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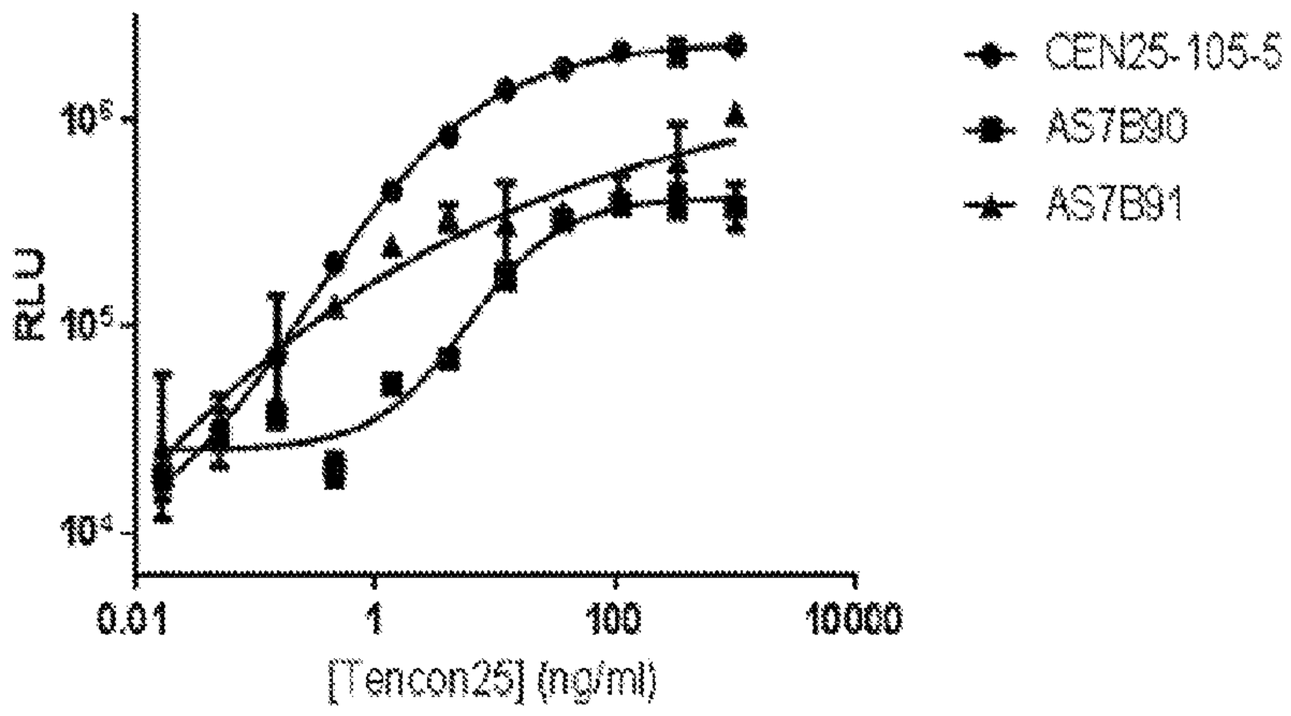
(54) Titre : REGIONS DE LIAISON A UN ANTIGENE DIRIGEES CONTRE LES DOMAINES DE LA FIBRONECTINE DE TYPE III ET LEURS PROCEDES D'UTILISATION

(54) Title: ANTIGEN BINDING REGIONS AGAINST FIBRONECTIN TYPE III DOMAINS AND METHODS OF USING THE SAME

Figure 1A



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EC50	0.2898	5.94	~ 3.553e-015

(57) Abrégé/Abstract:
Fibronectin type III (FN3) domain antibodies, polynucleotides capable of encoding the FN3 domain antibodies or antigen-binding fragments, cells expressing FN3 domain antibodies or antigen-binding fragments, as well as associated vectors and detectably labeled FN3 domain antibodies or antigen-binding fragments may be used to engineer FN3 domain-targeting chimeric antigen receptors (CARs). Methods of making the FN3 domain antibodies, CARs, and engineered immune cells, and methods of using the engineered immune cells are applicable to treat diseases including cancer.

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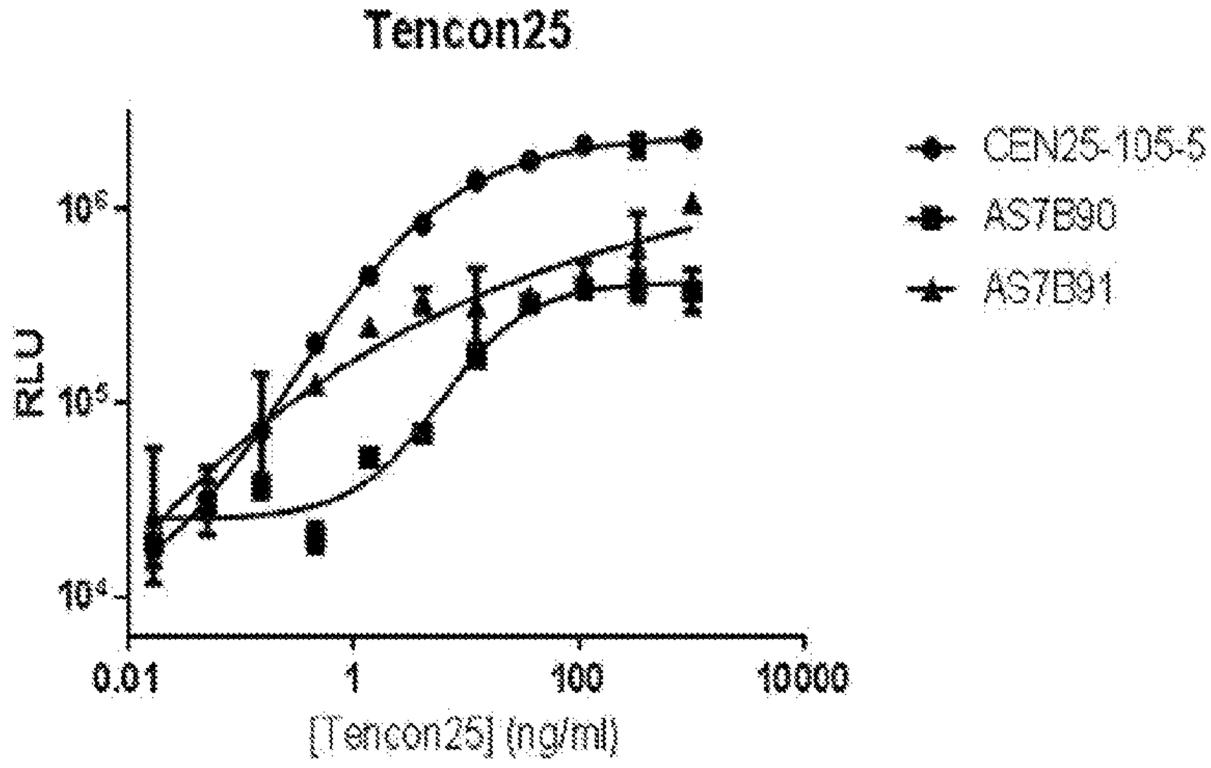

	CEN25-105-5	AS7B90	AS7B91
EC50	0.2896	5.94	~ 3.553e-015

Figure 1A

(57) Abstract: Fibronectin type III (FN3) domain antibodies, polynucleotides capable of encoding the FN3 domain antibodies or antigen-binding fragments, cells expressing FN3 domain antibodies or antigen-binding fragments, as well as associated vectors and detectably labeled FN3 domain antibodies or antigen-binding fragments may be used to engineer FN3 domain-targeting chimeric antigen receptors (CARs). Methods of making the FN3 domain antibodies, CARs, and engineered immune cells, and methods of using the engineered immune cells are applicable to treat diseases including cancer.

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ANTIGEN BINDING REGIONS AGAINST FIBRONECTIN TYPE III DOMAINS AND METHODS OF USING THE SAME

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on June 29, 2018, is named JBI5032WOPCTSL.txt and is 88,120 bytes in size.

TECHNICAL FIELD

The present invention relates to antibodies that bind specifically to fibronectin type III domains (FN3 domains) and methods of producing and using the described antibodies.

BACKGROUND

Fibronectin based scaffolds are a family of proteins capable of evolving to bind any compound of interest. These proteins, which generally make use of a scaffold derived from a fibronectin type III (FN3) or FN3-like domain, function in a manner characteristic of natural or engineered antibodies (that is, polyclonal, monoclonal, or single-chain antibodies) and, in addition, possess structural advantages. Specifically, the structure of these antibody mimics has been designed for optimal folding, stability, and solubility, even under conditions that normally lead to the loss of structure and function in antibodies. An example of fibronectin-based scaffold proteins is the Centyrin™ (Jacobs *et al.,* Protein Engineering, Design, and Selection, 25:107-117, 2012; US2010/0216708).

Fibronectin type III (Fn3) domains comprise, in order from N-terminus to C-terminus, a beta or beta-like strand, A; a loop, AB; a beta or beta-like strand, B; a loop, BC; a beta or beta-like strand C; a loop CD; a beta or beta-like strand D; a loop DE; a beta or beta-like strand, E; a loop, EF; a beta or beta-like strand F; a loop FG; and a beta or beta-like strand G. Any or all of loops AB, BC, CD, DE, EF and FG may participate in target binding. The BC, DE, and FG loops are both structurally and functionally analogous to the complementarity determining regions (CDRs) from immunoglobulins.

Given the small size, lack of disulfide bonds, high stability, and ability to be expressed in prokaryotic hosts, the FN3 domains have gained biopharmaceutical interest. FN3 domains can be easily conjugated to drugs/toxins, penetrate efficiently into tissues and are readily formatted into multispecific binders and fusion proteins, including chimeric antigen receptors (CARs).

Despite the versatility, there is presently no antibody available which specifically binds to FN3 domains for detection, assays, or biopharmaceutical purposes.

SUMMARY OF THE PRESENT INVENTION

The present invention includes antibodies and antigen-binding fragments that bind to a non-randomized region of a fibronectin type III (FN3) domain. Also described are related polynucleotides capable of encoding the provided FN3 domain antibodies and antigen-binding fragments, cells expressing the provided antibodies and antigen-binding fragments, as well as associated vectors and detectably labeled FN3 domain antibodies and antigen-binding fragments. The antibody or antigen binding fragment thereof does not selectively bind to a randomized region of the FN3 domain as measured by ELISA under the conditions shown in Example 3.

In addition, methods of using the provided FN3 domain antibodies and antigen-binding fragments are described. The described FN3 domain antibodies and antigen-binding fragments can be used to detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains. In another embodiment, the described FN3 domain antibodies and antigen-binding fragments can be used to activate T-cell expressing CARs comprising FN3 domains. In yet another embodiment, the described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the present invention comprises isolated antibodies and antigen-binding fragments wherein the antibody or antigen binding fragment specifically binds to a non-randomized region of an FN3 domain. These FN3 domain antibodies, or antigen-binding fragments thereof may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains. In some embodiments, the FN3 domain antibodies, or antigen-binding fragments activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain antibodies and antigen-binding fragments bind to an FN3 domain that is modified in the

loop regions. Table 1 provides CDR sequences of an FN3 domain-specific antibody described herein.

Table 1. CDR sequences of FN3 domain specific antibodies (SEQ ID NO:)

Delineation	HC-CDR1	HC-CDR2	HC-CDR3	LC-CDR1	LC-CDR2	LC-CDR3
IMGT	GIDLSTSV (1)	IYTNVNT (4)	ARAVYAGAMDL (7)	ERIYSN (9)	KAS (11)	QYTSYGSGYVGT (13)
Kabat	TSVMG (2)	FIYTNVNTYYASWAKG (5)	AVYAGAMDL (8)	QASERIYSNLA (10)	KASTLAS (12)	QYTSYGSGYVGT (13)
Chothia	GIDLSTS (3)	YTNVN (6)	AVYAGAMDL (8)	QASERIYSNLA (10)	KASTLAS (12)	QYTSYGSGYVGT (13)

Delineation	HC-CDR1	HC-CDR2	HC-CDR3	LC-CDR1	LC-CDR2	LC-CDR3
IMGT	GFSLNTSGTG (35)	IWWDDDK (41)	VRIKGRMDY (44)	QSVLFGSKQKNY (46)	WAS (48)	HQYLSLFT (50)
Kabat	TSGTGVG (36)	HIWWDDDKGYNPALKS (42)	IKGRMDY (45)	KSSQSVLFGSKQKNYLA (47)	WASTRES (49)	HQYLSLFT (50)
Chothia	GFSLNTSGT (37)	WWDDD (43)	IKGRMDY (45)	KSSQSVLFGSKQKNYLA (47)	WASTRES (49)	HQYLSLFT (50)

Delineation	HC-CDR1	HC-CDR2	HC-CDR3	LC-CDR1	LC-CDR2	LC-CDR3
IMGT	GIDFSSVAY (38)	IYAGSSSSI (51)	ARGLFTSGSGYYIDM (54)	QSIGSD (56)	SAS (58)	QCTYSSSTGYNA (60)
Kabat	SVAYMC (39)	CIYAGSSSSIYYASWAKG (52)	GLFTSGSGYYIDM (55)	QASQSIGSNLA (57)	GASNLAA (59)	QRGYISSAVDFFV (61)
Chothia	GIDFSSVA (40)	YAGSSSS (53)	GLFTSGSGYYIDM (55)	QASQSIGSNLA (57)	GASNLAA (59)	QRGYISSAVDFFV (61)

In some embodiments, the FN3 antibody, or an antigen-binding fragment thereof, comprises a heavy chain comprising a CDR1, a CDR2, and a CDR3 of any one of the amino acid sequences described in Table 1 and a light chain comprising a CDR1, a CDR2, and a CDR3 of any one of the amino acid sequences described in Table 1. The FN3 domain antibodies of the invention may comprise the heavy chain variable regions sequence of SEQ ID NO: 14 and may comprise the light chain variable region sequence of SEQ ID NO: 15. In other embodiments, the FN3 domain antibodies of the invention may comprise the heavy chain variable regions sequence of SEQ ID NO: 74 and may comprise the light chain variable region sequence of SEQ ID NO: 75. In other embodiments, the FN3 domain antibodies of the invention may comprise the heavy chain variable regions sequence of SEQ ID NO: 78 and may comprise the light chain

variable region sequence of SEQ ID NO: 79.

The FN3 domain antibodies described herein include antibodies with the described features of the CDRs and variable domains in combination with any of the IgG isotypes, including modified versions in which the Fc sequence has been modified to effect different effector functions.

In addition to the described FN3 domain antibodies and antigen-binding fragments, also provided are polynucleotide sequences capable of encoding the FN3 domain antibodies and antigen-binding fragments. Vectors comprising the described polynucleotides are also provided, as are cells expressing the FN3 domain antibodies or antigen-binding fragments provided herein. Also described are cells capable of expressing the disclosed vectors. These cells may be mammalian cells (such as 293F cells, CHO cells), insect cells (such as Sf9 cells), yeast cells, plant cells, or bacteria cells (such as E. coli). A process for the production of the FN3 domain antibodies or antigen-binding fragments is also provided.

The present invention also comprises a CAR of the invention comprising an isolated polypeptide comprising:

(a) an extracellular domain having an scFv that specifically binds to a non-randomized region of an FN3 domain;

(b) a transmembrane domain; and

(c) an intracellular signaling domain.

The CAR can further comprise a hinge region connecting the extracellular domain and the transmembrane domain.

In some embodiments, the CAR isolated polypeptide comprises:

(a) an extracellular domain comprising an FN3 domain of the invention, such as an FN3 domain having an amino acid sequence that is at least 90% identical to one of SEQ ID NOs: 68-73, preferably one of SEQ ID NOs: 68-73;

(b) a hinge region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 24, preferably SEQ ID NO:24;

(c) a transmembrane domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 25, preferably SEQ ID NO:25; and

(d) an intracellular signaling domain comprising a co-stimulatory domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 26,

preferably SEQ ID NO:26, and a primary signaling domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 27, preferably SEQ ID NO:27.

The present invention also comprises an isolated polynucleotide encoding a CAR of the invention comprising:

(a) an extracellular domain having an scFv that specifically binds to a non-randomized region of an FN3 domain;

(b) a transmembrane domain; and

(c) an intracellular signaling domain.

The CAR can further comprise a hinge region connecting the extracellular domain and the transmembrane domain.

In some embodiments, the isolated polynucleotide encoding a CAR comprises:

(a) an extracellular domain comprising an FN3 domain of the invention, such as an FN3 domain having an amino acid sequence that is at least 90% identical to one of SEQ ID NOs: 68-73, preferably one of SEQ ID NOs: 68-73;

(b) a hinge region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 24, preferably SEQ ID NO:24;

(c) a transmembrane domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 25, preferably SEQ ID NO:25; and

(d) an intracellular signaling domain comprising a co-stimulatory domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 26, preferably SEQ ID NO:26, and a primary signaling domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 27, preferably SEQ ID NO:27.

In another general aspect, the present invention relates to a CAR of the invention, a vector comprising a polynucleotide encoding a CAR of the invention, and a host cell comprising the vector or the isolated polynucleotide encoding a CAR of the invention. The invention also relates to a method of producing a CAR of the invention, comprising culturing a host cell comprising a polynucleotide sequence encoding the CAR under conditions to produce the CAR, and recovering the CAR. The CAR can be associated with the host cell or an isolated cell membrane from the host cell.

According to another general aspect, the invention relates to engineered immune cells comprising a CAR of the invention. Preferably, the engineered immune cells are T cell receptor knockout immune cells. Preferably, the engineered immune cells are HLA I/B2-microglobulin knockout immune cells. Optionally, the HLA I/B2-

microglobulin knockout immune cells are additionally HLA II knockout immune cells that are devoid of allogeneic immune responses from the host patient. The engineered immune cells can comprise a second CAR having an extracellular domain binding specifically to a target different from an FN3 domain. The engineered immune cells can also be resistant to at least one anti-cancer chemotherapy.

In another general aspect, the invention relates to pharmaceutical compositions comprising engineered immune cells of the invention.

In another general aspect, the invention relates to a method of treating a B cell-related condition in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition of the invention. In a preferred embodiment, the B cell-related condition is multiple myeloma.

In another general aspect, the invention relates to a method of engineering an immune cell of the invention, comprising providing an immune cell, and introducing into the cell a polypeptide encoding a CAR of the invention.

In another general aspect, the invention relates to a method of producing a pharmaceutical composition, comprising combining an engineered immune cell of the invention with a pharmaceutically acceptable carrier to obtain the pharmaceutical composition

Within the scope of the invention are kits including the disclosed FN3 domain antibodies or antigen-binding fragments thereof. The described kits may be used to carry out the methods of using the FN3 domain antibodies or antigen-binding fragments provided herein, or other methods known to those skilled in the art. In some embodiments, the described kits may include the FN3 domain antibodies or antigen-binding fragments described herein and reagents for use in detecting the presence of FN3 domains in a biological sample and, optionally, a vessel for containing the FN3 domain antibody or fragment when not in use, instructions for use of the FN3 domain antibody or fragment, the FN3 domain antibody or fragment affixed to a solid support, and/or detectably labeled forms of the FN3 domain antibody or fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A-1C shows testing of recombinant AS7B90 and AS7B91 binding to immobilized FN3 domains or negative control proteins, and comparison of each to the

original rabbit hybridoma derived antibody CEN25-105-5. Fig. 1A shows binding data for tencon25 which has no target specificity. Fig. 1B shows binding data for A3, which is an FN3 domain specific for human cMET. Fig. 1C shows binding data for 83v2-ABD, which is an FN3 domain specific for human EGFR with an albumin binding domain.

Fig. 2A-2M shows that AS7B91 was able to detect all CARTyrins expressed on the surface of T cells.

Fig. 3 shows detection of AS7B91 scFv CAR expression on the surface of primary T cells using labeled tencon 25.

Fig.4A-4C show AS7B91 scFv CAR-BCMA specific FN3 domain degranulation in response to BCMA-expressing target cells. Fig. 4A shows H929 (BCMA high-expressing cells). Fig. 4B shows ARH77 (BCMA low-expressing cells). Fig. 4C shows K562 (BCMA negative cells). Different FN3 domains were incubated at a concentration of 50 nM with AS7B91 scFv CAR T cells, washed, and then added to target cells at a 1:1 ratio. BAR-T variant 1= AS7B91 scFv L-H CAR; BAR-T variant 2= AS7B91 scFv H-L CAR.

Fig. 5 shows AS7B91 scFv CAR-BCMA killing of BCMA expressing target cells. H929, BCMA high expressing cells; ARH77, BCMA low expressing cells; K562, BCMA negative cells. Different FN3 domains were incubated at a concentration of 25 nM with AS7B91 scFv CAR T cells, washed, and then added to target cells at a 1:1 ration. BAR-T variant 1= AS7B91 scFv L-H CAR; BAR-T variant 2= AS7B91 scFv H-L CAR.

Fig. 6 shows binding of labeled Tencon-25 to AS7B16 scFv CAR constructs on T cells. L2H, AS7B16 light chain-heavy chain orientation. H2L, AS7B16 heavy chain-light chain orientation.

Fig. 7 shows binding of labeled EGFR 83v2 to AS7B16 scFv CAR constructs on T cells. L2H, AS7B16 light chain-heavy chain orientation. H2L, AS7B16 heavy chain-light chain orientation.

Fig. 8 shows binding of labeled Tencon-25 to AS7B82 scFv CAR constructs on T cells. L2H, AS7B82 light chain-heavy chain orientation. H2L, AS7B16 heavy chain-light chain orientation.

Fig. 9 shows binding of labeled EGFR 83v2 to AS7B82 scFv CAR constructs on T

cells. L2H, AS7B16 light chain-heavy chain orientation. H2L, AS7B16 heavy chain-light chain orientation.

Fig. 10 Generation of FcgR expressing cell lines. Purified lentiviral expression plasmids encoding for human FcgRs (CD16a, CD32 and CD64) were packaged for transfection of 293T cells using the Lenti-Pac HIV Expression Packaging System.

Fig. 11A-11B. Conjugation of Citrullinated Cyclic Peptides to Tencon25 centyrin was performed at a 1:5 ratio (centyrin to peptide) via sortase chemistry. Conjugates were purified manually over a Ni Sepharose column (GE) to remove free sortase and peptide. Following purification, the conjugates were buffered exchanged in to PBS and concentrated. Conjugates were QC'd by (a) mass spectrometry (LC-MS) and (b) size exclusion chromatography (superdex 75) and sterile filtered.

Fig. 12. Detection of anti-citrullinated antibody binding to centyrin-CCP1-peptide conjugates. FcgR expressing HEK293 cells were incubated with 200ug/mL of human anti-citrullinated fibrinogen antibody or human IgG1 isotype control. Binding was assessed by flow cytometry.

Fig. 13. AS7B91 scFv CAR-T cell mediated killing of anti-citrullinated mAb bound FcR expressing cells.

Fig. 14A-14C. Evaluation of potential peptide/antibody combinations in multiple auto-immune diseases for BAR-T platform expansion. Peptides specific for a) Myasthenia Gravis (MG), b) Multiple Sclerosis (MS) and c) systemic lupus erythematosus (SLE) were tested for their ability to bind to anti-AChR, anti-MOG, or anti dsDNA antibodies, respectively. Fig. 14A discloses SEQ ID NOS 85-87, respectively, in order of appearance. Fig. 14B discloses SEQ ID NOS 88-90, respectively, in order of appearance. Fig. 14C discloses SEQ ID NOS 91-93, respectively, in order of appearance.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Various publications, articles and patents are cited or described in the background and throughout the specification; each of these references is herein incorporated by reference in its entirety. Discussion of documents, acts, materials,

devices, articles or the like which has been included in the present specification is for the purpose of providing context for the invention. Such discussion is not an admission that any or all of these matters form part of the prior art with respect to any inventions disclosed or claimed.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a cell" includes a combination of two or more cells, and the like.

The term "about" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of up to ±10% from the specified value, as such variations are appropriate to perform the disclosed methods. Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

"Isolated" means a biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins that have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. "Isolated" nucleic acids, peptides and proteins can be part of a composition and still be isolated if such composition is not

part of the native environment of the nucleic acid, peptide, or protein. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids. An "isolated" antibody or antigen-binding fragment, as used herein, is intended to refer to an antibody or antigen-binding fragment which is substantially free of other antibodies or antigen-binding fragments having different antigenic specificities (for instance, an isolated antibody that specifically binds to an FN3 domain is substantially free of antibodies that are not specific to FN3 domains).

As used herein, the term "fibronectin type III domain" or "FN3 domain" refers to a domain occurring frequently in proteins including fibronectins, tenascin, intracellular cytoskeletal proteins, cytokine receptors and prokaryotic enzymes (Bork and Doolittle, PNAS USA 89:8990-8994, 1992; Meinke *et al.*, J Bacteriol 175:1910-1918, 1993; Watanabe *et al.,* J Biol Chem 265:15659-15665, 1990), or a derivative thereof. Exemplary FN3 domains are the 15 different FN3 domains present in human tenascin C, the 15 different FN3 domains present in human fibronectin (FN), and non-natural synthetic FN3 domains, for example, in US8278419. Individual FN3 domains are referred to by domain number and protein name, e.g., the 3rd FN3 domain of tenascin (TN3), or the 10th FN3 domain of fibronectin (FN10).

"Antibody" refers to all isotypes of immunoglobulins (IgG, IgA, IgE, IgM, IgD, and IgY) including various monomeric, polymeric and chimeric forms, unless otherwise specified. Specifically encompassed by the term "antibody" are polyclonal antibodies, monoclonal antibodies (mAbs), and antibody-like polypeptides, such as chimeric antibodies and humanized antibodies.

"Antigen-binding fragments" are any proteinaceous structure that may exhibit binding affinity for a particular antigen. Antigen-binding fragments include those provided by any known technique, such as enzymatic cleavage, peptide synthesis, and recombinant techniques. Some antigen-binding fragments are composed of portions of intact antibodies that retain antigen-binding specificity of the parent antibody molecule. For example, antigen-binding fragments may comprise at least one variable region (either a heavy chain or light chain variable region) or one or more CDRs of an antibody known to bind a particular antigen. Examples of suitable antigen-binding fragments include, without limitation diabodies and single-chain molecules as well as Fab, F(ab')2, Fc, Fabc, and Fv molecules, single chain (Sc) antibodies, individual

antibody light chains, individual antibody heavy chains, chimeric fusions between antibody chains or CDRs and other proteins, protein scaffolds, heavy chain monomers or dimers, light chain monomers or dimers, dimers consisting of one heavy and one light chain, a monovalent fragment consisting of the VL, VH, CL and CH1 domains, or a monovalent antibody as described in WO2007059782, bivalent fragments comprising two Fab fragments linked by a disulfide bridge at the hinge region, a Fd fragment consisting essentially of the V.sub.H and C.sub.H1 domains; a Fv fragment consisting essentially of the VL and VH domains of a single arm of an antibody, a dAb fragment (Ward et al., Nature 341, 544-546 (1989)), which consists essentially of a VH domain and also called domain antibodies (Holt et al; Trends Biotechnol. 2003 Nov.; 21(11):484-90); camelid or nanobodies (Revets et al; Expert Opin Biol Ther. 2005 Jan.; 5(1):111-24); an isolated complementarity determining region (CDR), and the like. All antibody isotypes may be used to produce antigen-binding fragments. Additionally, antigen-binding fragments may include non-antibody proteinaceous frameworks that may successfully incorporate polypeptide segments in an orientation that confers affinity for a given antigen of interest, such as protein scaffolds. Antigen-binding fragments may be recombinantly produced or produced by enzymatic or chemical cleavage of intact antibodies. The phrase "an antibody or antigen-binding fragment thereof" may be used to denote that a given antigen-binding fragment incorporates one or more amino acid segments of the antibody referred to in the phrase.

The terms "CDR", and its plural "CDRs", refer to a complementarity determining region (CDR) of which three make up the binding character of a light chain variable region (CDRL1, CDRL2 and CDRL3) and three make up the binding character of a heavy chain variable region (CDRH1, CDRH2 and CDRH3). CDRs contribute to the functional activity of an antibody molecule and are separated by amino acid sequences that comprise scaffolding or framework regions. The exact definitional CDR boundaries and lengths are subject to different classification and numbering systems. CDRs may therefore be referred to by IMGT, Kabat, Chothia, or any other boundary definitions. Despite differing boundaries, each of these systems has some degree of overlap in what constitutes the so called "hypervariable regions" within the variable sequences. CDR definitions according to these systems may therefore differ in length and boundary areas with respect to the adjacent framework region. See for example Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th ed.

NIH Publication No. 91-3242 (1991); Chothia et al., "Canonical Structures For the Hypervariable Regions of Immunoglobulins," *J. Mol. Biol.* 196:901 (1987); and MacCallum et al., "Antibody-Antigen Interactions: Contact Analysis and Binding Site Topography," J. Mol. Biol. 262:732 (1996)), each of which is hereby incorporated by reference in its entirety.

Typically, CDRs form a loop structure that can be classified as a canonical structure. The term "canonical structure" refers to the main chain conformation that is adopted by the antigen binding (CDR) loops. From comparative structural studies, it has been found that five of the six antigen binding loops have only a limited repertoire of available conformations. Each canonical structure can be characterized by the torsion angles of the polypeptide backbone. Correspondent loops between antibodies may, therefore, have very similar three dimensional structures, despite high amino acid sequence variability in most parts of the loops (Chothia et al., "Canonical Structures For the Hypervariable Regions of Immunoglobulins," *J. Mol. Biol.* 196:901 (1987); Chothia et al., "Conformations of Immunoglobulin Hypervariable Regions," I 342:877 (1989); Martin and Thornton, "Structural Families in Loops of Homologous Proteins: Automatic Classification, Modelling and Application to Antibodies," *J. Mol. Biol.* 263:800 (1996), each of which is incorporated by reference in its entirety). Furthermore, there is a relationship between the adopted loop structure and the amino acid sequences surrounding it. The conformation of a particular canonical class is determined by the length of the loop and the amino acid residues residing at key positions within the loop, as well as within the conserved framework (i.e., outside of the loop). Assignment to a particular canonical class can therefore be made based on the presence of these key amino acid residues.

"Specifically binds" or "binds specifically" or derivatives thereof when used in the context of antibodies, or antibody fragments, represents binding via domains encoded by immunoglobulin genes or fragments of immunoglobulin genes to one or more epitopes of a protein of interest, without preferentially binding other molecules in a sample containing a mixed population of molecules. Typically, an antibody binds to a cognate antigen with a K_d of less than about $1x10^{-8}$ M, as measured by a surface plasmon resonance assay, or a cell-binding assay. In a preferred embodiment, binding specificity is measure using biolayer interferometry. Phrases such as "[antigen]-specific" antibody are meant to convey that the recited antibody specifically binds the

recited antigen.

"Polynucleotide," synonymously referred to as "nucleic acid molecule," "nucleotides" or "nucleic acids," refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short nucleic acid chains, often referred to as oligonucleotides.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus in which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

As used herein, the term "host cell" can be any type of cell, e.g., a primary cell, a cell in culture, or a cell from a cell line. In specific embodiments, the term "host cell" refers to a cell transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell. Progeny of such a cell may not be identical to the parent cell transfected with the nucleic acid molecule, e.g., due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome. The terms "expression" and "production" are used synonymously herein, and refer to the biosynthesis of a gene product. These terms encompass the transcription of a gene into RNA. These terms also encompass translation of RNA into one or more polypeptides, and further encompass all naturally occurring post-transcriptional and post-translational modifications. The expression or production of an antibody or antigen-binding fragment thereof may be within the

cytoplasm of the cell, or into the extracellular milieu such as the growth medium of a cell culture. The meaning of "substantially the same" can differ depending on the context in which the term is used. Because of the natural sequence variation likely to exist among heavy and light chains and the genes encoding them, one would expect to find some level of variation within the amino acid sequences or the genes encoding the antibodies or antigen-binding fragments described herein, with little or no impact on their unique binding properties (e.g., specificity and affinity). Such an expectation is due in part to the degeneracy of the genetic code, as well as to the evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, in the context of nucleic acid sequences, "substantially the same" means at least 65% identity between two or more sequences. Preferably, the term refers to at least 70% identity between two or more sequences, more preferably at least 75% identity, more preferably at least 80% identity, more preferably at least 85% identity, more preferably at least 90% identity, more preferably at least 91% identity, more preferably at least 92% identity, more preferably at least 93% identity, more preferably at least 94% identity, more preferably at least 95% identity, more preferably at least 96% identity, more preferably at least 97% identity, more preferably at least 98% identity, and more preferably at least 99% or greater identity. The percent identity between two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The percent identity between two nucleotide or amino acid sequences may e.g. be determined using the algorithm of E. Meyers and W. Miller, Comput. Appl. Biosci 4, 11-17 (1988) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences may be determined using the Needleman and Wunsch, J. Mol. Biol. 48, 444-453 (1970) algorithm.

The degree of variation that may occur within the amino acid sequence of a protein without having a substantial effect on protein function is much lower than that of a nucleic acid sequence, since the same degeneracy principles do not apply to amino acid sequences. Accordingly, in the context of an antibody or antigen-binding

fragment, "substantially the same" means antibodies or antigen-binding fragments having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the antibodies or antigen-binding fragments described. Other embodiments include FN3 domain antibodies, or antigen-binding fragments, that have framework, scaffold, or other non-binding regions that do not share significant identity with the FN3 domain antibodies and antigen-binding fragments described herein, but do incorporate one or more CDRs or other sequences needed to confer binding that are 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to such sequences described herein.

As used herein, the term "chimeric antigen receptor" (CAR) refers to a recombinant polypeptide comprising at least an extracellular domain that binds specifically to an antigen or a target, a transmembrane domain and an intracellular T cell receptor-activating signaling domain. Engagement of the extracellular domain of the CAR with the target antigen on the surface of a target cell results in clustering of the CAR and delivers an activation stimulus to the CAR-containing cell. CARs redirect the specificity of immune effector cells and trigger proliferation, cytokine production, phagocytosis and/or production of molecules that can mediate cell death of the target antigen-expressing cell in a major histocompatibility (MHC)-independent manner.

As used herein, the term "extracellular antigen binding domain," "extracellular domain," or "extracellular ligand binding domain" refers to the part of a CAR that is located outside of the cell membrane and is capable of binding to an antigen, target or ligand.

As used herein, the term "hinge region" refers to the part of a CAR that connects two adjacent domains of the CAR protein, e.g., the extracellular domain and the transmembrane domain.

As used herein, the term "transmembrane domain" refers to the portion of a CAR that extends across the cell membrane and anchors the CAR to cell membrane.

As used herein, the term "intracellular T cell receptor-activating signaling domain", "cytoplasmic signaling domain," or "intracellular signaling domain" refers to the part of a CAR that is located inside of the cell membrane and is capable of transducing an effector signal.

As used herein, the term "stimulatory molecule" refers to a molecule expressed by a T cell that provides the primary cytoplasmic signaling sequence(s) that regulate primary activation of the T cell receptor (TCR) complex in a stimulatory way for at

least some aspect of the T cell signaling pathway. Stimulatory molecules comprise two distinct classes of cytoplasmic signaling sequence, those that initiate antigen-dependent primary activation (referred to as "primary signaling domains"), and those that act in an antigen-independent manner to provide a secondary of co-stimulatory signal (referred to as "co-stimulatory signaling domains").

The term "expression" as used herein, and refer to the biosynthesis of a gene product. The term encompasses the transcription of a gene into RNA. The term also encompasses translation of RNA into one or more polypeptides, and further encompasses all naturally occurring post-transcriptional and post-translational modifications. The expressed FN3 domain or CAR can be within the cytoplasm of a host cell, into the extracellular milieu such as the growth medium of a cell culture, or anchored to the cell membrane.

As used herein, the term "immune cell" or "immune effector cell" refers to a cell that is involved in an immune response, e.g., in the promotion of an immune effector response. Examples of immune cells include T cells, B cells, natural killer (NK) cells, mast cells, and myeloid-derived phagocytes. According to particular embodiments, the engineered immune cells are T cells, and are referred to as CAR-T cells because they are engineered to express CARs of the invention.

As used herein, the term "engineered immune cell" refers to an immune cell, also referred to as an immune effector cell, that has been genetically modified by the addition of extra genetic material in the form of DNA or RNA to the total genetic material of the cell. According to embodiments herein, the engineered immune cells have been genetically modified to express a FN3 domain-targeting CAR according to the invention.

As used herein, the term "carrier" refers to any excipient, diluent, filler, salt, buffer, stabilizer, solubilizer, oil, lipid, lipid containing vesicle, microsphere, liposomal encapsulation, or other material well known in the art for use in pharmaceutical formulations. It will be understood that the characteristics of the carrier, excipient or diluent will depend on the route of administration for a particular application. As used herein, the term "pharmaceutically acceptable carrier" refers to a non-toxic material that does not interfere with the effectiveness of a composition according to the invention or the biological activity of a composition according to the invention.

As used herein, the term "subject" refers to an animal, and preferably a

mammal. According to particular embodiments, the subject is a mammal including a non-primate (e.g., a camel, donkey, zebra, cow, pig, horse, goat, sheep, cat, dog, rat, rabbit, guinea pig or mouse) or a primate (e.g., a monkey, chimpanzee, or human). In particular embodiments, the subject is a human.

As used herein, the term "therapeutically effective amount" refers to an amount of an active ingredient or component that elicits the desired biological or medicinal response in a subject. A therapeutically effective amount can be determined empirically and in a routine manner, in relation to the stated purpose.

As used herein, the terms "treat," "treating," and "treatment" are all intended to refer to an amelioration or reversal of at least one measurable physical parameter related to a cancer or autoimmunity, which is not necessarily discernible in the subject, but can be discernible in the subject. The terms "treat," "treating," and "treatment," can also refer to causing regression, preventing the progression, or at least slowing down the progression of the disease, disorder, or condition. In a particular embodiment, "treat," "treating," and "treatment" refer to an alleviation, prevention of the development or onset, or reduction in the duration of one or more symptoms associated with the disease, disorder, or condition, such as a tumor or more preferably a cancer. In a particular embodiment, "treat," "treating," and "treatment" refer to prevention of the recurrence of the disease, disorder, or condition. In a particular embodiment, "treat," "treating," and "treatment" refer to an increase in the survival of a subject having the disease, disorder, or condition. In a particular embodiment, "treat," "treating," and "treatment" refer to elimination of the disease, disorder, or condition in the subject.

FN3 domain libraries

Tencon is a non-naturally occurring FN3 domain designed from a consensus sequence of fifteen FN3 domains from human tenascin-C (Jacobs *et al.*, Protein Engineering, Design, and Selection, 25:107-117, 2012; US2010/0216708). The crystal structure of Tencon shows six surface-exposed loops that connect seven beta-strands as is characteristic to the FN3 domains, the beta-strands referred to as A, B, C, D, E, F, and G, and the loops referred to as AB, BC, CD, DE, EF, and FG loops (Bork and Doolittle, PNAS USA 89:8990-8992, 1992; US6673901). These loops, or selected residues within each loop, can be randomized in order to construct libraries of FN3 domains that can be used to select novel molecules that bind a particular antigen.

Therefore, as described herein, a "non-randomized" region of an FN3 domain refers to a region within the FN3 domain that is conserved among all FN3 domains. Table 2 shows positions and sequences of each loop and beta-strand in Tencon (SEQ ID NO: 33).

Table 2

FN3 domain	**Tencon (SEQ ID NO: 33)**
A strand	1-12
AB loop	13-16
B strand	17-21
BC loop	22-28
C strand	29-37
CD loop	38-43
D strand	44-50
DE loop	51-54
E strand	55-59
EF loop	60-64
F strand	65-74
FG loop	75-81
G strand	82-89

Libraries designed based on the Tencon sequence can thus have randomized sequence in one or more of the loops or strands. For example, libraries based on Tencon can have randomized sequence in one or more of the AB loop, BC loop, CD loop, DE, EF loop and FG loop. For example, the Tencon BC loop is 7 amino acids long, thus 1, 2, 3, 4, 5, 6 or 7 amino acids can be randomized in a library based on Tencon sequence, diversified at the BC loop. The Tencon CD loop is 6 amino acids long, thus 1, 2, 3, 4, 5 or 6 amino acids can be randomized in a library based on Tencon sequence, diversified at the CD loop. The Tencon EF loop is 5 amino acids long, thus 1, 2, 3, 4 or 5 amino acids can be randomized in a library based on Tencon sequence, diversified at the EF loop. The Tencon FG loop is 7 amino acids long, thus 1, 2, 3, 4, 5, 6 or 7 amino acids can be randomized in a library based on Tencon sequence, diversified at the FG loop. Further diversity at loops in the Tencon libraries can be

achieved by insertion and/or deletions of residues at loops. For example, the BC, CD, EF and/or FG loops can be extended by 1-22 amino acids, or decreased by 1-3 amino acids. The FG loop in Tencon is 7 amino acids long, whereas the corresponding loop in antibody heavy chains ranges from 4-28 residues. To provide maximum diversity, the FG loop can be diversified in sequence as well as in length to correspond to the antibody CDR3 length range of 4-28 residues. For example, the FG loop can be further diversified in length by extending the loop by an additional 1, 2, 3, 4 or 5 amino acids.

Libraries designed based on the Tencon sequence can also have randomized alternative surfaces that form on a side of the FN3 domain and comprise two or more beta strands, and at least one loop. One such alternative surface is formed by amino acids in the C and the F beta-strands and the CD and the FG loops (a C-CD-F-FG surface). A library design based on Tencon alternative C-CD-F-FG surface is described in US2013/0226834. Libraries designed based on the Tencon sequence also includes libraries designed based on Tencon variants, such as Tencon variants having substitutions at residues positions 11, 17, 46 and/or 86 (residue numbering corresponding to SEQ ID NO: 33), and which variants display improve thermal stability. Exemplary Tencon variants are described in US2011/0274623, and include Tencon27 (SEQ ID NO: 34) having substitutions E11R, L17A, N46V and E86I when compared to Tencon of SEQ ID NO: 33.

Tencon libraries and other FN3 sequence-based libraries can be randomized at chosen residue positions using a random or defined set of amino acids. For example, variants in the library having random substitutions can be generated using NNK codons, which encode all 20 naturally occurring amino acids. In other diversification schemes, DVK codons can be used to encode amino acids Ala, Trp, Tyr, Lys, Thr, Asn, Lys, Ser, Arg, Asp, Glu, Gly, and Cys. Alternatively, NNS codons can be used to give rise to all 20 amino acid residues while simultaneously reducing the frequency of stop codons. Libraries of FN3 domains with biased amino acid distribution at positions to be diversified can be synthesized, for example, using Slonomics® technology (http:_//www_sloning_com). This technology uses a library of pre-made double stranded triplets that act as universal building blocks sufficient for thousands of gene synthesis processes. The triplet library represents all possible sequence combinations necessary to build any desired DNA molecule. The codon designations are according to the well known IUB code.

FN3 Domain Antibodies and Antigen-Binding Fragments

Described herein are isolated monoclonal antibodies or antigen-binding fragments that specifically bind to a non-randomized region of FN3 domains. The general structure of an antibody molecule comprises an antigen binding domain, which includes heavy and light chains, and the Fc domain, which serves a variety of functions, including complement fixation and binding antibody receptors.

The described FN3 domain-specific antibodies or antigen-binding fragments include all isotypes, IgA, IgD, IgE, IgG and IgM, and synthetic multimers of the four-chain immunoglobulin structure. The described antibodies or antigen-binding fragments also include the IgY isotype generally found in hen or turkey serum and hen or turkey egg yolk.

The FN3 domain-specific antibodies and antigen-binding fragments may be derived from any species by recombinant means. For example, the antibodies or antigen-binding fragments may be mouse, rat, goat, horse, swine, bovine, chicken, rabbit, camelid, donkey, human, or chimeric versions thereof. For use in administration to humans, non-human derived antibodies or antigen-binding fragments may be genetically or structurally altered to be less antigenic upon administration to a human patient.

In some embodiments, the antibodies or antigen-binding fragments are chimeric. As used herein, the term "chimeric" refers to an antibody, or antigen-binding fragment thereof, having at least some portion of at least one variable domain derived from the antibody amino acid sequence of a non-human mammal, a rodent, or a reptile, while the remaining portions of the antibody, or antigen-binding fragment thereof, are derived from a human.

In some embodiments, the antibodies are humanized antibodies. Humanized antibodies may be chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and

capacity. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody may include at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The antibodies or antigen-binding fragments described herein can occur in a variety of forms, but will include one or more of the antibody CDRs shown in Table 1.

Described herein are isolated antibodies and antigen-binding fragments that specifically bind to FN3 domains. In some embodiments, the FN3 domain-specific antibodies or antigen-binding fragments are derived from rabbits. While the FN3 domain-specific antibodies or antigen-binding fragments exemplified herein are rabbit-derived, the antibodies or antigen-binding fragments exemplified may be chimerized.

In some embodiments are provided an FN3 domain-specific antibody, or an antigen-binding fragment thereof, comprising a heavy chain comprising a CDR1, a CDR2, and a CDR3 of any one of the antibodies described in Table 1 and a light chain comprising a CDR1, a CDR2, and a CDR3 of any one of the antibodies described in Table 1.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 comprising SEQ ID NO: 1, a heavy chain CDR2 comprising SEQ ID NO: 4, a heavy chain CDR3 comprising SEQ ID NO: 7, a light chain CDR1 comprising SEQ ID NO: 9, a light chain CDR2 comprising SEQ ID NO: 11, and a light chain CDR3 comprising SEQ ID NO: 13. This FN3 domain-specific antibody or antigen-binding fragment may comprise non-rabbit framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 14 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 15. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 comprising SEQ ID NO: 2, a heavy chain CDR2 comprising SEQ ID NO: 5, a heavy chain CDR3 comprising SEQ ID NO: 8, a light chain CDR1 comprising SEQ ID NO: 10, a light chain CDR2 comprising SEQ ID NO: 12, and a light chain CDR3 comprising SEQ ID NO: 13. This FN3 domain-specific antibody or antigen-binding fragment may comprise non-rabbit framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 14 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 15. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 comprising SEQ ID NO: 3, a heavy chain CDR2 comprising SEQ ID NO: 6, a heavy chain CDR3 comprising SEQ ID NO: 8, a light chain CDR1 comprising SEQ ID NO: 10, a light chain CDR2 comprising SEQ ID NO: 12, and a light chain CDR3 comprising SEQ ID NO: 13. This FN3 domain-specific antibody or antigen-binding fragment may comprise non rabbit framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 14 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 15. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 35, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 41, a

heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 44, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 46, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 48, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50. This FN3 domain-specific antibody or antigen-binding fragment may comprise non mouse framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 74 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 75. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 36, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 42, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 45, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 47 a light chain CDR2 having the amino acid sequence of SEQ ID NO: 49, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50. This FN3 domain-specific antibody or antigen-binding fragment may comprise non mouse framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 74 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 75. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 having the amino acid sequence of SEQ ID

NO: 37, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 43, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 45, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 47, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 49, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50. This FN3 domain-specific antibody or antigen-binding fragment may comprise non mouse framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 74 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 75. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 38, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 51, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 54, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 56, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 58, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 60. This FN3 domain-specific antibody or antigen-binding fragment may comprise non mouse framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 78 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 79. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 39, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 52, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 55, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 57 a light chain CDR2 having the amino acid sequence of SEQ ID NO: 59, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 61. This FN3 domain-specific antibody or antigen-binding fragment may comprise non mouse framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 78 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 79. The described FN3 domain antibodies or antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 40, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 53, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 55, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 57, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 59, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 61. This FN3 domain-specific antibody or antigen-binding fragment may comprise non mouse framework sequences. This FN3 domain-specific antibody or antigen-binding fragment may bind to non-randomized regions of an FN3 domain, may detect the T-cell surface expression of chimeric antigen receptors (CARs) comprising FN3 domains, and may activate T-cell expressing CARs comprising FN3 domains. In some embodiments, the FN3 domain-specific antibodies and antigen-binding fragments comprise a variable heavy chain region substantially the same as, or identical to, SEQ ID NO: 78 and a variable light chain substantially the same as, or identical to, SEQ ID NO: 79. The described FN3 domain antibodies or

antigen binding fragments can be used to generate CARs comprising the described antigen binding fragments.

Also disclosed are isolated polynucleotides that encode the antibodies or antigen-binding fragments that specifically bind to an FN3 domain. The isolated polynucleotides capable of encoding the variable domain segments provided herein may be included on the same, or different, vectors to produce antibodies or antigen-binding fragments.

Polynucleotides encoding recombinant antigen-binding proteins also are within the scope of the disclosure. In some embodiments, the polynucleotides described (and the peptides they encode) include a leader sequence. Any leader sequence known in the art may be employed. The leader sequence may include, but is not limited to, a restriction site or a translation start site.

The FN3 domain-specific antibodies or antigen-binding fragments described herein include variants having single or multiple amino acid substitutions, deletions, or additions that retain the biological properties (e.g., binding affinity or immune effector activity) of the described FN3 domain-specific antibodies or antigen-binding fragments. These variants may include: (a) variants in which one or more amino acid residues are substituted with conservative or nonconservative amino acids, (b) variants in which one or more amino acids are added to or deleted from the polypeptide, (c) variants in which one or more amino acids include a substituent group, and (d) variants in which the polypeptide is fused with another peptide or polypeptide such as a fusion partner, a protein tag or other chemical moiety, that may confer useful properties to the polypeptide, such as, for example, an epitope for an antibody, a polyhistidine sequence, a biotin moiety and the like. Antibodies or antigen-binding fragments described herein may include variants in which amino acid residues from one species are substituted for the corresponding residue in another species, either at the conserved or nonconserved positions. In other embodiments, amino acid residues at nonconserved positions are substituted with conservative or nonconservative residues. The techniques for obtaining these variants, including genetic (deletions, mutations, etc.), chemical, and enzymatic techniques, are known to persons having ordinary skill in the art.

The FN3 domain-specific antibodies or antigen-binding fragments described herein may embody several antibody isotypes, such as IgM, IgD, IgG, IgA and IgE. In some embodiments, the antibody isotype is IgG. Antibody or antigen-binding fragment

thereof specificity is largely determined by the amino acid sequence, and arrangement, of the CDRs. Therefore, the CDRs of one isotype may be transferred to another isotype without altering antigen specificity. Alternatively, techniques have been established to cause hybridomas to switch from producing one antibody isotype to another (isotype switching) without altering antigen specificity. Accordingly, such antibody isotypes are within the scope of the described antibodies or antigen-binding fragments.

The affinity of the described FN3 domain-specific antibodies, or antigen-binding fragments, may be determined by a variety of methods known in the art, such as surface plasmon resonance or ELISA-based methods. Assays for measuring affinity by SPR include assays performed using a BIAcore 3000 machine, where the assay is performed at room temperature (*e.g.* at or near 25°C), wherein the antibody capable of binding to FN3 domains is captured on the BIAcore sensor chip by an anti-Fc antibody (*e.g.* goat anti-human IgG Fc specific antibody Jackson ImmunoResearch laboratories Prod # 109-005-098) to a level around 75RUs, followed by the collection of association and dissociation data at a flow rate of 40µl/min.

Methods of detecting FN3 domains

Provided herein are methods for detecting FN3 domains in a biological sample by contacting the sample with an antibody, or antigen-binding fragment thereof, described herein. As described herein, the sample may be derived from urine, blood, serum, plasma, saliva, ascites, circulating cells, circulating tumor cells, cells that are not tissue associated (*i.e.*, free cells), tissues (*e.g.*, surgically resected tumor tissue, biopsies, including fine needle aspiration), histological preparations, and the like. In some embodiments, the described methods include detecting FN3 domains in a biological sample by contacting the sample with any of the FN3 domain-specific antibodies or antigen-binding fragments thereof described herein.

In some embodiments, the sample may be contacted with more than one of the FN3 domain-specific antibodies or antigen-binding fragments described herein. For example, a sample may be contacted with a first FN3 domain-specific antibody, or antigen-binding fragment thereof, and then contacted with a second FN3 domain-specific antibody, or antigen-binding fragment thereof, wherein the first antibody or antigen-binding fragment and the second antibody or antigen-binding fragment are not the same antibody or antigen-binding fragment. In some embodiments, the first

antibody, or antigen-binding fragment thereof, may be affixed to a surface, such as a multiwell plate, chip, or similar substrate prior to contacting the sample. In other embodiments the first antibody, or antigen-binding fragment thereof, may not be affixed, or attached, to anything at all prior to contacting the sample.

The described FN3 domain-specific antibodies and antigen-binding fragments may be detectably labeled. In some embodiments labeled antibodies and antigen-binding fragments may facilitate the detection of FN3 domains via the methods described herein. Many such labels are readily known to those skilled in the art. For example, suitable labels include, but should not be considered limited to, radiolabels, fluorescent labels, epitope tags, biotin, chromophore labels, ECL labels, or enzymes. More specifically, the described labels include ruthenium, ^{111}In-DOTA, ^{111}In-diethylenetriaminepentaacetic acid (DTPA), horseradish peroxidase, alkaline phosphatase and beta-galactosidase, poly-histidine (HIS tag), acridine dyes, cyanine dyes, fluorone dyes, oxazin dyes, phenanthridine dyes, rhodamine dyes, Alexafluor® dyes, and the like.

The described FN3 domain-specific antibodies and antigen-binding fragments may be used in a variety of assays to detect FN3 domains in a biological sample. Some suitable assays include, but should not be considered limited to, western blot analysis, radioimmunoassay, surface plasmon resonance, immunofluorimetry, immunoprecipitation, equilibrium dialysis, immunodiffusion, electrochemiluminescence (ECL) immunoassay, immunohistochemistry, fluorescence-activated cell sorting (FACS) or ELISA assay.

Kits for Detecting FN3 domains

Provided herein are kits for detecting FN3 domains in a biological sample. These kits include one or more of the FN3 domain antibodies described herein, or an antigen-binding fragment thereof, and instructions for use of the kit.

The provided FN3 domain antibody, or antigen-binding fragment, may be in solution; lyophilized; affixed to a substrate, carrier, or plate; or detectably labeled.

The described kits may also include additional components useful for performing the methods described herein. By way of example, the kits may comprise means for obtaining a sample from a subject, a control or reference sample, *e.g.,* a sample from a subject having slowly progressing cancer and/or a subject not having

cancer, one or more sample compartments, and/or instructional material which describes performance of a method of the invention and tissue specific controls or standards.

The means for determining the level of FN3 domains can further include, for example, buffers or other reagents for use in an assay for determining the level of FN3 domains. The instructions can be, for example, printed instructions for performing the assay and/or instructions for evaluating the level of expression of FN3 domains.

The described kits may also include means for isolating a sample from a subject. These means can comprise one or more items of equipment or reagents that can be used to obtain a fluid or tissue from a subject. The means for obtaining a sample from a subject may also comprise means for isolating blood components, such as serum, from a blood sample. Preferably, the kit is designed for use with a human subject.

FN3 domain-targeting chimeric antigen receptors (CARs)

In other general aspects, the invention relates to an FN3 domain-targeting CAR comprising an FN3 domain-specific scFv.

In one aspect, the invention relates to a CAR comprising:

a. an extracellular domain having an scFv that specifically binds to a non-randomized region of an FN3 domain;
b. a transmembrane domain; and
c. an intracellular signaling domain.

In some embodiments, in a nascent CAR, the extracellular domain is preceded by a signal peptide at the N-terminus. Any suitable signal peptide can be used in the invention. The signal peptide can be derived from a natural, synthetic, semi-synthetic or recombinant source.

According to embodiments of the invention, the extracellular domain of a CAR comprises an scFv that specifically binds to a non-randomized region of an FN3 domain. Any scFv that specifically binds to an FN3 domain according to embodiments of the invention, including but not limited to those described herein, can be used in the extracellular domain of the CAR.

According to embodiments of the invention, a CAR can further comprise a hinge region connecting the extracellular domain and the transmembrane domain. The

hinge region functions to move the extracellular domain away from the surface of the engineered immune cell to enable proper cell/cell contact, binding to the target or antigen and activation (Patel et al., Gene Therapy, 1999; 6: 412-419). Any suitable hinge region can be used in a CAR of the invention. It can be derived from a natural, synthetic, semi-synthetic or recombinant source. According to some embodiments, the hinge region of the CAR is a 6x GS peptide (SEQ ID NO: 84), or a fragment thereof, or a hinge region from a CD8 protein, or a derivative thereof. In particular embodiments, the hinge region has an amino acid sequence at least 90% identical to SEQ ID NO: 24, preferably the amino acid sequence of SEQ ID NO: 24.

Any suitable transmembrane domain can be used in a CAR of the invention. The transmembrane domain can be derived from a natural, synthetic, semi-synthetic or recombinant source. According to some embodiments, the transmembrane domain is a transmembrane domain from molecules such as CD8, CD28, CD4, CD2, GMCSFR and the like. In particular embodiments, the transmembrane domain has an amino acid sequence at least 90% identical to SEQ ID NO: 25, preferably the amino acid sequence of SEQ ID NO: 25.

Any suitable intracellular signaling domain can be used in a CAR of the invention. In particular embodiments, the entire intracellular signaling domain is used. In other particular embodiments, a truncated portion of the signaling domain that transduces the effector signal is used. According to embodiments of the invention, the intracellular signaling domain generates a signal that promotes an immune effector function of the CAR-containing cell, e.g. a CAR-T cell, including, but not limited to, proliferation, activation, and/or differentiation. In particular embodiments, the signal promotes, e.g., cytolytic activity, helper activity, and/or cytokine secretion of the CAR-T cell. In other embodiments, no intracellular signaling domain is used in a CAR of the invention and the CAR comprising an scFv that specifically binds to an FN3 domain of the invention is used along with an FN3 domain for targeting the effector cell to target cells.

According to some embodiments, the intracellular signaling domain comprises a functional signaling domain derived from CD3 zeta, TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD16, CD22, CD27, CD28, CD30, CD79a, CD79b, CD134 (also known as TNFRSF4 or OX-40), 4-1BB (CD137), CD278 (also known as ICOS), FcεRI, DAP10, DAP12, ITAM domains or CD66d, and the like.

According to particular embodiments, the intracellular signaling domain comprises a primary signaling domain and one or more co-stimulatory signaling domains.

In one embodiment, the intracellular signaling domain comprises a primary intracellular signaling domain having a functional signaling domain derived from human CD3zeta. In particular embodiments, the primary intracellular signaling domain has an amino acid sequence at least 90% identical to SEQ ID NO: 27, preferably the amino acid sequence of SEQ ID NO: 27.

According to some embodiments, the intracellular signaling domain further comprises the co-stimulatory intracellular signaling domain derived from human 4-1BB. In particular embodiments, the co-stimulatory intracellular signaling domain has an amino acid sequence at least 90% identical to SEQ ID NO: 26, preferably the amino acid sequence of SEQ ID NO: 26.

In one embodiment, a CAR of the invention is associated with a host cell expressing the CAR.

In another embodiment, a CAR of the invention is present in an isolated cell membrane of the host cell expressing the CAR.

In yet another embodiment, a CAR of the invention is purified or isolated from other components of the host cell expressing the CAR.

Polynucleotides, vectors and host cells

In other general aspects, the invention relates to isolated polynucleotides and vectors encoding FN3 domain antibodies or CARs of the invention, and recombinant cells containing the vectors.

Nucleic acids encoding any of the various proteins or polypeptides disclosed herein can be synthesized chemically or using other methods in the art in view of the present disclosure. Codon usage can be selected so as to improve expression in a cell. Such codon usage will depend on the cell type selected. Specialized codon usage patterns have been developed for E. coli and other bacteria, as well as mammalian cells, plant cells, yeast cells and insect cells. See for example: Mayfield et al., PNAS USA. 2003 100(2):438-42; Sinclair et al. Protein Expr Purif. 2002 (1):96-105; Connell, Curr Opin Biotechnol. 2001 (5):446-9; Makrides et al. Microbiol Rev. 1996 60(3):512-38; and Sharp et al. Yeast. 1991 7(7):657-78.

General techniques for nucleic acid manipulation are within the purview of one skilled in the art and are also described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Laboratory Press, 2 ed., 1989, or Ausubel et al., eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, and periodic updates, herein incorporated by reference. The DNA encoding a protein is operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, viral, or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants are additionally incorporated. Suitable regulatory elements are well-known in the art.

It will be appreciated by those skilled in the art that the coding sequence of a protein can be changed without changing the amino acid sequence of the protein. Accordingly, it will be understood by those skilled in the art that nucleic acid sequences encoding FN3 domain antibodies or CARs of the invention can be altered without changing the amino acid sequences of the proteins.

In one embodiment, the invention relates to a vector comprising an isolated nucleic acid encoding an FN3 domain antibody or a CAR of the invention. Any vector known to those skilled in the art in view of the present disclosure can be used, such as a plasmid, cosmid, a phage vector or a viral vector. In one embodiment, the vector is an expression vector comprising a polynucleotide sequence encoding an FN3 domain or a CAR of the invention operably linked to a promoter sequence, optionally one or more other regulatory sequences.

In another embodiment, the invention relates to transient expression of a CAR of the invention by an mRNA encoding the CAR. In one aspect, the mRNA encoding the CAR is introduced into an immune effector cell as a form of transient transfection, wherein the expression of the non-integrated transgene is expressed for a period of hours, days or weeks, wherein the period of time of expression is less than the period of time for expression of the gene if integrated into the genome or contained within a stable plasmid replicon in the host cell. In one aspect, the mRNA is produced by in vitro transcription using a PCR-generated template.

In another general aspect, the invention relates to a host cell comprising an isolated nucleic acid encoding an FN3 domain antibody or a CAR of the invention. The host cell can be stably or transiently transfected with a nucleic acid molecule of the invention.

Suitable host cells include prokaryotes, yeast, mammalian cells, or bacterial cells. Suitable bacteria include gram negative or gram positive organisms, for example, E. coli or Bacillus spp. Yeast, preferably from the Saccharomyces species, such as S. cerevisiae, can also be used for production of polypeptides. Various mammalian or insect cell culture systems can also be employed to express recombinant proteins. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, (Bio/Technology, 6:47, 1988). In some instances, it will be desired to produce proteins in vertebrate cells, such as for glycosylation, and the propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of suitable mammalian host cell lines include endothelial cells, COS-7 monkey kidney cells, CV-1, L cells, C127, 3T3, Chinese hamster ovary (CHO), human embryonic kidney cells, HeLa, 293, 293T, and BHK cell lines.

A host cell of the invention can be an engineered FN3 domain-targeting immune cell, which is described in detail *infra*.

Protein production

In another general aspect, the invention relates to a method of producing an FN3 domain antibody of the invention, comprising culturing a host cell comprising a nucleic acid encoding the FN3 domain antibody under conditions to produce the FN3 domain antibody of the invention, and recovering the FN3 domain antibody from the cell or cell culture (e.g., from the supernatant). Expressed FN3 domain antibodies can be harvested from the cells or cell culture and purified according to conventional techniques known in the art in view of the present disclosure.

In another general aspect, the invention relates to a method of producing a CAR of the invention, comprising culturing a host cell comprising a nucleic acid encoding the CAR under conditions to produce the CAR of the invention, and recovering the CAR. Expressed CARs can be harvested from the cells and purified according to conventional techniques known in the art and as described herein.

Host cells are transformed with the expression or cloning vectors for protein production and cultured in conventional nutrient media modified as appropriate, e.g., for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the proteins of this invention can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al, Meth. Enz. 58:44 (1979); Barnes et al, Anal. Biochem. 102:255 (1980); US4767704; US4657866; US4927762; US4560655; US5122469; WO90/03430; WO87/00195 or USRE30985 can be used as culture media for the host cells. Any of these media can be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements can also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Proteins disclosed herein can also be produced using cell-translation systems *in vitro*. For such purposes, the nucleic acids encoding the proteins must be modified to allow *in vitro* transcription to produce mRNA and to allow cell-free translation of the mRNA in the particular cell-free system being utilized. Exemplary eukaryotic cell-free translation systems include, for example, mammalian or yeast cell-free translation systems, and exemplary prokaryotic cell-free translation systems include, for example, bacterial cell-free translation systems.

Proteins disclosed herein can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL). Modifications to the protein can also be produced by chemical synthesis.

The proteins disclosed herein can be purified by isolation/purification methods for proteins generally known in the field of protein chemistry. Non-limiting examples include extraction, recrystallization, salting out (e.g., with ammonium sulfate or sodium sulfate), centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion exchange chromatography, hydrophobic chromatography, normal phase chromatography, reversed-phase chromatography, gel filtration, gel permeation chromatography, affinity chromatography, electrophoresis, countercurrent distribution or any combinations of these. After purification, proteins can be exchanged into different buffers and/or concentrated by any of a variety of methods known to the art, including, but not limited to, filtration and dialysis.

The purified proteins are preferably at least 85% pure, more preferably at least 95% pure, and most preferably at least 98% pure.

Engineered FN3 domain-targeting CAR-T cells, compositions and methods thereof

In another general aspect, the invention relates to engineered FN3 domain-targeting immune cells comprising an FN3 domain-targeting CAR of the invention, methods of making the engineered immune cells, compositions comprising the engineered immune cells, and methods of using the engineered immune cells to treat diseases such as multiple myeloma.

In one general aspect, the invention relates to engineered immune cells comprising FN3 domain-targeting CARs of the invention.

According to some embodiments, the immune cell can be made less allogeneic, for instance, by inactivating at least one gene expressing one or more component of T cell receptor (TCR) as described in WO 2013/176915, which can be combined with the inactivation of a gene encoding or regulating HLA I/B2-microglobulin (B2M) protein expression. Accordingly, the risk of graft versus host syndrome and graft rejection is significantly reduced. A T cell lacking a functional TCR, referred to as a "TCR knockout" or a "TCR-KO" cell, can be engineered such that it does not express any functional TCR on its surface, engineered such that it does not express one or more subunits that comprise a functional TCR (e.g., engineered such that it does not express (or exhibits reduced expression) of TCR alpha, TCR beta, TCR gamma, TCR delta, TCR epsilon, and/or TCR zeta) or engineered such that it produces very little functional

TCR on its surface. Alternatively, the T cell can express a substantially impaired TCR (i.e., a TCR that will not elicit an adverse immune reaction in a host), e.g., by expression of mutated or truncated forms of one or more of the subunits of the TCR. Modified T cells that lack expression of a functional TCR and/or B2M can be obtained by any suitable means, including a knock out or knock down of one or more subunit of TCR and/or B2M. For example, the T cell can include a knock down of TCR and/or B2M using siRNA, shRNA, clustered regularly interspaced short palindromic repeats (CRISPR), transcription-activator like effector nuclease (TALEN), megaTAL, meganuclease, or zinc finger endonuclease (ZFN).

In particular embodiments, the immune effector cell comprising an FN3 domain-targeting CAR of the invention is a T cell, a NKT cell or a NK cell, preferably, a human T cell or human NK cell, more preferably a TCR knockout cell, most preferably a human TCR knockout cell and/or an HLA I/B2M knockout cell. In other embodiments, the immune effector cell comprising a FN3 domain-targeting CAR of the invention is an engineered T cell line, such as a TALL-104 T cell line (i.e., a IL-2-dependent human non-restricted cytotoxic T cell line that expresses CD8 and CD3 but not CD16).

Immune effector cells of the invention can be autologous (i.e., "self," e.g., autogenic) or non-autologous (i.e., "non-self," e.g., allogeneic, syngenic, xenogenic). Autologous refers to any material derived from the same individual into whom it is later to be re-introduced. Non-autologous refers to any material derived from a different individual of the same species as the individual into whom the material is later to be introduced

In another general aspect, the invention relates to methods of making the engineered FN3 domain-targeting immune cells comprising FN3 domain-targeting CARs of the invention. A vector encoding the CAR can be directly transduced into an immune cell. Alternatively, *in vitro* transcribed RNA or synthetic RNA encoding the CAR can be introduced into an immune cell.

According to particular embodiments, the method of making the engineered FN3 domain-targeting immune cells comprises transfecting or transducing immune effector cells isolated from an individual such that the immune effector cells express one or more CAR(s) according to embodiments of the invention. Methods of preparing immune cells for immunotherapy are described, e.g., in WO2014/130635,

WO2013/176916 and WO2013/176915, which are incorporated herein by reference. Individual steps that can be used for preparing engineered immune cells are disclosed, e.g., in WO2014/039523, WO2014/184741, WO2014/191128, WO2014/184744 and WO2014/184143, which are incorporated herein by reference.

In a particular embodiment, the immune effector cells, such as T cells, are genetically modified with CARs of the invention (e.g., transduced with a viral vector comprising a nucleic acid encoding a CAR) and then are activated and expanded in vitro. In various embodiments, T cells can be activated and expanded before or after genetic modification to express a CAR, using methods as described, for example, in US6352694, US6534055, US6905680, US6692964, US5858358, US6887466, US6905681, US7144575, US7067318, US7172869, US7232566, US7175843, US5883223, US6905874, US6797514, US6867041, US2006/121005, which are incorporated herein by reference. T cells can be expanded in vitro or in vivo. Generally, the T cells of the invention can be expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex-associated signal and a ligand that stimulates a co-stimulatory molecule on the surface of the T cells. As non-limiting examples, T cell populations can be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore, or by activation of the CAR itself. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. Conditions appropriate for T cell culture include, e.g., an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 5 (Lonza)) that can contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), cytokines, such as IL-2, IL-7, IL-15, and/or IL-21, insulin, IFN-g, GM-CSF, TGFb and/or any other additives for the growth of cells known to the skilled artisan. In other embodiments, the T cells can be activated and stimulated to proliferate with feeder cells and appropriate antibodies and cytokines using methods such as those described in US6040177, US5827642, and WO2012129514, which are incorporated herein by reference.

In some embodiments, a CAR-expressing cell of the invention can further comprise a second CAR having an extracellular domain that specifically binds to the same target or a different target. Preferably, the immune cell expresses two CARs that specifically bind to two different targets, or the immune cell expresses a bispecific receptor, such as a CAR comprising two FN3 domains that specifically bind to two different targets, i.e., an FN3 domain and another target, associated with a disease of interest. For example, the other target can also be associated with a type of cancer. More preferably, the two CARs also have different intracellular signaling domains, for example, the first CAR has a costimulatory signaling domain but not a primary signaling domain, and the second CAR has a primary signaling domain but not a costimulatory signaling domain, or vice versa. By the placement of a costimulatory signaling domain, such as that from 4-1BB, CD28, CD27 ICOS, or OX-40, onto one CAR, and the primary signaling domain, such as that from CD3 zeta, on the other CAR, one can limit the CAR activity to cells where both targets are expressed, e.g., for enhanced specificity.

In some embodiments, a CAR-expressing cell of the invention can further comprise an inhibitory CAR as a self-regulating safety switch to constrain T cell–based therapies and avoid off-tumor toxicity. For example, the inhibitory CAR can include an extracellular domain that binds specifically to a target found on normal cells but not on the target cancer cells. The inhibitory CAR also includes an intracellular domain having an inhibitory receptor signaling domain, such as an intracellular domain of an inhibitory molecule including, but not limited to, PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta. Cells expressing an FN3 domain-targeting CAR and an inhibitory CAR are suppressed when encountering a normal cell, but activated when encountering a tumor cell not expressing the normal cell target.

In some other embodiments, a CAR-expressing cell of the invention can further comprise an agent that enhances the activity of a CAR-expressing cell. For example, the agent can inhibit the activity of an inhibitory molecule, such as those described herein, in the host cell.

According to particular embodiments, the engineered immune CAR-expressing cells are further genetically engineered to be chemoresistant. This chemoresistance can allow the engineered immune cells to survive in the presence of drugs while selectively targeting an FN3 domain of interest.

According to some embodiments, drug resistance can be conferred on the CAR-expressing cells by genetically engineering them to express at least one drug resistance gene. A drug resistance gene of the invention can encode resistance to anti-metabolite, methotrexate, vinblastine, cisplatin, alkylating agents, anthracyclines, cytotoxic antibiotics, anti-immunophilins, their analogs or derivatives, etc. Several drug resistance genes have been identified that can be used to confer drug resistance to engineered immune CAR-expressing cells of the invention. See, e.g., Takebe et al., Mol Ther. 2001 Jan;3(1):88-96.; Sugimoto et al, J Gene Med. 2003 May;5(5):366-76; Zielske et al., J Clin Invest. 2003 Nov;112(10):1561-70; Nivens et al, Cancer Chemother Pharmacol. 2004 Feb;53(2):107-15; Bardenheuer et al., Leukemia. 2005 Dec;19(12):2281-8; Kushman et al., Carcinogenesis. 2007 Jan;28(1):207-14. Examples of drug resistance genes that can be expressed in the cells include a mutant or modified form of Dihydrofolate reductase (DHFR), a mutant or modified form of ionisine-5'-monophosphate dehydrogenase II (IMPDH2), multidrug resistance protein 1 (MDR1), calcineurin, methylguanine transferase (MGMT), microRNA-21, the antibiotic resistance genes ble and mcrA, etc. According to particular embodiments, said drug resistance genes can be expressed in the cell by any suitable means, including, e.g., introducing a transgene encoded by at least one vector into a cell.

Resistance to anti-cancer chemotherapies can also be conferred, e.g., by inactivating genes that are responsible for the cell's sensitivity to the drug. Examples of genes that can be inactivated to confer drug resistance to the cells include, e.g., CD52, glucocorticoid receptors, CD3, human hypoxanthine-guanine phosphoribosyl transferase (HPRT), human deoxycytidine kinase (dCK), etc. Genes responsible for the cell's sensitivity to anti-cancer drugs can be inactivated by any suitable means, including a knock out or knock down of the gene, e.g., using siRNA, shRNA, CRISPR, TALEN, or ZFN.

In another general aspect, the invention relates to a pharmaceutical composition, comprising an engineered FN3 doamin-targeting immune cell of the invention and a pharmaceutically acceptable carrier. In view of the present disclosure, any

pharmaceutically acceptable carrier suitable for use in CAR-T pharmaceutical composition can be used in the invention.

In another general aspect, the invention relates to a method of treating a cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition of the invention.

According to embodiments of the invention, a therapeutically effective amount of the pharmaceutical composition stimulates an immune response in a subject in need thereof, preferably results in treatment of a disease, disorder, or condition; prevents or slows the progression of the disease, disorder, or condition; or reduces or completely alleviates symptoms associated with the immune disease, disorder, or condition.

According to particular embodiments, the disease, disorder or condition to be treated is a hyperproliferative disease. According to other particular embodiments, the disease, disorder or condition to be treated is a cancer or tumor, or a malignant hyperproliferative disease, preferably a cancer selected from the group consisting of a solid tumor, a hematologic cancer, bladder cancer, biliary cancer, brain cancer, breast cancer, colon cancer, esophageal cancer, gastric cancer, glioma, head cancer, leukemia, liver cancer, lung cancer, lymphoma, multiple myeloma, neck cancer, ovarian cancer, melanoma, pancreatic cancer, renal cancer, salivary cancer, stomach cancer, thymic epithelial cancer, and thyroid cancer.

According to particular embodiments, a therapeutically effective amount of the FN3 domain-targeting immune cell composition is sufficient to achieve one, two, three, four, or more of the following effects in a subject in need thereof: (i) reduce or ameliorate the severity of the disease, disorder or condition to be treated or a symptom associated therewith; (ii) reduce the duration of the disease, disorder or condition to be treated, or a symptom associated therewith; (iii) prevent the progression of the disease, disorder or condition to be treated, or a symptom associated therewith; (iv) cause regression of the disease, disorder or condition to be treated, or a symptom associated therewith; (v) prevent the development or onset of the disease, disorder or condition to be treated, or a symptom associated therewith; (vi) prevent the recurrence of the disease, disorder or condition to be treated, or a symptom associated therewith; (vii) reduce hospitalization of a subject having the disease, disorder or condition to be treated, or a symptom associated therewith; (viii) reduce hospitalization length of a subject having the disease, disorder or condition to be treated, or a symptom associated

therewith; (ix) increase the survival of a subject with the disease, disorder or condition to be treated, or a symptom associated therewith; (xi) inhibit or reduce the disease, disorder or condition to be treated, or a symptom associated therewith in a subject; and/or (xii) enhance or improve the prophylactic or therapeutic effect(s) of another therapy. In particular embodiments, a therapeutically effective amount refers to the amount of therapy which is sufficient to achieve one, two, three, four, or more of the following effects: (i) decrease the tumor volume; (ii) decrease the number of tumor cells; (iii) decrease the number of metastases; (iv) increase the life expectancy; (v) decrease tumor cell proliferation; (vi) decrease tumor cell survival; (vii) ameliorate physiological symptoms associated with the cancerous condition; and/or (viii) prevent the occurrence of tumor.

The therapeutically effective amount or dosage can vary according to various factors, such as the disease, disorder or condition to be treated, the means of administration, the target site, the physiological state of the subject (including, e.g., age, body weight, health), whether the subject is a human or an animal, other medications administered, and whether the treatment is prophylactic or therapeutic. Treatment dosages are optimally titrated to optimize safety and efficacy. The exact dose can be ascertained by one skilled in the art using known techniques. In general, the FN3 domain-targeting immune cells are administered at a dose of about 10^5 to 10^8 cells/kg body weight. According to some embodiments, the total dose of cells can be administered to the subject by dose fractionation, e.g., one, two, three or more separate administration of a partial dose.

According to particular embodiments, the compositions described herein are formulated to be suitable for the intended route of administration to a subject. For example, the compositions described herein can be formulated to be suitable for intravenous, subcutaneous, or intramuscular administration.

According to particular embodiments, a composition used in the treatment of a cancer can be used in combination with another treatment including, but not limited to, a chemotherapy, a lympho-depleting therapy, a radiation therapy, other immune-oncology drug, a targeted therapy, a cancer vaccine, or other anticancer drugs.

As used herein, the term "in combination," in the context of the administration of two or more therapies to a subject, refers to the use of more than one therapy. The use of the term "in combination" does not restrict the order in which therapies are

administered to a subject. For example, a first therapy (e.g., a composition described herein) can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy to a subject.

In another general aspect, the invention relates to a method of redirecting immune cells to target their killing of FN3 domain binding cells in a subject in need thereof, comprising administering to the subject a pharmaceutical composition of the invention.

In another general aspect, the invention relates to a method of producing a pharmaceutical composition comprising FN3 domain-targeting immune cells of the invention, comprising combining FN3 domain-targeting immune cells with a pharmaceutically acceptable carrier to obtain the pharmaceutical composition.

The invention provides also the following non-limiting embodiments.

EMBODIMENTS

1. An isolated antibody, or antigen-binding fragment thereof, that specifically binds to a non-randomized region of a fibronectin type III (FN3) domain.
2. The isolated antibody, or an antigen-binding fragment of embodiment 1, comprising:

    a. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 1, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 4, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 7, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 9, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 11, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 13;

    b. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 2, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 5,

a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 8, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 10, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 12, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 13;

c. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 3, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 8, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 10, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 12, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 13;

d. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 35, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 41, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 44, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 46, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 48, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50;

e. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 36, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 42, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 45, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 47 a light chain CDR2 having the amino acid sequence of SEQ ID NO: 49, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50;

f. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 37, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 43, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 45, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 47, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 49, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50;

g. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 38, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 51, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 54, a

light chain CDR1 having the amino acid sequence of SEQ ID NO: 56, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 58, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 60;

h. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 39, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 52, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 55, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 57 a light chain CDR2 having the amino acid sequence of SEQ ID NO: 59, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 61; or

i. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 40, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 53, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 55, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 57, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 59, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 61.

3. The antibody or antigen-binding fragment of embodiment 1, wherein

a. the variable heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 14, and the variable light chain of the antibody comprises the amino acid sequence of SEQ ID NO:15;

b. the variable heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 74, and the variable light chain of the antibody comprises the amino acid sequence of SEQ ID NO:75; or

c. the variable heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 78, and the variable light chain of the antibody comprises the amino acid sequence of SEQ ID NO:79;

4. The antibody or antigen-binding fragment of embodiment 1, wherein

a. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 18 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 19;

b. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 20 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 21;

c. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 62 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 63; or

d. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 64 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 65.

5. The antigen binding fragment of any one of embodiments 1 to 4 wherein the antigen binding fragment is a Fab fragment, a Fab2 fragment, or a single chain antibody.

6. The antibody or antigen-binding fragment of any one of embodiments 1 to 5 wherein the antibody or antigen-binding fragment thereof is an IgG.

7. An isolated polynucleotide encoding the antibody or antigen-binding fragment of any one of embodiments 1 to 6.

8. A vector comprising the polynucleotide of embodiment 7

9. A host cell comprising the isolated polynucleotide of embodiment 7

10. A host cell comprising the vector of embodiment 8.

11. An isolated polynucleotide encoding a chimeric antigen receptor (CAR) comprising:

(a) an extracellular domain comprising an scFv that specifically binds to a non-randomized region of an FN3 domain;

(b) a transmembrane domain; and

(c) an intracellular signaling domain,

wherein the CAR optionally further comprises a hinge region connecting the extracellular domain and the transmembrane domain.

12. The isolated polynucleotide of embodiment 11, wherein the CAR comprises:

(a) an extracellular domain comprising an scFv having an amino acid sequence that is at least 90% identical to one of SEQ ID NOs: 68-73;

(b) a hinge region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 24;

(c) a transmembrane domain comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 25; and

(d) an intracellular signaling domain comprising a co-stimulatory domain having an amino acid sequence that is at least 90% identical to SEQ ID

NO: 26, and a primary signaling domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 27.

13. The isolated polynucleotide of embodiment 12, wherein:

(a) the extracellular domain comprises the amino acid sequence of one of SEQ ID NOs: 68-73;

(b) the hinge region comprises the amino acid sequence of SEQ ID NO: 24;

(c) the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 25; and

(d) the intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 26 and the amino acid sequence of SEQ ID NO: 27.

14. A vector comprising the polynucleotide of any of embodiments 11-13.

15. A host cell comprising the polynucleotide of any of embodiments 11-13.

16. A host cell comprising the vector of embodiment 14.

17. A chimeric antigen receptor (CAR) comprising:

(a) an extracellular domain comprising an scFv that specifically binds to a non-randomized region of an FN3 domain;

(b) a transmembrane domain; and

(c) an intracellular signaling domain,

wherein the CAR optionally further comprises a hinge region connecting the extracellular domain and the transmembrane domain.

18. The CAR of embodiment 16, comprising:

(a) an extracellular domain comprising an scFv having an amino acid sequence that is at least 90% identical to one of SEQ ID NOs: 68-73;

(b) a hinge region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 24;

(c) a transmembrane domain comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 25; and

(d) an intracellular signaling domain comprising a co-stimulatory domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 26, and a primary signaling domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 27.

19. The CAR of embodiment 17, wherein:

(a) the extracellular domain comprises the amino acid sequence of one of SEQ ID NOs: 68-73;

(b) the hinge region comprises the amino acid sequence of SEQ ID NO: 24;

(c) the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 25; and

(d) the intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 26 and the amino acid sequence of SEQ ID NO: 27.

EXAMPLES

The following examples are provided to supplement the prior disclosure and to provide a better understanding of the subject matter described herein. These examples should not be considered to limit the described subject matter. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be apparent to persons skilled in the art and are to be included within, and can be made without departing from, the true scope of the invention.

EXAMPLE 1: IMMUNIZATION OF RABBITS TO GENERATE ANTI-FN3 DOMAIN ANTIBODIES

Rabbit monoclonal antibody generation was performed using the Tencon25 antigen (SEQ ID NO: 28).

LPAPKNLVVSEVTEDSARLSWTAPDAAFDSFLIQYQESEKVGEAIVLTVPGSER SYDLTGLKPGTEYTVSIYGVKGGHRSNPLSAIFTT

Animals: 3 months old New Zealand white rabbits with IDs E4831and E4832 and immunization was performed.

Immunization protocol:

Rabbits were immunized using standard protocol of five injections and two test bleeds per rabbit. At the time of each injection, the antigen aliquot was thawed and combined with Complete Freund's Adjuvant (CFA) (for the first injection) or with incomplete Freund's Adjuvant (IFA) for the subsequently injections. The injection

route was subcutaneous (SC). The immunization details are summarized in Table 3:

Table 3: Rabbit Immunization Details

Antigen ID	Rabbit ID	Type	number	amount	Note
Tencon25	E4831				
		Bleed	0	5ml	
		Injection	1	0.4	
		Injection	2	0.2	
		Injection	3	0.2	
		Bleed	1	5ml	
		Injection	4	0.2	
		Bleed	2	5ml	
Tencon25	E4832				
		Bleed	0	5ml	
		Injection	1	0.4	
		Injection	2	0.2	
		Injection	3	0.2	
		Bleed	1	5ml	
		Injection	4	0.2	
		Bleed	2	5ml	
		Injection	5	0.4	

ELISA titer examination:

The serum titer against Tencon25 as well as the counter screening antigens Tencon28 (SEQ ID NO: 29) and P114-83 (SEQ ID NO: 30) was evaluated using test bleeds 1 and 2 with Epitomics, Inc. standard protocols. In conclusion, both rabbits had good immune response against the immunogen and had met the standard cut-off for splenectomy (O.D. > 0.3 at 1:64K dilution). Rabbit E4832 was chosen as the candidate for splenectomy and monoclonal fusion.

Splenectomy:

IV boost was administered to rabbit E4832, followed by splenectomy The spleen was delivered and splenocytes were isolated, according to Epitomics'

standard procedure (Table 3).

Table 4: Details of Splenocyte Isolation

Antigen id	Rabbit id	Tissue type	Weight (g)	Size (cm)	Viability (%)	Total Cells in Mil
Tencon25	E4832	Spleen	2.14	6	80	1300

Fusion:

Two hundred million lymphocyte cells were fused with 100 million fusion partner cells and plated on 20X 96-well plates respectively (Table 4). The plates were kept in tissue culture incubators under standard conditions.

Table 5: Fusion Information

Antigen ID	Fusion Code	Fusion date	# of Plates	Fusion Efficiency (%)
CEN-11	F1	8/10/2011	20	46
CEN-11	F2	8/11/2011	20	59

Cell growth was examined 2-3 weeks after fusion and fusion efficiency computed using the number of wells with growth divided by the total number of wells examined. A minimum of two plates were examined for each fusion.

The screening process is summarized below:

- *Pre-screen*: plates #5 & #25;
- *Primary screen*: the remaining 38 plates;
- *Screen method*: standard ELISA, plates coated with 50 ng of Tencon25/well;
- *Positive control*: bleed 2 of E4832 at 1:10K dilution;
- *Results*: 158 clones with O.D. greater than 0.5 were considered putatively positive and were further expanded to 24-well plate;
- *Confirmatory screen*: plates coated with 50 ng of Tencon25/well or coated with 50 ng of Tencon28 or P114-83.
- *Results*: 151 clones were confirmed positive against Tencon25. Among them, 78 were Tencon25 specific as they are Tencon28 negative.

EXAMPLE 2: SELECTION OF ANTI-FN3 DOMAIN ANTIBODIES

Production:

Clone supernatant was evaluated for binding to all centyrins and no binding to negative control protein. From this evaluation, one clone, CEN-25-105-5, was picked for production. The hybridoma cells were cultured and adapted into serum-free medium and inoculated into 1L integra flasks. After harvesting, the antibodies were purified using protein A resins and QCed. Yield was 20 mg for CEN-25-105-5.

Table 6. CDR amino acid sequences of CEN-25-105-5

(SEQ ID NO:)

Delineation	HC-CDR1	HC-CDR2	HC-CDR3	LC-CDR1	LC-CDR2	LC-CDR3
IMGT	GIDLSTSV (1)	IYTNVNT (4)	ARAVYAGAMDL (7)	ERIYSN (9)	KAS (11)	QYTSYGSGYVGT (13)
Kabat	TSVMG (2)	FIYTNVNTYYASWAKG (5)	AVYAGAMDL (8)	QASERIYSNLA (10)	KASTLAS (12)	QYTSYGSGYVGT (13)
Chothia	GIDLSTS (3)	YTNVN (6)	AVYAGAMDL (8)	QASERIYSNLA (10)	KASTLAS (12)	QYTSYGSGYVGT (13)

VH and VL of CEN-25-105-5 is shown below in Table 7.

Table 7: Variable heavy chain and variable light chain sequences of CEN-25-105-5

Variable regions of heavy chain and light chains for CEN-25-105-5 were cloned into rat and mouse IgGK expression vectors for expression and characterization. CEN-25-105-5 is now referred to as AS7B91 as a mouse IgG2a kappa antibody and AS7B90 as the rat IgG1 kappa antibody. AS7B16 and AS7B82 were also generated from mouse and rabbit V regions, respectively. Tables 8 and 9 show the sequences of the CDRs and the sequences of the V regions of AS7B16 and AS7B82. The heavy chain and light chain sequences for all antibodies are shown below in Table 10.

Sequence type	VH Sequence	SEQ ID NO:	VL Sequence	SEQ ID NO:
Amino acid	QSLEESGGRLVTPGTPLTLTCTVSGIDLSTSVMGWVRQAPGKGLESIGFIYTNVNTYYASWAKGRFTISRTSTTVDLKITSPTTGDTATYFCARAVYAGAMDLWGQGTLVTVSS	14	DVVMTQTPASVSGPVGGTVTIKCQASERIYSNLAWYQQKPGQPPKLLIYKASTLASGVSSRFKGSGSGTEFTLTIRDLECADAATYSCQYTSYGSGYVGTFGGGTEVVVEG	15
DNA	Ctggaggagtccgggggtcgcctggtcacgcctgggacacccctgacactcacctgcacagtctctggaatcgacctcagtacctctgtcatgggttgggtccgccaggctccaggggaaggggctggaatccatcggattcatttatactaatgttaacacatactacgcgagctgggcaaaaggccgattcaccatctccagaacctcgaccacggtggatctgaaaatcaccagtccgacaaccggggacacggccacctatttctgtgccagagctgttttatgctggtgctatggacttgtgggggccaaggcaccctggtcaccgtctcctca	16	gatgttgtgatgacccagactccagcctccgtgtctggacctgtgggaggcacagtcaccatcaagtgccaggccagtgagagaatttatagcaatttagcctggtatcagcagaaaccagggcagcctcccaaactcctgatctacaaggcatccactctggcatctggggtctcatcgcggttcaaaggcagtggatctgggacagagttcactctcaccatcagggaccttgagtgtgccgatgctgccacttactcctgtcaatatacttcttatggcagtggttatgttggtactttcggcggagggaccgaggtggtggtcgaaggt	17

Table 8. CDR amino acid sequences of AS7B16 and AS7B82

AS7B16

Delineation	HC-CDR1	HC-CDR2	HC-CDR3	LC-CDR1	LC-CDR2	LC-CDR3

Delineation	HC-CDR1	HC-CDR2	HC-CDR3	LC-CDR1	LC-CDR2	LC-CDR3
IMGT	GFSLNTSG TG (35)	IWWDDDK (41)	VRIKGRMDY (44)	QSVLFGSKQKNY (46)	WAS (48)	HQYLSLFT (50)
Kabat	TSGTGVG (36)	HIWWDDDKGYNPAL KS (42)	IKGRMDY (45)	KSSQSVLFGSKQKN YLA (47)	WASTRES (49)	HQYLSLFT (50)
Chothia	GFSLNTSG T (37)	WWDDD (43)	IKGRMDY (45)	KSSQSVLFGSKQKN YLA (47)	WASTRES (49)	HQYLSLFT (50)

5

AS7B82

Delineation	HC-CDR1	HC-CDR2	HC-CDR3	LC-CDR1	LC-CDR2	LC-CDR3
IMGT	GIDFSSVA Y (38)	IYAGSSSSI (51)	ARGLFTSGSG YYIDM (54)	QSIGSD (56)	SAS (58)	QCTYSSSTGY NA (60)
Kabat	SVAYMC (39)	CIYAGSSSSIYYASWA KG (52)	GLFTSGSGYY IDM (55)	QASQSIGSNLA (57)	GASNLAA (59)	QRGYISSAVD FFV (61)
Chothia	GIDFSSVA (40)	YAGSSSS (53)	GLFTSGSGYY IDM (55)	QASQSIGSNLA (57)	GASNLAA (59)	QRGYISSAVD FFV (61)

Table 9: Variable heavy chain and variable light chain sequences of AS7B16 and AS7B82

Sequence type	VH Sequence	SEQ ID NO:	VL Sequence	SEQ ID NO:
Amino acid AS7B16	QVTLKESGPGILQPSQ TLSLTCSFSGFSLNTS GTGVGWIRQPSGKGL EWLAHIWWDDDKGY NPALKSRLTISKNTSS NLVFLKIASVDTADT ATYYCVRIKGRMDY WGQGTSVTVSS	74	NIMMTQSPSSLAVSAG EKVTMNCKSSQSVLFG SKQKNYLAWYQQKPG QSPKLLIYWASTRESG VPDRFTGSGSGTDFILT ISNVQAEDLAVYYCHQ YLSLFTFGSGTKLEIK	75
DNA AS7B16	AGGTTACTCTGAAA GAGTCTGGCCCTGG GATATTGCAGCCCTC CCAGACCCTCAGTCT GACTTGTTCTTTCTC TGGGTTTTCACTGAA CACTTCTGGTACGGG TGTAGGCTGGATTCG TCAGCCTTCAGGGA AGGGTCTGGAGTGG CTGGCACACATTTGG TGGGATGATGACAA GGGGTATAACCCAG	76	AACATTATGATGACA CAGTCGCCATCCTCTC TGGCTGTGTCTGCAG GAGAAAAGGTCACTA TGAACTGTAAGTCCA GTCAAAGTGTTTTATT CGGTTCAAAACAGAA GAACTATTTGGCCTG GTACCAGCAGAAACC AGGGCAGTCTCCTAA ATTGCTGATCTACTGG GCATCCACTAGGGAA TCTGGTGTCCCTGATC	77

	CCCTGAAGAGCCGA CTGACAATCTCCAA AAACACCTCCAGCA ACCTGGTATTCCTCA AGATCGCCAGTGTG GACACTGCAGATAC TGCCACATATTACTG TGTTCGAATCAAAG GCCGGATGGACTAC TGGGGTCAAGGAAC CTCAGTCACCGTCTC CTCA		GCTTCACAGGCAGTG GATCTGGGACAGATT TTATACTTACCATCAG CAATGTACAAGCTGA AGACCTGGCAGTTTA TTACTGTCATCAATAC CTCTCCCTATTCACGT TCGGCTCGGGGACAA AGTTGGAAATAAAA	
Amino acid AS7B82	QEQQKESGGGLVKPG ASLTLTCTASGIDFSS VAYMCWVRQAPGK GLEWIACIYAGSSSSI YYASWAKGRFTVSR TSSTTVTLQMTSLTA ADTATYFCARGLFTS GSGYYIDMWGPGTL VTVSS	78	DVVMTQTPSSVEVAV GGTVTIKCQASQSIGSN LAWYQQKPGQRPKLLI YGASNLAAGVPSRFSG SGSGTQFTLTISDVECA DAATYYCQRGYISSAV DFFVFGGGTEVVVKG	79
DNA AS7B82	CAGGAGCAGCAGAA GGAGTCCGGGGGAG GCCTGGTCAAGCCT GGGGCATCCCTGAC ACTCACCTGCACAG CTTCTGGAATCGACT TCAGTAGTGTCGCCT ACATGTGTTGGGTCC GCCAGGCTCCAGGG AAGGGGCTGGAGTG GATCGCATGCATTTA TGCTGGTAGTAGTA GTAGCATCTACTACG CGAGCTGGGCGAAA GGCCGATTCACCGTC TCCAGAACCTCGTCT ACCACGGTGACTCT GCAAATGACCAGTC TGACAGCCGCGGAC ACGGCCACCTATTTC TGTGCGAGAGGTCT ATTTACTAGTGGTAG TGGATATTATATAGA CATGTGGGGCCCAG GCACCCTGGTCACC	80	GATGTCGTGATGACC CAGACTCCATCCTCTG TGGAGGTAGCTGTGG GAGGCACAGTCACCA TCAAGTGCCAGGCCA GTCAGAGCATTGGTA GTAATTTAGCCTGGT ATCAGCAGAAACCAG GGCAGCGTCCCAAGC TCCTGATCTATGGTGC ATCCAATCTGGCCGC TGGGGTCCCATCGCG GTTCAGTGGCAGTGG ATCTGGGACACAGTT CACTCTCACCATCAG CGACGTGGAGTGTGC CGATGCTGCCACTTA CTACTGTCAACGGGG TTATATTAGCAGTGCT GTTGATTTTTTTGTTT TCGGCGGAGGGACCG AGGTGGTGGTCAAAG GT	81

	GTCTCCTCA			

Table 10: Heavy chain and light chain sequences of AS7B91

Clone	Heavy Chain Amino Acid Sequence	SEQ ID NO:	Light Chain Amino Acid Sequence	SEQ ID NO:
AS7B91	QSLEESGGRLVTPGTP LTLTCTVSGIDLSTSVM GWVRQAPGKGLESIGF IYTNVNTYYASWAKG RFTISRTSTTVDLKITSP TTGDTATYFCARAVY AGAMDLWGQGTLVT VSSAKTTAPSVYPLAP VCGDTTGSSVTLGCLV KGYFPEPVTLTWNSGS LSSGVHTFPAVLQSDL YTLSSSVTVTSSTWPS QSITCNVAHPASSTKV DKKIEPRGPTIKPCPPC KCPAPNLLGGPSVFIFP PKIKDVLMISLSPIVTC VVVDVSEDDPDVQIS WFVNNVEVHTAQTQT HREDYNSTLRVVSALP IQHQDWMSGKEFKCK VNNKDLPAPIERTISKP KGSVRAPQVYVLPPPE EEMTKKQVTLTCMVT DFMPEDIYVEWTNNG KTELNYKNTEPVLDSD GSYFMYSKLRVEKKN WVERNSYSCSVVHEG LHNHHTTKSFSRTPGK	18	DVVMTQTPASVSGPV GGTVTIKCQASERIYSN LAWYQQKPGQPPKLLI YKASTLASGVSSRFKG SGSGTEFTLTIRDLECA DAATYSCQYTSYGSG YVGTFGGGTEVVVEG RADAAPTVSIFPPSSEQ LTSGGASVVCFLNNFY PKDINVKWKIDGSERQ NGVLNSWTDQDSKDS TYSMSSTLTLTKDEYE RHNSYTCEATHKTSTS PIVKSFNRNEC	19
AS7B90	QSLEESGGRLVTPGTP LTLTCTVSGIDLSTSVM GWVRQAPGKGLESIGF IYTNVNTYYASWAKG RFTISRTSTTVDLKITSP	20	DVVMTQTPASVSGPV GGTVTIKCQASERIYSN LAWYQQKPGQPPKLLI YKASTLASGVSSRFKG SGSGTEFTLTIRDLECA	21

	TTGDTATYFCARAVY AGAMDLWGQGTLVT VSSAETTAPSVYPLAP GTALKSNSMVTLGCL VKGYFPEPVTVTWNS GALSSGVHTFPAVLQS GLYTLTSSVTVPSSTW PSQTVTCNVAHPASST KVDKKIVPRNCGGDC KPCICTGSEVSSVFIFPP KPKDVLTITLTPKVTC VVVDISQDDPEVHFSW FVDDVEVHTAQTRPPE EQFNSTFRSVSELPILH QDWLNGRTFRCKVTS AAFPSPIEKTISKPEGRT QVPHVYTMSPTKEEM TQNEVSITCMVKGFYP PDIYVEWQMNGQPQE NYKNTPPTMDTDGSYF LYSKLNVKKEKWQQG NTFTCSVLHEGLHNHH TEKSLSHSPGK		DAATYSCQYTSYGSG YVGTFGGGTEVVVEG RADAAPTVSIFPPSSEQ LASGGASVVCFINKFY PKDISVKWKIDGSERQ NDVLNSVTDQDSKDST YSMSSTLTLTKADYER HNLYTCEVVHKTSASP VVKSFNRNEC	
AS7B16	QVTLKESGPGILQPSQT LSLTCSFSGFSLNTSGT GVGWIRQPSGKGLEW LAHIWWDDDKGYNPA LKSRLTISKNTSSNLVF LKIASVDTADTATYYC VRIKGRMDYWGQGTS VTVSSKTTPPSVYPLAP GSAAQTNSMVTLGCL VKGYFPEPVTVTWNS GSLSSGVHTFPAVLES DLYTLSSSVTVPSSPRP SETVTCNVAHPASSTK VDKKIVPRDCGCKPCI CTVPEVSSVFIFPPKPK DVLTITLTPKVTCVVV DISKDDPEVQFSWFVD DVEVHTAQTQPREEQF NSTFRSVSELPIMHQD WLNGKEFKCRVNSAA FPAPIEKTISKTKGRPK APQVYTIPPPKEQMAK DKVSLTCMITDFFPEDI TVEWQWNGQPAENY KNTQPIMNTNGSYFVY	62	NIMMTQSPSSLAVSAG EKVTMNCKSSQSVLFG SKQKNYLAWYQQKPG QSPKLLIYWASTRESG VPDRFTGSGSGTDFILT ISNVQAEDLAVYYCHQ YLSLFTFGSGTKLEIKR ADAAPTVSIFPPSSEQL TSGGASVVCFLNNFYP KDINVKWKIDGSERQN GVLNSWTDQDSKDST YSMSSTLTLTKDEYER HNSYTCEATHKTSTSPI VKSFNRNEC	63

	SKLNVQKSNWEAGNT FTCSVLHEGLHNHHTE KSLSHSPGK			
AS7B82	QEQQKESGGGLVKPG ASLTLTCTASGIDFSSV AYMCWVRQAPGKGLE WIACIYAGSSSSIYYAS WAKGRFTVSRTSSTTV TLQMTSLTAADTATYF CARGLFTSGSGYYIDM WGPGTLVTVSSASTKG PSVFPLAPSSKSTSGGT AALGCLVKDYFPEPVT VSWNSGALTSGVHTFP AVLQSSGLYSLSSVVT VPSSSLGTQTYICNVN HKPSNTKVDKKVEPKS CDKTHTCPPCPAPELL GGPSVFLFPPKPKDTL MISRTPEVTCVVVDVS HEDPEVKFNWYVDGV EVHNAKTKPREEQYNS TYRVVSVLTVLHQDW LNGKEYKCKVSNKAL PAPIEKTISKAKGQPRE PQVYTLPPSRDELTKN QVSLTCLVKGFYPSDI AVEWESNGQPENNYK TTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFS CSVMHEALHNHYTQK SLSLSPGK	64	DVVMTQTPSSVEVAV GGTVTIKCQASQSIGSN LAWYQQKPGQRPKLLI YGASNLAAGVPSRFSG SGSGTQFTLTISDVECA DAATYYCQRGYISSAV DFFVFGGGTEVVVKG RTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFY PREAKVQWKVDNALQ SGNSQESVTEQDSKDS TYSLSSTLTLSKADYE KHKVYACEVTHQGLS SPVTKSFNRGEC	65

EXAMPLE 3: BINDING OF RECOMBINANT AS7B90 AND AS7B91 TO FN3 DOMAINS AS COMPARED TO ORIGINAL CEN-25-105-5 RABBIT ANTIBODY

Supernatants for AS7B90 and AS7B91, which are the rat and mouse chimeras, respectively of CEN-25-105-5 were evaluated for their ability to bind recombinant FN3 domains by ELISA. Three plates were coated with dose-response curves of, FN3 domain A3 specific for human cMET (SEQ ID NO: 31), 83v2-ABD specific for human EGFR with an albumin binding domain (SEQ ID NO: 32), tencon 25 that has no specificity (SEQ ID NO: 28), or negative control protein, all at 50 μL/well in 50mM

PBS. Plates were incubated over night at 4°C. Supernatants were dumped from plates and 200 µL/well Superblock was added to block plates for 1 hour at RT. Plates were washed 3X with TBS-T. Supernatants and CEN25-105-5 were prepared at 1 µg/ml in Superblock and added to the plates. Plates were incubated for 1 hour at RT. Plates were washed 3X with TBS-T. Secondary antibodies (HRP-goat anti-rabbit, rat or mouse) were prepared at 1:10,000 in Superblock and added to the plates. Plates were incubated for 1 hour at RT. Plates were washed 3X with TBS-T. 50 µL/well POD reagent (prepared according to Sigma-Aldrich manufacturer's instructions and incubated in the dark at RT for at least 30 minutes prior to use) was added to plates. After 3-5 minute incubation, protected from light, the M5 was used to read luminescence. The results were plotted in Prism (Figs. 1A-C) using a Log/Log transformation, and then a four-parameter curve fit. As shown in Figs. 1A-C, both AS7B90 and AS7B91showed specificity to the three different FN3 domains, confirming that the binding epitope of these anti FN3 domain antibodies are specific to the conserved framework regions of the, and not the variable loops which render FN3 domain specificity. It should be noted that there was no non-specific binding to negative control antibody. The mouse version of the antibody, AS7B91, appeared to have binding characteristics more similar to the original rabbit hybridoma antibody.

EXAMPLE 4: TESTING AS7B91 FOR DETECTION OF ANTI-BCMA CARTYRINS EXPRESSED ON THE SURFACE OF PRIMARY T CELLS.

100 µl per well were plated of $1x10^6$ anti-BCMA CARTyrin T cells/ml. Wells were washed 2x with PBS. eFlour 506 Fixable Viability Dye was added to samples at a 1:2000 final concentration for LIVE-DEAD sample staining and allowed to incubate for 30 minutes at 4°C. Staining reaction was quenched with FACS buffer, and samples were washed two times with FACS buffer. Fc Block was added (33 µl Fc/ml FACS) for 10 minutes at room temperature. 100 µl of primary AS7B91, FACS buffer, or isotype control were added and incubated 20 minutes on ice. Samples were then washed one time with FACS buffer. Secondary antibody was added and incubated for 20 minutes on ice (Anti-Mouse IgG-AF647 used at 1:50 for final concentration of 10 µg/ml). After secondary antibody incubation, wells were washed one time with FACS buffer followed by one time with PBS. After washing, samples were fixed in 2% PFA for 10 minutes at room temperature. Samples were washed and resuspended in FACS buffer.

As shown in Fig. 2, the recombinant AS7B91 was able to detect the expression of CARTyrins on the surface of primary T cells and can thus be used as a general CARTyrin detection reagent. In a homogeneous population of CARTyrin expressing T cells, the AS7B91 antibody could be used to quantitate the number of CARTyrins on the surface of the cells.

EXAMPLE 5: USE OF AS7B91 TO ACTIVATE T CELL-EXPRESSING CARTYRINS

Since the CARTyrins are attached to T cell signaling domains, the binding and clustering of these CARTyrins by the AS7B91 antibody could result in activation of those signaling domains and the activation of the T cells expressing them. To test this, primary T cells expressing CARTyrins were incubated with AS7B91 or anti-CD3 antibody, that is known to induce activation. Briefly, 12 different BCMA RNA-expressing CARTyrin clones were electroporated in primary pan T cells derived from normal human blood (Normal Blood Donor Service - TSRI) using the ECM 830 Square Wave Electroporation System (BTX). $5x10^6$ pan T-cells received a single electric pulse (500V, 750 us) per the manufacturer's protocol, either with or without 10 μg of BCMA-targeting CAR mRNA. Surface expression of CARs was assessed 24 hours later using a polyclonal anti-FN3 domain Ab. AS7B91 or an anti-CD3 antibody were plated both at 5 μg/ml in the presence of a soluble anti-CD28 antibody (2ug/ml). Cells and supernatants were harvested on day 2 and day 6 post stimulation. Cells were Stained for T cell subset markers (CD4, 8), activation markers (CD25, 69, 71, 137, HLA-DR) and Fixable Viability Dye. Cells gated Live (FVD negative) -> doublet exclusion -> CD4 or CD8 single positive -> CD4s and CD8s assessed for activation marker expression individually. LSR Voltages set based on antibody conjugated beads and single color controls. Gates were set based on Fluorescence Minus One (FMO) and isotype controls

As seen in Table 11, the AS7B91 monoclonal anti-FN3 domain antibody is able to stimulate CARTyrin + primary panT cells in the presence of co-stimulation through CD28 (analogous to anti-CD3/CD28 procedures). This activation is dependent on CARTyrin expression, and is not as robust or as long lived as that observed with anti-CD3. In addition, treatment with the AS7B91 and anti-CD28 results in an 11-fold increase in cell number compared to unstimulated cells. In summary, the anti-FN3

domain antibody can induce T cell proliferation and activation of cells expressing CARTyrins.

Table 11. Activation of CARTyrin expressing pan T cells using the anti-centryin antibody AS7B91 in conjunction with anti-CD28 co-stimulation. Compared to unstimulated control cells expressing , pos=positive staining (neg in unstim), hi=high level of staining, lo=low level of staining (neg in unstim), neg=no staining.

	Day 2					
CD8	CD25	DR	CD71	CD69	CD137	
	pos	hi	hi	lo	neg	a-Centyrin
	pos	hi	hi	pos	pos	a-CD3

EXAMPLE 6: ENGINEERING OF AS7B91 INTO A SCFV CHIMERIC ANTIGEN RECEPTOR:

To generate a CAR construct of the AS7B91, AS7B16, and AS7B82 antibodies, the variable regions were constructed into scFvs. For AS7B91, scFvs were generated in two different orientations: HCv-LCv, or LCv-HCv.

AS7B91 H-L scFv (SEQ ID NO: 22)
METGLRWLLLVAVLKGVQCQSLEESGGRLVTPGTPLTLTCTVSGIDLSTSVMG
WVRQAPGKGLESIGFIYTNVNTYYASWAKGRFTISRTSTTVDLKITSPTTGDTA
TYFCARAVYAGAMDLWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDVVM
TQTPASVSGPVGGTVTIKCQASERIYSNLAWYQQKPGQPPKLLIYKASTLASGV
SSRFKGSGSGTEFTLTIRDLECADAATYSCQYTSYGSGYVGTFGGGTEVVVEG

AS7B91 L-H scFv (SEQ ID NO: 23)
MDTRAPTQLLGLLLLWLPGARCDVVMTQTPASVSGPVGGTVTIKCQASERIYS
NLAWYQQKPGQPPKLLIYKASTLASGVSSRFKGSGSGTEFTLTIRDLECADAAT
YSCQYTSYGSGYVGTFGGGTEVVVEGGGGGSGGGGSGGGGSGGGGSLEESGG
RLVTPGTPLTLTCTVSGIDLSTSVMGWVRQAPGKGLESIGFIYTNVNTYYASW
AKGRFTISRTSTTVDLKITSPTTGDTATYFCARAVYAGAMDLWGQGTLVTVSS

AS7B16 H-L scFv (SEQ ID NO: 66)
QVTLKESGPGILQPSQTLSLTCSFSGFSLNTSGTGVGWIRQPSGKGLEWLAHIW
WDDDKGYNPALKSRLTISKNTSSNLVFLKIASVDTADTATYYCVRIKGRMDY
WGQGTSVTVSSGGGGSGGGGSGGGGSGGGGSNIMMTQSPSSLAVSAGEKVT
MNCKSSQSVLFGSKQKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSG
SGTDFILTISNVQAEDLAVYYCHQYLSLFTFGSGTKLEIK

AS7B16 L-H scFv (SEQ ID NO: 82)

NIMMTQSPSSLAVSAGEKVTMNCKSSQSVLFGSKQKNYLAWYQQKPGQSPKL
LIYWASTRESGVPDRFTGSGSGTDFILTISNVQAEDLAVYYCHQYLSLFTFGSGT
KLEIKGGGGSGGGGSGGGGSGGGGSQVTLKESGPGILQPSQTLSLTCSFSGFSL
NTSGTGVGWIRQPSGKGLEWLAHIWWDDDKGYNPALKSRLTISKNTSSNLVFL
KIASVDTADTATYYCVRIKGRMDYWGQGTSVTVSS

AS7B82 H-L scFv (SEQ ID NO: 67)

QEQQKESGGGLVKPGASLTLTCTASGIDFSSVAYMCWVRQAPGKGLEWIACIY
AGSSSSIYYASWAKGRFTVSRTSSTTVTLQMTSLTAADTATYFCARGLFTSGSG
YYIDMWGPGTLVTVSSGGGGGSGGGGSGGGGSGGGGSDVVMTQTPSSVEVA
VGGTVTIKCQASQSIGSNLAWYQQKPGQRPKLLIYGASNLAAGVPSRFSGSGS
GTQFTLTISDVECADAATYYCQRGYISSAVDFFVFGGGTEVVVKG

AS7B82 L-H scFv (SEQ ID NO: 83)

DVVMTQTPSSVEVAVGGTVTIKCQASQSIGSNLAWYQQKPGQRPKLLIYGASN
LAAGVPSRFSGSGSGTQFTLTISDVECADAATYYCQRGYISSAVDFFVFGGGTE
VVVKGGGGGGSGGGGSGGGGSGGGGSQEQQKESGGGLVKPGASLTLTCTAS
GIDFSSVAYMCWVRQAPGKGLEWIACIYAGSSSSIYYASWAKGRFTVSRTSSTT
VTLQMTSLTAADTATYFCARGLFTSGSGYYIDMWGPGTLVTVSS

The amino acid sequence of the different scFv sequences were back-translated and engineered with hinge sequence, TM domain, and signaling domains. The completed construct was cloned into a T7 in vitro transcription vector to generate mRNA using the commercially available mMESSAGE mMACHINE® T7 ULTRA Transcription Kit. The amino acid sequences for the components of the CAR constructs were as shown in Table 12:

Table 12. Amino Acid Sequence of the two different AS7B91, AS7B16 and AS7B82 CAR constructs (H-L orientation and L-H orientation).

Domain	Sequence
Extracellular AS7B91	SEQ ID NO: 72 (H-L orientation) QSLEESGGRLVTPGTPLTLTCTVSGIDLSTSVMGWVRQAPG KGLESIGFIYTNVNTYYASWAKGRFTISRTSTTVDLKITSPTT GDTATYFCARAVYAGAMDLWGQGTLVTVSSGGGGSGGGG SGGGGSGGGGSDVVMTQTPASVSGPVGGTVTIKCQASERIY SNLAWYQQKPGQPPKLLIYKASTLASGVSSRFKGSGSGTEF TLTIRDLECADAATYSCQYTSYGSGYVGTFGGGTEVVVEG SEQ ID NO: 73 (L-H orientation) DVVMTQTPASVSGPVGGTVTIKCQASERIYSNLAWYQQKP

	GQPPKLLIYKASTLASGVSSRFKGSGSGTEFTLTIRDLECADA ATYSCQYTSYGSGYVGTFGGGTEVVVEGGGGGSGGGGSGG GGSGGGGSLEESGGRLVTPGTPLTLTCTVSGIDLSTSVMGW VRQAPGKGLESIGFIYTNVNTYYASWAKGRFTISRTSTTVDL KITSPTTGDTATYFCARAVYAGAMDLWGQGTLVTVSS
Extracellular AS7B16	SEQ ID NO: 68 (H-L orientation) QVTLKESGPGILQPSQTLSLTCSFSGFSLNTSGTGVGWIRQPS GKGLEWLAHIWWDDDKGYNPALKSRLTISKNTSSNLVFLKI ASVDTADTATYYCVRIKGRMDYWGQGTSVTVSSGGGGSG GGGSGGGGSGGGGSNIMMTQSPSSLAVSAGEKVTMNCKSS QSVLFGSKQKNYLAWYQQKPGQSPKLLIYWASTRESGVPD RFTGSGSGTDFILTISNVQAEDLAVYYCHQYLSLFTFGSGTK LEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFK QPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAP AYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPR RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGL YQGLSTATKDTYDALHMQALPPR SEQ ID NO: 69 (L-H orientation) NIMMTQSPSSLAVSAGEKVTMNCKSSQSVLFGSKQKNYLA WYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFILTIS NVQAEDLAVYYCHQYLSLFTFGSGTKLEIKGGGGSGGGGS GGGGSGGGGSQVTLKESGPGILQPSQTLSLTCSFSGFSLNTS GTGVGWIRQPSGKGLEWLAHIWWDDDKGYNPALKSRLTIS KNTSSNLVFLKIASVDTADTATYYCVRIKGRMDYWGQGTS VTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR GLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIF KQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADA PAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR
Extracellular AS7B82	SEQ ID NO: 70 (H-L orientation) QEQQKESGGGLVKPGASLTLTCTASGIDFSSVAYMCWVRQ APGKGLEWIACIYAGSSSSIYYASWAKGRFTVSRTSSTTVTL QMTSLTAADTATYFCARGLFTSGSGYYIDMWGPGTLVTVSS GGGGGSGGGGSGGGGSGGGGSDVVMTQTPSSVEVAVGGT VTIKCQASQSIGSNLAWYQQKPGQRPKLLIYGASNLAAGVP SRFSGSGSGTQFTLTISDVECADAATYYCQRGYISSAVDFFV FGGGTEVVVKGTTTPAPRPPTPAPTIASQPLSLRPEACRPAA GGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRG RKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVK FSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR GKGHDGLYQGLSTATKDTYDALHMQALPPR SEQ ID NO: 71 (L-H orientation) DVVMTQTPSSVEVAVGGTVTIKCQASQSIGSNLAWYQQKP

	GQRPKLLIYGASNLAAGVPSRFSGSGSGTQFTLTISDVECAD AATYYCQRGYISSAVDFFVFGGGTEVVVKGGGGGGSGGGG SGGGGSGGGGSEQQKESGGGLVKPGASLTLTCTASGIDFSS VAYMCWVRQAPGKGLEWIACIYAGSSSSIYYASWAKGRFT VSRTSSTTVTLQMTSLTAADTATYFCARGLFTSGSGYYIDM WGPGTLVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKF SRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPE MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRG KGHDGLYQGLSTATKDTYDALHMQALPPR
human CD8 hinge	SEQ ID NO: 24 TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDIY
human CD8 TM domain	SEQ ID NO: 25 IWAPLAGTCGVLLLSLVITLYCK
human 4-1BB intracellular domain	SEQ ID NO: 26 RGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
human CD3 zeta intracellular domain	SEQ ID NO: 27 RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGER RRGKGHDGLYQGLSTATKDTYDALHMQALPPR

EXAMPLE 7: GENERATION AND ANALYSIS OF ENGINEERED IMMUNE CELLS EXPRESSING AS7B91, AS7B16, AND AS7B82 CARS

mRNA was electroporated into pan T-cells derived from normal human blood (Normal Blood Donor Service - TSRI) using the ECM 830 Square Wave Electroporation System (BTX). $5x10^6$ pan T-cells received a single electric pulse (500V, 750 us) per the manufacturer's protocol, either with or without 10 μg of AS7B91 CAR mRNA. Surface expression of CARs was assessed 24 hours later using a polyclonal anti-FN3 domain Ab. The results are shown in Fig. 3.

The functionality of the AS7B91 scFv CAR cells were tested for their ability to bind to FN3 domains and induce T cell killing in response to FN3 domain binding to target cells. This was first determined in a degranulation assay using BCMA-specific FN3 domains along with $BCMA^{hi}$ (H929 cells), $BCMA^{lo}$ (D0HH-2 cells) $BCMA^{neg}$ (EXPI293 cells) target cells, and then in a killing assay.

In the degranulation assay (CD107a mobilization) T-cells were incubated in 96-well plates, together with 1:1 or 1:10 amount of cells expressing or not the BCMA protein. Co-cultures were maintained in a final volume of OpTmizer "complete" media containing 1:1500 Golgi Stop (BD) and 1:1300 anti-CD107a APC (Biolegend) for 4 hours at 37 C. After the 4h incubation period, cells were stained with a fixable viability dye (eFluor 780, from eBioscience) and fluorochrome-conjugated anti-CD8 (PE conjugated, Biologend) and analyzed by flow cytometry. The degranulation activity was determined by determining the mean fluorescence intensity signal (MFI) for CD107a staining among CD8+ cells. Degranulation assays were carried out 24h after mRNA transfection. As seen in Fig. 4, upon the addition of an anti-BCMA specific FN3 domain, the AS7B91 scFv CAR expressing T cells underwent degranulation in an anti-BCMA and AS7B91 specific manner. These data show that the AS7B91 scFv CAR is functional in its ability to bind to and signal in response to a target specific FN3 domain, in which the target is expressed on the surface of other cells.

AS7B91 scFv CAR-T cell killing in response to BCMA-specific FN3 domains along with CFSE labeled- $BCMA^{hi}$ (U-2932 cells), $BCMA^{lo}$ (D0HH-2 cells) $BCMA^{neg}$ (EXPI293 cells) target cells was evaluated. CAR-T/mock T cells were preincubated with the BCMA-specific FN3 domains for 1 hour at 37 ºC prior to a 48-hour incubation with the BCMA target cells at E:T ratios ranging from 0 to 1. At the end of the experiment, dead cells were labeled with CFSE-FL1, SYTOX-red-FL4. The percentage of cell death was analyzed by a flow cytometer (FACSCalibur) and the data was analyzed with FlowJo v 10. As seen in Fig. 5, upon the addition of a BCMA-specific FN3 domain, the AS7B91 scFv CAR expressing T cells killed BCMA expressing target cells in an anti-BCMA FN3 domain and AS7B91 scFv CAR-specific manner. These data show that the AS7B91 scFv CAR is functional in its ability to bind to and signal in response to a target specific FN3 domain, in which the target is expressed on the surface of other cells.

For AS7B16 and AS7B82, binding was assessed using flow cytometry. Briefly, Twenty-four hours post-electroporation, cells were centrifuged at 100x g for 10 minutes and washed two times in FACS stain buffer (BD Biosciences Catalog# 554657). Cells were incubated with either APC labeled anti-Tencon-25 or AF647 labeled anti-EGFR 83v2 FN3 domains at a final concentration of 50 nM at 4°C for 1 hour. The labeled cells were washed twice in FACS stain buffer and re-suspended in 200 uL of the same

buffer. Data was collected using a BD LSRFortessa flow cytometer and analysis was performed using Flowjo software. The data is shown in Fig. 6 to Fig. 9. All CARTs tested, AS7B16, AS7B82 and AS7B91 bind to both FN3 domains tested, Tencon-T25 and EGFR 83v2. Binding of CARTs occurs in the following manner: ASS7B91>AS7B82>AS7B16.

EXAMPLE 8: *IN VITRO* KILLING OF ANTI-CITRULLINATED PROTEIN ANTIBODY-EXPRESSING CELLS MEDIATED BY AS7B91 SCFV CAR-T PRE-COMPLEXED WITH FN3 DOMAIN-CONJUGATED TO CYCLIC CITRULLINATED PEPTIDES

Autoimmune diseases are characterized by the dysregulated production of antibodies to self-antigens (auto-antibodies). These autoantibodies can be directed against a variety of molecules including, but not limited to, proteins and nucleic acids. In the case of proteins, these autoantibodies often recognize post-translationally modified antigens. Shown below is the *in vitro* killing of anti-citrullinated protein antibody (ACPA) expressing cells mediated by AS7B91 scFv CAR-T cells ("BAR-T" cells) pre-complexed with centyrin conjugated to cyclic citrullinated peptides (CCP-1). This finding suggests that self-antigens can be conjugated to the Tencon25 centyrin for engagement with BAR-T cells leading to targeted elimination of antigen-specific, auto-reactive B cells.

Generation of FcgR expressing cell lines

Purified lentiviral expression plasmids encoding for human FcgRs (CD16a, CD32 and CD64) were packaged for transfection of 293T cells using the Lenti-Pac HIV Expression Packaging System (GeneCopoeia). Seventy-two hours post transfection, lenti-containing supernatant was harvested and used to transduce HEK293 cells. Media was supplemented with polybrene (final concentration-8ug/mL). The next day, polybrene containing media was replaced with RPMI Media 1640 supplemented with 10% fetal bovine serum. Seventy-two hours post transduction, cells were harvested and stained for FcgR expression. The cells were then sorted based on FcgR expression (SH800S Cell Sorter-Sony Biotechnology). High expressing cells

were cultured and used as target cells for future studies.

Conjugation of Citrullinated Cyclic Peptides to Tencon25 centyrin

Glycine tagged (GGG-) citrullinated cyclic peptide-1 (CCP-1-Cit) and arginine control peptide (CCP-1-Arg) were obtained from Peptides International. Sortase-v5 tagged Tencon25 (Tencon25_sort_v5) was desalted into TBS and concentrated prior to conjugation. Each peptide was conjugated at a 1:5 ratio (FN3 domain to peptide) via sortase chemistry. Conjugates were purified manually over a Ni Sepharose column (GE) to remove free sortase and peptide. Following purification, the conjugates were buffered exchanged in to PBS and concentrated. Conjugates were QC'd by (a) mass spectrometry (LC-MS) and (b) size exclusion chromatography (superdex 75) and sterile filtered.

Detection of anti-citrullinated antibody binding to centyrin-CCP1-peptide conjugates

FcgR expressing HEK293 cells were incubated with 200ug/mL of human anti-citrullinated fibrinogen antibody (clone 1F11-Modiquest) or human IgG1 isotype control (Abcam) for 30mins on ice. Cells were washed 2X with FACS Buffer then incubated with centyrin-CCP1-Cit conjugate (576nM) for 1hr on ice. Cells were washed 2X with FACS buffer then incubated with PE-labeled anti-centyrin (AS7B91) antibody for 30mins on ice. Binding was assessed by flow cytometry (BD FACSCanto II) and analyzed with BD FACSDiva 6.1.3 software.

AS7B91 scFv CAR-T cell mediated killing of anti-citrullinated mAb bound FcR expressing cells

Mock electroporated T cells (Mock) or AS7B91 scFv CAR-T cells ("BAR-T") were pre-complexed with centyrin-conjugated CCP1 (cit-CCP). HEK293 cells expressing CD16a or CD64 were labeled with Cell Tracker Green (CTG) dye (ThermoFisher) and pre-complexed with either human IgG1 isotype control or anti-citrullinated fibrinogen antibody (anti-CCP Ab). BAR-T and HEK293 cells were then

plated at a 1:1 or 5:1 ratio for ~18hrs. At the end of the experiment, cells were stained with Zombie Dye Violet (Biolegend). Dead cells were considered as CTG+/Zombie+ cells as assessed by flow cytometry (BD FACSCanto II). The data was analyzed with BD FACSDiva 6.1.3 and Graphpad Prism 5 software. A 2-fold increase in cell death of target cells bound to the anti-citrullinated antibody when incubated with BAR-T cells pre-complexed with centyrin-CCP-1-cit relative to incubation with mock T cells or isotype control antibodies was observed.

Evaluation of potential peptide/antibody combinations in multiple auto-immune diseases for BAR-T platform expansion

Peptides specific for a) Myasthenia Gravis (MG), b) Multiple Sclerosis (MS) and c) systemic lupus erythematosus (SLE) were tested for their ability to bind to anti-AChR, anti-MOG, or anti dsDNA antibodies, respectively. Three high affinity binding neutravidin plates were coated with 10ug/mL of indicated peptides. Plates were washed 3X with PBS-T and blocked with 2X Assay Buffer A (eBioscience) for 2hrs at room temperature. Plates were washed 3X with PBS-T and 100ug/mL of indicated antibody was added for 1hr at room temperature. Plates were washed 3X with PBS-T and the appropriate secondary antibody was added for 1hr at room temperature. Plates were washed 3X with PBS-T and TMB Substrate was added for 5-8 mins prior to addition of Stop Solution (ThermoFisher). The SpectraMax340PC instrument was used to read the absorbance and the data was analyzed using Graphpad Prism 5 software. The binding results identified one potential lead peptide/antibody combination for each disease indication for further evaluation.

5

Brief Description of the Sequence Listing

SEQ ID NO:	Type	Species	Description	Sequence
1	PRT	rabbit	CEN-25-105-5 HCDR1 IMGT	GIDLSTSV
2	PRT	rabbit	CEN-25-105-5 HCDR1 Kabat	TSVMG
3	PRT	rabbit	CEN-25-105-5 HCDR1 Chothia	GIDLSTS
4	PRT	rabbit	CEN-25-105-5 HCDR2 IMGT	IYTNVNT
5	PRT	rabbit	CEN-25-105-5 HCDR2 Kabat	FIYTNVNTYYASWAKG
6	PRT	rabbit	CEN-25-105-5 HCDR2 Chothia	YTNVN
7	PRT	rabbit	CEN-25-105-5 HCDR3 IMGT	ARAVYAGAMDL
8	PRT	rabbit	CEN-25-105-5 HCDR3 Kabat and Chothia	AVYAGAMDL
9	PRT	rabbit	CEN-25-105-5 LCDR1 IMGT	ERIYSN
10	PRT	rabbit	CEN-25-105-5 LCDR1 Kabat and Chothia	QASERIYSNLA
11	PRT	rabbit	CEN-25-	KAS

			105-5 LCDR2 IMGT	
12	PRT	rabbit	CEN-25-105-5 LCDR2 Kabat and Chothia	KASTLAS
13	PRT	rabbit	CEN-25-105-5 LCDR3 IMGT, Kabat and Chothia	QYTSYGSGYVGT
14	PRT	rabbit	CEN-25-105-5 VH	QSLEESGGRLVTPGTPLTLTCTVSGID LSTSVMGWVRQAPGKGLESIGFIYTN VNTYYASWAKGRFTISRTSTTVDLKI TSPTTGDTATYFCARAVYAGAMDL WGQGTLVTVSS
15	PRT	rabbit	CEN-25-105-5 VL	DVVMTQTPASVSGPVGGTVTIKCQA SERIYSNLAWYQQKPGQPPKLLIYKA STLASGVSSRFKGSGSGTEFTLTIRDL ECADAATYSCQYTSYGSGYVGTFGG GTEVVVEG
16	DNA	rabbit	CEN-25-105-5 VH	Ctggaggagtccgggggtcgcctggtcacgcctgggac acccctgacactcacctgcacagtctctggaatcgacctc agtacctctgtcatgggttgggtccgccaggctccaggga aggggctggaatccatcggattcatttatactaatgttaaca catactacgcgagctgggcaaaaggccgattcaccatctc cagaacctcgaccacggtggatctgaaaatcaccagtcc gacaaccggggacacggccacctatttctgtgccagagc tgtttatgctggtgctatggacttgtggggccaaggcaccc tggtcaccgtctcctca
17	DNA	rabbit	CEN-25-105-5 VL	gatgttgtgatgacccagactccagcctccgtgtctggac ctgtgggaggcacagtcaccatcaagtgccaggccagtg agagaatttatagcaatttagcctggtatcagcagaaacca gggcagcctcccaaactcctgatctacaaggcatccactc tggcatctggggtctcatcgcggttcaaaggcagtggatc tgggacagagttcactctcaccatcagggaccttgagtgt gccgatgctgccacttactcctgtcaatatacttcttatggc agtggttatgttggtactttcggcggagggaccgaggtgg tggtcgaaggt
18	PRT	artificial	AS7B91-Heavy Chain	QSLEESGGRLVTPGTPLTLTCTVSGID LSTSVMGWVRQAPGKGLESIGFIYTN VNTYYASWAKGRFTISRTSTTVDLKI TSPTTGDTATYFCARAVYAGAMDL WGQGTLVTVSSAKTTAPSVYPLAPV CGDTTGSSVTLGCLVKGYFPEPVTLT WNSGSLSSGVHTFPAVLQSDLYTLSS

				SVTVTSSTWPSQSITCNVAHPASSTK VDKKIEPRGPTIKPCPPCKCPAPNLLG GPSVFIFPPKIKDVLMISLSPIVTCVVV DVSEDDPDVQISWFVNNVEVHTAQT QTHREDYNSTLRVVSALPIQHQDWM SGKEFKCKVNNKDLPAPIERTISKPK GSVRAPQVYVLPPPEEEMTKKQVTL TCMVTDFMPEDIYVEWTNNGKTELN YKNTEPVLDSDGSYFMYSKLRVEKK NWVERNSYSCSVVHEGLHNHHTTKS FSRTPGK
19	PRT	artificial	AS7B91-Light Chain	DVVMTQTPASVSGPVGGTVTIKCQA SERIYSNLAWYQQKPGQPPKLLIYKA STLASGVSSRFKGSGSGTEFTLTIRDL ECADAATYSCQYTSYGSGYVGTFGG GTEVVVEGRADAAPTVSIFPPSSEQL TSGGASVVCFLNNFYPKDINVKWKI DGSERQNGVLNSWTDQDSKDSTYS MSSTLTLTKDEYERHNSYTCEATHK TSTSPIVKSFNRNEC
20	PRT	artificial	AS7B90-Heavy Chain	QSLEESGGRLVTPGTPLTLTCTVSGID LSTSVMGWVRQAPGKGLESIGFIYTN VNTYYASWAKGRFTISRTSTTVDLKI TSPTTGDTATYFCARAVYAGAMDL WGQGTLVTVSSAETTAPSVYPLAPG TALKSNSMVTLGCLVKGYFPEPVTV TWNSGALSSGVHTFPAVLQSGLYTL TSSVTVPSSTWPSQTVTCNVAHPASS TKVDKKIVPRNCGGDCKPCICTGSEV SSVFIFPPKPKDVLTITLTPKVTCVVV DISQDDPEVHFSWFVDDVEVHTAQT RPPEEQFNSTFRSVSELPILHQDWLN GRTFRCKVTSAAFPSPIEKTISKPEGR TQVPHVYTMSPTKEEMTQNEVSITC MVKGFYPPDIYVEWQMNGQPQENY KNTPPTMDTDGSYFLYSKLNVKKEK WQQGNTFTCSVLHEGLHNHHTEKSL SHSPGK
21	PRT	artificial	AS7B90-Light Chain	DVVMTQTPASVSGPVGGTVTIKCQA SERIYSNLAWYQQKPGQPPKLLIYKA STLASGVSSRFKGSGSGTEFTLTIRDL ECADAATYSCQYTSYGSGYVGTFGG GTEVVVEGRADAAPTVSIFPPSSEQL ASGGASVVCFINKFYPKDISVKWKID GSERQNDVLNSVTDQDSKDSTYSMS STLTLTKADYERHNLYTCEVVHKTS ASPVVKSFNRNEC
22	PRT	artificial	AS7B91 H-L scFv	METGLRWLLLVAVLKGVQCQSLEES GGRLVTPGTPLTLTCTVSGIDLSTSV

				MGWVRQAPGKGLESIGFIYTNVNTY YASWAKGRFTISRTSTTVDLKITSPTT GDTATYFCARAVYAGAMDLWGQGT LVTVSSGGGGSGGGGSGGGGSGGGG SDVVMTQTPASVSGPVGGTVTIKCQ ASERIYSNLAWYQQKPGQPPKLLIYK ASTLASGVSSRFKGSGSGTEFTLTIRD LECADAATYSCQYTSYGSGYVGTFG GGTEVVVEG
23	PRT	artificial	AS7B91 L-H scFv	MDTRAPTQLLGLLLLWLPGARCDVV MTQTPASVSGPVGGTVTIKCQASERI YSNLAWYQQKPGQPPKLLIYKASTL ASGVSSRFKGSGSGTEFTLTIRDLECA DAATYSCQYTSYGSGYVGTFGGGTE VVVEGGGGSGGGGSGGGGSGGGG SLEESGGRLVTPGTPLTLTCTVSGIDL STSVMGWVRQAPGKGLESIGFIYTNV NTYYASWAKGRFTISRTSTTVDLKIT SPTTGDTATYFCARAVYAGAMDLW GQGTLVTVSS
24	PRT	human	CD8 hinge	TTTPAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACDIY
25	PRT	human	CD8 TM domain	IWAPLAGTCGVLLLSLVITLYCK
26	PRT	human	4-1BB intracellular domain	RGRKKLLYIFKQPFMRPVQTTQEEDG CSCRFPEEEEGGCEL
27	PRT	human	CD3 zeta intracellular domain	RVKFSRSADAPAYKQGQNQLYNELN LGRREEYDVLDKRRGRDPEMGGKPR RKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR
28	PRT	artificial	Tencon25	LPAPKNLVVSEVTEDSARLSWTAPD AAFDSFLIQYQESEKVGEAIVLTVPGS ERSYDLTGLKPGTEYTVSIYGVKGGH RSNPLSAIFTT
29	PRT	artificial	Tencon28	MLPAPKNLVVSEVTHDSARLSWTAP DAAFDSFLIQYQESEKVGEAIVLTVP GSERSYDLTGLKHHTEYTVSIYGVKG GHRSNPLSAIFHTHHHHHH
30	PRT	artificial	P114-83	MLPAPKNLVVSRVTHDSARLSWTAP DAAFDSFWIRYFEFLGSGEAIVLTVP GSERSYDLTGLKHHTEYVVNIMGVK GGKISPPLSAIFTTHHHHHH

31	PRT	artificial	A3	MLPAPKNLVVSRVTEDSARLSWTAP DAAFDSFWIRYFEFLGSGEAIVLTVP GSERSYDLTGLKPGTEYVVNIMGVK GGKISPPLSAIFTTGGHHHHHH
32	PRT	artificial	83v2-ABD sequence	MLPAPKNLVVSEVTEDSARLSWDDP WAFYESFLIQYQESEKVGEAIVLTVP GSERSYDLTGLKPGTEYTVSIYGVHN VYKDTNMRGLPLSAIFTTGGGGSGG GGSLAEAKVLANRELDKYGVSDYY KNLINNAKTVEGVKALIDEILAALPG GHHHHHH
33	PRT	artificial	Tencon	LPAPKNLVVSEVTEDSLRLSWTAPDA AFDSFLIQYQESEKVGEAINLTVPGSE RSYDLTGLKPGTEYTVSIYGVKGGH RSNPLSAEFTT
34	PRT	artificial	Tencon27	LPAPKNLVVSRVTEDSARLSWTAPD AAFDSFLIQYQESEKVGEAIVLTVPGS ERSYDLTGLKPGTEYTVSIYGVKGGH RSNPLSAIFTT
35	PRT	mouse	AS7B16 HCDR1 IMGT	GFSLNTSGTG
36	PRT	mouse	AS7B16 HCDR1 KABAT	TSGTGVG
37	PRT	mouse	AS7B16 HCDR1 CHOTHIA	GFSLNTSGT
38	PRT	rabbit	AS7B82 HCDR1 IMGT	GIDFSSVAY
39	PRT	rabbit	AS7B82 HCDR1 KABAT	SVAYMC
40	PRT	rabbit	AS7B82 HCDR1 CHOTHIA	GIDFSSVA
41	PRT	mouse	AS7B16 HCDR2 IMGT	IWWDDDK
42	PRT	mouse	AS7B16 HCDR2 KABAT	HIWWDDDKGYNPALKS
43	PRT	mouse	AS7B16	WWDDD

			HCDR2 CHOTHIA	
44	PRT	mouse	AS7B16 HCDR3 IMGT	VRIKGRMDY
45	PRT	mouse	AS7B16 HCDR3 KABAT AND CHOTHIA	IKGRMDY
46	PRT	mouse	AS7B16 LCDR1 IMGT	QSVLFGSKQKNY
47	PRT	mouse	AS7B16 LCDR1 KABAT AND CHOTHIA	KSSQSVLFGSKQKNYLA
48	PRT	mouse	AS7B16 LCDR2 IMGT	WAS
49	PRT	mouse	AS7B16 LCDR2 KABAT AND CHOTHIA	WASTRES
50	PRT	mouse	AS7B16 LCDR2 IMGT, KABAT AND CHOTHIA	HQYLSLFT
51	PRT	rabbit	AS7B82 HCDR2 IMGT	IYAGSSSSI
52	PRT	rabbit	AS7B82 HCDR2 KABAT	CIYAGSSSSIYYASWAKG
53	PRT	rabbit	AS7B82 HCDR2 CHOTHIA	YAGSSSS
54	PRT	rabbit	AS7B82 HCDR3 IMGT	ARGLFTSGSGYYIDM
55	PRT	rabbit	AS7B82 HCDR3 KABAT AND CHOTHIA	GLFTSGSGYYIDM

56	PRT	rabbit	AS7B82 LCDR1 IMGT	QSIGSD
57	PRT	rabbit	AS7B82 LCDR1 KABAT AND CHOTHIA	QASQSIGSNLA
58	PRT	rabbit	AS7B82 LCDR2 IMGT	SAS
59	PRT	rabbit	AS7B82 LCDR2 KABAT AND CHOTHIA	GASNLAA
60	PRT	rabbit	AS7B82 LCDR3 IMGT	QCTYSSSTGYNA
61	PRT	rabbit	AS7B82 LCDR3 KABAT AND CHOTHIA	QRGYISSAVDFFV
62	PRT	artificial	AS7B16-Heavy Chain	QVTLKESGPGILQPSQTLSLTCSFSGF SLNTSGTGVGWIRQPSGKGLEWLAHI WWDDDKGYNPALKSRLTISKNTSSN LVFLKIASVDTADTATYYCVRIKGR MDYWGQGTSVTVSSKTTPPSVYPLA PGSAAQTNSMVTLGCLVKGYFPEPV TVTWNSGSLSSGVHTFPAVLESDLYT LSSSVTVPSSPRPSETVTCNVAHPASS TKVDKKIVPRDCGCKPCICTVPEVSS VFIFPPKPKDVLTITLTPKVTCVVVDI SKDDPEVQFSWFVDDVEVHTAQTQP REEQFNSTFRSVSELPIMHQDWLNGK EFKCRVNSAAFPAPIEKTISKTKGRPK APQVYTIPPPKEQMAKDKVSLTCMIT DFFPEDITVEWQWNGQPAENYKNTQ PIMNTNGSYFVYSKLNVQKSNWEAG NTFTCSVLHEGLHNHHTEKSLSHSPG K
63	PRT	artificial	AS7B16-Light Chain	NIMMTQSPSSLAVSAGEKVTMNCKS SQSVLFGSKQKNYLAWYQQKPGQSP KLLIYWASTRESGVPDRFTGSGSGTD FILTISNVQAEDLAVYYCHQYLSLFTF GSGTKLEIKRADAAPTVSIFPPSSEQL TSGGASVVCFLNNFYPKDINVKWKI DGSERQNGVLNSWTDQDSKDSTYS

				MSSTLTLTKDEYERHNSYTCEATHK TSTSPIVKSFNRNEC
64	PRT	artificial	AS7B82-Heavy Chain	QEQQKESGGGLVKPGASLTLTCTAS GIDFSSVAYMCWVRQAPGKGLEWIA CIYAGSSSSIYYASWAKGRFTVSRTSS TTVTLQMTSLTAADTATYFCARGLF TSGSGYYIDMWGPGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYT LPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK
65	PRT	artificial	AS7B82-Light Chain	DVVMTQTPSSVEVAVGGTVTIKCQA SQSIGSNLAWYQQKPGQRPKLLIYGA SNLAAGVPSRFSGSGSGTQFTLTISDV ECADAATYYCQRGYISSAVDFFVFG GGTEVVVKGRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYS LSSTLTLSKADYEKHKVYACEVTHQ GLSSPVTKSFNRGEC
66	PRT	artificial	AS7B16 H-L scFv	QVTLKESGPGILQPSQTLSLTCSFSGF SLNTSGTGVGWIRQPSGKGLEWLAHI WWDDDKGYNPALKSRLTISKNTSSN LVFLKIASVDTADTATYYCVRIKGR MDYWGQGTSVTVSSGGGGSGGGGS GGGGSGGGGSNIMMTQSPSSLAVSA GEKVTMNCKSSQSVLFGSKQKNYLA WYQQKPGQSPKLLIYWASTRESGVP DRFTGSGSGTDFILTISNVQAEDLAV YYCHQYLSLFTFGSGTKLEIK
67	PRT	artificial	AS7B82 H-L scFv	QEQQKESGGGLVKPGASLTLTCTAS GIDFSSVAYMCWVRQAPGKGLEWIA CIYAGSSSSIYYASWAKGRFTVSRTSS TTVTLQMTSLTAADTATYFCARGLF TSGSGYYIDMWGPGTLVTVSSGGGG GSGGGGSGGGGSGGGGSDVVMTQT PSSVEVAVGGTVTIKCQASQSIGSNL AWYQQKPGQRPKLLIYGASNLAAGV PSRFSGSGSGTQFTLTISDVECADAAT

				YYCQRGYISSAVDFFVFGGGTEVVV KG
68	PRT	artificial	AS7B16 H-L CAR ECD	QVTLKESGPGILQPSQTLSLTCSFSGF SLNTSGTGVGWIRQPSGKGLEWLAHI WWDDDKGYNPALKSRLTISKNTSSN LVFLKIASVDTADTATYYCVRIKGR MDYWGQGTSVTVSSGGGGSGGGGS GGGGSGGGGSNIMMTQSPSSLAVSA GEKVTMNCKSSQSVLFGSKQKNYLA WYQQKPGQSPKLLIYWASTRESGVP DRFTGSGSGTDFILTISNVQAEDLAV YYCHQYLSLFTFGSGTKLEIKTTTPAP RPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGV LLLSLVITLYCKRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCE LRVKFSRSADAPAYKQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR
69	PRT	artificial	AS7B16 L-H CAR ECD	NIMMTQSPSSLAVSAGEKVTMNCKS SQSVLFGSKQKNYLAWYQQKPGQSP KLLIYWASTRESGVPDRFTGSGSGTD FILTISNVQAEDLAVYYCHQYLSLFTF GSGTKLEIKGGGGSGGGGSGGGGSG GGGSQVTLKESGPGILQPSQTLSLTCS FSGFSLNTSGTGVGWIRQPSGKGLEW LAHIWWDDDKGYNPALKSRLTISKN TSSNLVFLKIASVDTADTATYYCVRI KGRMDYWGQGTSVTVSSTTTPAPRP PTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLL SLVITLYCKRGRKKLLYIFKQPFMRP VQTTQEEDGCSCRFPEEEEGGCELRV KFSRSADAPAYKQGQNQLYNELNLG RREEYDVLDKRRGRDPEMGGKPRRK NPQEGLYNELQKDKMAEAYSEIGMK GERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPR
70	PRT	artificial	AS7B82 H-L CAR ECD	QEQQKESGGGLVKPGASLTLTCTAS GIDFSSVAYMCWVRQAPGKGLEWIA CIYAGSSSSIYYASWAKGRFTVSRTSS TTVTLQMTSLTAADTATYFCARGLF TSGSGYYIDMWGPGTLVTVSSGGGG GSGGGGSGGGGSGGGGSDVVMTQT PSSVEVAVGGTVTIKCQASQSIGSNL AWYQQKPGQRPKLLIYGASNLAAGV PSRFSGSGSGTQFTLTISDVECADAAT

				YYCQRGYISSAVDFFVFGGGTEVVV KGTTTPAPRPPTPAPTIASQPLSLRPE ACRPAAGGAVHTRGLDFACDIYIWA PLAGTCGVLLLSLVITLYCKRGRKKL LYIFKQPFMRPVQTTQEEDGCSCRFP EEEEGGCELRVKFSRSADAPAYKQG QNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR
71	PRT	artificial	AS7B82 L-H CAR ECD	DVVMTQTPSSVEVAVGGTVTIKCQA SQSIGSNLAWYQQKPGQRPKLLIYGA SNLAAGVPSRFSGSGSGTQFTLTISDV ECADAATYYCQRGYISSAVDFFVFG GGTEVVVKGGGGGGSGGGGSGGGG SGGGGSEQQKESGGGLVKPGASLTL TCTASGIDFSSVAYMCWVRQAPGKG LEWIACIYAGSSSSIYYASWAKGRFT VSRTSSTTVTLQMTSLTAADTATYFC ARGLFTSGSGYYIDMWGPGTLVTVS STTTPAPRPPTPAPTIASQPLSLRPEAC RPAAGGAVHTRGLDFACDIYIWAPL AGTCGVLLLSLVITLYCKRGRKKLLY IFKQPFMRPVQTTQEEDGCSCRFPEE EEGGCELRVKFSRSADAPAYKQGQN QLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQG LSTATKDTYDALHMQALPPR
72	PRT	artificial	AS7B91 H-L CAR ECD	QSLEESGGRLVTPGTPLTLTCTVSGID LSTSVMGWVRQAPGKGLESIGFIYTN VNTYYASWAKGRFTISRTSTTVDLKI TSPTTGDTATYFCARAVYAGAMDL WGQGTLVTVSSGGGGSGGGGSGGG GSGGGGSDVVMTQTPASVSGPVGGT VTIKCQASERIYSNLAWYQQKPGQPP KLLIYKASTLASGVSSRFKGSGSGTEF TLTIRDLECADAATYSCQYTSYGSGY VGTFGGGTEVVVEG
73	PRT	artificial	AS7B91 L-H CAR ECD	DVVMTQTPASVSGPVGGTVTIKCQA SERIYSNLAWYQQKPGQPPKLLIYKA STLASGVSSRFKGSGSGTEFTLTIRDL ECADAATYSCQYTSYGSGYVGTFGG GTEVVVEGGGGGSGGGGSGGGGSG GGGSLEESGGRLVTPGTPLTLTCTVS GIDLSTSVMGWVRQAPGKGLESIGFI YTNVNTYYASWAKGRFTISRTSTTV DLKITSPTTGDTATYFCARAVYAGA MDLWGQGTLVTVSS

74	PRT	artificial	AS7B16 VH	QVTLKESGPGILQPSQTLSLTCSFSGF SLNTSGTGVGWIRQPSGKGLEWLAHI WWDDDKGYNPALKSRLTISKNTSSN LVFLKIASVDTADTATYYCVRIKGR MDYWGQGTSVTVSS
75	PRT	artificial	AS7B16 VL	NIMMTQSPSSLAVSAGEKVTMNCKS SQSVLFGSKQKNYLAWYQQKPGQSP KLLIYWASTRESGVPDRFTGSGSGTD FILTISNVQAEDLAVYYCHQYLSLFTF GSGTKLEIK
76	DNA	artificial	AS7B16 VH	AGGTTACTCTGAAAGAGTCTGGCCC TGGGATATTGCAGCCCTCCCAGACC CTCAGTCTGACTTGTTCTTTCTCTGG GTTTTCACTGAACACTTCTGGTACG GGTGTAGGCTGGATTCGTCAGCCTT CAGGGAAGGGTCTGGAGTGGCTGG CACACATTTGGTGGGATGATGACA AGGGGTATAACCCAGCCCTGAAGA GCCGACTGACAATCTCCAAAAACA CCTCCAGCAACCTGGTATTCCTCAA GATCGCCAGTGTGGACACTGCAGA TACTGCCACATATTACTGTGTTCGA ATCAAAGGCCGGATGGACTACTGG GGTCAAGGAACCTCAGTCACCGTCT CCTCA
77	DNA	artificial	AS7B16 VL	AACATTATGATGACACAGTCGCCAT CCTCTCTGGCTGTGTCTGCAGGAGA AAAGGTCACTATGAACTGTAAGTC CAGTCAAAGTGTTTTATTCGGTTCA AAACAGAAGAACTATTTGGCCTGG TACCAGCAGAAACCAGGGCAGTCT CCTAAATTGCTGATCTACTGGGCAT CCACTAGGGAATCTGGTGTCCCTGA TCGCTTCACAGGCAGTGGATCTGGG ACAGATTTTATACTTACCATCAGCA ATGTACAAGCTGAAGACCTGGCAG TTTATTACTGTCATCAATACCTCTC CCTATTCACGTTCGGCTCGGGGACA AAGTTGGAAATAAAA
78	PRT	artificial	AS7B82 VH	QEQQKESGGGLVKPGASLTLTCTAS GIDFSSVAYMCWVRQAPGKGLEWIA CIYAGSSSSIYYASWAKGRFTVSRTSS TTVTLQMTSLTAADTATYFCARGLF TSGSGYYIDMWGPGTLVTVSS
79	PRT	artificial	AS7B82 VL	DVVMTQTPSSVEVAVGGTVTIKCQA SQSIGSNLAWYQQKPGQRPKLLIYGA SNLAAGVPSRFSGSGSGTQFTLTISDV ECADAATYYCQRGYISSAVDFFVFG GGTEVVVKG

80	DNA	artificial	AS7B16 VH	CAGGAGCAGCAGAAGGAGTCCGGG GGAGGCCTGGTCAAGCCTGGGGCA TCCCTGACACTCACCTGCACAGCTT CTGGAATCGACTTCAGTAGTGTCGC CTACATGTGTTGGGTCCGCCAGGCT CCAGGGAAGGGGCTGGAGTGGATC GCATGCATTTATGCTGGTAGTAGTA GTAGCATCTACTACGCGAGCTGGG CGAAAGGCCGATTCACCGTCTCCA GAACCTCGTCTACCACGGTGACTCT GCAAATGACCAGTCTGACAGCCGC GGACACGGCCACCTATTTCTGTGCG AGAGGTCTATTTACTAGTGGTAGTG GATATTATATAGACATGTGGGGCCC AGGCACCCTGGTCACCGTCTCCTCA
81	DNA	artificial	AS7B16 VL	GATGTCGTGATGACCCAGACTCCAT CCTCTGTGGAGGTAGCTGTGGGAG GCACAGTCACCATCAAGTGCCAGG CCAGTCAGAGCATTGGTAGTAATTT AGCCTGGTATCAGCAGAAACCAGG GCAGCGTCCCAAGCTCCTGATCTAT GGTGCATCCAATCTGGCCGCTGGG GTCCCATCGCGGTTCAGTGGCAGTG GATCTGGGACACAGTTCACTCTCAC CATCAGCGACGTGGAGTGTGCCGA TGCTGCCACTTACTACTGTCAACGG GGTTATATTAGCAGTGCTGTTGATT TTTTTGTTTTCGGCGGAGGGACCGA GGTGGTGGTCAAAGGT

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WO2012129514

WE CLAIM:

1. An isolated antibody, or antigen-binding fragment thereof, that specifically binds to a non-randomized region of a fibronectin type III (FN3) domain.
2. The isolated antibody, or an antigen-binding fragment of claim 1, comprising:

a. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 1, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 4, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 7, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 9, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 11, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 13;

b. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 2, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 5, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 8, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 10, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 12, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 13;

c. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 3, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 8, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 10, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 12, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 13;

d. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 35, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 41, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 44, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 46, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 48, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50;

e. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 36, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 42, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 45, a

light chain CDR1 having the amino acid sequence of SEQ ID NO: 47 a light chain CDR2 having the amino acid sequence of SEQ ID NO: 49, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50;

f. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 37, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 43, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 45, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 47, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 49, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 50;

g. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 38, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 51, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 54, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 56, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 58, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 60;

h. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 39, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 52, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 55, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 57 a light chain CDR2 having the amino acid sequence of SEQ ID NO: 59, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 61; or

i. a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 40, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 53, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 55, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 57, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 59, and a light chain CDR3 having the amino acid sequence of SEQ ID NO: 61.

3. The antibody or antigen-binding fragment of claim 1, wherein

d. the variable heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 14, and the variable light chain of the antibody comprises the amino acid sequence of SEQ ID NO:15;

e. the variable heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 74, and the variable light chain of the antibody comprises the amino acid sequence of SEQ ID NO:75; or

f. the variable heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 78, and the variable light chain of the antibody comprises the amino acid sequence of SEQ ID NO:79;

4. The antibody or antigen-binding fragment of claim 1, wherein

e. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 18 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 19;

f. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 20 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 21;

g. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 62 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 63; or

h. the heavy chain of the antibody comprises the amino acid sequence of SEQ ID NO: 64 and light chain of the antibody comprises the amino acid sequence of SEQ ID NO: 65.

5. The antigen binding fragment of any one of claims 1 to 4 wherein the antigen binding fragment is a Fab fragment, a Fab2 fragment, or a single chain antibody.

6. The antibody or antigen-binding fragment of any one of claims 1 to 5 wherein the antibody or antigen-binding fragment thereof is an IgG.

7. An isolated polynucleotide encoding the antibody or antigen-binding fragment of any one of claims 1 to 6.

8. A vector comprising the polynucleotide of claim 7

9. A host cell comprising the isolated polynucleotide of claim 7

10. A host cell comprising the vector of claim 8.

11. An isolated polynucleotide encoding a chimeric antigen receptor (CAR) comprising:

(a) an extracellular domain comprising an scFv that specifically binds to a non-randomized region of an FN3 domain;

(b) a transmembrane domain; and

(c) an intracellular signaling domain,

wherein the CAR optionally further comprises a hinge region connecting the extracellular domain and the transmembrane domain.

12. The isolated polynucleotide of claim 11, wherein the encoded CAR comprises:

(a) an extracellular domain comprising an scFv having an amino acid sequence that is at least 90% identical to one of SEQ ID NOs: 68-73;

(b) a hinge region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 24;

(c) a transmembrane domain comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 25; and

(d) an intracellular signaling domain comprising a co-stimulatory domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 26, and a primary signaling domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 27.

13. The isolated polynucleotide of claim 12, wherein the encoded CAR comprises:

(a) an extracellular domain comprising the amino acid sequence of one of SEQ ID NOs: 68-73;

(b) a hinge region comprising the amino acid sequence of SEQ ID NO: 24;

(c) a transmembrane domain comprising the amino acid sequence of SEQ ID NO: 25; and

(d) an intracellular signaling domain comprising the amino acid sequence of SEQ ID NO: 26 and the amino acid sequence of SEQ ID NO: 27.

14. A vector comprising the polynucleotide of any of claims 11-13.

15. A host cell comprising the polynucleotide of any of claims 11-13.

16. A host cell comprising the vector of claim 14.

17. A chimeric antigen receptor (CAR) comprising:

(a) an extracellular domain comprising an scFv that specifically binds to a non-randomized region of an FN3 domain;

(b) a transmembrane domain; and

(c) an intracellular signaling domain,

wherein the CAR optionally further comprises a hinge region connecting the extracellular domain and the transmembrane domain.

18. The CAR of claim 17, comprising:

(a) an extracellular domain comprising an scFv having an amino acid sequence that is at least 90% identical to one of SEQ ID NOs: 68-73;

(b) a hinge region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 24;

(c) a transmembrane domain comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 25; and

(d) an intracellular signaling domain comprising a co-stimulatory domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 26, and a primary signaling domain having an amino acid sequence that is at least 90% identical to SEQ ID NO: 27.

19. The CAR of claim 18, wherein:

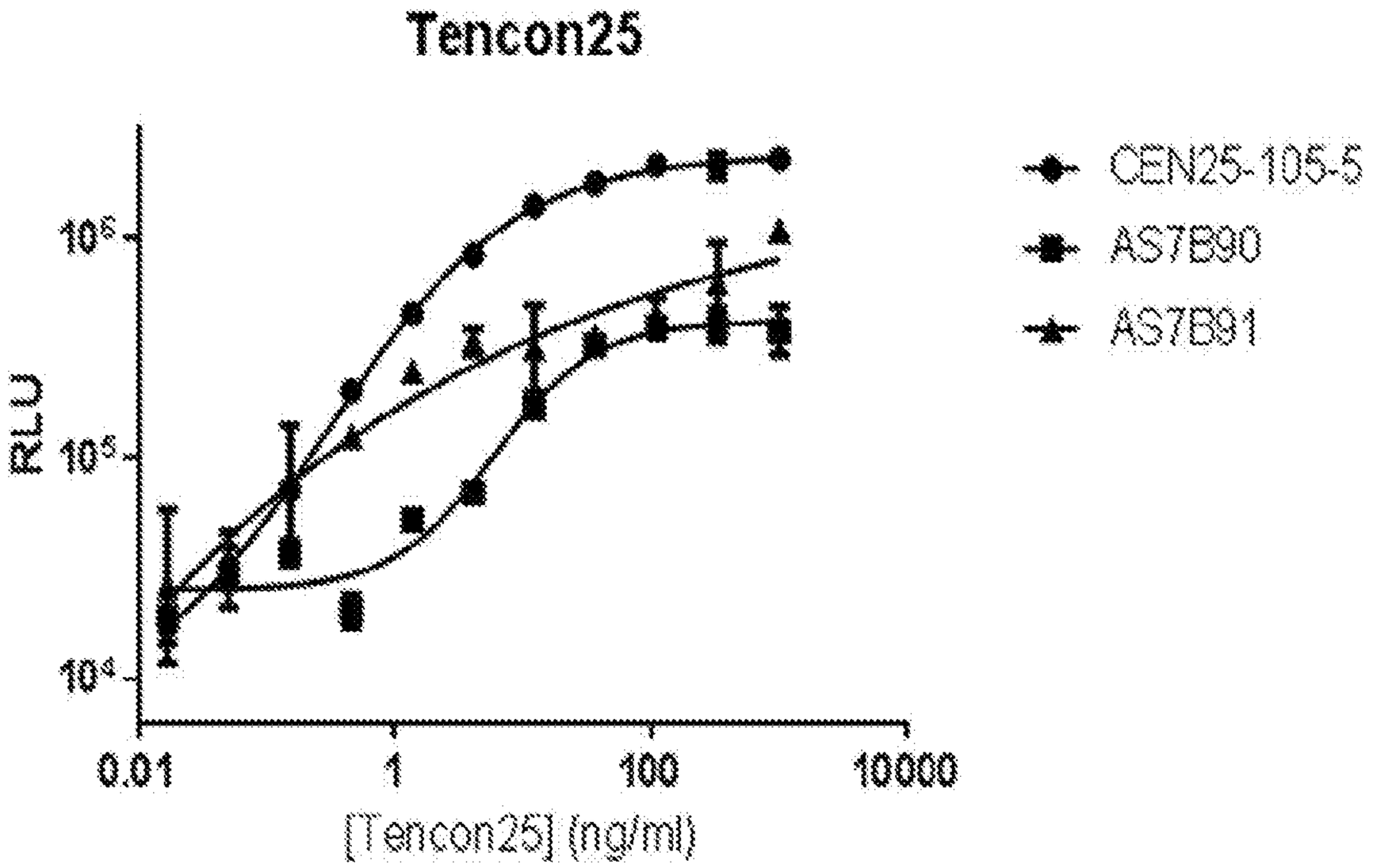
(a) the extracellular domain comprises the amino acid sequence of one of SEQ ID NOs: 68-73;

(b) the hinge region comprises the amino acid sequence of SEQ ID NO: 24;

(c) the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 25; and

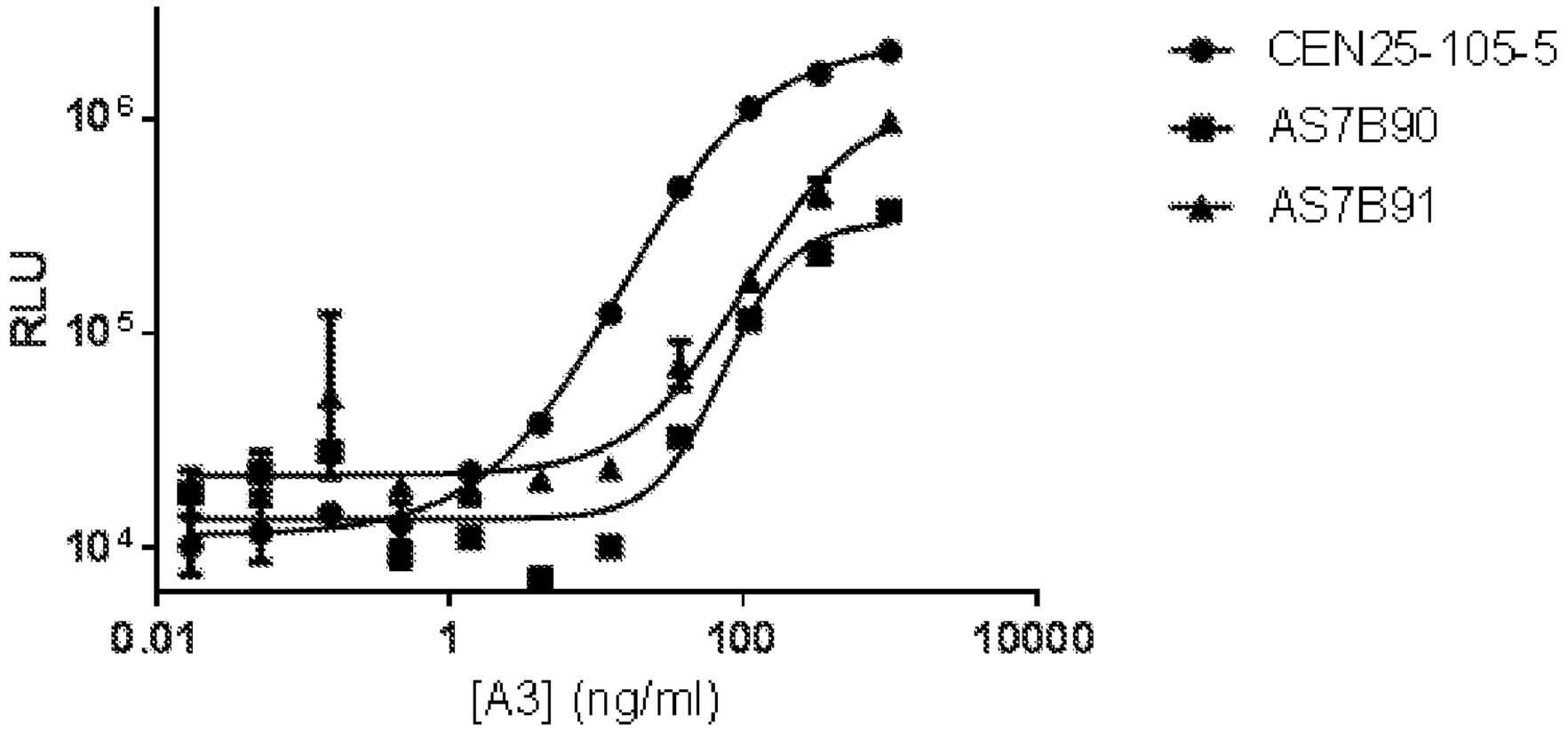
(d) the intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 26 and the amino acid sequence of SEQ ID NO: 27.

Figure 1A



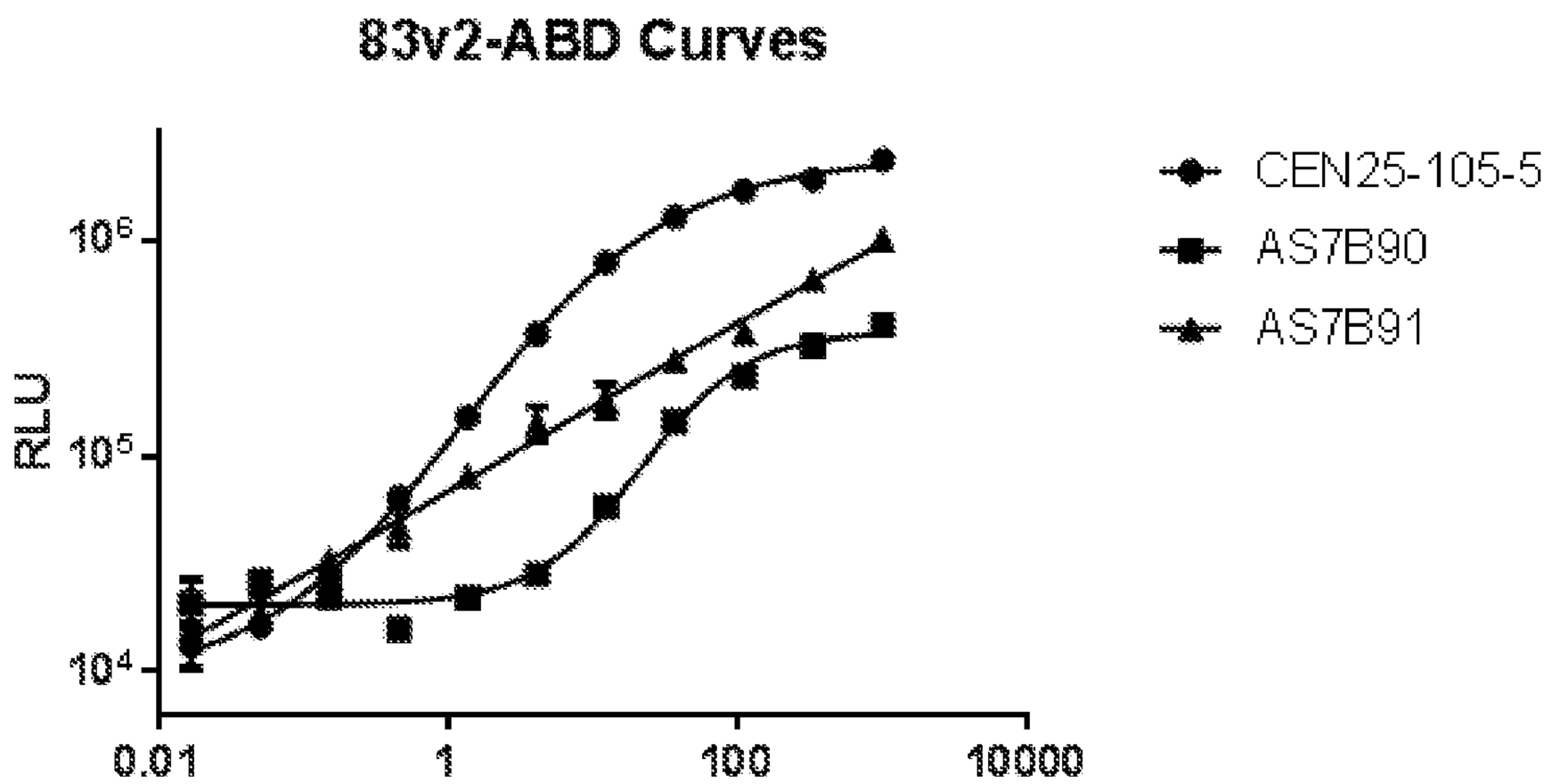
	CEN25-105-5	AS7B90	AS7B91
EC50	0.2898	5.94	~ 3.553e-015

Figure 1B



	CEN25-105-5	AS7B90	AS7B91
EC50	14.92	74.05	100.5

Figure 1C



[83v2-ABD] (ng/ml)

	CEN25-105-5	AS7B90	AS7B91
EC50	1.304	21.43	2.899

Figure 2A (EP control)

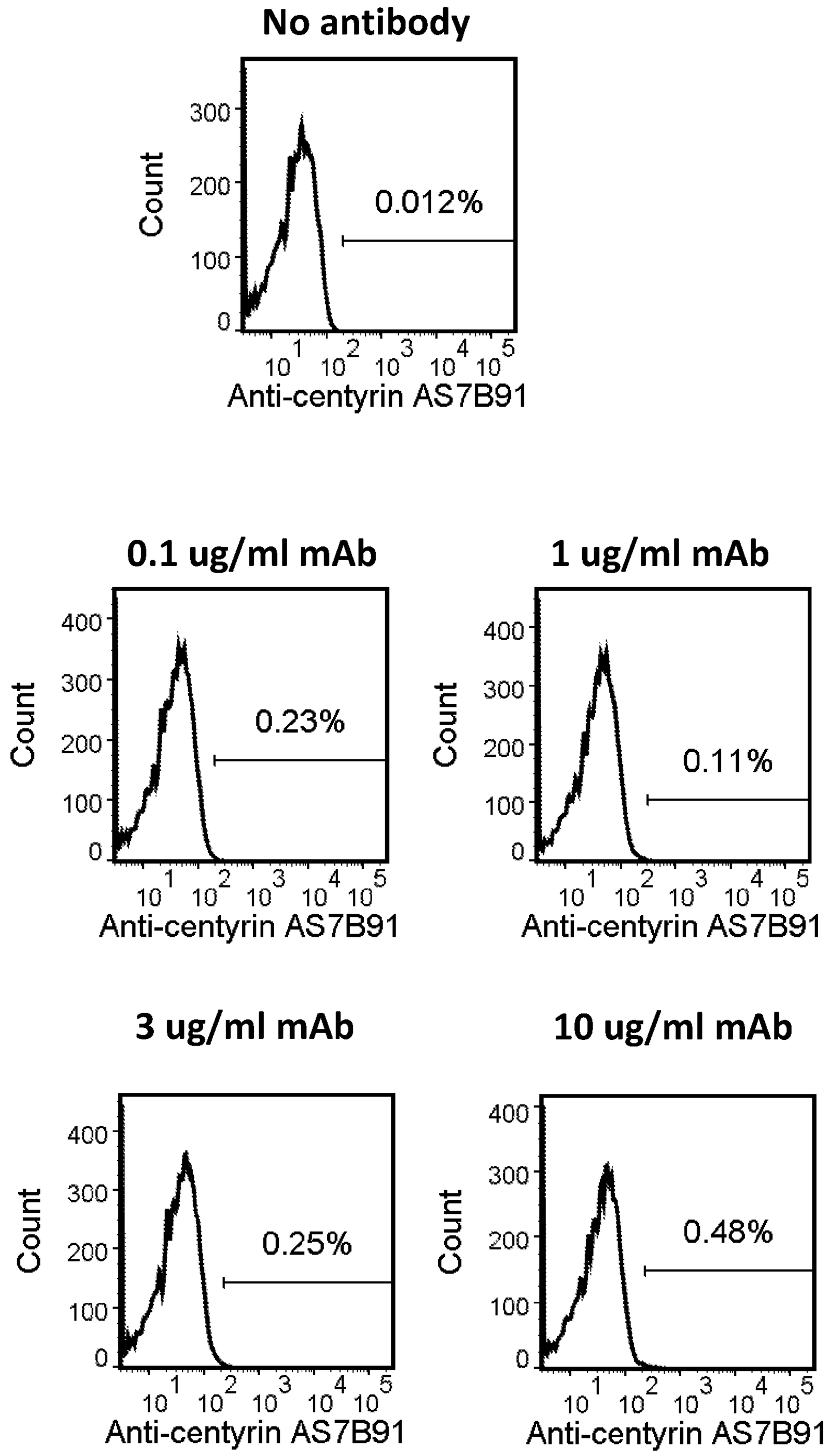


Figure 2B (AO8)

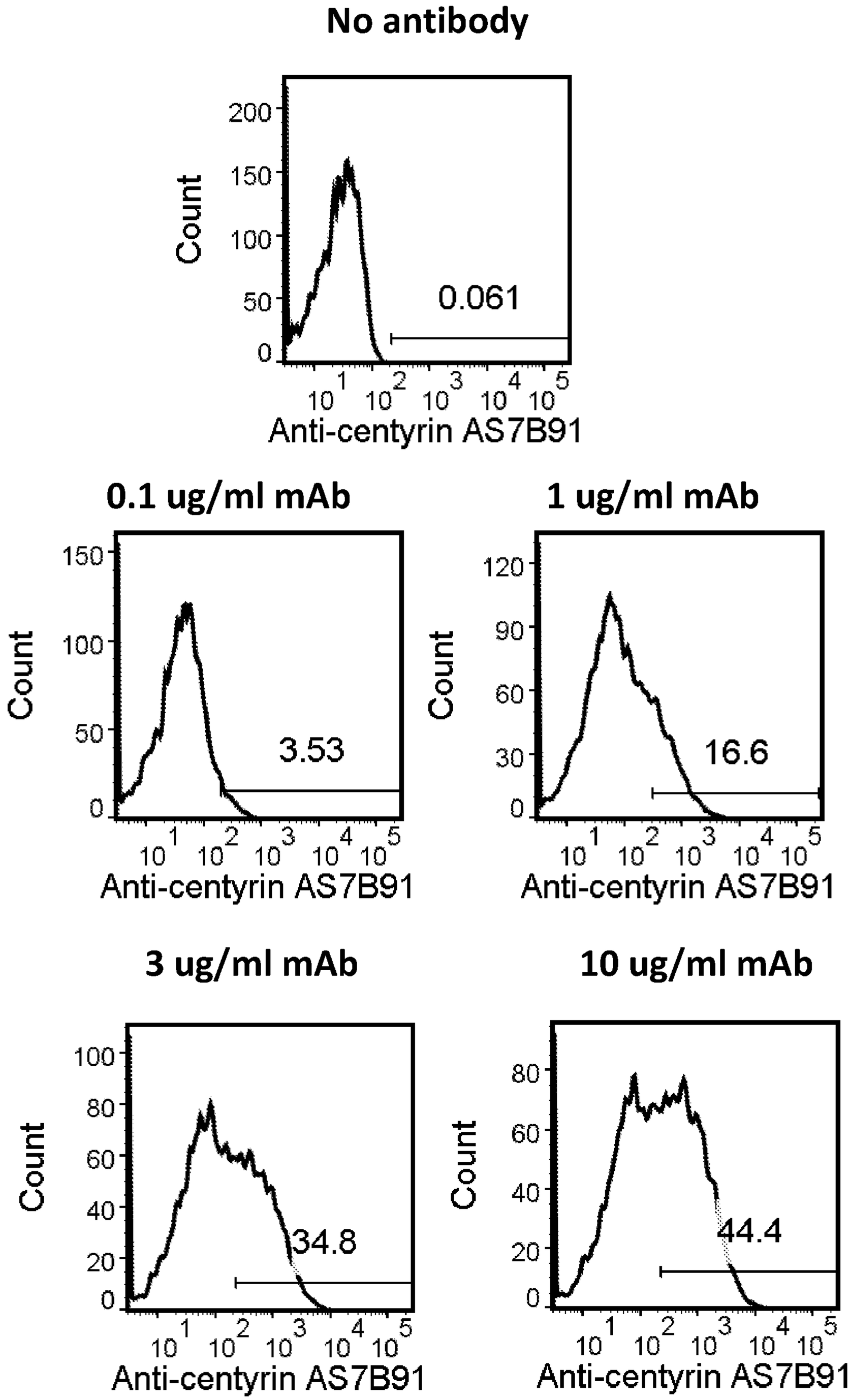


Figure 2C (A12)

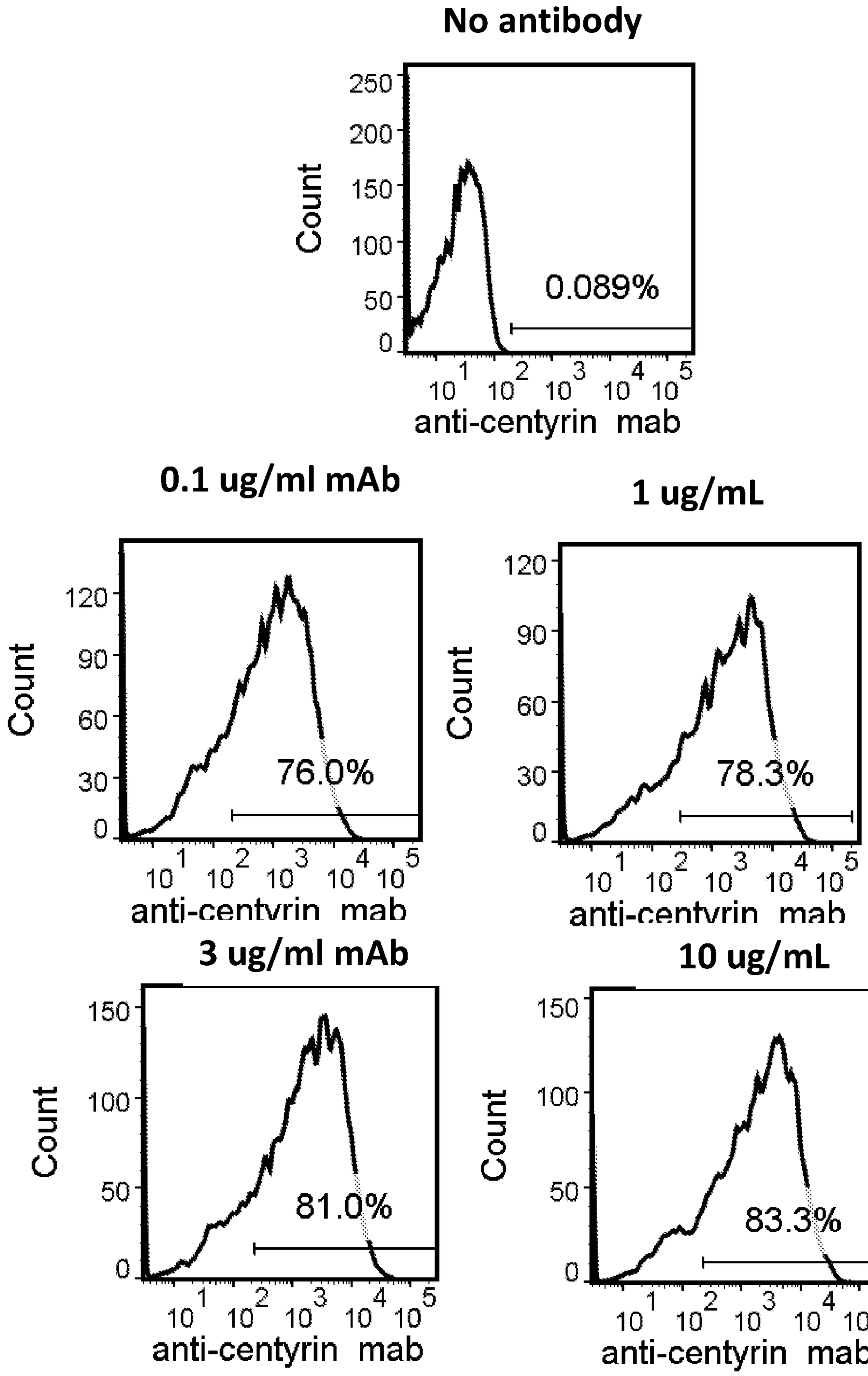


Figure 2D (BO3)

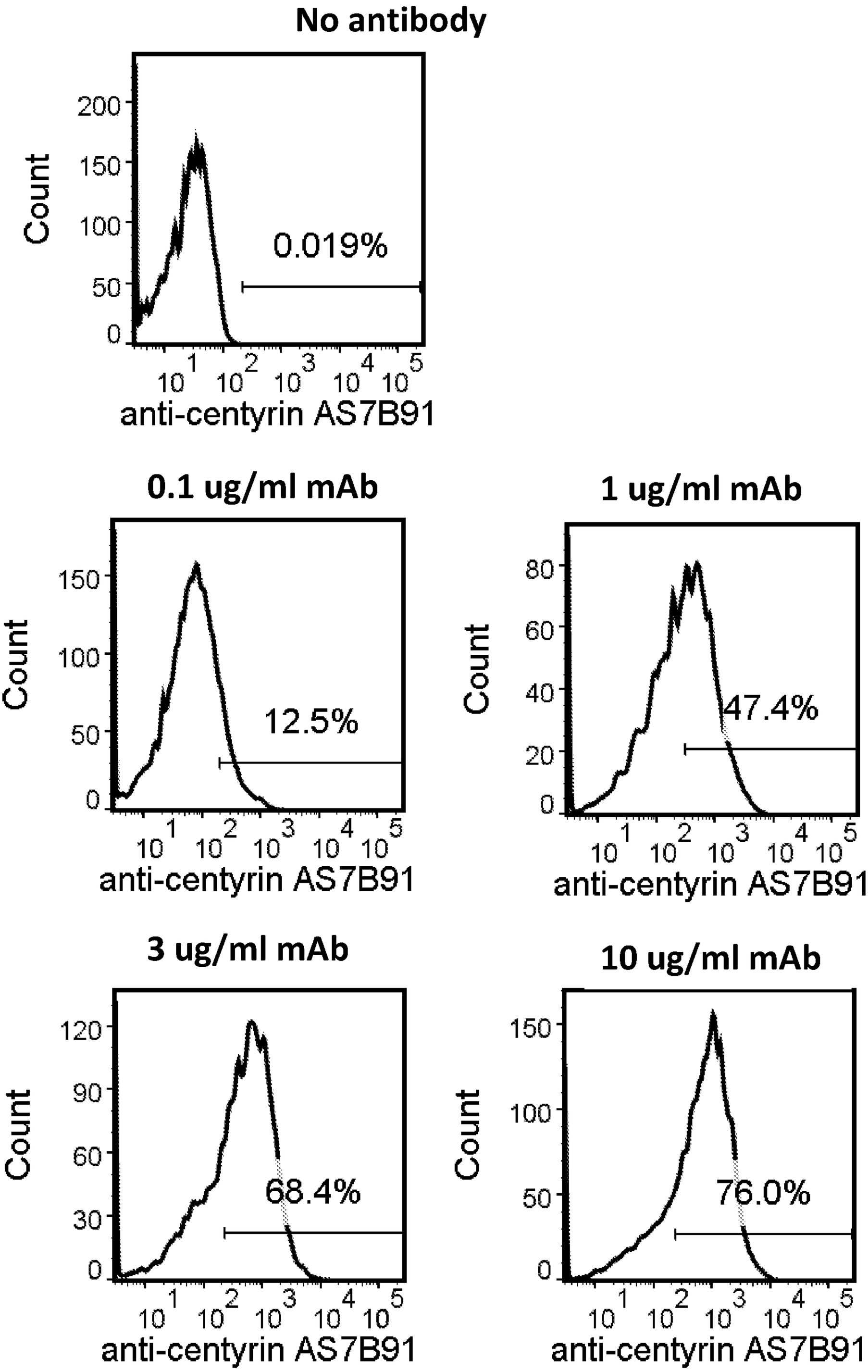


Figure 2E (CO8)

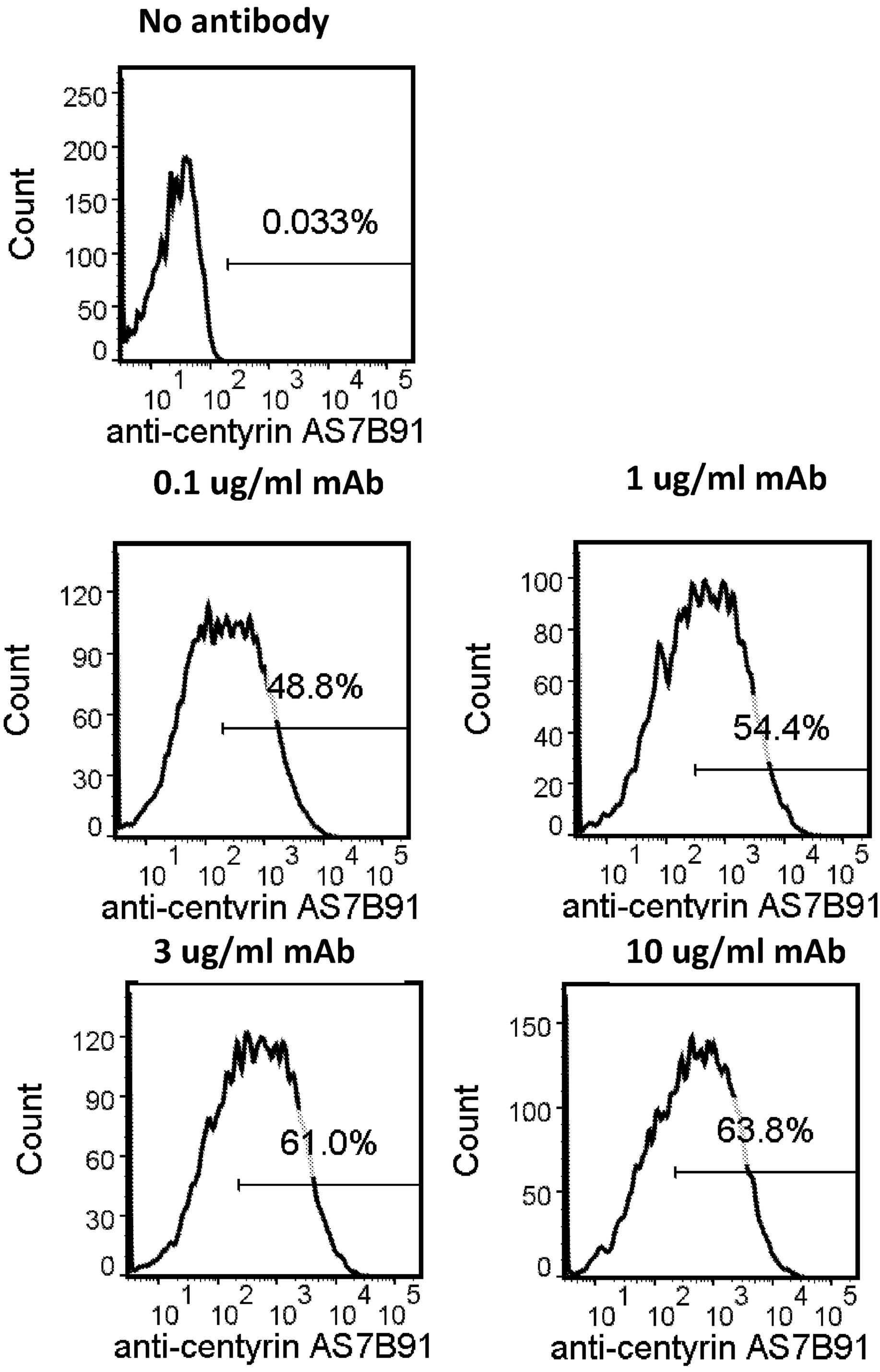


Figure 2F (DO8)

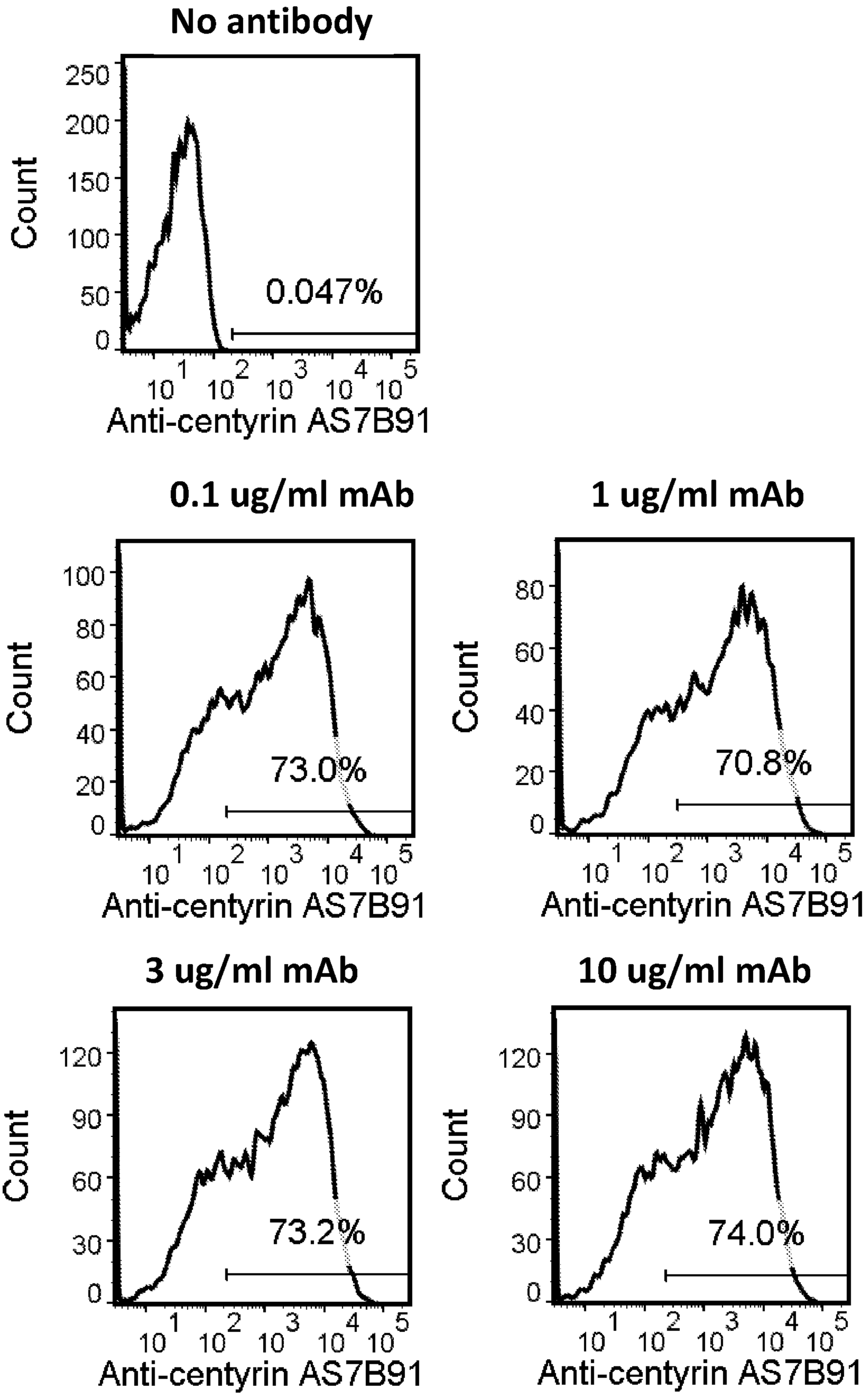


Figure 2G (D10)

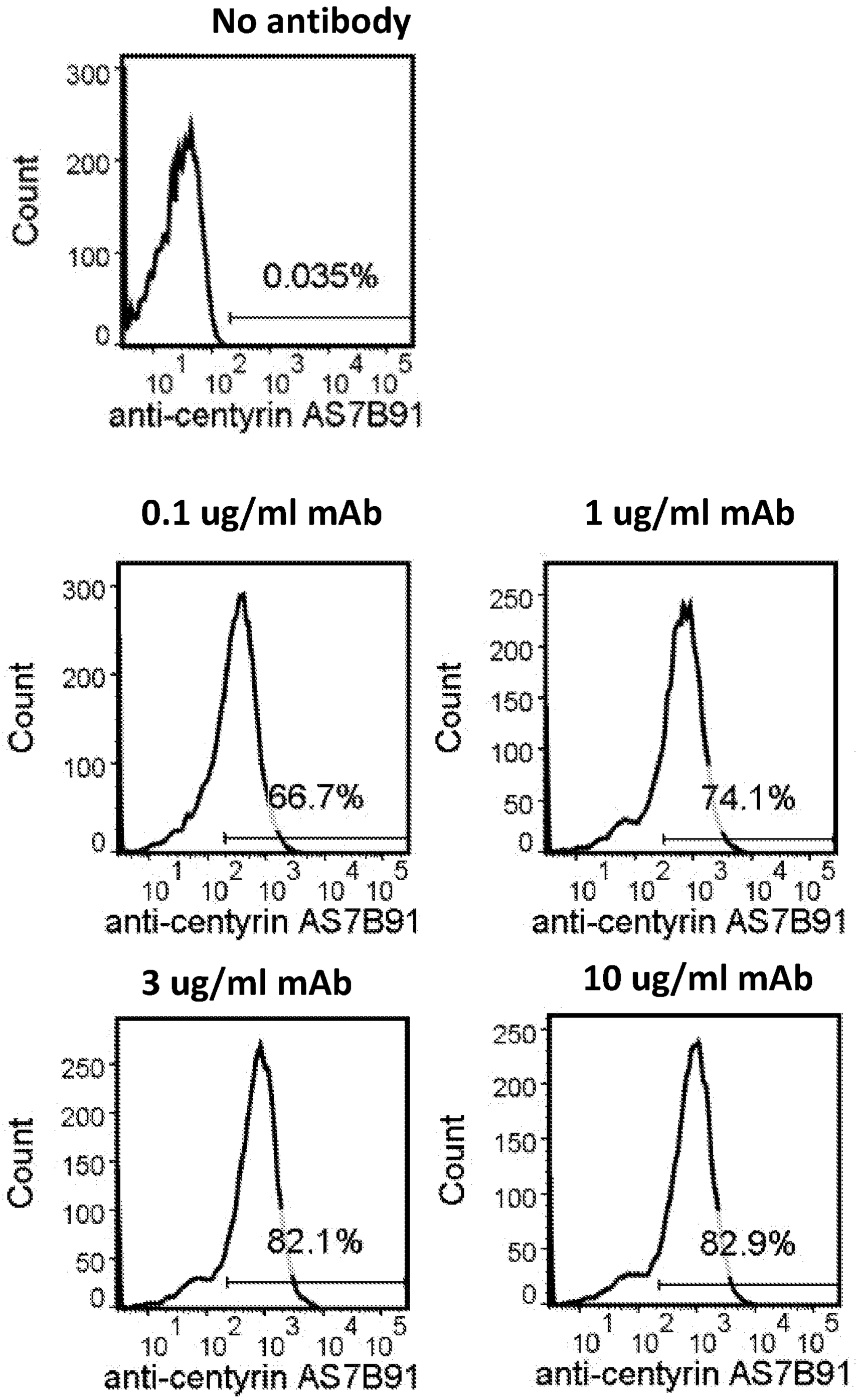


Figure 2H (EO2)

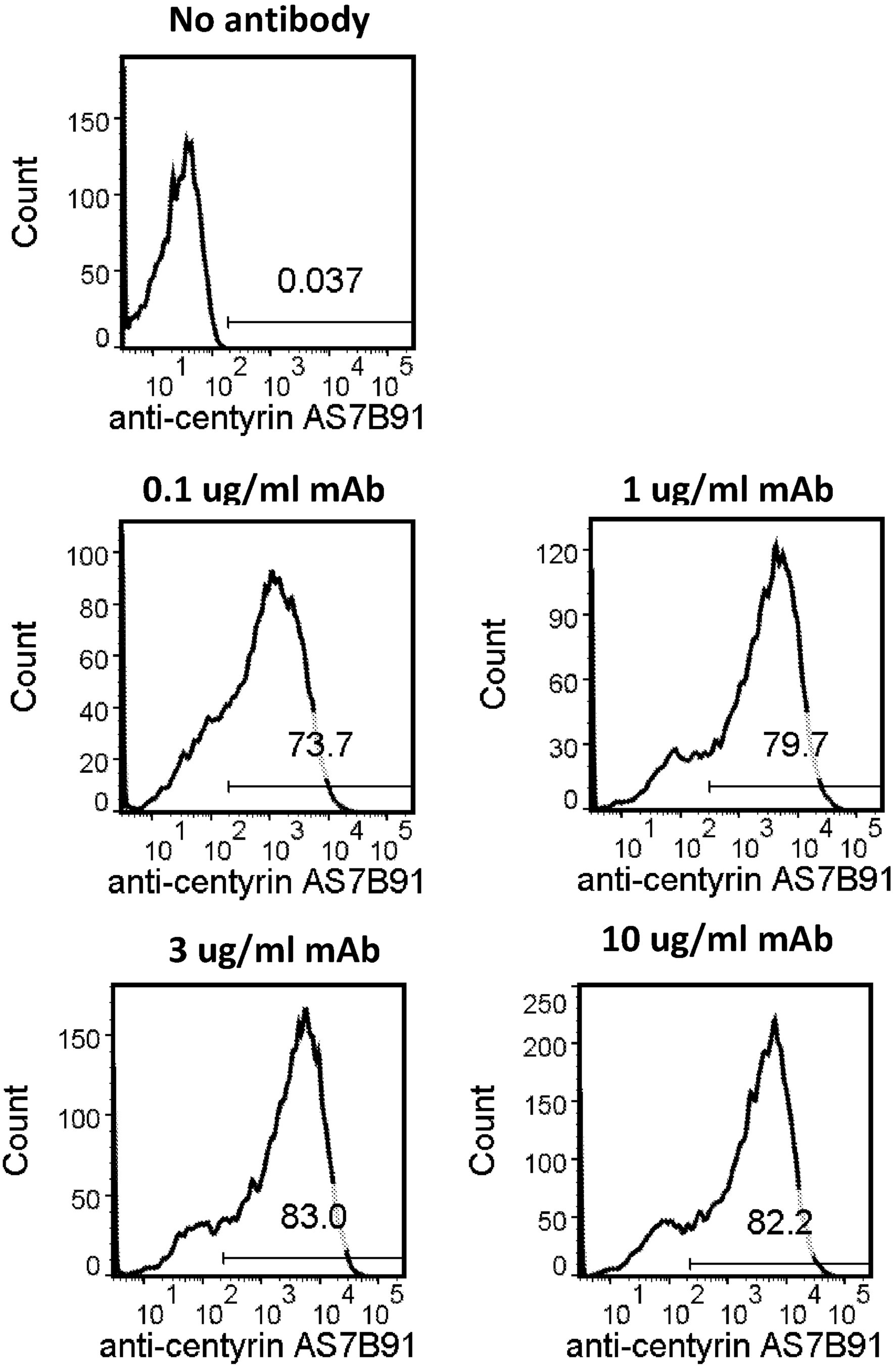


Figure 2I (EO9)

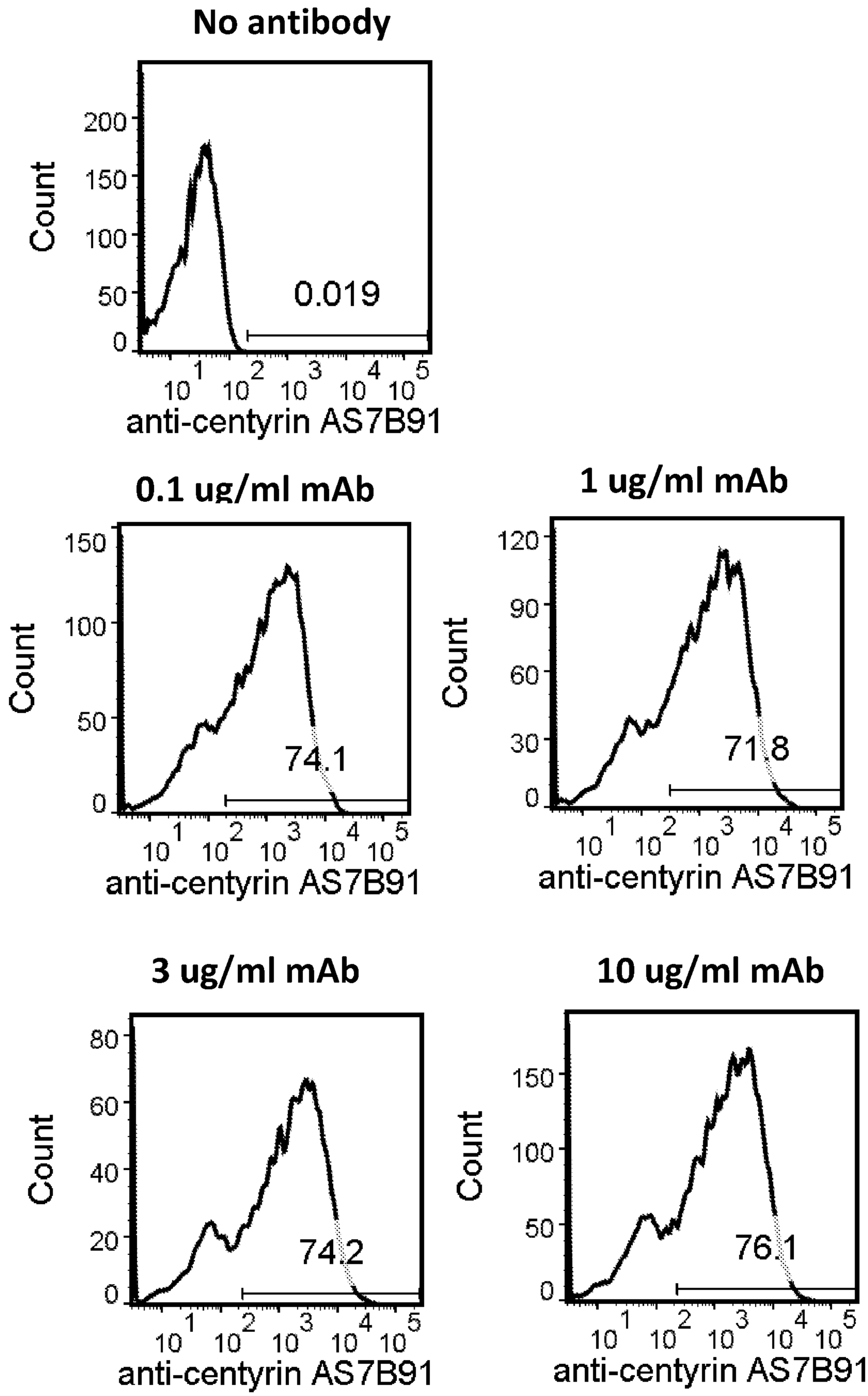


Figure 2J (FO1)

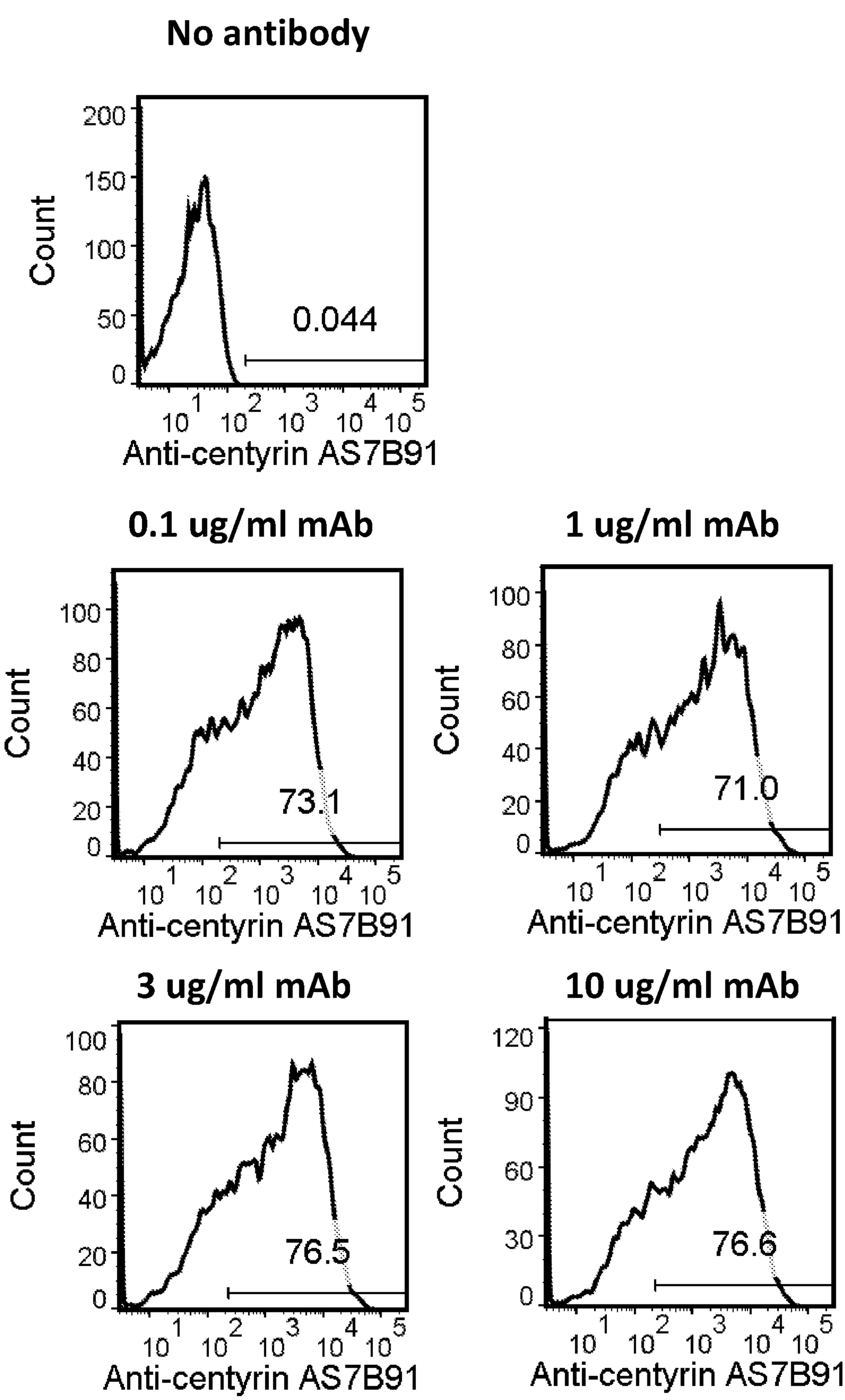


Figure 2K (F11)

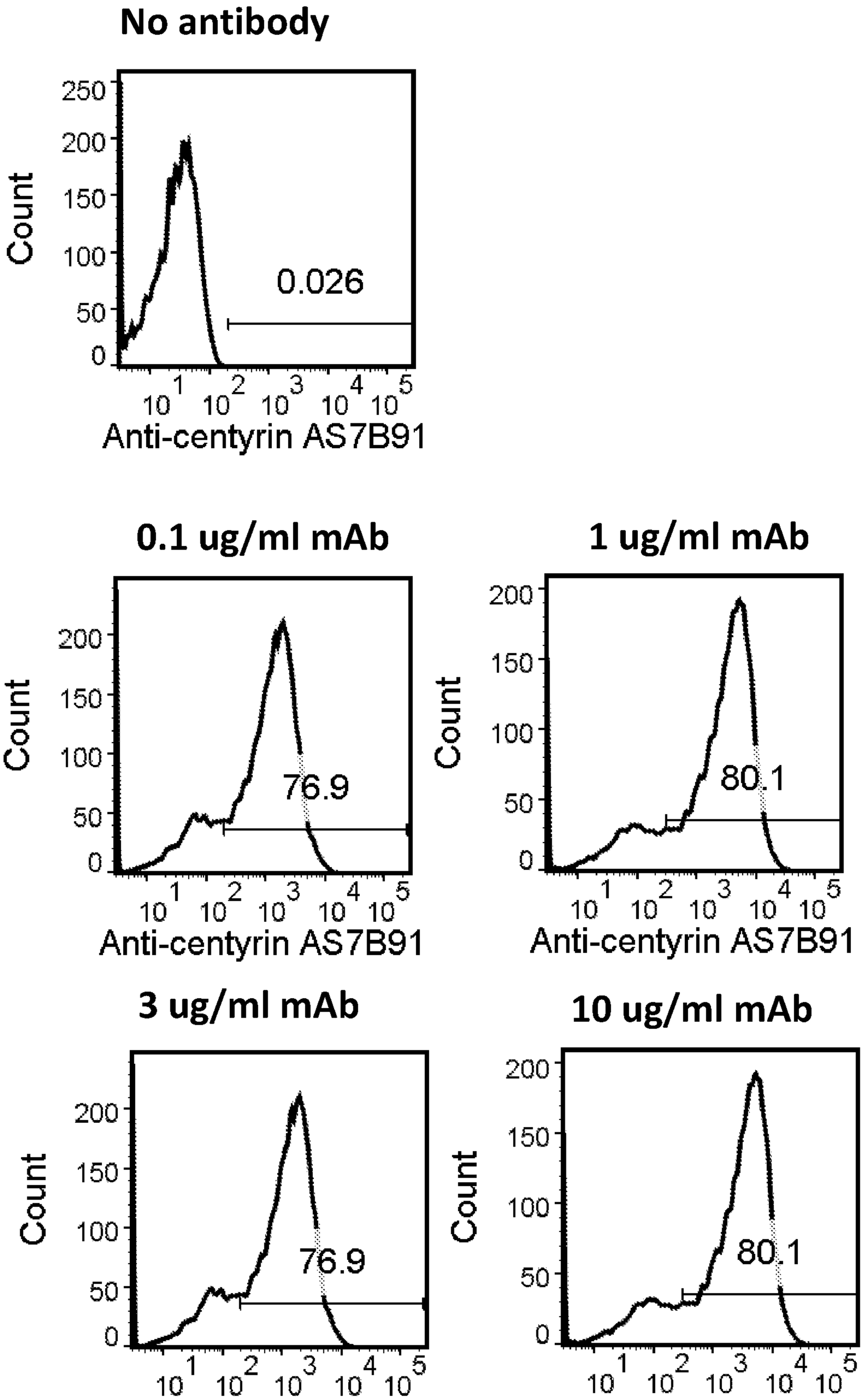
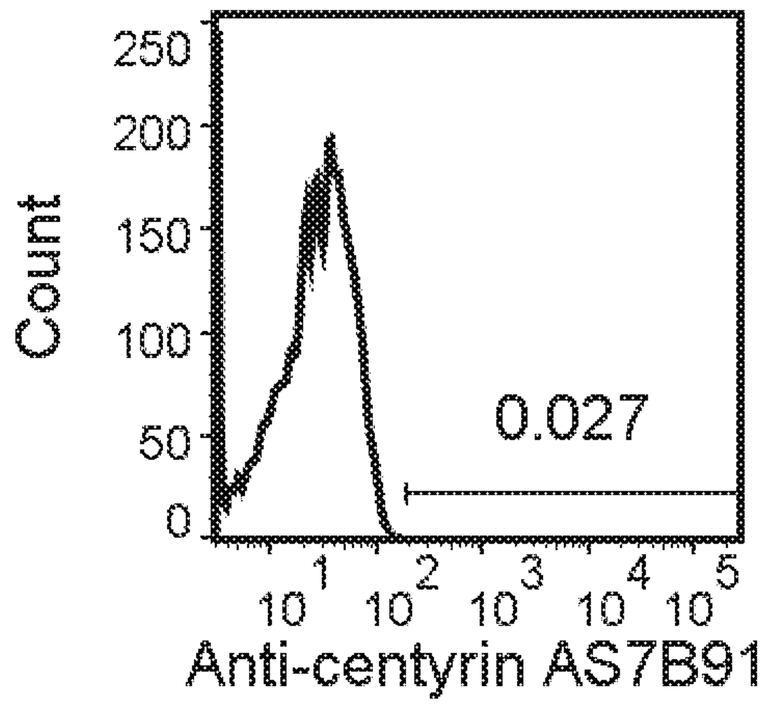
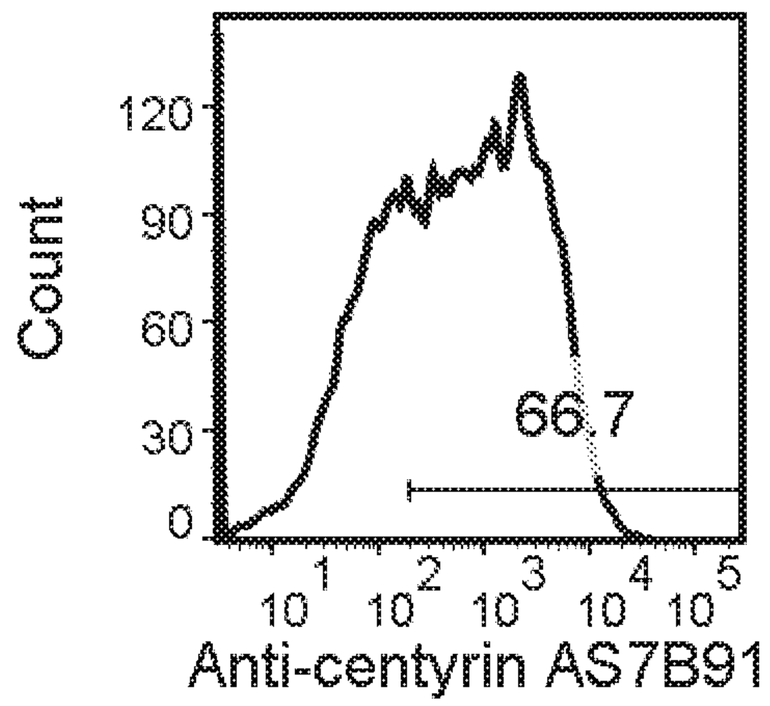


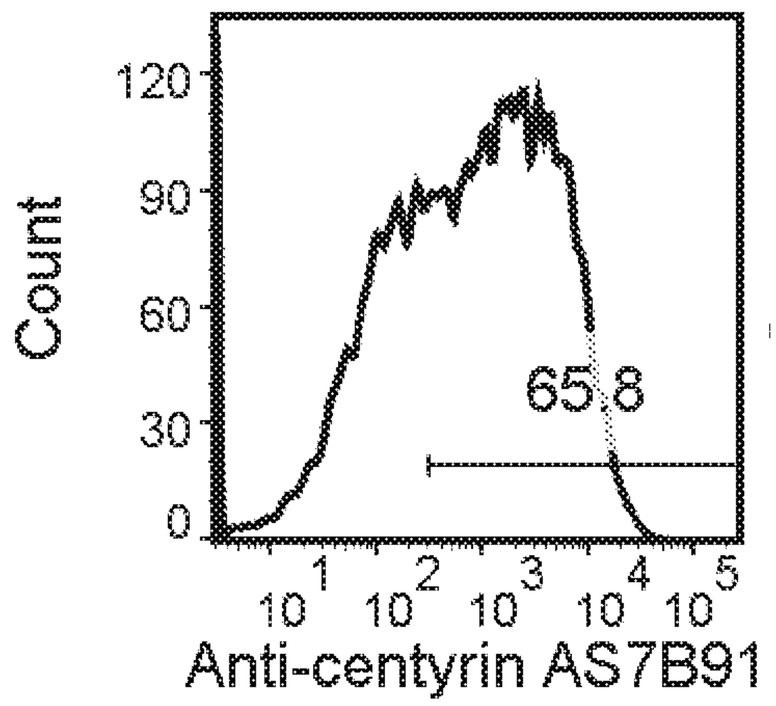
Figure 2L (G10)



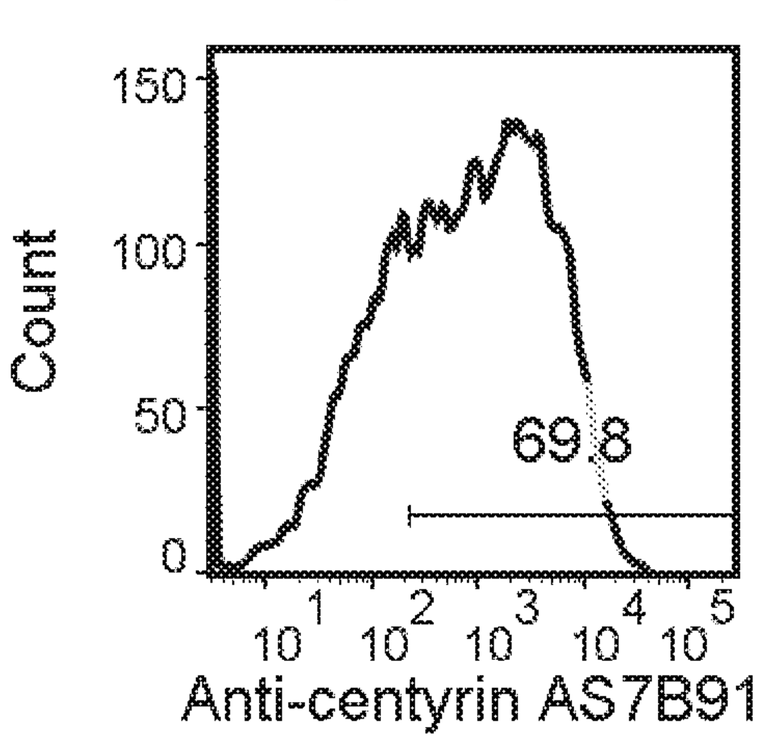
0.1 ug/ml mAb



1 ug/ml mAb



3 ug/ml mAb



10 ug/ml mAb

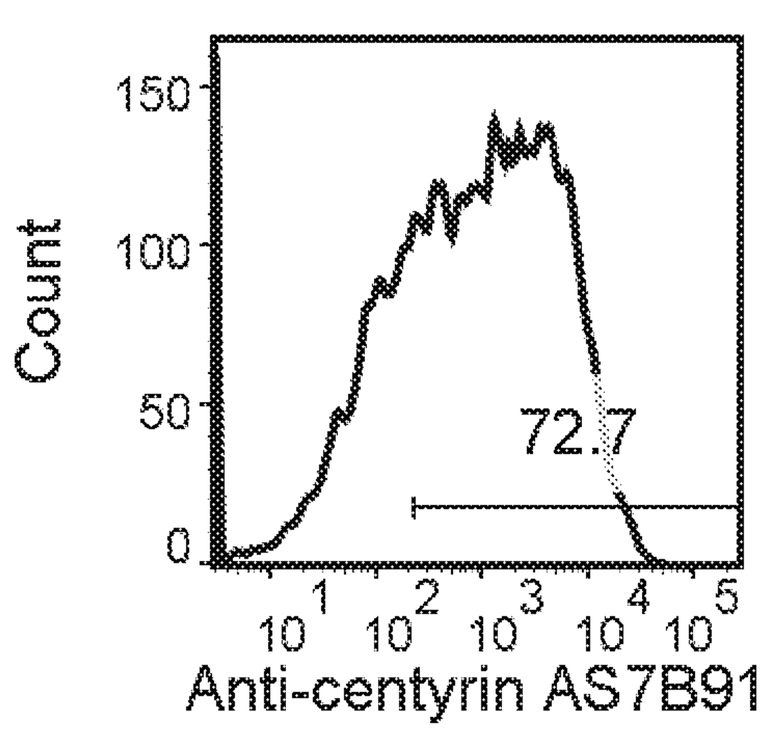


Figure 2M (DO8-89)

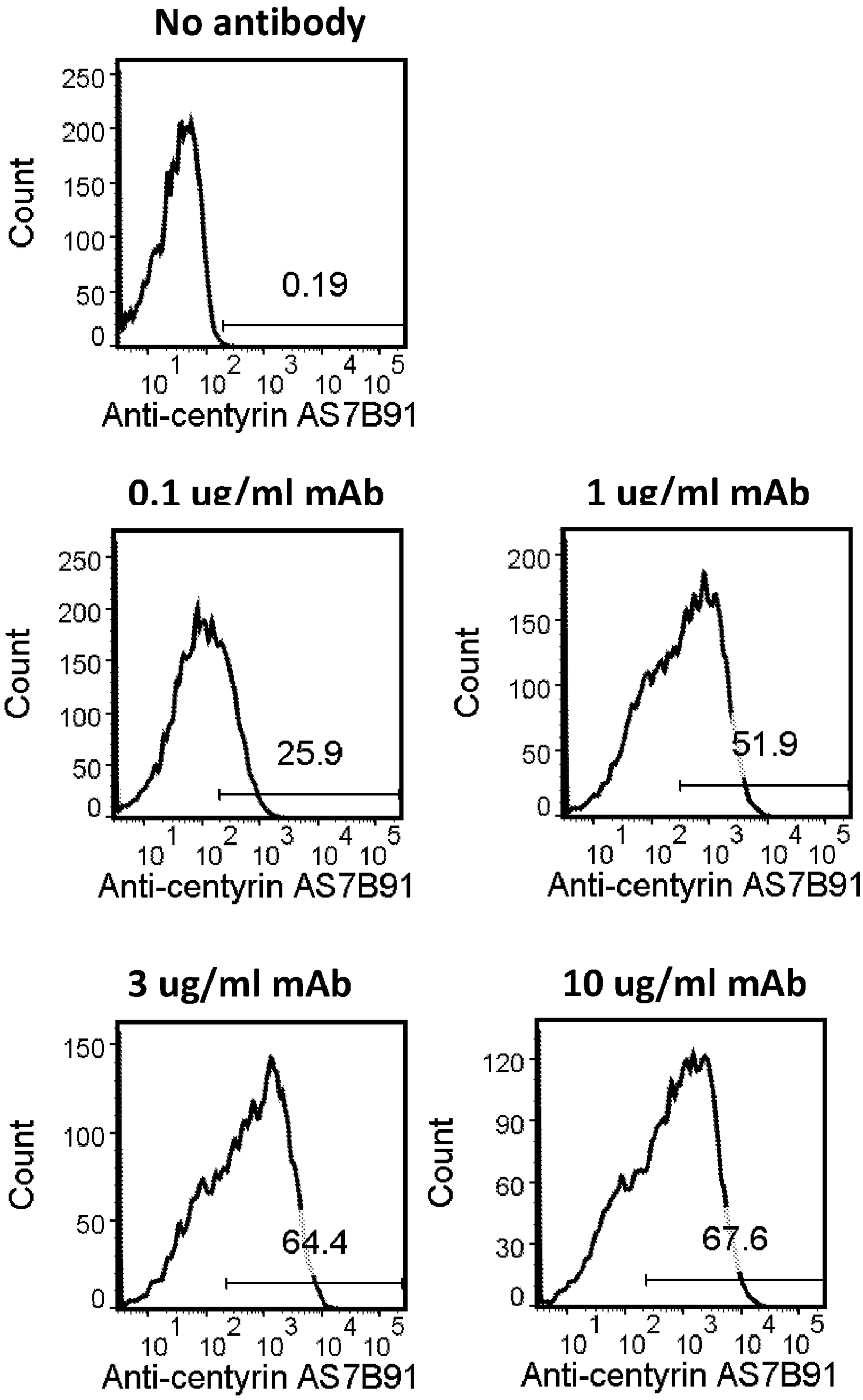


Figure 3

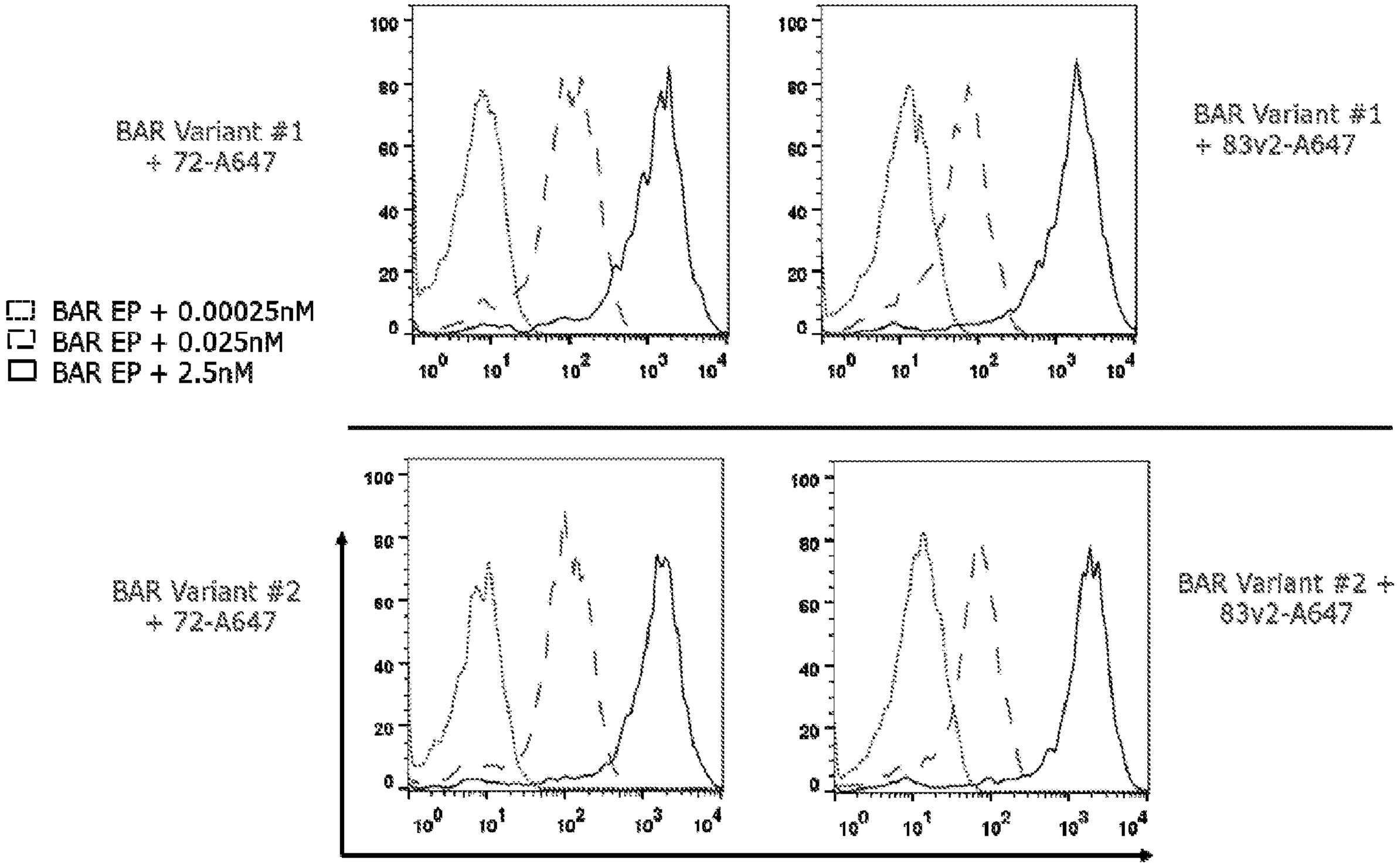

BAR Variant #1
+ 72-A647
BAR Variant #1
+ 83v2-A647
BAR EP + 0.00025nM
BAR EP + 0.025nM
BAR EP + 2.5nM
BAR Variant #2
+ 72-A647
BAR Variant #2 +
83v2-A647
Centyrin Binding
AS7B91 scFv L-H= BAR-T variant 1
AS7B91 scFv H-L=BAR-T variant 2

Figure 4A

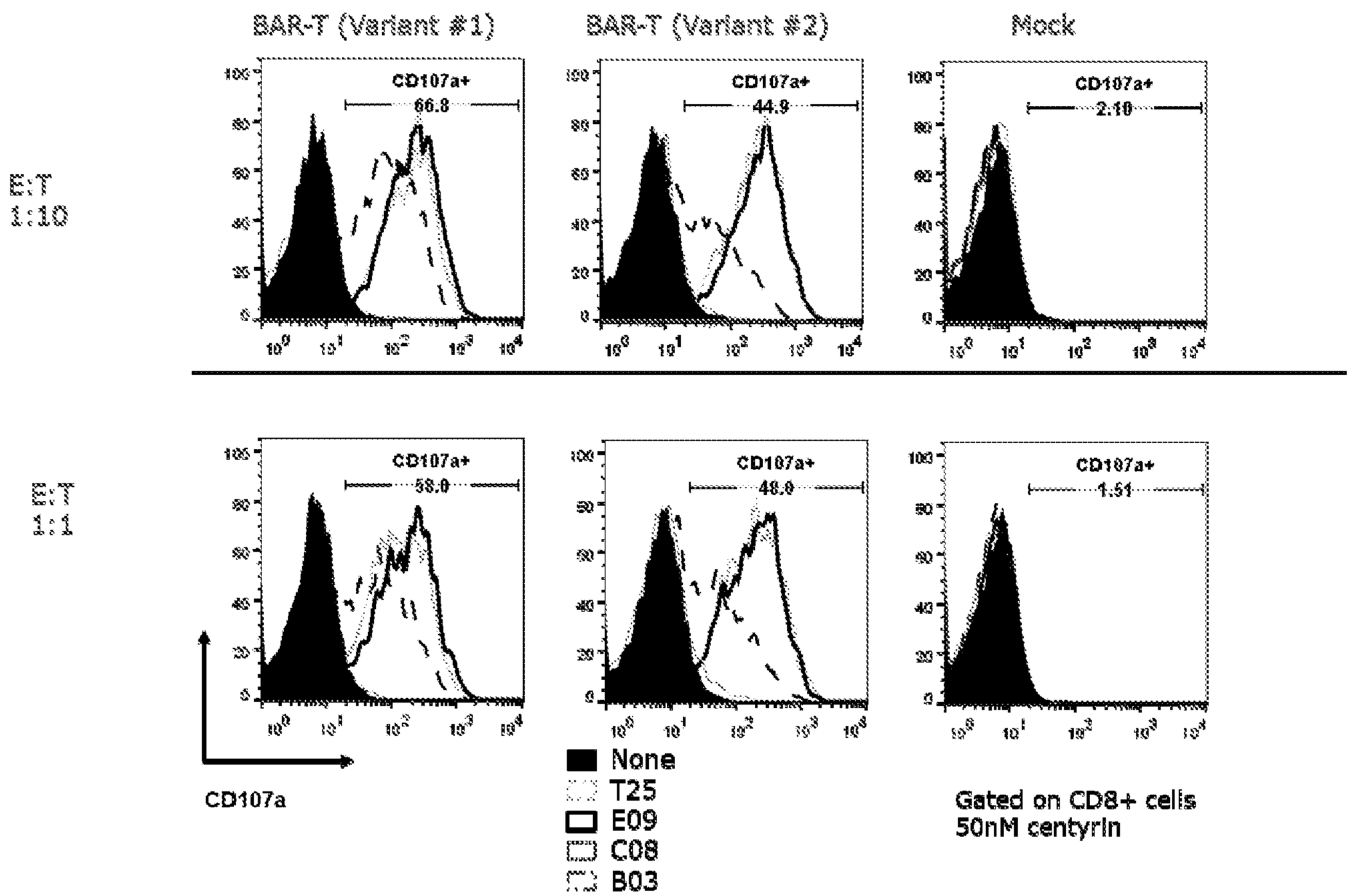

H929 cells (BCMA^hi)
BAR-T (Variant #1)
BAR-T (Variant #2)
Mock
E:T
1:10
CD107a+
66.8
44.9
2.10
E:T
1:1
58.0
48.0
1.51
CD107a
None
T25
E09
C08
B03
Gated on CD8+ cells
50nM centyrin

Figure 4B

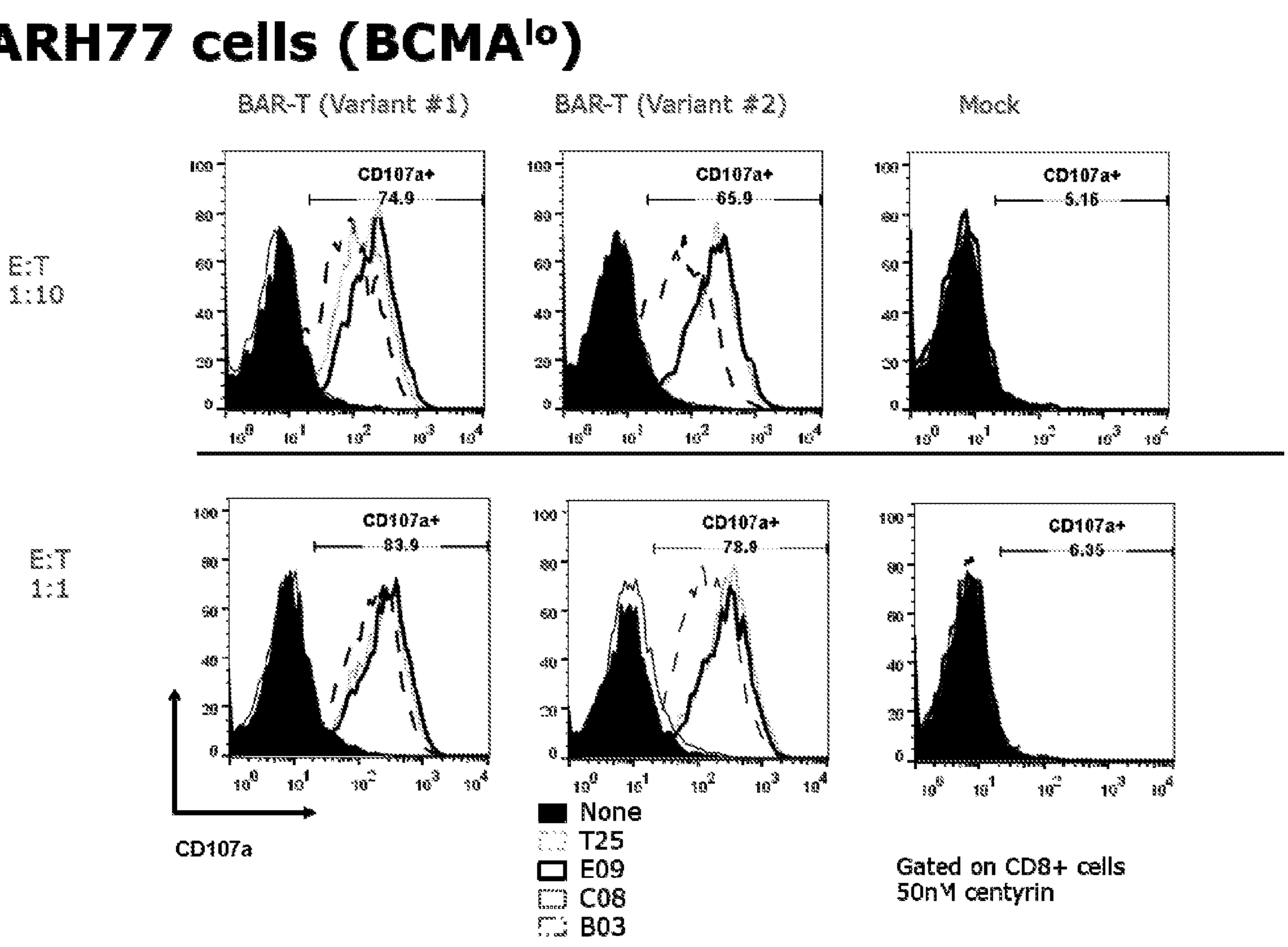

ARH77 cells (BCMA^lo)
BAR-T (Variant #1)
BAR-T (Variant #2)
Mock
E:T
1:10
E:T
1:1
CD107a+
74.9
65.9
5.16
83.9
78.9
6.35
CD107a
None
T25
E09
C08
B03
Gated on CD8+ cells
50nM centyrin

Figure 4C

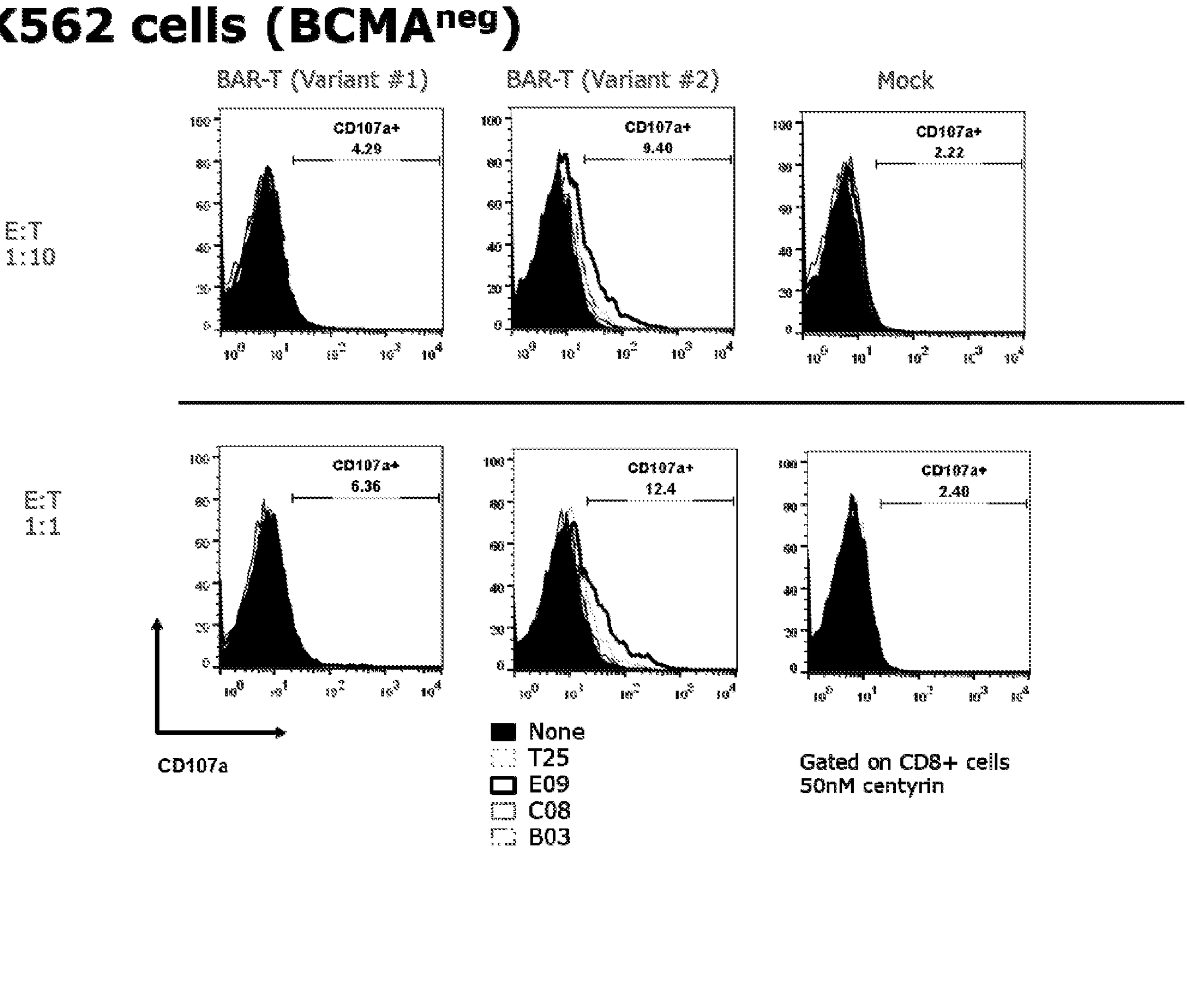

K562 cells (BCMA^neg)
BAR-T (Variant #1)
BAR-T (Variant #2)
Mock
E:T
1:10
CD107a+
4.29
CD107a+
9.40
CD107a+
2.22
E:T
1:1
CD107a+
6.36
CD107a+
12.4
CD107a+
2.48
CD107a
None
T25
E09
C08
B03
Gated on CD8+ cells
50nM centyrin

Figure 5

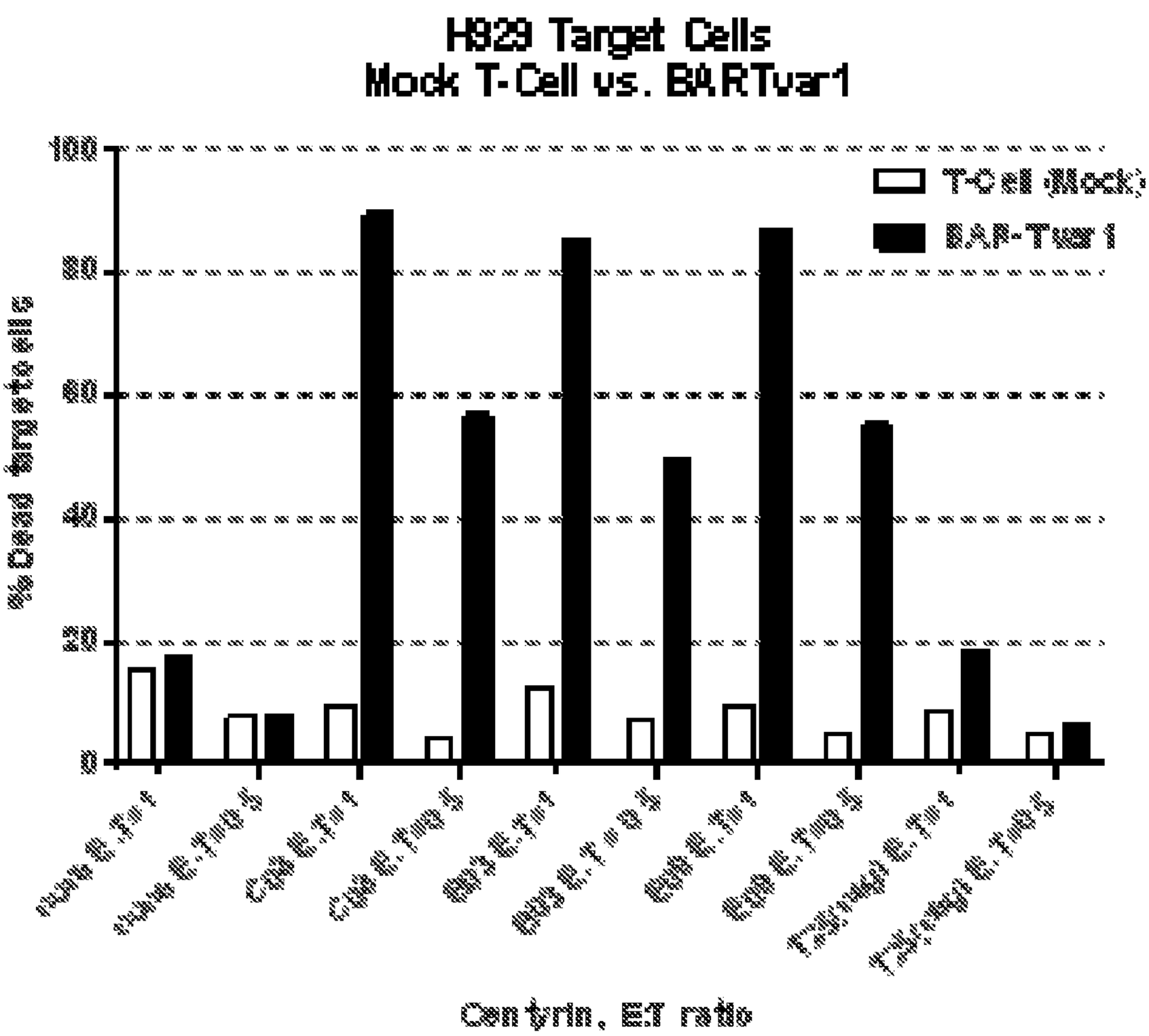

H929 Target Cells
Mock T-Cell vs. BARTvar1
% Dead Target Cells
T-Cell Mock
BAR-Tvar1
Cytotox. ET ratio

Figure 5 con't

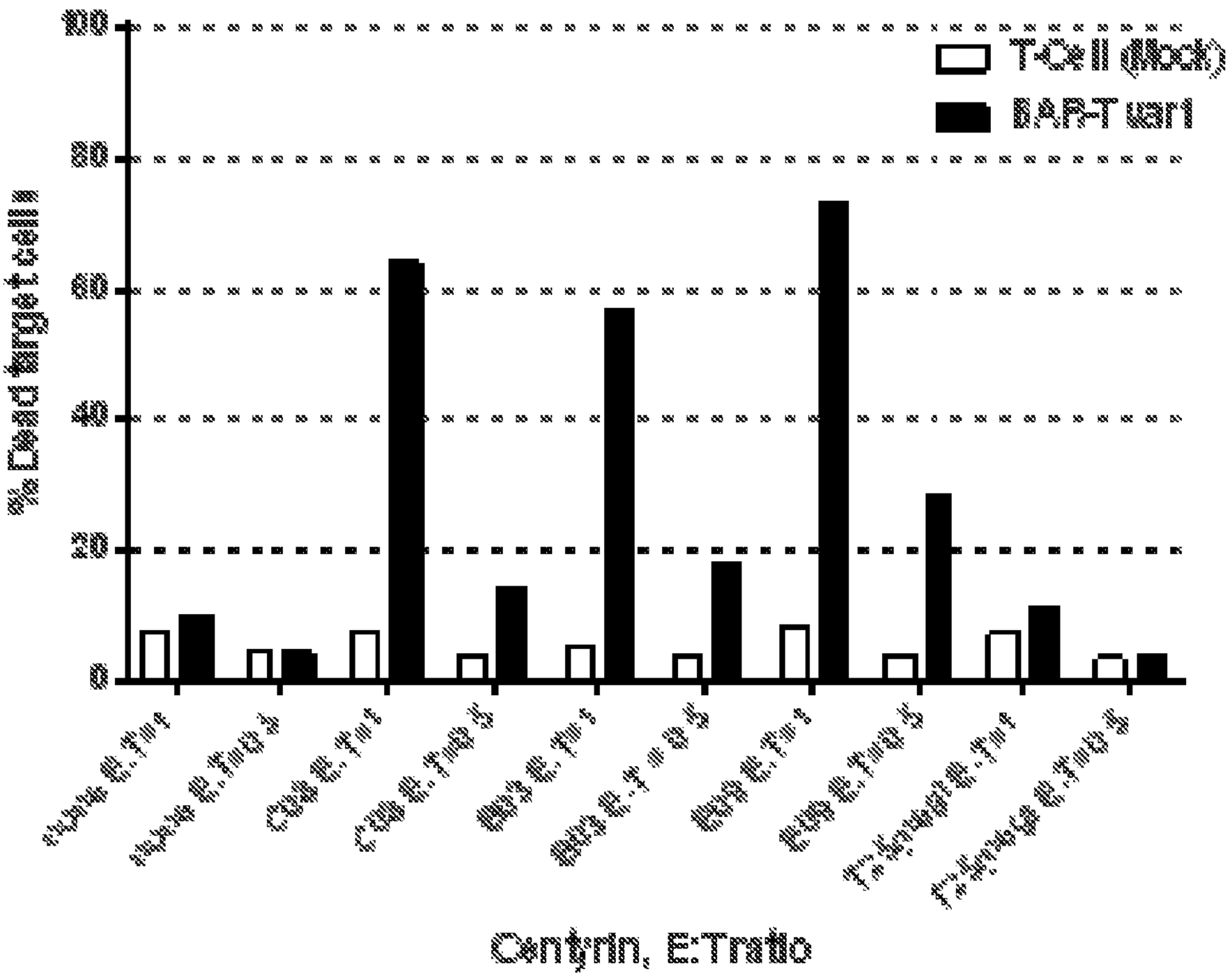


Figure 5 con't

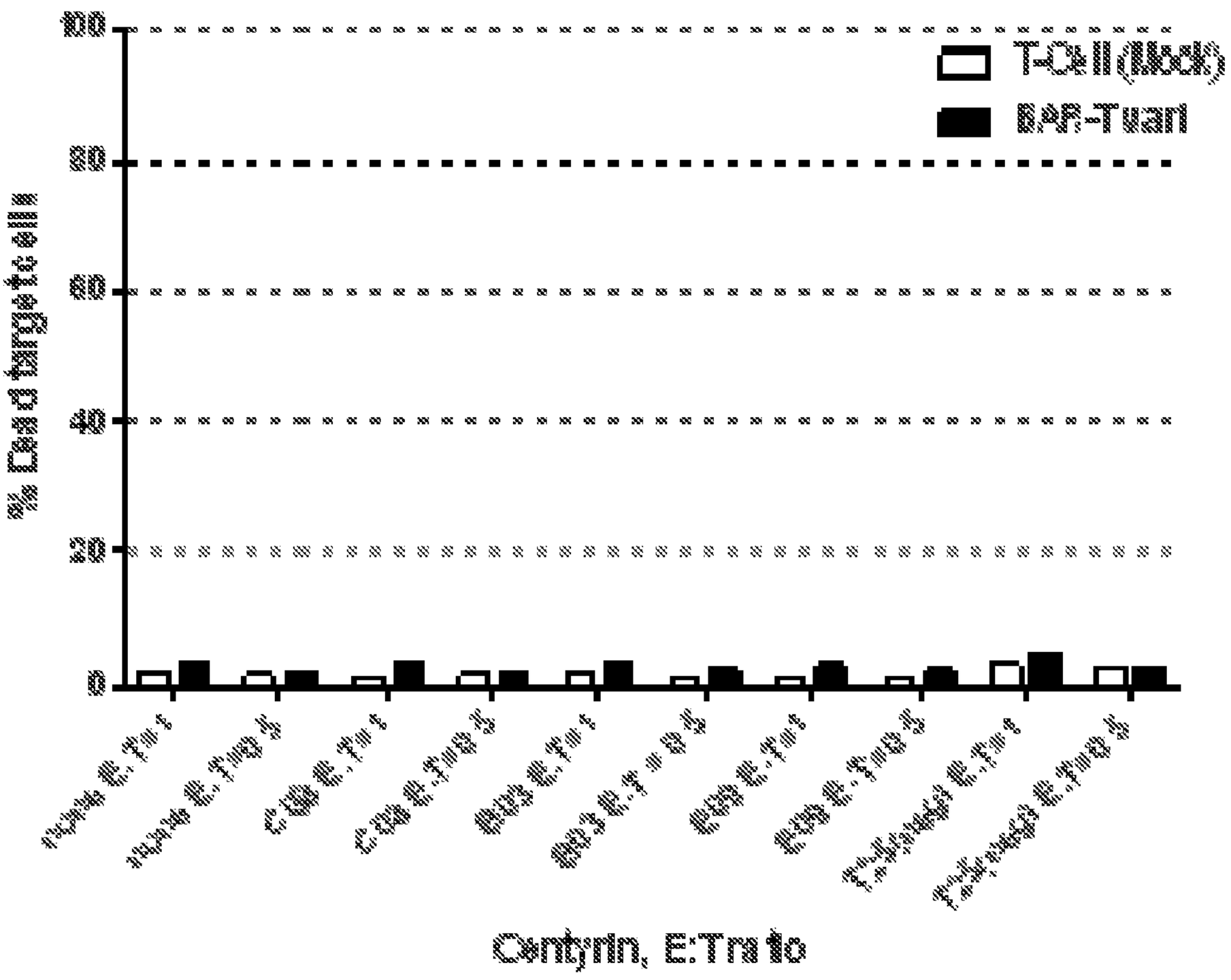


Figure 5 con't

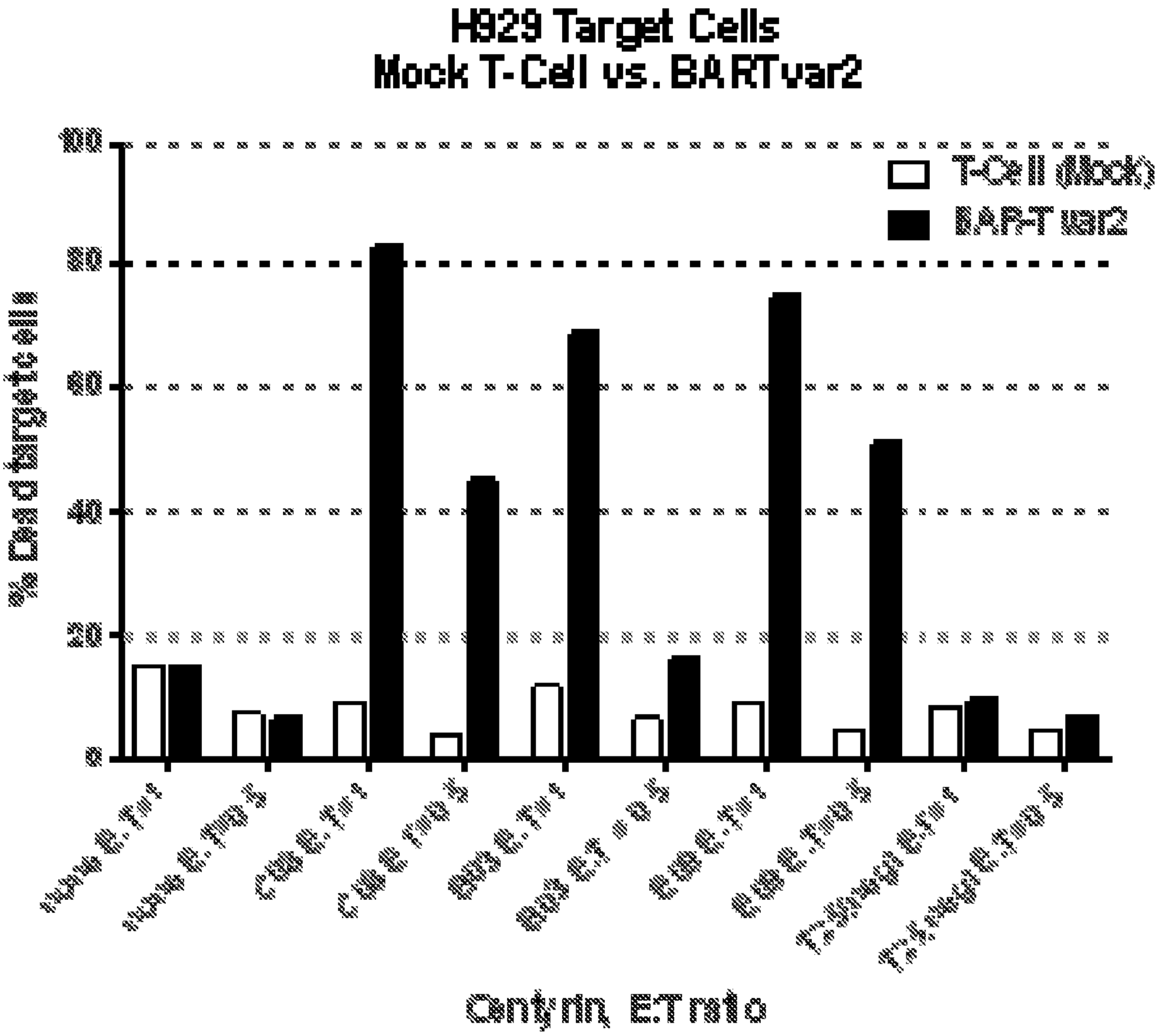


Figure 5 con't

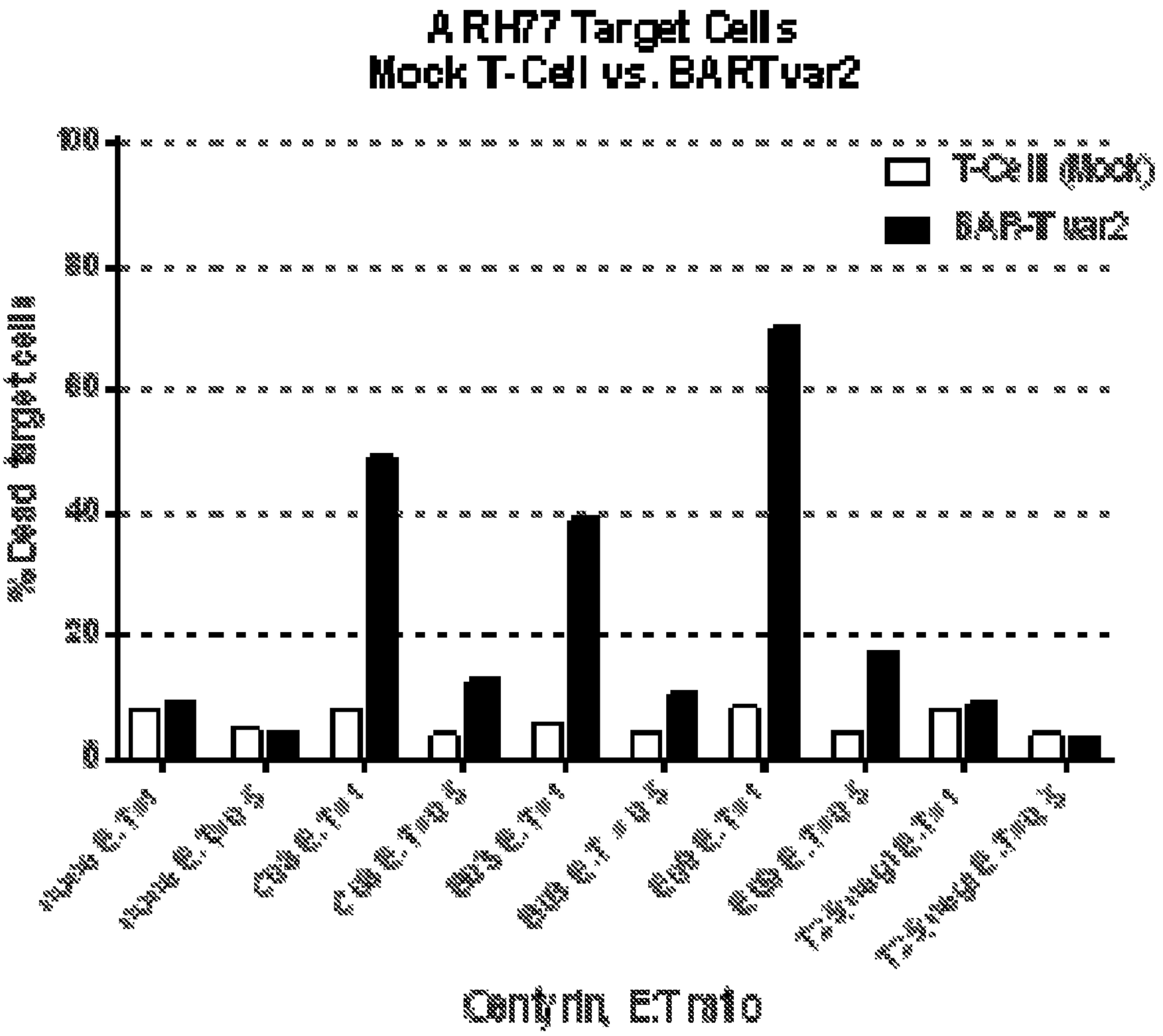


Figure 5 con't

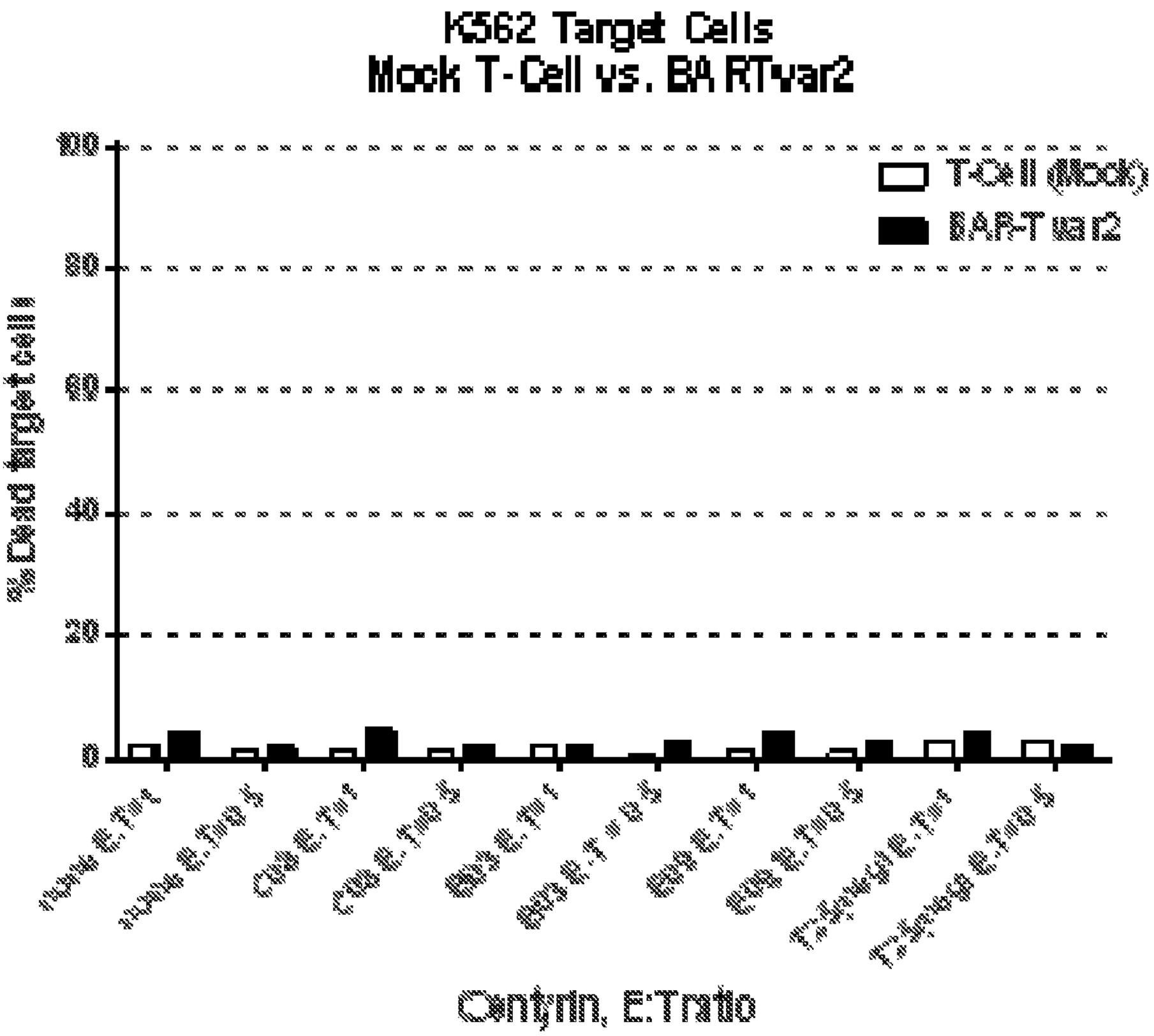
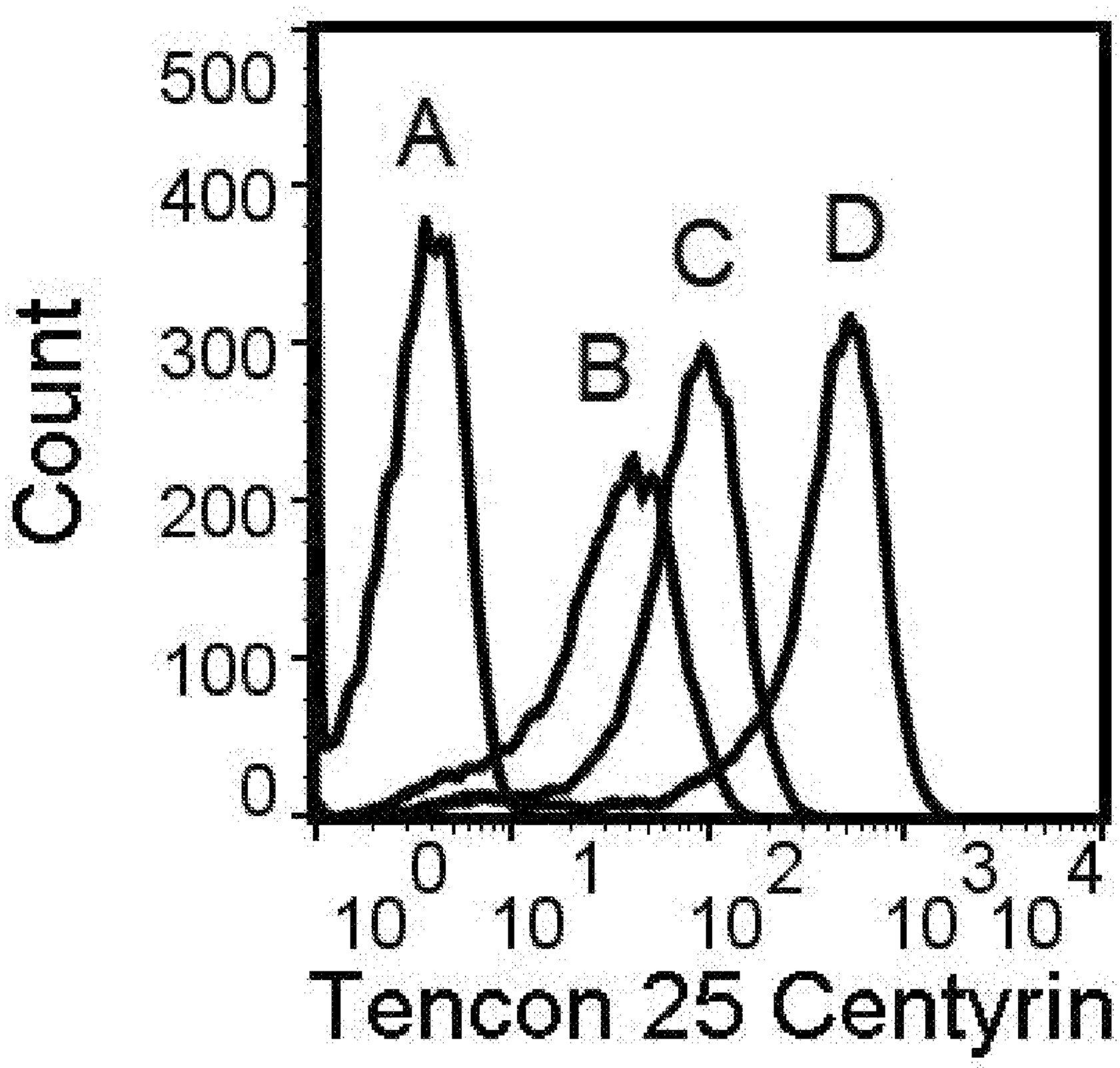


Figure 6



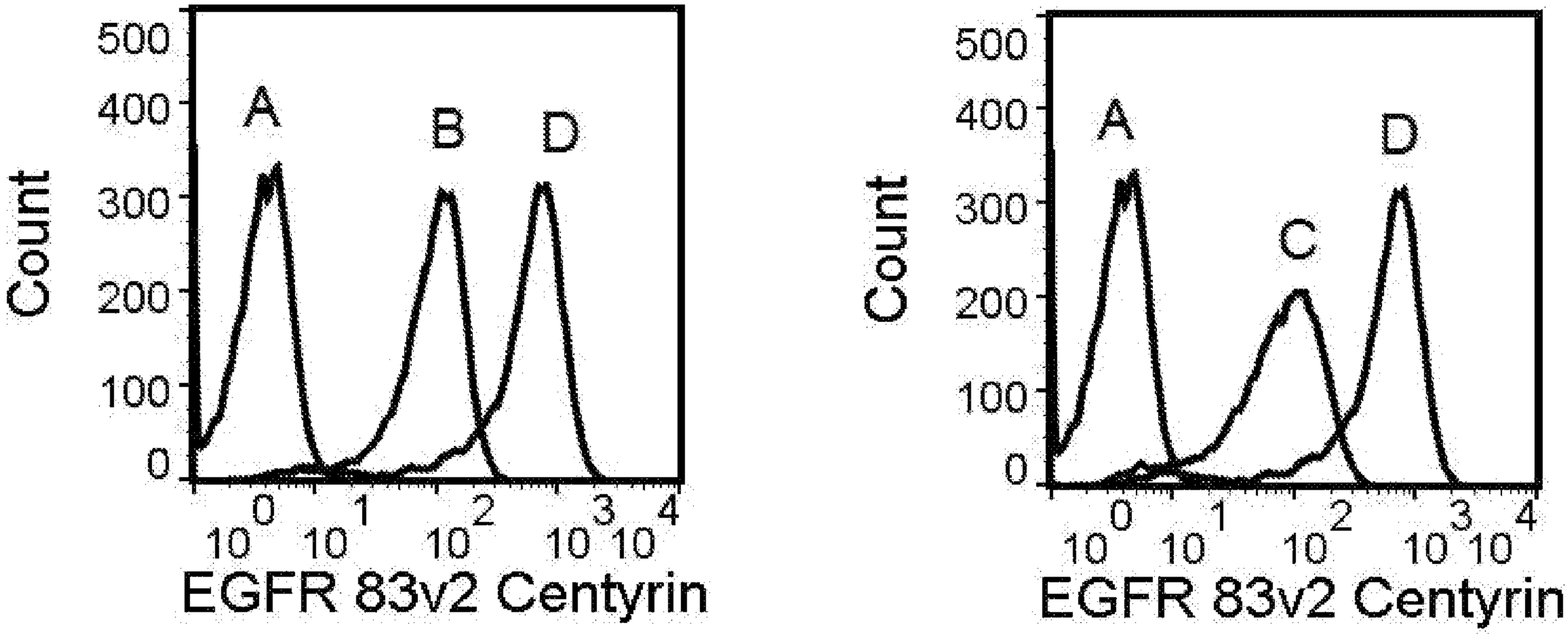
A. No CAR

B. AS7B16 L2H scFv CAR

C. AS7B16 H2L scFv CAR

D. AS7B91 H2L scFv CAR

Figure 7



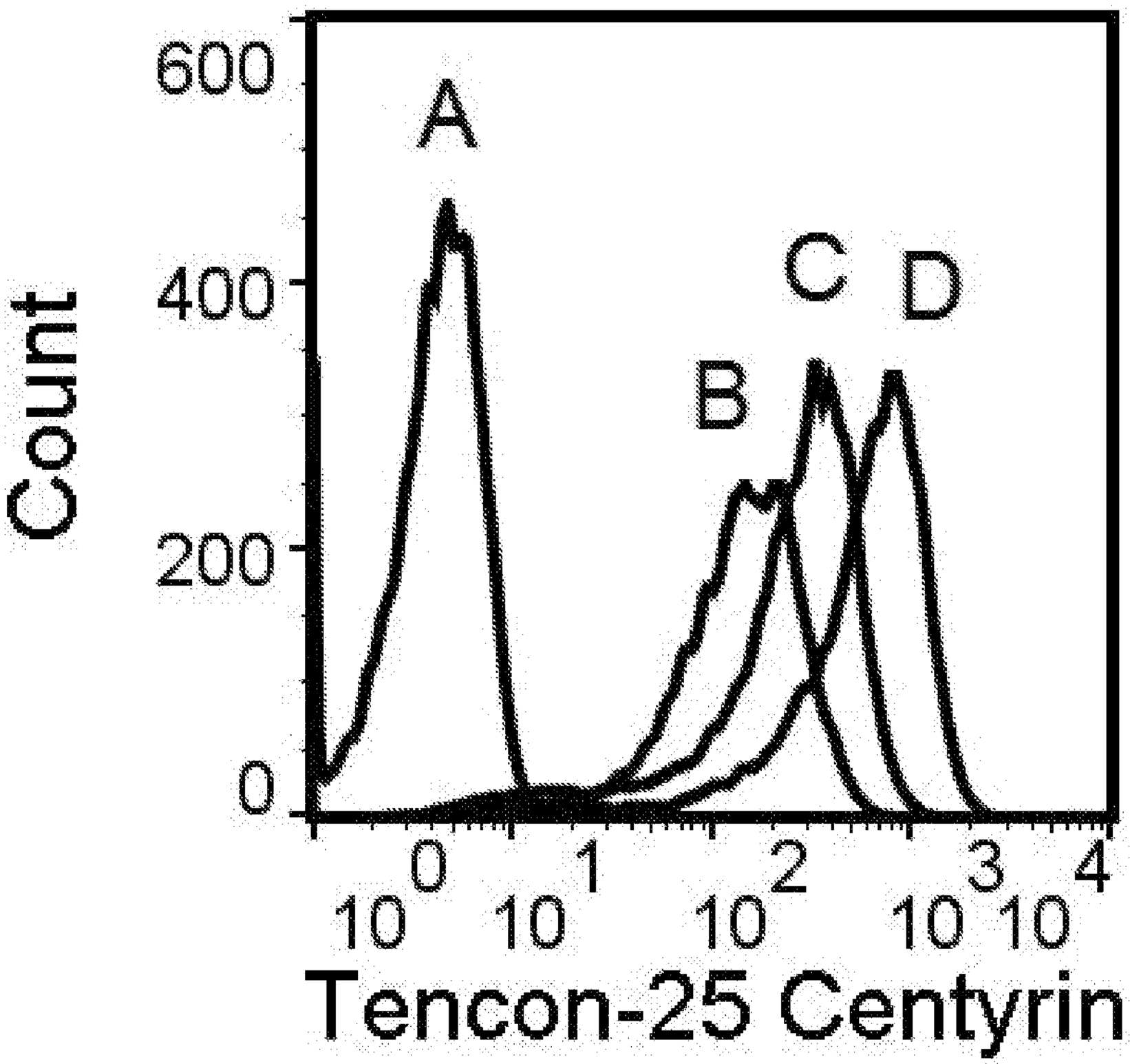
A. No CAR

B. AS7B16 L2H scFv CAR

C. AS7B16 H2L scFv CAR

D. AS7B91 H2L scFv CAR

Figure 8



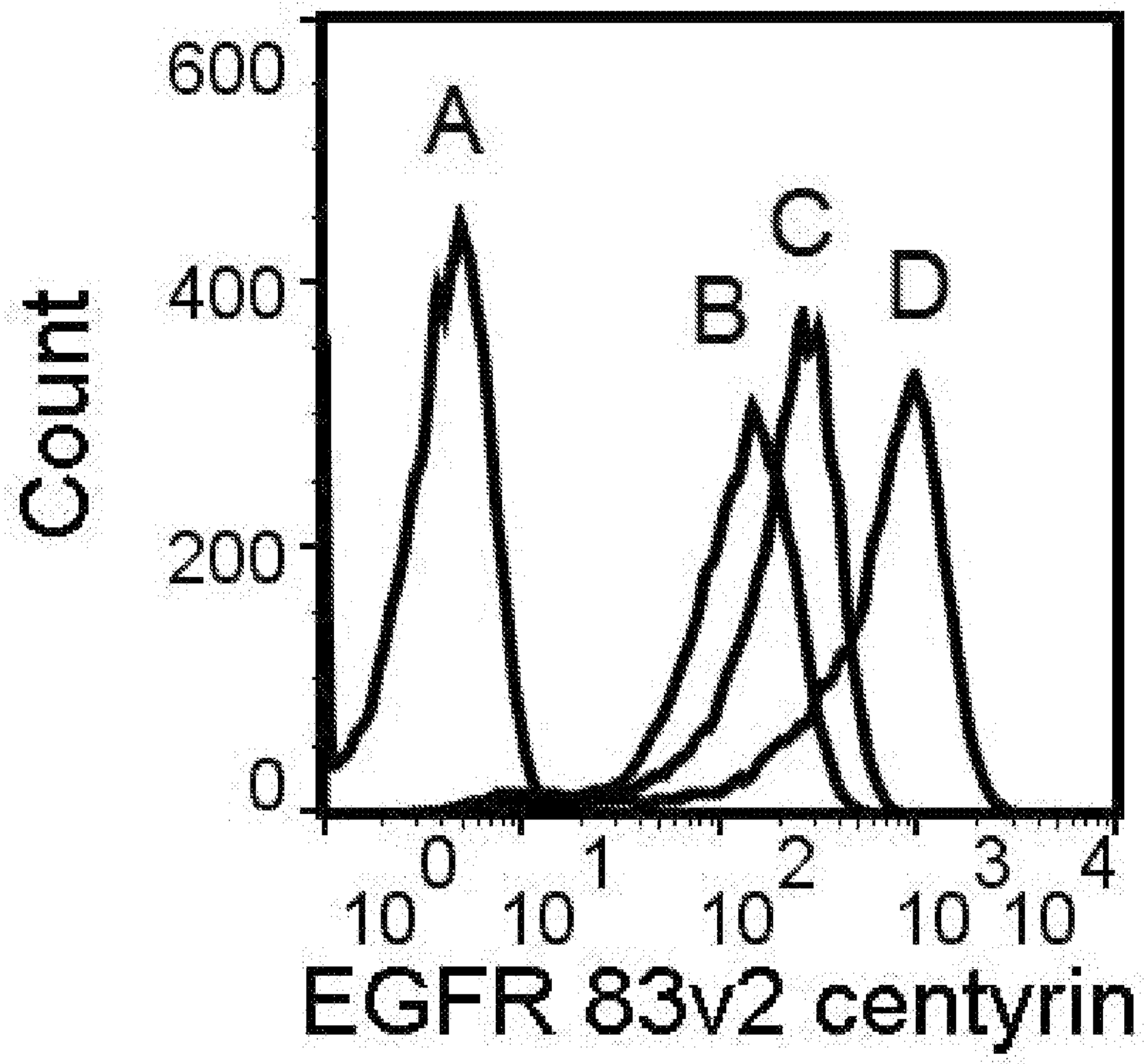
A. No CAR

B. AS7B82 L2H scFv CAR

C. AS7B82 H2L scFv CAR

D. AS7B91 H2L scFv CAR

Figure 9



A. No CAR

B. AS7B82 L2H scFv CAR

C. AS7B82 H2L scFv CAR

D. AS7B91 H2L scFv CAR

Figure 10

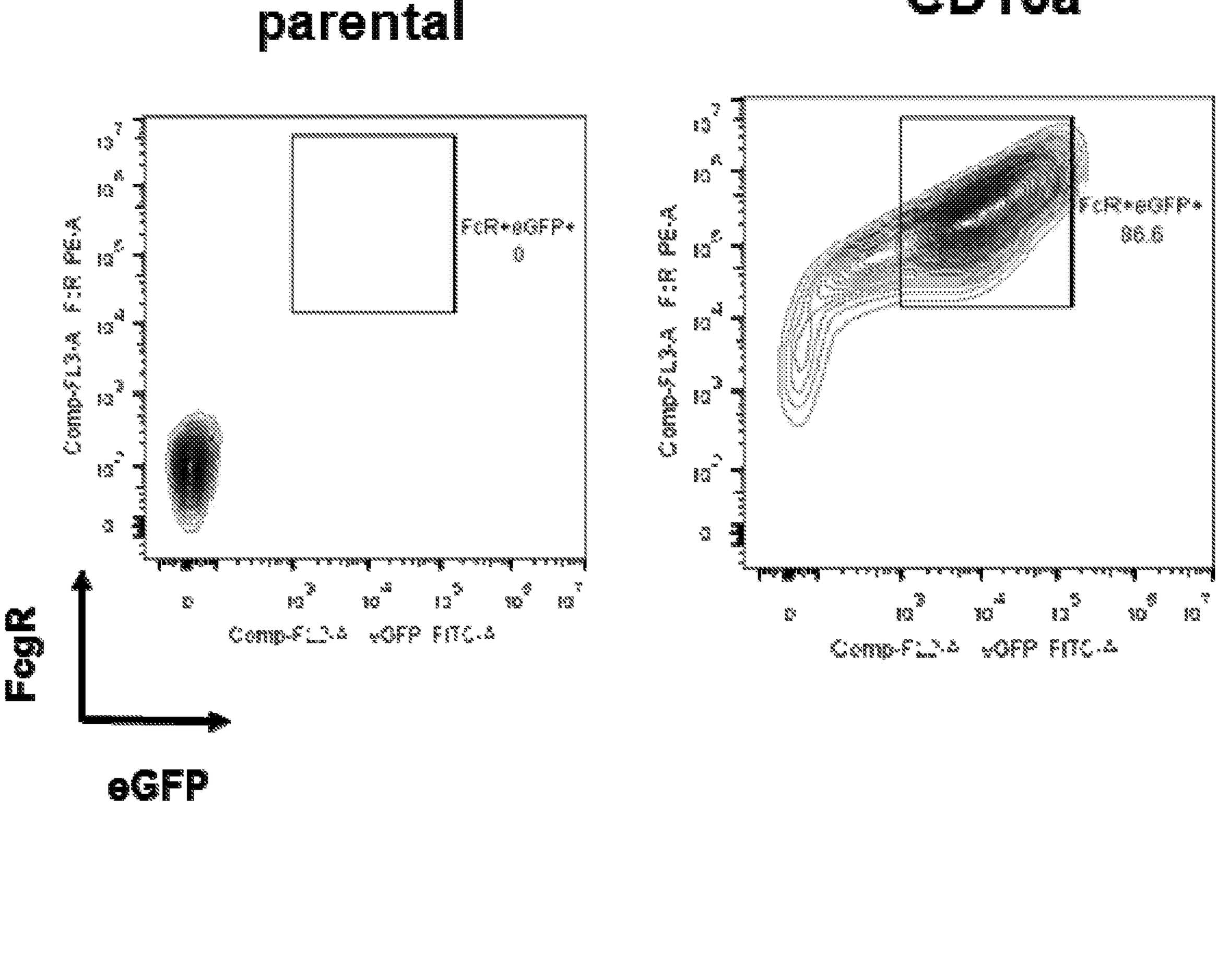

parental
CD16a
FcR+eGFP+
0
FcR+eGFP+
96.6
Comp-FL3-A :: FcR PE-A
Comp-FL1-A :: eGFP FITC-A
FcgR
eGFP

Figure 10 continued

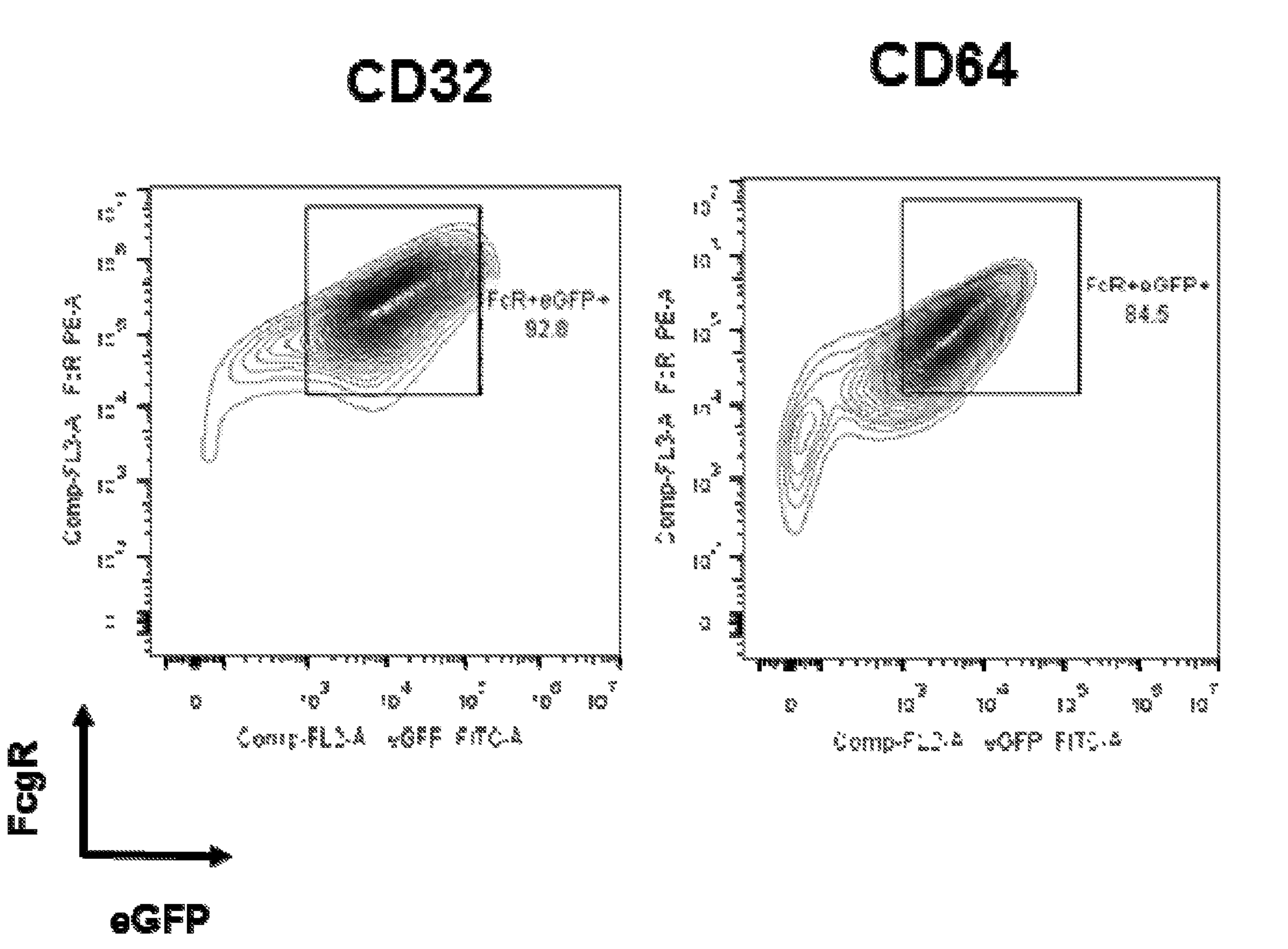
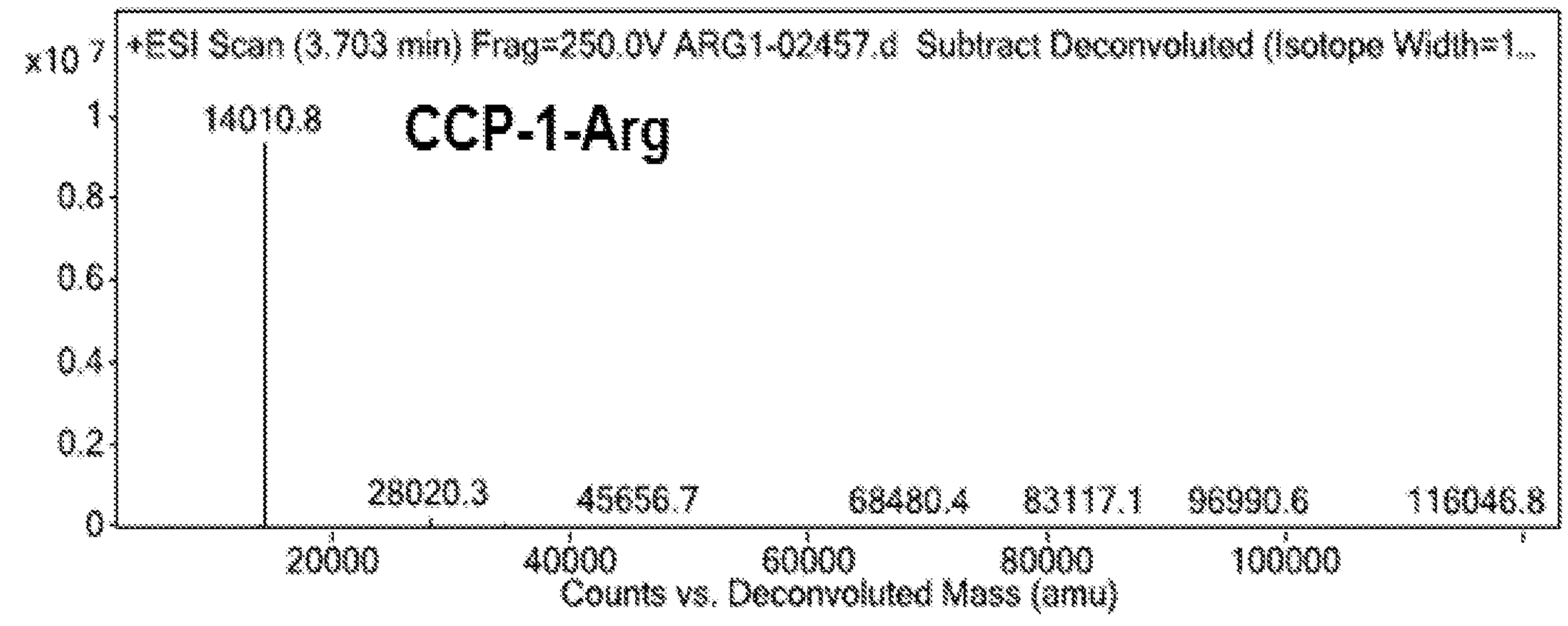


Figure 11A


x10 7
+ESI Scan (3.703 min) Frag=250.0V ARG1-02457.d Subtract Deconvoluted (Isotope Width=1...
14010.8
CCP-1-Arg
1
0.8
0.6
0.4
0.2
0
28020.3
45656.7
68480.4
83117.1
96990.6
116046.8
20000
40000
60000
80000
100000
Counts vs. Deconvoluted Mass (amu)

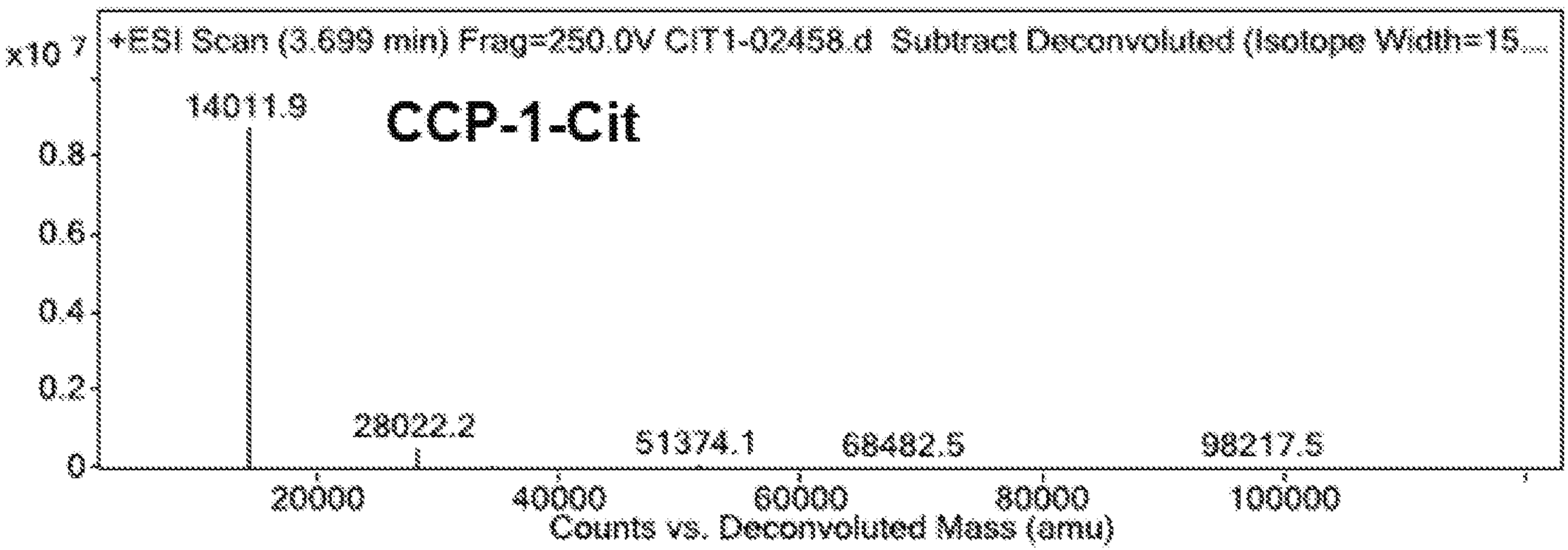
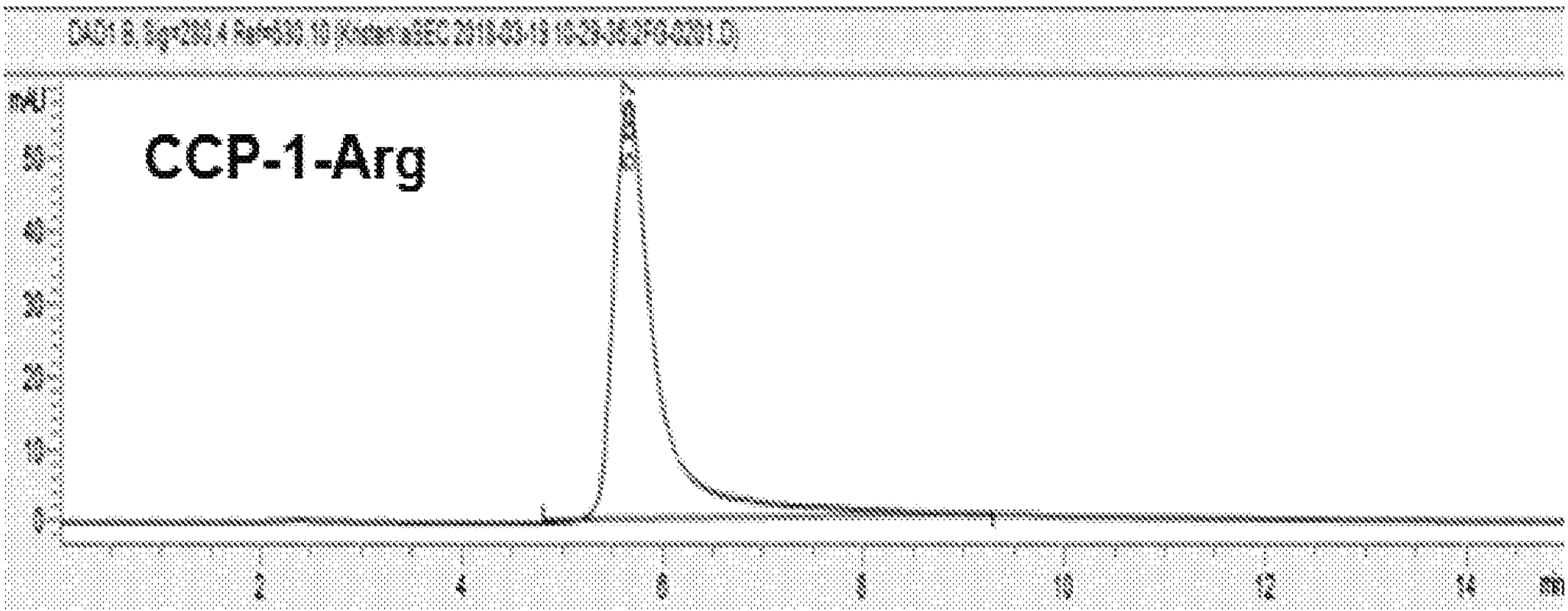

x10 7
+ESI Scan (3.699 min) Frag=250.0V CIT1-02458.d Subtract Deconvoluted (Isotope Width=15....
14011.9
CCP-1-Cit
0.8
0.6
0.4
0.2
0
28022.2
51374.1
68482.5
98217.5
20000
40000
60000
80000
100000
Counts vs. Deconvoluted Mass (amu)

Figure 11B



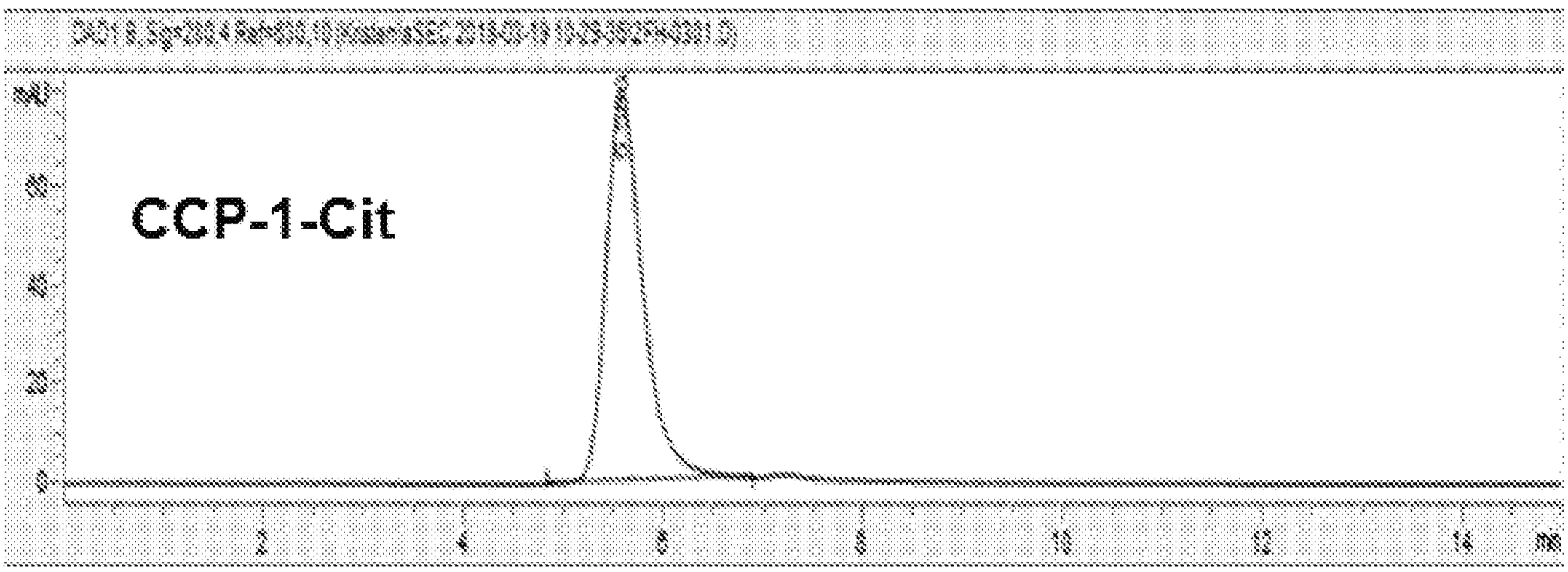


Figure 12

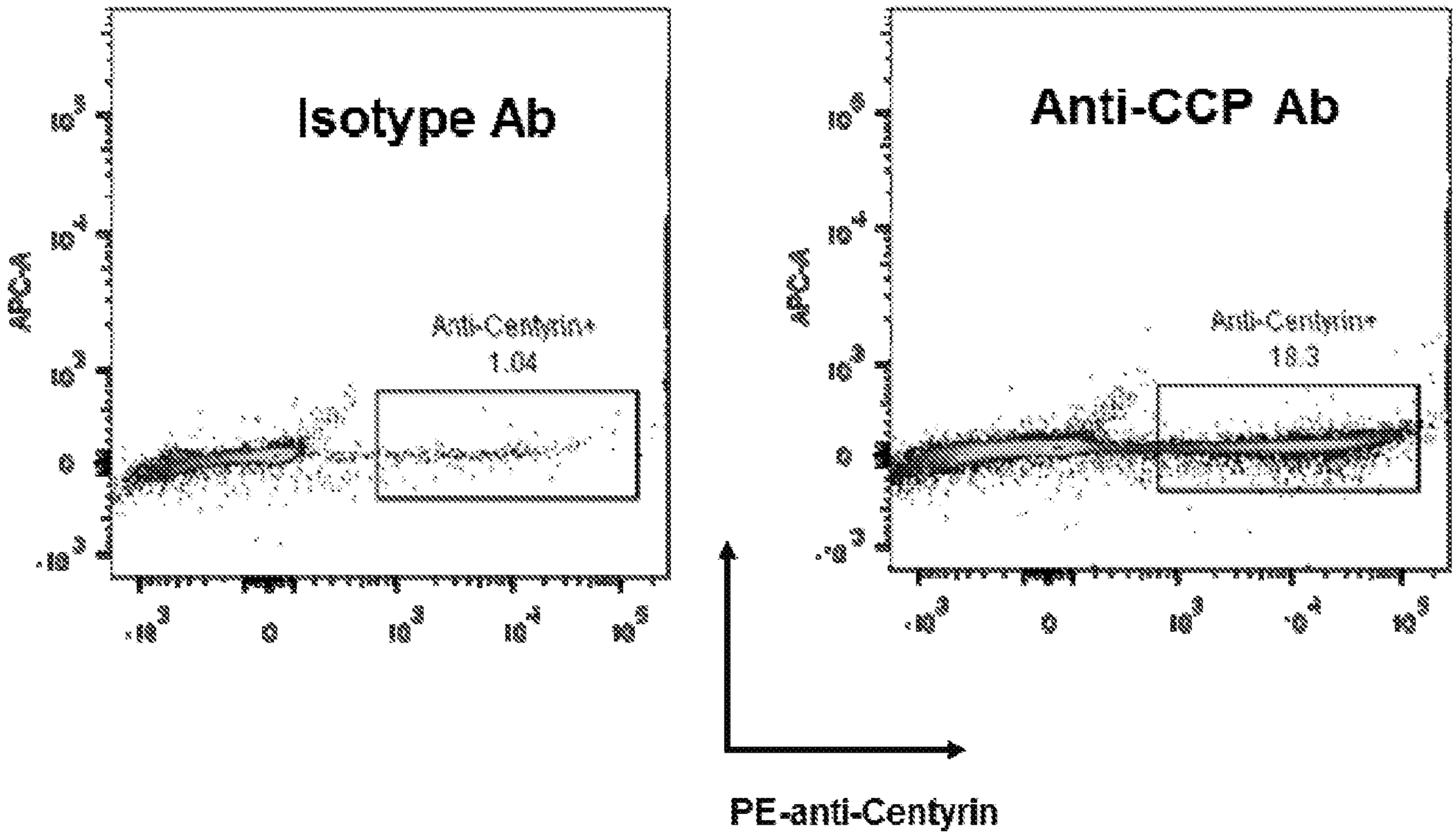

Isotype Ab
Anti-Centyrin+
1.04
APC-A
Anti-CCP Ab
Anti-Centyrin+
18.3
APC-A
PE-anti-Centyrin

Figure 13

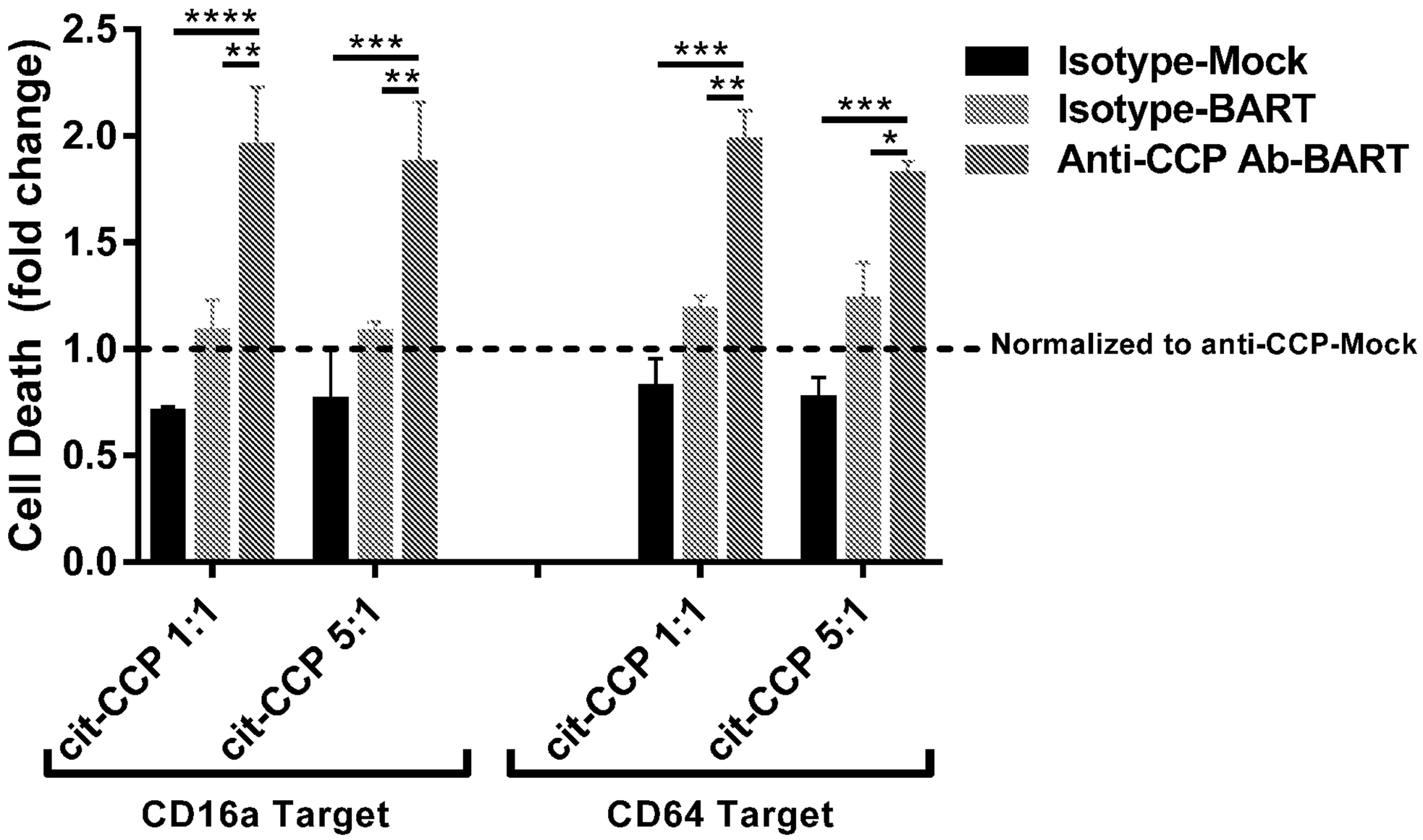

Cell Death (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
****
**
***
**
***
**
***
*
Isotype-Mock
Isotype-BART
Anti-CCP Ab-BART
Normalized to anti-CCP-Mock
cit-CCP 1:1
cit-CCP 5:1
cit-CCP 1:1
cit-CCP 5:1
CD16a Target
CD64 Target

Figure 14A

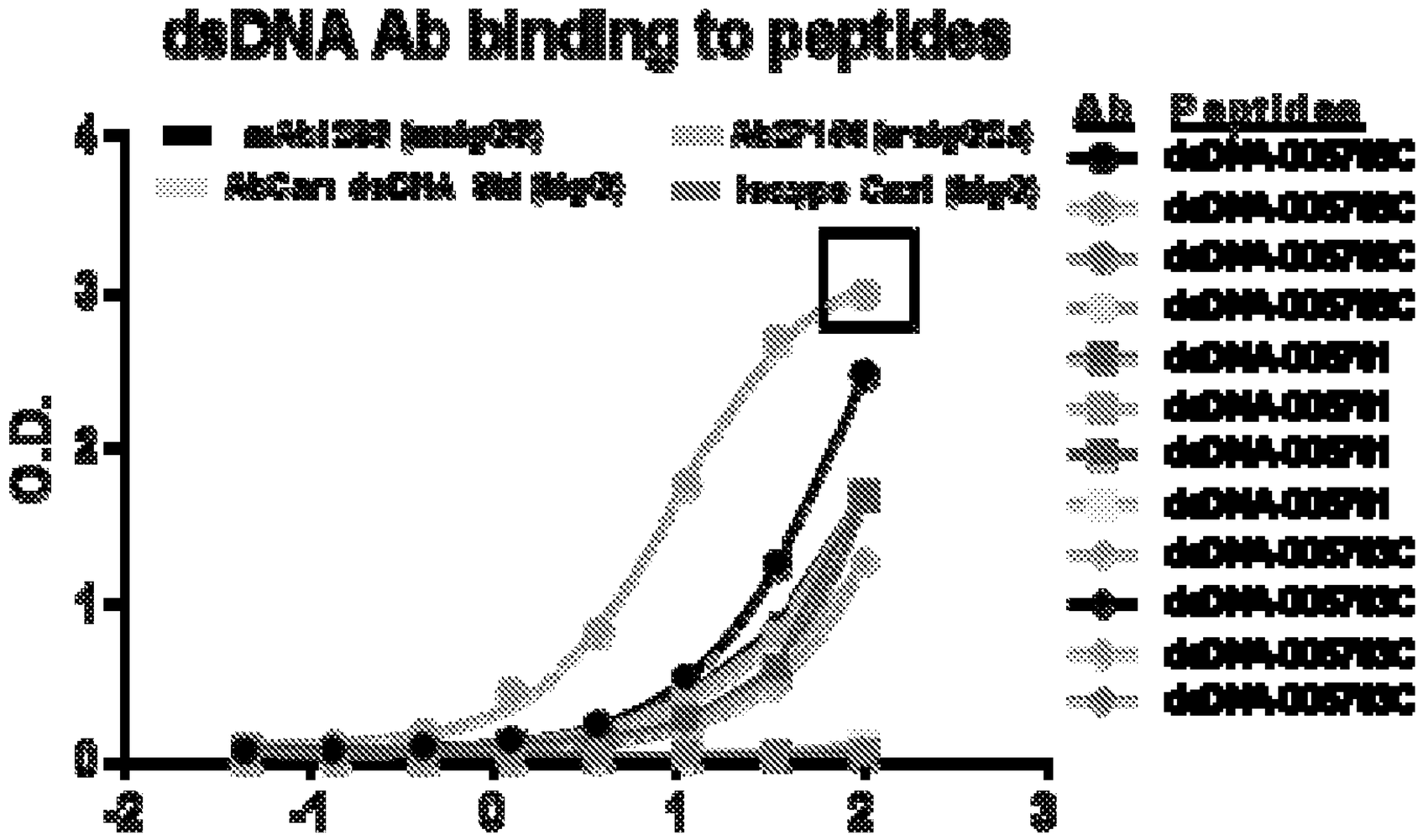

dsDNA Ab binding to peptides
O.D.
4
3
2
1
0
-2
-1
0
1
2
3
ug/mL Ab
Ab
Peptides
dsDNA-005765C
dsDNA-005765C
dsDNA-005765C
dsDNA-005765C
dsDNA-005PH
dsDNA-005PH
dsDNA-005PH
dsDNA-005PH
dsDNA-005765C
dsDNA-005765C
dsDNA-005765C
dsDNA-005765C
dsDNA-005765C = Asp-Trp-Glu-Tyr-Ser-Val-Trp-Leu-Ser
dsDNA-005PH = Ala-Ser-Pro-Val-Thr-Ala-Arg-Val-Leu
dsDNA-005PH = Arg-Leu-Thr-Ser-Ser-Leu-Arg-Tyr-Asn

Figure 14B

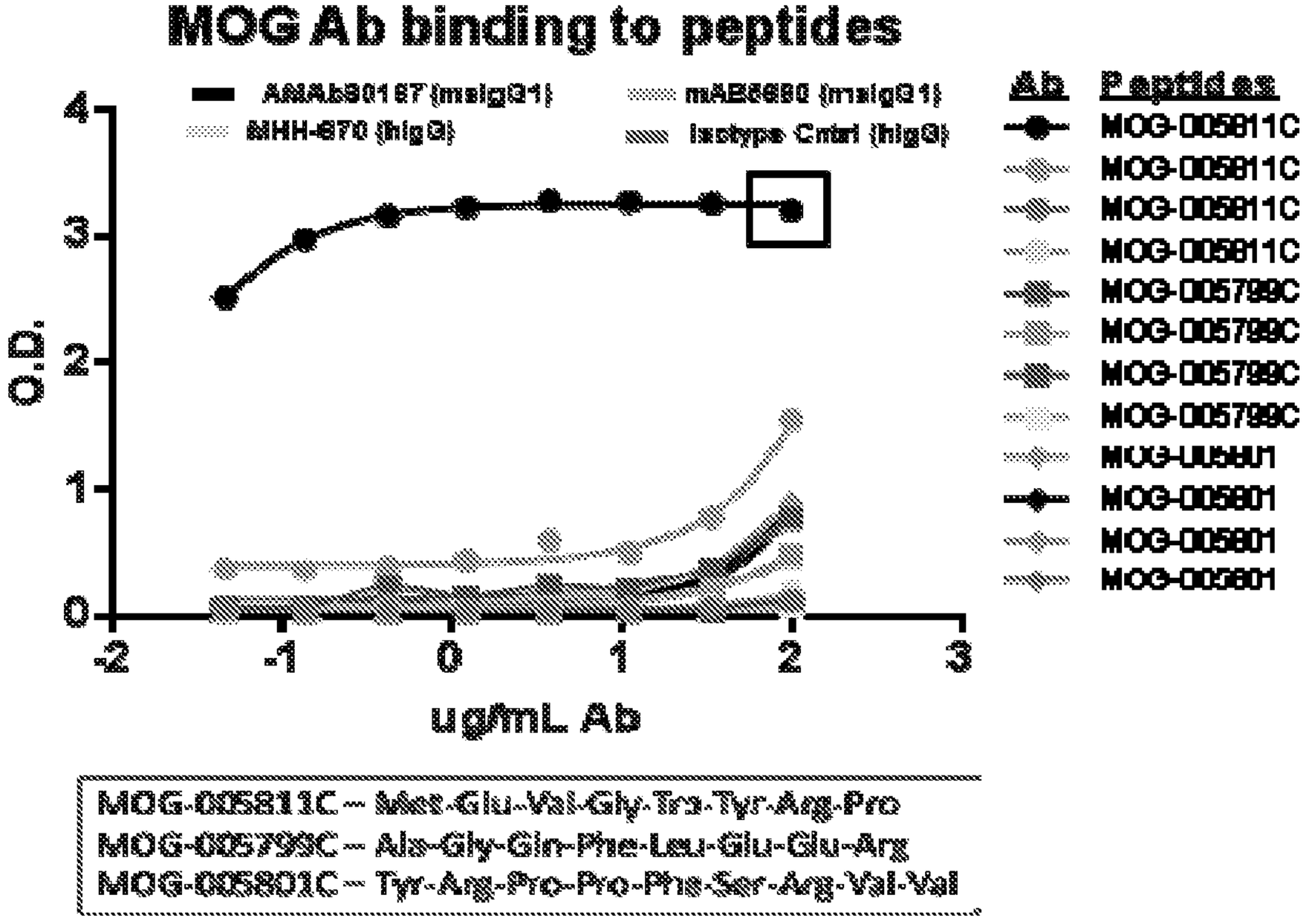

MOG Ab binding to peptides
AMAb90167 (mIgG1)
mAB6680 (mIgG1)
MHH-670 (hIgG)
Isotype Cntrl (hIgG)
O.D.
0
1
2
3
4
-2
-1
0
1
2
3
ug/mL Ab
Ab
Peptides
MOG-005811C
MOG-005811C
MOG-005811C
MOG-005811C
MOG-005799C
MOG-005799C
MOG-005799C
MOG-005799C
MOG-005801
MOG-005801
MOG-005801
MOG-005811C – Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro
MOG-005799C – Ala-Gly-Gln-Phe-Leu-Glu-Glu-Arg
MOG-005801C – Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val

Figure 14C

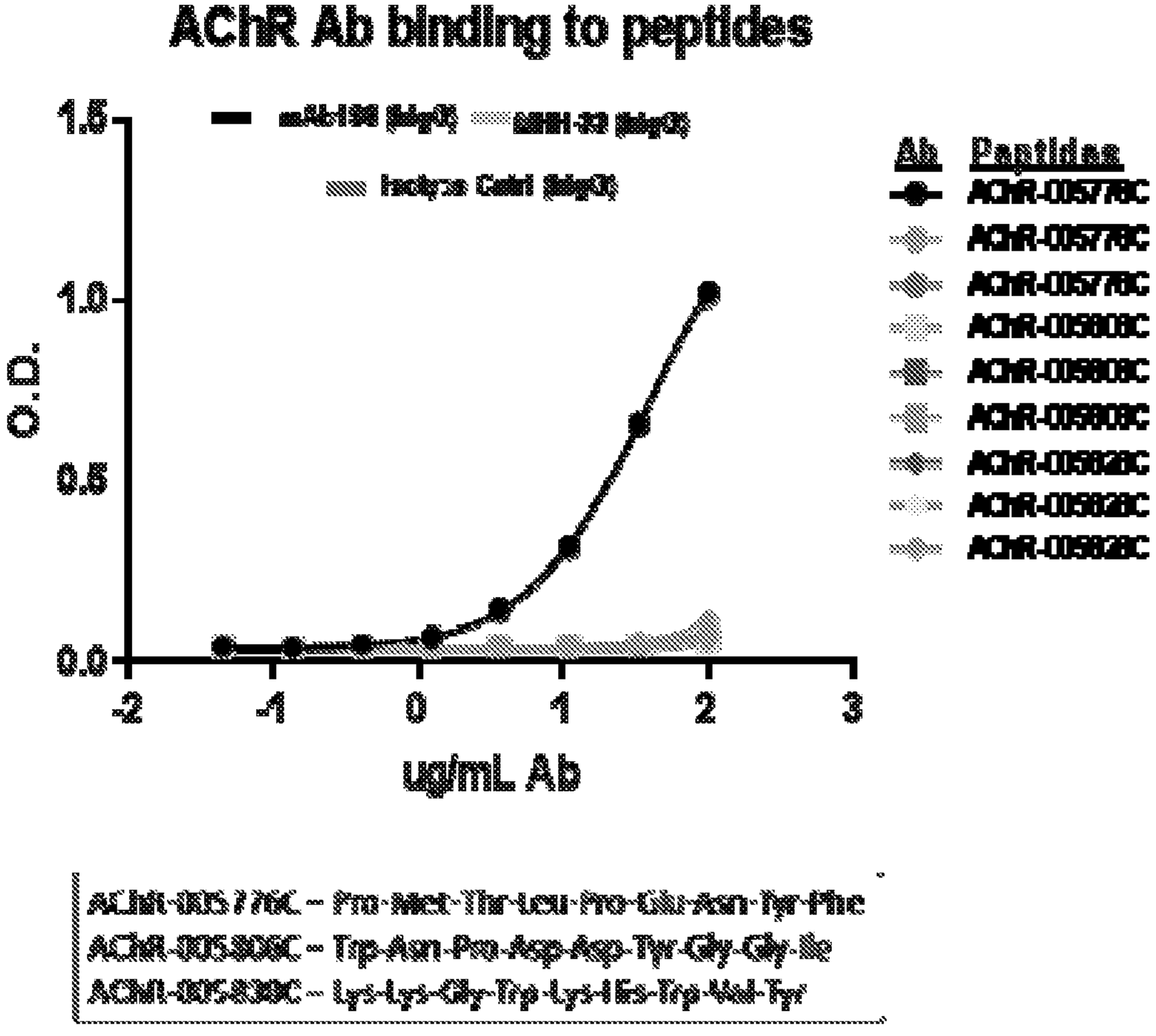
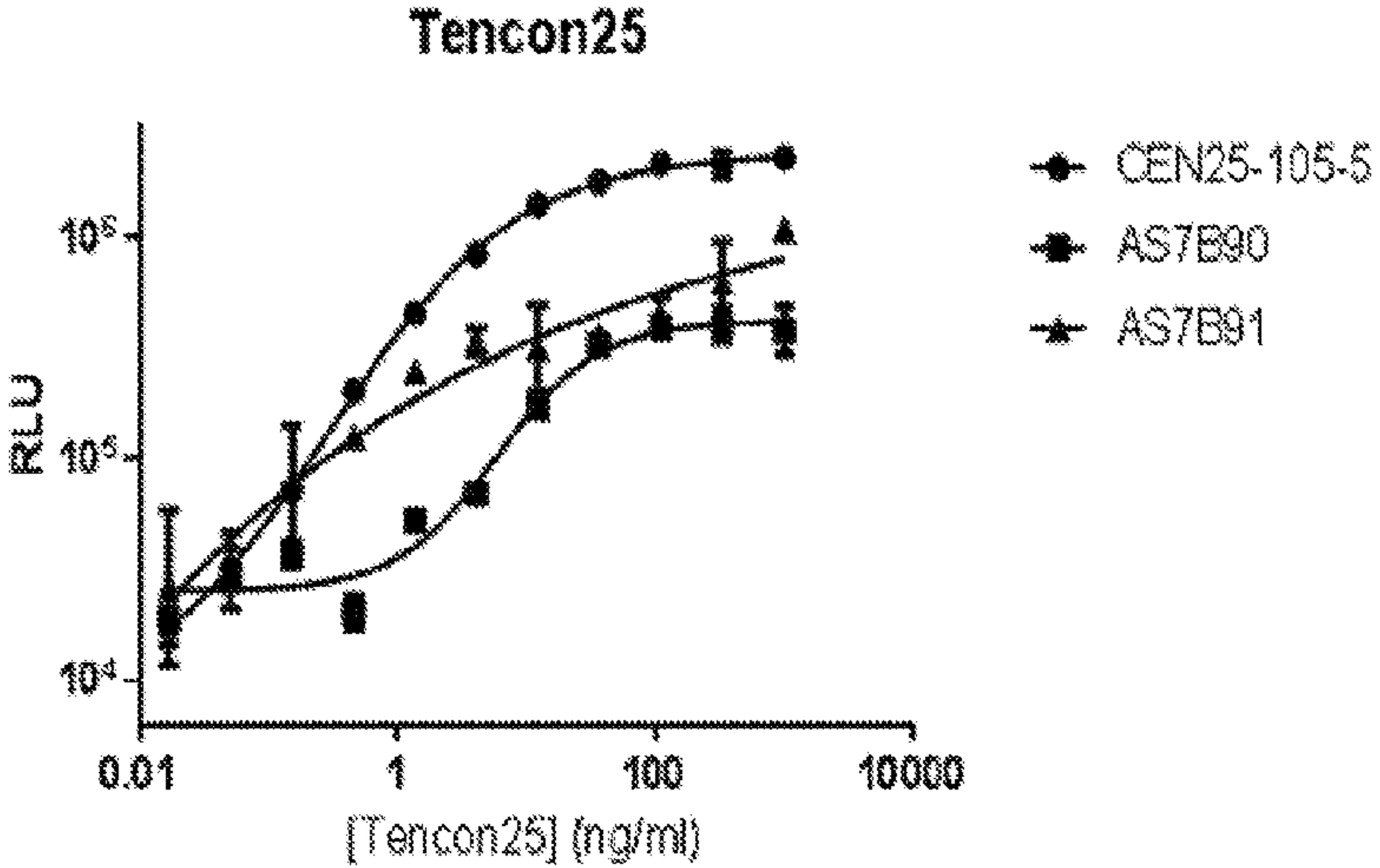

ACHR Ab binding to peptides
1.5
1.0
0.5
0.0
O.D.
-2
-1
0
1
2
3
ug/mL Ab
Ab
Peptides

Figure 1A



	CEN25-105-5	AS7B90	AS7B91
EC50	0.2896	5.94	~ 3.553e-015