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(54) Titre : ESSAI A BASE DE CELLULES POUR DETECTER DES HOMODIMERES ANTI-CD3
(54) Title: CELL-BASED ASSAY FOR DETECTING ANTI-CD3 HOMODIMERS

(57) Abrégé/Abstract:
The present invention provides a cell-based assay for identifying and/or quantifying anti-CD3 homodimers in a composition comprising a T cell-dependent Bispecific antibody (TDB). In some aspects, the invention T cells comprising a T cell activation responsive reporter are contacted with the TDB to detect the presence of anti-CD3 homodimers. Compositions of reporter T cells and kits are also contemplated.

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(54) **Title**: CELL-BASED ASSAY FOR DETECTING ANTI-CD3 HOMODIMERS

(57) **Abstract**: The present invention provides a cell-based assay for identifying and/or quantifying anti-CD3 homodimers in a composition comprising a T cell-dependent Bispecific antibody (TDB). In some aspects, the invention T cells comprising a T cell activation responsive reporter are contacted with the TDB to detect the presence of anti-CD3 homodimers. Compositions of reporter T cells and kits are also contemplated.

# CELL-BASED ASSAY FOR DETECTING ANTI-CD3 HOMODIMERS

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims the benefit of U.S. Provisional Patent Application No. 62/167,761, filed May 28, 2015, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

## SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

**[0002]** The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 146392022940SEQLISTING.txt, date recorded: May 26, 2016, size: 17 KB).

## FIELD OF THE INVENTION

**[0003]** The present invention provides methods for analyzing preparations of multispecific antibodies wherein at least one antigen binding fragment of the multispecific antibody binds CD3. In some embodiments, the invention provides methods for determining the presence of anti-CD3 homodimers in a composition of one or more multispecific antibodies wherein at least one antigen binding fragment of the multispecific antibody binds CD3.

## BACKGROUND OF THE INVENTION

**[0004]** T cell Dependent Bispecific (TDB) antibodies are designed to bind a target antigen expressed on a cell, and to bind to T cells, often by binding CD3e subunit of the T cell receptor. The binding of the bispecific antibody to the extracellular domains of both the target antigen and to the CD3 of T cell results in T cell recruitment to target cells resulting in T cell activation and target cell depletion. In the absence of the target cell, the single anti-CD3 arm is not able to cross-link TCRs to induce T cell activation and target cell killing. Anti-CD3 homodimer is a product related impurity that is formed during the manufacturing process of TDB antibodies and is capable of cross-linking TCR and inducing a low level of T cell activation in the presence or absence of target cells. Anti-CD3 homodimer may also impact therapeutic efficacy if present at high levels which can result in a decrease in TDB biological potency in vitro. Anti-CD3 homodimer can have off-target effects by inducing a low level of T cell activation and inflammatory cytokines by T cells in the absence of target cells. It is therefore desirable to control the levels of T cell activating product related variants present in the manufacturing

process of TDB and a sensitive, reproducible and quantitative impurity assay method is needed to detect anti-CD3 homodimers that may be present in the purified product in order to support the development of a safe and efficacious clinical drug candidate.

**[0005]** Impurity assays need to be able to distinguish between the product/process related impurity and the desired product. Many traditional approaches for Chinese Hamster Ovary Cell protein (CHOP) impurity detection use a binding assay format approach, where the presence of process related CHO proteins can be sensitively detected in the product to evaluate product purity and safety. These CHOP antibodies are specific for the CHOP proteins, but do not recognize the product, so there is in general no impact to sensitively detecting impurities in the presence of the final product. Similar approaches could be used for bi-specific antibodies, provided antibodies can be identified that would distinguish between the final product and the impurity. The implementation of a useful anti-CD3 homodimer binding assay format would require the development of highly specialized antibodies, which may not be possible for this antigen, or other bi-specifics in general. Alternative physiochemical based methods (RP-HPLC, Mass Spec) can also be used to detect product related impurities and rely on the ability to adequately separate product related impurities from the product, and thereby detect the amount of impurity present. The amount of the impurity is detected relative to the other species present in the material, or by spiking in a variant standard and comparing the percent of material present to the spiked standard. However, many of these methods may involve additional sample handling and processing steps in order to separate the variant from the desired product material and these steps may alter the material or limit the sensitivity and accuracy of the method. Moreover, it is also desirable to know that the structural isoforms of any anti-CD3 homodimer product related impurity, which may be present in the bi-specific Test Article (purified product, DS, DP, stability sample, stress sample), or other potential T cell activating impurities, are biologically active in order to assign appropriate risk to the impurity. The novel anti-CD3 homodimer assay approach described herein uses a cell-based approach to detect biologically active anti-CD3 homodimer impurities and thereby avoids the challenges and limitations for homodimer impurity detection in bi-specific preparations using binding assay or physiochemical based formats.

**[0006]** All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

## SUMMARY

**[0007]** The invention provides methods for detecting anti-CD3 homodimers in a composition comprising a T cell dependent bispecific antibody (TDB) wherein the bispecific antibody comprises a target antigen binding fragment and a CD3 binding fragment, the method comprising contacting a population of T cells with the composition, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a response element that is responsive to T cell activation, and wherein the population of T cells does not comprise the target antigen, wherein expression of the reporter indicates the presence of anti-CD3 homodimers.

**[0008]** In some embodiments of the above embodiment, the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, a beta lactamase, or a beta galactosidase. In further embodiments, the luciferase is a firefly luciferase, a *Renilla* luciferase, or a nanoluciferase. In some embodiments, the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter. In some embodiments, the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

**[0009]** In some embodiments of the above embodiments, the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells. In some embodiments, the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

**[0010]** In some embodiments of the above embodiments, the population of T-cells is contacted with a composition comprising the bispecific antibody at a concentration ranging from 0.01 ng/mL to 50 ng/mL. In some embodiments, the reporter is detected after any one or more of 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 or 24 hours after contacting the cells with the composition.

**[0011]** In some aspects, the invention provides methods for quantitating the amount of anti-CD3 homodimer antibodies in a composition comprising a TDB where the TDB comprises a target antigen binding fragment and CD3 binding fragment, the method comprising contacting population of T cells with the composition at one or more concentrations of the TDB, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a promoter responsive to T cell activation and wherein the population of T cells does not comprise the target antigen, correlating the expression of the reporter as a function of antibody concentration with a standard curve generated by contacting the T cells with different concentrations of purified anti-CD3 homodimers.

**[0012]** In some embodiments of the above quantitating the amount of anti-CD3 homodimer antibodies in a composition, the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, beta lactamase, or a beta galactosidase. In further embodiments, the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase.

**[0013]** In some embodiments of the above quantitating the amount of anti-CD3 homodimer antibodies in a composition, the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, a beta lactamase, or a beta galactosidase. In further embodiments, the luciferase is a firefly luciferase, a *Renilla* luciferase, or a nanoluciferase. In some embodiments, the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter. In some embodiments, the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

**[0014]** In some embodiments of the above embodiments, the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells. In some embodiments, the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

**[0015]** In some embodiments of the above embodiments, the population of T-cells is contacted with a composition comprising the bispecific antibody at a concentration ranging from 0.01 ng/mL to 50 ng/mL. In some embodiments, the reporter is detected after any one or more of 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 or 24 hours after contacting the cells with the composition.

**[0016]** In some aspects, the invention provides an engineered T cell for the detection of anti-CD3 homodimers in a composition comprising a bispecific antibody where the bispecific antibody comprises a target antigen binding fragment and CD3 binding fragment, wherein the T cell comprises a reporter operably linked to a response element that is responsive to T cell activation.

**[0017]** In some embodiments of the above aspect, the T cell comprises a reporter wherein the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, beta lactamase, or a beta galactosidase. In further embodiments, the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase.

**[0018]** In some embodiments of the above embodiment, the T cell comprises a reporter wherein the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, a beta lactamase, or a beta galactosidase. In further embodiments, the luciferase is a firefly luciferase, a *Renilla* luciferase, or a nanoluciferase. In some embodiments, the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO

promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter. In some embodiments, the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

**[0019]** In some embodiments of the above embodiments, the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells. In some embodiments, the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

**[0020]** In some aspects, the invention provides a kit for the detection of anti-CD3 homodimers in a composition comprising a bispecific antibody where the bispecific antibody comprises a target antigen binding fragment and CD3 binding fragment, wherein the kit comprises an engineered T cell comprising a reporter operably linked to a response element that is responsive to T cell activation. In some embodiments, the kit further comprises an anti-CD3 homodimer assay standard and/or an anti-CD3 homodimer control.

**[0021]** In some embodiments of the above kits, the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, beta lactamase, or a beta galactosidase. In further embodiments, the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase.

**[0022]** In some embodiments of the above kits, the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, a beta lactamase, or a beta galactosidase. In further embodiments, the luciferase is a firefly luciferase, a *Renilla* luciferase, or a nanoluciferase. In some embodiments, the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter. In some embodiments, the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

**[0023]** In some embodiments of the above kits, the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells. In some embodiments, the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0024]** Figs. 1A and 1B show that a CD20 TDB (αCD20 (Mab2; VH SEQ ID NO:31/VL SEQ ID NO:32)/αCD3(Mab1; VH SEQ ID NO:19/VL SEQ ID NO:20)) requires antigen CD20 expressing target cells to induce T cell activation and target cell killing. Fig. 1A shows activation of $CD8^+$ cells as measured by expression of CD69 and CD25. Circles represent samples including the target cells and squares represent samples containing T cells but no target

cells. Fig. 1B shows the killing of target cells in the presence of T cells (circles) or in samples where CD3+ T cells were depleted from the PBMC pool (squares).

**[0025]** Fig. 2 shows that anti-CD3 homodimers can activate human donor T cells. Human PBMCs from two different donors were treated with increasing amounts of purified anti-CD3 homodimers (triangles) or CD20 TDB (circles). T cell activation was measured by the % $CD69^+$ cells in the population of human $CD8^+$ cells. The $EC_{50}$ for CD20 TDB was 5.5 ng/ml for cells from Donor 1 and 4.4 ng/ml for cells from Donor 2. The $EC_{50}$ for anti-CD3 homodimer was 526 ng/ml for cells from Donor 1 and 169 ng/ml for cells from Donor 2.

**[0026]** Fig. 3A shows that an anti-CD3 homodimer can decrease CD20 TDB potency. CD20 TDB was spiked with various amounts of anti-CD3 homodimer and target cell (squares) and T cell (diamond) responses were measured. Fig. 3B shows that low levels of anti-CD3 homodimer spiked into CD20 TDB do not significantly reduce $CD8^+$ T cell activation (left panel) or $CD4^+$ T cell activation (right panel). CHO TDB represented by circles, CHO TBD + 2.5% HD represented by squares, and CHO TBD + 5% HD represented by triangles.

**[0027]** Fig. 4A shows that anti-CD3 homodimer can weakly activate human $CD8^-$ T cells from various human donors in the absence of target cells. Figs. 4B-4E show that anti-CD3 homodimer activation of human T cells shows a dose dependent trend for the increase of some representative cytokines. The mean average cytokine level response has been plotted.

**[0028]** Fig. 5A shows T cell activation by anti-CD3 homodimer can be monitored using a reporter gene assay. The human Jurkat $CD4^+$ T cell line was genetically engineered to stably express the firefly luciferase reporter gene driven by various T Cell Receptor (TCR) responsive transcriptional response elements (AP-1, NFAT, and NFκB), stable cell pools selected, and pools evaluated for response to treatment with 10 μg/mL of purified anti-CD3 homodimer for 4 hours. Luminescence responses (luciferase reporter gene activity) were plotted, with the highest response observed from the Jurkat/NFκBluciferase stable pool. Fig. 5B shows Jurkat/NFκBLuciferase stable clones.

**[0029]** Figs. 6A and 6B show that purified anti-CD3 homodimer can activate T cells in the presence of or absence of target cells. Fig. 6A shows a comparison of purified CD20 TDB and purified anti-CD3 homodimer potential to activate T cells. Jurkat T cells expressing a NFκBLuciferase reporter gene are activated dose-dependently by CD20 TDB in the presence of target antigen expressing cells. CD20 TDB activates Jurkat/NFκB-fireflyLuciferase cells in the presence of the target antigen expressing cell line. Purified CD20 TDB is 1000-fold more active than purified anti-CD3 homodimer, in the presence of co-stimulatory target antigen-expressing cells. Fig. 6B shows that in the absence of target antigen-expressing cells (squares), CD20 TDB

does not activate Jurkat/NFκBLuciferase cells, but purified anti-CD3 homodimer dose-dependently induces NFκB-dependent luciferase activity (diamonds).

**[0030]** Fig. 7 shows the calculation of anti-CD3 homodimer present in CD20 TDB samples. Luciferase activity observed from the Jurkat/NFκBLuc sample treated cells, as measured by a luminescence plate reader (RLU), is compared to the T cell activation response generated by a known amount of anti-CD3 homodimer. An equation derived from the curve fitted to the homodimer standard response is used to solve for the concentration of anti-CD3 homodimer present in a sample. The percentage of anti-CD3 homodimer present in a sample is then determined from the ratio of the detected homodimer in the sample divided by the total amount of CD20 TDB present in the sample.

**[0031]** Fig. 8 shows that the anti-CD3 T cell activation assay is accurate for CD20 TDB test samples from 0.25% to 35%. Spike recovery of anti-CD3 homodimer demonstrates that the analytical method is linear across the range of the method with an $R^2$ of 0.99, slope of 1.05 and y-int of 0.078 and shows minimal bias.

**[0032]** Fig. 9 shows that T cell activation assay is able to detect other product related impurities. The level of HMWS present in CD20 TDB material impacts T-cell activation in the homodimer assay.

## DETAILED DESCRIPTION OF THE INVENTION

**[0033]** The invention provides methods for detecting anti-CD3 homodimers in a composition comprising a T cell dependent bispecific antibody (TDB) where the bispecific antibody comprises a target antigen binding fragment and a CD3 binding fragment, the method comprising contacting a population of T cells with the composition, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a promoter responsive to T cell activation, and wherein the population of T cells does not comprise the target antigen, wherein expression of the reporter indicates the presence of anti-CD3 homodimers.

**[0034]** In some aspects, the invention provides methods for quantitating the amount of anti-CD3 homodimer antibodies in a composition comprising a TDB where the TDB comprises a target antigen binding fragment and CD3 binding fragment, the method comprising contacting population of T cells with the composition at one or more concentrations of the TDB, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a promoter responsive to T cell activation and wherein the population of T cells does not comprise the target antigen, correlating the expression of the reporter as a function of antibody concentration with a standard

curve generated by contacting the T cells with different concentrations of purified anti-CD3 homodimers.

**[0035]** In other aspects, the invention provides engineered T cells for the detection of anti-CD3 homodimers in a composition comprising a TDB where the TDB comprises a target antigen binding fragment and CD3 binding fragment, wherein the T cell comprises a reporter operably linked to a promoter responsive to T cell activation.

**[0036]** In other aspects, the invention provides kits for the detection of anti-CD3 homodimers in a composition comprising a TDB where the TDB comprises a target antigen binding fragment and CD3 binding fragment, wherein the kit comprises an engineered T cell comprising a reporter operably linked to a promoter responsive to T cell activation.

## I. Definitions

**[0037]** The term "polypeptide" or "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component or toxin. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art. The terms "polypeptide" and "protein" as used herein specifically encompass antibodies.

**[0038]** "Purified" polypeptide (*e.g.*, antibody or immunoadhesin) means that the polypeptide has been increased in purity, such that it exists in a form that is more pure than it exists in its natural environment and/or when initially synthesized and/or amplified under laboratory conditions. Purity is a relative term and does not necessarily mean absolute purity.

**[0039]** The term "antagonist" is used in the broadest sense, and includes any molecule that partially or fully blocks, inhibits, or neutralizes a biological activity of a native polypeptide. In a similar manner, the term "agonist" is used in the broadest sense and includes any molecule that mimics a biological activity of a native polypeptide. Suitable agonist or antagonist molecules specifically include agonist or antagonist antibodies or antibody fragments, fragments or amino acid sequence variants of native polypeptides, *etc.* Methods for identifying agonists or antagonists of a polypeptide may comprise contacting a polypeptide with a candidate agonist or

antagonist molecule and measuring a detectable change in one or more biological activities normally associated with the polypeptide.

**[0040]** A polypeptide "which binds" an antigen of interest, *e.g.* a tumor-associated polypeptide antigen target, is one that binds the antigen with sufficient affinity such that the polypeptide is useful as a diagnostic and/or therapeutic agent in targeting a cell or tissue expressing the antigen, and does not significantly cross-react with other polypeptides. In such embodiments, the extent of binding of the polypeptide to a "non-target" polypeptide will be less than about 10% of the binding of the polypeptide to its particular target polypeptide as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

**[0041]** With regard to the binding of a polypeptide to a target molecule, the term "specific binding" or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target.

**[0042]** The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies *(e.g.* bispecific antibodies including TDB) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity. The term "immunoglobulin" (Ig) is used interchangeable with antibody herein.

**[0043]** Antibodies are naturally occurring immunoglobulin molecules which have varying structures, all based upon the immunoglobulin fold. For example, IgG antibodies have two "heavy" chains and two "light" chains that are disulphide-bonded to form a functional antibody. Each heavy and light chain itself comprises a "constant" (C) and a "variable" (V) region. The V regions determine the antigen binding specificity of the antibody, whilst the C regions provide structural support and function in non-antigen-specific interactions with immune effectors. The antigen binding specificity of an antibody or antigen-binding fragment of an antibody is the ability of an antibody to specifically bind to a particular antigen.

**[0044]** The antigen binding specificity of an antibody is determined by the structural characteristics of the V region. The variability is not evenly distributed across the 110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches

called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called "hypervariable regions" (HVRs) that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (*see* Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

**[0045]** Each V region typically comprises three HVRs, *e.g.* complementarity determining regions ("CDRs", each of which contains a "hypervariable loop"), and four framework regions. An antibody binding site, the minimal structural unit required to bind with substantial affinity to a particular desired antigen, will therefore typically include the three CDRs, and at least three, preferably four, framework regions interspersed there between to hold and present the CDRs in the appropriate conformation. Classical four chain antibodies have antigen binding sites which are defined by $V_H$ and $V_L$ domains in cooperation. Certain antibodies, such as camel and shark antibodies, lack light chains and rely on binding sites formed by heavy chains only. Single domain engineered immunoglobulins can be prepared in which the binding sites are formed by heavy chains or light chains alone, in absence of cooperation between $V_H$ and $V_L$.

**[0046]** The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (*see* Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in

binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

**[0047]** The term "hypervariable region" (HVR) when used herein refers to the amino acid residues of an antibody that are responsible for antigen binding. The hypervariable region may comprise amino acid residues from a "complementarity determining region" or "CDR" (*e.g.*, around about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the $V_L$, and around about 31-35B (H1), 50-65 (H2) and 95-102 (H3) in the $V_H$ (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (*e.g.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the $V_L$, and 26-32 (H1), 52A-55 (H2) and 96-101 (H3) in the $V_H$ (Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)).

**[0048]** "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

**[0049]** As used herein, "T cell Dependent Bispecific" antibodies or "TDB" are bispecific antibodies designed to bind a target antigen expressed on a cell, and to bind to T cells, often by binding CD3e subunit of the T cell receptor.

**[0050]** "Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; tandem diabodies (taDb), linear antibodies (*e.g.*, U.S. Patent No. 5,641,870, Example 2; Zapata *et al.*, *Protein Eng*. 8(10):1057-1062 (1995)); one-armed antibodies, single variable domain antibodies, minibodies, single-chain antibody molecules; multispecific antibodies formed from antibody fragments (*e.g.*, including but not limited to, Db-Fc, taDb-Fc, taDb-CH3, (scFV)4-Fc, di-scFv, bi-scFv, or tandem (di,tri)-scFv); and Bi-specific T-cell engagers (BiTEs).

**[0051]** Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

**[0052]** "Fv" is the minimum antibody fragment that contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the $V_H$-$V_L$ dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising

only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

**[0053]** The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

**[0054]** The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

**[0055]** Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

**[0056]** "Single-chain Fv" or "scFv" antibody fragments comprise the $V_H$ and $V_L$ domains of antibody, wherein these domains are present in a single polypeptide chain. In some embodiments, the Fv polypeptide further comprises a polypeptide linker between the $V_H$ and $V_L$ domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv *see* Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

**[0057]** The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain ($V_H$) connected to a light chain variable domain ($V_L$) in the same polypeptide chain ($V_H$ - $V_L$). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

**[0058]** The term "multispecific antibody" is used in the broadest sense and specifically covers an antibody that has polyepitopic specificity. Such multispecific antibodies include, but are not

limited to, an antibody comprising a heavy chain variable domain ($V_H$) and a light chain variable domain ($V_L$), where the $V_HV_L$ unit has polyepitopic specificity, antibodies having two or more $V_L$ and $V_H$ domains with each $V_HV_L$ unit binding to a different epitope, antibodies having two or more single variable domains with each single variable domain binding to a different epitope, full length antibodies, antibody fragments such as Fab, Fv, dsFv, scFv, diabodies, bispecific diabodies, triabodies, tri-functional antibodies, antibody fragments that have been linked covalently or non-covalently. "Polyepitopic specificity" refers to the ability to specifically bind to two or more different epitopes on the same or different target(s). "Monospecific" refers to the ability to bind only one epitope. According to one embodiment the multispecific antibody is an IgG antibody that binds to each epitope with an affinity of 5 μM to 0.001 pM, 3 μM to 0.001 pM, 1 μM to 0.001 pM, 0.5 μM to 0.001 pM, or 0.1 μM to 0.001 pM.

**[0059]** In some examples, the multispecific antibody is a bispecific antibodies (e.g., a bispecific antibody that binds CD3 and another epitope). Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

**[0060]** Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, Nature 305: 537 (1983), WO 93/08829, and Traunecker et al., EMBO J. 10: 3655 (1991)), and "knob-in-hole" engineering (see, e.g., U.S. Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al., Science, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992)); using "diabody" technology for making bispecific antibody fragments (*see*, *e.g.*, Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (*see*, *e.g.*, Gruber *et al.*, *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, *e.g.*, in Tutt *et al. J. Immunol.* 147: 60 (1991).

**[0061]** The expression "single domain antibodies" (sdAbs) or "single variable domain (SVD) antibodies" generally refers to antibodies in which a single variable domain (VH or VL) can confer antigen binding. In other words, the single variable domain does not need to interact with another variable domain in order to recognize the target antigen. Examples of single domain antibodies include those derived from camelids (lamas and camels) and cartilaginous fish (*e.g.*, nurse sharks) and those derived from recombinant methods from humans and mouse antibodies (Ward, ES *et al.*, *Nature* (1989) 341:544-546; Dooley, H. *et al.*, *Dev Comp Immunol* (2006)

30:43-56; Muyldemans S *et al.*, *Trend Biochem Sci* (2001) 26:230-235; Holt, LJ *et al.*, *Trends Biotechnol* (2003):21:484-490; WO 2005/035572; WO 03/035694; Davies, J *et al.*, *Febs Lett* (1994) 339:285-290; WO00/29004; WO 02/051870).

**[0062]** The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the methods provided herein may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (*see*, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

**[0063]** The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, 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 (U.S. Patent No. 4,816,567; Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate *(e.g.* Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences (US Pat No. 5,693,780).

**[0064]** "Humanized" forms of non-human *(e.g.*, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a

non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine 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 hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence, except for FR substitution(s) as noted above. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region, typically that of a human immunoglobulin. For further details, *see* Jones *et al*., *Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

**[0065]** For the purposes herein, an "intact antibody" is one comprising heavy and light variable domains as well as an Fc region. The constant domains may be native sequence constant domains (*e.g.* human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

**[0066]** "Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ($V_H$) followed by a number of constant domains. Each light chain has a variable domain at one end ($V_L$) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

**[0067]** A "naked antibody" is an antibody (as herein defined) that is not conjugated to a heterologous molecule, such as a cytotoxic moiety or radiolabel.

**[0068]** In some embodiments, antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding;

antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors.

**[0069]** "Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) *(e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes *et al., Proc. Natl. Acad. Sci. (USA)* 95:652-656 (1998).

**[0070]** "Human effector cells" are leukocytes that express one or more FcRs and perform effector functions. In some embodiments, the cells express at least FcγRIII and carry out ADCC effector function. Examples of human leukocytes that mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred.

**[0071]** "Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.* polypeptide (*e.g.*, an antibody)) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996), may be performed.

**[0072]** The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. In some embodiments, the FcR is a native sequence human FcR. Moreover, a preferred FcR is one that binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its

cytoplasmic domain. (*see* Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al., Immunomethods* 4:25-34 (1994); and de Haas *et al., J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al., J. Immunol.* 117:587 (1976) and Kim *et al., J. Immunol.* 24:249 (1994)).

**[0073]** "Impurities" refer to materials that are different from the desired polypeptide product. In some embodiments of the invention, impurities include charge variants of the polypeptide. In some embodiments of the invention, impurities include charge variants of an antibody or antibody fragment. In other embodiments of the invention, the imprurities includes, without limitation: host cell materials, such as CHOP; leached Protein A; nucleic acid; a variant, fragment, aggregate or derivative of the desired polypeptide; another polypeptide; endotoxin; viral contaminant; cell culture media component, *etc.* In some examples, the impurity may be a host cell protein (HCP) from, for example but not limited to, a bacterial cell such as an *E. coli* cell, an insect cell, a prokaryotic cell, a eukaryotic cell, a yeast cell, a mammalian cell, an avian cell, a fungal cell. In some examples, the impurity is a homodimer (*e.g.*, an anti-CD3 homodimer).

**[0074]** As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous polypeptide with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (*i.e.*, is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

**[0075]** By "reporter molecule", as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

**[0076]** As used herein "essentially the same" indicates that a value or parameter has not been altered by a significant effect. For example, an ionic strength of a chromatography mobile phase

at column exit is essentially the same as the initial ionic strength of the mobile phase if the ionic strength has not changed significantly. For example, an ionic strength at column exit that is within 10%, 5% or 1% of the initial ionic strength is essentially the same as the initial ionic strength.

**[0077]** Reference to "about" a value or parameter herein includes (and describes) variations that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X".

**[0078]** As used herein and in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise. It is understood that aspects and variations of the invention described herein include "consisting" and/or "consisting essentially of" aspects and variations.

## II. Cell based reporter assays

**[0079]** The present invention provides cell-based assays to detect anti-CD3 homodimers present in a composition comprising a TDB wherein one antigen binding fragment of the TDB binds CD3 and activates T cells.

### *A. T cell activation*

**[0080]** The mechanism of action of a TDB is to specifically deplete a target antigen expressing cell. Simultaneous binding of the TDB to the CD3e subunit of the T cell receptor (TCR) and to the target antigen expressed on the surface of the target cell results in TCR clustering leading to T cell activation and the cytotoxic depletion of the target cell. There have been many TDBs in the clinic (αCD3/αCD19, αCD3/αCD20, αCD3/αHER2; de Gast GC, *et al.*, 1995, *Cancer Immunol Immunother.* 40(6):390-396; Buhmann R, *et al.*,2009 *Bone Marrow Transplant.* 43(5):383-397; Chan JK, *et al.*, 2006, *Clin Cancer Res.* 12(6):1859-1867) and new versions of TDB-like bispecifics are being evaluated to improve clinical efficacy (Chames, P. and Baty, D. 2009, *MAbs* 1(6):539-547; Fournier, P. and Schirrmacher, V., 2013, *BioDrugs* 27(1):35-53). TDB bi-specifics are capable of activating both $CD4^+$ and $CD8^+$ T cell lineages, provided the right target expressing cells are present. Activation of $CD4^+$ T cells will result in the induction of cytokine gene expression (IL-2, *etc.*) leading to the recruitment and activation of other immune cells, including the expansion and proliferation of $CD8^+$ T cells. $CD8^+$ CTL activation results from the formation of an immunological synapse-like structure with target cells via TDB-mediated cellular bridging leads to the activation of the CTL, induction of transcription of Perforin and Granzymes (A, B, C; depending on CTL subtype), degranulization, and the

localized release of Perforin and Granzymes across the 'immunological synapse'-like interface between the target and effector cell resulting in the killing of the target cell (Pores-Fernando, Pores-Fernando AT, Zweifach A, 2009, *Immunol Rev.*, 231(1):160-173; Pipkin, ME, *et al.*, 2010, *Immunol Rev.*, 235(1):55-72). Effector cell mediated cell killing is a relatively slow process requiring the stabilization of the synapse for several hours and requires the transcriptional dependent activation of the *prf1* gene and granzyme genes to ensure complete cell killing. Alternatively, CTL-mediated killing of target cells has also been shown to occur by Fas-mediated apoptosis (Pardo, J, *et al.*, 2003, *Int Immunol.,* 15(12):1441-1450). The transcriptional regulation of the *prf1*, *grB* and Fas-mediated cell killing machinery is dependent on NFAT, NFκB and STAT enhancer elements located within the promoters of the genes required to mediate B cell depletion (Pipkin, ME, *et al.*, 2010, *Immunol Rev.*, 235(1):55-72; Pardo, J, *et al.*, 2003, *Int Immunol.,* 15(12):1441-1450). The strength of the interaction between the target and effector cells (immunological synapse) is dependent on other co-stimulatory molecules from which signaling is also necessary to stabilize and maintain the interaction between target and effector cell (Krogsgaard M, *et al.*, 2003, *Semin Immunol.* 15(6):307-315; Pattu V, *et al.*, 2013, *Front Immunol.*, 4:411; Klieger Y, *et al.*, 2014, *Eur J Immunol.* 44(1):58-68; Schwartz JC, *et al.*, 2002, *Nat Immunol.* 3(5):427-434). The monitoring of the transcriptional induction of target genes, through the use of reporter gene assays, is therefore a MOA-reflective alternative assay system to observe the activation of T cells by TDB.

**[0081]** T cell activation requires the spatial and kinetic reorganization of cell surface proteins and signaling molecules at the contact site of the antigen presenting cell to form the immunological synapse. Coordination of the activation and signaling of the T Cell Receptor (TCR) and co-stimulatory receptors (CD28, CD40, ICOS, *etc.*) and ligands regulates both the duration and signaling that is required for T cell activation. Antigen presentation on the surface of the Antigen Presenting Cell (APC) through MHC is recognized by TCR on the surface of the T cell. MHC and TCR clustering initiates the recruitment and activation of signaling pathways that can lead to T cell activation, depending on the expression of co-stimulatory and immunomodulatory receptors, which play a key role in regulating T cell activation. Antibodies to subunits of the TCR, such as CD3e (OKT3; Brown, WM, 2006, *Curr Opin Investig Drugs* 7:381-388; Ferran, C *et al.*, 1993 *Exp Nephrol* 1:83-89), can induce T cell activation by cross-linking TCR and thereby mimicking the clustering of TCR at the immunological synapse, and have been used clinically, as well as for many years as a surrogate activators to study TCR signaling in vitro. TCR clustering by anti-CD3 antibodies without co-stimulation weakly activates T cells, but still leads to T cell activation and limited cytokine transcription and release.

Anti-CD3 mediated signaling has been shown to activate several transcription factors, including NFAT, AP1, and NFκB (MF *et al.*, 1995, *J. Leukoc. Biol.* 57:767-773; Shapiro VS *et al.*, 1998, *J. Immunol.* 161(12)6455-6458; Pardo, J, *et al.*, 2003, *Int Immunol.,* 15(12):1441-1450). Co-stimulation regulates the level and type of cytokine release via the modulation of signaling that impacts transcriptional regulation of cytokine expression which impacts the nature of the T cell activation response (Shannon, MF *et al.*, 1995, *J. Leukoc. Biol.* 57:767-773). The TDB bi-specific clusters TCR on the cell surface of the T cell as a result of the bridge formed between the T cell and the target antigen expressing cell. Transcriptional regulatory elements driving the expression of reporter genes that may be transcriptional induced by T cell activation were tested in T cell lines to determine which events are activated by the TDB in the presence and absence of target cells.

***B. Reporter molecules***

**[0082]** A reporter assay is an analytical method that enables the biological characterization of a stimulus by monitoring the induction of expression of a reporter in a cell. The stimulus leads to the induction of intracellular signaling pathways that result in a cellular response that typically includes modulation of gene transcription. In some examples, stimulation of cellular signaling pathways result in the modulation of gene expression via the regulation and recruitment of transcription factors to upstream non-coding regions of DNA that are required for initiation of RNA transcription leading to protein production. Control of gene transcription and translation in response to a stimulus is required to elicit the majority of biological responses such as cellular proliferation, differentiation, survival and immune responses. These non-coding regions of DNA, also called enhancers, contain specific sequences that are the recognition elements for transcription factors which regulate the efficiency of gene transcription and thus, the amount and type of proteins generated by the cell in response to a stimulus. In a reporter assay, an enhancer element and minimal promoter that is responsive to a stimulus is engineered to drive the expression of a reporter gene using standard molecular biology methods. The DNA is then transfected into a cell, which contains all the machinery to specifically respond to the stimulus, and the level of reporter gene transcription, translation, or activity is measured as a surrogate measure of the biological response.

**[0083]** In some aspects, the invention provides methods of detecting anti-CD3 homodimers in a composition comprising a TDB by contacting a population of T cells with the composition, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a promoter responsive to CD3 activation such that expression of the reporter indicates the presence of anti-

CD3 homodimers. A reporter molecule may be any molecule for which an assay can be developed to measure the amount of that molecule that is produced by the cell in response to the stimulus. For example, a reporter molecule may be a reporter protein that is encoded by a reporter gene that is responsive to the stimulus; for example, T cell activation. Commonly used examples of reporter molecules include but are not limited to luminescent proteins such as luciferase, which emit light as a by-product of the catalysis of substrate which can be measured experimentally. Luciferases are a class of luminescent proteins that are derived from many sources including firefly luciferase (from the species, *Photinus pyralis*), Renilla luciferase from sea pansy (*Renilla reniformis*), click beetle luciferase (from *Pyrearinus termitilluminans*), marine copepod Gaussia luciferase (from *Gaussia princeps*), and deep sea shrimp Nano luciferase (from *Oplophorus gracilirostris*). Firefly luciferase catalyzes the oxygenation of luciferin to oxyluciferin resulting in the emission of a photon of light while other luciferases such as Renilla emit light by catalyzing coelenterazine. The wavelength of light emitted by different luciferase forms and variants can be read using different filter systems, which facilitate multiplexing. The amount of luminescence is proportional to the amount of luciferase expressed in the cell and luciferase genes have been used as a sensitive reporter to evaluate the impact of a stimulus to elicit a biological response. Reporter gene assays have been used for many years for a wide range of purposes including basic research, HTS screening, and for potency (Brogan J, *et al*., 2012, *Radiat Res*. 177(4):508-513; Miraglia LJ, *et al*., 2011, *Comb Chem High Throughput Screen.* 14(8):648-657; Nakajima Y, and Ohmiya Y. 2010, *Expert Opin Drug Discovery*, 5(9):835-849; Parekh BS, *et al*., 2012, *Mabs*, 4(3):310-318; Svobodova K, and Cajtham L T., 2010, *Appl Microbiol Biotechnol*., 88(4): 839-847).

**[0084]** In some embodiments, the invention provides cell-based assays to detect anti-CD3 homodimers in TDB compositions where T cells encoding a reporter construct that is responsive to T cell activation. In some embodiments, the reporter construct comprises a luciferase. In some embodiments, the luciferase is a firefly luciferase (*e.g.*, from the species *Photinus pyralis*), Renilla luciferase from sea pansy (*e.g.*, from the species *Renilla reniformis*), click beetle luciferase (*e.g.*, from the species *Pyrearinus termitilluminans*), marine copepod Gaussia luciferase (*e.g.*, from the species *Gaussia princeps*), and deep sea shrimp Nano luciferase (*e.g.*, from the species *Oplophorus gracilirostris*). In some embodiments, expression of luciferase in the engineered T cell indicates the presence of anti-CD3 homodimers in the TDB composition. In other aspects, the reporter construct encodes a β-glucuronidase (GUS); a fluorescent protein such as Green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP) and variants thereof; a chloramphenicoal

acetyltransferase (CAT); a β-galactosidase; a β-lactamase; or a secreted alkaline phosphatase (SEAP).

**[0085]** In some aspects of the invention, nucleic acid encoding a reporter molecule (*e.g.*, a reporter protein) is operably linked to a promoter and/or enhancer responsive to T cell activation. In some embodiments, the promoter and/or enhancer responsive to T cell activation is an expression control sequence. Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the polypeptide (*e.g.*, the reporter polypeptide). Suitably, the expression control sequences are eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells (*e.g.*, T cells). Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences following T cell activation.

**[0086]** Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

**[0087]** In some aspects of the invention, the invention provides T cells comprising nucleic acid encoding a reporter molecule under the control of a promoter responsive to T cell activation. Promoters responsive to T cell activation are known in the art.

**[0088]** In other embodiments, the invention provides T cells comprising nucleic acid encoding a reporter molecule under the control of a minimal promoter operably linked to an enhancer element responsive to T cell activation. In some embodiments, the minimal promoter is a thymidine kinase (TK) minimal promoter, a minimal promoter from cytomegalovirus (CMV), an SV40-derived promoter, or a minimal elongation factor 1 alpha (EF1α) promoter. In some embodiments, nucleic acid encoding the reporter molecule is under the control of a minimal TK promoter regulated by T cell activation responsive DNA recognition elements. In some embodiments, the T cell activation responsive DNA recognition elements are NFAT (Nuclear Factor of Activated T cells) enhancers, AP-1 (Fos/Jun) enhancers, NFAT/AP1 enhancers, NFκB enhancers, FOXO enhancers, STAT3 enhancers, STAT5 enhancers and IRF enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the polypeptide-encoding sequence, but in some embodiments, is located at a site 5' from the promoter. In some

embodiments, the invention provides T cells wherein a luciferase gene is operably linked to a minimal TK promoter which in turn is operably linked to an NFκB responsive enhancer element.

**[0089]** In some embodiments of the invention, expression reporter vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

**[0090]** In some embodiments, the invention provides vectors for the expression of the reporter molecule in T cells. Vector components generally include, but are not limited to, one or more of the following, a signal sequence, an origin of replication, one or more marker genes, a multiple cloning site containing recognition sequences for numerous restriction endonucleases, an enhancer element, a promoter (*e.g.*, an enhancer element and/or promoter responsive to T cell activation), and a transcription termination sequence. In some embodiments, the vector is a plasmid. In other embodiments, the vector is a recombinant viral genome; *e.g.*, a recombinant lentiviral genome, a recombinant retrovirus genome, a recombinant adeno-associated viral genome. The vectors containing the polynucleotide sequences (*e.g.*, the reporter gene operably linked to a T-cell responsive promoter/enhancer) can be transferred into a host T cell by well-known methods. For example, calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection may be used. (*See* generally Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press, 2nd ed., 1989). Other methods used to transform mammalian cells include the use of polybrene, protoplast fusion, liposomes, electroporation, and microinjection.

### *C. Cells*

**[0091]** In some aspects, the invention provides a cell-based assay to detect anti-CD3 homodimers in a composition comprising a TDB by contacting a population of T cells comprising a reporter complex responsive to T cell activation. In some embodiments the T cells of the population are $CD4^+$ T cells. In some embodiments, the T cells are $CD8^+$ T cells. In yet other embodiments, the T cell is a $CD4^+/CD8^+$ T cell. In some embodiments, the $CD4^+$ and/or $CD8^+$ T cells exhibit increased release of cytokines selected from the group consisting of IFN-γ, TNF-α, and interleukins. In some embodiments, the population of T cells is a population of immortalized T cells (*e.g.*, an immortalized T cell line). In some embodiments, the population

of T cells is a population of immortalized $CD4^+$ and/or $CD8^+$ cells that expressed TCR/CD3e. In some embodiments, the T cell is a Jurkat cell. In some embodiments, the T cell is a CTLL-2 T cell.

**[0092]** In some embodiments, T cells of the invention comprise a T cell receptor. T cell receptors exist as a complex of several proteins. The T cell receptor itself is composed of two separate peptide chains encoded by the independent T cell receptor alpha and beta (TCRα and TCRβ) genes. Other proteins in the complex include the CD3 proteins: CD3ε (also known as CD3e), CD3γ, CD3δ and CD3ζ. The CD3 proteins are found as CD3εγ and CD3εδ heterodimers and a CD3ζ homodimer. The CD3 ζ homodimer allows the aggregation of signaling complexes around these proteins. In some embodiments, one arm of the TDB binds a T cell receptor complex. In some embodiments, the TDB binds CD3. In some embodiments, the TDB binds the CD3ε (CD3e) protein.

**[0093]** In some embodiments, the invention provides compositions comprising T cells for use in a cell-based assay to detect and/or quantitate anti-CD3 homodimers in a composition of TDB. In some embodiments the T cells of the composition are $CD4^+$ T cell. In some embodiments, the T cells of the composition are $CD8^+$ T cell. In yet other embodiments, the T cells of the composition are $CD4^+/CD8^+$ T cells. In some embodiments, the T cells of the composition are immortalized T cells. In some embodiments, the T cells of the composition are Jurkat cells. In some embodiments, the T cells of the composition are CTLL-2 T cells. In some embodiments, the T cells of the composition comprise a reporter complex responsive to T cell activation. In some embodiments, the reporter complex comprises a polynucleotide encoding a luciferase. In some embodiments, the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase. In some embodiments, the polynucleotide encoding the reporter (*e.g.*, luciferase) is operably linked to a T cell activation responsive regulatory element (*e.g.*, a T cell activation responsive promoter and/or enhancer). In some embodiments, the promoter responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter.

**[0094]** In some embodiments, T cells in which T cell activation-responsive reporter constructs have been introduced (reporter T cells) are screened for activation by anti-CD3 homodimers. For example, stable clones can be isolated by limiting dilution and screened for their response to a purified anti-CD3 homodimer. In some embodiments, stable reporter T cells are screened with more than about any of 1 μg/mL, 2 μg/mL, 3 μg/mL, 4 μg/mL, 5 μg/mL, 6 μg/mL, 7 μg/mL, 8 μg/mL, 9 μg/mL, or 10 μg/mL purified anti-CD3 homodimer.

**[0095]** In some embodiments, the invention provides compositions of T cells engineered with a T cell activation reporter complex. In some embodiments, the reporter is a luciferase, a fluorescent protein (*e.g.*, a GFP, aYFP, *etc.*), an alkaline phosphatase, or a beta galactosidase. In some embodiments, the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase. In some embodiments, the promoter responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter. In some embodiments, the promoter responsive to T cell activation comprises T cell responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF. In some embodiments, the composition of T cells comprises $CD4^+$ T cells and/or $CD8^+$ T cells. In some embodiments, the T cells are Jurkat cells or CTLL-2 cells. In some embodiments, the T cells are Jurkat cells comprising a polynucleotide encoding a luciferase operably linked to an NFκB promoter.

***D. Methods of identifying CD3 homodimers***

**[0096]** In some aspects, the invention provides methods for detecting anti-CD3 homodimers in a composition comprising a TDB wherein the TDB antibody comprises a target antigen binding fragment and a CD3 binding fragment, the method comprising contacting a population of T cells with the composition, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a promoter responsive to T cell activation, and wherein the population of T cells does not comprise the target antigen, wherein expression of the reporter indicates the presence of anti-CD3 homodimers. In some embodiments, the population of T cells does not comprise cells expressing the target antigen (non-T cell antigen) of the TDB.

**[0097]** In some embodiments, the population of T-cells is contacted with a composition comprising the TDB at a concentration range of any one of about 0.01 ng/mL to about 50 ng/mL, about 0.05 ng/mL to about 50 ng/mL, about 0.1 ng/mL to about 50 ng/mL, about 0.5 ng/mL to about 50 ng/mL, about 1 ng/mL to about 50 ng/mL, about 5 ng/mL to about 50 ng/mL, about 10 ng/mL to about 50 ng/mL, about 0.01 ng/mL to about 40 ng/mL, about 0.01 ng/mL to about 30 ng/mL, about 0.01 ng/mL to about 20 ng/mL, about 0.01 ng/mL to about 10 ng/mL, about 0.01 ng/mL to about 5 ng/mL, about 0.01 ng/mL to about 1 ng/mL, about 0.01 ng/mL to about 0.5 ng/mL, about 0.01 ng/mL to about 0.1 ng/mL, about 0.01 ng/mL to about 0.05 ng/mL, about 0.1 ng/mL to about 10 ng/mL, about 0.5 ng/mL to about 10 ng/mL, about 1 ng/mL to about 10 ng/mL, or about 5 ng/mL to about 50 ng/mL.

**[0098]** In some embodiments, the reporter is detected after any one of more than about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about

10 hr, about 12 hr, about 16 hr, about 20 hr, or about 24 hr after contacting the cells with the composition. In some embodiments, the reporter is detected between any one of about 1 hr and about 24 hr, about 1 hr and about 12 hr, about 1 hr and about 8 hr, about 1 hr and about 6 hr, about 1 hr and about 4 hr, about 1 hr and about 2 hr, about 4 hr and about 24 hr, about 4 hr and about 12 hr, about 4 hr and about 8 hr, about 8 hr and about 24 hr, about 8 hr and about 12 hr, about 16 hr and about 24 hr, about 16 hr and about 20 hr, or about 20 hr and about 24 hr after contacting the cells with the composition.

**[0099]** In some aspects, the invention provides methods for quantitating the amount of anti-CD3 homodimer antibodies in a composition comprising a TDB where the TDB comprises a target antigen binding fragment and CD3 binding fragment, the method comprising contacting population of T cells with the composition at one or more concentrations of the TDB, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a promoter responsive to T cell activation and wherein the population of T cells does not comprise the target antigen, correlating the expression of the reporter as a function of antibody concentration with a standard curve generated by contacting the T cells with different concentrations of purified anti-CD3 homodimers. Dilutions of an anti-CD3 homodimer assay standard (a purified anti-CD3 homodimer of known concentration), an anti-CD3 homodimer control, and TDB test samples are prepared and added to reporter T cells. After a timed incubation, the amount of reporter activity that is induced by the homodimer assay standard, the homodimer control, and the TDB test samples are measured. The quantity of biologically active anti-CD3 homodimer in a TDB test sample is determined from a standard curve generated from the anti-CD3 homodimer assay standard. The percentage of anti-CD3 homodimer present in a test sample is determined by the ratio of the quantity of anti-CD3 homodimer present relative to the total amount of TDB present in the test sample.

**[0100]** In some embodiments, the population of T-cells is contacted with a composition comprising the TDB at a concentration range of any one of about 0.01 ng/mL to about 50 ng/mL, about 0.05 ng/mL to about 50 ng/mL, about 0.1 ng/mL to about 50 ng/mL, about 0.5 ng/mL to about 50 ng/mL, about 1 ng/mL to about 50 ng/mL, about 5 ng/mL to about 50 ng/mL, about 10 ng/mL to about 50 ng/mL, about 0.01 ng/mL to about 40 ng/mL, about 0.01 ng/mL to about 30 ng/mL, about 0.01 ng/mL to about 20 ng/mL, about 0.01 ng/mL to about 10 ng/mL, about 0.01 ng/mL to about 5 ng/mL, about 0.01 ng/mL to about 1 ng/mL, about 0.01 ng/mL to about 0.5 ng/mL, about 0.01 ng/mL to about 0.1 ng/mL, about 0.01 ng/mL to about 0.05 ng/mL, about 0.1 ng/mL to about 10 ng/mL, about 0.5 ng/mL to about 10 ng/mL, about 1 ng/mL to about 10 ng/mL, or about 5 ng/mL to about 50 ng/mL.

**[0101]** In some embodiments, the standard curve from the anti-CD3 homodimer assay standard is generated by contacting reporter T cells with anti-CD3 homodimer at a plurality of concentrations ranging from about any one of 0.01 ng/mL to 50 ng/mL. In some embodiments, the plurality of concentrations of anti-CD3 homodimer standard include any one of 100/mL ng, 150 ng/mL, 200 ng/mL, 250 ng/mL, 500 ng/mL, 750 ng/mL, 1 μg/mL, 2.5 μg/mL, 5 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 250 μg/mL, or 500 μg/mL. In some embodiments, the plurality of concentrations of anti-CD3 homodimer standard is about three, four, five, six, seven, eight, nine, ten or more than ten concentrations.

**[0102]** The accuracy of the method is evaluated by spiking in purified amounts of anti-CD3 homodimer of known quantities into a preparation of TDB and measuring the percent recovery of anti-CD3 homodimer. In some embodiments, one or more mixtures of anti-CD3 homodimer and TDB are generated by adding more than about any one of 100 ng, 150 ng, 200 ng, 250 ng, 500 ng, 750 ng, 1 μg, 2.5 μg, 5 μg, 10 μg, 25 μg, 50 μg, 100 μg, 250 μg, or 500 μg purified anti-CD3 homodimer to an about 1 mg/mL stock of αCD20/αCD3 TDB.

**[0103]** In some embodiments, the reporter is detected after any one of more than about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 12 hr, about 16 hr, about 20 hr, or about 24 hr after contacting the cells with the composition. In some embodiments, the reporter is detected between any one of about 1 hr and about 24 hr, about 1 hr and about 12 hr, about 1 hr and about 8 hr, about 1 hr and about 6 hr, about 1 hr and about 4 hr, about 1 hr and about 2 hr, about 4 hr and about 24 hr, about 4 hr and about 12 hr, about 4 hr and about 8 hr,, about 8 hr and about 24 hr, about 8 hr and about 12 hr, about 16 hr and about 24 hr, about 16 hr and about 20 hr, or about 20 hr and about 24 hr after contacting the cells with the composition.

### *E. Assay Development*

**[0104]** The following is an exemplary but non-limiting method of developing a cell-based assay to detect anti-CD3 homodimers in a preparation of TDB.

**[0105]** *DNA constructs*: Lentivirus is used to generate the stable cell lines used to evaluate the purity of the TDB bi-specific antibody. Lentiviral vectors are constructed that express the reporter gene firefly luciferase, Renilla luciferase, or Nanoluciferase under the control of a minimal TK promoter regulated by DNA recognition elements for NFAT (Nuclear Factor of Activated T cells), AP-1 (Fos/Jun), NFAT/AP1, NFκB, FOXO, STAT3,5, and IRF. The lentiviral expression cassettes used for the generation of the stable reporter cell lines may be third generation self-inactivating bi-cistronic vectors that expressed various antibiotic selection

markers under the control of constitutive promoters/enhancers (EF1alpha or SV40) to enable the generation of stable cell lines. The reporter lentiviral vectors used are modified from the pCDH.MCS.EF1a.Puro are commercially available vector (SBI biosciences; Cat No. CD510B-1). Promoter modifications include the removal of the CMV minimal promoter and substitution with various enhancer elements (NFAT, NFκB, etc.), addition of a minimal core RNA polymerase promoter (TATA box) from pRK5.CMV.Luciferase (Osaka, G *et al.*, 1996 *J Pharm Sci.* 1996, 85:612-618), and substitution of different selection cassettes from internal DNAs (Neomycin resistance gene from pRK5.tk.neo, Hygromycin resistance gene from pRK5.tk.hygro; and the blasticidin resistance gene from pRK5.tk.blastocidin). Impact of the constitutive promoters used for selection on the activation of the enhancer elements is minimal due to the incorporation of a non-coding stretch of DNA designed to minimize promoter/enhancer cross-talk. Firefly Luciferase from pRK5.CMV.Luciferase (Osaka, 1996) is cloned into the HindIII-NotI site of the modified lentiviral parent vector. Other luminescent proteins including Renilla Luciferase and NanoLuciferase may also be subcloned into the HindIII-NotI site. Lentiviral packaging constructs (pCMV.HIVdelta, pCMC.VSV-G, and pCMV.Rev) used to generate viral stocks from transient transfection of 293s (293 suspension adapted cell line) cells may be obtained (pCMV.VSV-G) or generated (pCMV.HIVdelta, pCMV.REV). HIV strain MN (Nakamura, GR *et al.*, 1993, *J. Virol.* 67(10):6179-6191) may be used to generate the pCMV.HIVdelta packaging vector and contains an internal EcoRI partial digest deletion to inactivate by deletion the HIV viral envelope and modifications to the 5' and 3'LTRs for safety purposes. HIV Rev is cloned from pCMV.HIVdelta transfected 293s cell RNA by RT-PCR and introduced into the ClaI-Xho site of pRK5.tk.neo. The use of VSV-G to pseudotype the lentiviral reporters (substituting VSV-G for HIV env) enables the infection of any cell type. Lentiviral expression plasmids and packaging constructs are amplified in Stbl2 competent cells (Life Technologies, Cat. No. 10268-019) and DNA purified using Qiagen Maxi Prep kit (Cat. No. 12662). All DNA constructs are confirmed by DNA sequencing.

**[0106]** Reporter gene assay cell line development: Jurkat CD4+ T cell line (DSMZ, Cat. No. ACC 282) and CTLL-2 $CD8^+$ T cell line (Life Technologies, Cat. No. K1653) are used to evaluate the feasibility of a reporter gene assay to monitor the activation of T cells by the TDB. Lentiviral vectors are constructed that express the reporter gene firefly luciferase, Renilla luciferase, or Nanoluciferase under the control of a minimal TK promoter regulated by DNA recognition elements for NFAT (Nuclear Factor of Activated T cells), AP-1 (Fos/Jun), NFAT/AP1, NFκB, FOXO, STAT3,5, and IRF. Reporter gene viral stocks are generated by transient transfection of 293s cells and pseudotyped with VSV-G, concentrated, and titered

using standard methods (Naldini, L., *et al.*, 1996 *Science*, 272:263-267). The Jurkat CTLL-2 cells are infected with the lentiviral reporter viral stock at an MOI of 10 by spinoculation and after 3 days infected cells are selected for antibiotic resistance. After 2 weeks, stable pools are generated and evaluated for the response to purified TDB. A qPCR method that evaluates copy number and integration is used to demonstrate that all stable pools are stably infected with the reporter constructs. Purified anti-CD3 homodimer is able to activate both the NFAT and NFκB Jurkat reporter pools. Similar responses were observed for the other TDBs. On the bases of these experiments, limiting dilution of Jurkat/NFκB-luciferase and Jurkat/NFAT-Luciferase are set up to enable single cell cloning and generation of single stable reporter cell lines.

**[0107]** Development and evaluation of the T cell activation impurity assay: Anti-CD3 homodimer is a product related impurity that may activate T cells in the absence of target cells and therefore represents a separate activity from the TDB. The anti-CD3 homodimer species present as an impurity in TDB purified preparations may be covalently or non-covalently linked and may therefore adopt a conformation that enables the variant to cross-link and thereby activate TCR on the surface of T cells. As the TDB has only one anti-CD3 arm, the TDB cannot cross-link TCR and has no activity when incubated with T cells alone. In vivo, the TDB may be able to cross-link TCR on T cells, via FcgR mediated cross-linking mediated by effector cells (monocytes, macrophages, NK cells).

**[0108]** To quantitate the amount of aCD3 homodimer variant present, the amount of luciferase activity observed in a TDB sample is calculated from the best fit curve of the luciferase activity observed when a purified aCD3 homodimer standard of known concentration is incubated with the Jurkat/NFκB-fireflyLuciferase clone 2 cell line (Fig. 7). To evaluate matrix effects and impact of concentration of impurity in each sample, serial dilutions are prepared, and the dilutional linearity assessed in the end-point assay. The total amount of homodimer present is determined by the mass of impurity present in the total mass of TDB and expressed as % antiCD3 homodimer. The method is able to quantitatively detect as little as 0.1 micrograms (0.1 ppm) of purified anti-CD3 homodimer in purified TDB preparations. The assay format has also been shown to be able to detect impurity activity in TDB material that has not been purified past the initial purification steps of the current process and has been used to assess the purification approach used for TDB to remove impurity. Spiking in purified anti-CD3 homodimer, the assay format shows accurate recovery to as low as 0.5% homodimer spiked material in TDB test material (Fig. 7). The method has been used in conjunction with other orthogonal assays to demonstrate that the current purification process of TDBs removes homodimer and other T cell activating species to below the limit of quantitation of the assay. However, during process

development, various samples were shown to have T cell activating activity in the assay that did not correlate with the anti-CD3 homodimer mass spec assay. Correlations with other orthogonal analytical methods suggest that these other species may be some form of aggregate, or HMWS (Fig. 9). Aggregates of the TDB would induce TCR clustering and activity. It has been observed that as little as 1.5% HMWS can induce significant activity in the assay, as observed during the evaluation of various formulation studies. Purification and assessment of these other species enable the development of an impurity assay using various impurity reference standards to assess the purity and safety of TDB. The use of different reporter gene cell lines may enable the classification of the different species present. The current reported value of % anti-CD3 homodimer may therefore be modified to another value as a result of these efforts. The sensitivity of a reporter gene assay approach to detect biologically active impurities is a useful general approach for classification of product variants and the assessment of acceptable levels that can be present in a therapeutic.

### *III. Kits*

**[0109]** In some aspects of the invention, a kit or article of manufacture is provided comprising a container which holds a composition comprising engineered T cells comprising a reporter complex responsive to T cell activation as described herein, and optionally provides instructions for its use. In some embodiments, the kit provides an anti-CD3 homodimer assay standard (a purified anti-CD3 homodimer of known concentration), and/or an anti-CD3 homodimer control. The container holds the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, cultureware, reagents for detecting reporter molecules, and package inserts with instructions for use.

### *IV. Polypeptides*

**[0110]** The polypeptides to be analyzed using the methods described herein are generally produced using recombinant techniques. Methods for producing recombinant proteins are described, *e.g.*, in U.S. Pat Nos. 5,534,615 and 4,816,567, specifically incorporated herein by reference. In some embodiments, the protein of interest is produced in a CHO cell (*see, e.g.* WO 94/11026). In some embodiments, the polypeptide of interest is produced in an *E. coli* cell. *See, e.g.*, U.S. Pat. No. 5,648,237; U.S. Pat. No. 5,789,199, and U.S. Pat. No. 5,840,523, which describes translation initiation region (TIR) and signal sequences for optimizing expression and secretion. *See* also Charlton, Methods in Molecular Biology, Vol. 248 (B. K. C. Lo, *ed.*, Humana

Press, Totowa, N.J., 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*. When using recombinant techniques, the polypeptides can be produced intracellularly, in the periplasmic space, or directly secreted into the medium.

**[0111]** The polypeptides may be recovered from culture medium or from host cell lysates. Cells employed in expression of the polypeptides can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter *et al*., *Bio/Technology* 10: 163-167 (1992) describe a procedure for isolating polypeptides which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available polypeptide concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

**[0112]** In some embodiments, the polypeptide in the composition comprising the polypeptide and one or more contaminants has been purified or partially purified prior to analysis by the methods of the invention. For example, the polypeptide of the methods is in an eluent from an affinity chromatography, a cation exchange chromatography, an anion exchange chromatography, a mixed mode chromatography and a hydrophobic interaction chromatography. In some embodiments, the polypeptide is in an eluent from a Protein A chromatography.

**[0113]** Examples of polypeptides that may be analyzed by the methods of the invention include but are not limited to immunoglobulins, immunoadhesins, antibodies, enzymes, hormones, fusion proteins, Fc-containing proteins, immunoconjugates, cytokines and interleukins.

*(A) Antibodies*

**[0114]** In some embodiments of any of the methods described herein, the polypeptide for use in any of the methods of analyzing polypeptides and formulations comprising the polypeptides by the methods described herein is an antibody. In some embodiments, the polypeptide is a T cell-dependent bispecific (TDB) antibody.

**[0115]** Molecular targets for antibodies include CD proteins and their ligands, such as, but not limited to: (i) CD3, CD4, CD8, CD19, CD11a, CD20, CD22, CD34, CD40, CD79α (CD79a), and CD79β (CD79b); (ii) members of the ErbB receptor family such as the EGF receptor,

HER2, HER3 or HER4 receptor; (iii) cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and αv/β3 integrin, including either alpha or beta subunits thereof (*e.g.*, anti-CD11a, anti-CD18 or anti-CD11b antibodies); (iv) growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; *mpl* receptor; CTLA-4; protein C, BR3, c-met, tissue factor, β7 etc; (v) cell surface and transmembrane tumor-associated antigens (TAA), such as those described in U.S. Patent No. 7,521,541, and (vi) other targets such as FcRH5, LyPD1, TenB2. In some embodiments, the antibody is an anti-CD20/anti-CD3 antibody. Exemplary bispecific antibodies are provided in Table 1.

Table 1. Exemplary antibodies

| CD3 Arm | Type | Seq |
|---|---|---|
| Mab1 | HVR-H1 | NYYIH (SEQ ID NO:1) |
| | HVR-H2 | WIYPGDGNTKYNEKFKG (SEQ ID NO:2) |
| | HVR-H3 | DSYSNYYFDY (SEQ ID NO:3) |
| | HVR-L1 | KSSQSLLNSRTRKNYLA (SEQ ID NO:4) |
| | HVR-L2 | WASTRES (SEQ ID NO:5) |
| | HVR-L3 | TQSFILRT (SEQ ID NO:6) |
| | | |
| 38E4v1 | HVR-H1 | SYYIH (SEQ ID NO:7) |
| | HVR-H2 | WIYPENDNTKYNEKFKD (SEQ ID NO:8) |
| | HVR-H3 | DGYSRYYFDY (SEQ ID NO:9) |
| | HVR-L1 | KSSQSLLNSRTRKNYLA (SEQ ID NO:10) |
| | HVR-L2 | WTSTRKS (SEQ ID NO:11) |
| | HVR-L3 | KQSFILRT (SEQ ID NO:12) |
| | | |
| UCHT1v9 | HVR-H1 | GYTMN (SEQ ID NO:13) |
| | HVR-H2 | LINPYKGVSTYNQKFKD (SEQ ID NO:14) |
| | HVR-H3 | SGYYGDSDWYFDV (SEQ ID NO:15) |
| | HVR-L1 | RASQDIRNYLN (SEQ ID NO:16) |
| | HVR-L2 | YTSRLES (SEQ ID NO:17) |
| | HVR-L3 | QQGNTLPWT (SEQ ID NO:18) |
| | | |
| Mab1 | VH (hu) | EVQLVQSGAEVKKPGASVKVSCKASGYTFTNYYIHWVRQAPGQGL EWIGWIYPGDGNTKYNEKFKGRATLTADTSTSTAYLELSSLRSEDTA VYYCARDSYSNYYFDYWGQGTLVTVSS (SEQ ID NO:19) |
| | VL (hu) | DIVMTQSPDSLAVSLGERATINCKSSQSLLNSRTRKNYLAWYQQKP GQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYY CTQSFILRTFGQGTKVEIK (SEQ ID NO:20) |
| | | |
| 38E4v1 | VH (hu) | EVQLVQSGAEVKKPGASVKVSCKASGFTFTSYYIHWVRQAPGQGL EWIGWIYPENDNTKYNEKFKDRVTITADTSTSTAYLELSSLRSEDTA VYYCARDGYSRYYFDYWGQGTLVTVSS (SEQ ID NO:21) |
| | VL (hu) | DIVMTQSPDSLAVSLGERATINCKSSQSLLNSRTRKNYLAWYQQKP GQSPKLLIYWTSTRKSGVPDRFSGSGSGTDFTLTISSLQAEDVAVYY CKQSFILRTFGQGTKVEIK (SEQ ID NO:22) |
| | | |
| UCHT1v9 | VH (hu) | EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQAPGKD LEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDT AVYYCARSGYYGDSDWYFDVWGQGTLVTVSS (SEQ ID NO:23) |
| | VL (hu) | DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKL LIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNT LPWTFGQGTKLELK (SEQ ID NO:24) |
| Target Arm | | |
| Mab2 | HVR-H1 | GYTFTSYNMH (SEQ ID NO:25) |

| | | |
|---|---|---|
| | HVR-H2 | AIYPGNGDTSYNQKFKG (SEQ ID NO:26) |
| | HVR-H3 | VVYYSNSYWYFDV (SEQ ID NO:27) |
| | HVR-L1 | RASSSVSYMH (SEQ ID NO:28) |
| | HVR-L2 | APSNLAS (SEQ ID NO:29) |
| | HVR-L3 | QQWSFNPPT (SEQ ID NO:30) |
| Mab2 | VH | EVQLVESGGGLVQPGGSLRLSCAAS GYTFTSYNMH WVRQA PGKGLEWVG AIYPGNGDTSYNQKFKG RFTISVDKSKNTLYL QMNSLRAEDTAVYYCAR VVYYSNSYWYFDV WGQGTLVTVSS (SEQ ID NO:31) |
| | VL | DIQMTQSPSSLSASVGDRVTITC RASSSVSYMH WYQQKP GKAPKPLIY APSNLAS GVPSRFSGSGSGTDFTLTISSLQP EDFATYYC QQWSFNPPT FGQGTKVEIKR (SEQ ID NO:32) |
| | | |
| 4D5 Her2 | HVR-H1 | DTYIH (SEQ ID NO:33) |
| | HVR-H2 | RIYPTNGYTRYADSVKG (SEQ ID NO:34) |
| | HVR-H3 | WGGDGFYAMDY (SEQ ID NO:35) |
| | HVR-L1 | RASQDVNTAVA (SEQ ID NO:36) |
| | HVR-L2 | SASFLYS (SEQ ID NO:37) |
| | HVR-L3 | QQHYTTPPT (SEQ ID NO:38) |
| 4D5 | VH (hu) | EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQA PGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYL QMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS (SEQ ID NO:39) |
| | VL (hu) | DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKP GKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPE DFATYYCQQHYTTPPTFGQGTKVEIK (SEQ ID NO:40) |

**[0116]** Other exemplary antibodies include those selected from, and without limitation, anti-estrogen receptor antibody, anti-progesterone receptor antibody, anti-p53 antibody, anti-HER-2/neu antibody, anti-EGFR antibody, anti-cathepsin D antibody, anti-Bcl-2 antibody, anti-E-cadherin antibody, anti-CA125 antibody, anti-CA15-3 antibody, anti-CA19-9 antibody, anti-c-erbB-2 antibody, anti-P-glycoprotein antibody, anti-CEA antibody, anti-retinoblastoma protein antibody, anti-ras oncoprotein antibody, anti-Lewis X antibody, anti-Ki-67 antibody, anti-PCNA antibody, anti-CD3 antibody, anti-CD4 antibody, anti-CD5 antibody, anti-CD7 antibody, anti-CD8 antibody, anti-CD9/p24 antibody, anti-CD10 antibody, anti-CD11a antibody, anti-CD11c antibody, anti-CD13 antibody, anti-CD14 antibody, anti-CD15 antibody, anti-CD19 antibody, anti-CD20 antibody, anti-CD22 antibody, anti-CD23 antibody, anti-CD30 antibody, anti-CD31 antibody, anti-CD33 antibody, anti-CD34 antibody, anti-CD35 antibody, anti-CD38 antibody, anti-CD41 antibody, anti-LCA/CD45 antibody, anti-CD45RO antibody, anti-CD45RA antibody, anti-CD39 antibody, anti-CD100 antibody, anti-CD95/Fas antibody, anti-CD99 antibody, anti-CD106 antibody, anti-ubiquitin antibody, anti-CD71 antibody, anti-c-myc antibody, anti-cytokeratins antibody, anti-vimentin antibody, anti-HPV proteins antibody, anti-kappa light chains antibody, anti-lambda light chains antibody, anti-melanosomes antibody, anti-prostate specific antigen antibody, anti-S-100 antibody, anti-tau antigen antibody, anti-fibrin antibody, anti-keratins antibody and anti-Tn-antigen antibody.

*(i) Monoclonal antibodies*

**[0117]** In some embodiments, the antibodies are monoclonal antibodies. Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope except for possible variants that arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete or polyclonal antibodies.

**[0118]** For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

**[0119]** In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as herein described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the polypeptide used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

**[0120]** The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

**[0121]** In some embodiments, the myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, in some embodiments, the myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol*. 133:3001 (1984); Brodeur *et al*., *Monoclonal Antibody Production Techniques and Application*s pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

**[0122]** Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. In some embodiments, the binding

specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

**[0123]** The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.* 107:220 (1980).

**[0124]** After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice* pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

**[0125]** The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, polypeptide A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

**[0126]** DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). In some embodiments, the hybridoma cells serve as a source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin polypeptide, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.* 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

**[0127]** In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature* 348:552-554 (1990). Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.* 21:2265-2266 (1993)). Thus, these

techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

**[0128]** The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison *et al.*, P*roc. Natl Acad. Sci. USA* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

**[0129]** Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

**[0130]** In some embodiments of any of the methods described herein, the antibody is IgA, IgD, IgE, IgG, or IgM. In some embodiments, the antibody is an IgG monoclonal antibody.

*(ii) Humanized antibodies*

**[0131]** In some embodiments, the antibody is a humanized antibody. Methods for humanizing non-human antibodies have been described in the art. In some embodiments, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These 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* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-327 (1988); Verhoeyen *et al.*, *Science* 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

**[0132]** The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence that is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.* 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.* 196:901

(1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chain variable regions. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA* 89:4285 (1992); Presta *et al.*, *J. Immunol.* 151:2623 (1993)).

**[0133]** It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, in some embodiments of the methods, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available that illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

*(iii) Human antibodies*

**[0134]** In some embodiments, the antibody is a human antibody. As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals *(e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region ($J_H$) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. *See*, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA* 90:2551 (1993); Jakobovits *et al., Nature* 362:255-258 (1993); Bruggermann *et al., Year in Immuno.* 7:33 (1993); and US Patent Nos. 5,591,669; 5,589,369; and 5,545,807.

**[0135]** Alternatively, phage display technology (McCafferty *et al.*, *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat polypeptide gene

of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review *see*, *e.g.*, Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al.*, *EMBO J.* 12:725-734 (1993). *See also*, US Patent Nos. 5,565,332 and 5,573,905.

**[0136]** Human antibodies may also be generated by *in vitro* activated B cells (*see* US Patents 5,567,610 and 5,229,275).

*(iv) Antibody fragments*

**[0137]** In some embodiments, the antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (*see*, *e.g.*, Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.*, *Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). *See* WO 93/16185; US Patent No. 5,571,894; and US Patent No. 5,587,458. The antibody fragment may also be a "linear antibody," *e.g.*, as described in US Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

**[0138]** In some embodiments, fragments of the antibodies described herein are provided. In some embodiments, the antibody fragment is an antigen binding fragment. In some

embodiments, the antigen binding fragment is selected from the group consisting of a Fab fragment, a Fab' fragment, a $F(ab')_2$ fragment, a scFv, a Fv, and a diabody.

*(v) Bispecific antibodies*

**[0139]** In some embodiments, the antibody is a bispecific antibody. Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes. Alternatively, a bispecific antibody binding arm may be combined with an arm that binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule *(e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the cell. Bispecific antibodies can be prepared as full length antibodies or antibody fragments *(e.g.* $F(ab')_2$ bispecific antibodies). In some embodiments, the antibody is a T cell-dependent bispecific (TDB) antibody. In some embodiments, the TDB comprises an target antigen binding fragment and a T cell receptor binding fragment. In some embodiments, the TDB comprises an target antigen binding fragment and a CD3 binding fragment. In some embodiments, the TDB comprises a target antigen binding fragment and a CD3e binding fragment.

**[0140]** Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

**[0141]** According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. In some embodiments, the fusion is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. In some embodiments, the first heavy chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum

yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

**[0142]** In some embodiments of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies *see*, for example, Suresh *et al., Methods in Enzymology* 121:210 (1986).

**[0143]** According to another approach described in US Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers that are recovered from recombinant cell culture. In some embodiments, the interface comprises at least a part of the $C_H3$ domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains *(e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones *(e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

**[0144]** Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 0308936). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

**[0145]** Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to

thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

**[0146]** Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy chain variable domain ($V_H$) connected to a light chain variable domain ($V_L$) by a linker that is too short to allow pairing between the two domains on the same chain. Accordingly, the $V_H$ and $V_L$ domains of one fragment are forced to pair with the complementary $V_L$ and $V_H$ domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. *See* Gruber *et al.*, *J. Immunol.* 152:5368 (1994).

**[0147]** Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al.*, *J. Immunol.* 147: 60 (1991).

*(v) Multivalent Antibodies*

**[0148]** In some embodiments, the antibodies are multivalent antibodies. A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies provided herein can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (*e.g.*, tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain(s)

comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)n-VD2-(X2) n-Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain. In some embodiments, the multivalent antibody comprises a T cell binding fragment. In some embodiments, the multivalent antibody comprises a T cell receptor binding fragment. In some embodiments, the multivalent antibody comprises a CD3 binding fragment. In some embodiments, the multivalent antibody comprises a CD3e binding fragment.

**[0149]** In some embodiments, the antibody is a multispecific antibody. Example of multispecific antibodies include, but are not limited to, an antibody comprising a heavy chain variable domain ($V_H$) and a light chain variable domain ($V_L$), where the $V_HV_L$ unit has polyepitopic specificity, antibodies having two or more $V_L$ and $V_H$ domains with each $V_HV_L$ unit binding to a different epitope, antibodies having two or more single variable domains with each single variable domain binding to a different epitope, full length antibodies, antibody fragments such as Fab, Fv, dsFv, scFv, diabodies, bispecific diabodies, triabodies, tri-functional antibodies, antibody fragments that have been linked covalently or non-covalently. In some embodiment that antibody has polyepitopic specificity; for example, the ability to specifically bind to two or more different epitopes on the same or different target(s). In some embodiments, the antibodies are monospecific; for example, an antibody that binds only one epitope. According to one embodiment the multispecific antibody is an IgG antibody that binds to each epitope with an affinity of 5 μM to 0.001 pM, 3 μM to 0.001 pM, 1 μM to 0.001 pM, 0.5 μM to 0.001 pM, or 0.1 μM to 0.001 pM.

*(vi) Other Antibody Modifications*

**[0150]** It may be desirable to modify the antibody provided herein with respect to effector function, *e.g.*, so as to enhance antigen-dependent cell-mediated cyotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing

interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). *See* Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. J., *Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.*, *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement mediated lysis and ADCC capabilities. *See* Stevenson *et al.*, *Anti-Cancer Drug Design* 3:219-230 (1989).

**[0151]** For increasing serum half the serum half life of the antibody, amino acid alterations can be made in the antibody as described in US 2006/0067930, which is hereby incorporated by reference in its entirety.

*(B) Polypeptide Variants and Modifications*

**[0152]** Amino acid sequence modification(s) of the polypeptides, including antibodies, described herein may be used in the methods of purifying polypeptides (*e.g.*, antibodies) described herein.

*(i) Variant Polypeptides*

**[0153]** "Polypeptide variant" means a polypeptide, preferably an active polypeptide, as defined herein having at least about 80% amino acid sequence identity with a full-length native sequence of the polypeptide, a polypeptide sequence lacking the signal peptide, an extracellular domain of a polypeptide, with or without the signal peptide. Such polypeptide variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N or C-terminus of the full-length native amino acid sequence. Ordinarily, a TAT polypeptide variant will have at least about 80% amino acid sequence identity, alternatively at least about any of 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity, to a full-length native sequence polypeptide sequence, a polypeptide sequence lacking the signal peptide, an extracellular domain of a polypeptide, with or without the signal peptide. Optionally, variant polypeptides will have no more than one conservative amino acid substitution as compared to the native polypeptide sequence, alternatively no more than about any of 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitution as compared to the native polypeptide sequence.

**[0154]** The variant polypeptide may be truncated at the N-terminus or C-terminus, or may lack internal residues, for example, when compared with a full length native polypeptide. Certain variant polypeptides may lack amino acid residues that are not essential for a desired biological activity. These variant polypeptides with truncations, deletions, and insertions may be prepared by any of a number of conventional techniques. Desired variant polypeptides may be chemically

synthesized. Another suitable technique involves isolating and amplifying a nucleic acid fragment encoding a desired variant polypeptide, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the nucleic acid fragment are employed at the 5' and 3' primers in the PCR. Preferably, variant polypeptides share at least one biological and/or immunological activity with the native polypeptide disclosed herein.

**[0155]** Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme or a polypeptide which increases the serum half-life of the antibody.

**[0156]** For example, it may be desirable to improve the binding affinity and/or other biological properties of the polypeptide. Amino acid sequence variants of the polypeptide are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the polypeptide. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide (*e.g.*, antibody), such as changing the number or position of glycosylation sites.

**[0157]** Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the polypeptide with that of homologous known polypeptide molecules and minimizing the number of amino acid sequence changes made in regions of high homology.

**[0158]** A useful method for identification of certain residues or regions of the polypeptide (*e.g.*, antibody) that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells, *Science* 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably Alanine or Polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be

predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

**[0159]** Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in the Table 2 below under the heading of "exemplary substitutions." If such substitutions result in a change in biological activity, then more substantial changes, denominated "substitutions" in the Table 2, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 2.

| Original Residue | Substitutions | Exemplary Substitutions |
|---|---|---|
| Ala (A) | Val; Leu; Ile | Val |
| Arg (R) | Lys; Gln; Asn | Lys |
| Asn (N) | Gln; His; Asp, Lys; Arg | Gln |
| Asp (D) | Glu; Asn | Glu |
| Cys (C) | Ser; Ala | Ser |
| Gln (Q) | Asn; Glu | Asn |
| Glu (E) | Asp; Gln | Asp |
| Gly (G) | Ala | Ala |
| His (H) | Asn; Gln; Lys; Arg | Arg |
| Ile (I) | Leu; Val; Met; Ala; Phe; Norleucine | Leu |
| Leu (L) | Norleucine; Ile; Val; Met; Ala; Phe | Ile |
| Lys (K) | Arg; Gln; Asn | Arg |
| Met (M) | Leu; Phe; Ile | Leu |
| Phe (F) | Trp; Leu; Val; Ile; Ala; Tyr | Tyr |
| Pro (P) | Ala | Ala |
| Ser (S) | Thr | Thr |
| Thr (T) | Val; Ser | Ser |
| Trp (W) | Tyr; Phe | Tyr |
| Tyr (Y) | Trp; Phe; Thr; Ser | Phe |
| Val (V) | Ile; Leu; Met; Phe; Ala; Norleucine | Leu |

**[0160]** Substantial modifications in the biological properties of the polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, *Biochemistry* second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

**[0161]** Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

**[0162]** Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

**[0163]** Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the polypeptide to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

**[0164]** A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.*, a humanized antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.*, 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent

fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.*, binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and target. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

**[0165]** Another type of amino acid variant of the polypeptide alters the original glycosylation pattern of the antibody. The polypeptide may comprise non-amino acid moieties. For example, the polypeptide may be glycosylated. Such glycosylation may occur naturally during expression of the polypeptide in the host cell or host organism, or may be a deliberate modification arising from human intervention. By altering is meant deleting one or more carbohydrate moieties found in the polypeptide, and/or adding one or more glycosylation sites that are not present in the polypeptide.

**[0166]** Glycosylation of polypeptide is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

**[0167]** Addition of glycosylation sites to the polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

**[0168]** Removal of carbohydrate moieties present on the polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid

residues that serve as targets for glycosylation. Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases.

**[0169]** Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains, acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

*(ii) Chimeric Polypeptides*

**[0170]** The polypeptide described herein may be modified in a way to form chimeric molecules comprising the polypeptide fused to another, heterologous polypeptide or amino acid sequence. In some embodiments, a chimeric molecule comprises a fusion of the polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl-terminus of the polypeptide. The presence of such epitope-tagged forms of the polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

**[0171]** In an alternative embodiment, the chimeric molecule may comprise a fusion of the polypeptide with an immunoglobulin or a particular region of an immunoglobulin. A bivalent form of the chimeric molecule is referred to as an "immunoadhesin."

**[0172]** As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous polypeptide with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (*i.e.*, is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

**[0173]** The Ig fusions preferably include the substitution of a soluble (transmembrane domain deleted or inactivated) form of a polypeptide in place of at least one variable region within an Ig molecule. In a particularly preferred embodiment, the immunoglobulin fusion includes the hinge, $CH_2$ and $CH_3$, or the hinge, $CH_1$, $CH_2$ and $CH_3$ regions of an IgG1 molecule.

*(iii) Polypeptide Conjugates*

**[0174]** The polypeptide for use in polypeptide formulations may be conjugated to a cytotoxic agent such as a chemotherapeutic agent, a growth inhibitory agent, a toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

**[0175]** Chemotherapeutic agents useful in the generation of such conjugates can be used. In addition, enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated polypeptides. Examples include $^{212}$Bi, $^{131}$I, $^{131}$In, $^{90}$Y, and $^{186}$Re. Conjugates of the polypeptide and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the polypeptide.

**[0176]** Conjugates of a polypeptide and one or more small molecule toxins, such as a calicheamicin, maytansinoids, a trichothene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

**[0177]** Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub *Maytenus serrata*. Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters. Synthetic maytansinol and derivatives and analogues thereof are also contemplated. There are many linking groups known in the art for making polypeptide-maytansinoid conjugates, including, for example, those disclosed in U.S. Pat. No. 5,208,020. The linking groups include disufide groups, thioether groups, acid labile groups, photolabile

groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred.

**[0178]** The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hyrdoxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

**[0179]** Another conjugate of interest comprises a polypeptide conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, *see*, *e.g.*, U.S. Pat. No. 5,712,374. Structural analogues of calicheamicin which may be used include, but are not limited to, $\gamma_1^I$, $\alpha_2^I$, $\alpha_3^I$, N-acetyl-$\gamma_1^I$, PSAG and $\theta_1^I$. Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through polypeptide (*e.g.*, antibody) mediated internalization greatly enhances their cytotoxic effects.

**[0180]** Other antitumor agents that can be conjugated to the polypeptides described herein include BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex, as well as esperamicins.

**[0181]** In some embodiments, the polypeptide may be a conjugate between a polypeptide and a compound with nucleolytic activity (*e.g.*, a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

**[0182]** In yet another embodiment, the polypeptide (*e.g.*, antibody) may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the polypeptide receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radionucleotide).

**[0183]** In some embodiments, the polypeptide may be conjugated to a prodrug-activating enzyme which converts a prodrug (*e.g.*, a peptidyl chemotherapeutic agent) to an active anti-cancer drug. The enzyme component of the immunoconjugate includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

**[0184]** Enzymes that are useful include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs into free active drugs.

*(iv) Other*

**[0185]** Another type of covalent modification of the polypeptide comprises linking the polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sc*iences*, 18th edition, Gennaro, A.R., Ed., (1990).

***V. Obtaining Polypeptides for Use in the Formulations and Methods***

**[0186]** The polypeptides used in the methods of analysis described herein may be obtained using methods well-known in the art, including the recombination methods. The following sections provide guidance regarding these methods.

*(A) Polynucleotides*

**[0187]** "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA.

**[0188]** Polynucleotides encoding polypeptides may be obtained from any source including, but not limited to, a cDNA library prepared from tissue believed to possess the polypeptide mRNA and to express it at a detectable level. Accordingly, polynucleotides encoding polypeptide can be

conveniently obtained from a cDNA library prepared from human tissue. The polypeptide-encoding gene may also be obtained from a genomic library or by known synthetic procedures (*e.g.*, automated nucleic acid synthesis).

**[0189]** For example, the polynucleotide may encode an entire immunoglobulin molecule chain, such as a light chain or a heavy chain. A complete heavy chain includes not only a heavy chain variable region ($V_H$) but also a heavy chain constant region ($C_H$), which typically will comprise three constant domains: $C_H1$, $C_H2$ and $C_H3$; and a "hinge" region. In some situations, the presence of a constant region is desirable. In some embodiments, the polynucleotide encodes one or more immunoglobulin molecule chains of a TDB.

**[0190]** Other polypeptides which may be encoded by the polynucleotide include antigen-binding antibody fragments such as single domain antibodies ("dAbs"), Fv, scFv, Fab' and $F(ab')_2$ and "minibodies." Minibodies are (typically) bivalent antibody fragments from which the $C_H1$ and $C_K$ or $C_L$ domain has been excised. As minibodies are smaller than conventional antibodies they should achieve better tissue penetration in clinical/diagnostic use, but being bivalent they should retain higher binding affinity than monovalent antibody fragments, such as dAbs. Accordingly, unless the context dictates otherwise, the term "antibody" as used herein encompasses not only whole antibody molecules but also antigen-binding antibody fragments of the type discussed above. Preferably each framework region present in the encoded polypeptide will comprise at least one amino acid substitution relative to the corresponding human acceptor framework. Thus, for example, the framework regions may comprise, in total, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen amino acid substitutions relative to the acceptor framework regions.

**[0191]** All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

**[0192]** Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all references in the specification are expressly incorporated herein by reference.

# EXAMPLES

**[0193]** The examples below are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way. The following examples and detailed description are offered by way of illustration and not by way of limitation.

## Example 1. Anti-CD3 homodimers activate T cells

**[0194]** A T Cell Dependent Bispecific (TDB) antibody (αCD20/αCD3 TDB, anti-CD20 (Mab2; VH SEQ ID NO:31/VL SEQ ID NO:32)/anti-CD3 (Mab1; VH SEQ ID NO:19/VL SEQ ID NO:20)) requires CD20 antigen expressing cells in order to induce T cell activation and antigen cell killing. As shown in Fig. 1A, $CD8^+$ T cells were isolated from human peripheral blood, incubated with an antigen expressing target cell line in a 1:1 ratio, and stimulated with increasing concentrations of purified αCD20/αCD3 TDB antibody. After a timed incubation of 24 hours after addition of the TDB to the cells T cells were evaluated for the amount of CD69 (C-type lectin protein) and CD25 (IL-2 receptor) that was induced on the surface of the T cell, which are markers of T cell activation (Shipkova M, 2012, *Clin. Chim. Acta*. 413:1338-49 and Ziegler SF, *et al*., 1994, *Stem Cells* 12(5): 465-465), by flow cytometry. CD69 and CD25 cell surface expression are increased dose-dependently upon stimulation with αCD20/αCD3 TDB. In the absence of target cells (blue rectangles) there is no T cell activation as evidenced by a lack of increase in CD69 and CD25 cell surface expression. As shown in Fig. 1B, T cells are required to mediate target cell killing by αCD20/αCD3 TDB. PBMCs, or PBMCs that were depleted of CD3+ (T Cell Receptor/CD3e subunit) by negative selection (Milteny Biotec), were incubated with a CD20 expressing target cell line at a 1:1 ratio, and then stimulated with increasing concentrations of αCD20/αCD3 TDB. PBMCs showed a dose-dependent decrease in the number of target cells by flow cytometry after 24 hours (red circles). However, no loss of target cells was detected when CD3+ T cells were depleted from the PBMC pool (Blue rectangles). CD20-expressing target cell depletion by αCD20/αCD3 TDB therefore requires activation of CD3+ T cells, and αCD20/αCD3 TDB is not capable of inducing target cell killing alone.

**[0195]** Purified anti-CD3 homodimer activates human donor T cells. Human donor PBMCs from two different donors were treated with increasing concentrations of purified anti-CD3 homodimer or αCD20/αCD3 TDB bi-specific antibody and tested for the level of T cell activation by FACS after 24 hours as described above. Donor 1 (Fig. 2, left hand panel), and donor 2 (Fig. 2, right hand panel) were stained with anti-CD8 antibody, anti-CD69, and anti-CD25 antibodies. The percentage of $CD8^+$ T cells positive for the T cell activation markers

CD69 and CD25 was plotted against the amount of anti-CD3 homodimer or αCD20/αCD3 TDB treatment. Anti-CD3 and αCD20/αCD3 TDB dose-dependently activates T cells in the presence of target cells, but αCD20/αCD3 TDB (EC50: 4-6 ng/mL) is a stronger activator of T cells than anti-CD3 homodimer (EC50: 169-526 ng/mL). In spite of donor variability, anti-CD3 homodimer can activate human T cells.

**[0196]** Anti-CD3 homodimer can decrease bispecific antibody potency. αCD20/αCD3 TDB was spiked with varying concentrations of purified anti-CD3 homodimer responses were measured. Anti-CD3 homodimer dose-dependently significantly decreases αCD20/αCD3 TDB potency, both at the level of T cell activation and at the level of the target cell response at levels of anti-CD3 homodimer above 20% (Fig. 3A and Table 3). Low levels of anti-CD3 homodimer (HD) spiked into αCD20/αCD3 TDB do not significantly reduce T cell activation ($CD8^+$, Fig. 3B left hand panel; $CD4^+$, Fig. 3B right hand panel) using PBMCs. PBMCs were stimulated with increasing levels of TDB that had been fixed with a constant amount (2.5% or 5%) of purified anti-CD3 homodimer and analyzed by flow cytometry (FACS) to evaluate T cell activation (staining for T cell activation markers CD69 and CD25). Anti-CD3 homodimer at levels below 5% do not impact αCD20/αCD3 TDB T cell activation potential of either $CD8^+$ or $CD4^+$ T cells.

Table 3

| Sample | T cell response (% relative potency) | Target cell response (% relative potency) |
|---|---|---|
| αCD20/αCD3 TDB + 0 homodimer | 100 | 100 |
| αCD20/αCD3 TDB + 40% homodimer | 62 | 49 |
| αCD20/αCD3 TDB + 30% homodimer | 71 | 56 |
| αCD20/αCD3 TDB + 20% homodimer | 82 | 69 |
| αCD20/αCD3 TDB + 10% homodimer | 93 | 83 |
| αCD20/αCD3 TDB + 5% homodimer | 102 | 98 |
| αCD20/αCD3 TDB + 2.5% homodimer | 99 | 95 |
| αCD20/αCD3 TDB + 1% homodimer | 104 | 101 |

**[0197]** Anti-CD3 homodimer can weakly activate human $CD8^+$ T cells from various human donors in the absence of target cells. In the presence of target cells αCD20/αCD3 TDB is able to strongly activate the majority of $CD8^+$ T cells from PBMCs isolated from 6 human donors, and low levels of anti-CD3 homodimer (2.5% or 5%) do not significantly activate mean T cell activation potential of the TDB (Fig. 4A; B+ condition). In the absence of target cells (Fig. 4A; B- condition), anti-CD3 homodimer can activate $CD8^+$ T cells weakly (slight mean activation

potential increase). Anti-CD3 homodimer activation of human T cells shows a dose dependent trend for the increase of some representative cytokines. PBMCs isolated from 6 human donors were stimulated with 1 mg/mL αCD20/αCD3 TDB in the presence (B+ condition) or absence (B- condition) of target cells, spiked with or without either 2.5% or 5% purified anti-CD3 homodimer, and evaluated for T cell activation potential by testing for secreted cytokines as an indication of T cell activation. After 24 hours, conditioned media was collected and tested for the presence of cytokines using a Luminex cytokine detection kit. Anti-CD3 homodimer treatment in the absence of target cells showed a significant dose-dependent increase in some cytokine levels (IL-10 and MCP-1) from some donor PBMCs (Figs. 4B-4E; B- condition). The mean average cytokine level response has been plotted.

**Example 2. Anti-CD3 Homodimer impurity assay**

**[0198]** A biological impurity assay has been developed to detect the presence of T cell activating impurities in the presence of a T Cell Dependent Bispecific (TDB) antibody. As anti-CD3 homodimer is bivalent, each arm of the impurity can potentially cross-link TCR leading to T cell activation. TCR mediated cross-linking by anti-CD3 bivalent antibodies, such as OKT3, activates T cell signal transduction cascades leading to the phosphorylation and nuclear localization of transcription factors, including NFAT and NFκB, resulting in the transcriptional induction of target genes such as cytokines or cell killing agents such as Fas, Granzyme B and Perforins (Brown, WM, 2006, *Curr Opin Investig Drugs* 7:381-388; Ferran, C *et al.*, 1993 *Exp Nephrol* 1:83-89; Shannon, MF *et al.*, 1995, *J. Leukoc. Biol.* 57:767-773; Shapiro, 1998; Pardo, J, *et al.*, 2003, *Int Immunol.,* 15(12):1441-1450). Reporter genes, such as firefly luciferase, under the transcriptional control of AP1, NFAT, or NFκB, have been used to monitor TCR activation of signaling pathways and T cell activation (Shannon, MF *et al.*, 1995, *J. Leukoc. Biol.* 57:767-773; Shapiro, 1998). As a TDB does not activate T cells in the absence of target cells (Figs. 1A and 1B), a reporter gene assay approach was evaluated as a potential assay strategy to detect anti-CD3 homodimer in the presence of the TDB. To initially evaluate if anti-CD3 homodimers can activate T cells in vitro, Jurkat T cells (DSMZ, ACC 282) were infected with recombinant TCR-responsive reporter gene lentiviral stocks (AP1-Luciferase, NFAT-Luciferase, or NFκB-Luciferase) and stable pools treated with purified anti-CD3 homodimer at 10 μg/mL for 4 hours. Jurkat/AP1Luciferase, Jurkat/NFATLuciferase, and Jurkat/NFκBLuciferase stable pools show a dose-dependent induction of luciferase upon stimulation with purified anti-CD3 homodimer. Luminescence responses (luciferase reporter gene activity) was plotted, with the highest response observed from the Jurkat/NFκBluciferase

stable pool. (Fig. 5A). Jurkat/NFκBLuciferase stable clones isolated by limiting dilution were screened for their response to 10 μg/mL of purified anti-CD3 homodimer. Jurkat T cell NFκBLuciferase pools demonstrated the highest response to anti-CD3 homodimer compared to other TCR-response elements, but the other response elements could also be potentially useful for detecting anti-CD3 homodimer. (Fig. 5B).

**[0199]** To determine the relative response of this clone to either αCD20/αCD3 TDB or to anti-CD3 homodimer, the Jurkat/NFκBLuciferase clone 2 cell line was treated with increasing concentrations of either αCD20/αCD3 TDB or anti-CD3 homodimer in the presence of a CD20 expressing target cell line, and luciferase activity plotted (Fig. 6A). The cells were stimulated with αCD20/αCD3 TDB or a CD3 homodimer for 4 hours in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum. Purified is 1000-fold more active than purified anti-CD3 homodimer, in the presence of co-stimulatory target cells. The level of T cell activation by anti-CD3 homodimer is lower than that of αCD20/αCD3 TDB, but is detectable in the presence of target cells. In the absence of target cells, αCD20/αCD3 TDB does not result in T cell activation at even high levels of the TDB, as measured by NFκB-dependent activation of luciferase transcription in this cell line, but the anti-CD3 homodimer is able to induce luciferase induction even at low levels of the product related impurity (Fig. 6B). These T cell activation responses observed for the engineered Jurkat/NFκBLuciferase clone 2 reporter gene cell line is comparable to that observed using human T cells isolated from donor Peripheral Blood Mononuclear Cells (PBMCs) using other measures of T cell activation, indicating that the use of a reporter gene to monitor T cell activation response is comparable (Table 4). The Jurkat/NFκBluciferase clone 2 cell line (Jurkat-NFκBLuc), was used to develop and optimize a cell-based assay method for the detection of anti-CD3 homodimer impurity in αCD20/αCD3 TDB. Collectively, these data demonstrate that an engineered T cell reporter gene cell line can be used to detect biologically active anti-CD3 homodimer product related impurity in a TDB.

Table 4

| | Anti-CD3 homodimer ($EC_{50}$) in absence of target cells | αCD20/αCD3 TDB ($EC_{50}$) in presence of target cells |
|---|---|---|
| Human PBMC ($CD69^+/CD25^+$) Donor 1 | 526 ng/mL | 5.5 ng/mL |
| Human PBMC ($CD69^+/CD25^+$) Donor 2 | 169 ng/mL | 4.4 ng/mL |
| Jurkat/NFκBLuc | 210 ng/mL | 1.3 ng/mL |

**Example 3. Quantiative method to detect anti-CD3 homodimers**

**[0200]** A sensitive and quantitative analytical method to detect biologically active impurities present in the presence of αCD20/αCD3 TDB has been developed. The αCD20/αCD3 TDB T Cell Activation Assay detects anti-CD3 homodimer present in TDB test samples by measuring CD3e/TCR cross-linking-induced activation of the Rel/NFκB signaling pathway using an engineered T cell reporter gene cell line, Jurkat-NFκBLuc. Since there are no target cells present in the assay, only anti-CD3 homodimer can activate the T cell reporter cell line. Activated NFκB translocates to the nucleus, binds to the 8 NFκB response elements in the synthetic promoter which drive the transcription of Luciferase. In the assay, dilutions of anti-CD3 homodimer Assay Standard, anti-CD3 homodimer Control, and αCD20/αCD3 TDB test samples were prepared and added to cultured Jurkat-NFκBLuc reporter gene cells in a 96 well assay plate. The aCD3 Homodimer Standard is a purified lot of aCD3 homodimer isolated from the αCD20/αCD3 TDB purification process. The aCD3 homodimer control is αCD20/αCD3 TDB spiked with purified aCD3 homodimer and is used as a system suitability criteria in the assay. The use of the aCD3 homodimer control in the assay is specific to the method used for the impurity assay run. After a timed incubation of four hours, the amount of luciferase activity that had been induced by the Homodimer Assay Standard, Homodimer Control, and αCD20/αCD3 TDB test samples was measured using a luminescence plate reader. The quantity of biologically active anti-CD3 homodimer in a αCD20/αCD3 TDB test sample was determined from a standard curve of luminescence generated from the anti-CD3 Homodimer Assay Standard in a separate set of plate wells (Fig. 7). The percentage of anti-CD3 homodimer present in a test sample was determined by the ratio of the quantity of anti-CD3 homodimer present relative to the total amount of αCD20/αCD3 TDB present in the test sample. The accuracy of the method was evaluated by spiking in known quantities of purified anti-CD3 homodimer into a preparation of αCD20/αCD3 TDB and measuring the percent recovery of anti-CD3 homodimer. The method shows good overall linearity (Fig. 8) and has an overall precision of 6.8% (Table 5). In a 1 mg/mL stock of αCD20/αCD3 TDB, the method was able to reproducibly detect spiked levels of anti-CD3 homodimer to as low as 150 nanograms, or 0.02%. Based on the recovery studies performed, the optimized method was able to reliably quantitate anti-CD3 homodimer levels from 0.25% to 35% anti-CD3 homodimer present in various preparations of TDB and had an overall precision of 6.8%. Precision was determined as the average %CV of recovery at each spike level.

Table 5

| Level (%) | 0.25 | 0.5 | 0.75 | 1.0 | 2.5 | 5.0 | 10.0 | 20.0 | 30.0 | 35.0 |
|---|---|---|---|---|---|---|---|---|---|---|
| % recovery | 104 | 103 | 105 | 107 | 97 | 109 | 110 | 114 | 97 | 102 |
| % CV | 3.14 | 5.37 | 6.33 | 12.33 | 3.32 | 12.87 | 4.61 | 7.76 | 5.79 | 6.53 |

**[0201]** The αCD20/αCD3 TDB T cell activation assay is also sensitive to the presence of another product related impurity, anti-CD3 aggregates and αCD20/αCD3 TDB high molecular weight species. Samples that contain above 2% HWMS as detected using SEC, could result in T cell activation of the Jurkat/NFκB-Luc cell line. This activation by HMWS was quantified as % homodimer from the T-cell activation assay (Fig. 9).

CLAIMS

WHAT IS CLAIMED IS:

1. A method for detecting anti-CD3 homodimers in a composition comprising a T cell dependent bispecific antibody (TDB) wherein the bispecific antibody comprises a target antigen binding fragment and a CD3 binding fragment, the method comprising

contacting a population of T cells with the composition, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a response element that is responsive to T cell activation, and wherein the population of T cells does not comprise the target antigen,

wherein expression of the reporter indicates the presence of anti-CD3 homodimers.

2. The method of claim 1, wherein the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, a beta lactamase, or a beta galactosidase.

3. The method of claim 2, wherein the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase.

4. The method of any one of claims 1-3, wherein the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter.

5. The method of claim 4, wherein the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

6. The method of any one of claims 1-5, wherein the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells.

7. The method of any one of claims claim 1-5, wherein the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

8. The method of any one of claims 1-7, wherein population of T-cells is contacted with a composition comprising the bispecific antibody at a concentration ranging from 0.01 ng/mL to 50 ng/mL.

9. The method of any one of claims 1-8, wherein the reporter is detected after any one or more of 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 or 24 hours after contacting the cells with the composition.

10. Method for quantitating the amount of anti-CD3 homodimer antibodies in a composition comprising a TDB where the TDB comprises a target antigen binding fragment and CD3 binding fragment, the method comprising

contacting population of T cells with the composition at one or more concentrations of the TDB, wherein the T cells comprise nucleic acid encoding a reporter operably linked to a promoter responsive to T cell activation and wherein the population of T cells does not comprise the target antigen,

correlating the expression of the reporter as a function of antibody concentration with a standard curve generated by contacting the T cells with different concentrations of purified anti-CD3 homodimers.

11. The method of claim 10, wherein the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, beta lactamase, or a beta galactosidase.

12. The method of claim 11, wherein the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase.

13. The method of any one of claims 10-12, wherein the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter.

14. The method of claim 13, wherein the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

15. The method of any one of claims 10-14, wherein the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells.

16. The method of any one of claims claim 10-14, wherein the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

17. The method of any one of claims 10-16, wherein population of T-cells is contacted with a composition comprising the bispecific antibody at a concentration ranging from about 0.01 ng/mL to about 50 ng/mL.

18. The method of any one of claims 10-17, wherein the standard curve is generated by contacting the T cells with different concentrations of purified anti-CD3 antibody ranging from about 0.01 ng/mL to about 50 ng/mL.

19. The method of any one of claims 10-18, wherein the reporter is detected after any one or more of 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 or 24 hours after contacting the cells with the composition.

20. An engineered T cell for the detection of anti-CD3 homodimers in a composition comprising a bispecific antibody where the bispecific antibody comprises a target antigen binding fragment and CD3 binding fragment, wherein the T cell comprises a reporter operably linked to a response element that is responsive to T cell activation.

21. The engineered T cell of claim 20, wherein the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, beta lactamase, or a beta galactosidase.

22. The engineered T cell of claim 21, wherein the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase.

23. The engineered T cell of any one of claims 20-22, wherein the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter.

24. The engineered T cell of claim 23, wherein the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

25. The engineered T cell of any one of claims 20-24, wherein the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells.

26. The engineered T cell of any one of claims claim 20-24, wherein the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

27. A kit for the detection of anti-CD3 homodimers in a composition comprising a bispecific antibody where the bispecific antibody comprises a target antigen binding fragment and CD3 binding fragment, wherein the kit comprises an engineered T cell comprising a reporter operably linked to a response element that is responsive to T cell activation.

28. The kit of claim 27, further comprising an anti-CD3 homodimer assay standard and/or an anti-CD3 homodimer control.

29. The kit of claim 27 or 28, wherein the reporter is a luciferase, a fluorescent protein, an alkaline phosphatase, a beta lactamase, or a beta galactosidase.

30. The kit of claim 29, wherein the luciferase is a firefly luciferase, a Renilla luciferase, or a nanoluciferase.

31. The kit of any one of claims 27-30, wherein the response element that is responsive to T cell activation is an NFAT promoter, an AP-1 promoter, an NFκB promoter, a FOXO promoter, a STAT3 promoter, a STAT5 promoter or an IRF promoter.

32. The kit of claim 31, wherein the response element that is responsive to T cell activation comprises T cell activation responsive elements from any one or more of NFAT, AP-1, NFκB, FOXO, STAT3, STAT5 and IRF.

33. The kit of any one of claims 27-32, wherein the population of T cells is population of $CD4^+$ T cells or $CD8^+$ T cells.

34. The kit of any one of claims claim 27-32, wherein the population of T cells is population of Jurkat T cells or CTLL-2 T cells.

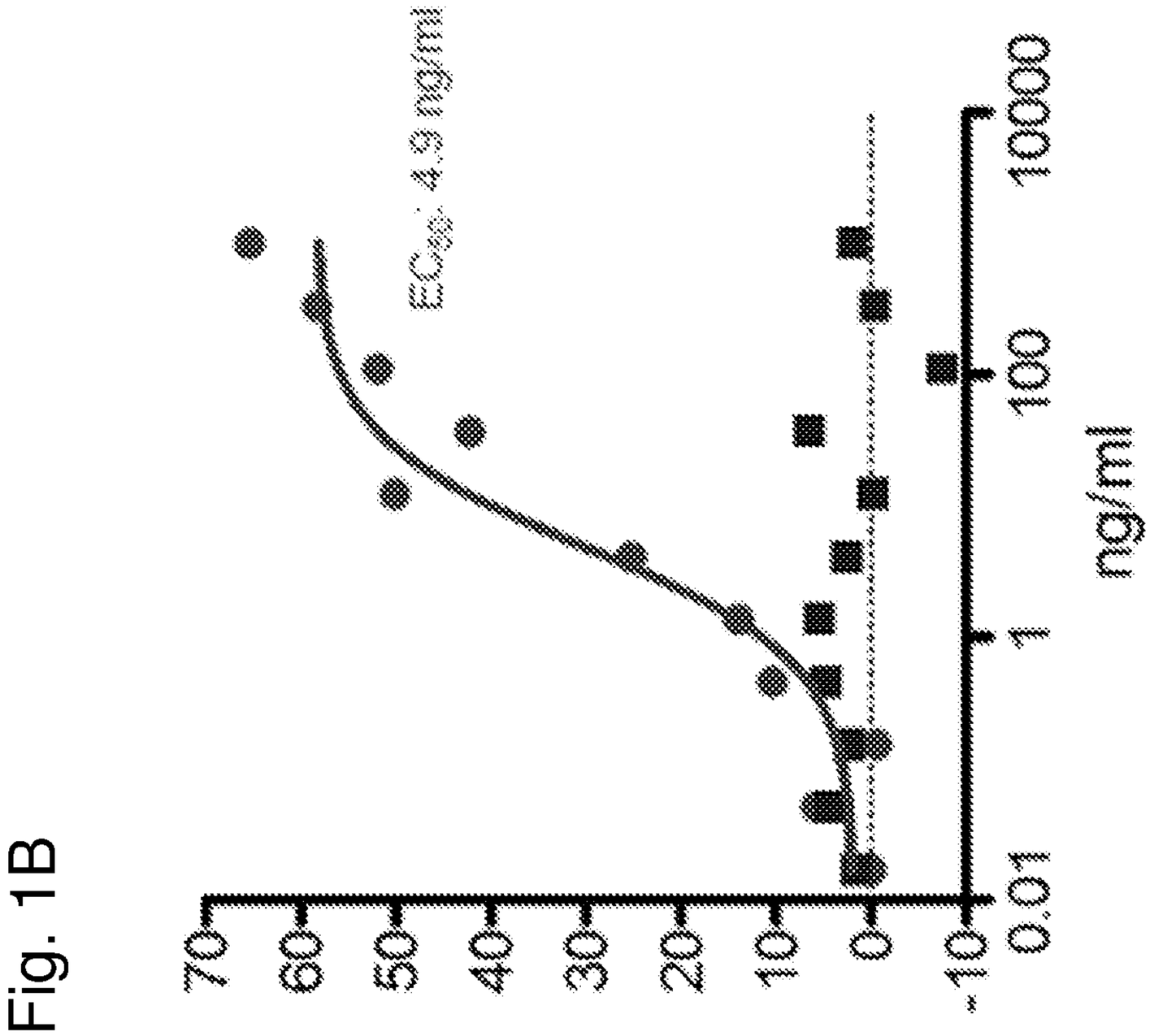

EC50: 4.9 ng/ml
% of target cells killing
70
60
50
40
30
20
10
0
-10
0.01
1
100
10000
ng/ml

Fig. 1B

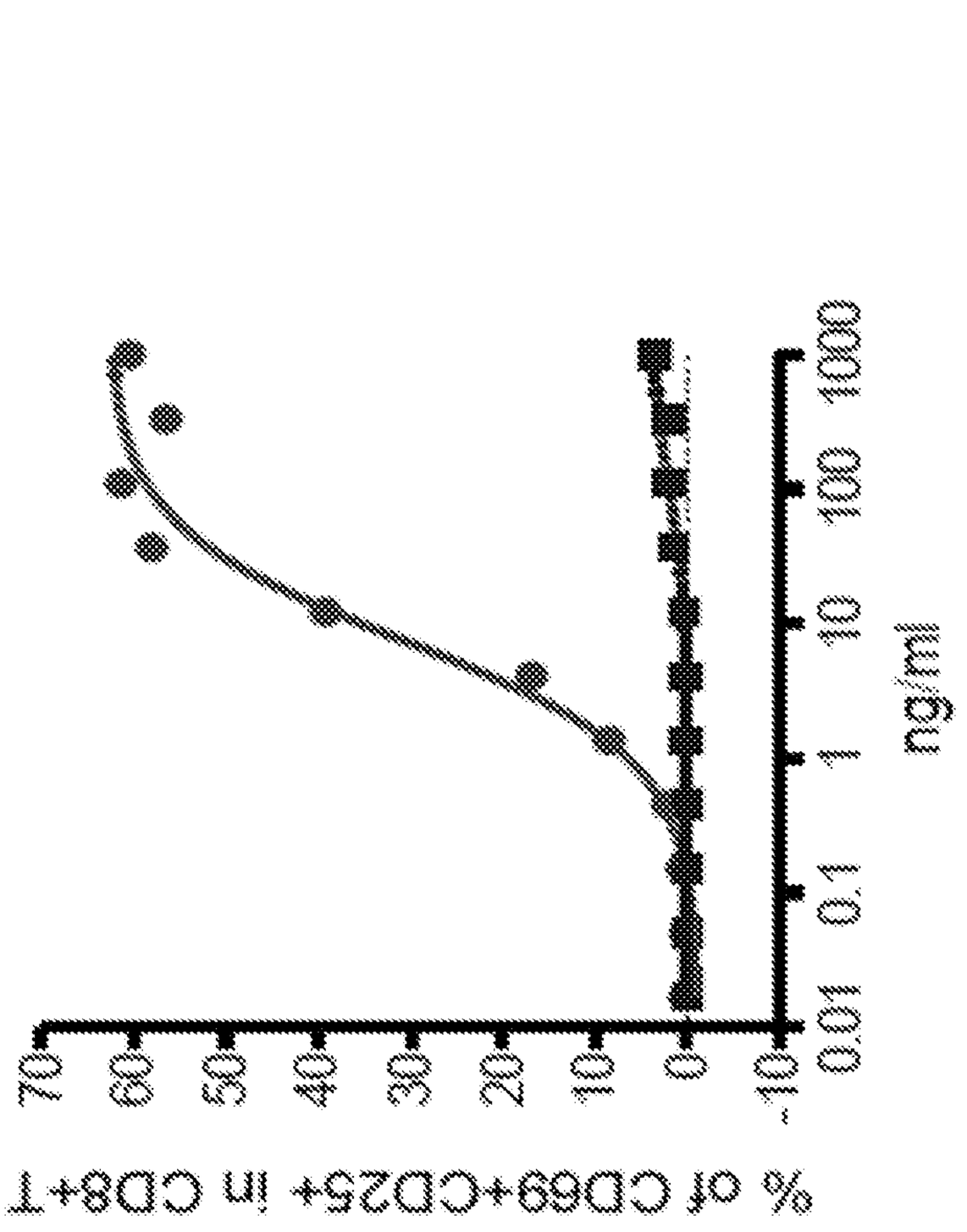

% of CD69+CD25+ in CD8+T
70
60
50
40
30
20
10
0
-10
0.01
0.1
1
100
1000
ng/ml

Fig. 1A

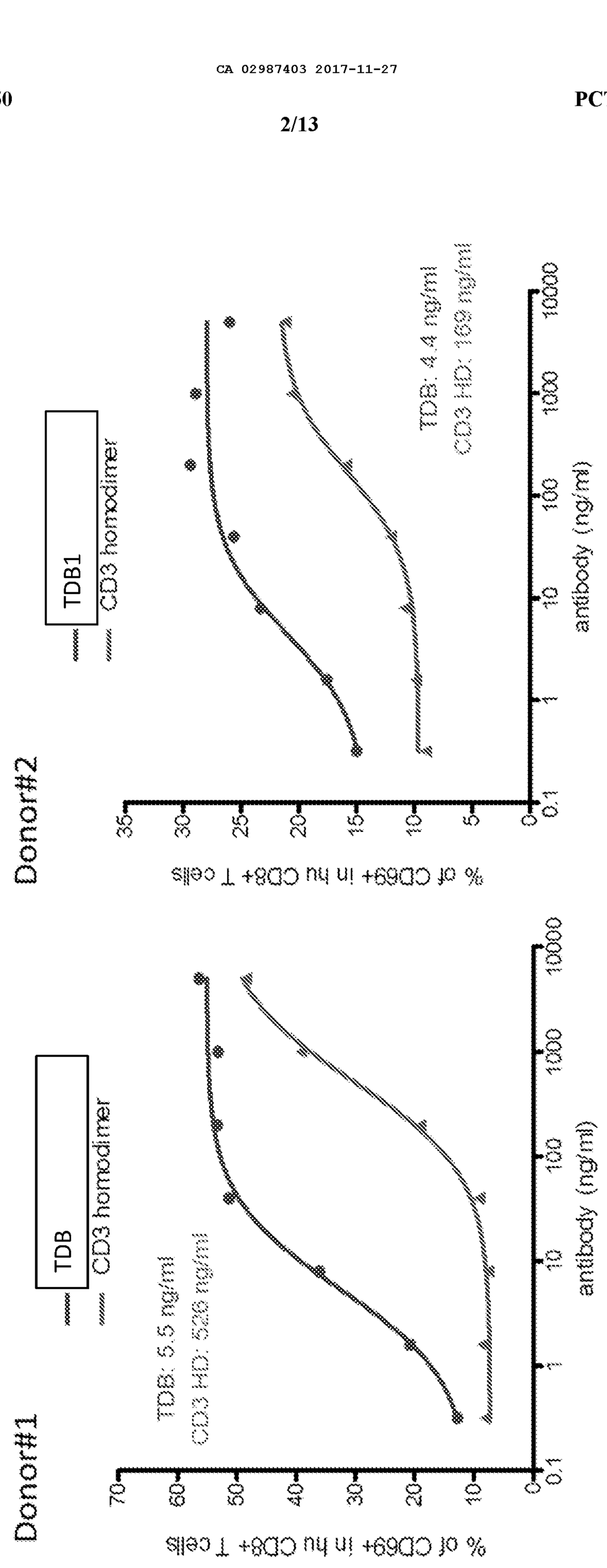

Donor#2
TDB1
CD3 homodimer
TDB1: 4.4 ng/ml
CD3 HD: 169 ng/ml
% of CD69+ in hu CD8+ T cells
antibody (ng/ml)
0.1
1
10
100
1000
10000
0
5
10
15
20
25
30
35
Donor#1
TDB
CD3 homodimer
TDB: 5.5 ng/ml
CD3 HD: 529 ng/ml
% of CD69+ in hu CD8+ T cells
antibody (ng/ml)
0.1
1
10
100
1000
10000
0
10
20
30
40
50
60
70

Fig. 2

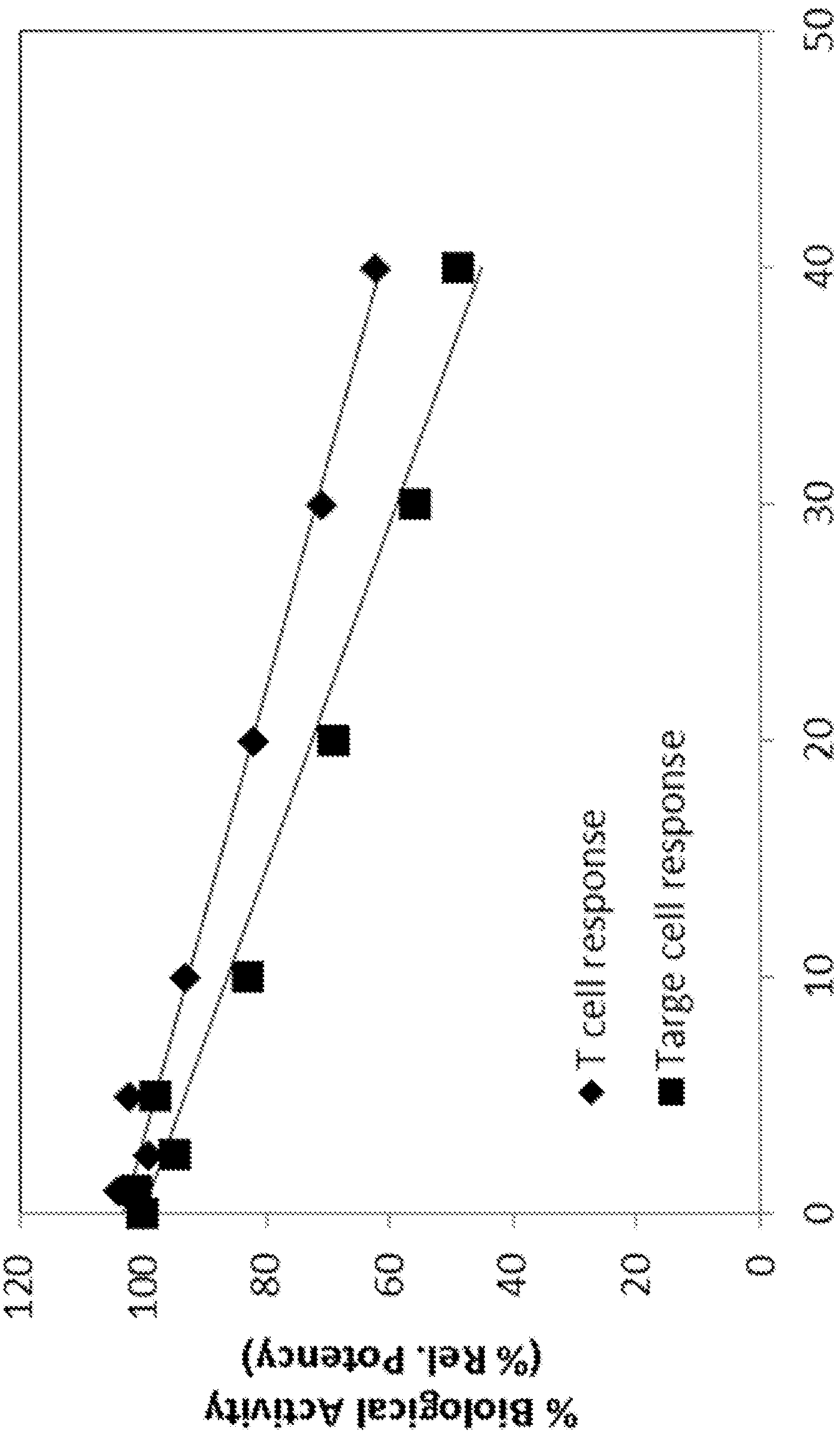

% Biological Activity
(% Rel. Potency)
120
100
80
60
40
20
0
% aCD3 Homodimer Spike
0
10
20
30
40
50
T cell response
Targe cell response

Fig. 3A

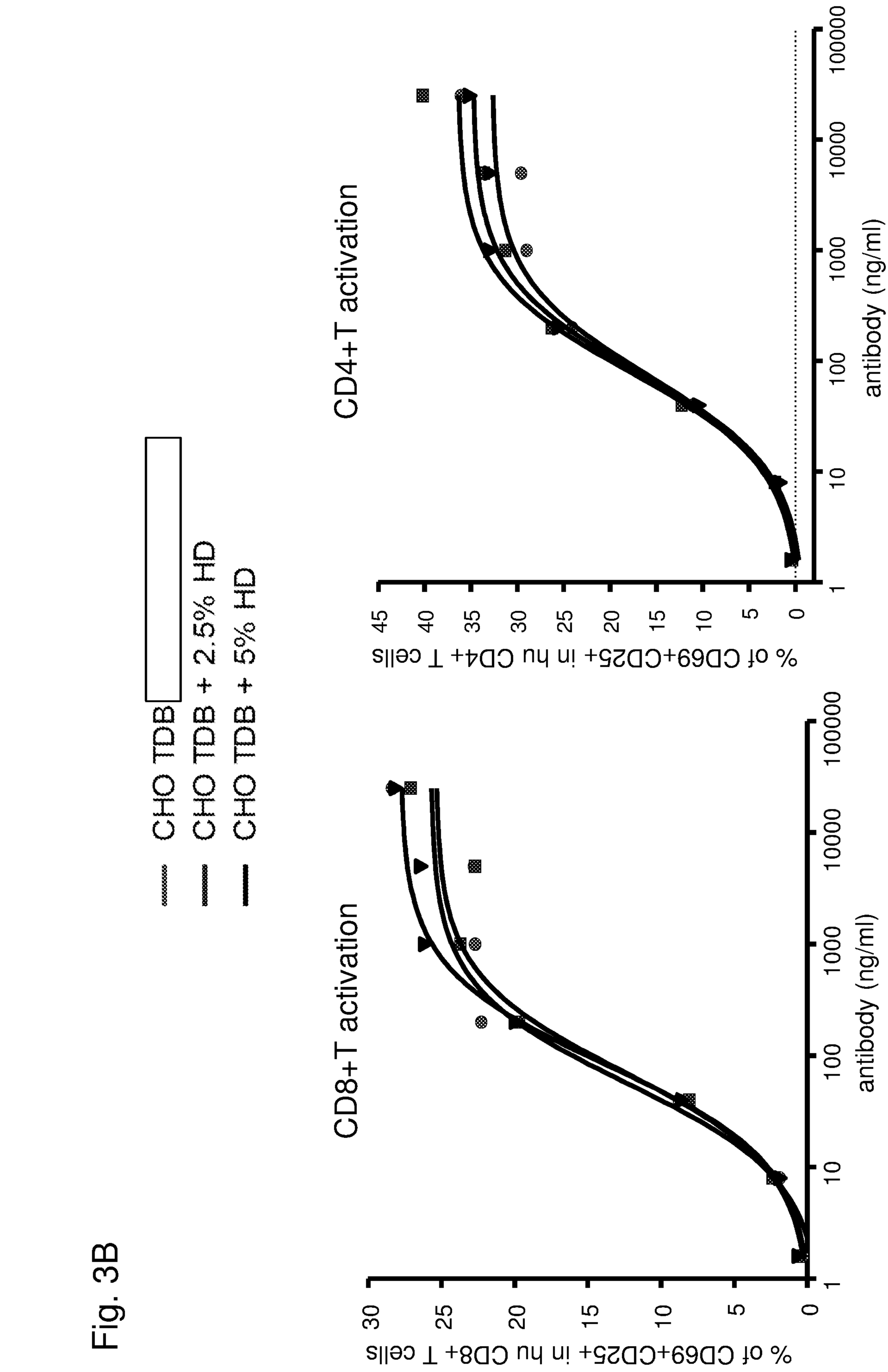

CHO TDB
+ 2.5% HD
+ 5% HD
CD4+T activation
% of CD69+CD25+ in hu CD4+ T cells
0
5
10
15
20
25
30
35
40
45
1
10
100
1000
10000
100000
antibody (ng/ml)
CD8+T activation
% of CD69+CD25+ in hu CD8+ T cells
0
5
10
15
20
25
30
1
10
100
1000
10000
antibody (ng/ml)

Fig. 3B

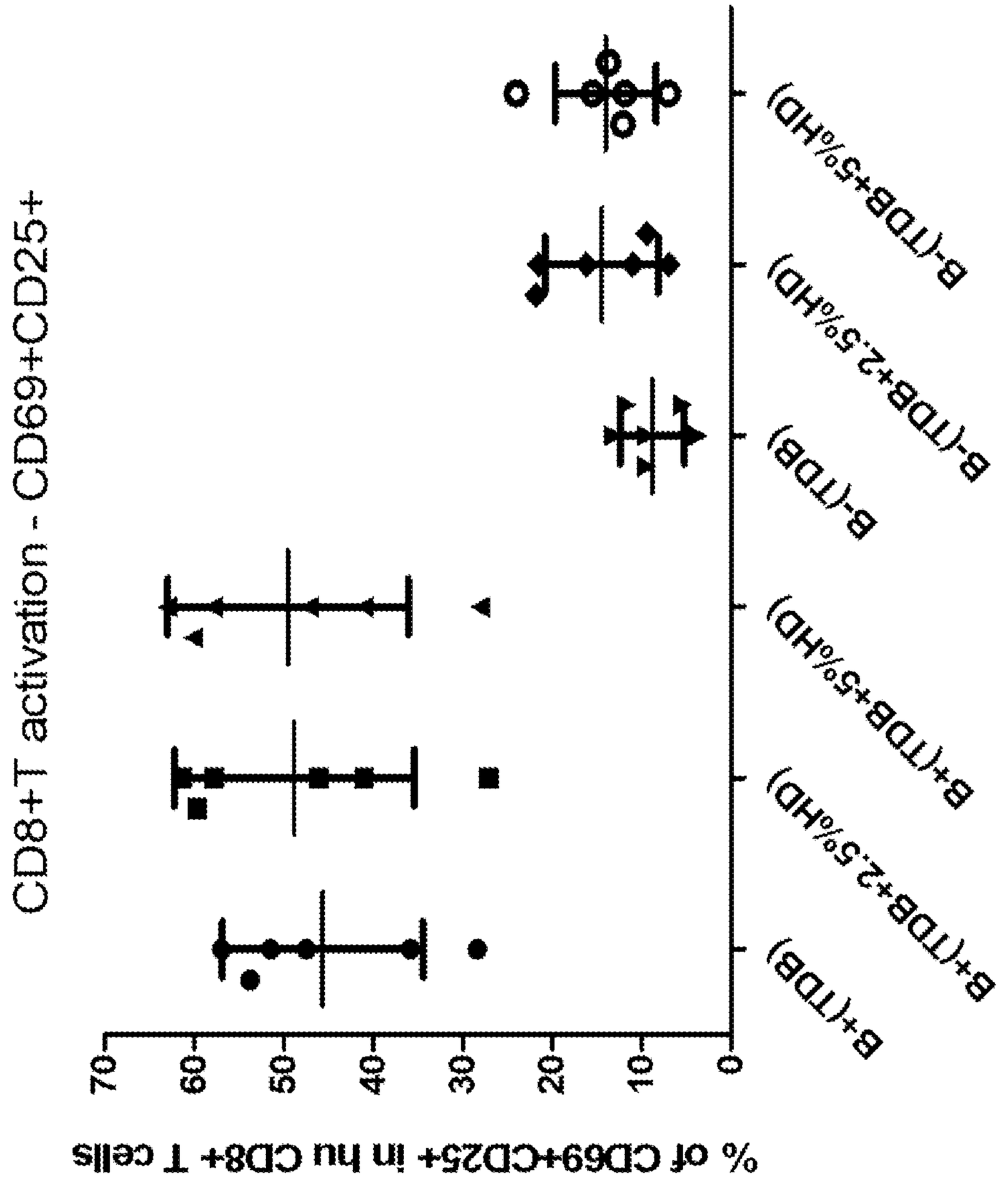

CD8+T activation - CD69+CD25+
% of CD69+CD25+ in hu CD8+ T cells
70
60
50
40
30
20
10
0
B+(TDB)
B+(TDB+2.5%HD)
B+(TDB+5%HD)
B-(TDB)
B-(TDB+2.5%HD)
B-(TDB+5%HD)

Fig. 4A

Fig. 4B

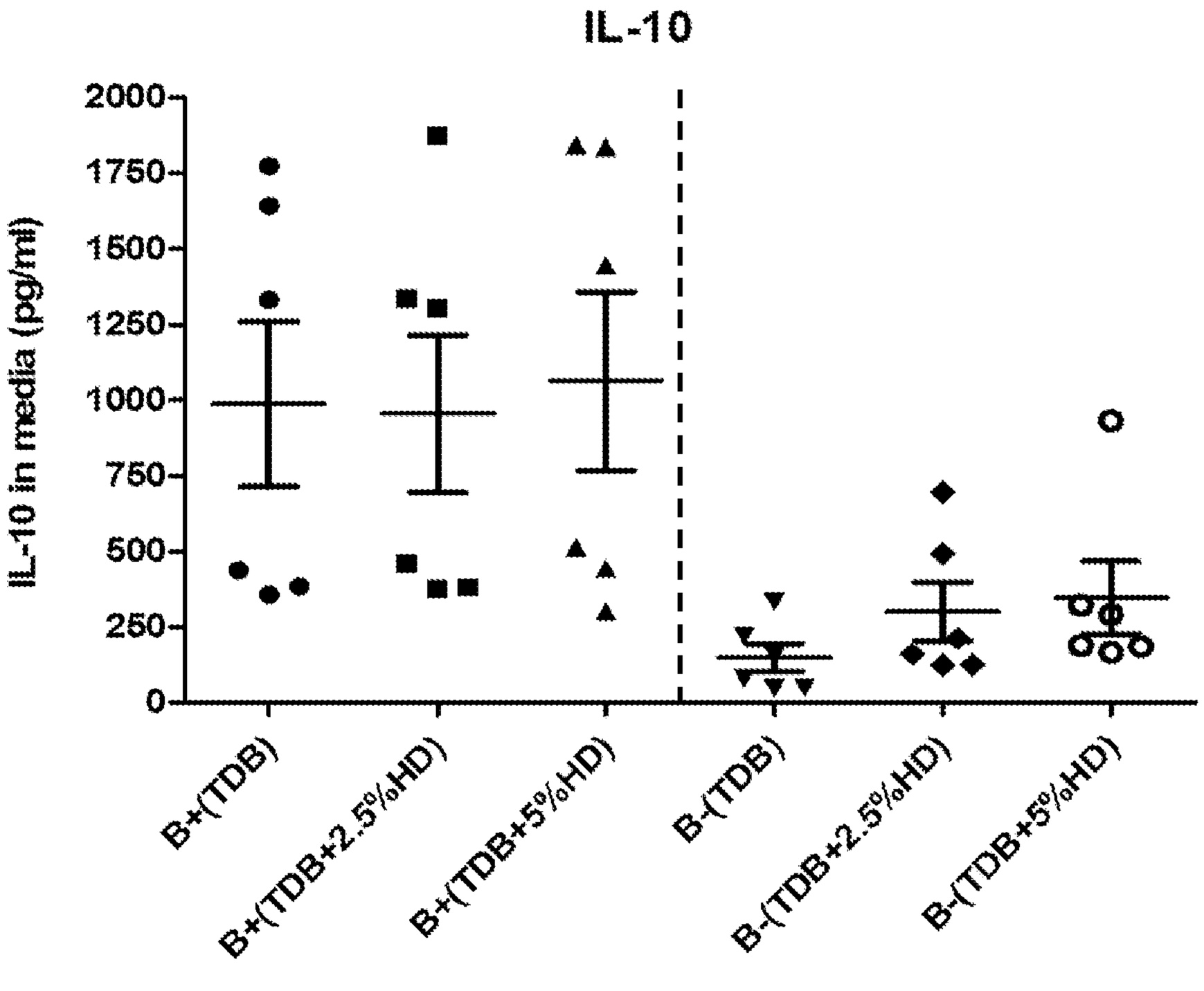

IL-10
IL-10 in media (pg/ml)
2000
1750
1500
1250
1000
750
500
250
0
B+(TDB)
B+(TDB+2.5%HD)
B+(TDB+5%HD)
B-(TDB)
B-(TDB+2.5%HD)
B-(TDB+5%HD)

Fig. 4C

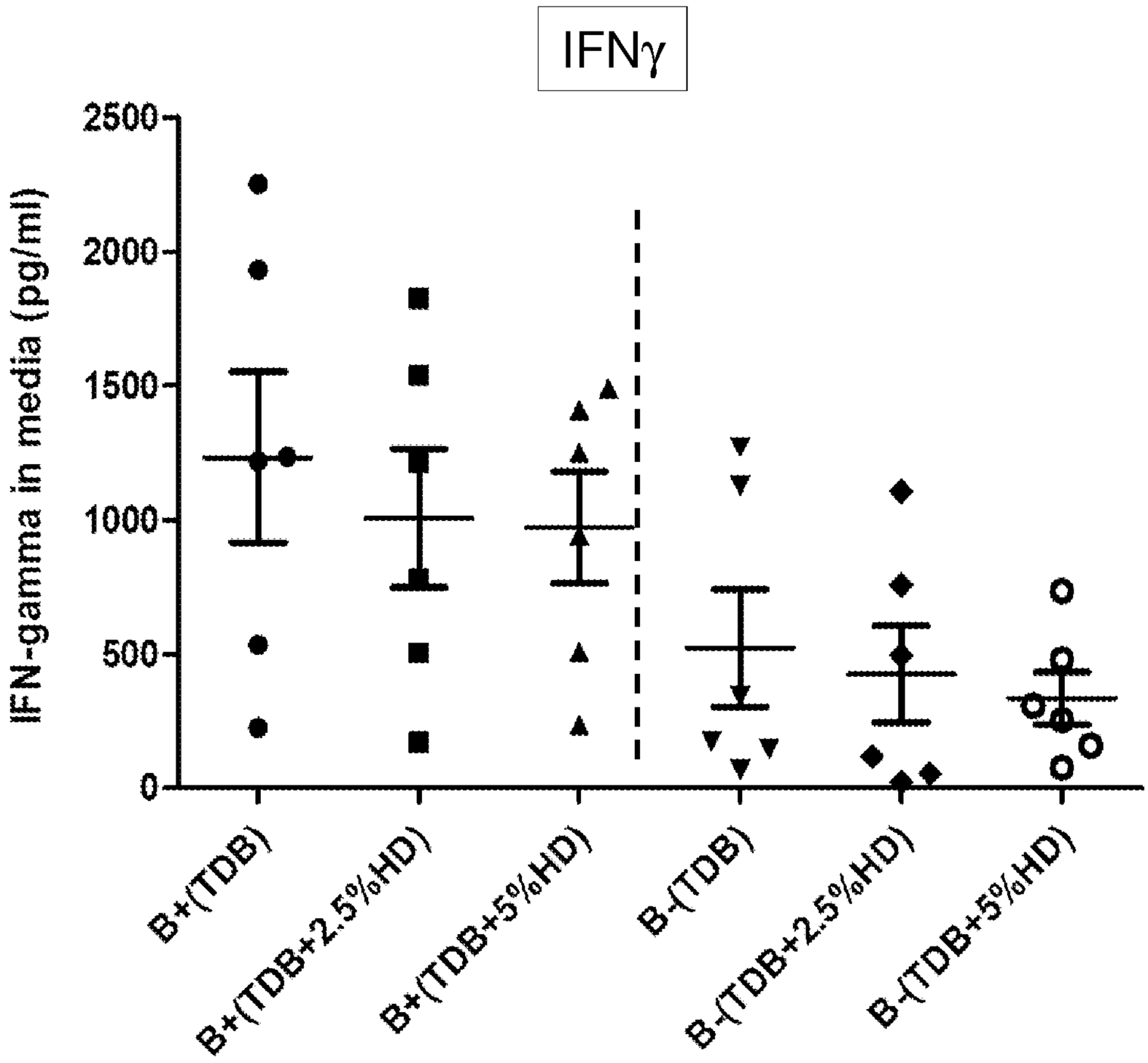

IFNγ
IFN-gamma in media (pg/ml)
2500
2000
1500
1000
500
0
B+(TDB)
B+(TDB+2.5%HD)
B+(TDB+5%HD)
B-(TDB)
B-(TDB+2.5%HD)
B-(TDB+5%HD)

Fig. 4D

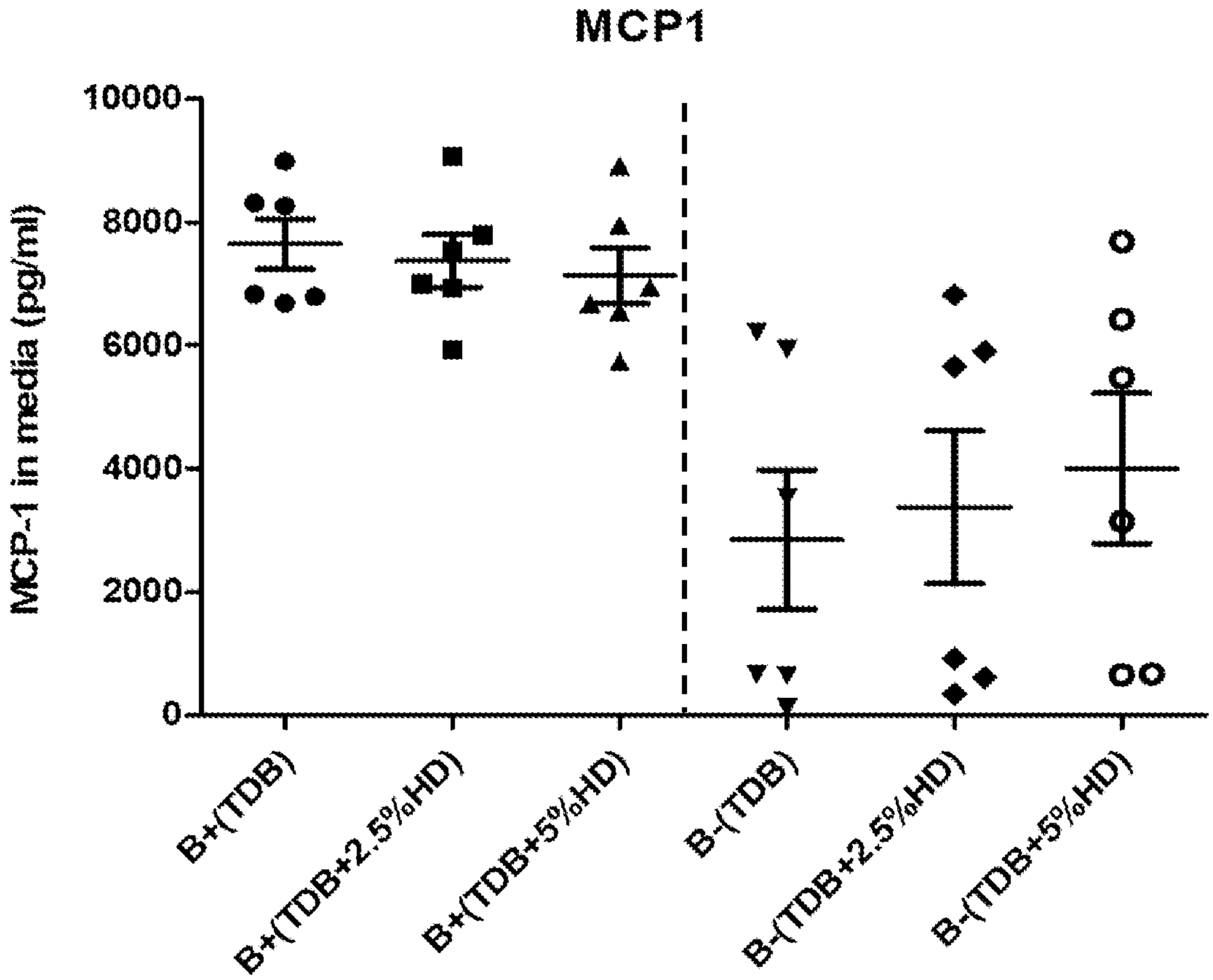
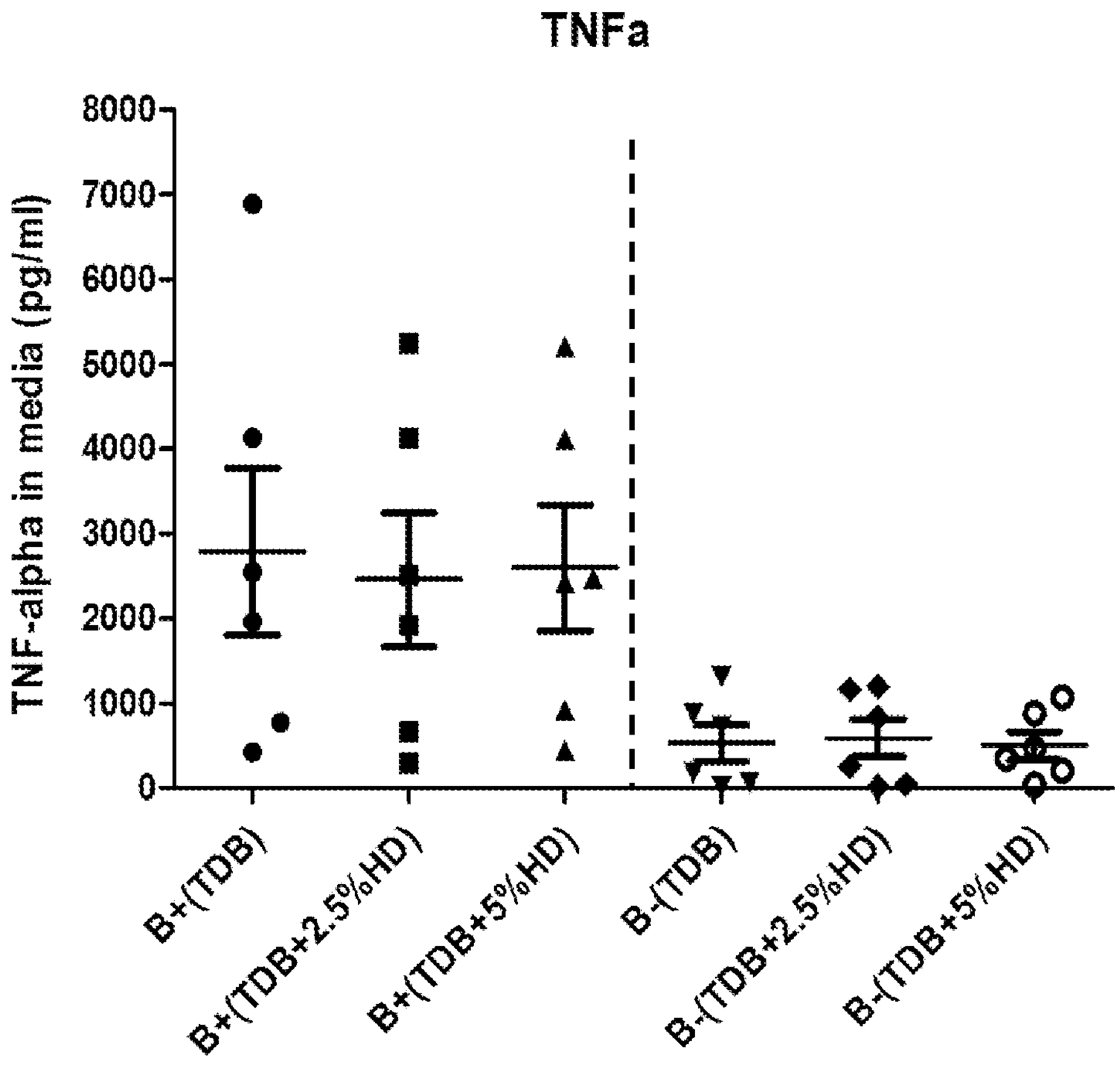

MCP1
MCP-1 in media (pg/ml)
10000
8000
6000
4000
2000
0
B+(TDB)
B+(TDB+2.5%HD)
B+(TDB+5%HD)
B-(TDB)
B-(TDB+2.5%HD)
B-(TDB+5%HD)

Fig. 4E


TNFa
TNF-alpha in media (pg/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
B+(TDB)
B+(TDB+2.5%HD)
B+(TDB+5%HD)
B-(TDB)
B-(TDB+2.5%HD)
B-(TDB+5%HD)

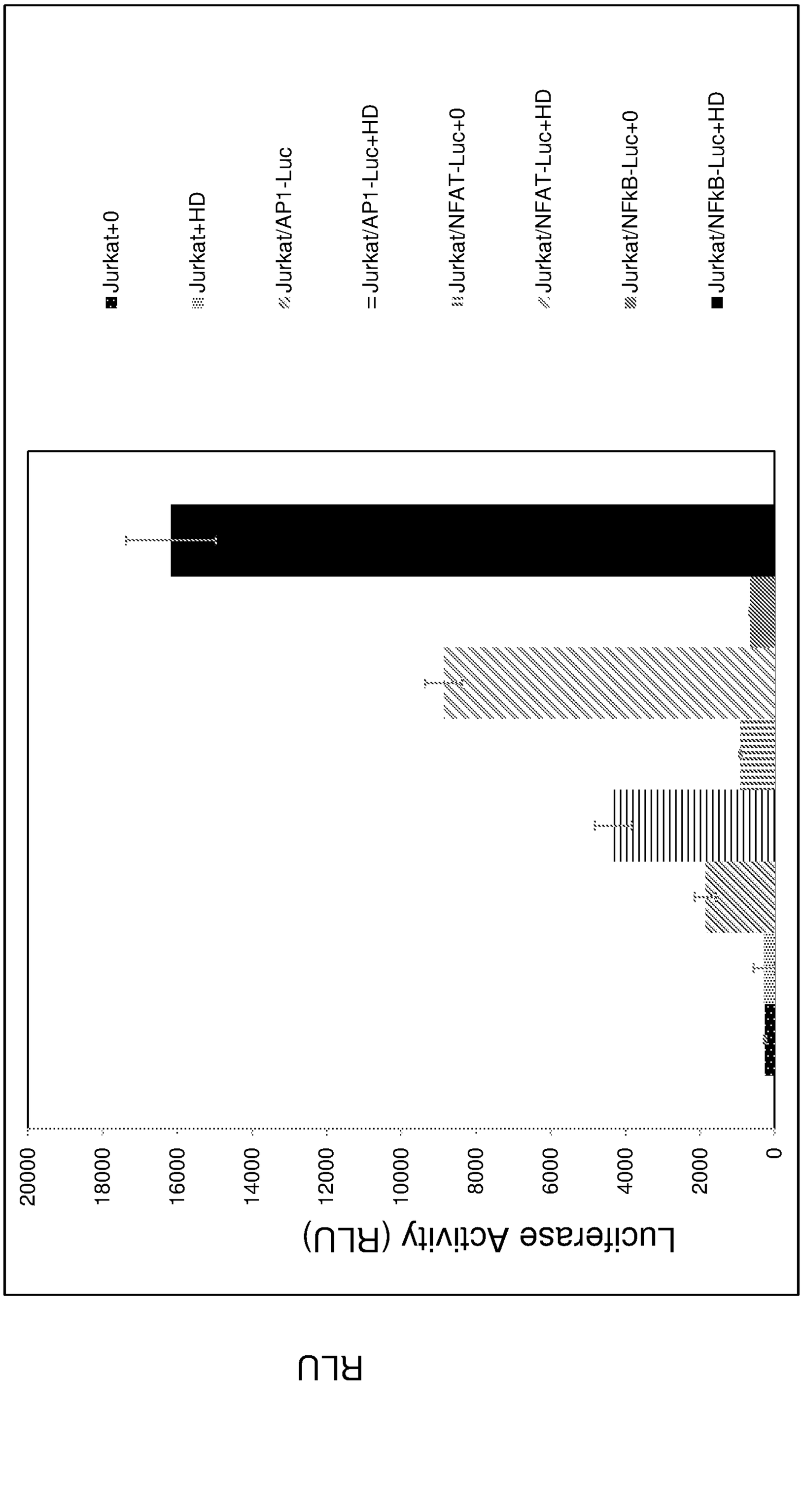

Jurkat+0
Jurkat+HD
Jurkat/AP1-Luc
Jurkat/AP1-Luc+HD
Jurkat/NFAT-Luc+0
Jurkat/NFAT-Luc+HD
Jurkat/NFkB-Luc+0
Jurkat/NFkB-Luc+HD
Luciferase Activity (RLU)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
RLU

Fig. 5A

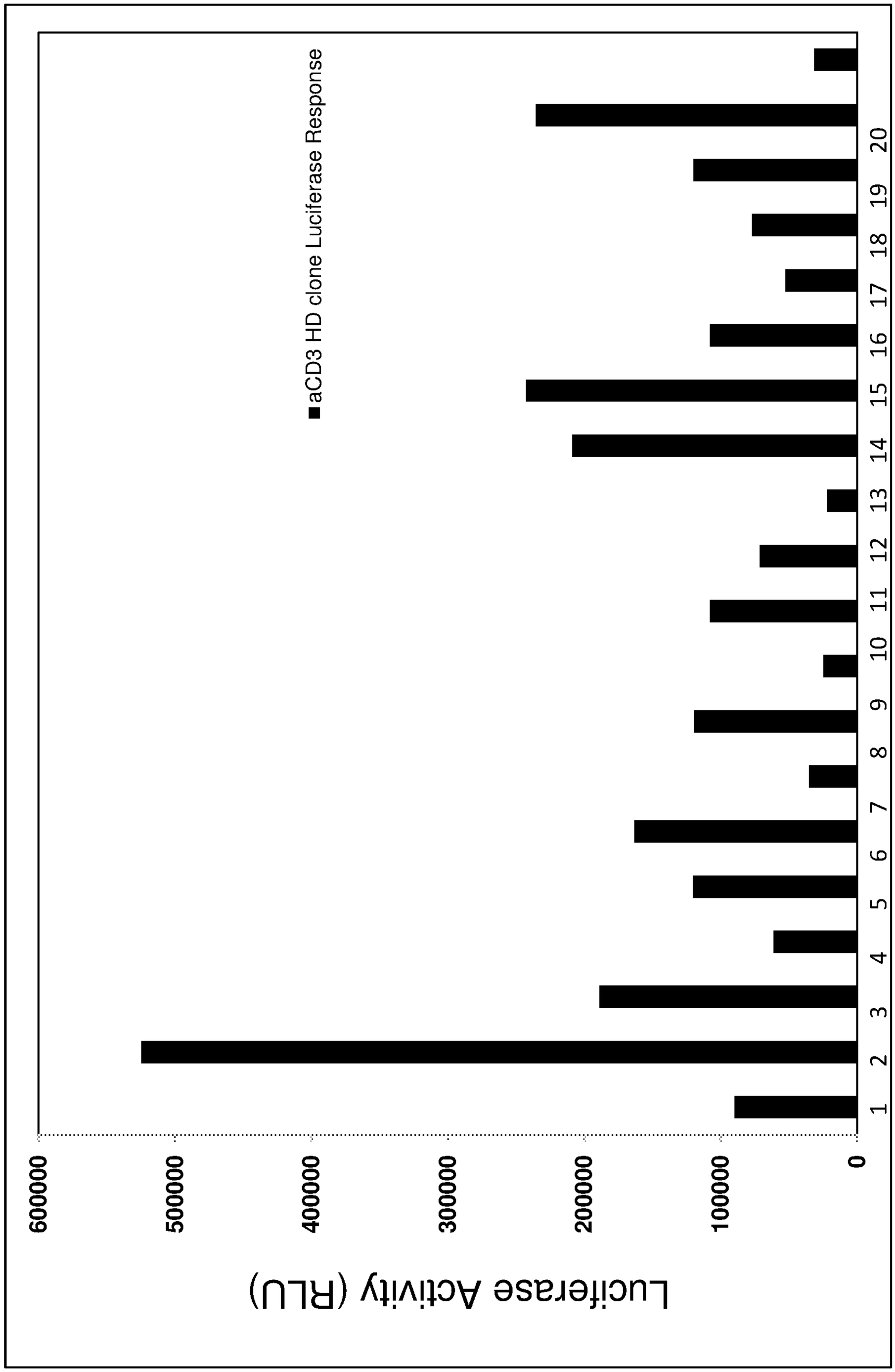

Jurkat/NFkBLuciferase Stable Clones
aCD3 HD clone Luciferase Response
Luciferase Activity (RLU)
600000
500000
400000
300000
200000
100000
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Fig. 5B

Fig. 6A

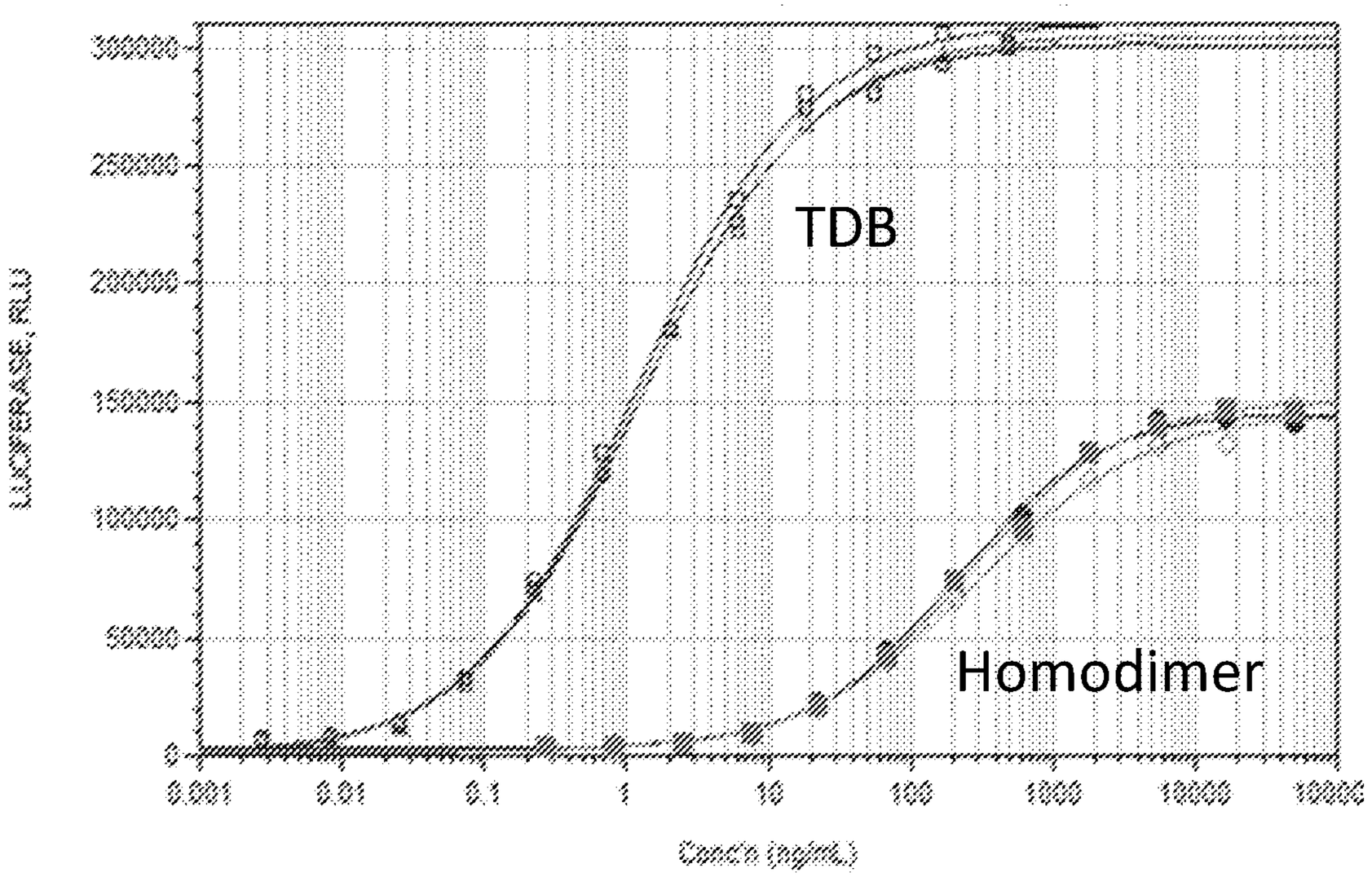
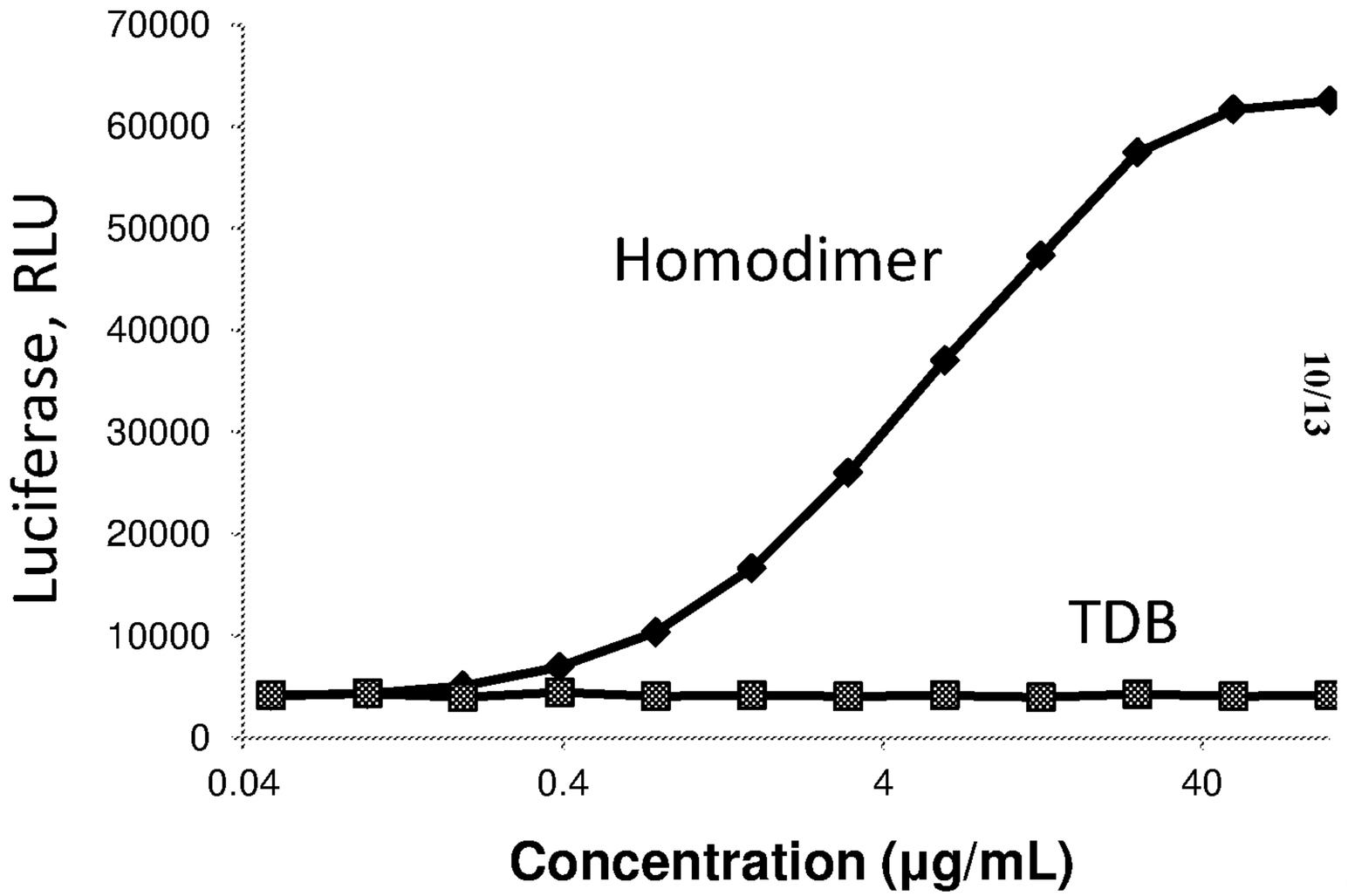

TDB
Homodimer
0.001
0.01
0.1
1
10
100
1000
10000
100000

Fig. 6B


Homodimer
TDB
70000
60000
50000
40000
30000
20000
10000
0
0.04
0.4
4
40
Concentration (µg/mL)

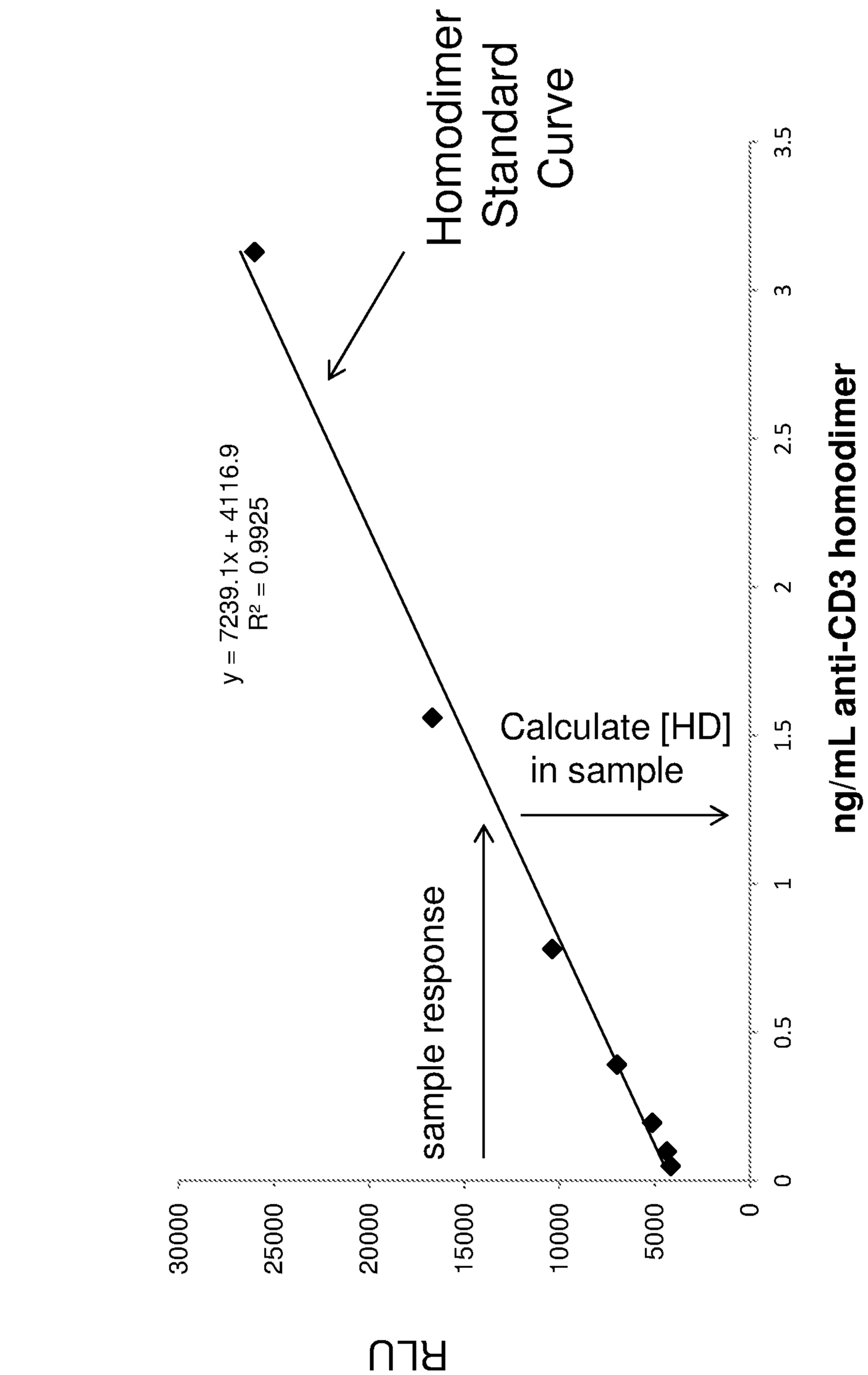

Homodimer Standard Curve
y = 7239.1x + 4116.9
R² = 0.9925
Calculate [HD] in sample
sample response
RLU
ng/mL anti-CD3 homodimer
0
5000
10000
15000
20000
25000
30000
0
0.5
1
1.5
2
2.5
3
3.5

Fig. 7

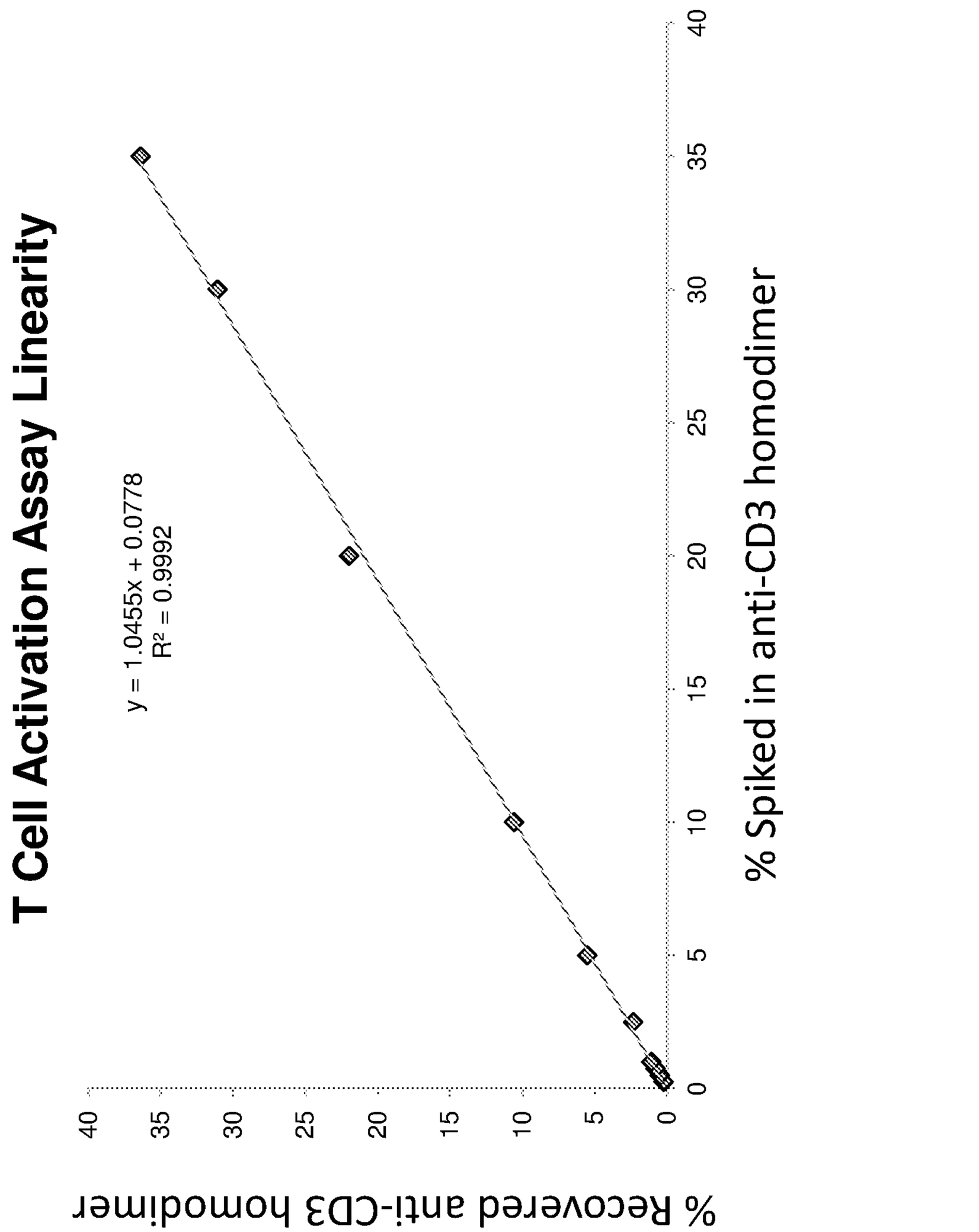

T Cell Activation Assay Linearity
y = 1.0455x + 0.0778
R² = 0.9992
% Recovered anti-CD3 homodimer
% Spiked in anti-CD3 homodimer
0
5
10
15
20
25
30
35
40

Fig. 8

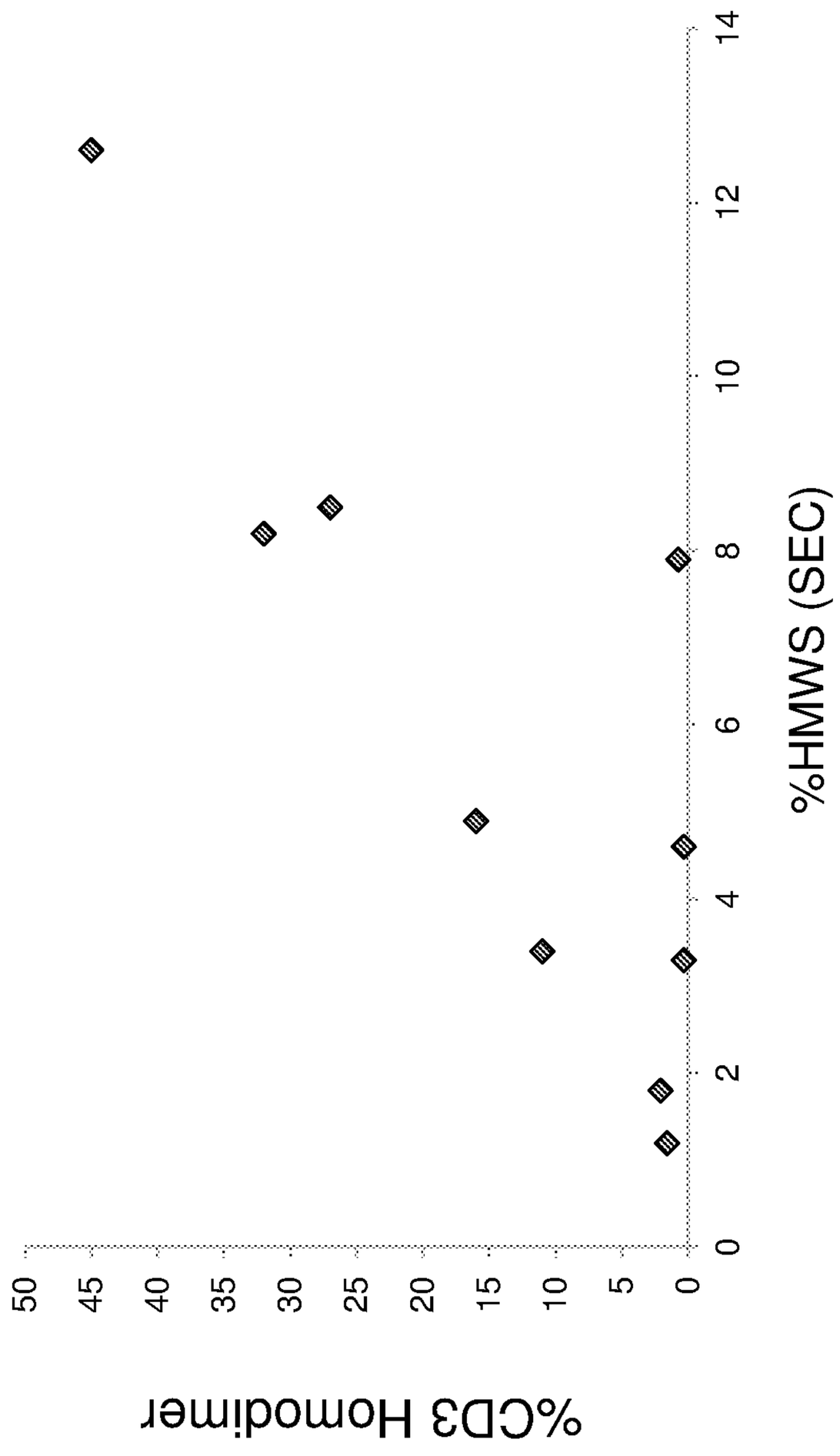

%CD3 Homodimer
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
%HMWS (SEC)

Fig. 9