10 or Clone 19 antibody, and enough anti-CD3 antibody to induce ~25% proliferation in the absence of anti-PD-1 antibody. Proliferation was measured by CFSE dilution at day 5. In experiment 1, visual inspection indicates that inhibition of proliferation is seen with all three antibodies, with Clones 2 and 19 now giving the highest levels of inhibition. For the data that can be analyzed using automated analysis software (FlowJo), the amount of inhibition of proliferation by Clone 19 is of the order of 80%. In experiment 2, although the degree of proliferation in the presence of OKT3 only is somewhat reduced (to ~15%), it is clear that Clones 2 and 19 are profoundly inhibiting proliferation; at most the cells that start proliferating undergo one or two rounds of proliferation only. Clone 10 is without any inhibitory effect in experiment 2.

Clone 10 antibodies were further tested for their ability to inhibit TCR-derived activating signals by covalently coupling the antibodies, along with anti-CD3 antibodies, to tosyl-activated DYNALBEADS. The beads were then added to cultures of human CD4⁺ T cells and proliferation measured by ³H-thymidine incorporation.

Tosyl-activated 4.5 µm DYNALBEADS (M450; Invitrogen) were washed in 0.1M sterile phosphate buffer (pH 7.5) and loaded with 2 µg of anti-human CD3 (clone OKT3) per 1x10⁷ beads at 37°C for 8h with continuous inversion mixing. Beads were then washed to remove un-conjugated anti-CD3. Aliquots of the anti-CD3 conjugated beads were then secondarily coated with 3 µg of anti-PD-1 antibody or control per 1x10⁷ beads at 37°C for 19h with continuous inversion mixing. Beads were washed and then incubated in 0.2M Tris / 0.1% BSA (pH 8.5) for 3 hours to inactivate free tosyl groups, followed by washing and re-suspension of beads in PBS / 0.1% BSA / 2mM EDTA (pH 7.4). Equal anti-CD3 and antibody coating of the bead sets was confirmed by staining the beads with fluorochrome-labelled isotype-specific antibodies and analysing by flow cytometry.

Fresh heparinized blood was diluted 1:1 with RPMI and the lymphocytes isolated by density gradient separation (Ficoll™ Hypaque). CD4⁺ T cells were purified from the whole PBLs by negative selection using MACS (CD4⁺ T cell isolation Kit II; Miltenyi Biotec).

1×10^5 human CD4⁺ T cells / well were cultured at a 1:1 ratio with the coated beads in 96-well round-bottomed plates and incubated at 37°C for 6 days. Proliferation was measured at day 6 by addition of 0.5 μ Ci/well ³H-thymidine for the last 6 hours of culture. Cells were harvested onto glass-fibre filters and incorporated ³H-thymidine was measured by β -scintillation counting.

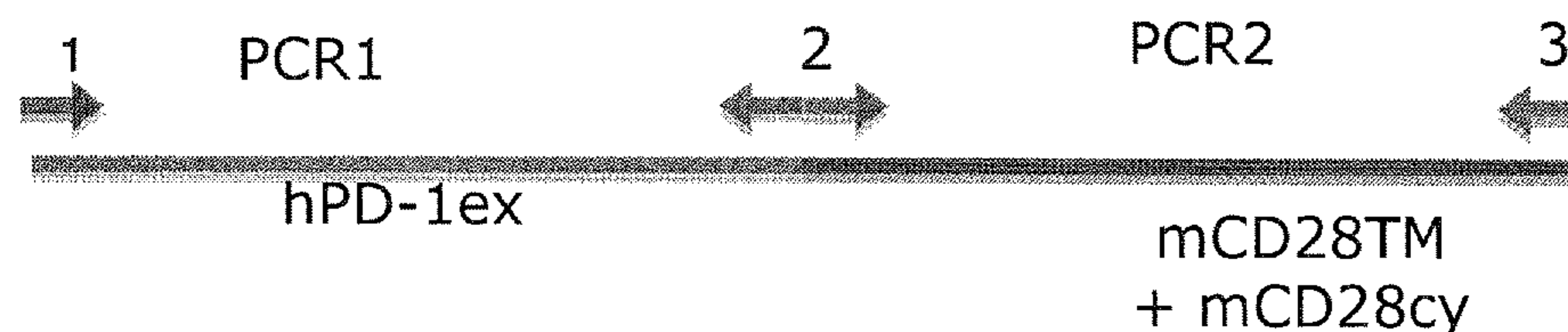
The results in Figure 13 show the day 6 proliferative response by human CD4⁺ T cells measured in the presence of anti-CD3 plus Clone 10 antibody or control coated beads. The data are expressed as percentage of the maximal response (anti-CD3 plus BSA control) and are the mean of 4 different donor responses. CD4⁺ T cell proliferation was inhibited in the presence of Clone 10, so that the average proliferation observed was only 37.7 % of the maximum.

Clone 19 generally induces stronger signaling by the hPD-1/mCD3 ζ WT/mCD28 chimera than Clone 10 (Fig. 4B) but in some experiments it gives weaker inhibitory signaling by native PD-1 (see, e.g. Fig. 8). It is possible that this is because, in some experiments, Clone 19 but not Clone 10 ligation results in the phosphorylation of both the ITIM (inhibitory) and the ITSM (activating) tyrosine-based signaling motifs of PD-1 (see Fig. 9).

EXAMPLE 5—USING TWO ANTIBODIES WITH NON-OVERLAPPING EPITOPES TO ENHANCE SIGNALING BY A MONOMERIC RECEPTOR

Individual anti-PD-1 antibodies working alone, e.g. Clone 10, are already inhibitory but it should be possible to significantly enhance these effects by using pairs of anti-PD-1 antibodies. Initial characterization of the signaling properties of the antibodies relied on an assay in which PD-1 was expressed in the form of the hPD-1/mCD3 ζ WT/mCD28 chimera, which forms a homodimer. This was done in order to facilitate comparisons with anti-CD28 superagonistic antibodies, since CD28 is also a homodimer. A question that arises is: To what extent is signaling by the hPD-1/mCD3 ζ WT/mCD28 chimera dependent on its bivalency, and resulting cross-linking? To test this, a monomeric, monovalent form of PD-1, hPD-1/mCD28, that consisted of the extracellular (antibody-binding) and transmembrane regions of human PD-1 spliced to the cytoplasmic region of CD28 (in order to have an “active” readout consisting of IL-2 secretion; Fig. 10a), was generated.

5.1 Construction of a monomeric form of PD-1 for detecting anti-PD1 antibody-induced activating signaling in a T-cell hybridoma



The hPD-1/mCD28 construct was created in a series of three steps. In step 1, oligonucleotide 1 (left arrow; sequence 5'-TAGTAGAGATCTCTCAAGCAGGCCACCA TGCAAATCCCACAGGCGCCGTGG-3', SEQ ID NO: 33), which encodes a BglII restriction site and the rat ribosome binding site followed by the initiating codon and the first 24 bases of the signal peptide-encoding sequence of human PD-1, was used in a polymerase chain reaction (PCR1) with the complement of oligonucleotide 2 (5'-GCCCAGCCGGCCAG TTCAAACCTTTTGGGTGCTGGTGGTGGTTGGT-3', SEQ ID NO: 37). Oligonucleotide 2 encodes the last 23 bases of the human PD-1 extracellular domain (up to residue 149 of the mature polypeptide), followed by 24 bases encoding the NH₂-terminal sequence of the mouse CD28 transmembrane region. PCR reactions were carried out under standard conditions. In step 2, oligonucleotide 2 was used in a PCR reaction (PCR2) with the complement of oligonucleotide 3 (5'-TTTGCAGCGTACCGCCCCACGCGTTAGTAGCTCGAG-3', SEQ ID NO: 38) which encodes the COOH-terminal end of the cytoplasmic domain of mouse CD28, a Mlu I restriction site followed by a stop codon and a Xho I restriction site. In step 3, the purified PCR2 product was fused with the purified PCR1 product from step 1 by annealing the two products, extending the annealed hybrid, and then amplifying it with oligonucleotides 1 and 3.

Mouse CD28 sequence was amplified using pCR4®-TOPO®rCD28/mCD28 as template, which was originally amplified from DO11.10 mouse T cell hybridoma cDNA. The human extracellular PD-1 was amplified from pE14hPD-1Long, a gift from Dr Chao Yu of the MRC Human Immunology Unit, Oxford. The fusion PCR products were cloned into pCR4®-TOPO®(Invitrogen) and the final products sequenced by the dideoxy method. The constructs were cut with BglII and XhoI and inserted into the lentiviral vector pHR-SIN-BX-IRES-Em for infection of DO11.10 cells. Activation of the DO11.10 cells expressing the hPD-1/mCD28 chimera by anti-PD-1 antibodies was examined using IL-2 secretion as a read-out.

5.2 Lack of signaling by hPD-1/mCD28 suggests that agonistic signaling may be enhanced by cross-linking a monomeric receptor with two antibodies that bind to non-overlapping epitopes

Clone 10 and Clone 19 were not agonistic for a chimeric form of human PD-1, *i.e.* hPD-1/mCD28, consisting of the monomeric extracellular region of PD-1 attached to the transmembrane and intracellular signaling domains of CD28 (Fig. 10), in contrast to the equivalent CD28 construct (containing the homodimeric extracellular domain of rat CD28). The likeliest explanation for this is that, because PD-1 is monomeric and CD28 is a homodimer, the attachment of bivalent antibody leads to the assembly of a multimeric array of “cross-linked” CD28 molecules and a very high density of signaling domains (Fig. 11a), whereas the binding of Clone 10 or Clone 19 brings together only pairs of PD-1 molecules (Fig. 11b). *In vivo*, therefore, antibodies binding to homodimeric receptors will generally produce stronger signaling than an antibody that binds to a monomeric receptor. In the case of PD-1, if a multimeric assembly of PD-1 molecules could be generated this would be predicted to lead to much more potent signaling (Fig. 11c). The positions of the epitopes of Clone 10 (or Clone 2) and of Clone 19 on opposite “sides” of PD-1 (Fig. 3) imply that the two antibodies are likely to bind to non-overlapping surfaces, *i.e.* that each native PD-1 monomer would be capable of binding both antibodies. This suggests that pairs of the antibodies could be used *in vivo* to “cross-link” native PD-1 monomers as shown in Figure 11c. The high-density arrays of sequestered PD-1 molecules thus generated are expected to produce more potent signaling than would be possible using single antibodies.

5.3 Agonistic signalling is enhanced by cross-linking a monomeric receptor with two antibodies that bind to non-overlapping epitopes

To test the idea that pairs of antibodies could be used to “cross-link” native PD-1 monomers and induce enhanced agonistic signaling, DO11.10 cells expressing the hPD-1/mCD28 chimeric protein were used in a Clone 10/Clone 19 antibody stimulation assay as follows. 96-well flat-bottomed plates (Costar EIA/RIA plates) were coated overnight at 4°C with 500 μ g/ml donkey anti-mouse IgG (Jackson Immunoresearch) in coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, pH 9.6). Prior to the addition of cells, the plates were washed three times with 200 μ l chilled PBS. 5×10^5 cells were centrifuged at 1200 rpm for 3 minutes

and resuspended in 100 μ l complete medium containing the Clone 19 antibody at various concentrations for 30 minutes. The cells then washed and subjected to an additional 30 minute incubation with Clone 10 at various concentrations, before the cells were plated out in triplicate onto the donkey anti-mouse IgG pre-coated 96 well plates. Cells were incubated at 37°C, in 5% CO₂ for 48 hours before the cell culture supernatant was removed and assayed for mouse interleukin-2 (IL-2) by ELISA.

The results of this experiment (Fig. 7) show that at the highest concentrations, *i.e.* 100 μ g/ml, neither Clone 10 nor Clone 19 initiate signalling (IL-2 production) in DO11.10 cells expressing the hPD-1/mCD28 chimeric protein. However, successive incubations of the antibodies at 10-100 μ g/ml induced significant levels of IL-2 production. This suggests that cross-linking pairs of antibodies could be used to induce enhanced signalling *in vivo*.

**Sequence information for Clones 2, 10 and 19 antibodies
(CDRs indicated by underlining in amino acid sequences)**

Clone 2

VK DNA (SEQ ID NO: 1)

gacattgtgctgacacagtctctctctttagctgtatctctggggcagagggccaccatctcatgcagggccagcaaaagtgtcagt
acatctggcttaattatatacactggtaccaacagaaaccaggacagccacccaaactcctcatctatcttgcacccaacctagaatctg
gggtccctgccaggttcagtggtggtctgggacagacttcacctcaacatccatcctgtggaggacgaggatgctgcaaccta
ttactgtcagcacagtagggagcttccgctcacgttcggtgctgggaccaagctggaataaaa

VK protein (SEQ ID NO: 2)

DIVLTQSPASLA VSLGQRATISCRASKSVSTSGFNYIHWYQQKPGQPPKLLIYLASNLE
SGVPARFSGSGSGTDFTLNIHPVEDEDAATYYCQHSRELPLTFGAGTKLEIK

VH DNA (original cloned) (SEQ ID NO: 3)

caggtccaactgcagcagcctggggctgaactggtgaagcctggggcttcagtgaagttgtcctgcaaggcttctggctacacctca
ccacctactattgtactgggtgaggcagaggcctggacaaggccttgagtggattggggggattaatcctagcaatggtggtactaac
tcaatgagaagttcaagagcaaggccacactgactgtagacaaatcctccagcacagcctacatgcaactcaacagcctgacatctg
aggactctgcggtctattactgtacaagacgggactataggtacgacagaggcttgactactggggccaaggcacctcagtcacagt
c

VH DNA (mutated to remove splice site) (SEQ ID NO: 5)

caggtccaactgcagcagcctggggctgaactggtgaagcctggggcttcagtgaagttgtcctgcaaggcttctggctacacctca
ccacctactattgtactgggtgaggcagaggcctggacaaggccttgagtggattggggggattaatcctagcaatggtggtactaac

ttcaatgagaagtcaagagcaaggccacactgactgtagacaaatcctcctctacagcctacatgcaactcaacagcctgacatctga
ggactctgcggtctattactgtacaagacgggactataggtacgacagaggctttgactactggggccaaggcacctcagtcacagtc

VH protein (SEQ ID NO: 4 or SEQ ID NO: 6)

QVQLQQPGAELVKPGASVKLSCKASGYTFTTYLYWVRQRPQGLEWIGGINPSNG
GINFNEKFKSKATLTVDKSSSTAYMQLNSLTSEDSAVYYCTRRRDYRYDRGFDYWG
QGTSVT

Clone 10

VK DNA (SEQ ID NO: 7)

gatgtttgatgacccaaactccactctccctgcctgtcagtttgagatcaagcctccatctcttcagatctggcagaacattgtacat
agtaatgaaacacctatttagaatggtacctacagaaaccaggccagctccaaagctcctgatctacaaagctccaaccgatttttg
gggtcccagacaggatcagtggcagtgatcaggacagattcacactcaagatcagcagagtgaggctgaggatctgggagttt
attctgctttcaagggtcacatgtccattcacgttcggctcggggacaaagctggaataaaa

VK protein (SEQ ID NO: 8)

DVLMTQTPLSLPVSLGDQASISCRSGQNIVHSNGNTYLEWYLQKPGQSPKLLIYKVS
NRFFGVPDRISGSGSGTDFTLKISRVEAEDLGVYFCFQGSHVPFTFGSGTKLEIK

VH DNA (SEQ ID NO: 9)

gatgtgcagcttcaggagtcgggacctggcctggtgaaacctctcagctctgtccctcacctgcactgtcactggctactcaatcacc
agtgattatgcctggaactggatccggcagttccaggaacaaactggagtggatgggctacataaactacagtggtagcactagcta
caaccatctctcaaaagtcgaatctctatcactcgagacacatccaagaaccagttctcctgcagttgaattctgtgactactgaggac
acagccacatattactgtgcaagatggatcggtagtagcgcctggtacttcgatgtctggggcgcaggaccacggtcacagtc

VH protein (SEQ ID NO: 10)

DVQLQESGPGLVKPSQSLSLTCTVTGYSITSDYAWNWIRQFPGNKLEWMGYINYSSGS
TSYNPSLKSRISITRDTSKNQFFLQLNSVTTEDTATYYCARWIGSSAWYFDVWGAGT
TDTV

Clone 19

VK DNA (SEQ ID NO: 11)

gaaaatgtgctcaccagctccagcaatcatgtctgcatctccaggggaaaagggtaccatgacctgcagggccagctcaagtgtaa
ttccagttactgcactggtagcagcagaagtcaggtgcctcccccaactctggattatagcacttccaactggcttctggagtcct
gatcgttcagtggcagtggtctgggacctcttactctctcaaatcagcagtggtggaggctgaagatgctgccacttattactgccag
cagtacaatggttaccgctcacgttcggtgctgggaccaagctggaataaaa

VK protein (SEQ ID NO: 12)

ENVLTQSPA~~IM~~SASPGEKVTMT~~CR~~ASSSVISSYLHWYQQKSGASPKLWIYSTSNLASG
VPDRFSGSGSGTSYSLTISSVEAEDAATYYCQQYNGYPLTFGAGTKLEIK

VH DNA (SEQ ID NO: 13)

caggctcagctacagcagctctggggctgagctggtgaagcctggggcctcagtgaagatgtcctgcaaggctttggctacaccttac
tacctatccaatagagtggatgaagcagaatcatgggaagagcctagagtgattggaaatttcctcctacaatgatgataactaagtac
aatgaaaaattcaagggaaggccaaattgactgtagaaaaatcctctaccacagctctacttgagctcagccgattaacatctgacga
ctctgctgtttactgtgcaagggagaactacggtagtcacgggggtttgttactggggccaagggactctggtcacctgc

VH protein (SEQ ID NO: 14)

QVQLQQSGAELVKPGASVKMSCKAFGYTFTTYPIEWMKQNHGKSLEWIGNFHPYN
DDTKYNEKFKGKAKLTVEKSSTTVYLELSRLTSDDSA VYYCARENYGSHGGFVYW
GQGLVTV

TABLE 1. CDR SEQUENCES FOR CLONES 2, 10 AND 19 ANTIBODIES			
	CDR1	CDR2	CDR3
Clone 2 V κ	RASKSVSTSGFN <u>YIH</u> (SEQ ID NO: 15)	LASNLES (SEQ ID NO: 16)	QHSRELPLT (SEQ ID NO: 17)
Clone 2 VH	<u>GYTFTTYLY</u> (SEQ ID NO: 18)	GINPSNGGTNFNEKFKS (SEQ ID NO: 19)	RDYRYDRGFDY (SEQ ID NO: 20)
Clone 10 V κ	RSGQNIVHSNGNT <u>YLE</u> (SEQ ID NO: 21)	KVSNRFF (SEQ ID NO: 22)	FQGSHVPFT (SEQ ID NO: 23)
Clone 10 VH	<u>GYSITSDYAWN</u> (SEQ ID NO: 24)	YINYSGSTSYNPSLKS (SEQ ID NO: 25)	WIGSSAWYFDV (SEQ ID NO: 26)
Clone 19 V κ	RASSSVISSYLH (SEQ ID NO: 27)	STSNLAS (SEQ ID NO: 28)	QQYNGYPLT (SEQ ID NO: 29)
Clone 19 VH	<u>GYTFTTYPIE</u> (SEQ ID NO: 30)	NFHPYNDDTKYNEKFKG (SEQ ID NO: 31)	ENYGSHGGFVY (SEQ ID NO: 32)

In Table 1 and the sequences provided above, the heavy chain CDR1s for clones 2, 10 and 19 have been identified according to both the combined Kabat/Chothia numbering system and the Kabat numbering system. All other CDRs have been identified according to the Kabat numbering system (Kabat *et al.*, 1987, "In sequences of proteins of immunological interest", U.S. Dept. Health and Human Services, NIH USA. Heavy chain CDR1s for clones 2, 10 and 19, as identified by the Kabat numbering system, are identified (underlined amino acids) in Table 1.

THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated antibody selected from the group consisting of:
 - a) a monoclonal antibody that specifically binds to PD-1 produced by the hybridoma clone 19 or antigen binding fragment thereof, said hybridoma clone being deposited with the European Collection of Cell Cultures (ECACC) under accession number 08090901;
 - b) an isolated antibody or antigen binding fragment that binds to PD-1 and comprising a light chain comprising SEQ ID NO: 27 (light chain CDR1), SEQ ID NO:28 (light chain CDR2), SEQ ID NO:29 (light chain CDR3) and a heavy chain comprising SEQ ID NO:30 (heavy chain CDR1), SEQ ID NO:31 (heavy chain CDR2) and SEQ ID NO:32 (heavy chain CDR3);
 - c) an isolated antibody or antigen binding fragment that binds to PD-1 and comprising a light chain comprising SEQ ID NO: 27 (light chain CDR1), SEQ ID NO:28 (light chain CDR2), SEQ ID NO:29 (light chain CDR3) and a heavy chain comprising amino acids 6-10 of SEQ ID NO:30 (heavy chain CDR1; Kabat numbering system), SEQ ID NO:31 (heavy chain CDR2) and SEQ ID NO:32 (heavy chain CDR3) and
 - d) an isolated antibody or antigen binding fragment that binds to PD-1, said isolated antibody or antigen binding fragment comprising a heavy variable region (VH) of SEQ ID NO: 12 and a light variable region (VL) of SEQ ID NO: 14.
2. The isolated antibody according to claim 1, wherein said antibody is the monoclonal antibody that specifically binds to PD-1 produced by the hybridoma clone 19 or antigen binding fragment thereof, said hybridoma clone being deposited with the European Collection of Cell Cultures (ECACC) under accession number 08090901.
3. The isolated antibody according to claim 1, wherein said antibody binds PD-1 and is a humanized, chimeric or single chain antibody comprising: i) a light chain comprising SEQ ID NO: 27 (light chain CDR1), SEQ ID NO:28 (light chain CDR2), SEQ ID NO:29 (light chain CDR3) and a heavy chain comprising SEQ ID NO:30 (heavy chain CDR1), SEQ ID NO:31

(heavy chain CDR2) and SEQ ID NO:32 (heavy chain CDR3); or ii) a light chain comprising SEQ ID NO: 27 (light chain CDR1), SEQ ID NO:28 (light chain CDR2), SEQ ID NO:29 (light chain CDR3) and a heavy chain comprising amino acids 6-10 of SEQ ID NO:30 (heavy chain CDR1; Kabat numbering system), SEQ ID NO:31 (heavy chain CDR2) and SEQ ID NO:32 (heavy chain CDR3).

4. The isolated antibody according to claim 1, wherein said antibody binds PD-1 and comprises a heavy variable region (VH) of SEQ ID NO: 12 and a light variable region (VL) of SEQ ID NO: 14.

5. The isolated antibody according to claim 4, wherein SEQ ID NO: 12 and SEQ ID NO: 14 are fused to human constant regions.

6. The isolated antibody according to claim 1, wherein said antibody is tagged with a detectable label.

7. The isolated antibody according to claim 6, wherein said detectable label is a radiolabel or an enzyme.

8. A pharmaceutical composition comprising an antibody according to any one of claims 1 to 7 and a carrier.

9. The pharmaceutical composition according to claim 8, wherein said antibody is the monoclonal antibody that specifically binds to PD-1 produced by the hybridoma clone 19 or antigen binding fragment thereof, said hybridoma clone being deposited with the European Collection of Cell Cultures (ECACC) under accession number 08090901.

10. The pharmaceutical composition according to claim 8, wherein said antibody binds PD-1 and is a humanized, chimeric or single chain antibody comprising a light chain

comprising: i) SEQ ID NO: 27 (light chain CDR1), SEQ ID NO:28 (light chain CDR2), SEQ ID NO:29 (light chain CDR3) and a heavy chain comprising SEQ ID NO:30 (heavy chain CDR1), SEQ ID NO:31 (heavy chain CDR2) and SEQ ID NO:32 (heavy chain CDR3); or ii) a light chain comprising SEQ ID NO: 27 (light chain CDR1), SEQ ID NO:28 (light chain CDR2), SEQ ID NO:29 (light chain CDR3) and a heavy chain comprising amino acids 6-10 of SEQ ID NO:30 (heavy chain CDR1; Kabat numbering system), SEQ ID NO:31 (heavy chain CDR2) and SEQ ID NO:32 (heavy chain CDR3).

11. The pharmaceutical composition according to claim 8, wherein said antibody binds PD-1 and comprises a heavy variable region (VH) of SEQ ID NO: 12 and a light variable region (VL) of SEQ ID NO: 14.

12. The pharmaceutical composition according to claim 11, wherein SEQ ID NO: 12 and SEQ ID NO: 14 are fused to human constant regions.

13. The pharmaceutical composition according to claim 8, wherein said antibody is tagged with a detectable label.

14. The pharmaceutical composition according to claim 13, wherein said detectable label is a radiolabel or an enzyme.

15. One or more isolated nucleic acids encoding an antibody according to any one of claims 3 to 5.

16. The isolated nucleic acid according to claim 15, wherein said antibody is encoded by SEQ ID NO: 11 and SEQ ID NO: 13.

17. A vector comprising one or more nucleic acids according to claims 15 or 16.

18. A host cell comprising the vector according to claim 17 or the one or more nucleic acids according to claim 15 or 16.

19. A method of isolating PD-1 comprising contacting a sample with the antibody according to any one of claims 1 to 5 under conditions that allow for formation of an antibody-antigen complex.

20. A method of isolating PD-1-expressing cells comprising contacting cells expressing PD-1 with the antibody according to any one of claims 1-5 under conditions that allow for formation of an antibody-antigen complex.

21. Use of a specific antigen and the antibody of any one of claims 1 to 5 to induce tolerance to said specific antigen.

22. Use of a specific antigen and the antibody of any one of claims 1 to 5 in the manufacture of a medicament to induce tolerance to said specific antigen.

23. Use of the antibody of any one of claims 1 to 5 to reduce immune responses mediated by activated lymphocytes in a subject.

24. Use of the antibody of any one of claims 1 to 5 in the manufacture of a medicament to reduce immune responses mediated by activated lymphocytes in a subject.

25. Use of the antibody according to any one of claims 1 to 5 to treat allergies, rheumatoid arthritis, type I diabetes mellitus, multiple sclerosis, inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, tissue transplant rejection, skin transplant rejection, organ transplant rejection or graft-versus-host disease (GVHD).

26. Use of the antibody according to any one of claims 1 to 5 in the manufacture of a medicament to treat allergies, rheumatoid arthritis, type I diabetes mellitus, multiple sclerosis,

inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, tissue transplant rejection, skin transplant rejection, organ transplant rejection or graft-versus-host disease (GVHD).

27. The use according to claim 21, 23 or 25, wherein said use further comprises use of an additional PD-1 specific monoclonal antibody that binds to an epitope that is distinct and non-overlapping to the epitope bound by the antibody according to any one of claims 1 to 5.

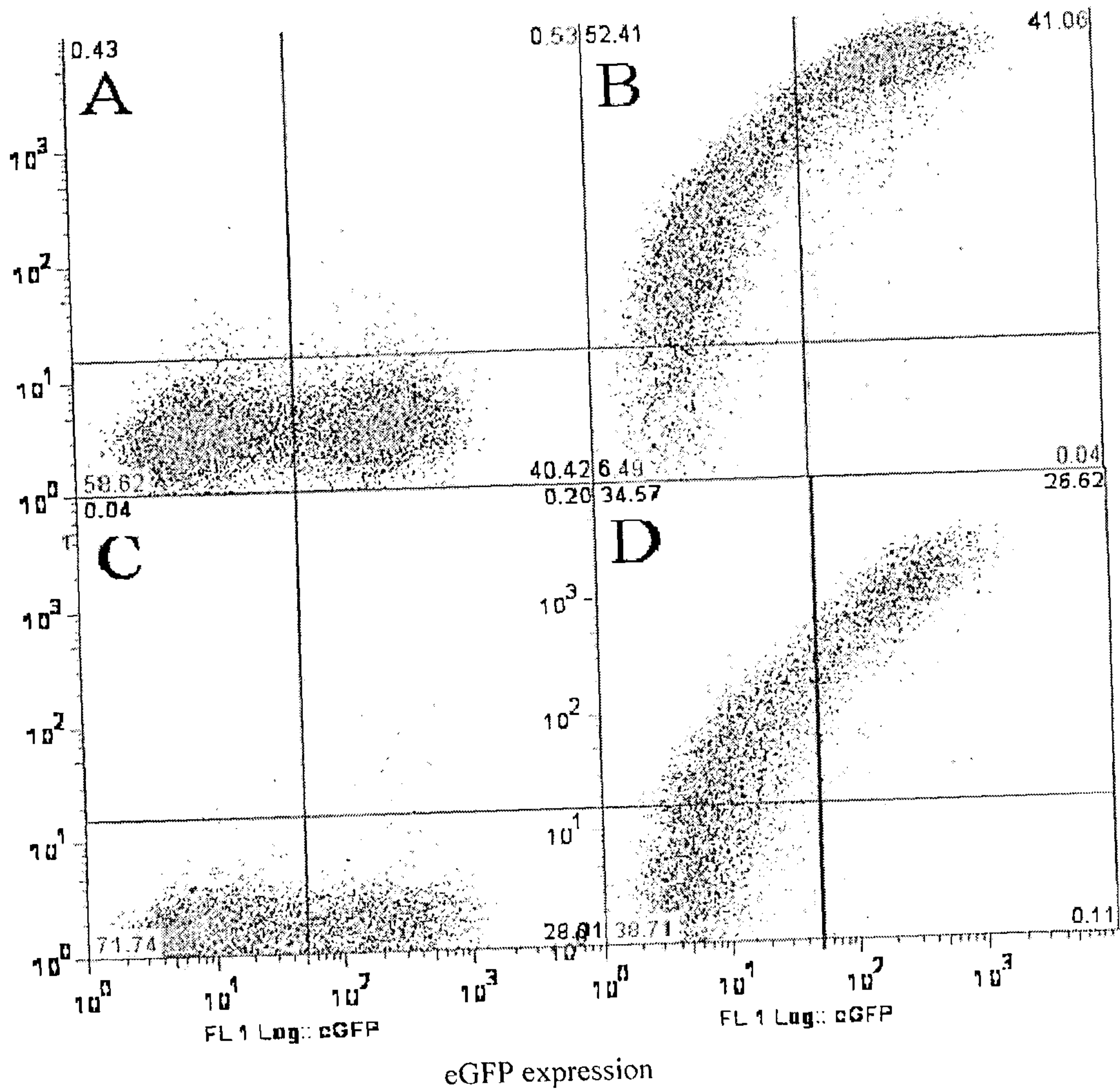
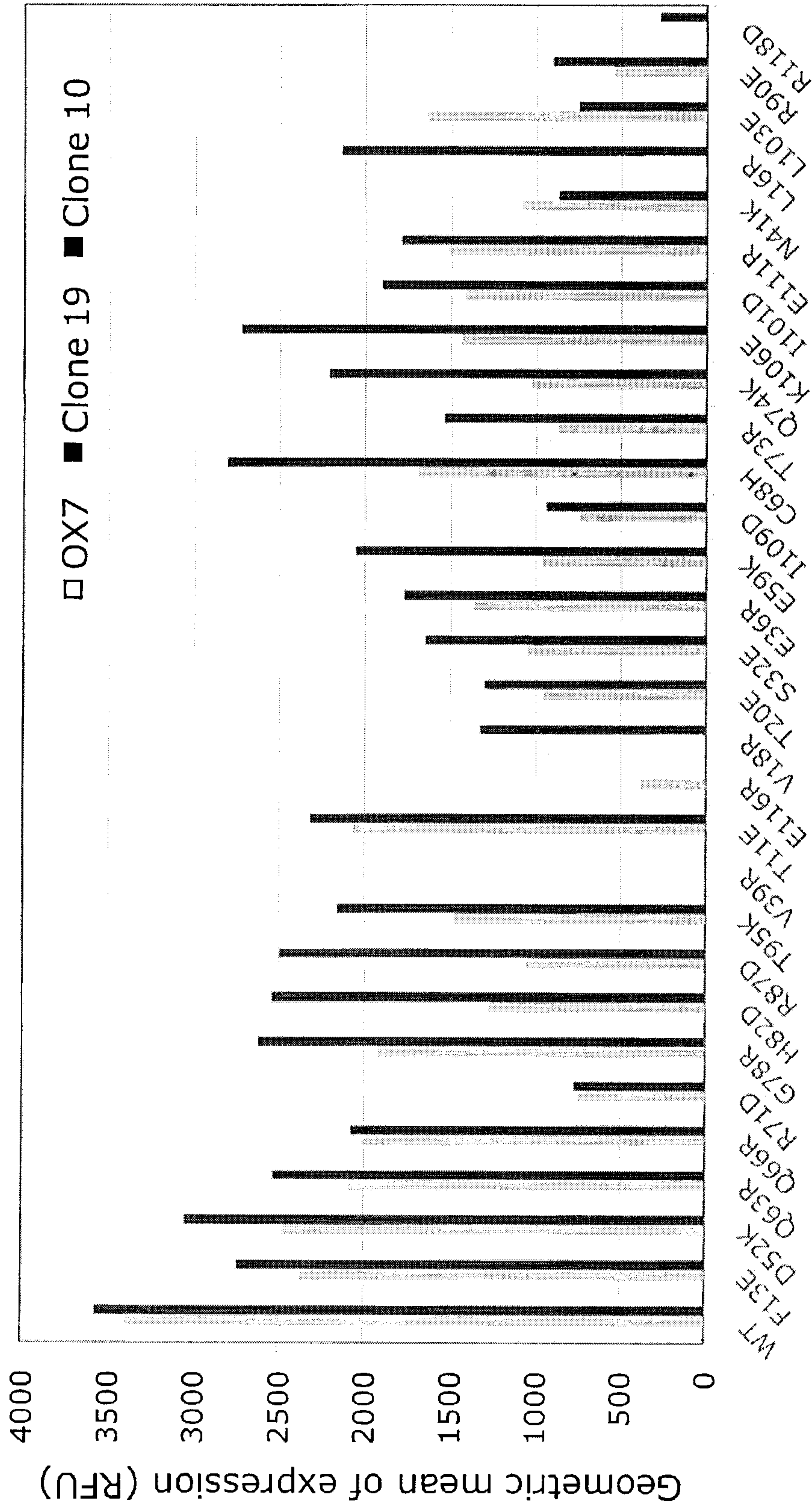


FIG. 1

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Mutations
FIG. 2

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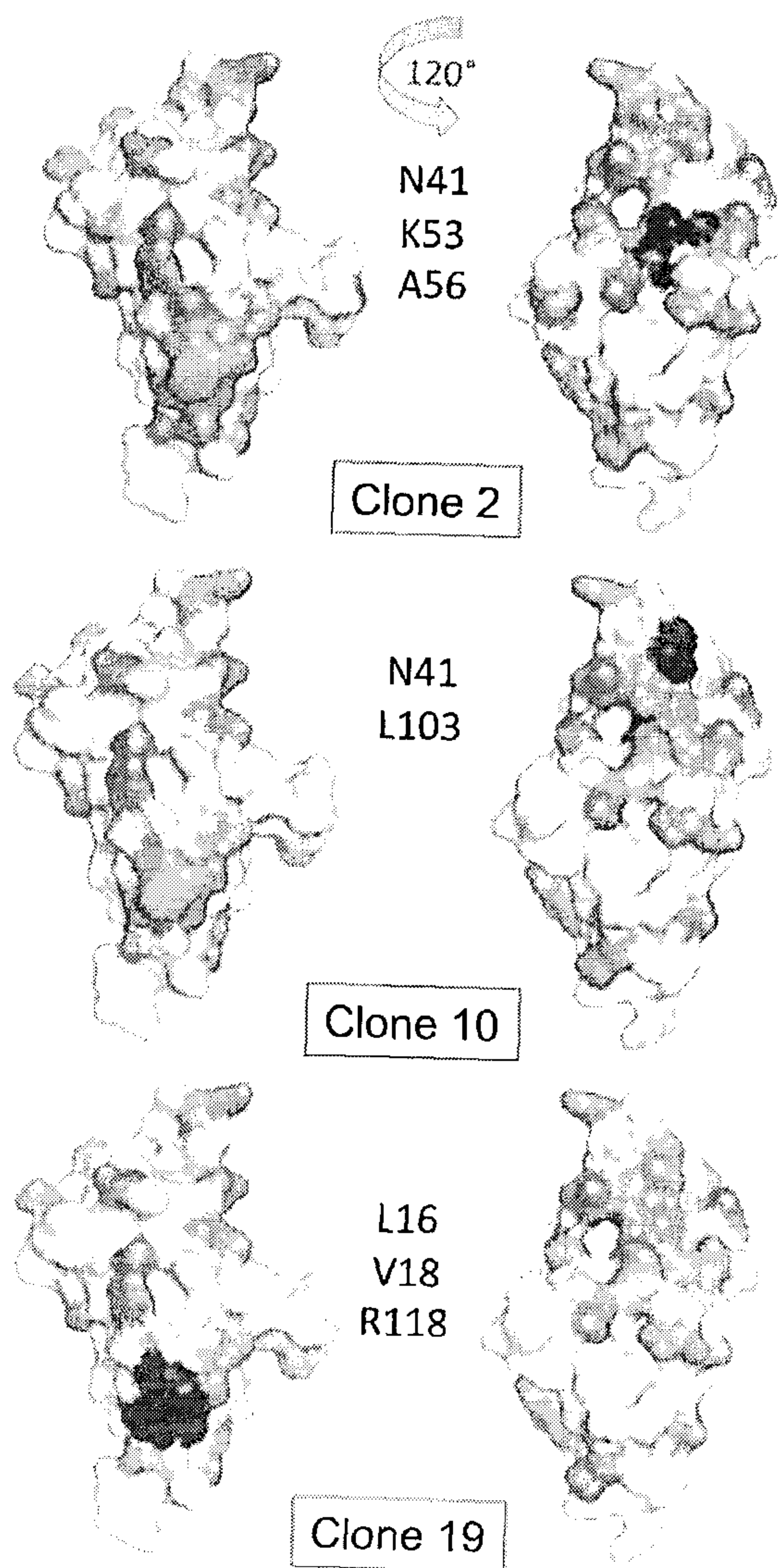


FIG. 3

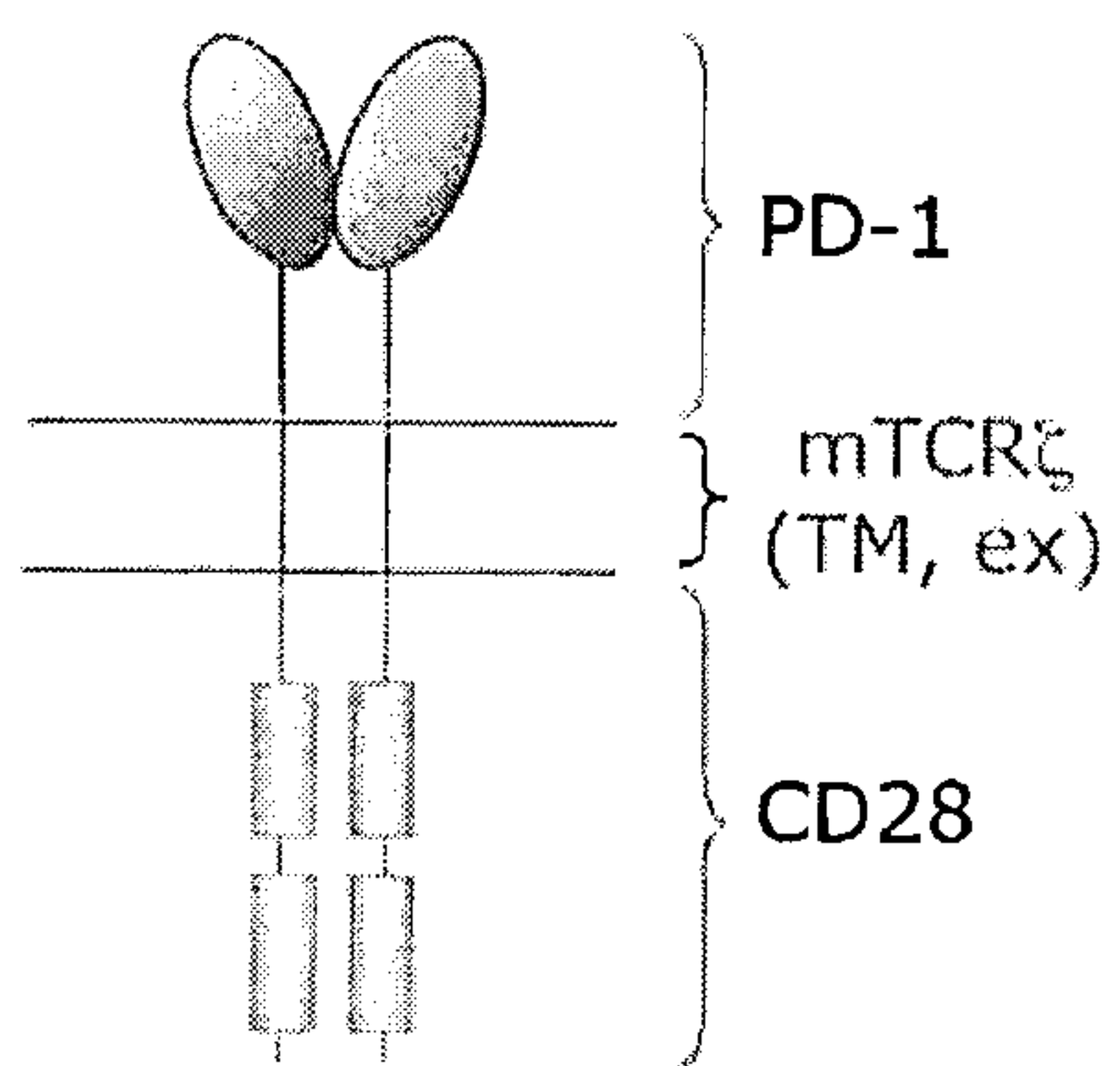


FIG. 4A

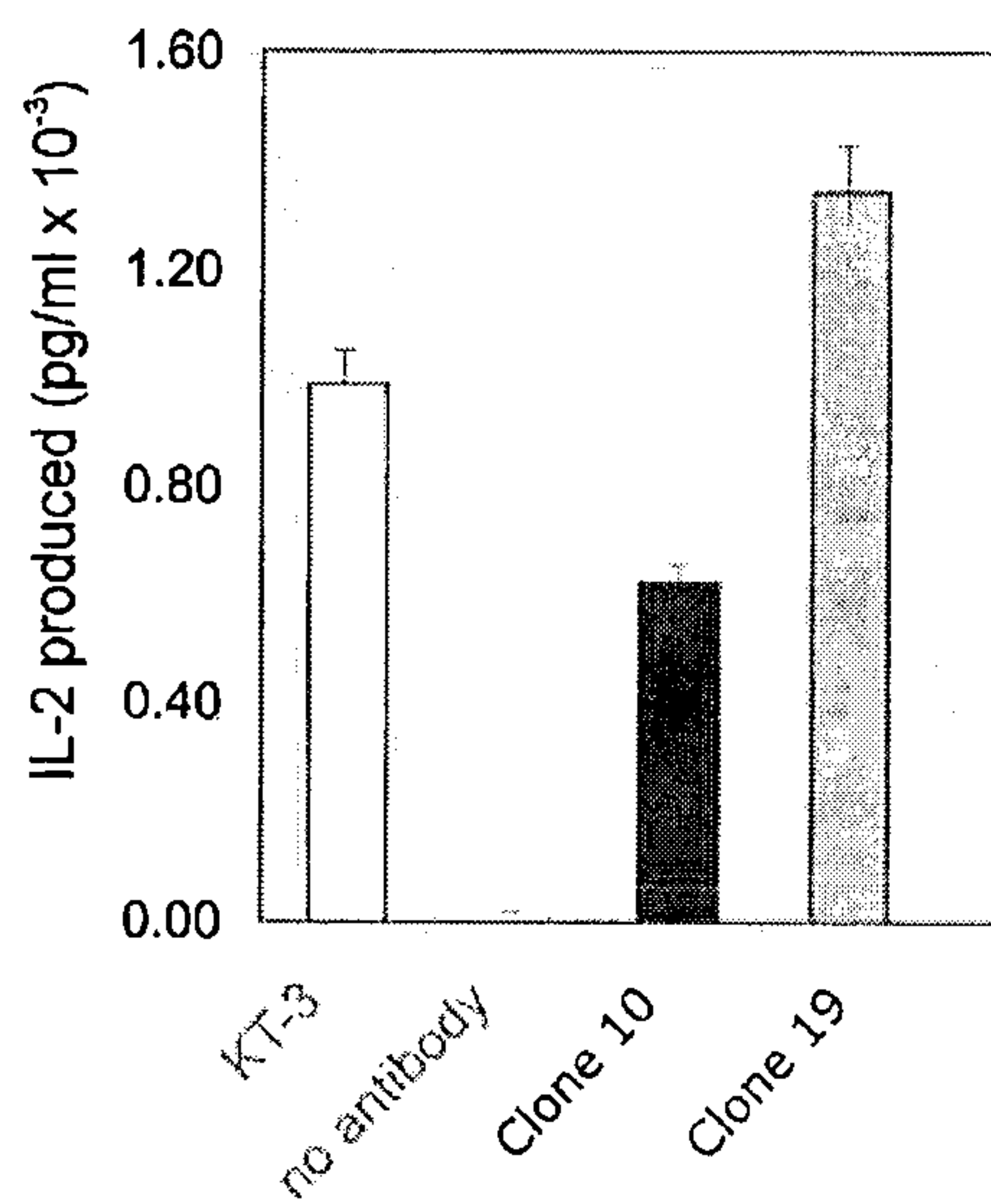
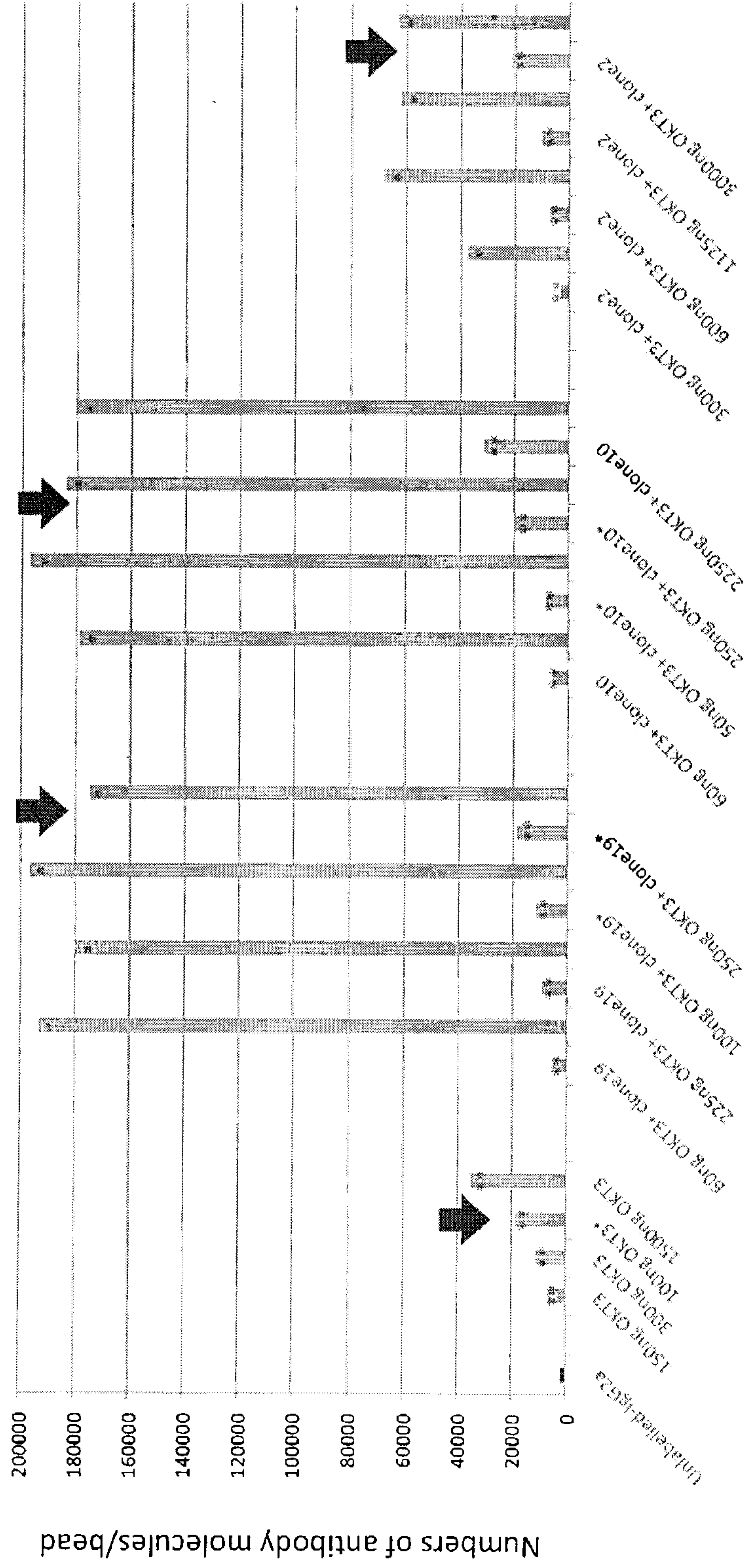


FIG. 4B

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* Total mAb: 2500ng

FIG. 5

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Experiment 1 - No beads

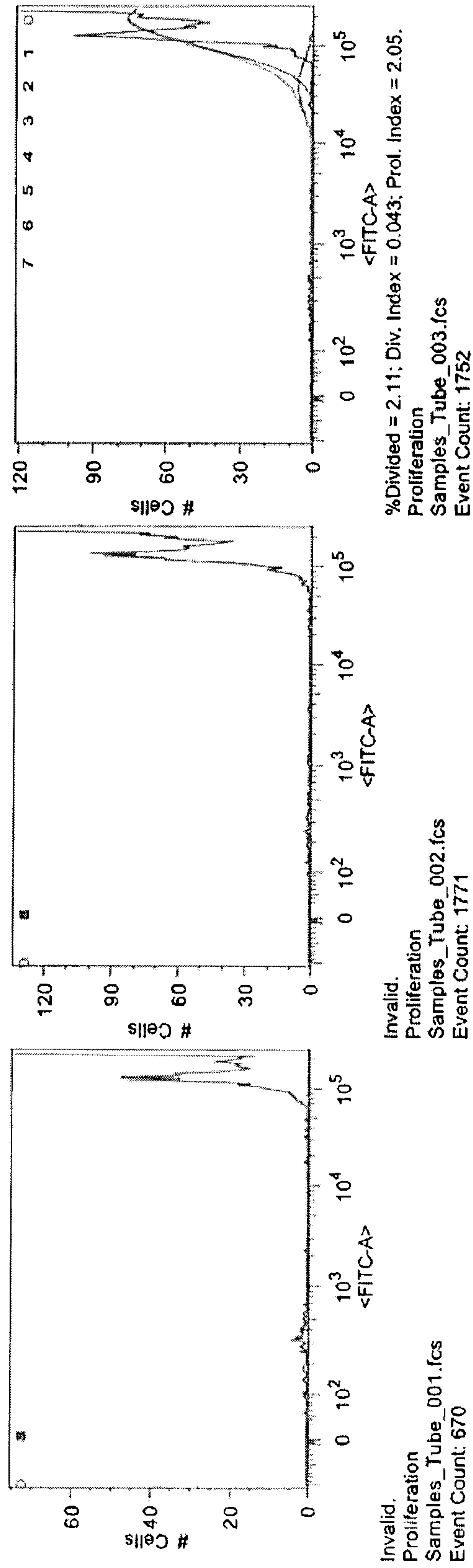


FIG. 6

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Experiment 1 - OKT3 beads

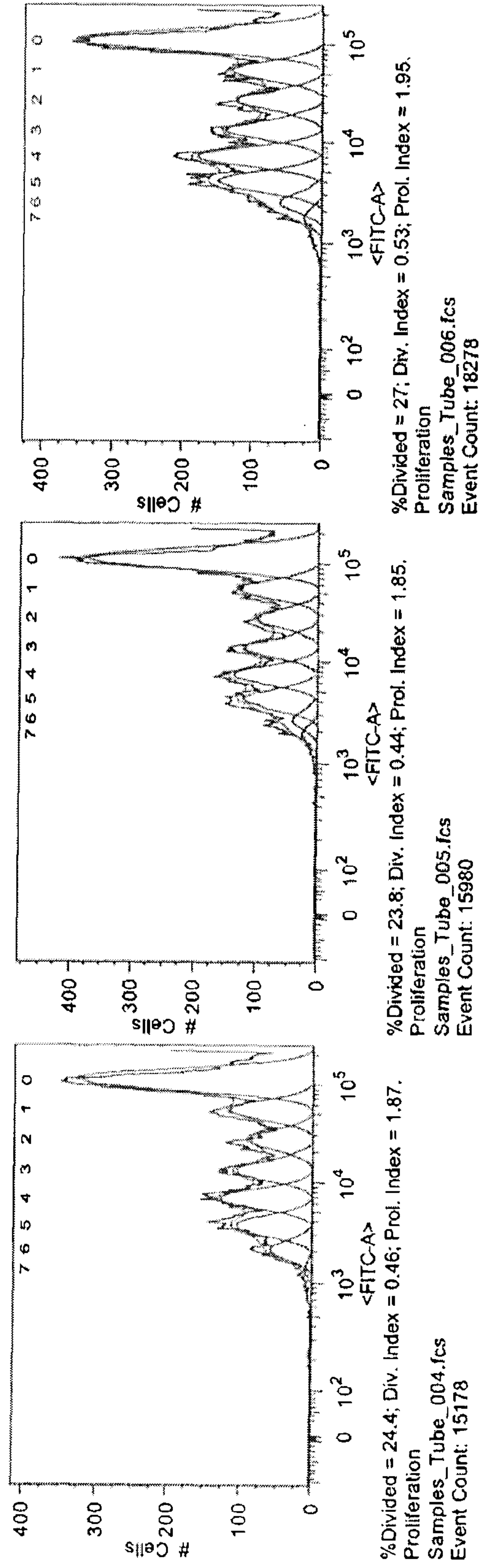


FIG. 6 (continued)

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Experiment 1 - Cl 19 beads

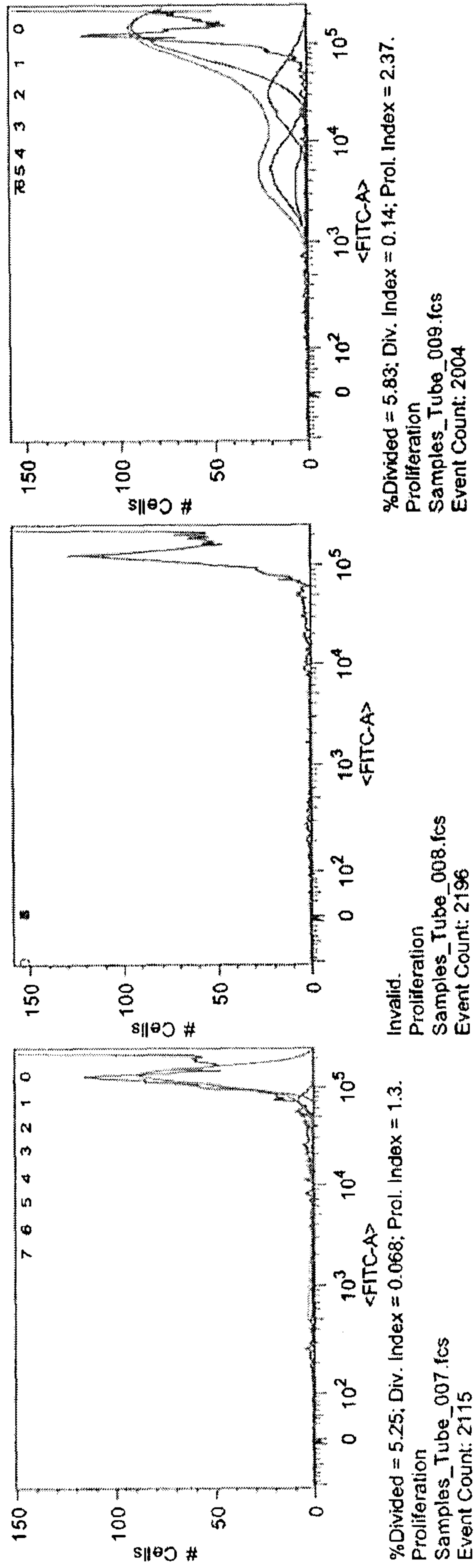


FIG. 6 (continued)

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Experiment 1 - Cl 10 beads

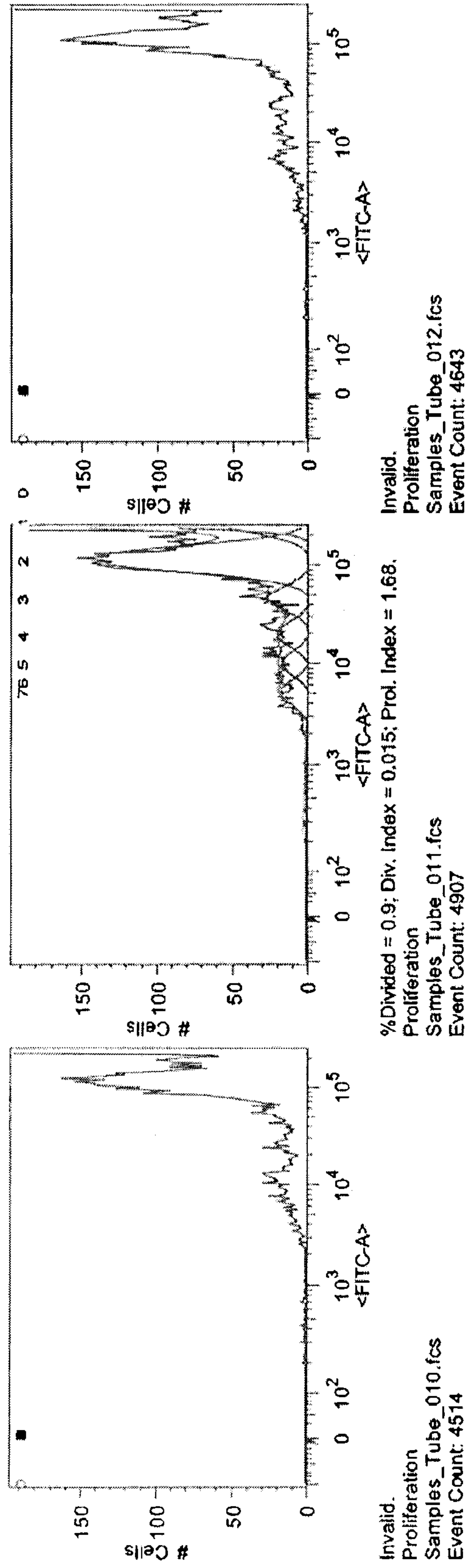


FIG. 6 (continued)

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Experiment 1 - Cl 2 beads

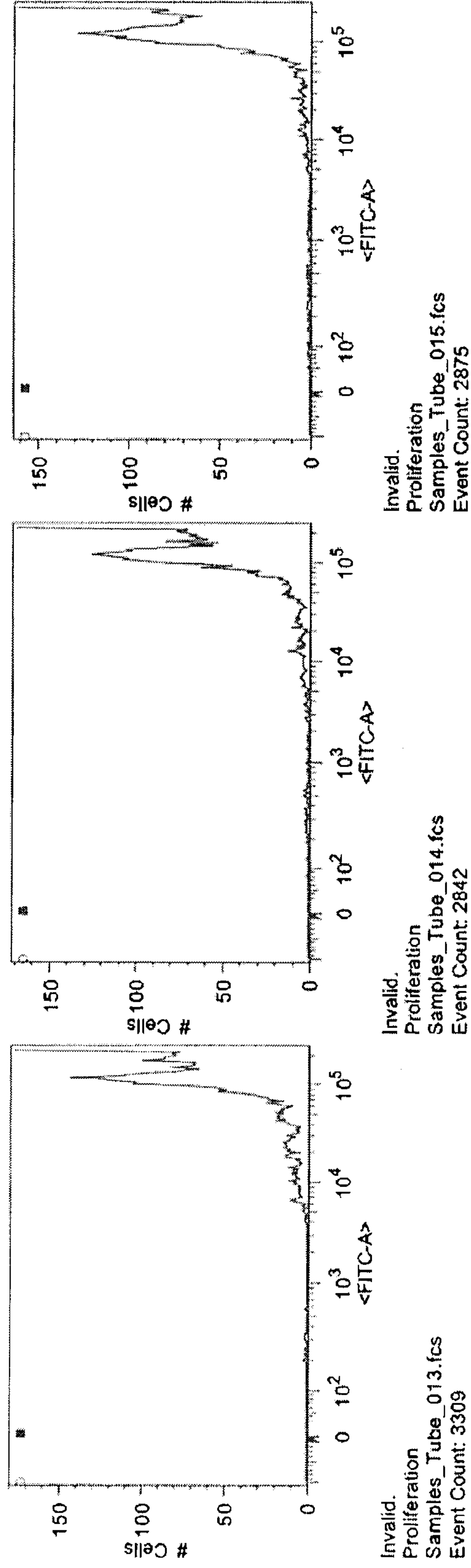


FIG. 6 (continued)

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Experiment 2 - No beads

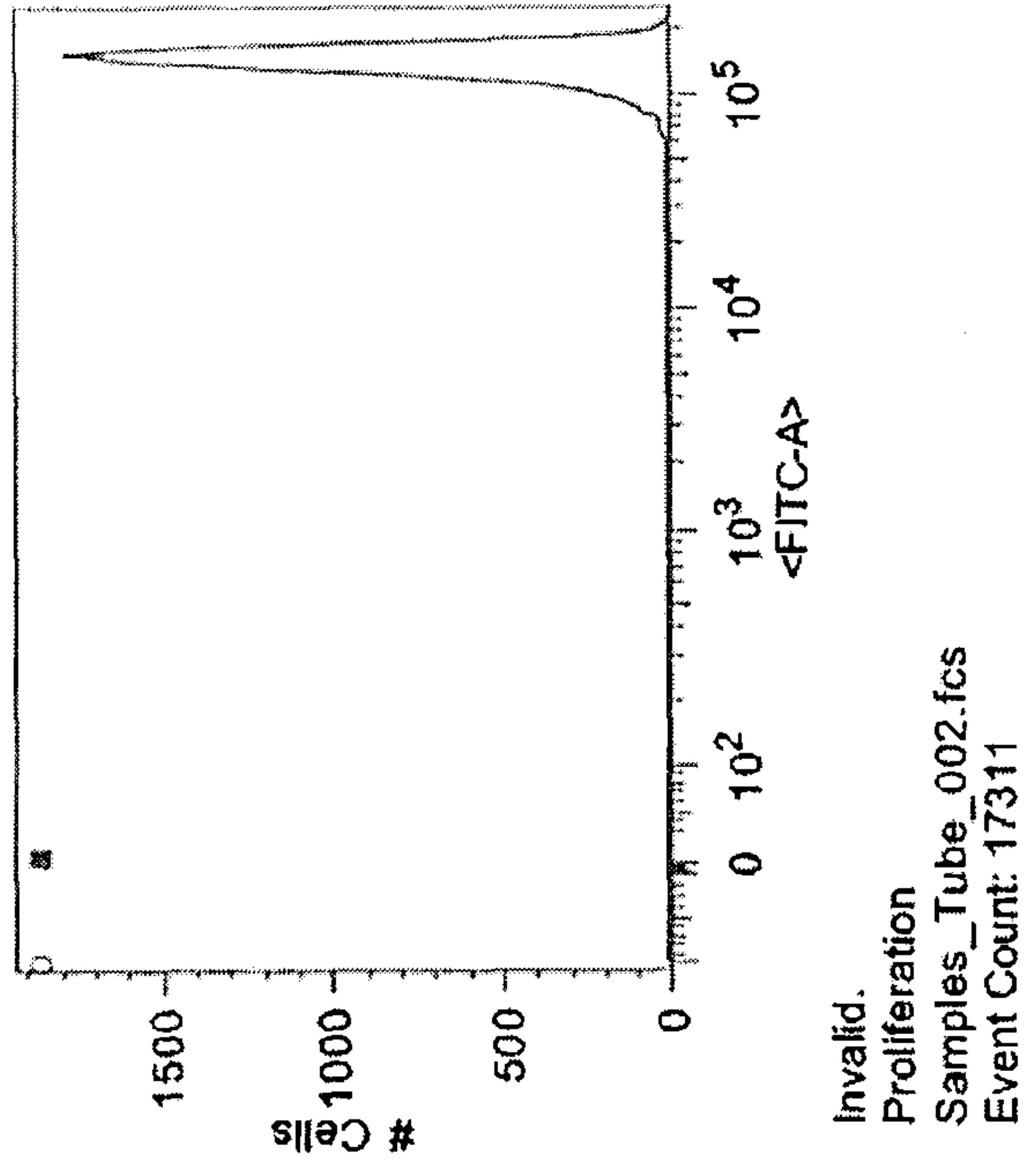
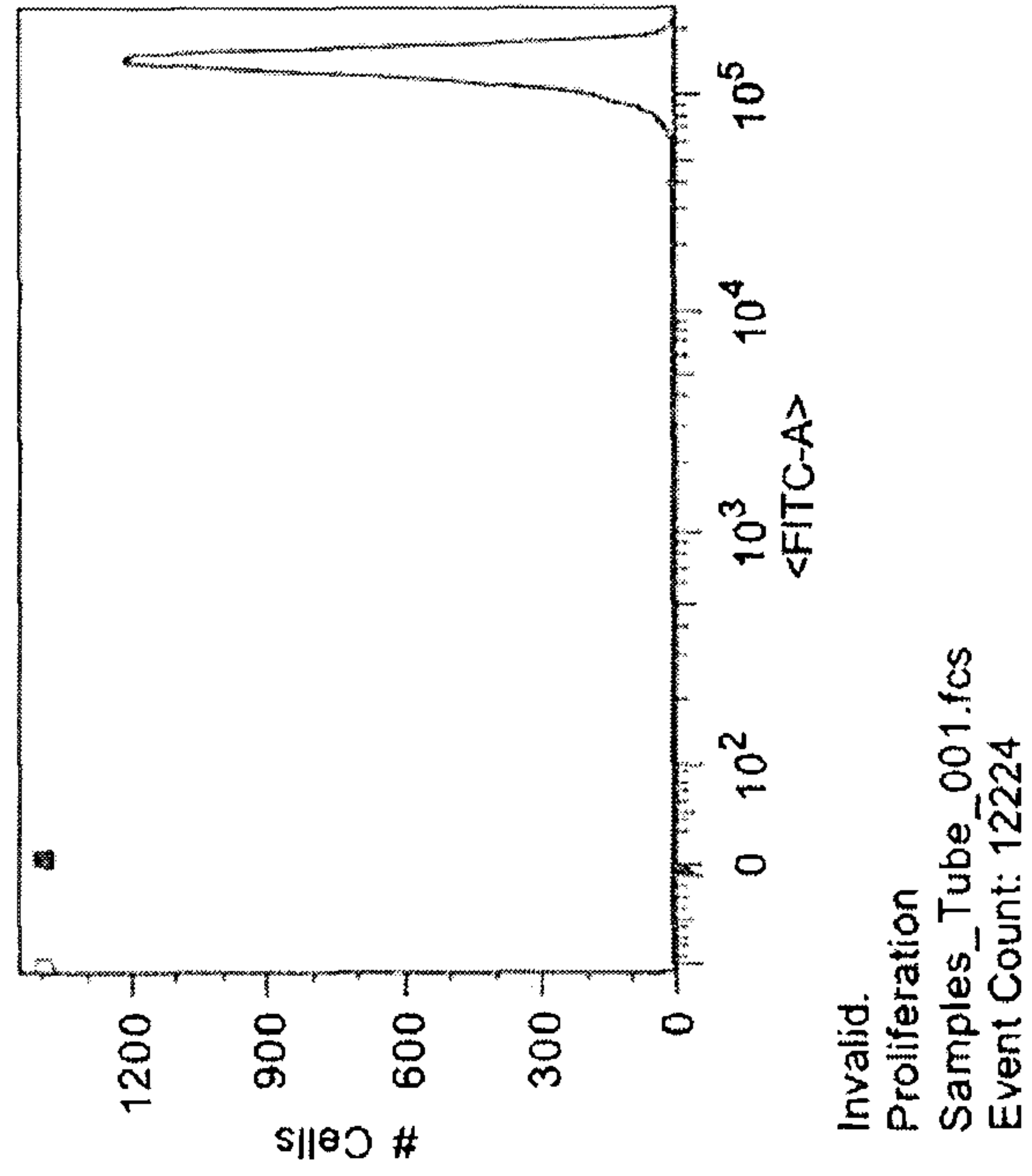


FIG. 6 (continued)

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Experiment 2 - OKT3 beads

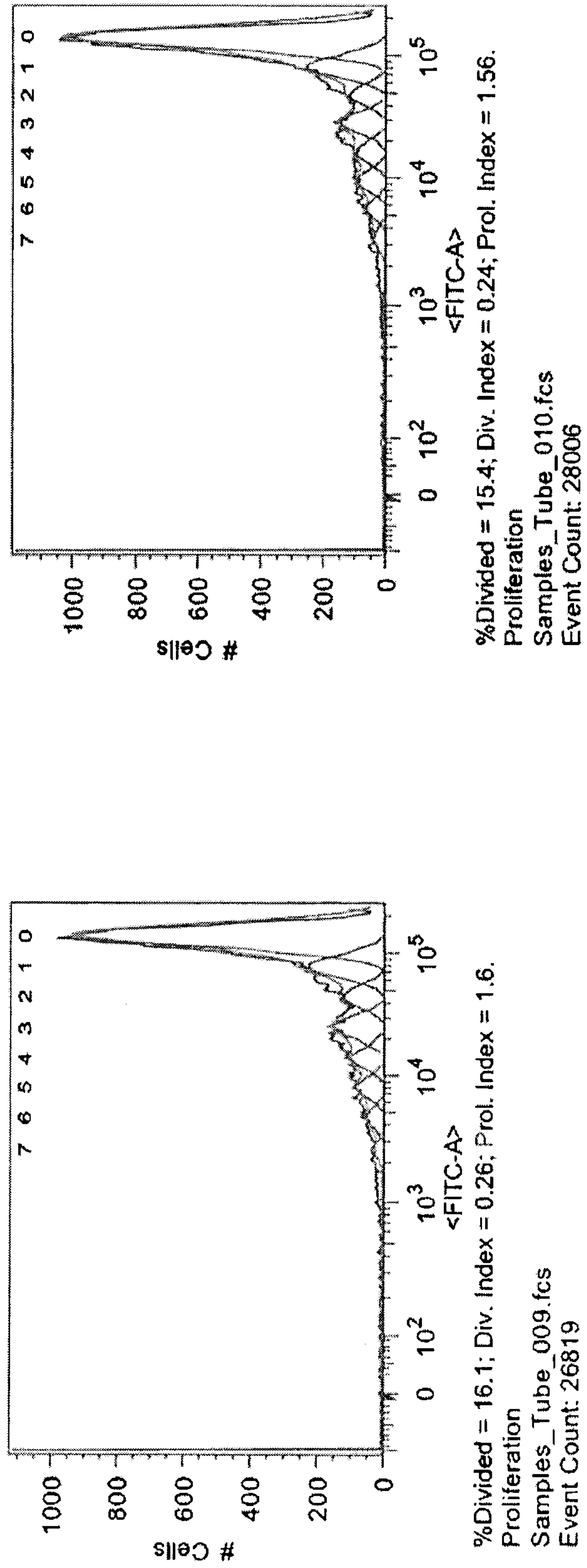


FIG. 6 (continued)

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Experiment 2 - CI 19 beads

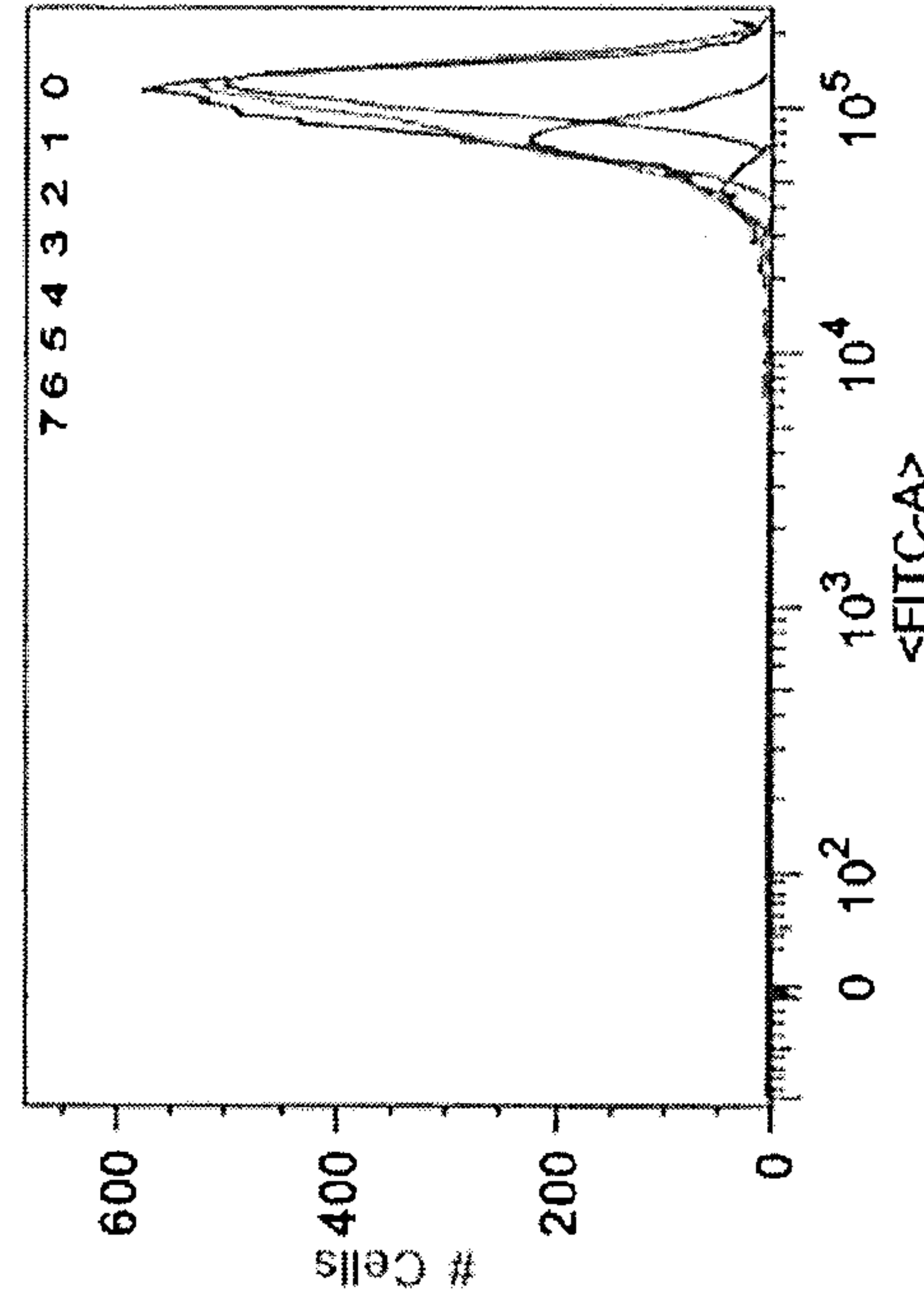
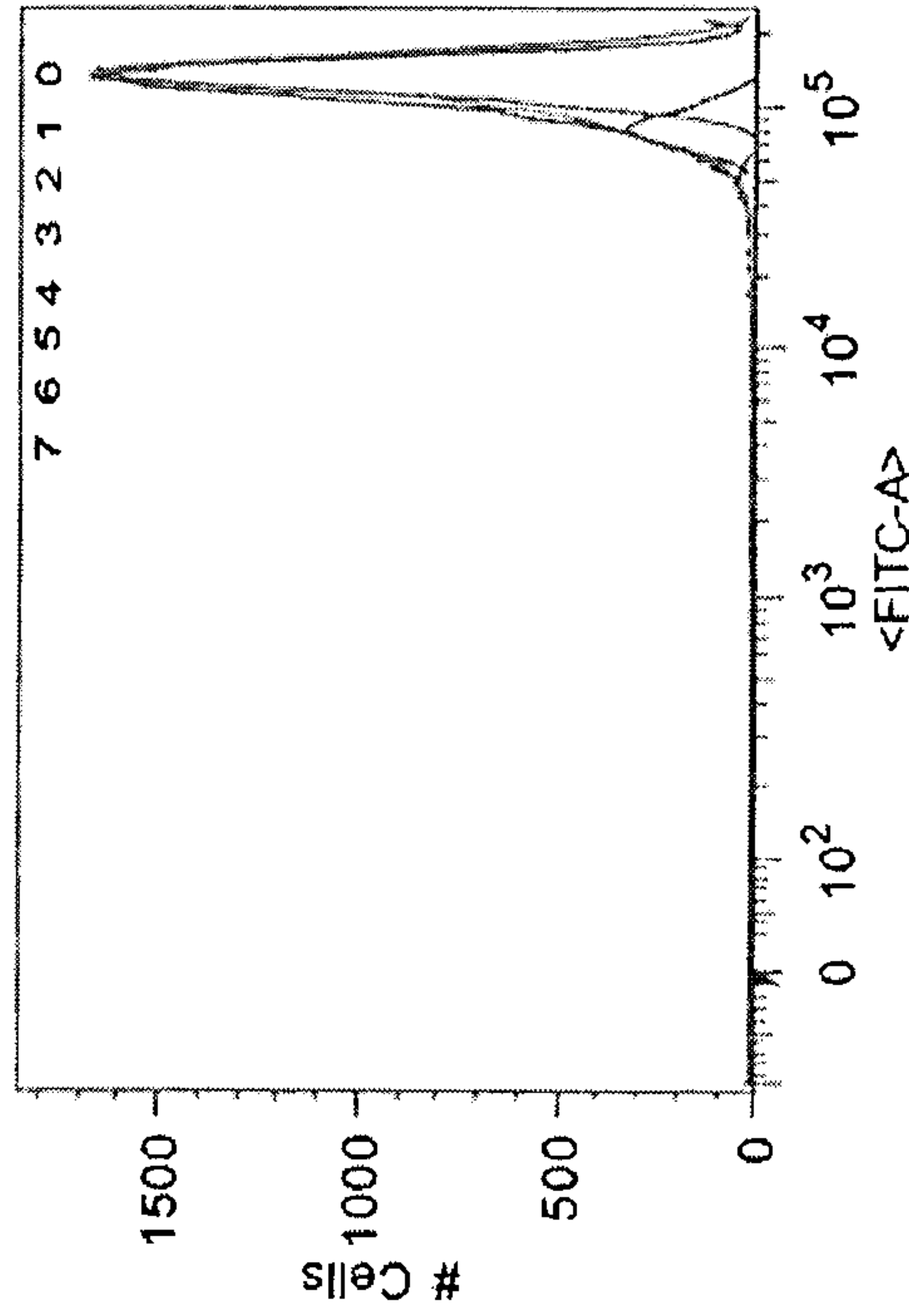


FIG. 6 (continued)

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Experiment 2 - Cl 10 beads

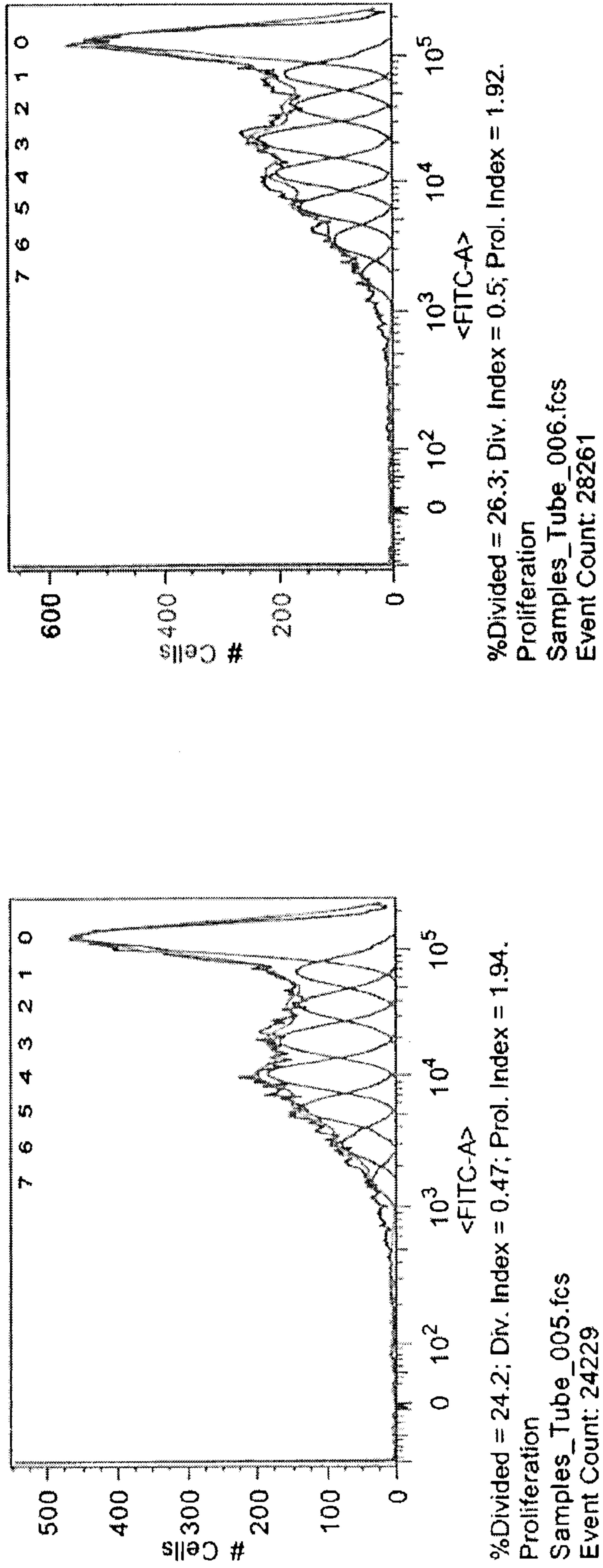


FIG. 6 (continued)

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Experiment 2 - CI 2 beads

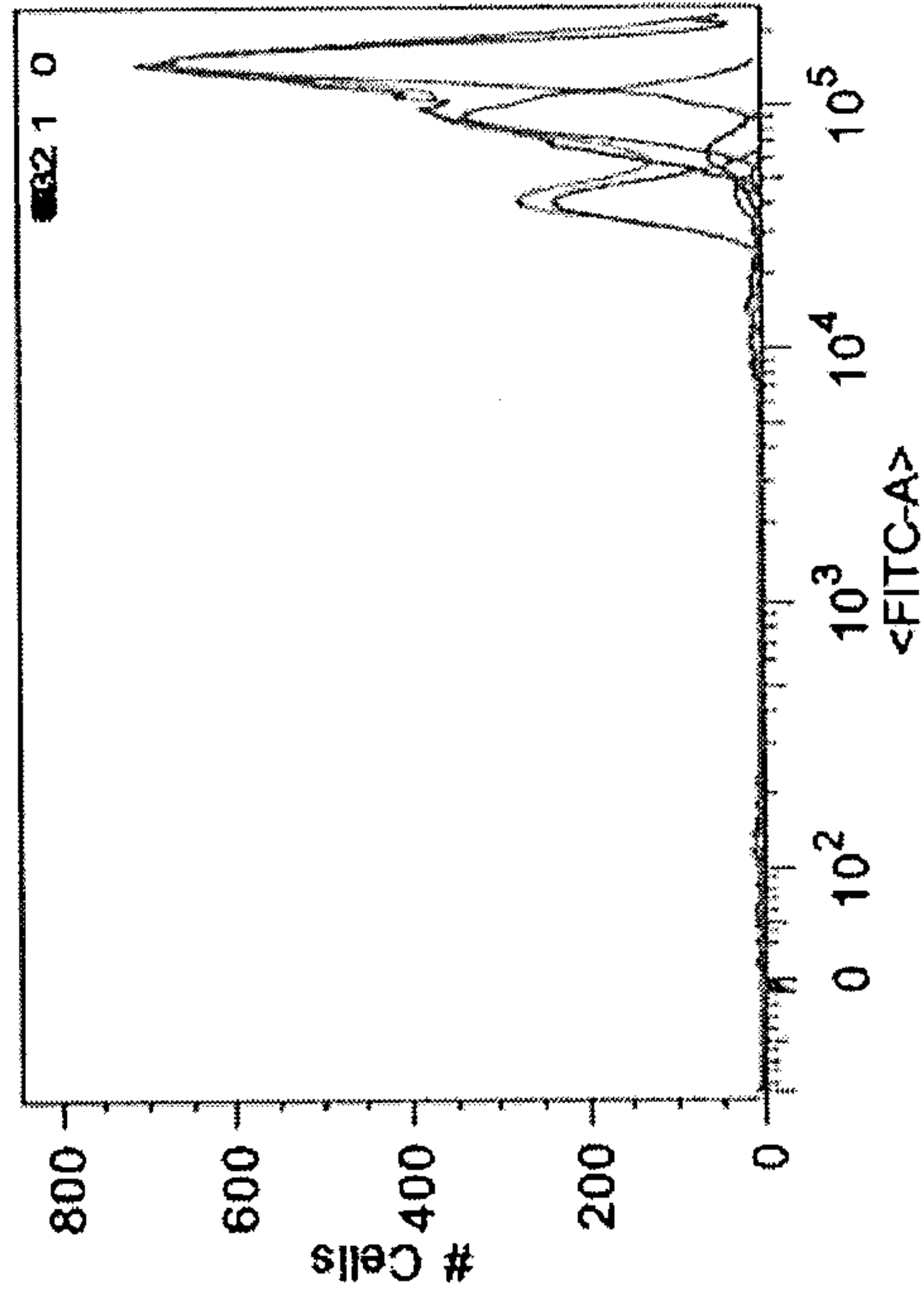
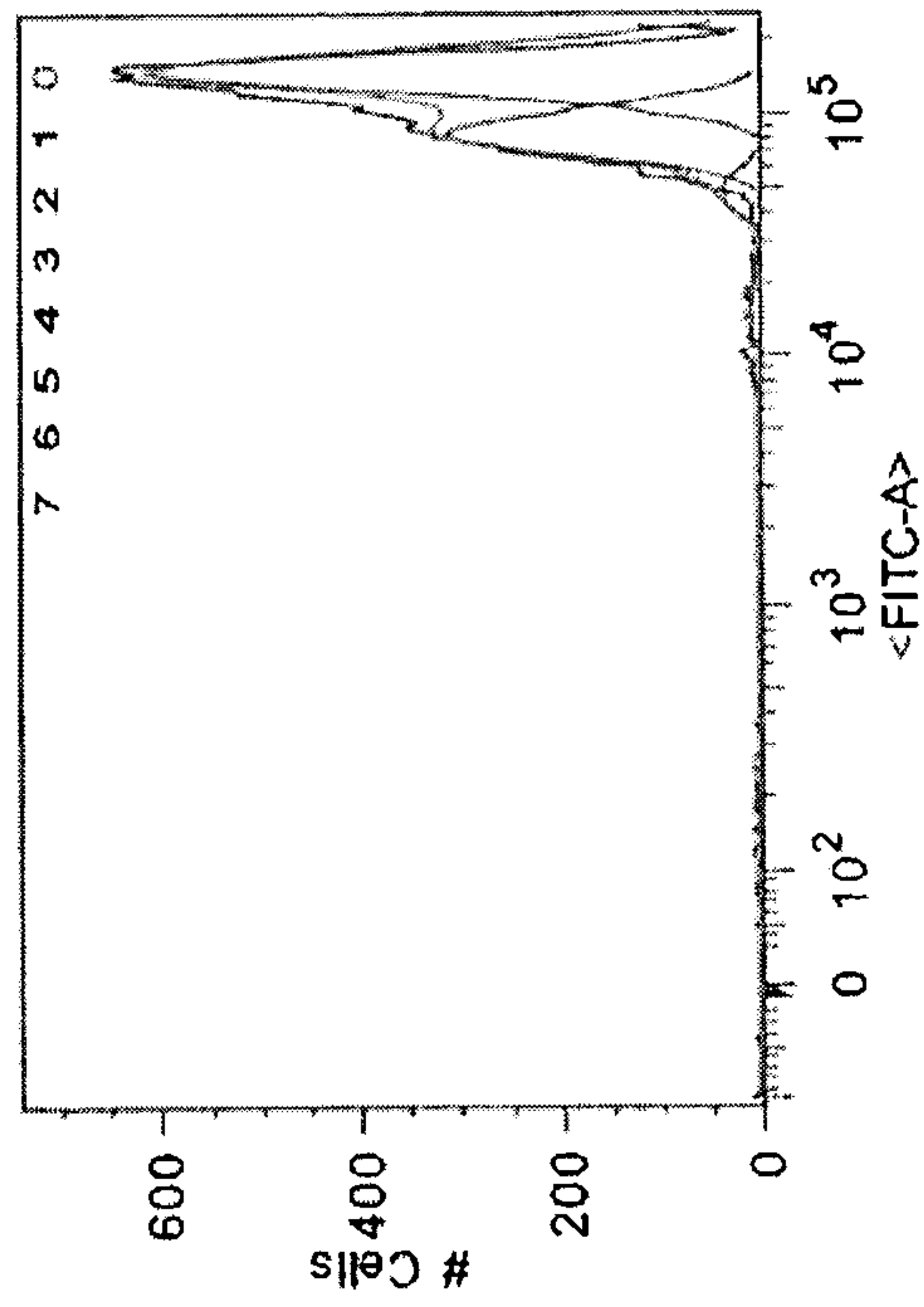


FIG. 6 (continued)

■ 0 - 100 pg/ml IL-2 ■ 100 - 200 pg/ml IL-2 ■ 200 - 300 pg/ml IL-2

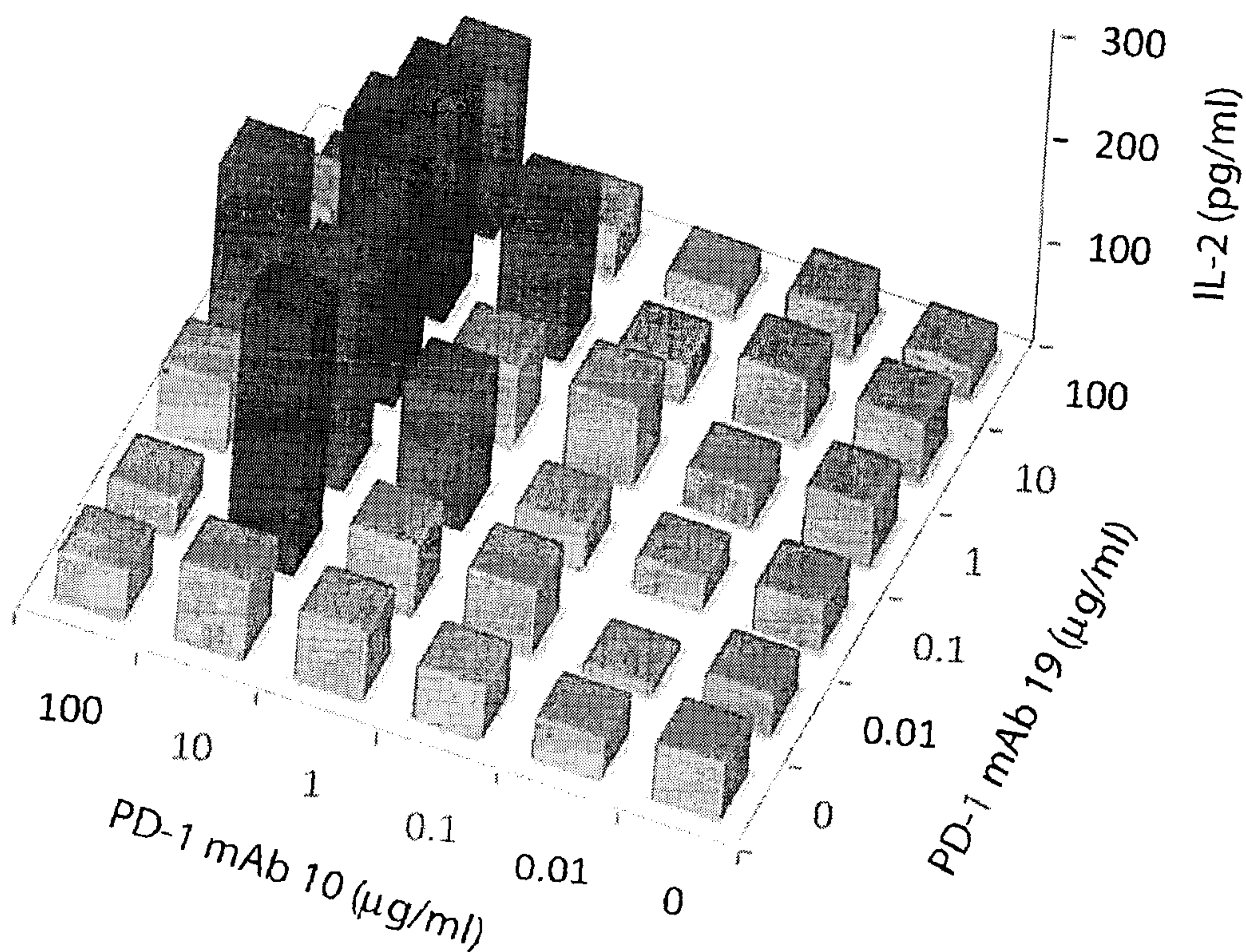


FIG. 7

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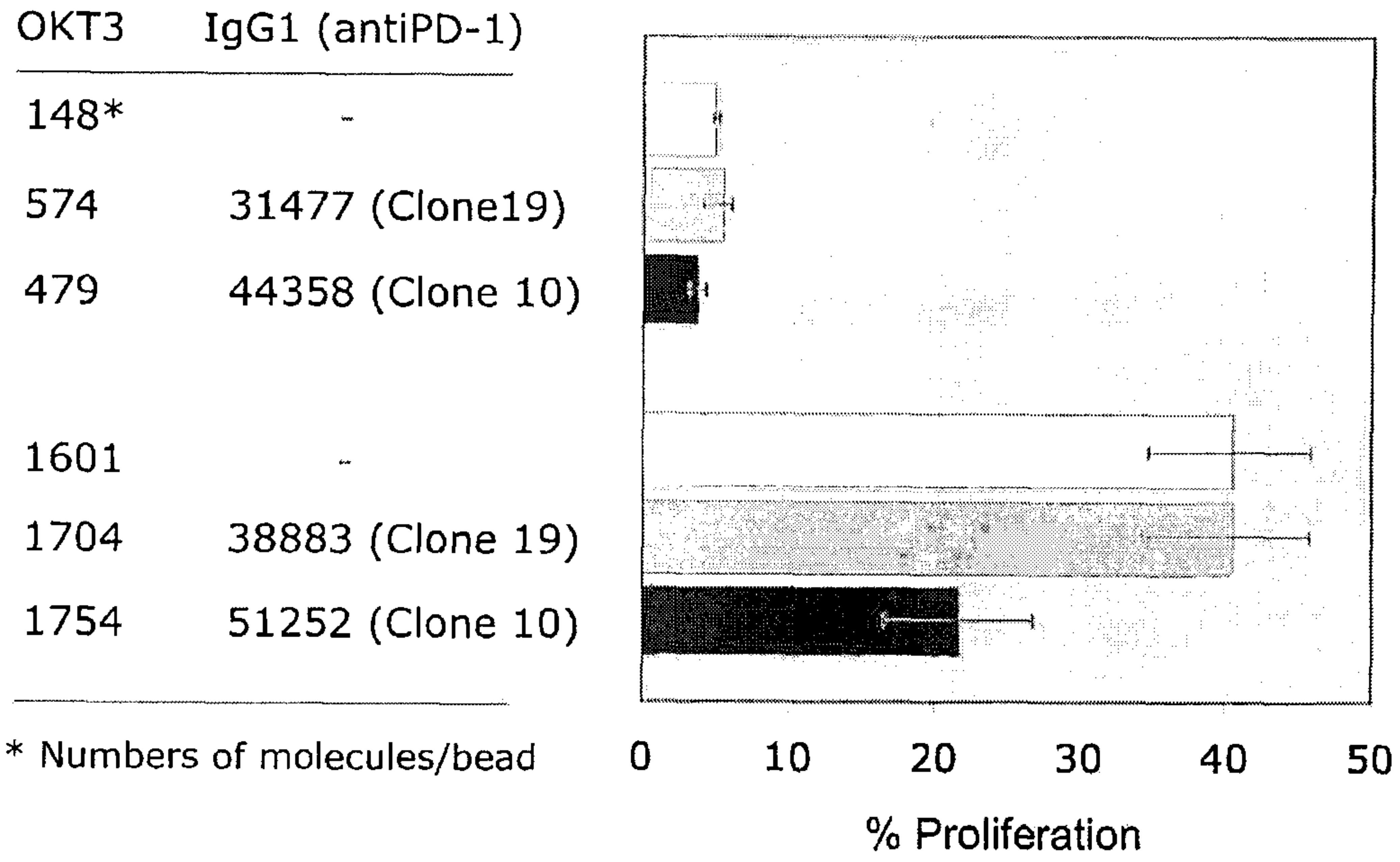


FIG. 8

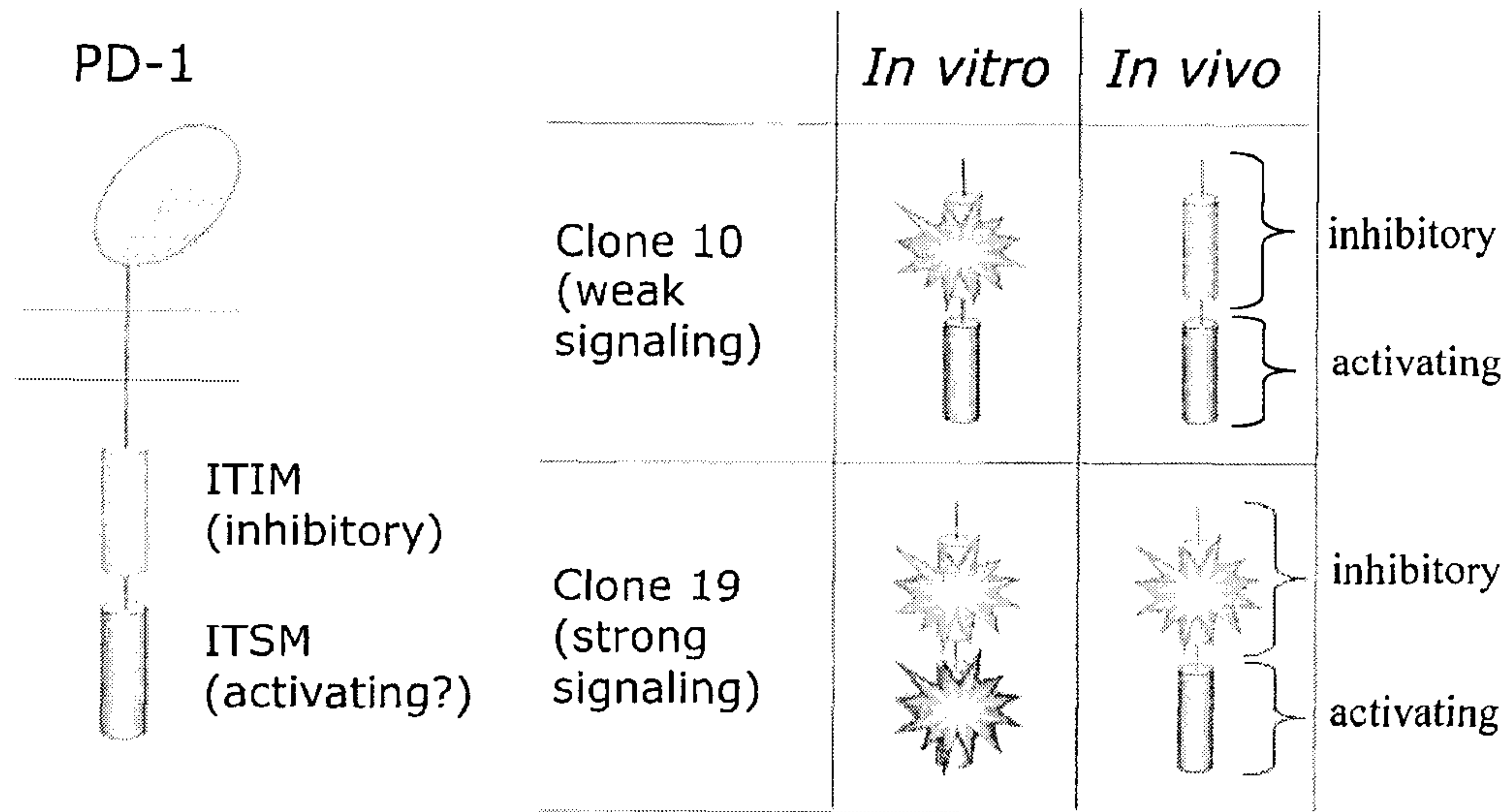


FIG. 9

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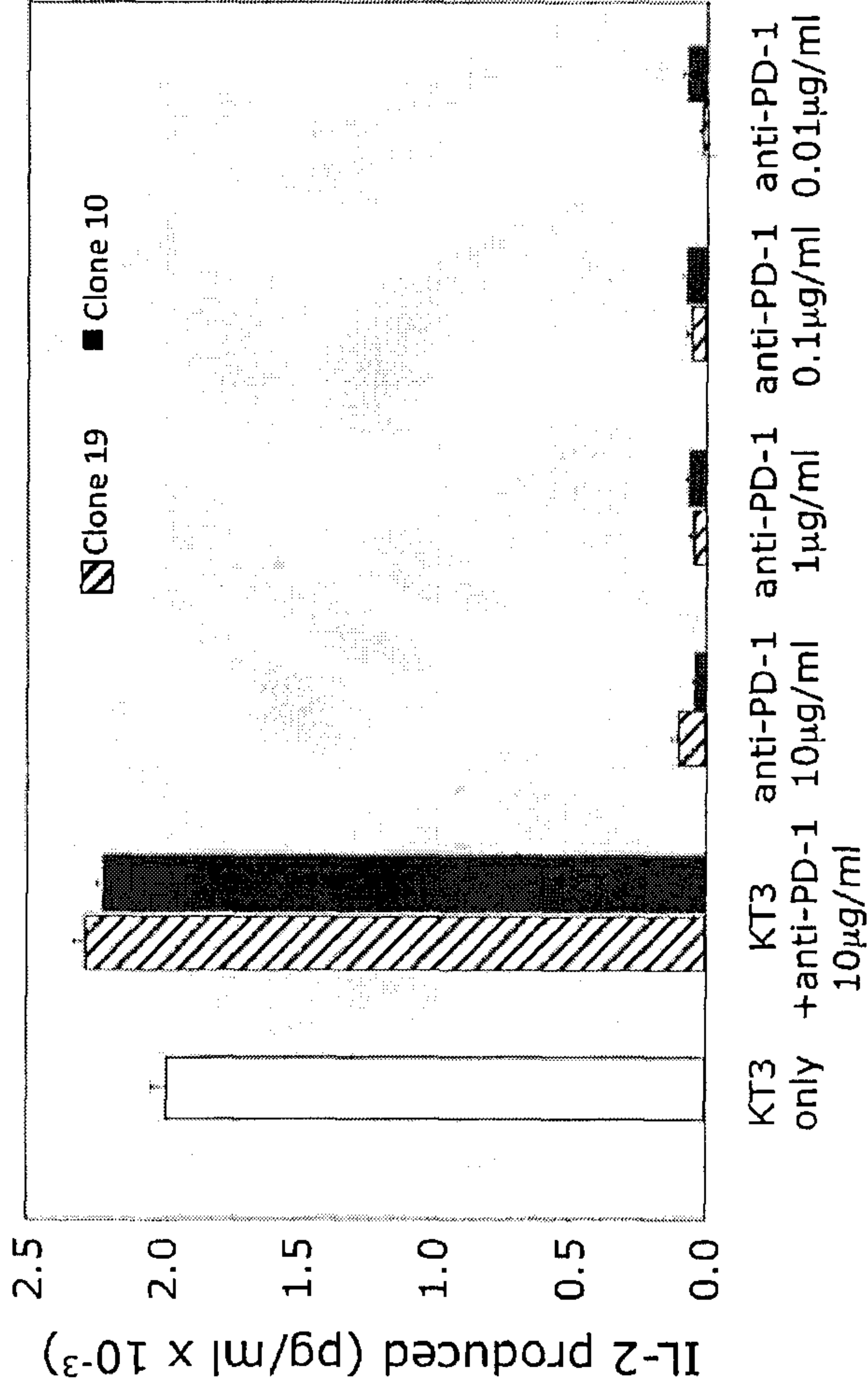
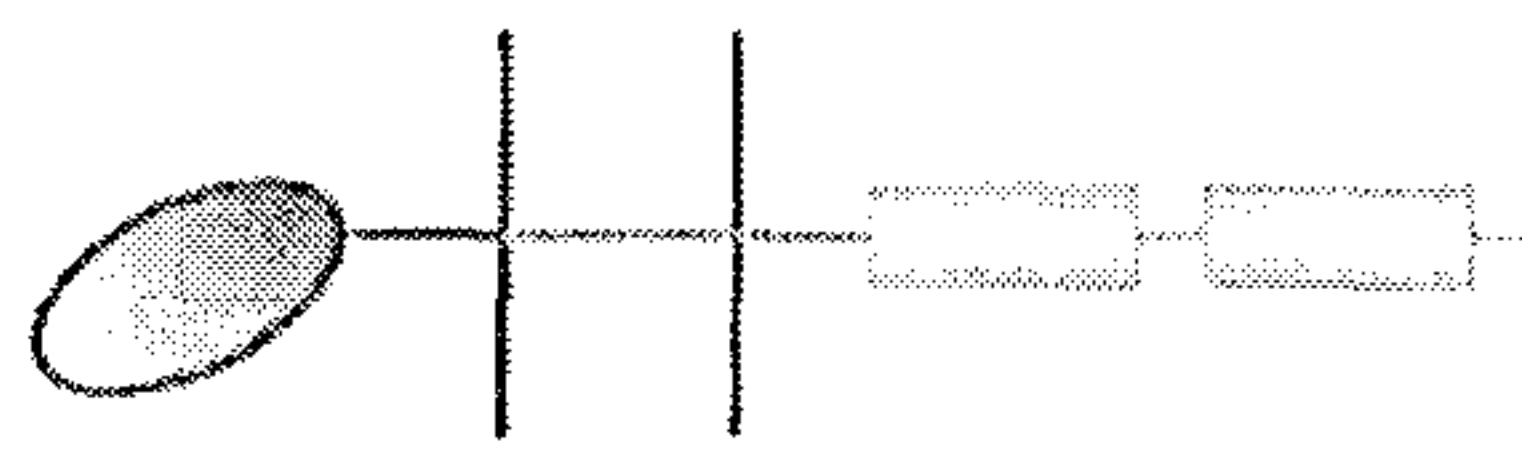
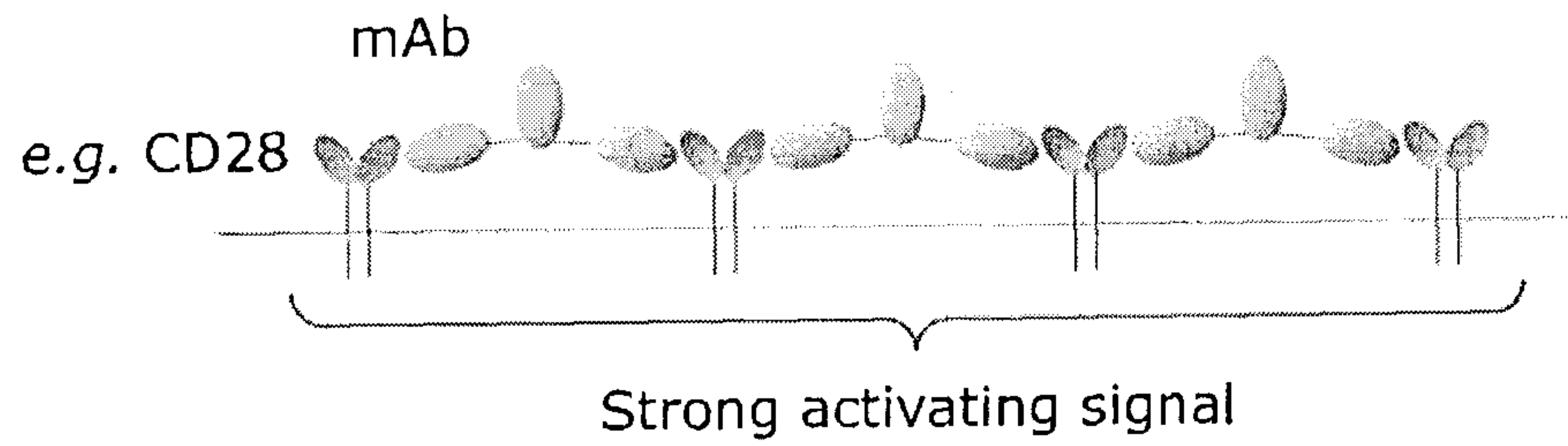
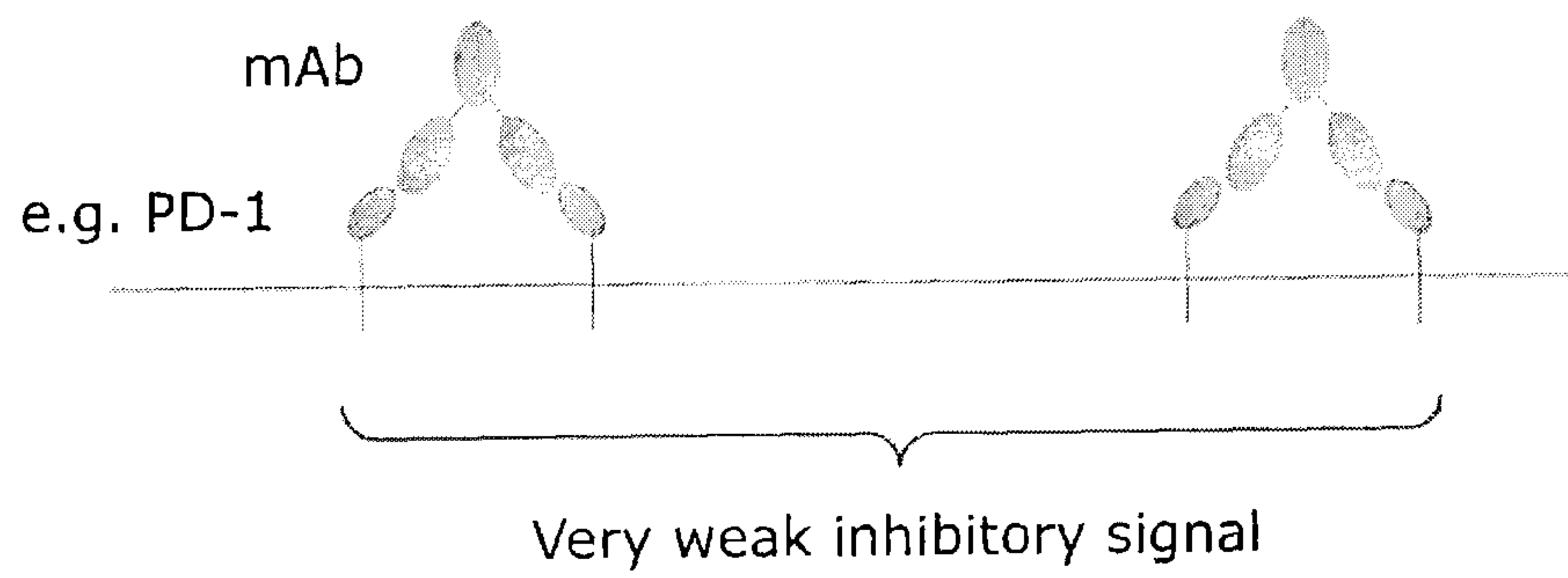
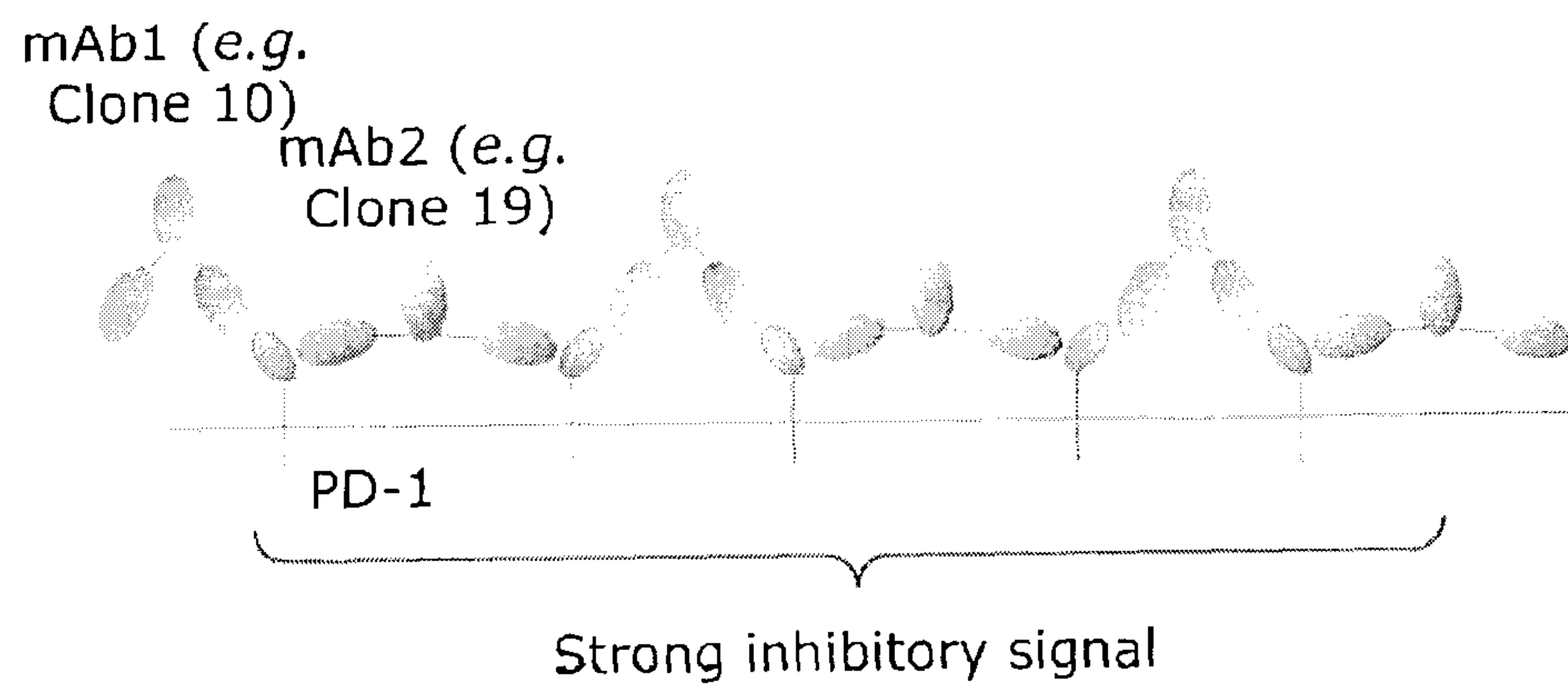


FIG. 10B



hPD-1/
mCD28

FIG. 10A

FIG. 11A Single anti-homodimer mAb**FIG. 11B** Single anti-monomer mAb**FIG. 11C** Two anti-monomer mAbs binding to two non-overlapping epitopes

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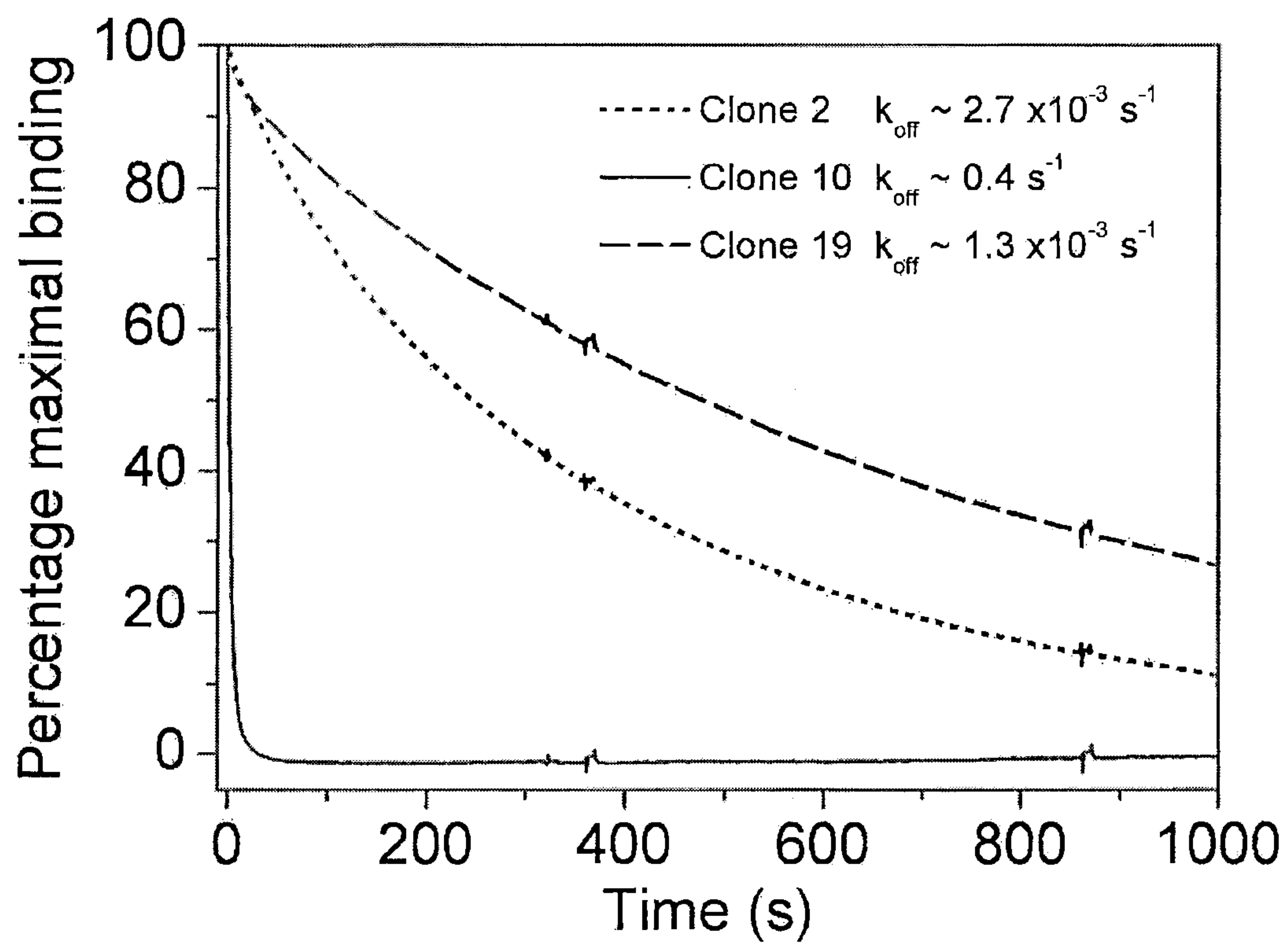


FIG. 12

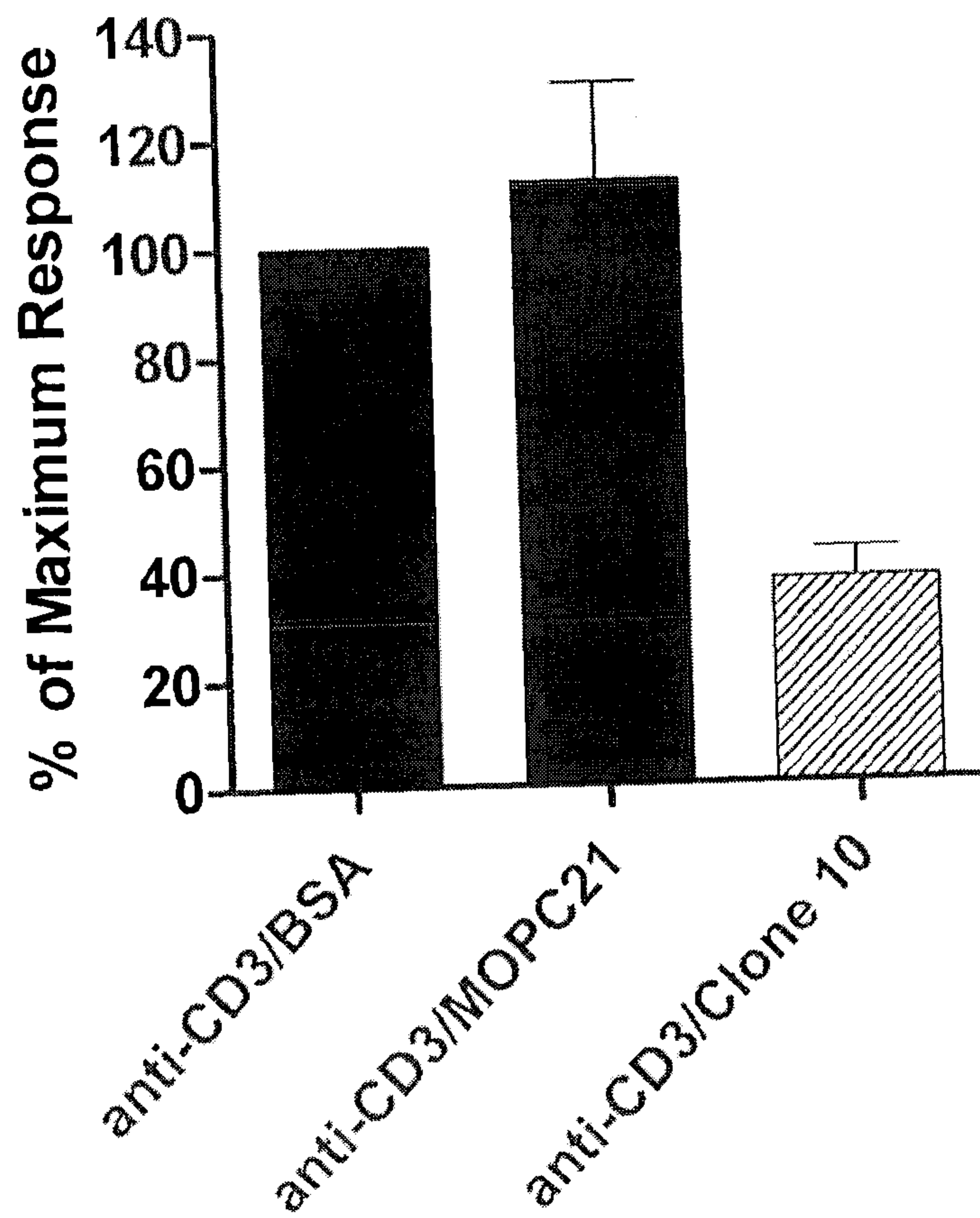


FIG. 13