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(54) **Titre :** MOLECULES DE LIAISON MOINS IMMUNOGENES

(54) **Title:** HUMANIZED ANTI-CD3 BISPECIFIC BINDING MOLECULES HAVING REDUCED IMMUNOGENICITY, USES AND COMPOSITIONS RELATING THERETO

(57) **Abrégé/Abstract:**

Humanized anti-CD3 bispecific binding molecules having reduced immunogenicity are provided. Humanized antibody constructs derived from OKT3 in the format of bispecific binding molecules can have reduced specific activities and related antibody-derived compounds having reduced side-effects that are useful in the treatment of human diseases are derisible. Accordingly, there is provided a bispecific binding molecule having a first domain which contains a humanized antibody-derived light chain that specifically binds to/interacts with the human CD3 complex. The bispecific binding molecule has a second domain which contains a different antigen-interaction-site or an effector domain. Specific amino acid substitutions in light chain of OKT3 - specifically at Ser24, Val54 and Leu96 according to the Kabat system - in the first domain of the bispecific binding molecule result in the humanization, reduced immunogenicity, and enhanced cytotoxicity. Uses, methods, compositions, cells and vectors relating thereto are also described.

Abstract

Humanized anti-CD3 bispecific binding molecules having reduced immunogenicity are provided. Humanized antibody constructs derived from OKT3 in the format of bispecific binding molecules can have reduced specific activities and related antibody-derived compounds having reduced side-effects that are useful in the treatment of human diseases are derisible. Accordingly, there is provided a bispecific binding molecule having a first domain which contains a humanized antibody-derived light chain that specifically binds to/interacts with the human CD3 complex. The bispecific binding molecule has a second domain which contains a different antigen-interaction-site or an effector domain. Specific amino acid substitutions in light chain of OKT3 – specifically at Ser24, Val54 and Leu96 according to the Kabat system – in the first domain of the bispecific binding molecule result in the humanization, reduced immunogenicity, and enhanced cytotoxicity. Uses, methods, compositions, cells and vectors relating thereto are also described.

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Humanized anti-CD3 bispecific binding molecules having reduced immunogenicity, uses and compositions relating thereto

The present invention provides a bispecific binding molecule, wherein said molecule comprises or consists of at least two domains whereby one of said at least two domains specifically binds to/interacts with the human CD3 complex and said domain comprises an amino acid sequence of an antibody derived light chain, wherein said amino acid sequence is a particularly identified amino acid sequence comprising specific amino acid substitutions, and a second domain is or contains at least one further antigen-interaction-site and/or at least one further effector domain. The invention further provides nucleic acid molecules encoding the bispecific binding molecules of the invention, vectors comprising said nucleic acid molecules and host cells transformed or transfected with said vectors. Moreover, the invention concerns a method for the production of bispecific binding molecules of the invention and compositions comprising the bispecific binding molecules of the invention, the nucleic acid molecules of the invention or the host cells of the invention.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are believed to be available to the skilled person.

Since the development of genetic engineering, immune therapy has been used to treat a number of serious diseases, e.g. tumorous diseases. However, the use of antibodies derived from non-human sources leads to several problems when using as a part of a therapeutic regimen in humans.

Firstly, non-human source antibodies may cause "cytokine release syndrome (CRS)". CRS is a clinical syndrome, which has been observed following the administration of the first few doses of anti-CD3 antibodies and is related to the fact

that many antibodies directed against CD3 are mitogenic. In vitro, mitogenic antibodies directed against CD3 induce T cell proliferation and cytokine production. In vivo this mitogenic activity leads to the large-scale release of cytokines, including many T cell-derived cytokines, within the initial hours after the first injection of antibody. The mitogenic capacity of CD3-specific antibodies is monocyte/macrophage dependent and it involves the production of IL-6 and IL-1 β by these cells. CRS symptoms range from frequently reported mild "flu-like" symptoms to less frequently reported severe "shock-like" reactions (which may include cardiovascular and central nervous system manifestations). Symptoms include, inter alia, headache, tremor, nausea/vomiting, diarrhoea, abdominal pain, malaise and muscle/joint aches and pains, generalized weakness, cardiorespiratory events as well as neuro-psychiatric events. Severe pulmonary oedema has occurred in patients with fluid overload and in those who appeared not to have a fluid overload. (Chatenoud, 2003 Nat. Rev. Immunol. 3:123-132)

Secondly, murine antibodies were recognized by a human anti-murine-antibody humoral immune-response (HAMAs) leading to small therapeutic window (Schroff (1985) Cancer Res.45:879-885, Shawler (1985) J. Immunol. 135:1530-1535). HAMAs are typically generated during the second week of treatment with the murine therapeutic antibody and neutralize the murine antibodies by blocking the binding to their intended target. The HAMA response can depend on the murine constant ("Fc") antibody regions or/and the nature of the murine variable ("V") regions. This host response dramatically alters the pharmacokinetic profile of the antibody, leading to a rapid clearance of the antibody and prevents repeated dosing (Reff, 2002 Cancer Control 9:152-166).

Four basic antibody strategies have been adapted to tackle the immunogenicity of therapeutic antibodies; chimerization, providing fully human V-regions, deimmunization and humanization. In chimeric antibodies, the murine constant regions are replaced with human constant regions on the basis that the constant region contributes a significant component to the immunogenicity. There are two approaches to generate fully human V-regions: selecting human antibody V-regions from a phage library and providing transgenic mice which have their own

immunoglobulin genes replaced with human immunoglobulin genes. In deimmunization, specific immunogenic peptides are changed with ones having reduced or no immunogenicity according to specific algorithms.

5 In general, humanization entails substitutions of non-human antibody framework sequences in the variable region for corresponding human sequences, as for example is the case with CDR-grafting. The prior art describes several approaches to humanize antibodies. One of these methods is CDR grafting into foreign framework, wherein CDRs from one species are grafted into human frameworks (EP
10 239400). However, such humanized antibodies have often problems of insufficient binding affinity (Riechmann, 1988, Nature 332:323-327). This can be overcome by modifying the above-mentioned approach by introducing additional mutations into human frameworks. Examples where such method has been used are described in EP469167, EP 971959, EP 940468. Other approaches to humanize antibodies, are
15 humanization by phage display (US 5,565,322) and humanization by resurfacing/veneering, wherein surface exposed amino acids of the antibody are identified and substituted with amino acids similar or identical to human frameworks (see e.g. EP 519596, EP 592106).

Human CD3 denotes an antigen which is expressed on T cells as part of the
20 multimolecular T cell complex and which consists of three different chains: CD3- ϵ , CD3- δ , and CD3- γ . Clustering of CD3 on T cells, e.g., by immobilized anti-CD3 antibodies leads to T cell activation similar to the engagement of the T cell receptor but independent of its clone-typical specificity; (see WO 99/54440 or Hoffman (1985) J. Immunol. 135:5-8).

25 Antibodies which specifically recognize CD3 antigen are described in the prior art, e.g. in Traunecker, EMBO J 10 (1991), 3655-9 and Kipriyanov, Int. J. Cancer 77 (1998), 763-772. Lately, antibodies directed against CD3 have been proposed in the treatment of a variety of diseases. These antibodies or antibody constructs act as
30 either T-cell depleting agents or as mitogenic agents, as disclosed in EP 1 025 854. Human/rodent hybrid antibodies which specifically bind to the human CD3 antigen complex are disclosed in WO 00/05268 and are proposed as immunosuppressive agents, for example for the treatment of rejection episodes following the

transplantation of the renal, septic and cardiac allografts. WO 03/04648 discloses a bispecific antibody directed against CD3 and to an ovarian cancer antigen. Furthermore, Kufer (1997) Cancer Immunol Immunother 45:193-7 relates to a bispecific antibody specific for CD3 and EpCAM for the therapy of minimal residual
5 cancer.

Several attempts to humanize an antibody binding to CD3 have been performed. US 5,929,212, US 5,859,205, WO 91/09968, WO 91/09967 and Adair, 1994 Hum. Antibod. Hybridomas, 5:41-48 describe a humanization method for the murine anti-
10 human CD3 monoclonal antibody OKT3, wherein mouse (donor) CDRs are grafted into human (acceptor) frameworks and donor amino acid residues are introduced into the frameworks. US 6,407,213 and WO 92/22653 describe a humanized UCHT1 antibody, wherein a minimum number of murine CDR and FR residues have been introduced into the context of consensus human variable domain sequences
15 as required to achieve antigen-binding affinity and biological properties comparable to the murine parent antibody. Additional examples of humanized CD3 antibodies are EP 0626390 (OKT3), US 5,885,573 (OKT3), US 5,834,597 (OKT3), US 5,585,097 (YTH 12.5) and US2002131968 (YTH 12.5).

20 However, it has been observed that humanized antibody constructs derived from OKT3 in the format of bispecific binding molecules have reduced specific activities such as the capacity to induce a signal via binding to/interacting with CD3.

Thus, the technical problem underlying the invention was to provide means and
25 methods for the provision of highly efficient antibody-derived compounds which may be useful in the treatment of human diseases with reduced side-effects. In particular, the reduction of side effects is targeted, wherein the side effects are induced by the immunogenicity of the compound and result in a reduction of the activity of the compound.

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The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a bispecific binding molecule, whereby said molecule comprises or consists of at least two domains,

(a) wherein one of said at least two domains specifically binds to/interacts with the human CD3 complex, wherein said domain comprises an amino acid sequence of an antibody derived light chain, wherein said amino acid sequence is

- (i) an amino acid sequence of SEQ ID NO: 2;
- (ii) an amino acid sequence encoded by a nucleic acid sequence corresponding to SEQ ID NO: 1;
- (iii) an amino acid sequence encoded by a nucleotide sequence hybridizing with the complementary strand of a nucleic acid sequence as defined in (ii) under stringent conditions; and
- (iv) an amino acid sequence encoded by a nucleic acid sequence which is degenerate as a result of the genetic code to a nucleotide sequence of any one of (ii) and (iii)

with the proviso that amino acid sequences according to (i) to (iv) comprise amino acid substitutions in the CDR regions of the light chain in positions L24, L54 and L96 according to the Kabat system; and

(b) wherein a second domain is or contains at least one further antigen-interaction-site and/or at least one further effector domain.

The term "binding to/interacting with" as used in the context with the present invention defines a binding/interaction of at least two "antigen-interaction-sites" with each other. The term "antigen-interaction-site" defines, in accordance with the present invention, a motif of a polypeptide which shows the capacity of specific interaction with a specific antigen or a specific group of antigens. Said binding/interaction is also understood to define a "specific recognition". The term "specifically recognizing" means in accordance with this invention that the antibody molecule is capable of specifically interacting with and/or binding to at least two amino acids of each of the human target molecule as defined herein. Antibodies can recognize, interact and/or bind to different epitopes on the same target molecule. Said term relates to the specificity of the antibody molecule, i.e. to its ability to discriminate between the specific regions of the human target molecule as defined herein. The specific interaction of the antigen-interaction-site with its specific antigen

may result in an initiation of a signal, e.g. due to the induction of a change of the conformation of the antigen, an oligomerization of the antigen, etc. Thus, specific motifs in the amino acid sequence of the antigen-interaction-site are a result of their primary, secondary or tertiary structure as well as the result of secondary
5 modifications of said structure.

The term "specific interaction" as used in accordance with the present invention is understood to define that the CD3 specific domain of the bispecific binding molecule of the invention does not or essentially does not cross-react with (poly)peptides of similar structures. Cross-reactivity of a panel of binding molecules under
10 investigation may be tested, for example, by assessing binding of said panel of single-chain binding molecules under conventional conditions (see, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1988 and Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1999) to the (poly)peptide of interest as well as to a number of more or less
15 (structurally and/or functionally) closely related (poly)peptides. These methods may comprise, inter alia, binding studies, blocking and competition studies with structurally and/or functionally closely related molecules. These binding studies also comprise FACS analysis, surface plasmon resonance (SPR, e.g. with BIAcore®), analytical ultracentrifugation, isothermal titration calorimetry, fluorescence
20 anisotropy, fluorescence spectroscopy or by radiolabeled ligand binding assays. Accordingly, examples for the specific interaction of an antigen-interaction-site with a specific antigen may comprise the specificity of a ligand for its receptor. Said definition particularly comprises the interaction of ligands which induce a signal upon binding to its specific receptor. Examples for corresponding ligands comprise
25 cytokines which interact/bind with/to its specific cytokine-receptors. Another example for said interaction, which is also particularly comprised by said definition, is the interaction of an antigenic determinant (epitope) with the antigenic binding site of an antibody. Said interaction is also characterized by no or essentially no cross-reactivity of the antigenic binding site of an antibody with other epitopes of similar
30 structures.

It is understood that the definition of the term "binding to/interacting with" comprises a binding/interacting of the binding domain to/with linear epitopes as well as a binding to/interacting with conformational epitopes, which may also be designated

as structural epitope or discontinuous epitope. The definition of corresponding epitopes is known in the art. Said epitopes e.g. may consist of two regions of the human target molecules or parts thereof. In context of this invention, a conformational epitope is defined by two or more discrete amino acid sequences
5 separated in the primary sequence which come together on the surface of the molecule when the polypeptide folds to the native protein (Sela, (1969) Science 166, 1365 and Laver, (1990) Cell 61, 553-6).

The term "discontinuous epitope" is particularly understood in context of the invention to define non-linear epitopes that are assembled from residues from
10 distant portions of the polypeptide chain. These residues come together on the surface when the polypeptide chain folds into a three-dimensional structure to constitute a conformational/structural epitope.

The binding molecules of the present invention are also envisaged to specifically bind to/interact with at least one binding domain with a conformational epitope(s)
15 composed of and/or comprising at least two regions of the human CD3 complex, or composed of/comprising individual components, like CD3- ϵ , CD3- δ and CD3- γ and/or combinations of said components, such as CD3- ϵ / CD3- δ or CD3- ϵ / CD3- γ . Furthermore, it is envisaged that said conformational/structural epitope(s) described herein comprises individual parts/regions/stretches of at least two regions
20 of a single component of the human CD3 complex, preferably at least two parts/regions/stretches of CD3- ϵ , even more preferably of the extracellular domain of CD3- ϵ .

As defined herein above a second domain of the bispecific binding molecule of the invention binds to at least one further antigen-interaction-site and/or at least one
25 further effector domain. The term "effector domain" characterizes in the context of the present invention a domain of the molecule of the invention which initiates a biological effect such as the induction of a primary or secondary stimulation signal, the induction of a cytotoxic effect (including apoptosis inducing signals) or merely having the ability to specifically bind to/interact with a specific antigen-interaction-
30 site. "Cytotoxic effect" also comprises cellular cytotoxicity exerted by T cells. Accordingly, the bispecific binding molecule of the invention is characterized by at least two different specificities.

Specificity can be determined experimentally by methods known in the art and

methods as disclosed and described herein. Such methods comprise, but are not limited to Western blots, ELISA-, RIA-, ECL-, IRMA-tests and peptide scans.

The term "CDR" as employed herein relates to "complementary determining region", which is well known in the art. The CDRs are parts of immunoglobulins that determine the specificity of said molecules and make contact with specific ligand. The CDRs are the most variable part of the molecule and contribute to the diversity of these molecules. There are three CDR regions CDR1, CDR2 and CDR3 in each V domain. CDR-H characterizes a CDR region of a variable heavy chain and CDR-L relates to a CDR region of a variable light chain. H means the variable heavy chain and L means the variable light chain. The CDR regions of an Ig-derived region may be determined as described in Kabat (1991; Sequences of Proteins of Immunological Interest, 5th edit., NIH Publication no. 91-3242 U.S. Department of Health and Human Services), Chothia (1987; J. Mol. Biol. 196, 901-917) and Chothia (1989; Nature, 342, 877-883).

The "Kabat system" means in the context of the present invention the standard for numbering the residues in a consistent manner according to Kabat (1991; Sequences of Proteins of Immunological Interest, 5th edit., NIH publication no. 91-3242 U.S. Department of Health and Human services) and Chothia (1987; J. Mol. Biol. 196, 901-917). This numbering system is widely used by the skilled artisans and is based on sequence variability and three dimensional loops of the variable domain region which are important in antigen binding activity. All the residues of the light chains or heavy chains have distinct Kabat positions; i.e. the Kabat numbering system applies to CDRs as well as to frameworks. The positions of specific residues of any antibody may be numbered according to Kabat. The numbering system and Kabat positions of specific residues of antibodies are indicated in <http://www.bioinf.org.uk/abs>. For example, the position L24 as mentioned in the invention means the residue 24 in the light chain according to Kabat system. Accordingly, L54 and L96 refer to positions 54 and 96 in the light chain of the antibody according to the Kabat system.

The rules to identify the CDR regions of VH and VL chains according to Kabat are shown in www.bioinf.org.uk/abs and in Table 1.

Table 1.

Identification of the CDRs in the heavy chain (CDR-H regions) and in the light chain (CDR-L regions)

CDR-H1	Start	Approx residue 26 (always 4 after a Cys) [Chothia / AbM definition]; Kabat definition starts 5 residues later
	Residues before	always Cys-XXX-XXX-XXX
	Residues after	always a Trp. Typically Trp-Val, but also, Trp-Ile, Trp-Ala
	Length	10 to 12 residues [AbM definition]; Chothia definition excludes the last 4 residues
CDR-H2	Start	always 15 residues after the end of Kabat / AbM definition) of CDR-H1
	Residues before	typically Leu-Glu-Trp-Ile-Gly, but a number of variations
	Residues after	Lys/Arg-Leu/Ile/Val/Phe/Thr/Ala-Thr/Ser/Ile/Ala
	Length	Kabat definition 16 to 19 residues; AbM (and recent Chothia) definition ends 7 residues earlier
CDR-H3	Start	always 33 residues after end of CDR-H2 (always 2 after a Cys)
	Residues before	always Cys-XXX-XXX (typically Cys-Ala-Arg)
	Residues after	always Trp-Gly-XXX-Gly
	Length	3 to 25 residues

CDR-L1	Start	Approx residue 24
	Residue before	always a Cys
	Residue after	always a Trp. Typically Trp-Tyr-Gln, but also, Trp-Leu-Gln, Trp-Phe-Gln, Trp-Tyr-Leu
	Length	10 to 17 residues
CDR-L2	Start	always 16 residues after the end of L1
	Residues before	generally Ile-Tyr, but also, Val-Tyr, Ile-Lys, Ile-Phe
	Length	always 7 residues (except NEW (7FAB) which has a deletion in this region)
	Start	always 16 residues after the end of L1
CDR-L3	Start	always 33 residues after end of L2 (except NEW (7FAB) which has the deletion at the end of CDR-L2)
	Residue before	always Cys
	Residues after	always Phe-Gly-XXX-Gly
	Length	7 to 11 residues

In accordance with this invention, a framework region relates to a region in the V domain (VH or VL domain) of immunoglobulins and T-cell receptors that provides a

protein scaffold for the hypervariable complementarity determining regions (CDRs) that make contact with the antigen. In each V domain, there are four framework regions designated FR1, FR2, FR3 and FR4. Framework 1 encompasses the region from the N-terminus of the V domain until the beginning of CDR1, framework 2 relates to the region between CDR1 and CDR2, framework 3 encompasses the region between CDR2 and CDR3 and framework 4 means the region from the end of CDR3 until the C-terminus of the V domain; see, inter alia, Janeway, Immunobiology, Garland Publishing, 2001, 5th ed. Thus, the framework regions encompass all the regions outside the CDR regions in VH or VL domains.

10 The person skilled in the art is readily in a position to deduce from a given sequence the framework regions and, the CDRs; see Kabat (1991) Sequences of Proteins of Immunological Interest, 5th edit., NIH Publication no. 91-3242 U.S. Department of Health and Human Services, Chothia (1987). J. Mol. Biol. 196, 901-917 and Chothia (1989) Nature, 342, 877-883.

15 According to the present invention "bispecific binding molecules" are (poly)peptides which necessarily specifically bind with one domain to the human CD3 complex and/or its individual components. The term "(poly)peptide" as used herein describes a group of molecules which comprise the group of peptides, as well as the group of polypeptides. The group of peptides consists of molecules with up to 30 amino acids, the group of polypeptides consists of molecules with consisting of more than 20 30 amino acids. Most preferably, said "bispecific binding molecules" are selected from the group of antibodies, antibody fragments, antibody derivatives, specific binding peptides and specific binding proteins. Said antibody fragments are known in the art and comprise, but are not limited to, Fab-fragments, F(ab')₂ fragments, Fv fragments and the like. Antibody derivatives comprise but are not limited to labeled 25 antibodies/antibody fragments as well as chemically modified antibody molecules/antibody fragments. As will be detailed below, particularly preferred derivatives of antibodies in the context of this invention are scFv's.

One domain of the bispecific binding molecule of the invention is derived from a humanized CDR-grafted CD3-antibody. The term "humanized" as used herein in the 30 context with antibodies and antibody constructs may be defined as substitution of non-human sequences with corresponding human sequences. This can be achieved by grafting murine CDRs into human framework or replacing single murine amino

acids in the framework with single human amino acids at the corresponding position. The term humanization as used in the invention additionally encompasses introduction of further mutations in order to improve the binding or cytotoxic activity of the protein. These further mutations need not necessarily be replacements of
5 murine residues to human residues.

Methods for the substitution of amino acids and, particularly, of amino acids in specific positions by specifically selected amino acids in a given amino acid sequence are known to the person skilled in the art and represent standard
10 laboratory methods. An example of such a method is primer mutagenesis (Sambrook et al. 1989).

It has been surprisingly found that humanized CD3 specific antibody constructs which comprise additional amino acid substitutions in the CDRs of the light chain, as described herein above, in the context of bispecific binding molecules have cytotoxic
15 activity. These molecules have the capacity to induce cell death in target cells. In contrast humanized CD3 specific antibody constructs described in the art, e.g. in Adair, 1994 Hum. Antibod. Hybridomas, 5:41-48, show significantly impaired capacity to induce cell death in target cells when said constructs are expressed in the context of above defined bispecific binding molecules.

20 In particular, the bispecific molecule of the invention shows significant binding to its specific epitopes (see Example 4, Fig.2) and high cytotoxic activity (Example 6, Fig. 6). The bispecific humanized CD3 of the invention with substitutions in the CDRs of the light chain of the CD3 binding part shows an EC50 value of 50 pg/ml whereas the EC50 value of the bispecific antibody construct comprising the humanized
25 OKT3 described in Adair, 1994 Hum. Antibod. Hybridomas, 5:41-48 is 195 pg/ml. Due to the four-fold increase in cytotoxic activity the bispecific molecule of the invention may be used effectively in therapeutic activities. Furthermore, provision of a humanized bispecific molecule having high cytotoxic activity demonstrates a major advantage in the medical field because low amounts of the bispecific molecule of
30 the invention are needed to reach therapeutic effect for patients. Thus, the bispecific molecules of the invention provide an important advantage over the prior art antibodies when treating patients since they show at the same time a high cytotoxic activity and are less immunogenic due to humanization. They therefore offer a clear

improvement in the medical field.

The bispecific binding molecule of the invention differs from the humanized molecules described in the art by the above described three amino acid
5 substitutions in CDRs of the light chains.

Since antibodies bind to/interact with its specific antigens via intramolecular forces which are affected by the particular amino acid sequences of the CDRs, a person skilled in the art would not have substituted amino acids in the amino acid sequence of the CDR region in order to increase biologic activity of the antibody. Instead the
10 skilled person would have retained the original murine CDR sequence. Therefore, it is surprising that the bispecific binding molecule of the invention has such high cytotoxic activity.

It is particularly preferred that the domain which binds to/interacts with the human
15 CD3 complex is characterized by having a serine at position L24, a valine at position L54 and a leucine at position L96. The position L24 means the position 24 in the light chain as described in Kabat (1991; Sequences of Proteins of Immunological Interest, 5th edit., NIH publication no. 91-3242 U.S. Department of Health and Human services) and Chothia (1987; J. Mol. Biol. 196, 901-917) and in
20 <http://www.bioinf.org.uk/abs>. Similarly, the positions L54 and L96 represent the residues 54 and 96, respectively, of the light chain as described by Kabat and Chothia.

The bispecific binding molecule of the invention is further characterized in one
25 embodiment that said CDR region of the light chain comprises the amino acid sequence of SEQ ID NOs: 4, 6 or 8 or encoded by a nucleic acid sequence of SEQ ID NOs: 3, 5 or 7.

It is envisaged by the invention that the domain which binds to/interacts with the
30 human CD3 complex is a scFv.

The term "scFv " (single-chain Fv) is well understood in the art. ScFv's are preferred in context of this invention, due to their small size and the possibility of recombinantly producing these antibody derivative.

It is further envisaged, that the domain of the bispecific binding molecule of the invention which binds to/interacts with the human CD3 complex comprises or consists of the amino acid sequence of SEQ ID NO: 10 (light chain of the humanized CD3 binding molecule of the invention) or is encoded by a nucleic acid sequence of SEQ ID NO: 9.

Preferably the binding molecule of the invention is a binding molecule, wherein the domain which binds to/interacts with the human CD3 complex comprises or consists of the amino acid sequence as depicted in SEQ ID NO.: 14 or encoded by a nucleic acid sequence of SEQ ID NO: 13.

It is further envisaged by the invention that the bispecific binding molecule is a binding molecule, wherein said second domain is at least one further antigen-interaction-site specific for one or more cell surface molecule(s).

The term "cell surface molecule" as used herein denotes molecules which are presented or/and attached on/to the surface of a cell. Examples for said cell surface molecules are membrane and transmembrane proteins (including modified variants, such as glycosylated variants), molecules attached to said proteins or the cell surface as well as glycosylated moieties such as for example glycolipids. Attachment is to be understood as being effected preferably by way of an integral membrane protein, a GPI-linked (glycosyl phosphatidyl inositol-linked) protein, a proteinaceous or non-proteinaceous moiety bound covalently or non-covalently to another carrier molecule such as sugar moieties or ganglioside moieties. Preferably said cell surface molecule(s) is/are (a) tumor-specific molecule(s). A tumor-specific molecule is a tumor-associated cell surface antigen which is either found exclusively on tumor cells or is overexpressed on tumor cells as compared to non-malignant cells. Tumor-associated cell surface antigens can be expressed not only on tumor cells but also on cells/tissue which are/is not essential for survival or which can be replenished by stem cells not expressing tumor-associated cell surface antigen. Furthermore, tumor-associated cell surface antigen can be expressed on malignant cells and non-malignant cells but is better accessible by a therapeutic agent of interest on malignant cells. Examples of over-expressed tumor-associated cell

surface antigens are HER-2/neu, EGF-Receptor, HER-3 and HER-4. An example of a tumor-associated cell surface antigen which is tumor specific is EGFRV-III. An example of a tumor-associated cell surface antigen which is presented on a cell which is non-essential for survival is PSMA. Examples of tumor-associated cell surface antigens which are presented on cells which are replenished are CD19, CD20 and CD33. An example of a tumor-associated cell surface antigen which is better accessible in a malignant state than in a non-malignant state is EpCAM.

Preferably, said second domain which is at least one further antigen-interaction-site is an antibody-derived region comprises a polypeptide sequence which corresponds to at least one variable region of an antibody. More preferably, said second domain is a further scFv. A particularly preferred molecular format of the invention provides a polypeptide construct in the format of a bispecific single chain antibody construct wherein the antibody-derived region comprises one VH and one VL region. VH and VL regions may be ordered in any arrangement.

The term "bispecific single chain antibody construct" relates to a construct comprising one domain consisting of (at least one) variable light chain as defined above capable of specifically interacting with/binding to human CD3/human CD3 complex and comprising a second domain consisting of (at least one) variable region(s) (or parts thereof) as defined above capable of specifically interacting with/binding to a further antigen. A part of a variable region may be at least one CDR ("Complementary Determining Region"), most preferably at least the CDR3 region. Said two domains/regions in the single chain antibody construct are preferably covalently connected to one another as a single chain. This connection can be effected either directly (domain1 interacting with CD3 – domain2 interacting with the further antigen or domain1 interacting with the further antigen – domain2 interacting with CD3) or through an additional polypeptide linker sequence (domain1 – linker sequence – domain2 or domain2 – linker sequence – domain1). In the event that a linker is used, this linker is preferably of a length and sequence sufficient to ensure that each of the first and second domains can, independently from one another, retain their differential binding specificities. Most preferably and as documented in the appended examples, the "bispecific single chain antibody construct" is a bispecific single chain Fv (bscFv). The molecular format of bispecific

single chain molecules is known in the art and is described e.g. in WO 99/54440, Mack, J. Immunol. (1997), 158, 3965-3970, Mack, PNAS, (1995), 92, 7021-7025; Kufer, Cancer Immunol. Immunother., (1997), 45, 193-197; Löffler, Blood, (2000), 95, 6, 2098-2103; Brühl, Immunol., (2001), 166, 2420-2426. Particular examples for
 5 such bispecific single chain antibody constructs of the invention are provided herein below and illustrated in the appended examples.

In accordance with the invention are bispecific binding molecules, wherein said second domain specifically binds to/interacts with an antigen selected from the group consisting of EpCAM, CCR5, CD19, HER-2, HER-3, HER-4, EGFR, PSMA,
 10 CEA, MUC-1 (mucin), MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, bhCG, Lewis-Y, CD20, CD33, CD30, ganglioside GD3, 9-O-Acetyl-GD3, GM2, Globo H, fucosyl GM1, Poly SA, GD2, Carboanhydrase IX (MN/CA IX), CD44v6, Sonic Hedgehog (Shh), Wue-1, Plasma Cell Antigen, membrane-bound IgE, Melanoma Chondroitin Sulfate Proteoglycan (MCSP), CCR8, TNF-alpha precursor, STEAP,
 15 mesothelin, A33 Antigen, Prostate Stem Cell Antigen (PSCA), Ly-6, desmoglein 4, E-cadherin neoepitope, Fetal Acetylcholine Receptor, CD25, CA 19-9 marker, CA-125 marker, Muellierian Inhibitory Substance (MIS) Receptor type II, sTn (sialylated Tn antigen; TAG-72), FAP (fibroblast activation antigen), endosialin, EGFRvIII, L6, SAS, CD63, TF-antigen, Cora antigen, CD7, CD22, Igα, Igβ, gp100,
 20 MT-MMPs, Fi9-antigen or CO-29.

According to a preferred embodiment of the invention said second domain specifically binds to/interacts the CD19 molecule.

It is particularly envisaged that the bispecific binding molecule of the invention which
 25 specifically binds to/interacts with the CD3 and the CD19 molecule is characterized in that said second domain comprises or consists of an amino acid sequence selected from the group of:

- (a) an amino acid sequence corresponding to SEQ ID NO.: 16 or 18;
- (b) an amino acid sequence encoded by a nucleic acid sequence corresponding
 30 to SEQ ID NO.: 15 or 17;
- (c) an amino acid sequence encoded by a nucleic acid sequence hybridizing with the complementary strand of a nucleic acid sequence as defined in (b) under stringent hybridization conditions; and

(d) an amino acid sequence encoded by a nucleic acid sequence which is degenerate as a result of the genetic code to a nucleotide sequence of any one of (b) and (c).

5 More preferably, the bispecific binding molecule comprises or consists of an amino acid sequence selected from the group of:

(a) an amino acid sequence corresponding to SEQ ID NO.: 20;

(b) an amino acid sequence encoded by a nucleic acid sequence corresponding to SEQ ID NO.: 19;

10 (c) an amino acid sequence encoded by a nucleic acid sequence hybridizing with the complementary strand of a nucleic acid sequence as defined in (b) under stringent hybridization conditions; and

(d) an amino acid sequence encoded by a nucleic acid sequence which is degenerate as a result of the genetic code to a nucleotide sequence of any

15 one of (b) and (c).

Said bispecific binding molecule is preferably a bispecific scFv construct, whereby a first scFv specifically binds to/interacts with CD3 and a second scFv specifically binds to/interacts with CD19.

20 According to a preferred embodiment of the invention said second domain specifically binds to/interacts with the EpCAM molecule.

It is particularly envisaged that the bispecific binding molecule of the invention which specifically binds to/interacts with the CD3 and the EpCAM molecule is characterized in that said second domain comprises or consists of an amino acid
25 sequence selected from the group of:

(a) an amino acid sequence corresponding to SEQ ID NO.: 22, 24, 26, 28, 30 or 32;

(b) an amino acid sequence encoded by a nucleic acid sequence corresponding to SEQ ID NO.: 21, 23, 25, 27, 29 or 31;

30 (c) an amino acid sequence encoded by a nucleic acid sequence hybridizing with the complementary strand of a nucleic acid sequence as defined in (b) under stringent hybridization conditions; and

(d) an amino acid sequence encoded by a nucleic acid sequence which is

degenerate as a result of the genetic code to a nucleotide sequence of any one of (b) and (c).

More preferably, the bispecific binding molecule comprises or consists of an amino acid sequence selected from the group of:

- (a) an amino acid sequence corresponding to SEQ ID NO.: 34 or 36;
 - (b) an amino acid sequence encoded by a nucleic acid sequence corresponding to SEQ ID NO.: 33 or 35;
 - (c) an amino acid sequence encoded by a nucleic acid sequence hybridizing with the complementary strand of a nucleic acid sequence as defined in (b) under stringent hybridization conditions; and
 - (d) an amino acid sequence encoded by a nucleic acid sequence which is degenerate as a result of the genetic code to a nucleotide sequence of any one of (b) and (c).
- Said bispecific binding molecule is preferably a bispecific scFv construct, whereby a first scFv specifically binds to/interacts with CD3 and a second scFv specifically binds to/interacts with EpCAM.

It is further preferred that said at least one further antigen-interaction-site of the bispecific binding molecule of the invention is humanized.

In a further embodiment, the invention encompasses a nucleic acid sequence encoding an above defined bispecific binding molecule of the invention.

Preferably, said nucleic acid sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding the mature form of a protein comprising the amino acid sequence selected from the group of SEQ ID Nos: 20, 34 or 36;
- (b) a nucleotide sequence comprising or consisting of a DNA sequence selected from the group of SEQ ID NOs: 19, 33 or 35;
- (c) a nucleotide sequence hybridizing with the complementary strand of a nucleotide sequence as defined in (b) under stringent hybridization conditions;
- (d) a nucleotide sequence encoding a protein derived from the protein encoded by a nucleotide sequence of (a) or (b) by way of substitution, deletion and/or addition of one or several amino acids of the amino acid sequence encoded by

the nucleotide sequence of (a) or (b);

- (e) a nucleotide sequence encoding a protein having an amino acid sequence at least 60 %, preferably 70 %, more preferably 80 %, particularly preferably 90 %, even more preferably 95 % and most preferably 99 % identical to the amino acid sequence encoded by the nucleotide sequence of (a) or (b);
- (f) a nucleotide sequence which is degenerate as a result of the genetic code to a nucleotide sequence of any one of (a) to (e).

The term "hybridizing" as used herein refers to polynucleotides which are capable of hybridizing to the complementary strand of the recited nucleic acid sequence or

parts thereof or to the recited nucleic acid sequence or parts thereof. Therefore, said nucleic acid sequence may be useful as probes in Northern or Southern Blot analysis of RNA or DNA preparations, respectively, or can be used as oligonucleotide primers in PCR analysis dependent on their respective size.

Preferably, said hybridizing polynucleotides comprise at least 10, more preferably at least 15 nucleotides while a hybridizing polynucleotide of the present invention to be used as a probe preferably comprises at least 100, more preferably at least 200, or most preferably at least 500 nucleotides.

It is well known in the art how to perform hybridization experiments with nucleic acid molecules, i.e. the person skilled in the art knows what hybridization conditions s/he has to use in accordance with the present invention. Such hybridization conditions are referred to in standard text books such as Sambrook et al. (loc cit.) and other standard laboratory manuals known by the person skilled in the art or as recited above. Preferred in accordance with the present inventions are polynucleotides which are capable of hybridizing to the polynucleotides of the invention or parts thereof, under stringent hybridization conditions.

"Stringent hybridization conditions" refer, i.e. to an overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1 x SSC at about 65°C. Also contemplated are nucleic acid molecules that hybridize to the polynucleotides of the invention at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide

concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH₂PO₄; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA; followed by washes at 50°C with 1 X SSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). It is of note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

The recited nucleic acid molecules may be, e.g., DNA, cDNA, RNA or synthetically produced DNA or RNA or a recombinantly produced chimeric nucleic acid molecule or mixtures of chimeras thereof comprising any of those polynucleotides either alone or in combination.

It is evident to the person skilled in the art that regulatory sequences may be added to the nucleic acid molecule of the invention. For example, promoters, transcriptional enhancers and/or sequences which allow for induced expression of the polynucleotide of the invention may be employed. A suitable inducible system is for example tetracycline-regulated gene expression as described, e.g., by Gossen and Bujard (Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551) and Gossen et al. (Trends Biotech. 12 (1994), 58-62), or a dexamethasone-inducible gene expression system as described, e.g. by Crook (1989) EMBO J. 8, 513-519 .

Furthermore, it is envisaged for further purposes that nucleic acid molecules may contain, for example, thioester bonds and/or nucleotide analogues. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell. Said nucleic acid molecules may be transcribed by an appropriate vector containing a chimeric gene which allows for the transcription of said nucleic acid molecule in the cell. In this respect, it is also to be understood that such polynucleotide can be used for "gene targeting" or "gene

therapeutic" approaches. In another embodiment said nucleic acid molecules are labeled. Methods for the detection of nucleic acids are well known in the art, e.g., Southern and Northern blotting, PCR or primer extension. This embodiment may be useful for screening methods for verifying successful introduction of the nucleic acid molecules described above during gene therapy approaches.

Said nucleic acid molecule(s) may be a recombinantly produced chimeric nucleic acid molecule comprising any of the aforementioned nucleic acid molecules either alone or in combination. Preferably, the nucleic acid molecule is part of a vector.

10 The present invention therefore also relates to a vector comprising the nucleic acid molecule of the present invention.

Many suitable vectors are known to those skilled in molecular biology, the choice of which would depend on the function desired and include plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering.

15 Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook et al. (loc cit.) and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Alternatively, the polynucleotides and vectors of the invention can be reconstituted
20 into liposomes for delivery to target cells. As discussed in further details below, a cloning vector was used to isolate individual sequences of DNA. Relevant sequences can be transferred into expression vectors where expression of a particular polypeptide is required. Typical cloning vectors include pBluescript SK, pGEM, pUC9, pBR322 and pGBT9. Typical expression vectors include pTRE,
25 pCAL-n-EK, pESP-1, pOP13CAT.

Preferably said vector comprises a nucleic acid sequence which is a regulatory sequence operably linked to said nucleic acid sequence encoding a single chain antibody constructs defined herein.

Such regulatory sequences (control elements) are known to the artisan and may
30 include a promoter, a splice cassette, translation initiation codon, translation and insertion site for introducing an insert into the vector. Preferably, said nucleic acid molecule is operatively linked to said expression control sequences allowing expression in eukaryotic or prokaryotic cells.

It is envisaged that said vector is an expression vector comprising the nucleic acid molecule encoding a bispecific binding molecule of the invention.

The term "regulatory sequence" refers to DNA sequences, which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is preferably used.

Thus, the recited vector is preferably an expression vector. An "expression vector" is a construct that can be used to transform a selected host and provides for expression of a coding sequence in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotes and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise normally promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the P_L , *lac*, *trp* or *tac* promoter in *E. coli*, and examples of regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as

the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. Furthermore, depending on the expression system used leader sequences capable of directing the polypeptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the recited nucleic acid sequence and are well known in the art; see also, e.g., appended example 3. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product; see supra. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pEF-DHFR, pEF-ADA or pEF-neo (Mack et al. PNAS (1995) 92, 7021-7025 and Raum et al. Cancer Immunol Immunother (2001) 50(3), 141-150) or pSPORT1 (GIBCO BRL).

Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. 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, and as desired, the collection and purification of the bispecific binding molecule of the invention may follow; see, e.g., the appended examples.

An alternative expression system which could be used to express a cell cycle interacting protein is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The coding sequence of a recited nucleic acid molecule may be cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of said coding sequence will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein coat. The recombinant viruses are then used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which the protein of the invention is expressed (Smith, J. Virol. 46 (1983), 584; Engelhard,

Proc. Nat. Acad. Sci. USA 91 (1994), 3224-3227).

Additional regulatory elements may include transcriptional as well as translational enhancers. Advantageously, the above-described vectors of the invention comprises a selectable and/or scorable marker.

- 5 Selectable marker genes useful for the selection of transformed cells and, e.g., plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin
10 and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygromycin, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-
15 phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from *Aspergillus terreus* which confers resistance to Blasticidin S
20 (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

Useful scorable markers are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or β -glucuronidase
25 (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a recited vector.

As described above, the recited nucleic acid molecule can be used alone or as part of a vector to express the bispecific binding molecule of the invention in cells, for,
30 e.g., purification but also for gene therapy purposes. The nucleic acid molecules or vectors containing the DNA sequence(s) encoding any one of the above described bispecific binding molecule of the invention is introduced into the cells which in turn produce the polypeptide of interest. Gene therapy, which is based on introducing

therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors, methods or gene-delivery systems for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, *Nature Medicine* 2 (1996), 534-539; Schaper, *Circ. Res.* 79 (1996), 911-919; Anderson, *Science* 256 (1992), 808-813; Verma, *Nature* 389 (1994), 239; Isner, *Lancet* 348 (1996), 370-374; Muhlhauser, *Circ. Res.* 77 (1995), 1077-1086; Onodera, *Blood* 91 (1998), 30-36; Verma, *Gene Ther.* 5 (1998), 692-699; Nabel, *Ann. N.Y. Acad. Sci.* 811 (1997), 289-292; Verzeletti, *Hum. Gene Ther.* 9 (1998), 2243-51; Wang, *Nature Medicine* 2 (1996), 714-716; WO 94/29469; WO 97/00957, US 5,580,859; US 5,589,466; or Schaper, *Current Opinion in Biotechnology* 7 (1996), 635-640. The recited nucleic acid molecules and vectors may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g., adenoviral, retroviral) into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom, most preferably said cell is a stem cell. An example for an embryonic stem cell can be, inter alia, a stem cell as described in, Nagy, *Proc. Natl. Acad. Sci. USA* 90 (1993), 8424-8428.

The invention also provides for a host transformed or transfected with a vector of the invention. Said host may be produced by introducing said at least one of the above described vector of the invention or at least one of the above described nucleic acid molecules of the invention into the host. The presence of said at least one vector or at least one nucleic acid molecule in the host may mediate the expression of a gene encoding the above described single chain antibody constructs.

The described nucleic acid molecule or vector of the invention which is introduced in the host may either integrate into the genome of the host or it may be maintained extrachromosomally.

The host can be any prokaryote or eukaryotic cell.

The term "prokaryote" is meant to include all bacteria which can be transformed or transfected with DNA or RNA molecules for the expression of a protein of the invention. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*. The term "eukaryotic" is meant to include yeast, higher plant, insect

and preferably mammalian cells. Depending upon the host employed in a recombinant production procedure, the protein encoded by the polynucleotide of the present invention may be glycosylated or may be non-glycosylated. Especially preferred is the use of a plasmid or a virus containing the coding sequence of the polypeptide of the invention and genetically fused thereto an N-terminal FLAG-tag and/or C-terminal His-tag. Preferably, the length of said FLAG-tag is about 4 to 8 amino acids, most preferably 8 amino acids. An above described polynucleotide can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Furthermore, methods for preparing fused, operably linked genes and expressing them in, e.g., mammalian cells and bacteria are well-known in the art (Sambrook, loc cit.).

Preferably, said the host is a bacterium or an insect, fungal, plant or animal cell. It is particularly envisaged that the recited host may be a mammalian cell. Particularly preferred host cells comprise CHO cells, COS cells, myeloma cell lines like SP2/0 or NS/0. As illustrated in the appended examples, particularly preferred are CHO-cells as hosts.

More preferably said host cell is a human cell or human cell line, e.g. per.c6 (Kroos, Biotechnol. Prog., 2003, 19:163-168).

In a further embodiment, the present invention thus relates to a process for the production of bispecific binding molecule of the invention comprising cultivating a cell and/or the host of the invention under conditions suitable for the expression/allowing the expression of bispecific binding molecule and isolating/recovering the bispecific binding molecule from the cell or the culture/culture medium.

The transformed hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptide of the invention can then be isolated from the growth medium, cellular lysates, or cellular membrane fractions. The isolation and purification of the, e.g., microbially expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies directed,

e.g., against a tag of the polypeptide of the invention or as described in the appended examples.

The conditions for the culturing of a host which allow the expression are known in the art to depend on the host system and the expression system/vector used in such process. The parameters to be modified in order to achieve conditions allowing the expression of a recombinant polypeptide are known in the art. Thus, suitable conditions can be determined by the person skilled in the art in the absence of further inventive input.

Once expressed, the bispecific binding molecule of the invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like; see, Scopes, "Protein Purification", Springer-Verlag, N.Y. (1982). Substantially pure polypeptides of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the bispecific binding molecule of the invention may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures. Furthermore, examples for methods for the recovery of the bispecific binding molecule of the invention from a culture are described in detail in the appended examples.

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Furthermore, the invention provides for a composition comprising a bispecific binding molecule of the invention or a bispecific binding molecule as produced by the process disclosed above, a nucleic acid molecule of the invention, a vector or a host of the invention. Said composition may, optionally, also comprise a proteinaceous compound capable of providing an activation signal for immune effector cells. Most preferably, said composition is a pharmaceutical composition further comprising, optionally, suitable formulations of carrier, stabilizers and/or excipients.

In the light of the present invention, said "proteinaceous compounds" providing an activation signal for immune effector cells" may be, e.g. an activation signal for T cells. Preferred formats of proteinaceous compounds comprise bispecific antibodies and fragments or derivatives thereof, e.g. bispecific scFv. Preferably, said activation signal for T cells may be provided via the T cell receptor (TCR), more preferably via

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CD3 molecule of the TCR. Proteinaceous compounds can comprise, but are not limited to, scFv's specific for CD3, scFv's specific for the T cell receptor or superantigens. Superantigens directly bind to certain subfamilies of T cell receptor variable regions in an MHC-independent manner thus mediating the primary T cell activation signal. The proteinaceous compound may also provide an activation signal for an immune effector cell which is a non-T cell. Examples of immune effector cells which are non-T cells comprise, inter alia, B cells and NK cells.

In accordance with this invention, the term "pharmaceutical composition" relates to a composition for administration to a patient, preferably a human patient. In a preferred embodiment, the pharmaceutical composition comprises a composition for parenteral, transdermal, intraluminal, intra arterial, intrathecal administration or by direct injection into the tissue or tumour. It is in particular envisaged that said pharmaceutical composition is administered to a patient via infusion or injection.

Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The pharmaceutical composition of the present invention may further comprise a pharmaceutically acceptable carrier. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions, etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 μ g to 5 g units per day. However, a more preferred dosage for continuous infusion might be in the range of 0.01 μ g to 2 mg, preferably 0.01 μ g to 1 mg, more preferably 0.01 μ g to 100 μ g, even more preferably 0.01 μ g to 50 μ g and most preferably 0.01 μ g to 10 μ g units per kilogram of body weight per hour. Particularly preferred dosages are recited herein below. Progress can be monitored by periodic assessment. Dosages

will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. The compositions of the invention may be administered locally or systematically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directed to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishes, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. In addition, the pharmaceutical composition of the present invention might comprise proteinaceous carriers, like, e.g., serum albumine or immunoglobuline, preferably of human origin. It is envisaged that the pharmaceutical composition of the invention might comprise, in addition to the proteinaceous CD3 binding molecules or nucleic acid molecules or vectors encoding the same (as described in this invention), further biologically active agents, depending on the intended use of the pharmaceutical composition. Such agents might be drugs acting on the gastro-intestinal system, drugs acting as cytostatica, drugs preventing hyperurikemia, drugs inhibiting immunereactions (e.g. corticosteroids), drugs acting on the circulatory system and/or agents such as T-cell co-stimulatory molecules or cytokines known in the art.

Possible indications for administration of the composition(s) of the invention are tumorous diseases, cancers, especially epithelial cancers/carcinomas such as breast cancer, colon cancer, prostate cancer, head and neck cancer, non-melanotic skin cancer, cancers of the genito-urinary tract, e.g. ovarian cancer, endometrial cancer, cervix cancer and kidney cancer, lung cancer, gastric cancer, cancer of the small intestine, liver cancer, pancreas cancer, gall bladder cancer, cancers of the bile duct, esophagus cancer, cancer of the salivatory glands and cancer of the

thyroid gland or other tumorous diseases like haematological tumors, melanomas, gliomas, sarcomas, e.g. osteosarcomas. Further indications for administration of the composition(s) of the invention are proliferative diseases, an inflammatory diseases, an immunological disorders, an autoimmune diseases, an infectious diseases, viral
5 diseases, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

The composition of the invention as described above may also be a diagnostic composition further comprising, optionally, means and methods for detection of
10 proliferative diseases, tumorous diseases, inflammatory diseases, immunological disorders, autoimmune diseases, infectious diseases, viral diseases, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

The bispecific specific binding molecules of the invention are also suited for use in
15 immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. Examples of immunoassays which can utilize the polypeptide of the invention e.g. for diagnostic purposes are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the enzyme linked immunosorbent assay (ELISA), enzyme immunoassay (EIA),
20 radioimmunoassay (RIA), the sandwich (immunometric assay), dot blot and the Western blot assay. Further assays, which can be used for detecting the bispecific binding molecules e.g. in diagnostic assays are FACS-based assays, cytotoxic assays (Cr^{51} , fluorescence release) or dye release assays.

The bispecific specific binding molecules of the invention can be bound to many
25 different carriers and used to isolate cells specifically bound to said polypeptides. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble, e.g. as beads, for the purposes of the
30 invention.

Said diagnostic composition may be shipped in one or more container comprising, optionally (a) buffer(s), storage solutions and/or remaining reagents or materials required for the conduct of medical or scientific purposes. Furthermore, parts of the

diagnostic composition of the invention can be packaged individually in vials or bottles or in combination in containers or multicontainer units.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds.

In a most preferred embodiment of the present invention, the use of a bispecific binding molecule of the invention or a binding molecule produced by a process of the invention, of a vector or of a host of the invention for the preparation of a pharmaceutical composition is envisaged. Said pharmaceutical composition may be employed in the prevention, treatment or amelioration of a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, viral disease, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

The invention also relates to a method for the prevention, treatment or amelioration of a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, viral disease, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases comprising the administering an effective amount of a bispecific binding molecule of the invention or a binding molecule produced by a process of the invention, of a vector or of a host of the invention to a subject in need of such a prevention, treatment or amelioration. Preferably, said subject is a human. It is further envisaged, that the method of treatment further comprises the administration of an effective amount of a proteinaceous compound capable of providing an activation signal for immune effector cells. Preferably, said proteinaceous compound is administered simultaneously or non-simultaneously with a bispecific binding molecule of the invention or as produced by the process of the invention, a nucleic acid molecule, a vector or a host of the invention.

Finally, the invention provides for a kit comprising the bispecific binding molecule of the invention or as produced by the process of the invention, a nucleic acid

molecule, a vector or a host of the invention.

Said kit is particularly useful in the preparation of the pharmaceutical composition of the present invention and may, inter alia, consist of a container useful for injections or infusions. Advantageously, the kit of the present invention further comprises, optionally (a) buffer(s), storage solutions and/or remaining reagents or materials required for the conduct of medical or scientific purposes. Furthermore, parts of the kit of the invention can be packaged individually in vials or bottles or in combination in containers or multicontainer units. The kit of the present invention may be advantageously used, inter alia, for carrying out the method of the invention and could be employed in a variety of applications referred herein, e.g., as a research tools or medical tools. The manufacture of the kits preferably follows standard procedures which are known to the person skilled in the art.

In another aspect, the present invention relates to a bispecific binding molecule comprising or consisting of a first domain and a second domain, wherein: (a) said first domain specifically binds to/interacts with the human CD3 complex and comprises or consists of: (i) an antibody-derived light chain having the amino acid sequence of SEQ ID NO: 10, or (ii) an antibody-derived light chain encoded by the nucleic acid sequence of SEQ ID NO: 9; and (b) said second domain is or contains a further antigen-interaction-site.

In another aspect, the present invention relates to a nucleic acid molecule encoding the bispecific binding molecule mentioned above.

In another aspect, the present invention relates to a vector comprising the above mentioned nucleic acid molecule.

In another aspect, the present invention relates to a host cell transformed or transfected with the above mentioned vector.

In another aspect, the present invention relates to a process for the production of the above mentioned bispecific binding molecule, said process comprising culturing

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the above mentioned host cell under conditions allowing the expression of the bispecific binding molecule and recovering the produced bispecific binding molecule from the culture.

In another aspect, the present invention relates to a product comprising: (a) the above mentioned bispecific binding molecule, or as produced by the above mentioned process; (b) the above mentioned nucleic acid molecule; (c) the above mentioned vector; or (d) the above mentioned host cell.

In another aspect, the present invention relates to the use of: (a) the above mentioned bispecific binding molecule, or as produced by the above mentioned process; (b) the above mentioned nucleic acid molecule; (c) the above mentioned vector; or (d) the above mentioned host cell; for the preparation of a pharmaceutical composition for the prevention, treatment or amelioration of a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, viral disease, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

In another aspect, the present invention relates to the use of: (a) the above mentioned bispecific binding molecule, or as produced by the above mentioned process; (b) the above mentioned nucleic acid molecule; (c) the above mentioned vector; or (d) the above mentioned host cell; for the prevention, treatment or amelioration of a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, viral disease, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

In another aspect, the present invention relates to a kit comprising: (a) the above mentioned bispecific binding molecule, or as produced by the above mentioned process; (b) the above mentioned nucleic acid molecule; (c) the above mentioned vector; or (d) the above mentioned host cell; and a suitable container.

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These and other embodiments are disclosed and encompassed by the description and Examples of the present invention. Further literature concerning any one of the antibodies, methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries and databases, using for example electronic devices. For example, the public database "Medline", available on the Internet, may be utilized, for example under

<http://www.ncbi.nlm.nih.gov/PubMed/medline.html>. Further databases and addresses, such as <http://www.ncbi.nlm.nih.gov/>, <http://www.infobiogen.fr/>, http://www.fmi.ch/biology/research_tools.html, <http://www.tigr.org/>, are known to the person skilled in the art and can also be obtained using, e.g., <http://www.lycos.com> or <http://www.google.com>.

The figures show:

Figure 1.

A) The nucleotide and amino acid sequence of the humanized anti-CD3 antibody light chain and heavy chain (SEQ ID NOs.:9-12); **B)** the nucleotide and amino acid sequence of the bispecific anti-CD19xhum.anti-CD3 antibody (SEQ ID NO.:19, 20); **C)** the nucleotide and amino acid sequence of the bispecific anti-EpCAM (5-10) x hum. anti-CD3 antibody (SEQ ID NO.:35, 36); **D)** the nucleotide and amino acid

sequence of the bispecific anti-EpCAM (3-1) x hum. anti-CD3 antibody (SEQ ID NO.:33, 34),

Figure 2.

5 FACS analysis of the binding affinity of different constructs to CD3 and CD19 or EpCAM.

FACS analysis of CD3 binding was performed with CD3 positive Jurkat cells. **A)** Bispecific anti-CD19x hum. anti-CD3 antibody construct (SEQ ID NO.: 20). Binding to CD19 was shown with CD19 positive Nalm6 cells.; **B)** Bispecific anti-EpCAM (3-1)x hum. anti-CD3 antibody construct (SEQ ID NO.: 34). Binding to EpCAM was shown with EpCAM positive Katolll cells.; **C)** Bispecific anti-EpCAM (5-10)x hum. anti-CD3 antibody construct (SEQ ID NO.: 36). Binding to EpCAM was shown with EpCAM positive Katolll cells. A shift to the right shows binding.

15 **Figure 3:**

Elution pattern of bispecific anti-CD19x hum. anti-CD3 antibody containing protein fractions from a Zn-Chelating Fractogel® column.

High adsorption at 280 nm from 50-530 ml retention time was due to non-bound protein in the column flow-through. The arrow at the peak at 617.44 ml indicates the humanized bispecific construct containing protein fraction that was used or further purified.

Figure 4:

Protein elution pattern from a Sephadex S200® gel filtration column.

25 The protein peak at 82.42 ml containing bispecific antibody against anti-CD19xhum. anti- CD3 corresponds to a molecular weight of ca. 52 kD. Fractions were collected from 40-120 ml retention time.

Figure 5:

30 **A)** SDS-PAGE analysis of bispecific anti-CD19x hum. anti-CD3 antibody protein fractions. Lane M: Molecular weight marker, Lane 1: cell culture supernatant; lane 2: IMAC eluate; lane 3: gel filtration aggregate peak; lane 4: purified bispecific antibody anti- CD19x hum. anti-CD3;

B) Western blot analysis of purified bispecific anti-CD19xhum. anti-CD3 antibody
Lane M: Molecular weight marker, Lane 1: cell culture supernatant; lane 2: IMAC
eluate; lane 3: gel filtration aggregate peak; lane 4: purified bispecific antibody anti-
CD19 xhum. anti-CD3 obtained from gel filtration.

5

Figure 6

Cytotoxicity assay of bispecific anti-CD19x hum. anti-CD3 antibody (SEQ ID NO.:
20).

NALM-6 cells were used as target cells and CD4 positive CB15 T-cells as effector
10 cells in a E:T ratio of 1:10.

The invention will now be described by reference to the following biological
examples which are merely illustrative and are not to be construed as a limitation of
scope of the present invention.

15

Example 1.

Generation of humanized antibody specific for the CD3 antigen

The location of the CDRs of the CD3 specific antibody OKT3 was determined with
reference to Kabat, EA, et al. Sequences of Proteins of Immunological Interest. 5th
20 edition. 3 vols. Bethesda, MD: National Institutes of Health. National Center for
Biotechnology Information, 1991;2597. NIH publication no. 91-3242.

The human framework regions chosen to receive the transplanted CDRs were KOL
and REI for the heavy and light chains respectively. The structures of these proteins
have been solved crystallographically (REI: Palm(1975) Hoppe Seylers Z Physiol
25 Chem 356, 167-191, KOL: Schmidt (1983) Hoppe Seylers Z Physiol Chem 364, 713-
747.)

A number of additional, murine residues were introduced into the human variable
region frameworks according to Adair 1994 Hum. Antibod. Hybridomas, 5:41-48.
These residues that have been changed are important for retaining original antigen
30 specificity. Additional mutations were introduced in the CDR1, CDR2 and CDR3 of
the light chain. The CDR sequences of the humanized OKT and improved
humanized CD3 of the invention are shown in Table 2. The sequence of the
improved humanized CD3 binding molecule is shown in Figure 1A; SEQ ID No.9-12.

Table 2. The CDRs of the light chain of the CD3 specific antibody OKT3.

CDRs of anti-CD3	Amino acid sequence of humanized OKT3	Amino acid sequence of humanized CD3
L1	SASSSVSYMN	RASSSVSYMN (SEQ ID No.:4)
L2	DTSKLAS	DTSKVAS (SEQ ID No.:6)
L3	QQWSSNPFT	QQWSSNPLT (SEQ ID No.:8)

Example 2**5 Construction of a bispecific single chain antibody with humanized anti-CD3 part****Example 2.1****Construction of bispecific single-chain anti-CD19xanti-CD3 antibodies with humanized anti-CD3 part**

10 The DNA encoding the scFv of the resulting humanized antibody was obtained by gene synthesis and further subjected to genetic fusion with a CD19-specific scFv to obtain a bispecific single chain antibody (Fig 1B, SEQ ID NO.:19, 20). The bispecific single chain antibody was subcloned with the restriction enzymes EcoRI and Sall into the mammalian expression vector pEF-DHFR.

15

Example 2.2**Construction of bispecific single-chain anti-EpCAMxanti-CD3 antibodies with humanized anti-CD3 part**

In addition to the bispecific constructs described in Example 1.1 two further
 20 bispecific single chain antibodies with different tumor specificities were constructed. The CD19 specificity of the bispecific anti-CD19xhum. anti-CD3 was replaced by two selected EpCAM antibodies 5-10 and 3-1. Thus, two EpCAM-specific bispecific single chain antibody constructs anti-EpCAM(5-10)xhum. anti-CD3 (SEQ ID NO.:35, 36) and anti-EpCAM (3-1)xhum. anti-CD3 (SEQ ID NO.:33, 34) were obtained.

25

Example 3.**Expression of the bispecific single chain antibodies with humanized anti-CD3 part**

The anti-CD19xhum. anti-CD3 and anti-EpCAMxhum. anti-CD3 constructs (SEQ ID
 30 19, 20, 33, 34, 35, 36) were expressed by stable transfection into DHFR deficient Chinese hamster ovary (CHO) cells as described by Mack, M. et al. (1995) Proc

Natl Acad Sci USA 92, 7021-7025. Transfection of the expression vector was performed after calcium phosphate treatment of the cells (Sambrook et. al.1989).

Example 4.

5 FACS analysis of binding activity of the single chain bispecific antibodies with humanized anti-CD3 part

In order to test the functionality with regard to binding capability a FACS analysis was performed.

10 Example 4.1

Flow cytometric binding analysis of anti-CD19xhum. anti-CD3 bispecific antibody

CD19 positive Nalm 6 cells (human B cell precursor leukaemia) and CD3 positive Jurkat cells (human T cell leukemia) were used. 200,000 Nalm 6 cells and 200,000
15 Jurkat cells were incubated with 50 μ l the pure cell culture supernatant of CHO cells transfected with the anti-CD19xhum. anti-CD3 specific polypeptide for 30 min on ice. The cells were washed twice in PBS. Then the binding of the construct was detected via its C-terminal Histidin Tag with a murine Penta His antibody (diluted 1:20 in 50 μ l PBS with 2% FCS; Qiagen) followed by a washing step and a
20 Phycoerythrin conjugated Fc gamma specific antibody (Dianova), diluted 1:100 in 50 μ l PBS with 2% FCS (Figure 2A, thick line). As negative control fresh cell culture medium instead of cell culture supernatant was used (Figure 2, thin line).

Cells were analysed by flow cytometry on a FACS-Calibur (Becton Dickinson, Heidelberg). FACS staining and measuring of the fluorescence intensity were
25 performed as described in Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 2002). The binding activity of the bispecific binding molecule was compared to the binding activity of the corresponding control bispecific antibody with the humanized OKT3 part as described in prior art.

30 As shown in Fig. 2, both anti-CD19 xhum. OKT3 and anti-CD19xhum. anti-CD3 (improved hum. OKT3) bound well CD19 and CD3.

Example 4.2**Flow cytometric binding analysis of anti-EpCAMxhum. anti-CD3 bispecific antibody**

For testing of the binding abilities of the EpCAM specific bispecific antibodies the assay as described in Example 4.1 was repeated with following modifications: instead of Nalm 6 cells EpCAM positive Kato III cells were used (stomach carcinoma cell line; ATCC HTB-103) and the supernatants of the CHO cells tranfected with the EpCAM bispecific antibodies were applied. The results of the EpCAM binding assays are shown in Figs. 2B and 2C. A corresponding bispecific antibody with a humanized OKT3 as described in the prior art was used as a control.

As shown in Figs. 2B and 2C, the bispecific construct comprising the humanized anti-CD3 (SEQ ID Nos. 34, 36) of the invention show much better binding than the constructs with humanized OKT3.

Example 5.**Purification of the bispecific constructs with the improved humanized anti-CD3 part**

In order to purify the bispecific single chain constructs anti-CD19xhum. anti-CD3 stably transfected CHO cells were grown in roller bottles with HiClone® CHO modified DMEM medium (HiQ) for 7 days before harvest. The cells were removed by centrifugation and the supernatant, containing the expressed protein was stored at -20°C.

Äkta FPLC System® (Pharmacia) and Unicorn Software were used for chromatography. All chemicals were of research grade and purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

The humanized bispecific single chain construct proteins were isolated in a two step purification process including immobilized metal affinity chromatography (IMAC) and gelfiltration .

IMAC (immobilized metal affinity chromatography) was performed, using a Fractogel column® (Pharmacia) that was loaded with ZnCl₂ according to the manufacturers protocol. The column was equilibrated with buffer A2 (20 mM sodium phosphate pH 7.5, 0.4 M NaCl) and the cell culture supernatant (500ml) was applied to the column

(10 ml) with a flow rate of 3 ml/min. The column was washed with buffer A2 to remove unbound sample. Bound protein was eluted using a 2-step gradient of buffer B2 (20 mM sodium phosphate pH 7.5, 0.4 M NaCl, 0.5 M Imidazol) Step 1: 20% buffer B2 in 10 column volumes; Step2: 100% buffer B2 in 10 column volumes.

5 Eluted protein fractions from the 100% step were pooled for further purification.(Figure 3)

Gelfiltration chromatography was performed on a Sephadex S200 HiPrep column® (Pharmacia) equilibrated with PBS (Gibco). Eluted protein samples (flow rate 1ml/min) were subjected to SDS-PAGE and Western Blot for detection. The column
10 was previously calibrated for molecular weight determination (molecular weight marker kit, Sigma MW GF-200). (Fig. 4)

Protein concentrations of the purified constructs were determined using protein assay dye (Micro BCA, Pierce) and IgG (Biorad) as standard protein. The yields of the protein are shown in Table 2. All constructs could be purified from cell culture
15 supernatants. Comparable yields of purified protein were obtained for anti-CD19xhum. anti-CD3 (16 µg/ml) and anti-CD19xhum. OKT3 (13,6 µg/ml).

The purified product had a molecular weight of 52 kDa under native conditions as determined by gelfiltration in PBS.

SDS-PAGE of the purified bispecific protein was performed on precast 4-12% Bis
20 Tris gels (Invitrogen). Sample preparation and application were according to the manufacturers protocol. The molecular weight was determined with MultiMark protein standard® (Invitrogen). The gel was stained with colloidal Coomassie (Invitrogen protocol) showing a band at 52 kDa. The purity of the isolated protein was shown to be >95%.

25 Western Blot was performed with an Optitran BA-S83 membrane® and the Invitrogen Blot Module® according to the manufacturers protocol. The antibodies used were Penta His (Quiagen) and goat-anti-mouse-alkaline phosphatase (AP) (Sigma), the staining solution was BCIP/NBT (Sigma). The humanized bispecific protein was detected by Western Blot showing a 52kD band (Fig.5B). corresponding
30 to the purified bispecific protein in the Coomassie stained SDS-gel (Fig. 5A).

Example 6.

Bioactivity of bispecific antibodies with humanized anti-CD3 part

In order to certify the high cytotoxic activity of the constructed bispecific antibodies the following assays were performed.

Example 6.1

5 anti-CD19x hum. anti-CD3 bispecific antibody (SEQ ID NO.: 20)

Target NALM-6 cells (1.5×10^7) were labeled with 10 μ M calcein AM (Molecular Probes) for 30 min at 37°C in cell culture medium. After two washes in cell culture medium, cells were counted and mixed with CD4-positive CB15 T-cells. The resulting effector target cell mixture contained 2×10^5 Nalm6 cells and 2×10^6 CB15
10 cells per ml (E:T ratio of 1:10). Antibodies were diluted in RPMI/10% FCS to the required concentration. 50 μ l of this solution was added to the cell suspension and incubated at 37°C/5% CO₂ for 2 hours. After the cytotoxic reaction, the released dye in the incubation medium was quantitated in a fluorescence reader and compared with the fluorescence signal from a control reaction where the cytotoxic compound
15 was absent (negative control), and a reaction where the fluorescence signal was determined for totally lysed cells (for 10 min in 1% saponin) as positive control. On the basis of these readings, the specific cytotoxicity was calculated according to the following formula: [Fluorescence (Sample) - Fluorescence (Control)] : [Fluorescence (Total Lysis)- Fluorescence (Control)] x 100.

20 Sigmoidal dose response curves typically had R² values >0.97 as determined by Prism Software (GraphPad Software Inc., San Diego, USA). EC₅₀ values calculated by the analysis program were used for comparison of bioactivity. The cytotoxicity of the bispecific antibody against CD19 and CD3 with humanized CD3 part is shown in Figure 6. A corresponding bispecific antibody with a humanized OKT3 as described
25 in the prior art was used as a control.

In the bispecific format the bispecific humanized improved CD3 (hum. anti-CD3) (SEQ ID NO. 20) has clearly increased cytotoxic activity (EC₅₀ value 50 pg/ml) compared to the humanized OKT3 as described in Adair (EC₅₀ value 195 pg/ml). Thus, these results demonstrate the major advantage of the improved humanized
30 antibody binding to CD3 of the invention. Due to the about four-fold increase in cytotoxic activity of the improved humanized CD3 in the bispecific format this molecule is highly advantageous for therapeutic applications. Based on the stronger cytotoxic activity lower amounts of protein are required for therapy than of the prior

art molecules. Thus, the bispecific molecules of the invention provide an important advantage over the prior art antibodies when treating patients since they show at the same time a high cytotoxic activity and are less immunogenic due to humanization. They therefore offer a clear improvement in the medical field.

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JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

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Claims

1. A bispecific binding molecule comprising or consisting of a first domain and a second domain, wherein:
 - (a) said first domain specifically binds to/interacts with the human CD3 complex and comprises or consists of:
 - (i) an antibody-derived light chain having the amino acid sequence of SEQ ID NO: 10, or
 - (ii) an antibody-derived light chain encoded by the nucleic acid sequence of SEQ ID NO: 9; and
 - (b) said second domain is or contains a further antigen-interaction-site.
2. The bispecific binding molecule of claim 1, further comprising an effector domain.
3. The bispecific binding molecule of claim 1 or 2, wherein said first domain comprises or consists of:
 - (i) the amino acid sequence of SEQ ID NO: 14; or
 - (ii) the polypeptide encoded by the nucleic acid sequence of SEQ ID NO: 13.
4. The bispecific binding molecule of any one of claims 1 to 3, wherein said second domain is a further antigen-interaction-site specific for one or more cell surface molecules.
5. The bispecific binding molecule of claim 4, wherein said one or more cell surface molecule is a tumor specific molecule.
6. The bispecific binding molecule of claim 4 or 5, wherein said second domain is an scFv.
7. The bispecific binding molecule of any one of claims 4 to 6, wherein said

second domain specifically binds to/interacts with an antigen which is: EpCAM, CCR5, CD19, HER-2, HER-3, HER-4, EGFR, PSMA, CEA, MUC-1 (mucin), MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, bhCG, Lewis-Y, CD20, CD33, CD30, ganglioside GD3, 9-O-Acetyl-GD3, GM2, Globo H, fucosyl GM1, Poly SA, GD2, Carboanhydrase IX (MN/CA IX), CD44v6, Sonic Hedgehog (Shh), Wue-1, Plasma Cell Antigen, membrane-bound IgE, Melanoma Chondroitin Sulfate Proteoglycan (MCSP), CCR8, TNF-alpha precursor, STEAP, mesothelin, A33 Antigen, Prostate Stem Cell Antigen (PSCA), Ly-6, desmoglein 4, E-cadherin neoepitope, Fetal Acetylcholine Receptor, CD25, CA 19-9 marker, CA-125 marker, Muellierian Inhibitory Substance (MIS) Receptor type II, sTn (sialylated Tn antigen; TAG-72), FAP (fibroblast activation antigen), endosialin, EGFRvIII, L6, SAS, CD63, TF-antigen, Cora antigen, CD7, CD22, Ig α , Ig β , gp100, MT-MMPs, Fi9-antigen or CO-29.

8. The bispecific binding molecule of claim 7, wherein said second domain comprises or consists of:

- (a) amino acid sequences corresponding to SEQ ID NOs: 16 and 18; or
- (b) amino acid sequences encoded by the nucleic acid sequences corresponding to SEQ ID NOs: 15 and 17.

9. The bispecific binding molecule of claim 8, wherein said molecule comprises or consists of:

- (a) an amino acid sequence corresponding to SEQ ID NO: 20; or
- (b) an amino acid sequence encoded by the nucleic acid sequence corresponding to SEQ ID NO: 19.

10. The bispecific binding molecule of claim 7, wherein said second domain comprises or consists of:

- (a) amino acid sequences corresponding to SEQ ID NOs: 22 and 24, 26 and 28, or 30 and 32; or
- (b) amino acid sequences encoded by the nucleic acid sequences corresponding to SEQ ID NOs: 21 and 23, 25 and 27, or 29 and 31.

11. The bispecific binding molecule of claim 10, wherein said molecule comprises or consists of:
- (a) an amino acid sequence corresponding to SEQ ID NO: 34 or 36; or
 - (b) an amino acid sequence encoded by the nucleic acid sequence corresponding to SEQ ID NO: 33 or 35.
12. The bispecific binding molecule of any one of claims 4 to 8 or 10, wherein said second domain is humanized.
13. A nucleic acid molecule encoding the bispecific binding molecule of any one of claims 1 to 12.
14. The nucleic acid molecule of claim 13 comprising:
- (a) a nucleic acid sequence encoding the mature form of a protein comprising the amino acid sequence corresponding to SEQ ID NO: 20,34 or 36; or
 - (b) a nucleic acid sequence comprising or consisting of the nucleic acid sequence corresponding to SEQ ID NO: 19,33 or 35.
15. A vector comprising the nucleic acid molecule of claim 13 or 14.
16. The vector of claim 15, which further comprises a regulatory sequence operably linked to the nucleic acid molecule of claim 13 or 14.
17. The vector of claim 15 or 16, wherein the vector is an expression vector.
18. A host cell transformed or transfected with the vector of any one of claims 15 to 17.
19. A process for the production of the bispecific binding molecule of any one of claims 1 to 12, said process comprising culturing the host cell of claim 18 under conditions allowing the expression of the bispecific binding molecule and recovering the produced bispecific binding molecule from the culture.

20. A composition comprising:
- (a) the bispecific binding molecule of any one of claims 1 to 12, or as produced by the process of claim 19;
 - (b) the nucleic acid molecule of claim 13 or 14;
 - (c) the vector of any one of claims 15 to 17; or
 - (d) the host cell of claim 18; and

a pharmaceutically acceptable carrier, stabilizer and/or excipient.

21. The composition of claim 20, further comprising a proteinaceous compound for providing an activation signal for immune effector cells.

22. The composition of claim 20 or 21 which is a diagnostic composition further comprising a component for detection of proliferative diseases, tumorous diseases, inflammatory diseases, immunological disorders, autoimmune diseases, infectious diseases, viral diseases, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

23. Use of:
- (a) the bispecific binding molecule of any one of claims 1 to 12, or as produced by the process of claim 19;
 - (b) the nucleic acid molecule of claim 13 or 14;
 - (c) the vector of any one of claims 15 to 17; or
 - (d) the host cell of claim 18,

for the preparation of a pharmaceutical composition for the prevention, treatment or amelioration of a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, viral disease, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

24. Use of:
- (a) the bispecific binding molecule of any one of claims 1 to 12, or as produced by the process of claim 19;
 - (b) the nucleic acid molecule of claim 13 or 14;
 - (c) the vector of any one of claims 15 to 17; or

(d) the host cell of claim 18,
for the prevention, treatment or amelioration of a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, viral disease, allergic reactions, parasitic reactions, graft-versus-host diseases or host-versus-graft diseases.

25. A kit comprising:

- (a) the bispecific binding molecule of any one of claims 1 to 12, or as produced by the process of claim 19;
- (b) the nucleic acid molecule of claim 13 or 14;
- (c) the vector of any one of claims 15 to 17; or
- (d) the host cell of claim 18,

in a suitable container.

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Figure 1A**Hum. anti-CD3 VL nt**

GACATCCAGATGACCCAGTCTCCATCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCA
CTTGCAAGCAAGTTCAAGCGTAAGCTACATGAATTGGTATCAGCAGACACAGGAAAGCCCC
TAAGAGATGGATCTATGACACATCCAAGTGGCTTCTGGGTCCCATCAAGGTTCAGTGGCAGT
GGATCTGGGACAGATTACACTTTCACCATCAGCAGTCTGCAACCTGAAGATATTGCAACTTACT
ACTGTCAACAGTGGAGTAGTAACCTCTCACTTTGGCCAGGGACCAAGCTGCAGATCACC

Hum. anti-CD3 VL AA

DIQMTQSPSSLASVGDRTVTITCRASSSVSYMNWYQQTPGKAPKRWIYDTSKVASGVPSRFSGS
GSGTDYTFITISLQPEDIAITYYCQQWSSNPLTFGQGTKLQIT

Hum. anti-CD3 VH nt

CAGGTGCAGCTGGTGCAGTCTGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCCTGAGACTCTCCT
GTAAGTCTTCTGGATACACCTTCACTAGGTATACGATGCACTGGGTCCGCCAGGCTCCAGGGAA
GGGCTGGAGTGGATTGGATACATAAATCCTAGCCGTGGTTACTAATTATAATCAGAAGGTG
AAGGACCGATTCACCATCTCCAGAGACAACCTCCAAGAACACGGCCTTCTGCAAAATGGACAGCC
TGAGACCCGAGGACACGGGTGTGTATTCTGTGCGAGATATTATGATGATCATTTACTGCCCTTGA
CTACTGGGCCAGGGCACCCCGGTCAACCGTCTCCTCA

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Figure 1A (cont.)**Hum. anti-CD3 VH AA**

QVQLVQSGGCVVQPGRLSLCKSSGYTFTRYTMHWVRQAPGKLEWIGYINPSRGYTNYNQKV
KDRFTISRDN SKNTAF LQMDSLRPEDTGVYFCARYYDDHYCLDYWGQGTPTVTVSS

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Figure 1B

TGTACACTCCGATATCCAGCTGACCCAGTCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACCAT
CTCCTGCAAGGCCAGCCAAAGTGTGATTATGATGGTATAGTTATTGAACCTGGTACCAACAGATTCCAGG
ACAGCCACCCAAACTCCTCATCTATGATGCATCCAATCTAGTTTCTGGGATCCCACCCAGGTTTAGTGGCAG
TGGGTCTGGGACAGACTTCACCCCTCAACATCCATCCTGTGGAGAAGGTGGATGCTGCAACCCTATCACTGTCA
GCAAGTACTGAGGATCCGTGGACGTTCCGTGGAGGGACCAAGCTCGAGATCAAAGGTGGTGGTCTCTGG
CGCGGGCTCCGGTGGTGGTCTCAGGTGCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCCTGG
GTCCCTCAGTGAAGATTTCCTGCAAGGCTTCTGGCTATGCATTCACTAGTACTGGATGAACTGGGTGAAGCA
GAGGCCCTGGACAGGGTCTTGAGTGGATTGGACAGATTGGCCCTGGAGATGGTGATATACTAACTACAATGGAAA
GTTCAAGGGTAAAGCCACTCTGACTGCAGACGAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCCTAGC
ATCTGAGGACTCTGCGGTCTATTCTGTGCAAGACGGGAGACTACGACGGTAGGCCGTTATTACTATGCTAT
GGACTACTGGGGCCAAAGGGACCAACCGTCAACCGTCTCCTCCGGAGGTGGTGGCTCCAGGTGCAGCTGGTGCA
GTCTGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTAAGTCTTCTGGATACACCTTCAC
TAGGTATACGATGCACTGGGTCCGCCAGGCTCCAGGGAAGGGCTGGAGTGGATTGGATACATAAATCCTAG
CCGTGGTTATACTAATTATAATCAGAAAGGTGAAGGACCGATTCAACCATCTCCAGAGACAACCTCCAAGAACAC
GGCCTTTCTGCAAAATGGACAGCCTGAGACCCGAGACACGGGTGTGTATTCTGTGCGAGATATTATGATGA
TCATTACTGCCCTTGACTATTGGGGCCAGGGCACCCCGTCAACCGTCTCCTCAGTCGAAAGGTGGAAAGTGGAGG
TTCTGGTGGAAAGTGGAGGTTCAAGGTGGAGTGGACGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGC
ATCTGTAGGAGACAGAGTCAACCATCACTTGCAGAGCAAGTTCAAGCGTAAGCTACATGAATTGGTATCAGCA
GACACCAGGGAAGCCCCCTAAGAGATGGATCTATGACACATCCAAAGTGGCTTCTGGGGTCCCATCAAGGTT
CAGTGGCAGTGGATCTGGGACAGATTACACTTTCACCATCAGCAGTCTGCAACCTGAAGATATTGCAACTTA
CTACTGTCAACAGTGGAGTAGTAACCCCTCTCACTTTTGGCCAGGGGACCAAGCTGCAGATCACC

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Figure 1B (cont.)

DIQLTQSPASLAVSLGQRATISCKASQSVDDYDGD SYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGSGSG
TDFTLNIHPVEKVDAATYHCQQSTEDPWTFFGGGSKLEIKGGGGGGGQVQLQQSGAELVRPGSSV
KISCKASGYAFSSYWMNVVKQRPQGQGLEWIGQIWPGDGTNYNGKFKGKATLTADESSSTAYMQLSSLASED
SAVYFCARRETTVGRIYYAMDYWGQGTTVVSSGGGQVQLVQSGGGVQVQGRSLRLSCKSSGYTFFRYT
MHWVRQAPGKGLEWIGYINPSRGYTNYNQVKDRFTISRDNSKNTAFLQMDSLRPEDTGVYFCARYYDDHYC
LDYWGQGTPTVSSVEGGSGGGGGVDDIQMTQSPSSLSASVGDRTITCRASSSVSYMNWYQQTPG
KAPKRWIYDTSKVASGVPSRFSGSGGTDYFTISSLQPEDIATYYCQQWSSNPLTFGQGTKLQIT

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Figure 1C

TGTACACTCCGAGCTCGTGATGACACAGTCTCCATCCTCCCTGACTGTGACAGCAGGAGAGAAGGTCACTAT
GAGCTGCAAGTCCAGTCAGAGTCTGTTAAACAGTGGAAATCAAAAGAACTACTTGACCTGGTACCAGCAGAA
ACCAGGGCAGCCTCCTAAACTGTTGATCTACTGGGCATCCACTAGGGAATCTGGGTCCCTGATCGCTTCAC
AGGCAGTGGATCTGGAACAGATTTCACTCTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTA
CTGTCAGAAATGATTATAGTTATCCGCTCACGTTCTGGTGGGACCAAGCTTGAGATCAAAGGTGGTGGTGG
TTCTGGCGGCGGCTCCGGTGGTGGTCTGAGGTGCAGCTGCTCGAGCAGTCTGGAGCTGAGCTGGT
AAGCCTGGGACTTCAGTGAAGATATCCTGCAAGGCTTCTGGATACGCCCTTCACTAAGTGGTGGTGGTGG
GGTAAAGCAGAGGCCCTGGACATGGACTTGAGTGGATTGGAGATATTTTCCCTGGAGTGGTAATATCCACTA
CAATGAGAAGTTCAAGGGCAAGCCACACTGACTGCAGACAAATCTTCGAGCACAGCCTATATGCAGCTCAG
TAGCCTGACATTTGAGGACTCTGCTGTCTATTTCTGTGCAAGACTGAGGAACCTGGACGAGCCTATGGACTA
CTGGGGCCAAAGGACCACGGTCACCGTCTCCTCCGGAGGTGGTGGCTCCAGGTGCAGCTGGTGCAGTCTGG
GGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTAGTCTTCTGGATACACCTTCACTAGGTA
TAAGATGCACTGGTCCGCCAGGCTCCAGGGAAGGGCTGGAGTGGATTGGATACATAAATCCTAGCCGTGG
TTATACTAATTATAATCAGAAGGTGAAGGACCGATTACCATCTCCAGAGACAACTCCAAGAACACGGCCTT
TCTGCAATGGACAGCCTGAGACCCGAGGACACGGGTGTATTTCTGTGCGAGATATTATGATGATCATTA
CTGCCCTTGACTATTGGGGCCAGGGCACCCGGTCACCGTCTCCTCAGTCGAGGTGGAAGTGGAGGTCTCTGG
TGGAAGTGGAGGTTCAAGGTGGAGTGGACGACATCCAGATGACCCAGTCTCCATCCCTGCTGCATCTGT
AGGAGACAGAGTCACCATCACTTGCAGAGCAAGTTCAAGCGTAAGCTACATGAATTGGTATCAGCAGACACC
AGGGAAGCCCCTAAGAGATGGATCTATGACACATCCAAAGTGGCTTCTGGGTCCCATCAAGGTTCAAGTGG
CAGTGGATCTGGGACAGATTACACTTTCACCATCAGCAGTCTGCAACCTGAAGATATTGCAACTTACTACTG
TCAACAGTGGAGTAGTAACCCCTCTCACTTTTGGCCAGGGACCAAGCTGCAGATCACC

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Figure 1C (cont.)

ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNLSGNQKNYLTWYQQKPGQPPLLIIYWASTRESGVPDRFTGSG
SGTDFTLTISSVQAEDLAVYYCQNDYSYPLTFGAGTKLEIKGGGGSGGGSEVQLLEQSGAELVRPG
TSVKISCKASGYAFTNYWLGWVKQRPQGHLEWIGDIFPGSGNIHYNEKFKGKATLTADKSSSTAYMQLSLT
FEDSAVYFCARLRNWDPEPMDYWGQGTTVTVSSGGGSQVLVQSGGVVQPGRSLRLSCKSSGYTFTTRYTMH
WVRQAPGKGLEWIGYINPSRGYTNYNQVKDRFTISRDN SKNTAFLQMDSLRPEDTG VYFCARYYDDHYCLD
YWGQGTPTVTVSSVEGGSGGGSGGVDDIQMTQSPSSLSASVGDRTVITCRASSSVSYMNWYQQTPGKA
PKRWIYDTSKVASGVPSRFSGSGGTDYFTFTISSLQPEDIAITYCQQWSSNPLTFGQGTKLQIT

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Figure 1D

GAGCTCGTCATGACCCAGTCTCCATCTTATCTTGCTGCATCTCCTGGAGAAACCAATTACTATTAAATTGCAGG
GCAAGTAAGAGCATTAGCAAATATTTAGCCTGGTATCAAGAGAAACCTGGGAAACCTAATAAGCTTCTTATC
TACTCTGGATCCACTTTTGCAATCTGGAATTCACATCAAGTTCAGTGGAGTCTGGTACAGATTTTCACT
CTCACCATCAGTAGCCTGGAGCCTGAAGATTTTGCAATGTATTACTGTCAACAGCATAAATGAATATCCGTAC
ACGTTGAGGGGGACCAAGCTTGAGATCAAAGTTGGTGGTCTGGCCGGCGGCTCCGGTGGTGGT
GGTTCAGGTGCAGCTGCTCGAGCAGTCTGGAGCTGAGCTGGTGAACCTGGGCTCAGTGAAGATATCC
TGCAAGGCTTCTGGATACGCCCTTCACTAACTAGCTGGTAAAGCAGAGGCCCTGGACATGGACTT
GAGTGGATTGGAGATCTTTCCCTGGAAGTGGTAATACTCACTACAATGAGAGGTTCAAGGGCAAGCCACA
CTGACTGCAGACAAATCCTCGAGCACAGCCTTTATGCAGCTCAGTAGCCTGACATCTGAGGACTCTGTGTC
TATTTCTGTGCAAGATTGAGGAACCTGGGACGAGGCTATGGACTACTGGGCCAAGGACCAACGGTCAACCGTC
TCCTCCGGAGGTGGATCCAGGTGCAGCTGGTGCAGTCTGGGGAGGCGGTGTCAGCCCTGGGAGGTCC
CTGAGACTCTCCTGTAAGTCTTCTGGATACACCTTCACTAGGTATACGATGCACCTGGTCCGCCAGGCTCCA
GGGAAGGGCTGGAGTGGATTGGATACATAAAATCCTAGCCGTGGTTATACTAATTAATCAGAAAGGTGAAG
GACCGATTCAACCATCTCCAGAGACAACCTCCAAGAACACGGCCTTCTGCAAAATGGACAGCCTGAGACCCGAG
GACACGGGTGTATTTCTGTGCGAGATATTAATGATGATCATTAATGACTTATGGGGCCAGGCAACC
CCGGTCACCGTCTCCTCAGTCGAAGGTGGAAGTTCCTGGTGAAGTGGAGGTTTCAGGTGGAGTGGAC
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCATCTGTAGGAGACAGAGTCACCATCACTTGCAGA
GCAAGTTCAGCGTAAGCTACATGAATTGGTATCAGCAGACACCAAGGAAAGCCCTAAGAGATGGATCTAT
GACACATCCAAAGTGGCTTCTGGGGTCCCATCAAGGTTCAAGTGGCAGTGGATCTGGACAGATTACACTTTC
ACCATCAGCAGTCTGCAACCTGAAGATATTGCAACTTACTACTGTCAACAGTGGAGTAGTAACCCCTCTCACT
TTTGCCAGGGGACCAAGCTGCAGATCACCC

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Figure 1D (cont.)

ELVMTQSPSYLAASPGETITINCRASKSISKYLAWYQEKPGKTNKLLIYSGSTLQSGIPSRFSGSGSGTDFT
LTISSEPEDEFAMYYCQQHNEYPYTFGGGTKLEIKGGGGGGGGSEVQLLEQSGAELVKPGASVKIS
CKASGYAFTNYWLGWVKQRPGHGLEWIGDLFPGSGNTHYNERFRGKATLTADKSSSTAFMQLSLTSEDSAV
YFCARLRNWDYWGQGTFTVSSGGGSQVQLVQSGGVVQPGRSLRLSCKSSGYTFTTRYTMHWVRQAP
GKGLEWIGYINPSRGYTNYNQVKDRFTISRDNSTAFMQMDSLRLPDTGVYFCARYYDDHYCLDYWGQGT
PVTVSSVEGGSGGGSGGVDDIQMTQSPSSLSASVGDRTITCRASSSVSYMNWYQQTPGKAPKRWIY
DTSKVASGVPSRFSGSGGTDTFTISSLQPEDIAITYCQQWSSNPLTFGGQGTKLQIT

Figure 2A

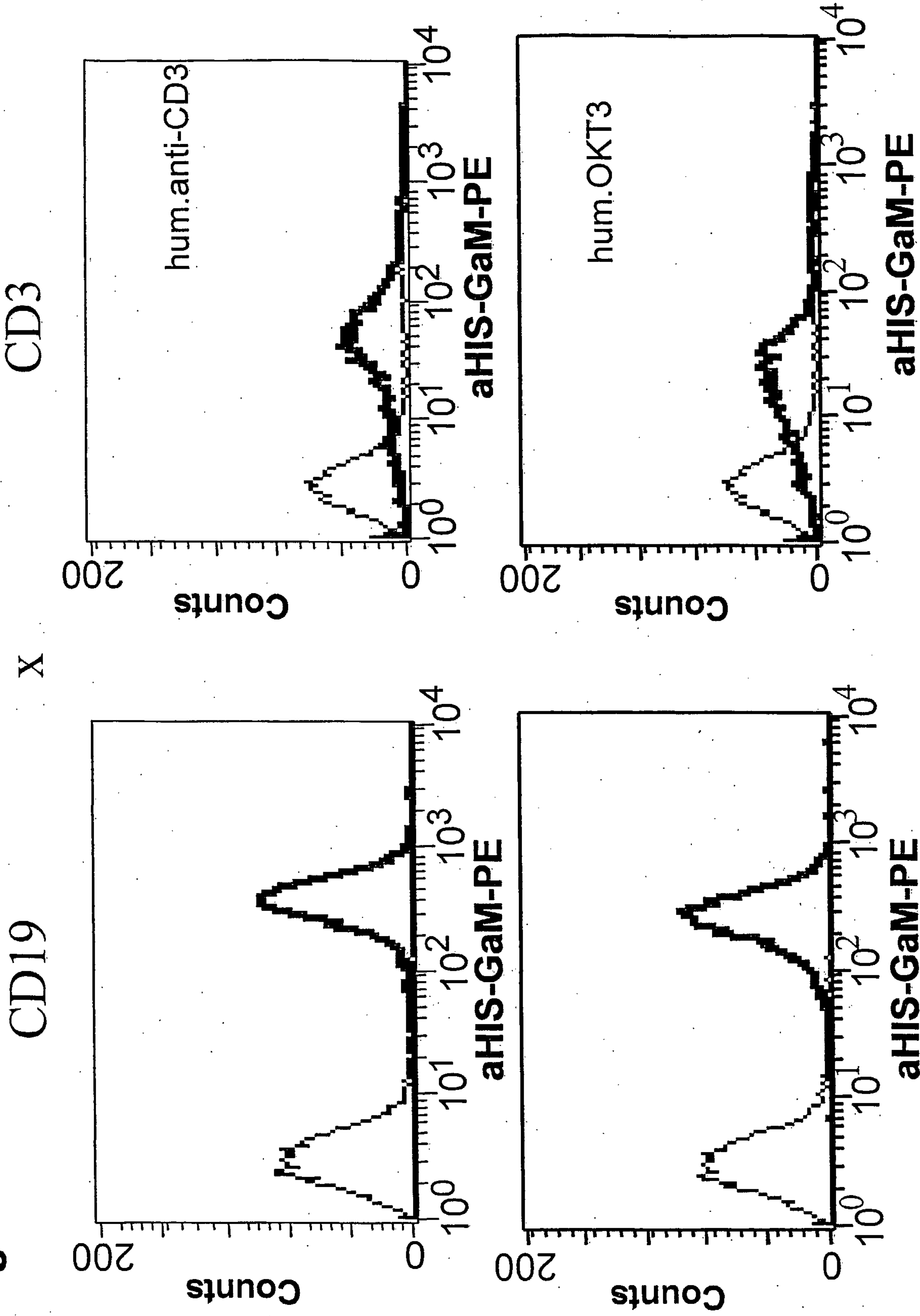
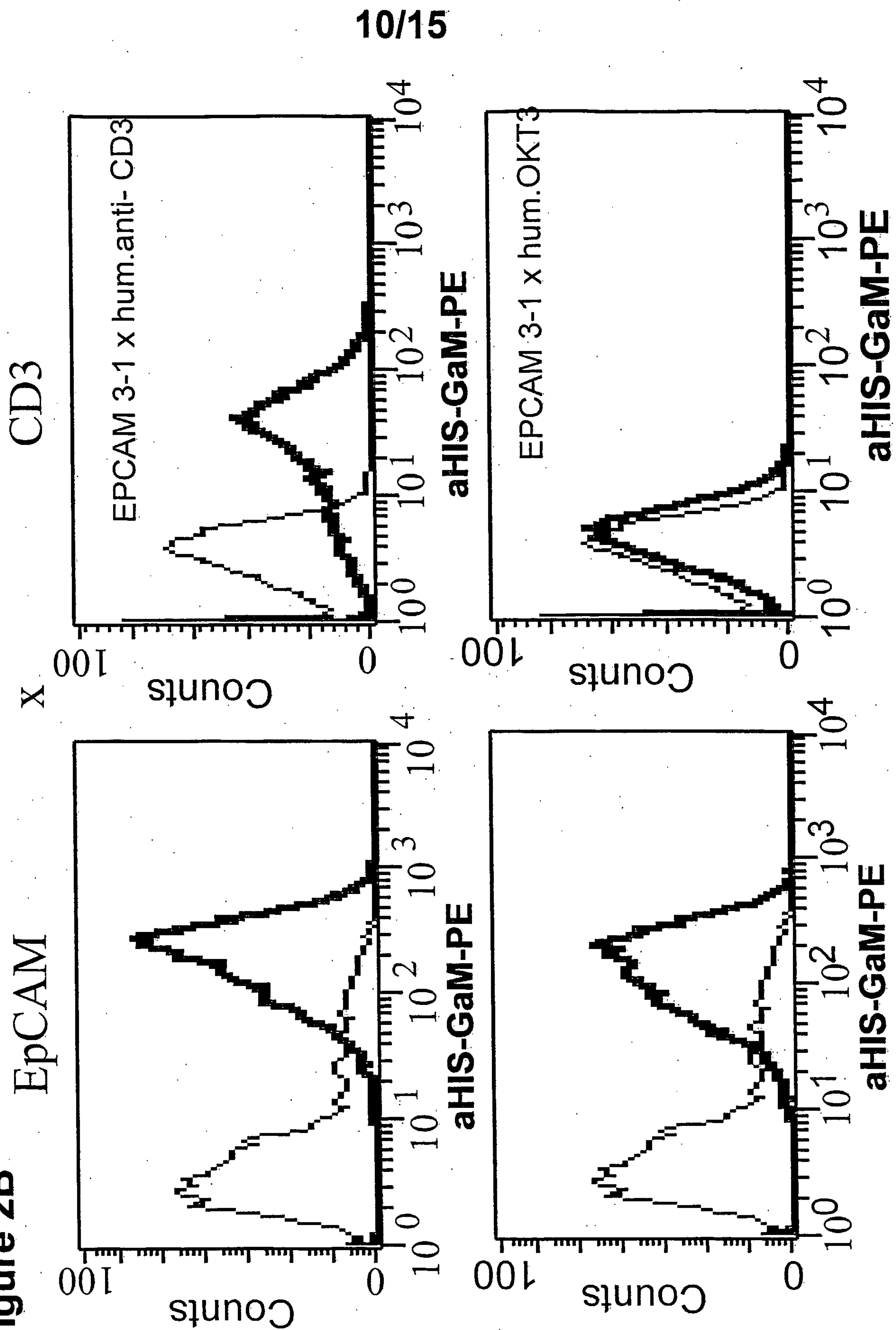
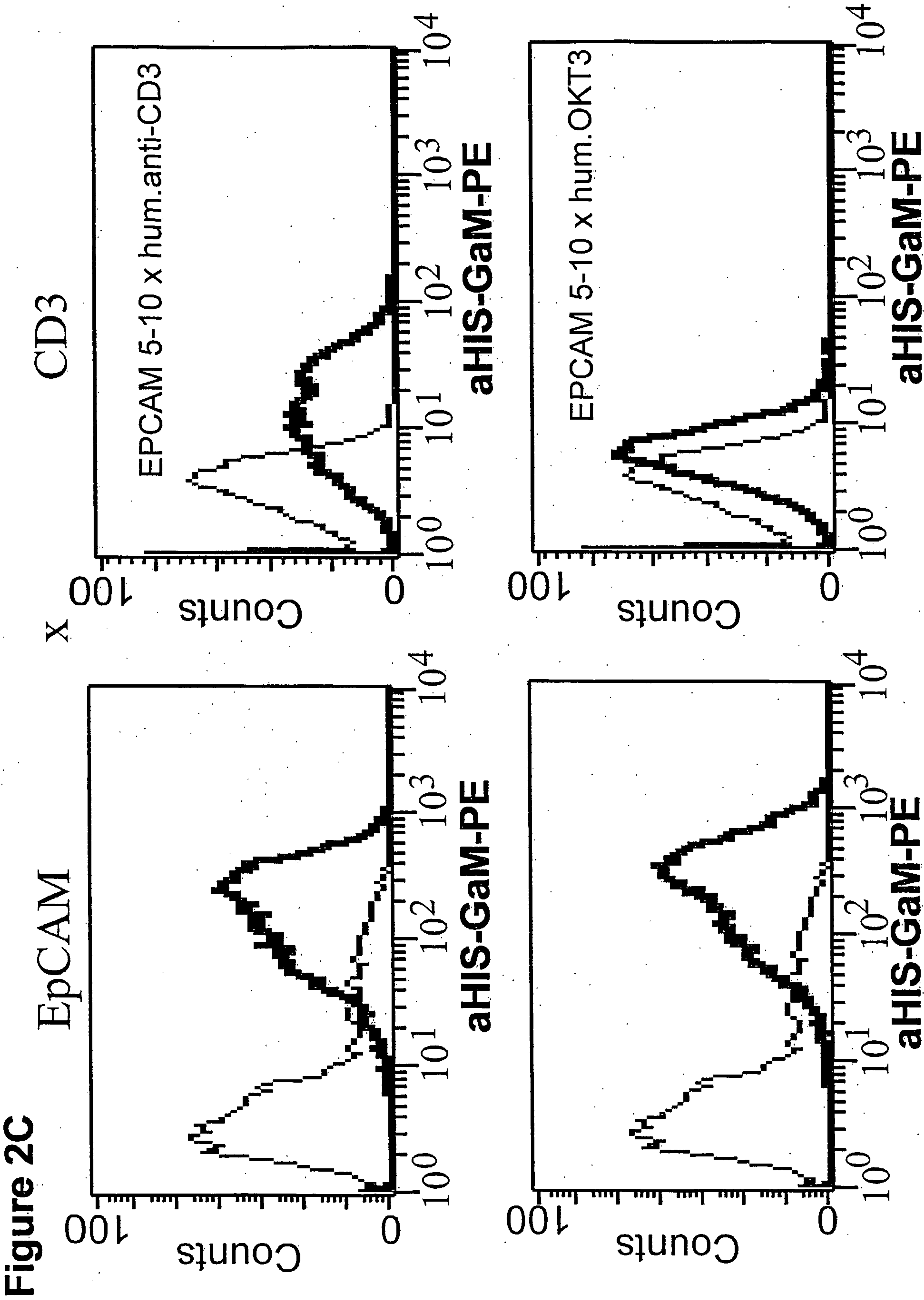


Figure 2B



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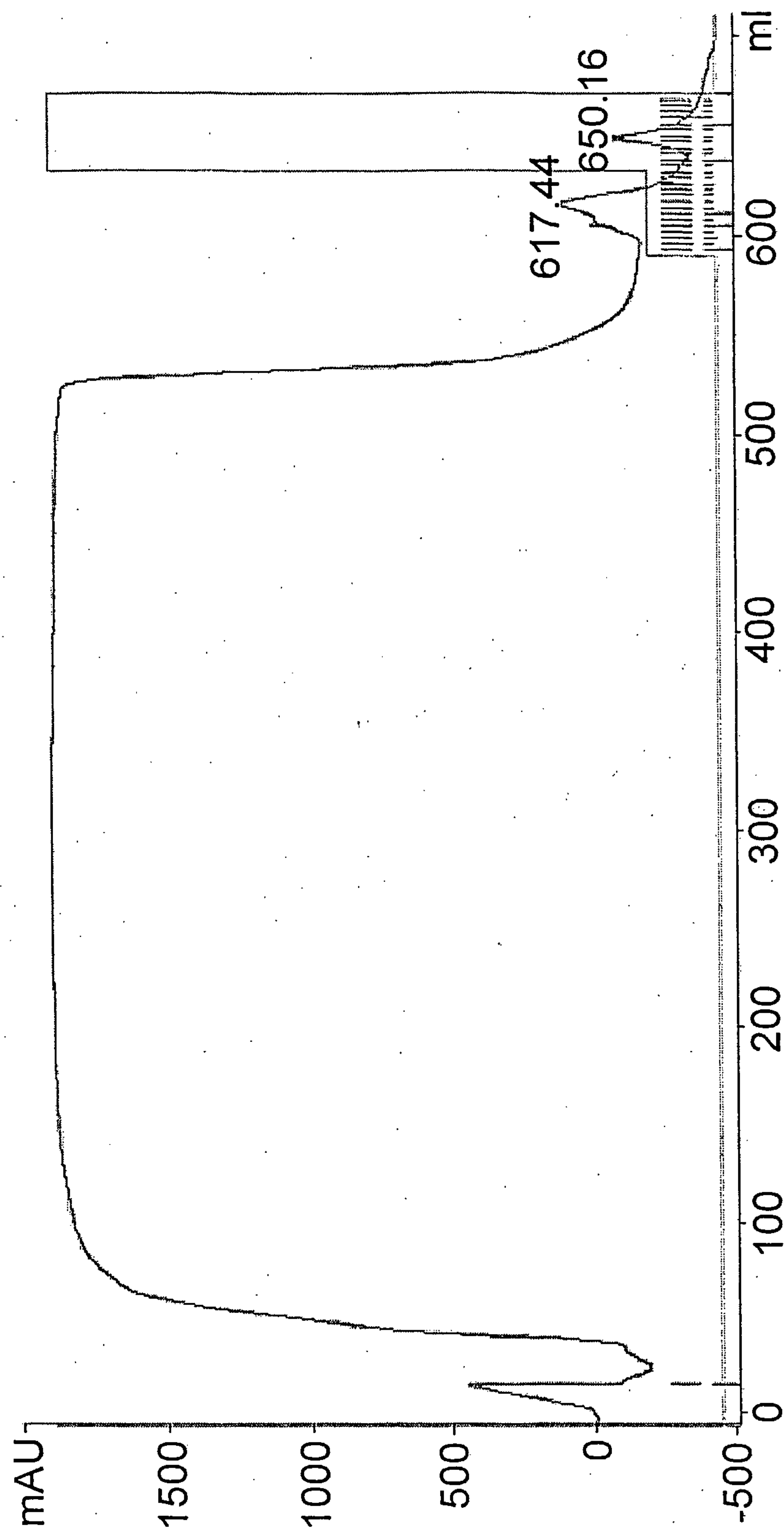


Figure 3

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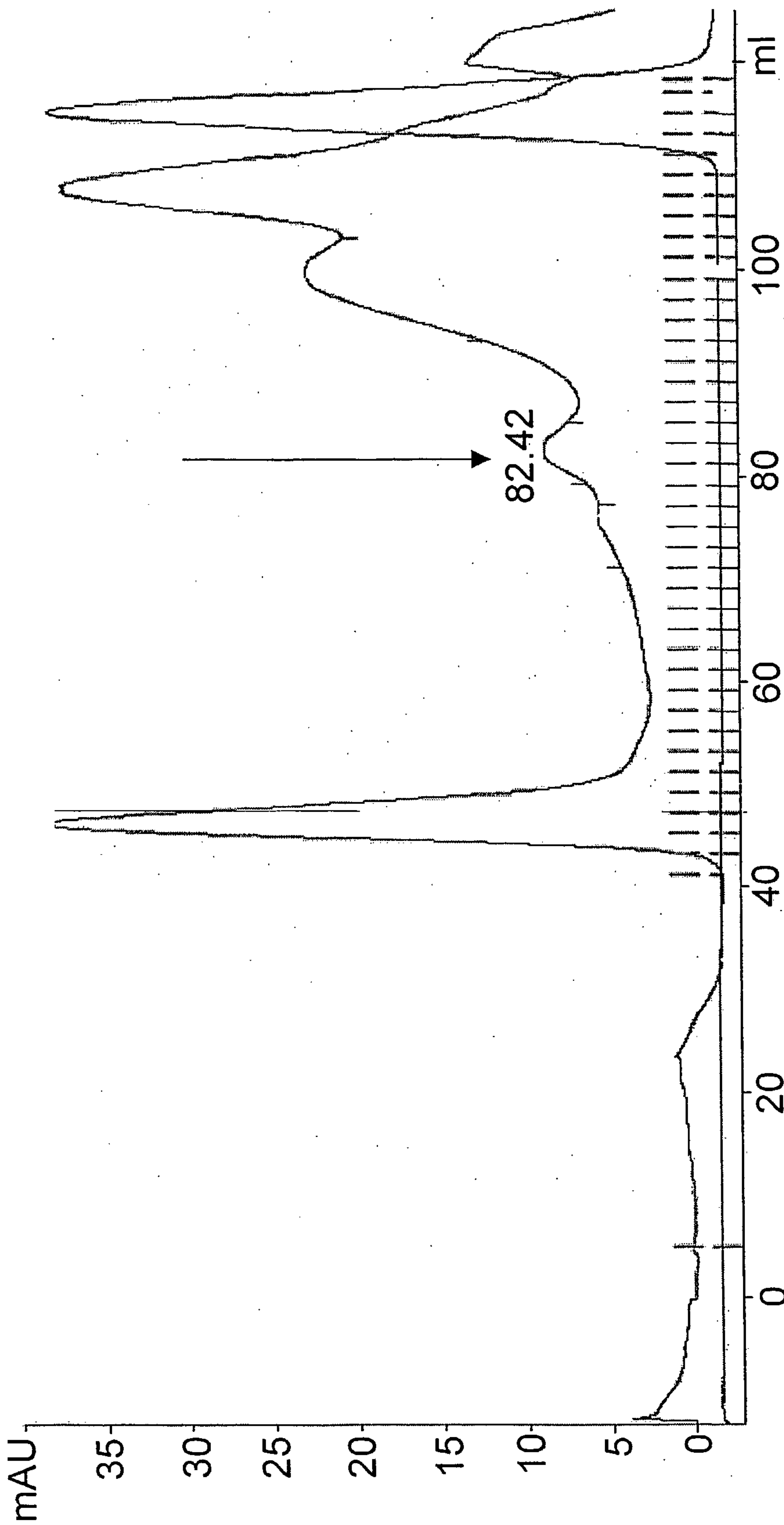
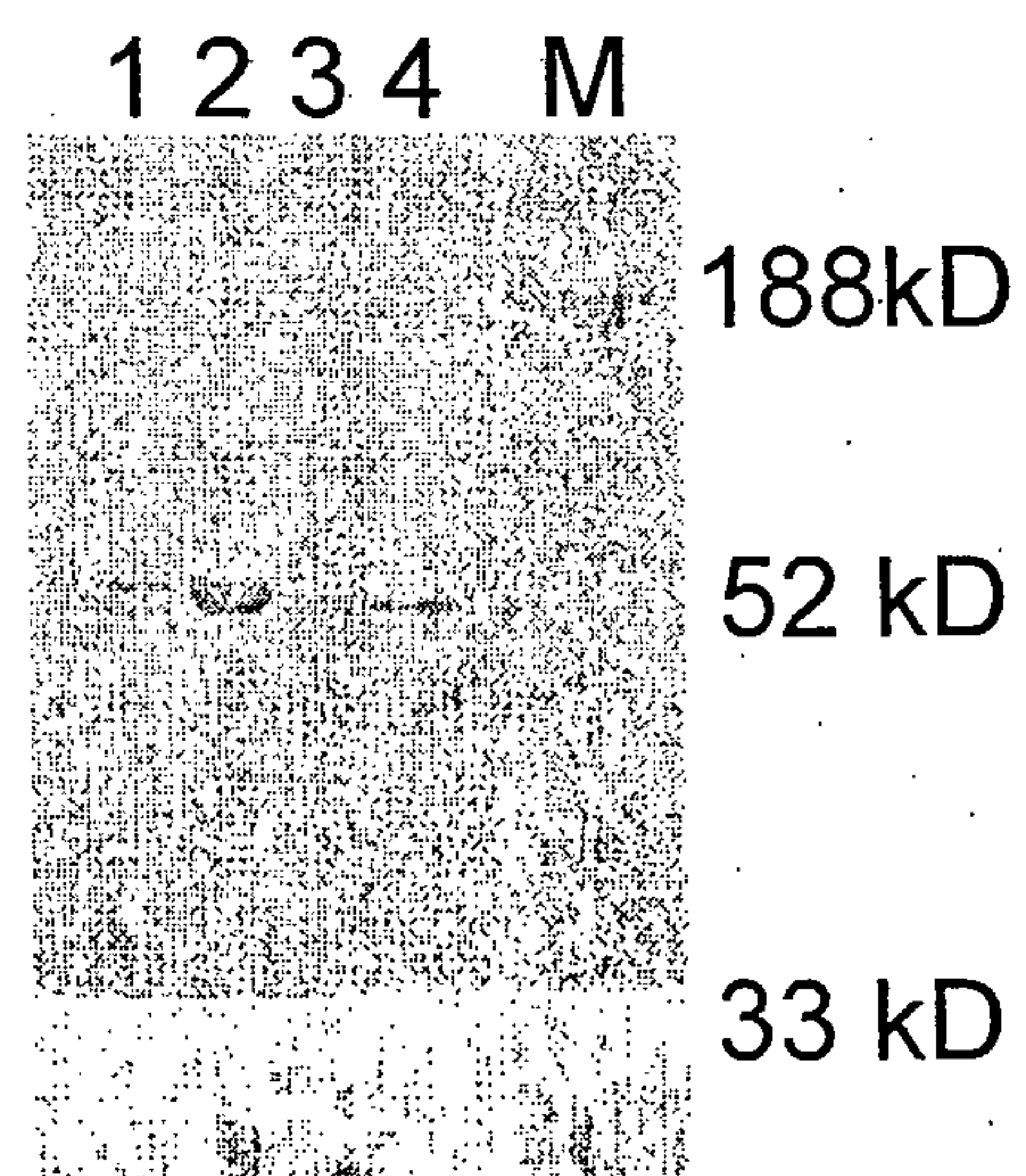
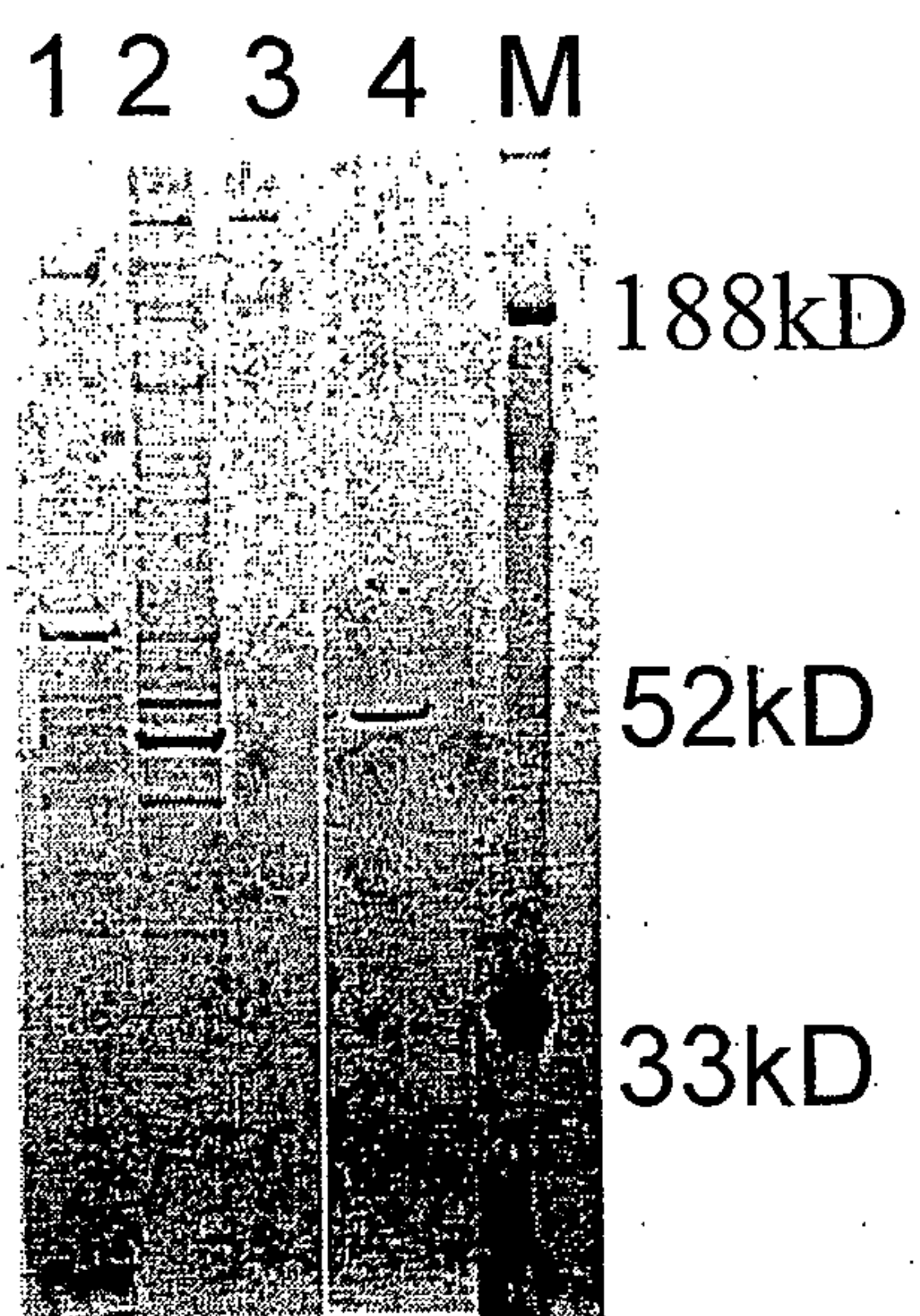


Figure 4

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Figure 6

