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(54) Titre : ANTICORPS ANTI-MUC16 ET LEURS PROCEDES D'UTILISATION
(54) Title: ANTIBODIES TO MUC16 AND METHODS OF USE THEREOF

Peptide 1 near Cleavage Site:
NFSPLARRVDRVAIYEE (SEQ ID NO:01)

Peptide 2 before Transmembrane:
TLDRSSVLVDGYSPNRNE (SEQ ID NO:02)

Peptide 3 inside Transmembrane:
CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03)

(57) **Abrégé/Abstract:**

The invention provides antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications for diseases in which MUC16 is overexpressed, such as cancer.

ABSTRACT

The invention provides antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications for diseases in which MUC16 is overexpressed, such as cancer.

ANTIBODIES TO MUC16 AND METHODS OF USE THEREOF

This invention was made with United States government support under PO1-CA52477-16 awarded by the United States Public Health Service (US PHS). The United States government has certain rights in this invention.

FIELD OF THE INVENTION

10 The invention relates to antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications
15 for diseases in which MUC16 is overexpressed, such as cancer.

BACKGROUND OF THE INVENTION

Cell surface markers and shed antigens are used in the diagnosis of several cancers. For example, the CA125 antigen, recognized by the OC125 antibody, is a tissue-specific, circulating
20 antigen expressed in ovarian cancer. The CA125 antigen is encoded by the MUC16 gene, cloned by Lloyd and Yin. The full-length gene describes a complex tethered mucin protein present primarily in a variety of gynecologic tissues, especially neoplasms. OC125 and other related antibodies react with glycosylation-dependent antigens present exclusively in the cleaved portion of the molecule.

25 A serum assay can detect elevated levels of the circulating CA125 antigen in many epithelial ovarian cancer patients, and this antigen, derived using the ovarian cell line OVCA433, is recognized by the OC125 antibody (1-2). The detection of circulating CA125 in serum has proven to be a useful tool for the management of ovarian cancer patients and clinical trials (3-4). However, CA125 is neither sufficiently sensitive nor specific for general cancer screening (5-6). A
30 variety of CA125 linked antibodies including VK8 and M11 have subsequently been defined as present on ovarian cancer cells (7-9). Although these antibodies have been used to develop serum assays and various other studies in ovarian cancer, they have significant shortcomings for clinical use in screening or tissue delivery. These antibodies are not useful as screening tools, nor can they

detect the proximal residual MUC16 protein fragment after cleavage. This has limited their diagnostic and therapeutic applications.

For example, OC125, M11, and most other antibodies prepared against ovarian cancer cell extracts are directed at complex, glycosylation-dependent antigens. These antigens are exclusively present in the shed portion of MUC16 and cannot be employed to follow the biology of the proximal portion of MUC16 and may not accurately reflect tissue distribution since the glycosylation patterns can vary substantially among tissues. Because the vast majority of MUC16-reactive antibodies, including OC125, react with the glycosylation-dependent antigen present exclusively in the cleaved portion of the molecule, the true distribution of MUC16 expression is not known (21). There is currently no antibody available to track the fate of the remaining MUC16 protein fragment after cleavage and CA125 release.

Thus, there remains a need for the identification of antibodies that are directed against sequences in the peptide backbone of MUC16, and that are useful for diagnosis and treatment of cancers in which MUC16 is expressed and/or overexpressed.

SUMMARY OF THE INVENTION

The invention provides an antibody, or an antigen-binding fragment thereof, that specifically binds to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from the group of a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSL (SEQ ID NO:19). In one embodiment, the antibody internalizes into a cell. While not intending to limit the invention to a particular sequence of MUC 16 ectodomain, in one embodiment, the MUC16 ectodomain polypeptide comprises a polypeptide selected from the group of Polypeptide 1 NFSPLARRVDRVAIYEE (SEQ ID NO:01) and Polypeptide 2 TLDRSSVLVDGYSPNRNE (SEQ ID NO:02). In another embodiment, the antibody lacks specific binding to a glycosylated MUC16 extracellular domain. In yet a further embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID NO:02) of the MUC16 ectodomain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:06, and a variable light (V_L) chain encoded by SEQ ID NO:07. In yet another alternative embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID NO:02) of the MUC16 ectodomain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:04, and a variable light (V_L) chain encoded by SEQ ID NO:05. In a further embodiment, the antibody specifically binds to the Polypeptide 1 (SEQ ID NO:01) of the MUC16 ectodomain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain

encoded by SEQ ID NO:08, and a variable light (V_L) chain encoded by at least one of SEQ ID NO:09 and SEQ ID NO:10. In one embodiment, the MUC16 cytoplasmic domain polypeptide comprises VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18). More preferably, but without limitation, the MUC16 cytoplasmic domain polypeptide comprises Polypeptide 3

5 CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03). In an alternative embodiment, the MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide comprises CQVSTFRSVPNRHHTGVDSL (SEQ ID NO:19). More preferably, but without limitation, the MUC16 extracellular domain polypeptide comprises Polypeptide 4 KSYF SDCQVSTFRS VPNRHHTGVD SLCNFSPL (SEQ ID NO:15). In yet another alternative embodiment, the

10 antibody specifically binds to the Polypeptide 4 (SEQ ID NO:15) of the MUC16 extracellular domain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:11, and a variable light (V_L) chain encoded by SEQ ID NO:12. In a further alternative embodiment, the antibody is selected from the group of a chimeric antibody, a monoclonal antibody, a recombinant antibody, an antigen-binding fragment of a recombinant antibody, a

15 humanized antibody, and an antibody displayed upon the surface of a phage. In another embodiment, the antigen-binding fragment is selected from the group of a Fab fragment, a $F(ab')_2$ fragment, and a Fv fragment. In an alternative embodiment, the antibody, or antigen-binding fragment thereof, is covalently linked to a cytotoxic agent or a prodrug of a cytotoxic agent. In a preferred embodiment, the antibody is a monoclonal antibody produced by a hybridoma cell line.

20 The invention also provides an isolated monoclonal antibody, or an antigen-binding fragment thereof, produced by a hybridoma cell line, wherein the antibody specifically binds to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from the group of a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide

25 CQVSTFRSVPNRHHTGVDSL (SEQ ID NO:19). In one embodiment, the MUC16 ectodomain polypeptide comprises Polypeptide 1 (SEQ ID NO:01) and the antibody is selected from the group of 9B11.20.16, 10A2, 2F4, 23D3, 30B1, and 31B2. In an alternative embodiment, the MUC16 ectodomain polypeptide comprises Polypeptide 2 (SEQ ID NO:02), and wherein the antibody is selected from the group of 4H11.2.5, 13H1, 29G9, 9C9.21.5.13, 28F8, 23G12, 9C7.6, 11B6, 25G4,

30 5C2.17, 4C7, 26B2, 4A5.37, 4A2, 25H3, and 28F7.18.10. In yet a further embodiment, the MUC16 cytoplasmic domain polypeptide comprises Polypeptide 3 CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03), and wherein the antibody is selected from the group of 31A3.5.1, 19D1, 10F6, 22E10, 22F1, 3H8, 22F11, 4D7, 24G12, 19G4, 9A5, 4C2, 31C8, 27G4, and 6H2. In another alternative embodiment, the MUC16 extracellular domain

polypeptide comprises Polypeptide 4 KSYF SDCQVSTFRS VPNRHHTGVD SLCNFSPL (SEQ ID NO:15), and wherein the antibody is selected from the group of 24B3 and 9C7.

The invention additionally provides a composition comprising (a) any one or more of the antibodies, or antigen-binding fragments thereof, that are described herein, and (b) a
5 pharmaceutically acceptable carrier.

Also provided by the invention is a hybridoma cell line that produces a monoclonal antibody that specifically binds to a polypeptide, or antigenic portion thereof, selected from the group of a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide
10 CQVSTFRSVPNRHHTGVDSL (SEQ ID NO:19).

The invention additionally provides a method for detecting a disease that comprises overexpression of MUC16 in a subject, comprising a) providing i) a sample from a subject, and ii) any one or more of the antibodies, or antigen-binding fragments thereof, that are described herein, b) contacting the sample with the antibody under conditions for specific binding of the antibody
15 with its antigen, and c) detecting an increased level of binding of the antibody to the sample compared to a control sample lacking the disease, thereby detecting the disease in the subject. In one embodiment, the disease is cancer. In a preferred embodiment, the cancer is selected from the group of ovarian cancer and breast cancer. While not intending to limit the method of detection, in one embodiment, detecting binding of the antibody to the sample is immunohistochemical,
20 enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, immunoprecipitation, and/or radiographic imaging.

Also provided herein is a method for treating a disease that comprises overexpression of MUC16, comprising administering to a subject having the disease a therapeutically effective amount of any one or more of the antibodies, or antigen-binding fragments thereof, that are
25 described herein. In one embodiment, the disease is cancer, as exemplified by ovarian cancer and breast cancer.

The invention also provides an isolated antibody, or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is selected from the group of a) TLDRKSVFVDGYSQNRDD (SEQ ID
30 NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQNRDD, and f) TLDRSSVLVDGYSQNRDD. In one embodiment, the antibody is selected from the group of a monoclonal antibody, a chimeric antibody, a recombinant antibody, an antigen-binding fragment of a recombinant antibody, a humanized antibody, and an

antibody displayed upon the surface of a phage. In a preferred embodiment, the antibody is a monoclonal antibody produced by hybridoma cells selected from the group of 12B10-3G10, 10C4-3H5, 10C4-1F2, 10C4-2H8, 10C4-1G7, 17F2-3G5, 17F2-3F6, 17F2-2F9, 17F2-1E11, 12B10-3F7, 12B10-2F6, 12B10-2F10, 25E9-3, 25E9-5, 25E9-1, 25E9-16, 21B8-1H11, 21B8-3G6, 21B8-3H9, 21B8-1G8, 21E1-1E3, 21E1-1G9, 21E1-2G7, 21E1-3G12, 4H1-2E1, 4H1-2E3, 4H1-3E1, 4H1-3H3, 15A8-2E2, 15A8-2E10, 15A8-2E11, 15A8-3D2, 22B5-1F6, 22B5-3G9, 22B5-2G8, and 22B5-3F11. In a particular embodiment, the MUC16 polypeptide is TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), and the antibody comprises a variable heavy (V_H) chain sequence SEQ ID NO:27, and a variable light (V_L) chain sequence SEQ ID NO:29, such as the monoclonal antibody produced by hybridoma cell 12B10-3G10. In an alternative embodiment, the antigen-binding fragment is selected from the group of a Fab fragment, a $F(ab')_2$ fragment, and a Fv fragment. In a more preferred embodiment, the antibody, or antigen-binding fragment thereof, is covalently linked to a cytotoxic agent and/or to a prodrug of a cytotoxic agent. In a further embodiment, the antibody specifically binds to human MUC16 (SEQ ID NO:25). In another embodiment, the antibody internalizes into a cell. In an alternative embodiment, the antibody lacks specific binding to a glycosylated MUC16 extracellular domain.

The invention also provides a composition comprising (a) any one or more of the invention's antibodies and/or antigen-binding fragments thereof, and (b) a pharmaceutically acceptable carrier.

The invention further provides a hybridoma cell that produces an antibody, or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is selected from the group of a) TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSL CNFSPL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQNRDD, and f) TLDRSSVLVDGYSQNRDD.

The invention also provides an isolated nucleotide sequence comprising a polynucleotide that encodes at least one of a variable heavy (V_H) chain sequence and the variable light (V_L) chain sequence of an antibody that specifically binds to a MUC16 polypeptide, wherein the MUC16 polypeptide is selected from the group of a) TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSL CNFSPL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQNRDD, and f) TLDRSSVLVDGYSQNRDD. In one embodiment, the MUC16 polypeptide is TLDRKSVFVDGYSQNRDD (SEQ ID NO:21) and the polynucleotide

encoding the variable heavy (V_H) chain sequence comprises SEQ ID NO:26, and wherein the polynucleotide encoding the variable light (V_L) chain sequence comprises SEQ ID NO:28.

The invention also provides a method for producing an antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, comprising administering to a subject an immunologically effective amount of a MUC16 polypeptide selected from the group of a) TLDRKSVFVDGYSQLNRDD (SEQ ID NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSL CNFSPL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQLNRDD, and f) TLDRSSVLVDGYSQLNRDD.

The invention additionally provides a method for identifying a subject as having disease, comprising determining the level, in a sample from the subject, of specific binding of any one or more of the invention's antibodies and/or antigen-binding fragments thereof, with the MUC16 polypeptide or with the antigenic portion thereof, wherein detecting an altered level of the specific binding relative to a control sample identifies the subject as having disease. In one embodiment, the disease is cancer exemplified by ovarian cancer and breast cancer. In another embodiment, the method further comprises detecting an altered level of binding of the antibody to the sample compared to a control sample. Optionally, the detecting is selected from the group of immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, immunoprecipitation, and radiographic imaging.

The invention also provides a method for reducing one or more symptoms of disease comprising administering to a subject in need thereof a therapeutically effective amount of any one or more of the invention's antibodies and/or antigen-binding fragment thereof. In one embodiment, the disease is cancer, exemplified by ovarian cancer and breast cancer. Optionally, the method further comprises detecting a reduction in one or more symptoms of the disease after the administration step.

Various aspects of the disclosure relate to an isolated antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is: TLDRSSVLVDGYSPNRNE (SEQ ID NO:2) wherein the antibody comprises a variable heavy (" V_H ") chain encoded by SEQ ID NO:06 and a variable light (" V_L ") chain encoded by SEQ ID NO:07.

Various aspects of the disclosure relate to an isolated antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is: TLDRSSVLVDGYSPNRNE (SEQ ID NO:2) wherein the antibody comprises a V_H chain encoded by SEQ ID NO:04 and a V_L chain encoded by SEQ ID NO:05.

Various aspects of the disclosure relate to a humanized antibody or antigen-binding fragment thereof made by substituting the complementarity determining regions of the antibody as described herein into a human framework domain, wherein the humanized antibody or antigen-binding fragment thereof specifically binds to the MUC16 polypeptide of SEQ ID NO:02 or to an antigenic portion thereof.

Various aspects of the disclosure relate to a humanized antibody or antigen-binding fragment thereof made by substituting the complementarity determining regions of the antibody as described herein into a human framework domain, wherein the humanized antibody or antigen-binding fragment thereof specifically binds to the MUC16 polypeptide of SEQ ID NO:02 or to an antigenic portion thereof.

Various aspects of the disclosure relate to a composition comprising (a) an antibody, or antigen-binding fragment thereof, as described herein, and (b) a pharmaceutically acceptable carrier.

Various aspects of the disclosure relate to a hybridoma cell that produces an antibody as described herein.

Various aspects of the disclosure relate to the use of the antibody as described herein, for identifying a subject as having a cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to an *ex vivo* method for identifying a subject as having a cancer in which MUC16 is expressed, wherein said method comprises (a) contacting a sample obtained from the subject with the antibody as described herein; and (b) determining whether the antibody has an increased level of binding to the sample as compared to a control sample lacking the cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to a method for indicating a subject as having disease, comprising determining the level, in a sample from the subject, of specific binding of an antibody of the present invention with a MUC16 polypeptide or with an antigenic portion thereof,

wherein detecting an altered level of the specific binding relative to a control sample indicates the subject as having disease.

Various aspects of the disclosure relate to a single chain variable fragment (scFv) comprising a V_H chain sequence encoded by SEQ ID NO:06 and a V_L chain sequence encoded by SEQ ID NO:07.

Various aspects of the disclosure relate to a chimeric antigen receptor (CAR) comprising the scFv as described herein.

Various aspects of the disclosure relate to a T cell expressing the chimeric antigen receptor (CAR) as described herein.

Various aspects of the disclosure relate to the use of the antibody, or antigen-binding fragment thereof, as described herein, for treating a cancer in a subject, wherein the cancer expresses MUC16.

Various aspects of the disclosure relate to a scFv comprising a V_H chain and a V_L chain, wherein the V_H chain and the V_L chain are of a humanized antibody or antigen-binding fragment thereof, wherein the humanized antibody or antigen-binding fragment thereof is made by substituting the complementarity determining regions of an antibody comprising a V_H chain encoded by SEQ ID NO:04 and a V_L chain encoded by SEQ ID NO:05 into a human framework domain, wherein the humanized antibody or antigen-binding fragment thereof specifically binds to the MUC16 polypeptide of SEQ ID NO:02 or to an antigenic portion thereof.

Various aspects of the disclosure relate to a CAR comprising the scFv as claimed.

Various aspects of the disclosure relate to a CAR comprising the scFv as claimed fused to a transmembrane domain fused to a T cell receptor ζ chain cytoplasmic signaling domain.

Various aspects of the disclosure relate to an isolated antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is: CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

Various aspects of the disclosure relate to a composition comprising (a) an antibody, or antigen-binding fragment thereof, as disclosed herein, and (b) a pharmaceutically acceptable carrier.

Various aspects of the disclosure relate to a hybridoma cell that produces an antibody as disclosed herein.

Various aspects of the disclosure relate to a use of the antibody or antigen-binding fragment thereof, as disclosed herein, for identifying a subject as having a cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to an *ex vivo* method for identifying a subject as having a cancer in which MUC16 is expressed, wherein said method comprises: (a) contacting a sample obtained from the subject with the antibody as disclosed herein; and (b) determining whether the antibody has an increased level of binding to the sample as compared to a control sample lacking the cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to an isolated polynucleotide comprising a nucleotide sequence that encodes at least one of a variable heavy (VH) chain sequence and the variable light (VL) chain sequence of an antibody that specifically binds to a MUC16 polypeptide, wherein the MUC16 polypeptide is: CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

Various aspects of the disclosure relate to a use of an antibody or antigen-binding fragment thereof, as disclosed herein, for reducing one or more symptoms of a cancer.

Various aspects of the disclosure relate to a use of an antibody or antigen-binding fragment thereof, as disclosed herein, for formulating a medicament for reducing one or more symptoms of a cancer.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: Three MUC16 carboxy terminus peptides were synthesized at the MSKCC Microchemistry Core Facility. Polypeptide 1 is near the putative cleavage site, Polypeptide 2 is before the transmembrane, and Polypeptide 3 is the internal peptide, which is inside the transmembrane.

Figure 2: Comparison staining of high-grade serous ovarian carcinomas using OC125 (left panel) and 4H11 (right panel).

Figure 3: Immunohistochemical scoring of OC125 and 4H11 on tissue microarrays of high-grade ovarian serous carcinoma. Only membranous and/or cytoplasmic staining was considered positive. Score 0: No staining; Score 1: <5% strong or weak; Score 2: 5-50% strong or weak; Score 3: 51-75% strong or 51-100% weak; Score 4: 76-99% strong; Score 5: 100% strong. Figure 3A: OC125 (Score 0); Figure 3B: OC125 (Score 1); Figure 3C: OC125 (Score 2); Figure 3D: OC125 (Score 3); Figure 3E: OC125 (Score 4); Figure 3F: OC125 (Score 5); Figure 3G: 4H11 (Score 0); Figure 3H: 4H11 (Score 1); Figure 3I: 4H11 (Score 2); Figure 3J: 4H11 (Score 3); Figure 3K: 4H11 (Score 4); Figure 3L: 4H11 (Score 5).

Figure 4: Western blot analysis. Figure 4A: Western blot analysis of GST- Δ MUC16^{c114} fusion protein with monoclonal antibodies 9C9.21.5.13 and 4H11.2.5. Figure 4B: Western blot analysis of SKOV3-phrGFP- Δ MUC16^{c114} and SKOV3-phrGFP- Δ MUC16^{c334} protein extract and probed with monoclonal antibodies 9C9.21.5.13 and 4H11.2.5.

Figure 5A: MUC16 carboxy terminus monoclonal antibodies binding affinity on OVCAR3 cells (Panels A-D). Figure 5B: Internalization of radio-labeled 4H11 and OC125 monoclonal antibodies on SKOV3-phrGFP- Δ MUC16^{c334} stable transfected cells.

Figure 6A-D: Comparison staining intensities of OC125 and 4H11 monoclonal antibodies on tissue microarrays containing cancers of the prostate (2A, concordant), lung (2B, discordant), breast (2C, discordant), and pancreas (2D, discordant).

Figure 7: FACS analysis as described in the Material and Methods section was performed with commercial antibodies and MUC16 carboxy terminus monoclonal antibodies on OVCAR3 wt, SKOV3-phrGFP- Δ MUC16^{c114} and SKOV3-phrGFP- Δ MUC16^{c334} stable transfected cell lines.

Figure 8: Nucleotide sequence encoding antibody variable heavy (V_H) chain and antibody variable light (V_L) chain. (A) 4A5 V_H (SEQ ID NO:04), (B) 4A5 V_L (SEQ ID NO:05), (C) 4H11 V_H (SEQ ID NO:06), (D) 4H11 V_L (SEQ ID NO:07), (E) 9B11 V_H (SEQ ID NO:08), (F) 9B11 V_{LA} (SEQ ID NO:09), (G) 9B11 V_{LB} (SEQ ID NO:10), (H) 24B3 V_H (SEQ ID NO:11), (I) 24B3 V_L (SEQ ID NO:12).

Figure 9: (A) Homo sapiens MUC16 (GenBank NP_078966) (SEQ ID NO:13), (B) Polypeptide 1 (SEQ ID NO:01), (C) Polypeptide 2 (SEQ ID NO:02), (D) Polypeptide 3 (SEQ ID NO:03), (E) Transmembrane domain (SEQ ID NO:14), (F) Polypeptide 4 (SEQ ID NO:15) containing a cysteine loop polypeptide (SEQ ID NO:19).

Figure 10: Schematic of MUC16 structure.

Figure 11. Design and *in vitro* analysis of MUC-CD targeted CARs. (A) Schematic diagram of the first generation 4H11z and second generation 4H11-28z retroviral vectors. 4H11scFv: MUC16 specific scFv derived from the heavy (V_H) and light (V_L) chain variable regions

of the monoclonal antibody 4H11; CD8: CD8 hinge and transmembrane domains; CD28: CD28 transmembrane and cytoplasmic signaling domains; ζ chain: T cell receptor ζ chain cytoplasmic signaling domain; LTR: long terminal repeat; black box: CD8 leader sequence; grey box:

(Gly₄Ser)₃ linker; arrows indicate start of transcription. (B) FACS analysis of human T cells

- 5 retrovirally transduced to express either the 4H11z or 19z1 CAR. (C) 4H11z⁺ but not 19z1⁺ T cells expand on 3T3(MUC-CD/B7.1) AAPC. CAR⁺ were co-cultured on 3T3(MUC-CD/B7.1) AAPC monolayers at 3×10^6 CAR⁺ T cells/well of a 6 well plate. Proliferation of CAR⁺ T cells, normalized to the CAR⁺ T cell fraction as assessed by FACS for the CAR⁺ fraction in combination with viable T cell counts obtained on days 2, 4 and 7, as assessed by trypan blue exclusion assays.

- 10 Figure 12. *In vitro* comparison of T cells modified to express the first generation 4H11z CAR to T cells modified to express the second generation co-stimulatory 4H11-28z CAR. (A) CAR⁺ T cells were co-cultured on MUC-CD monolayers with (right panel) or without B7.1 (left panel). 3×10^6 CAR⁺ T cells were co-cultured on AAPC monolayers in 6 well tissue culture plates in cytokine-free medium. Total viable T cell counts were assessed on days 2, 4 and 7, by trypan
- 15 blue exclusion assays. 4H11-28z⁺ T cells markedly expanded when compared to 4H11z⁺ T cells upon co-culture with 3T3(MUC-CD) AAPCs, **p=0.0023 (4H11z compared to 4H11-28z). In contrast, both 4H11z⁺ and 4H11-28z⁺ T cells expanded similarly on 3T3(MUC-CD/B7.1) AAPCs, p=0.09, (4H11z compared to 4H11-28z). Control 19-28z⁺ T cells did not proliferate on 3T3(MUC-CD), **p=0.0056 (19-28z compared to 4H11z), **p=0.0011 (19-28z compared to 4H11-28z), or on
- 20 3T3(MUC-CD/B7.1), **p=0.0026 (19-28z compared to 4H11z), **p=0.0087 (19-28z compared to 4H11-28z). (B) 4H11-28z⁺ but not 4H11z⁺ T cells secrete IL-2 upon co-culture with 3T3(MUC-CD) AAPCs. Tissue culture supernatants at day 2 following activation on 3T3(MUC-CD) AAPCs were analyzed for cytokine secretion. 4H11-28z⁺ T cells, in contrast to 4H11z⁺ T cells, demonstrated enhanced secretion of IL-2 consistent with T cell co-stimulation mediated through the
- 25 4H11-28z CAR. ***p=0.0008 (19z1 or 19-28z compared to 4H11z), **p=0.0026 (19z1 or 19-28z compared to 4H11-28z), **p=0.0046 (4H11z compared to 4H11-28z). Furthermore, both 4H11-28z⁺ and 4H11z⁺ T cells secreted IFN γ . *p=0.011 (4H11z compared to 4H11-28z). Control 19z1 and 1928z transduced T cells failed to secrete either IL-2 or IFN γ . **p=0.0034 (19z1 compared to 4H11z), **p=0.036 (19-28z compared to 4H11z), ***p=0.0008 (19-28z compared to 4H11-28z).
- 30 (C) Expansion of CAR⁺ T cells following 3 cycles of stimulation on 3T3(MUC-CD/B7.1). Human T cells transduced to express either 4H11z or 4H11-28z CARs demonstrated a >2 log expansion over 2 cycles of stimulation on 3T3(MUC-CD/B7.1) AAPCs. Arrows indicate 1st and 2nd cycles of restimulation on AAPCs. (D) FACS analysis of the CAR⁺ T cell fraction of 4H11-28z⁺ T cells increased following each weekly cycle of stimulation. (I) FACS following initial transduction, (II)

FACS at 7 days following first stimulation on AAPCs, (III) FACS at 7 days following second stimulation on AAPCs. These data are representative of one of three different experiments using three different healthy donor T cell populations, all of which demonstrated similar proliferation and cytokine secretion patterns.

5 Figure 13. MUC-CD targeted T cells specifically expand and lyse MUC-CD⁺ tumor cells. (A) Cytotoxicity assay of 4H11z⁺ and 4H11-28z⁺ T cells targeting OV-CAR(MUC-CD) tumor cells demonstrates efficient cytotoxicity mediated by T cells from healthy donors modified to express the first and second generation MUC-CD targeted CARs. Control T cells modified to express the first and second generation CD19-targeted 19z1 and 19-28z CARs failed to demonstrate significant lysis
10 of target tumor cells. (B) Healthy donor T cells modified to express the 4H11-28z CAR equally lyse primary patient ascites-derived MUC-CD⁺ tumor cells when compared to T cells modified to express the control 19-28z CAR. This data represents 1 or 3 experiments targeting primary tumor cells from 3 ovarian carcinoma patients with similar results. (C) Autologous T cells isolated from peripheral blood, when modified with the 4H11-28z CAR, exhibit significant lysis of autologous
15 MUC-CD⁺ ascites-derived tumor cells when compared to control 19-28z⁺ autologous T cells. These data represent 1 of 3 experiments utilizing T cells and autologous tumor cells from 3 different ovarian carcinoma patients with similar results. (D) Antigen specific proliferation of MUC-CD targeted CFSE labeled T cells after co-culture with OV-CAR3(MUC-CD) tumor cells. CFSE labeled CAR⁺ T cells were co-cultured with MUC-CD expressing OV-CAR3 tumor cells at
20 1:1 ratio for 5 days. Proliferation of CFSE labeled T cells was assessed by FACS demonstrating efficient proliferation of both 4H11z⁺ and 4H11-28z⁺ T cells but not control 19-28z⁺ T cells. (E) CFSE results were further confirmed by absolute T cell numbers assessed on days 2, 4 and 7 following co-culture with OV-CAR3(MUC-CD) tumor cells. (F) FACS analysis of the expression of 4-1BBL on OVCAR3(MUC-CD) cells. OV-CAR3(MUC-CD) cells were stained with anti-
25 human 4-1BBL antibody (thick line) or with isotype control (thin line). FACS analysis demonstrated expression of 4-1BBL on OV-CAR3(MUC-CD) tumor cells. Further FACS analyses failed to reveal expression of the co-stimulatory ligands B7.1, B7.2, or OX-40L.

 Figure 14. Eradication of OV-CAR3(MUC-CD) tumors after intra-peritoneal treatment with first and second generation of MUC-CD targeted T cells. (A) Intraperitoneal injection of OV-
30 CAR3(MUC-CD) tumors in untreated SCID-Beige mice results in abdominal distension and nodular peritoneal tumors. SCID-Beige mice were injected intraperitoneally with 3x10⁶ OV-CAR3(MUC-CD) cells. At 5 weeks post intraperitoneal injection of OV-CAR3(MUC-CD) tumor cells mice developed ascities as evidenced by a distended abdomen (center panel) when compared to a tumor free mouse (left panel). Post mortem visualization of the peritoneum demonstrates

nodular tumor masses (arrows) within the abdominal cavity (right panel). (B) Intraperitoneal injection of 4H11z⁺ and 4H11-28z⁺ T cells either delay tumor progression or fully eradicate disease. Kaplan-Meier survival curve of SCID-Beige mice treated with first or second generation of MUC-CD targeted T cells. SCID-Beige mice were infused ip with 3x10⁶ OV-CAR3(MUC-CD) tumor cells on day 1 followed by 3x10⁷ 4H11z⁺ or 4H11-28z⁺ T cells on day 2. All untreated mice or mice treated with control 19z1⁺ T cells developed established tumors and were sacrificed by day 50. In contrast, 27% of mice treated with either 4H11z⁺ or 4H11-28z⁺ T cells remained without clinical evidence of disease by day 120. *p=0.01 (4H11z compared to 19z1), **p=0.0023 (4H11-28z compared to 19z1), p=0.63 (4H11z compared to 4H11-28z).

Figure 15. MUC-CD targeted 4H11-28z⁺ T cells successfully traffic to ip OV-CAR3(MUC-CD/GFP-FFLuc) tumors following systemic intravenous infusion resulting in equally efficient anti-tumor efficacy when compared to ip 4H11-28z⁺ treated tumor bearing mice. (A) Kaplan-Meier survival curve of SCID-Beige mice treated ip or iv with 4H11-28z⁺ T cells. SCID-Beige mice were injected intraperitoneally with 3x10⁶ OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells followed by either iv or ip infusion of 3x10⁷ 4H11-28z⁺ T cells. Tumor eradication is enhanced after either ip or iv infusion of 4H11-28z⁺ T cells when compared to control treated mice. Both ip and iv 4H11-28z⁺ T cell treated mice exhibited statistically enhanced survival (**p<0.0001 and **p=0.0038, respectively) when compared to 19-28z⁺ T cell treated control cohorts. Conversely, difference in survival between the ip and iv 4H11-28z⁺ T cell cohorts was not statistically significant (p=0.22). (B) BLI of tumor progression of representative ip and iv 4H11-28z⁺ T cell treated mice with ultimately progressive disease following treatment compared to BLI of tumor progression in a representative control 19-28z⁺ T cell treated mouse. (C) Systemically injected CFSE stained 4H11-28z⁺ T cells traffic to advanced ip OV-CAR(MUC-CD) tumors. Presence of iv injected CFSE labeled 19-28z⁺ control T cells (left panel) and 4H11-28z⁺ T cells (right panel) 1 day following infusion into SCID-Beige mice with advanced OV-CAR(MUC-CD) tumors (injected 7 days earlier), as assessed by FACS analysis of single cell OV-CAR3(MUC-CD) tumor suspensions, reveals a marked population of 4H11-28z⁺ but not control 19-28z⁺ T cells within peritoneal OV-CAR3(MUC-CD) tumors.

Figure 16. Eradication of advanced OV-CAR3(MUC-CD) tumors in SCID-Beige mice by ip infusion of 4H11-28z⁺ T cells. SCID-Beige mice were injected ip with 3x10⁶ OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells 7 days prior to ip treatment with 3x10⁷ 4H11-28z⁺ T cells. (A) BLI of 4H11-28z⁺ T cell treated mice with either relapsed disease (middle row) or eradicated disease (bottom row) compared to a representative 19-28z⁺ T cell treated control mouse. (B) Kaplan-Meier survival curve of SCID-Beige mice with advanced OV-CAR3(MUC-CD/GFP-FFLuc) tumors

treated ip with 4H11-28z⁺ T cells. All 4H11-28z⁺ T cell treated mice demonstrated enhanced survival when compared to control 19-28z⁺ T cell treated mice (**p=0.0011), with an overall long-term survival of 25% at day 120.

Figure 17: CD8 leader sequence, CD3 zeta chain intracellular domain sequence, (G4S)₃ serine-glycine linker sequence, CD8 transmembrane domain sequence, and CD28 transmembrane + intracellular domains (-STOP) sequence.

Figure 18: SFG_4H11z sequence.

Figure 19: SFG-4H11-28z sequence.

Figure 20: (A) Mouse MUC16-CD Peptide 1 (SEQ ID NO:21), Mouse first Cysteine Loop Peptide 2 (SEQ ID NO:22), and Mouse second Cysteine Loop Peptide 3 (SEQ ID NO:23). (B) Alignment of mouse MUC16 (SEQ ID NO:24) and human MUC16 (SEQ ID NO:25) amino acid sequences. A cysteine was added to the peptide sequence at the N terminus of Peptide 1 and Peptide 3 for better conjugation with KLH.

Figure 21: ID8 extract with 1:10 dilution of Mouse MUC16 monoclonal Primary Supernatants.

Figure 22: BR5-FVB1 extract with 1:10 dilution of Mouse MUC16 monoclonal Primary Supernatants

Figure 23: Western Blot showing 38 hamster's monoclonal antibody Supernatants on ID8 cell extracts.

Figure 24 (A) Nucleotide sequence encoding 12B10-3G10-V_H (SEQ ID NO:26), (B) 12B10-3G10-V_H Amino Acid sequence (SEQ ID NO:27), (C) Nucleotide sequence encoding 12B10-3G10-V_L (SEQ ID NO:28) (Note the VL has an optional *NotI* site added by the primer for cloning, and (D) 12B10-3G10-V_L Amino Acid sequence (SEQ ID NO:29).

Figure 25: FACS Analysis with Purified 12B10-3G10 mAb on ID8 (mouse), OVCAR-3 (human) and BR5-FVB1 (mouse) cell lines.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The terms "purified," "isolated," and grammatical equivalents thereof as used herein, refer to the reduction in the amount of at least one undesirable component (such as cell, protein, nucleic acid sequence, carbohydrate, *etc.*) from a sample, including a reduction by any numerical percentage of from 5% to 100%, such as, but not limited to, from 10% to 100%, from 20% to 100%, from 30% to 100%, from 40% to 100%, from 50% to 100%, from 60% to 100%, from 70% to 100%, from 80% to 100%, and from 90% to 100%. Thus purification results in an "enrichment,"

i.e., an increase in the amount of a desirable component cell, protein, nucleic acid sequence, carbohydrate, *etc.*).

The term "antibody" refers to an immunoglobulin (*e.g.*, IgG, IgM, IgA, IgE, IgD, *etc.*). The basic functional unit of each antibody is an immunoglobulin (Ig) monomer (containing only one immunoglobulin ("Ig") unit). Included within this definition are polyclonal antibody, monoclonal antibody, and chimeric antibody.

The variable part of an antibody is its "V domain" (also referred to as "variable region"), and the constant part is its "C domain" (also referred to as "constant region") such as the kappa, lambda, alpha, gamma, delta, epsilon and mu constant regions. The "variable domain" is also referred to as the "F_V region" and is the most important region for binding to antigens. More specifically, variable loops, three each on the light (V_L) and heavy (V_H) chains are responsible for binding to the antigen. These loops are referred to as the "complementarity determining regions" ("CDRs" and "idiotypes."

The immunoglobulin (Ig) monomer of an antibody is a "Y"-shaped molecule that contains four polypeptide chains: two light chains and two heavy chains, joined by disulfide bridges.

Light chains are classified as either (λ) or kappa (κ). A light chain has two successive domains: one constant domain ("C_L") and one variable domain ("V_L"). The variable domain, V_L, is different in each type of antibody and is the active portion of the molecule that binds with the specific antigen. The approximate length of a light chain is 211 to 217 amino acids.

Each heavy chain has two regions, the *constant region* and the *variable region*. There are five types of mammalian Ig heavy denoted α, δ, ε, γ, and μ. The type of heavy chain present defines the *class* of antibody; these chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively. Distinct heavy chains differ in size and composition; α and γ contain approximately 450 amino acids, while μ and ε have approximately 550 amino acids. Each heavy chain has two regions, the constant region ("C_H") and the variable ("V_H") region. The constant region (C_H) is identical in all antibodies of the same isotype, but differs in antibodies of different isotypes. Heavy chains γ, α and δ have a constant region composed of *three* tandem (in a line) Ig domains, and a hinge region for added flexibility. Heavy chains μ and ε have a constant region composed of *four* immunoglobulin domains. The variable region (V_H) of the heavy chain differs in antibodies produced by different B cells, but is the same for all antibodies produced by a single B cell or B cell clone. The variable region of each heavy chain is approximately 110 amino acids long.

The term "specifically binds" and "specific binding" when made in reference to the binding of two molecules (*e.g.* antibody to an antigen, *etc.*) refer to an interaction of the two molecules that is dependent upon the presence of a particular structure on one or both of the molecules. For

example, if an antibody is specific for epitope "A" on the molecule, then the presence of a protein containing epitope A (or free, unlabelled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

5 The term "capable of binding" when made in reference to the interaction between a first molecule (such as antibody, polypeptide, glycoprotein, nucleic acid sequence, *etc.*) and a second molecule (such as antigen, polypeptide, glycoprotein, nucleic acid sequence, *etc.*) means that the first molecule binds to the second molecule in the presence of suitable concentration of salts, and suitable temperature, and pH. The conditions for binding molecules may be determined using routine and/or commercially available methods

10 The terms "antigen," "immunogen," "antigenic," "immunogenic," "antigenically active," "immunologic," and "immunologically active" when made in reference to a molecule, refer to any substance that is capable of inducing a specific humoral immune response (including eliciting a soluble antibody response) and/or cell-mediated immune response (including eliciting a CTL response). Antigenic peptides preferably contain at least 5, at least 6, at least 7, at least 8, at least 9,
15 and more preferably at least 10 amino acids. To elicit antibody production, in one embodiment, antigens may be conjugated to keyhole limpet hemocyanin (KLH) or fused to glutathione-S-transferase (GST).

A "cognate antigen" when in reference to an antigen that binds to an antibody, refers to an antigen that is capable of specifically binding to the antibody.

20 In one embodiment, the antigen comprises an epitope. The terms "epitope" and "antigenic determinant" refer to a structure on an antigen, which interacts with the binding site of an antibody or T cell receptor as a result of molecular complementarity. An epitope may compete with the intact antigen, from which it is derived, for binding to an antibody.

As used herein the terms "portion" and "fragment" when made in reference to a nucleic
25 acid sequence or protein sequence refer to a piece of that sequence that may range in size from 2 contiguous nucleotides and amino acids, respectively, to the entire sequence minus one nucleotide and amino acid, respectively.

A "subject" that may benefit from the invention's methods includes any multicellular animal, preferably a mammal. Mammalian subjects include humans, non-human primates,
30 murines, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, *etc.*). Thus, mammalian subjects are exemplified by mouse, rat, guinea pig, hamster, ferret and chinchilla. The invention's compositions and methods are also useful for a subject "in need of reducing one or more symptoms of" a disease, *e.g.*, in need of reducing cancer metastasis and/or in need of reducing one or more symptoms of cancer, includes a subject that exhibits and/or is at risk

of exhibiting one or more symptoms of the disease. For Example, subjects may be at risk based on family history, genetic factors, environmental factors, *etc.* This term includes animal models of the disease. Thus, administering a composition (which reduces a disease and/or which reduces one or more symptoms of a disease) to a subject in need of reducing the disease and/or of reducing one or more symptoms of the disease includes prophylactic administration of the composition (i.e., before the disease and/or one or more symptoms of the disease are detectable) and/or therapeutic administration of the composition (i.e., after the disease and/or one or more symptoms of the disease are detectable). The invention's compositions and methods are also useful for a subject "at risk" for disease (such as cancer) refers to a subject that is predisposed to contracting and/or expressing one or more symptoms of the disease. This predisposition may be genetic (*e.g.*, a particular genetic tendency to expressing one or more symptoms of the disease, such as heritable disorders, *etc.*), or due to other factors (*e.g.*, environmental conditions, exposures to detrimental compounds, including carcinogens, present in the environment, *etc.*). The term subject "at risk" includes subjects "suffering from disease," i.e., a subject that is experiencing one or more symptoms of the disease. It is not intended that the present invention be limited to any particular signs or symptoms. Thus, it is intended that the present invention encompass subjects that are experiencing any range of disease, from sub-clinical symptoms to full-blown disease, wherein the subject exhibits at least one of the indicia (*e.g.*, signs and symptoms) associated with the disease.

"Cancer cell" refers to a cell undergoing early, intermediate or advanced stages of multi-step neoplastic progression as previously described (Pitot et al., Fundamentals of Oncology, 15-28 (1978)). This includes cells in early, intermediate and advanced stages of neoplastic progression including "pre-neoplastic cells (i.e., "hyperplastic cells and dysplastic cells), and neoplastic cells in advanced stages of neoplastic progression of a dysplastic cell.

"Metastatic" cancer cell refers to a cancer cell that is translocated from a primary cancer site (*i.e.*, a location where the cancer cell initially formed from a normal, hyperplastic or dysplastic cell) to a site other than the primary site, where the translocated cancer cell lodges and proliferates.

"Cancer" refers to a plurality of cancer cells that may or may not be metastatic, such as ovarian cancer, breast cancer, lung cancer, prostate cancer, cervical cancer, pancreatic cancer, colon cancer, stomach cancer, esophagus cancer, mouth cancer, tongue cancer, gum cancer, skin cancer (*e.g.*, melanoma, basal cell carcinoma, Kaposi's sarcoma, *etc.*), muscle cancer, heart cancer, liver cancer, bronchial cancer, cartilage cancer, bone cancer, testis cancer, kidney cancer, endometrium cancer, uterus cancer, bladder cancer, bone marrow cancer, lymphoma cancer, spleen cancer, thymus cancer, thyroid cancer, brain cancer, neuron cancer, mesothelioma, gall bladder cancer, ocular cancer (*e.g.*, cancer of the cornea, cancer of uvea, cancer of the choroids, cancer of the

macula, vitreous humor cancer, etc.), joint cancer (such as synovium cancer), glioblastoma, lymphoma, and leukemia.

“Sample” and “specimen” as used herein are used in their broadest sense to include any composition that is obtained and/or derived from a biological source, as well as sampling devices
5 (e.g., swabs), which are brought into contact with biological or environmental samples. “Biological samples” include those obtained from a subject, including body fluids (such as urine, blood, plasma, fecal matter, cerebrospinal fluid (CSF), semen, sputum, and saliva), as well as solid tissue. Biological samples also include a cell (such as cell lines, cells isolated from tissue whether or not the isolated cells are cultured after isolation from tissue, fixed cells such as cells fixed for
10 histological and/or immunohistochemical analysis), tissue (such as biopsy material), cell extract, tissue extract, and nucleic acid (e.g., DNA and RNA) isolated from a cell and/or tissue, and the like. These examples are illustrative, and are not to be construed as limiting the sample types applicable to the present invention.

“Overexpression of MUC16” by a cell of interest (such as a cancer cell) refers to a higher
15 level of MUC16 protein and/or mRNA that is expressed by the cell of interest compared to a control cell (such as a non-cancerous cell, normal cell, etc.).

“Internalize” when in reference to a cell refers to entry from the extracellular medium into the cell membrane and/or cytoplasm.

“Glycosylated” when in reference to a sequence (e.g., an amino acid sequence or nucleotide
20 sequence) refers to a sequence that is covalently linked to one or more saccharides.

“Pharmaceutical” and “physiologically tolerable” composition refers to a composition that contains pharmaceutical molecules, *i.e.*, molecules that are capable of administration to or upon a subject and that do not substantially produce an undesirable effect such as, for example, adverse or allergic reactions, dizziness, gastric upset, toxicity and the like, when administered to a subject.
25 Preferably also, the pharmaceutical molecule does not substantially reduce the activity of the invention's compositions. Pharmaceutical molecules include “diluent” (*i.e.*, “carrier”) molecules and excipients.

“Immunogenically effective” and “antigenically effective” amount of a molecule interchangeably refer to an amount of the molecule that is capable of inducing a specific humoral
30 immune response (including eliciting a soluble antibody response) and/or cell-mediated immune response (including eliciting a cytotoxic T-lymphocyte (CTL) response).

“Treating” a disease refers to reducing one or more symptoms (such as objective, subjective, pathological, clinical, sub-clinical, etc.) of the disease.

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents (including "lower," "smaller," *etc.*) when in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, antibody, *etc.*), cell, and/or phenomenon (*e.g.*, disease symptom, binding to a molecule, specificity of binding of two molecules, affinity of binding of two molecules, specificity to cancer, sensitivity to cancer, affinity of binding, enzyme activity, *etc.*) in a first sample (or in a first subject) relative to a second sample (or relative to a second subject), mean that the quantity of molecule, cell and/or phenomenon in the first sample (or in the first subject) is lower than in the second sample (or in the second subject) by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the quantity of molecule, cell and/or phenomenon in the first sample (or in the first subject) is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). In another embodiment, the quantity of molecule, cell, and/or phenomenon in the first sample (or in the first subject) is lower by any numerical percentage from 5% to 100%, such as, but not limited to, from 10% to 100%, from 20% to 100%, from 30% to 100%, from 40% to 100%, from 50% to 100%, from 60% to 100%, from 70% to 100%, from 80% to 100%, and from 90% to 100% lower than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). In one embodiment, the first subject is exemplified by, but not limited to, a subject that has been manipulated using the invention's compositions and/or methods. In a further embodiment, the second subject is exemplified by, but not limited to, a subject that has not been manipulated using the invention's compositions and/or methods. In an alternative embodiment, the second subject is exemplified by, but not limited to, a subject to that has been manipulated, using the invention's compositions and/or methods, at a different dosage and/or for a different duration and/or via a different route of administration compared to the first subject. In one embodiment, the first and second subjects may be the same individual, such as where the effect of different regimens (*e.g.*, of dosages, duration, route of administration, *etc.*) of the invention's compositions and/or methods is sought to be determined in one individual. In another embodiment, the first and second subjects may be different individuals, such as when comparing the effect of the invention's compositions and/or methods on one individual participating in a clinical trial and another individual in a hospital.

The terms "increase," "elevate," "raise," and grammatical equivalents (including "higher," "greater," *etc.*) when in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, antibody, *etc.*), cell, and/or phenomenon (*e.g.*, disease symptom, binding to a molecule, specificity of binding of two molecules, affinity of binding of two molecules,

specificity to cancer, sensitivity to cancer, affinity of binding, enzyme activity, *etc.*) in a first sample (or in a first subject) relative to a second sample (or relative to a second subject), mean that the quantity of the molecule, cell and/or phenomenon in the first sample (or in the first subject) is higher than in the second sample (or in the second subject) by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the quantity of the molecule, cell and/or phenomenon in the first sample (or in the first subject) is at least 10% greater than, at least 25% greater than, at least 50% greater than, at least 75% greater than, and/or at least 90% greater than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). This includes, without limitation, a quantity of molecule, cell, and/or phenomenon in the first sample (or in the first subject) that is at least 10% greater than, at least 15% greater than, at least 20% greater than, at least 25% greater than, at least 30% greater than, at least 35% greater than, at least 40% greater than, at least 45% greater than, at least 50% greater than, at least 55% greater than, at least 60% greater than, at least 65% greater than, at least 70% greater than, at least 75% greater than, at least 80% greater than, at least 85% greater than, at least 90% greater than, and/or at least 95% greater than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). In one embodiment, the first subject is exemplified by, but not limited to, a subject that has been manipulated using the invention's compositions and/or methods. In a further embodiment, the second subject is exemplified by, but not limited to, a subject that has not been manipulated using the invention's compositions and/or methods. In an alternative embodiment, the second subject is exemplified by, but not limited to, a subject to that has been manipulated, using the invention's compositions and/or methods, at a different dosage and/or for a different duration and/or via a different route of administration compared to the first subject. In one embodiment, the first and second subjects may be the same individual, such as where the effect of different regimens (e.g., of dosages, duration, route of administration, etc.) of the invention's compositions and/or methods is sought to be determined in one individual. In another embodiment, the first and second subjects may be different individuals, such as when comparing the effect of the invention's compositions and/or methods on one individual participating in a clinical trial and another individual in a hospital.

The terms "alter" and "modify" when in reference to the level of any molecule and/or phenomenon refer to an increase or decrease.

Reference herein to any numerical range expressly includes each numerical value (including fractional numbers and whole numbers) encompassed by that range. To illustrate, and without limitation, reference herein to a range of "at least 50" includes whole numbers of 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, *etc.*, and fractional numbers 50.1, 50.2 50.3, 50.4, 50.5, 50.6, 50.7, 50.8,

50.9, *etc.* In a further illustration, reference herein to a range of “less than 50” includes whole numbers 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, *etc.*, and fractional numbers 49.9, 49.8, 49.7, 49.6, 49.5, 49.4, 49.3, 49.2, 49.1, 49.0, *etc.* In yet another illustration, reference herein to a range of from “5 to 10” includes each whole number of 5, 6, 7, 8, 9, and 10, and each fractional number such as 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, *etc.*

DESCRIPTION OF THE INVENTION

The invention provides antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications for diseases in which MUC16 is overexpressed, such as cancer.

Using synthetic peptides, the inventors raised novel-specific antibodies to the carboxy-terminal portion of MUC16, retained by the cell, proximal to the putative cleavage site. These antibodies were characterized using fluorescence-activated cell-sorting analysis, enzyme-linked immunoassay, Western blot analysis, and immunohistochemistry. Each of the selected monoclonal antibodies was reactive against recombinant GST-ΔMUC16^{c114} protein and the MUC16 transfected SKOV3 cell line. Three antibodies, 4H11, 9C9, and 4A5 antibodies demonstrated high affinities by Western blot analysis and saturation-binding studies of transfected SKOV3 cells, and displayed antibody internalization. Immunohistochemical positivity with novel antibody 4H11 was similar to OC125, but with important differences, including diffuse positivity in lobular breast cancer and a small percentage of OC125-negative ovarian carcinomas which showed intense and diffuse 4H11 antibody binding.

The invention's compositions and methods are useful for diagnostic and therapeutic applications, as well as biologic studies such as membrane receptor trafficking and intracellular events. Diagnostic applications include, for example, detection of cancer using immunohistochemical, radiographic imaging, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, and/or immunoprecipitation detection.

The invention is further described under (A) MUC16, (B) Prior Art Antibodies, (C) Invention's Antibodies, (D) Hybridoma Cell Lines, (E) Conjugates Of The Invention's Antibodies Linked To Cytotoxic Agents And/Or Prodrugs, (F) Detecting Muc16 Portions And Diagnostic Applications, and (G) Therapeutic Applications.

A. MUC16

“MUC16,” “MUC-16” and “Mucin 16” interchangeably refer to a type I membrane protein that is part of a family of tethered mucins. A schematic of Muc16 is in Figure 10, and an exemplary human Muc16 amino acid sequence (SEQ ID NO:13) is shown in Figure 9A. An alignment of mouse MUC16 (SEQ ID NO:24) and human MUC16 (SEQ ID NO:25) amino acid sequences is shown in Figure 20B. The term “type 1 protein” refers to a “membrane protein” that is at least partially embedded in the lipid bilayer of a cell, virus and the like, and that contains a transmembrane domain (TM) sequence embedded in the lipid bilayer of the cell, virus and the like. The portion of the protein on the NH₂-terminal side of the TM domain is exposed on the exterior side of the membrane, and the COOH-terminal portion is exposed on the cytoplasmic side.

Recently, the sequence of the cDNA-encoding MUC16/CA125 was described by Yin and Lloyd in 2001 and completed by O’Brien in 2002 (10-12). The complete MUC16 protein has various components consisting of a cytoplasmic tail with potential phosphorylation sites, a transmembrane domain, and an external domain proximal to an apparent cleavage site. Distal to the cleavage site, the released external domain contains 16-20 tandem repeats of 156 amino acids, each with many potential glycosylation sites (11). The overall repeat structure (Figure 10) is well conserved across mammals, but the repeats are not completely identical in exact amino acid composition.

The MUC16 protein is part of a family of tethered mucins that includes both MUC1 and MUC4 (13). MUC1 is present in a variety of tissues and appears to signal through a beta catenin pathway, interact with EGF receptor, mediates drug resistance and can act as an oncogene (14-17). The MUC4 protein is also expressed in a variety of tissues but is common on neoplasms of the gastrointestinal track (18-20). In contrast, the CA125 antigen has been more restricted in its distribution and is present primarily in gynecologic tissues and overexpressed in Müllerian neoplasms (21). However, the CA125 antigen, recognized by the OC125 antibody, is a heavily glycosylated antigen expressed in the tandem repeat region of the larger MUC16 protein. This glycoprotein is typically shed from a putative cleavage site in the extracellular domain of the MUC16 peptide backbone.

Thus, “MUC16” protein contains (a) a “cytoplasmic domain,” (b) a “transmembrane domain,” and (c) a “extracellular domain.” The MUC16 extracellular domain contains a cleavage site between a non-glycosylated ectodomain and a large glycosylated ectodomain of tandem repeats.

The terms “cytoplasmic domain,” “cytoplasmic tail,” and “CT” are used interchangeably to refer to a protein sequence, and portions thereof, that is on the cytoplasmic side of the lipid bilayer

of a cell, virus and the like. Methods for determining the CT of a protein are known in the art (Elofsson et al. (2007) *Annu. Rev. Biochem.* 76:125-140; Bernsel et al. (2005) *Protein Science* 14:1723-1728).

5 The terms "transmembrane domain" and "TM" are used interchangeably to refer to a protein sequence, and portions thereof, that spans the lipid bilayer of a cell, virus and the like. Methods for determining the TM of a protein are known in the art (Elofsson et al. (2007) *Annu. Rev. Biochem.* 76:125-140; Bernsel et al. (2005) *Protein Science* 14:1723-1728).

10 The terms "ectodomain" and "extracellular domain" are interchangeably used when in reference to a membrane protein to refer to the portion of the protein that is exposed on the extracellular side of a lipid bilayer of a cell, virus and the like. Methods for determining the ectodomain of a protein are known in the art (Singer (1990) *Annu. Rev. Cell Biol.* 6:247-296 and High et al. (1993) *J. Cell Biol.* 121:743-750, and McVector software, Oxford Molecular).

The exemplary Muc16 of Figure 9 contains (a) a "MUC16 cytoplasmic domain" from amino acid 14476 to 14507, vttr rkkegeynvq qqcpgyyqsh ldledlq (SEQ ID NO:16), that interacts with the intracellular signal transduction machinery; (b) a "MUC16 transmembrane domain" from amino acid 14452 to 14475, fwaviligl agllgvitcl icgvl (SEQ ID NO:14) that spans the plasma membrane; and (c) a "MUC16 extracellular domain" amino acid 1 to 14392 (SEQ ID NO:13) that contains a cleavage site between an non-glycosylated ectodomain and a large glycosylated ectodomain of tandem repeats. The "MUC16 ectodomain" is exemplified by nfsplar rvdraiyeec flrmtrngtq lqnftldrss vlvdgyspnr nepltgnsdl p (SEQ ID NO:17) from amino acid 14394 to 14451 of SEQ ID NO:13 of Figure 9A.

25 The exemplary MUC16 ectodomain contains both Polypeptide 1 (nfsplar rvdraiyeec (SEQ ID NO:01), which is from amino acid 14394 to 14410 of SEQ ID NO:13), and Polypeptide 2 (tldrss vlvdgyspnr ne (SEQ ID NO:02), which is from amino acid 14425 to 14442 of SEQ ID NO:13), against which the invention's exemplary antibodies were produced. Polypeptide 3, cgvlttrr rkkegeynvq qq (SEQ ID NO:03) is from amino acid 14472 to 14492 of SEQ ID NO:13, and contains both a transmembrane domain portion (cgvl) and a cytoplasmic domain portion (vttr rkkegeynvq qq (SEQ ID NO:18)). Thus, the CGVL is optional in SEQ ID NO:03, as it is part of the transmembrane domain.

30 Polypeptide 4 (ksyf sdcqvstfrs vprnhhtgvd slcnfspl (SEQ ID NO:15), is located in a non-glycosylated portion of the Muc16 extracellular domain, is from amino acid 14367 to 14398 of SEQ ID NO:13, and contains a cysteine loop polypeptide cqvstfrsvprnhhtgvdslc (SEQ ID NO:13).

B. Prior Art Antibodies

The expression of the MUC16/CA125 antigen has long been associated with gynecologic tissues. "CA125," "CA-125," "Cleaved CA125," and "cleaved CA-125," interchangeably refer to the glycosylated external domain of the tethered mucin MUC16, that is distal to the cleavage site
5 (Payne et al., U.S. Pat. No. 7,202,346). This released external domain contains 16-20 tandem repeats of 156 amino acids, each with potential glycosylation sites. An apparent cysteine-based disulfide loop of 19 amino acids is present in all repeats and the N-terminal end contains a hairbrush structure that is heavily O-glycosylated (11). The deduced size would be 2.5 MD for the protein part, and with added carbohydrates, this could increase to 5 MD (10, 26).

10 CA125, though it is not sensitive or specific enough to be used as a general screening tool, is routinely used to monitor patients with ovarian carcinoma. The tests used to measure CA125 are antibody based detection methods, as are the immunohistochemical stains routinely performed for diagnostic purposes. The epitope specificity of 26 antibodies to MUC16 was studied in the first report from the International Society of Oncodevelopmental Biology and Medicine (ISOBM) TD-1
15 Workshop and the application of 22 antibodies to immunohistochemistry was reported in the second report from the TD-1 workshop (7, 21). The existing antibodies were grouped as OC125-like, M11-like, or OV197-like and all of the known antibodies recognized CA125 epitopes in the repeating, glycosylated elements in the external domain of the tethered mucin MUC16, distal to the putative cleavage site.

20 The vast majority of MUC16-reactive antibodies, including OC125, react with the glycosylation-dependent antigen present exclusively in the cleaved portion of the molecule so the true distribution of MUC16 expression is not known (21). There is currently no antibody available to track the fate of the remaining MUC16 protein fragment after cleavage and CA125 release.

25 C. Invention's Antibodies

In order to better explore the biology of human MUC16, the inventors have derived monoclonal antibodies against the extracellular portion of the MUC16-carboxy terminus, proximal to the putative cleavage site, as well as one monoclonal antibody against the internal cytoplasmic domain. In contrast to prior antibodies, these are derived against the peptide backbone of MUC16
30 and are not directed at complex glycoprotein epitopes. Since these epitopes are proximal to the cleavage site, they are unlikely to be found in the circulation and provide novel targets for diagnostic methods and therapeutic interventions. Data herein demonstrate the identification and characterization of exemplary antibodies developed against the MUC16 peptide backbone.

The inventors have developed novel antibodies that are directed at the non-cleaved, non-glycosylated peptide backbone of MUC16. These are exemplified by both 4H11 and 9C9 antibodies, which react with peptide sequences in the non-cleaved ectodomain of MUC16 and are detectable on the surface of ovarian cancer cell lines and in paraffin-fixed tissues from human ovarian cancer surgical specimens. The antibodies show high affinity and are readily internalized by ovarian cancer cells when bound to the ectodomain of MUC16. This suggests that the proximal portion of MUC16 has an independent biology from the more distal, cleaved portion of the mucin. It also suggests that the proximal portions of MUC16 could provide convenient targets for diagnostic and therapeutic interventions. Targeting the peptide backbone of MUC16 provides highly specific tissue delivery for genetically engineered cells, liposomes, or antibody conjugates, including conjugates with the invention's antibodies.

The invention's antibodies, exemplified by antibody 4H11, are useful as tools in immunohistochemistry. Data herein show that 4H11 is relatively specific to high-grade ovarian serous carcinoma. Invasive lobular breast carcinoma is the major exception and shows extensive MUC16 protein as detected by 4H11. Lobular carcinoma of the breast has unique biology which is characterized by a propensity to metastasize to serosal surfaces (27). Since MUC16 is the cognate binding partner of mesothelin, this may have important implications for lobular cancer (28). The discordance rates for OC125 and 4H11 also suggest that 4H11 might provide additional, independent information from OC125 in a subset of ovarian carcinomas. Some tumors that are negative with OC125 retain cytoplasmic and extracellular portions of the MUC16 glycoprotein, portions of the molecule that are likely involved in transduction of signals potentially important in the malignant phenotype.

Thus, in one embodiment, the invention provides an isolated antibody, or an antigen-binding fragment thereof, that specifically binds to a polypeptide, or antigenic portion thereof, wherein the polypeptide is exemplified by a) MUC16 ectodomain polypeptide (exemplified by NFSPLAR RVDRVAIYEE FLRMTRNGTQ LQNFTLDRSS VLVDGYSPNR NEPLTGNSDL P (SEQ ID NO:17)), b) MUC16 cytoplasmic domain polypeptide (exemplified by VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18), which is contained within each of CGVLVTTRR RKKEGEYNVQ QQ (SEQ ID NO:03) and LVTTRR RKKEGEYNVQ QQ (SEQ ID NO:20)), and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSL C (SEQ ID NO:19).

One advantage of the invention's antibodies is that the antibody internalizes into a cell, thereby being useful in applications for delivery inside a cell, such as disease therapy.

"Internalized" when in reference to a molecule that is internalized by a cell refers to passage of the

molecule that is in contact with the extracellular surface of a cell membrane across the cell membrane to the intracellular surface of the cell membrane and/or into the cell cytoplasm. Methods for determining internalization are disclosed herein, including the detection of radiolabeled molecule inside the cell (Figure 5B).

5 In one embodiment, the invention's antibodies specifically bind to MUC16 ectodomain polypeptide that comprises a polypeptide selected from the group consisting of Polypeptide 1 NFSPLARRVDRVAIYEE (SEQ ID NO:01) and Polypeptide 2 TLDRSSVLVDGYSPNRNE (SEQ ID NO:02). Data herein show that the invention's antibodies specifically bind to GST-ΔMUC16^c
114 (Example 2, Table 1A). The specificity of the invention's antibodies is in contrast to prior art
10 antibodies (e.g., VK8, M11 and OC125 antibodies) that did not bind to GST-ΔMUC16^{c114} purified protein or cell lysates of the SKOV3-phrGFP-ΔMUC16^{c114} cell line (Example 2, Figure 2).

 In a further embodiment, the invention's antibodies lack specific binding to a glycosylated MUC16 extracellular domain, exemplified by the cleaved CA-125 described in Payne et al., U.S. Pat. No. 7,202,346.

15 While not intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID NO:02) of the MUC16 ectodomain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:06 (i.e., the antibody 4H11 variable heavy (VH) chain amino acid sequence of Figure 8), and a variable light (V_L) chain encoded by SEQ ID NO:07 (i.e., the
20 antibody 4H11 variable light (V_L) chain amino acid sequence of Figure 8). In a particular embodiment, the antibody is chimeric, wherein at least one of the V_L and V_H chains is fused to a human immunoglobulin constant region.

 Also without intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID
25 NO:02) of the MUC16 ectodomain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:04 (i.e., the antibody 4A5 variable heavy (V_H) chain nucleotide sequence of Figure 8), and a variable light (V_L) chain encoded by SEQ ID NO:05 (i.e., the antibody 4A5 variable light (V_L) chain nucleotide sequence of Figure 8). In a particular embodiment, the antibody is chimeric wherein at least one of the V_L and V_H chains is covalently linked to a human
30 immunoglobulin constant region.

 Still without intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to the Polypeptide 1 (SEQ ID NO:01) of the MUC16 ectodomain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:08 (i.e., the antibody 9B11 variable heavy (VH) chain

nucleotide sequence of Figure 8), and a variable light (V_L) chain encoded by at least one of SEQ ID NO:09 (i.e., antibody 9B11 variable light (V_{LA}) chain nucleotide sequence of Figure 8), and SEQ ID NO:10 (i.e., the antibody 9B11 variable light (V_{LB}) chain nucleotide sequence of Figure 8). In a particular embodiment, the antibody is chimeric wherein at least one of the V_L and V_H chains is covalently linked to a human immunoglobulin constant region.

While not intending to restrict the source of antigen to which the invention's antibodies bind, in one embodiment, the MUC16 ectodomain polypeptide is expressed by a cell. Data herein show that the invention's exemplary antibodies bind to SKOV3 cells transduced with phrGFP- Δ MUC16^{c114} (Example 2).

While not limiting the sequence of antigen to which the invention's antibodies bind, in a further embodiment, the invention's antibodies specifically bind to a MUC16 cytoplasmic domain polypeptide that comprises VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18). In a particular embodiment, the MUC16 cytoplasmic domain polypeptide comprises Polypeptide 3 CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03). In some embodiment, the MUC16 cytoplasmic domain polypeptide is expressed by a cell. For example, data herein show that the invention's exemplary antibody binds to SKOV3 cells transduced with phrGFP- Δ MUC16^{c114} (Example 2). In a particular embodiment, the cell is permeabilized to facilitate internalization of the antibody into the cell so that it comes into contact with its cytoplasmic antigen.

Still without limiting the sequence of antigen to which the invention's antibodies bind, in a further embodiment, the invention's antibodies bind to a MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSL (SEQ ID NO:19). In a more preferred embodiment, the MUC16 extracellular domain polypeptide comprises Polypeptide 4 KSYF SDCQVSTFRS VPNRHHTGVD SLCNFSPL (SEQ ID NO:15).

Still without intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to Polypeptide 4 (SEQ ID NO:15) of the MUC16 extracellular domain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:11 (i.e., the antibody 24B3 variable heavy (V_H) chain amino acid sequence of Figure 8), and a variable light (V_L) chain encoded by SEQ ID NO:12 (i.e., the antibody 24B3 variable light (V_L) chain amino acid sequence of Figure 8).

The invention contemplates chimeric antibodies (see U.S. Pat. No. 7,662,387), monoclonal antibodies, recombinant antibodies, an antigen-binding fragment of a recombinant antibody, a humanized antibody, and an antibody displayed upon the surface of a phage (U.S. Pat. No. 7,202,346). In particular, the invention contemplates antibody fragments that contain the idiotype ("antigen-binding region" or "antigen-binding fragment") of the antibody molecule. For example,

such antigen-binding fragments include, but are not limited to, the Fab region, F(ab')₂ fragment, pFc' fragment, and Fab' fragments.

The "Fab region" and "fragment, antigen binding region," interchangeably refer to portion of the antibody arms of the immunoglobulin "Y" that function in binding antigen. The Fab region is
5 composed of one constant and one variable domain from each heavy and light chain of the antibody. Methods are known in the art for the construction of Fab expression libraries (Huse et al., Science, 246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. In another embodiment, Fc and Fab fragments can be generated by using the enzyme papain to cleave an immunoglobulin monomer into two Fab
10 fragments and an Fc fragment. The enzyme pepsin cleaves below the hinge region, so a "F(ab')₂ fragment" and a "pFc' fragment" is formed. The F(ab')₂ fragment can be split into two "Fab' fragments" by mild reduction.

The invention also contemplates a "single-chain antibody" fragment, i.e., an amino acid sequence having at least one of the variable or complementarity determining regions (CDRs) of the
15 whole antibody, and lacking some or all of the constant domains of the antibody. These constant domains are not necessary for antigen binding, but constitute a major portion of the structure of whole antibodies. Single-chain antibody fragments are smaller than whole antibodies and may therefore have greater capillary permeability than whole antibodies, allowing single-chain antibody fragments to localize and bind to target antigen-binding sites more efficiently. Also, antibody
20 fragments can be produced on a relatively large scale in prokaryotic cells, thus facilitating their production. Furthermore, the relatively small size of single-chain antibody fragments makes them less likely to provoke an immune response in a recipient than whole antibodies. Techniques for the production of single-chain antibodies are known (U.S. 4,946,778). The variable regions of the heavy and light chains can be fused together to form a "single-chain variable fragment" ("scFv
25 fragment"), which is only half the size of the Fab fragment, yet retains the original specificity of the parent immunoglobulin.

The "Fc region" and "Fragment, crystallizable region" interchangeably refer to portion of the base of the immunoglobulin "Y" that function in role in modulating immune cell activity. The Fc region is composed of two heavy chains that contribute two or three constant domains
30 depending on the class of the antibody. By binding to specific proteins, the Fc region ensures that each antibody generates an appropriate immune response for a given antigen. The Fc region also binds to various cell receptors, such as Fc receptors, and other immune molecules, such as complement proteins. By doing this, it mediates different physiological effects including opsonization, cell lysis, and degranulation of mast cells, basophils and eosinophils. In an

experimental setting, Fc and Fab fragments can be generated in the laboratory by cleaving an immunoglobulin monomer with the enzyme papain into two Fab fragments and an Fc fragment.

The invention contemplates polyclonal antibodies and monoclonal antibodies. "Polyclonal antibody" refers to an immunoglobulin produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an immunoglobulin produced from a single clone of plasma cells. Generic methods are available for making polyclonal and monoclonal antibodies that are specific to a desirable polypeptide. For the production of monoclonal and polyclonal antibodies, various host animals can be immunized by injection with the peptide corresponding to any molecule of interest in the present invention, including but not limited to hamsters, rabbits, mice, rats, sheep, goats, *etc.* For preparation of monoclonal antibodies, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (See e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). These include, but are not limited to, the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, *Nature*, 256:495-497 (1975)), techniques using germ-free animals and utilizing technology such as that described in PCT/US90/02545, as well as the trioma technique, the human B-cell hybridoma technique (See e.g., Kozbor et al., *Immunol. Today*, 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). In some particularly preferred embodiments of the present invention, the present invention provides monoclonal antibodies.

Also contemplated are chimeric antibodies. As used herein, the term "chimeric antibody" contains portions of two different antibodies, typically of two different species. See, e.g.: U.S. Pat. No. 4,816,567 to Cabilly et al.; U.S. Pat. No. 4,978,745 to Shoemaker et al.; U.S. Pat. No. 4,975,369 to Beavers et al.; and U.S. Pat. No. 4,816,397 to Boss et al. Chimeric antibodies include monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is tetramer (H₂L₂) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a Hc region that aggregates (e.g., IgM H chain).

The invention also contemplates "humanized antibodies," i.e., chimeric antibodies that have constant regions derived substantially or exclusively from human antibody constant regions, and variable regions derived substantially or exclusively from the sequence of the variable region from a mammal other than a human. Humanized antibodies preferably have constant regions and variable regions other than the complement determining regions (CDRs) derived substantially or

exclusively from the corresponding human antibody regions and CDRs derived substantially or exclusively from a mammal other than a human. Thus, in one embodiment, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are generally made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a nonhuman immunoglobulin and all or substantially all of the FR residues are those of a human immunoglobulin sequence. The humanized antibody may also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Humanized antibodies may be generated using methods known in the art, e.g., U.S. Pat. No. 5,225,539 to Winter et al., including using human hybridomas (Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030 (1983)) or by transforming human B cells with EBV virus in vitro (Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96 (1985)). Additional methods include, for example, generation of transgenic non-human animals which contain human immunoglobulin chain genes and which are capable of expressing these genes to produce a repertoire of antibodies of various isotypes encoded by the human immunoglobulin genes (U.S. Pat. Nos. 5,545,806; 5,569,825 and 5,625,126). Humanized antibodies may also be made by substituting the complementarity determining regions of, for example, a mouse antibody, into a human framework domain (PCT Pub. No. WO92/22653).

Importantly, early methods for humanizing antibodies often resulted in antibodies with lower affinity than the non-human antibody starting material. More recent approaches to humanizing antibodies address this problem by making changes to the CDRs. See U.S. Patent Application Publication No. 20040162413. In some embodiments, the invention's humanized antibodies contain an optimized heteromeric variable region (e.g. that may or may not be part of a full antibody other molecule) having equal or higher antigen binding affinity than a donor heteromeric variable region, wherein the donor heteromeric variable region comprises three light chain donor CDRs, and wherein the optimized heteromeric variable region comprises: a) a light chain altered variable region comprising; i) four unvaried human germline light chain framework regions, and ii) three light chain altered variable region

CDRs, wherein at least one of the three light chain altered variable region CDRs is a light chain donor CDR variant, and wherein the light chain donor CDR variant comprises a different amino acid at only one, two, three or four positions compared to one of the three light chain donor CDRs (e.g. the at least one light chain donor CDR variant is identical to one of the light chain donor CDRs except for one, two, three or four amino acid differences).

Chimeric antibodies containing amino acid sequences that are fused to constant regions from human antibodies, or to toxins or to molecules with cytotoxic effect, are known in the art (e.g., U.S. Pat. Nos. 7,585,952; 7,227,002; 7,632,925; 7,501,123; 7,202,346; 6,333,410; 5,475,092; 5,585,499; 5,846,545; 7,202,346; 6,340,701; 6,372,738; 7,202,346; 5,846,545; 5,585,499; 5,475,092; 7,202,346; 7,662,387; 6,429,295; 7,666,425; and 5,057,313).

Antibodies that are specific for a particular antigen may be screened using methods known in the art (e.g., U.S. Pat. No. 7,202,346) and disclosed herein. For example, In the production of antibodies, screening for the desired antibody can be accomplished by radioimmunoassay, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (e.g., using colloidal gold, enzyme or radioisotope labels), Western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*

In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. As is well known in the art, the immunogenic peptide should be provided free of the carrier molecule used in any immunization protocol. For example, if the peptide was conjugated to KLH, it may be conjugated to BSA, or used directly, in a screening assay.

In one embodiment, the invention's antibodies are monoclonal antibodies produced by a hybridoma cell line. In a particular embodiment, the monoclonal antibody specifically binds to a MUC16 ectodomain polypeptide that comprises Polypeptide 1 (SEQ ID NO:01), as exemplified by the antibody selected from the group consisting of 9B11.20.16, 10A2, 2F4, 23D3, 30B1, and 31B2 (Tables 1 and 2). In a preferred embodiment, the antibody is 9B11.

In another embodiment, the monoclonal antibody specifically binds to a MUC16 ectodomain polypeptide that comprises Polypeptide 2 (SEQ ID NO:02), wherein the antibody is exemplified by 4H11.2.5, 13H1, 29G9, 9C9.21.5.13, 28F8, 23G12, 9C7.6, 11B6, 25G4, 5C2.17,

4C7, 26B2, 4A5.37, 4A2, 25H3, and 28F7.18.10 (Tables 1 and 2). In a preferred embodiment, the antibody is exemplified by 4H11.2.5, 4A5.37, 9C9.21.5.13, 28F7.18.10, 9C7.6, and 5C2.17.

In a further embodiment, the monoclonal antibody specifically binds to a MUC16 cytoplasmic domain polypeptide that comprises Polypeptide 3 CGVLVTTRRRKKEGEYNVQQQ
5 (SEQ ID NO:03), wherein the antibody is exemplified by 31A3.5.1, 19D1, 10F6, 22E10, 22F1, 3H8, 22F11, 4D7, 24G12, 19G4, 9A5, 4C2, 31C8, 27G4, and 6H2 (Tables 1 and 2). In a preferred embodiment, the antibody is 31A3.5.1.

In another embodiment, the monoclonal antibody specifically binds to a MUC16 extracellular domain polypeptide that comprises Polypeptide 4 KSYF SDCQVSTFRS
10 VPNRHHTGVD SLCNFSPL (SEQ ID NO:15), wherein the antibody is exemplified by 24B3 and 9C7 (Table 2).

The invention's antibodies and methods for their use (both diagnostic and therapeutic) are disease specific. "Specificity" of a method and/or molecule for disease, such as "specificity for cancer" which is interchangeably used with "cancer specificity", refers to the proportion (e.g.,
15 percentage, fraction, etc.) of negatives (i.e., healthy individuals not having disease) that are correctly identified, i.e., the percentage of healthy subjects who are correctly identified as not having disease. Specificity may be calculated according to the following equation:

Specificity = number of true negatives / (number of true negatives + number of false positives).
20

Thus, in some embodiments, the invention's compositions and/or methods have a "cancer specificity" greater than 50%, including any numerical value from 51% to 100%, such as 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%,
25 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%. While a 100% specificity is most desirable, i.e., not predicting anyone from the healthy group as having cancer, it is not necessary. Data herein demonstrate the invention's cancer specificity (Table 3).

In alternative embodiments, specificity is expressed (together with sensitivity) as a statistical measure of the performance of a binary classification test, such as using a Receiver
30 Operator Characteristic (ROC) curve". For any test, there is usually a trade-off between specificity and sensitivity. For example: in cancer screening tests of human subjects, it is undesirable to risk falsely identifying healthy people as having cancer (low specificity), due to the high costs. These costs are both physical (unnecessary risky procedures) and financial. This trade-off can be represented graphically using a ROC curve. "Receiver Operator Characteristic curve" and "ROC

curve" refer to a plot of the true positive rate (AKA sensitivity) versus true negative rate (AKA 1-specificity). The measured result of the test is represented on the x axis while the y axis represents the number of control (e.g., healthy) or case (e.g., cancer) subjects. For any given cut point (each point along the x axis) a sensitivity and specificity of the assay can be measured. The range of sensitivity and specificity for any given assay can range from 0% to 100%, depending on the selected cut point. For this reason, in some preferred embodiments, the AUC is used as the standard measure of an assay's specificity and/or sensitivity. The "area under the curve" ("AUC") for the ROC curve plot is equal to the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. Thus, AUC is a general measure of a tests ability to successfully discriminate between case (e.g., cancer) and control (e.g., healthy) subjects. Random chance would generate an AUC of 0.5. Therefore, in one embodiment, useful tests preferably have AUC's greater than 0.50, including any value from 0.51 to 1.00, such as from 0.55 to 1.00, from 0.60 to 1.00, from 0.65 to 1.00, from 0.70 to 1.00, from 0.75 to 1.00, from 0.80 to 1.00, from 0.85 to 1.00, from 0.90 to 1.00, from 0.95 to 1.00, and most preferably 1.00. AUC values greater than 0.50 include 0.51, 0.52, 0.52, 0.54, 0.55, 0.56, 0.57, 0.58, 0.59, 0.60, 0.61, 0.62, 0.63, 0.64, 0.65, 0.66, 0.67, 0.68, 0.69, 0.70, 0.71, 0.72, 0.73, 0.74, 0.75, 0.76, 0.77, 0.78, 0.79, 0.80, 0.81, 0.82, 0.83, 0.84, 0.85, 0.86, 0.87, 0.88, 0.89, 0.90, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, and 0.99.

The invention's antibodies and methods for their use (both diagnostic and therapeutic) are disease sensitive. "Sensitivity" of a method and/or molecule for disease, such as "sensitivity for cancer" which is interchangeably used with "cancer sensitivity," refers to the proportion (e.g., percentage, fraction, etc.) of positives (i.e., individuals having cancer) that are correctly identified as such (e.g. the percentage of people with cancer who are identified as having the condition). Sensitivity may be calculated according to the following equation; Sensitivity = number of true positives / (number of true positives + number of false negatives).

Thus, in some embodiments, the invention's compositions and/or methods have a "disease sensitivity," such as "cancer sensitivity," greater than 50%, including any numerical value from 51% to 100%, such as 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%. While a 100% sensitivity is most desirable (i.e., predicting all subjects from the cancer group as having cancer), it is not necessary.

In alternative embodiments, the invention's compositions and/or methods have a "disease sensitivity," such as "cancer sensitivity," equal to or lower than 50%, including any numerical

value from 0% to 50%, such as 1%, 2%, 3%, 4%, 6%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, and 49%.

- 5 In some embodiments, sensitivity is expressed (together with specificity) as a statistical measure of the performance of a binary classification test, such as using AUC of a ROC curve, as discussed above with respect to specificity.

D. Hybridoma Cell Lines

- 10 In addition to the invention's novel antibodies, the invention also provides hybridoma cell lines that produce these antibodies. "Hybridoma cell" refers to a cell line produced by fusing a specific antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture and for an absence of antibody chain synthesis. The antibodies produced by the hybridoma cell are all of a single specificity and are therefore monoclonal antibodies (in
15 contrast to polyclonal antibodies).

- In a particular embodiment, the invention provides hybridoma cell lines that produce a monoclonal antibody that specifically binds to a polypeptide, or antigenic portion thereof, selected from the group consisting of a) MUC16 ectodomain polypeptide (e.g., NFSPLAR RVDRVAIYEE FLRMTRNGTQ LQNFTLDRSS VLVDGYSPNR NEPLTGNSDL P (SEQ ID NO:17)), b)
20 MUC16 cytoplasmic domain polypeptide (e.g., VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18)), and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSL C (SEQ ID NO:19). The MUC16 polypeptide SEQ ID NO:18 is contained within LVTTRR RKKEGEYNVQ QQ (SEQ ID NO:20). Thus, SEQ ID NO:20 contains both a transmembrane domain amino acid (L) and a cytoplasmic domain portion VTTRR
25 RKKEGEYNVQ QQ (SEQ ID NO:18), i.e., the L is optional, as it is part of the transmembrane domain. The MUC16 polypeptide SEQ ID NO:18 is also contained within CGVLVTTRR RKKEGEYNVQ QQ (SEQ ID NO:03). Thus, SEQ ID NO:03 contains both a transmembrane domain portion (CGVL) and a cytoplasmic domain portion VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18), i.e., the CGVL is optional, as it is part of the transmembrane domain.

30

E. Conjugates Of The Invention's Antibodies Linked To Cytotoxic Agents And/Or Prodrugs

The invention contemplates conjugate antibodies. A "conjugate" antibody refers to an antibody of the present invention covalently linked to a cytotoxic agent and/or a prodrug of a cytotoxic agent.

"Cytotoxic agent" refers any agent that is capable of reducing the growth of, and/or killing, a target cell. A "prodrug" represents an analog of a cytotoxic agent that substantially lacks cytotoxic activity until subjected to an activation step. Activation steps may include enzymatic cleavage, a chemical activation step such as exposure to a reductant, or a physical activation step such as photolysis.

The covalent linkage between the invention's antibodies and the cytotoxic agent or prodrug can include cleavable linkages such as disulfide bonds, which may advantageously result in cleavage of the covalent linkage within the reducing environment of the target cell. Such conjugates are useful as tumor-cell specific therapeutic agents.

In one embodiment, the cytotoxic agent is a small drug molecule (Payne et al., U.S. Pat. No. 7,202,346). In another embodiment, the cytotoxic agent a maytansinoid, an analog of a maytansinoid, a prodrug of a maytansinoid, or a prodrug of an analog of a maytansinoid (U.S. Pat. Nos. 6,333,410; 5,475,092; 5,585,499; 5,846,545; 7,202,346). In another embodiment, the cytotoxic agent may be a taxane (see U.S. Pat. Nos. 6,340,701 & 6,372,738 & 7,202,346) or CC-1065 analog (see U.S. Pat. Nos. 5,846,545; 5,585,499; 5,475,092 & 7,202,346).

In another embodiment, the cytotoxic agent is exemplified by an auristatin, a DNA minor groove binding agent, a DNA minor groove alkylating agent, an enediyne, a duocarmycin, a maytansinoid, and a vinca alkaloid (U.S. Pat. No. 7,662,387).

In a further embodiment, the cytotoxic agent is an anti-tubulin agent (U.S. Pat. No. 7,662,387). In yet another embodiment, the cytotoxic agent is exemplified by dimethylvaline-valine-dolaisoleuine-dolaproine-phenylalanine-p-phenylenediamine (AFP), dovaline-valine-dolaisoleuine-dolaproine-phenylalanine (MMAF), and monomethyl auristatin E (MAE) (U.S. Pat. No. 7,662,387).

In an additional embodiment the toxic agent is exemplified by radioisotope emitting radiation, immunomodulator, lectin, and toxin (U.S. Pat. No. 6,429,295). In particular, the radioisotope emitting radiation is an alpha-emitter selected from the group consisting of ^{212}Bi , ^{213}Bi , and ^{211}At , or a beta-emitter selected from the group consisting of ^{186}Re and ^{90}Y , or a gamma-emitter ^{131}I (U.S. Pat. No. 7,666,425).

In an alternative embodiment, the toxin is exemplified by ricin, the A-chain of ricin, and pokeweed antiviral protein (U.S. Pat. No. 5,057,313).

In yet another embodiment, the cytotoxic agent is an anti-cancer drug selected from the group consisting of methotrexate, 5-fluorouracil, cycloheximide, daunomycin, doxorubicin,
5 chlorambucil, trenimon, phenylenediamine mustard, adriamycin, bleomycin, cytosine arabinoside or Cyclophosphamide (U.S. Pat. No. 5,057,13).

F. Detecting Muc16 Portions And Diagnostic Applications

The invention provides a method for detecting a disease that comprises overexpression of
10 MUC16 in a subject, wherein the method comprises a) providing i) a sample from a subject, and ii) any one or more of the invention's antibodies, b) contacting the sample with the antibody under conditions for specific binding of the antibody with its cognate antigen, and c) detecting an increased level of binding of the antibody to the sample compared to a control sample lacking the disease, thereby detecting the disease in the subject. Generic methods for detecting disease using
15 antibodies are known in the art (Payne et al., U.S. Pat. No. 7,202,346). The invention's methods are particularly useful in detecting cancer, such as ovarian cancer and breast cancer.

The invention's methods are not limited to a particular approach to detecting binding of the invention's antibodies to their antigens. In one embodiment, detecting binding to the invention's antibodies typically involves using antibodies that are labeled with a detectable moiety, such as
20 radioisotope (e.g., ^3H , ^{14}C , ^{32}P , ^{35}S , and/or ^{125}I), fluorescent or chemiluminescent compound (e.g., fluorescein isothiocyanate, rhodamine, and/or luciferin) and/or an enzyme (e.g., alkaline phosphatase, beta-galactosidase and/or horseradish peroxidase).

Methods for conjugating antibodies to a detectable moiety are known in the art (e.g., Hunter, et al., Nature 144:945 (1962); David, et al., Biochemistry 13:1014 (1974); Pain, et al., J.
25 Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

Thus, the invention's antibodies may be employed in immunoassays, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays, including immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), and Western blots.

30 For example, with respect to immunohistochemical detection, data herein demonstrate that antibody 4H11 is useful in detecting high-grade ovarian serous carcinoma, lobular cancer (28), and a subset of ovarian carcinomas that are negative with OC125 and that retain cytoplasmic and extracellular portions of the MUC16 glycoprotein.

The antibodies of the invention also are useful for radiographic *in vivo* imaging, wherein an antibody labeled with a detectable moiety such as a radio-opaque agent or radioisotope is administered to a subject, preferably into the bloodstream, and the presence and location of the labeled antibody in the host is assayed. This imaging technique is useful in the staging and
5 treatment of malignancies.

The invention's antibodies are additionally useful as affinity purification agents. In this process, the antibodies are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art, to capture and purify molecules that contain antigens that specifically bind to the invention's antibodies.
10

G. Therapeutic Applications

The invention provides methods for treating a disease that comprises overexpression of MUC16, comprising administering to a subject having the disease a therapeutically effective amount of any one or more of the invention's antibodies. Generic methods for treating disease with
15 antibodies are known in the art (Payne et al., U.S. Pat. No. 7,202,346). The invention's methods are particularly useful in treating cancer, such as ovarian cancer and breast cancer. These methods are also applicable to primary cancer, metastatic cancer, and recurrent cancer.

The term "administering" to a subject means providing a molecule to a subject. This may be done using methods known in the art (*e.g.*, Erickson et al., U.S. Patent 6,632,979; Furuta et al., U.S.
20 Patent 6,905,839; Jacobsen et al., U.S. Patent 6,238,878; Simon et al., U.S. Patent 5,851,789). The invention's compositions may be administered prophylactically (*i.e.*, before the observation of disease symptoms) and/or therapeutically (*i.e.*, after the observation of disease symptoms). Administration also may be concomitant with (*i.e.*, at the same time as, or during) manifestation of one or more disease symptoms. Also, the invention's compositions may be administered before,
25 concomitantly with, and/or after administration of another type of drug or therapeutic procedure (*e.g.*, surgery). Methods of administering the invention's compositions include, without limitation, administration in parenteral, oral, intraperitoneal, intranasal, topical and sublingual forms. Parenteral routes of administration include, for example, subcutaneous, intravenous, intramuscular, intrasternal injection, and infusion routes.

30 In one embodiment, the invention's compositions comprise a lipid for delivery as liposomes. Methods for generating such compositions are known in the art (Borghouts et al. (2005). *J Pept Sci* 11, 713-726; Chang et al. (2009) *PLoS One* 4, e4171; Faisal et al. (2009) *Vaccine* 27, 6537-6545; Huwyler et al. (2008) *Int J Nanomedicine* 3, 21-29; Song et al. (2008) *Int J Pharm* 363, 155-161; Voinea et al. *J Cell Mol Med* 6, 465-474).

Antibody treatment of human beings with cancer is known in the art, for example in U.S. Pat. Nos. 5,736,137; 6,333,410; 5,475,092; 5,585,499; 5,846,545; 7,202,346; 6,340,701; 6,372,738; 7,202,346; 5,846,545; 5,585,499; 5,475,092; 7,202,346; 7,662,387; 7,662,387; 6,429,295; 7,666,425; 5,057,313.

5 The invention's antibodies may be administered with pharmaceutically acceptable carriers, diluents, and/or excipients. Examples of suitable carriers, diluents and/or excipients include: (1) Dulbecco's phosphate buffered saline, pH about 7.4, containing about 1 mg/ml to 25 mg/ml human serum albumin, (2) 0.9% saline (0.9% w/v NaCl), and (3) 5% (w/v) dextrose.

10 The invention's antibodies are typically administered in a therapeutic amount. The terms "therapeutic amount," "pharmaceutically effective amount," "therapeutically effective amount," and "biologically effective amount," are used interchangeably herein to refer to an amount that is sufficient to achieve a desired result, whether quantitative or qualitative. In particular, a pharmaceutically effective amount is that amount that results in the reduction, delay, and/or elimination of undesirable effects (such as pathological, clinical, biochemical and the like) that are
15 associated with disease. For example, a "therapeutic amount that reduces cancer" is an amount that reduces, delays, and/or eliminates one or more symptoms of cancer.

For example, specific "dosages" of a "therapeutic amount" will depend on the route of administration, the type of subject being treated, and the physical characteristics of the specific subject under consideration. These factors and their relationship to determining this amount are
20 well known to skilled practitioners in the medical, veterinary, and other related arts. This amount and the method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors, which those skilled in the art will recognize. The dosage amount and frequency are selected to create an effective level of the compound without substantially harmful effects.

25 When present in an aqueous dosage form, rather than being lyophilized, the antibody typically will be formulated at a concentration of about 0.1 mg/ml to 100 mg/ml.

Depending on the type and severity of the disease, about 0.015 to 15 mg of antibody/kg of patient weight is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. For repeated administrations
30 over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs.

The methods of the present invention can be practiced *in vitro*, *in vivo*, or *ex vivo*.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1**5 Materials And Methods**

The following is a brief description of the exemplary materials and methods used in the subsequent Examples.

Cell Cultures:

OVCAR3, SKOV3, and A2780 cell lines were obtained through the American Type Culture
 10 Collection (ATCC, Manassas, VA) and sustained in culture according to the ATCC literature. For the creation of MUC16+ transfected cell lines, the carboxyterminus portion of the MUC16 cDNA was introduced as green fluorescent protein fusion proteins using the Vitality® phrGFP vector expression system (Stratagene, La Jolla, CA). Stable cell lines were selected using geneticin (G418, Invitrogen, Grand Island, NY) in their respective culture media and isolated by expression of Green Fluorescence
 15 Protein. Stable transfectants were routinely maintained in G418 in their culture media respectively. The Δ MUC16^{c114} transfectants have cell surface expression of MUC16 protein from the putative cleavage site to the carboxyterminus (AA 1776 to 1890) (12).

Monoclonal Preparation:

Using the MUC16 sequence, peptide sequences encoding elements of the Δ MUC16^{c114} amino
 20 acid sequence were synthesized at the Memorial Sloan-Kettering Cancer Center (MSKCC) Microchemistry Core Facility. The inventors synthesized 3 polypeptides (Figure 1) and modified Polypeptide 1 and Polypeptide 2 with a cysteine at the N-terminus for better conjugation to KLH. Equal concentrations of the KLH-conjugated peptides were mixed and then used as the immunogen for 5 BALB/c mice. The inventors selected 1 of the 5 mice whose serum showed the highest reactivity to
 25 individual peptides by ELISA, and the MSKCC Monoclonal Antibody Core Facility performed the fusion and selected the antibodies using standard protocols. After 10 days of fusion, supernatants were selected and screened for reactivity by ELISA against the individual synthetic peptides.

ELISA:

Sandwich ELISA was performed to see the positivity of the antibodies to individual peptides
 30 and GST- Δ MUC16^{c114} fusion protein following routine core facility protocol for ELISA assay.

FACS Analyses:

Adherent target cells were removed by 0.05% Trypsin and 0.1% EDTA, washed, and counted by a hemocytometer. Cells were distributed into multiple Eppendorf tubes with at least $0.5-1 \times 10^6$ cells per tube. Cells were washed with phosphate buffered saline (PBS) containing 1% FCS and 0.025% Sodium Azide (FACS buffer). For internal FACS staining, cells in the Eppendorf tubes were permeabilized with 1:10 diluted FACS Permeabilizing Solution 2 (BD BioSciences, San Jose, CA) for 10 minutes at room temperature and then washed twice with ice cold FACS buffer. Then they were incubated either without (for second antibody control) or with 1 μ g/tube of bioreactive supernatants of mouse MUC16 monoclonals for 30 minutes on ice. For surface FACS staining, cells were incubated either without (for second antibody control) or with 1 μ g/tube of bioreactive supernatants of MUC16 monoclonals (9B11.20.16, 9C9.21.5.13 and 4H11.2.5), Mouse anti-human OC125 (M3519), Mouse anti-human M11 (M3520) (DakoCytomation, Dako North America Inc., Carpinteria, CA) or VK8 (kindly provided by Dr. Beatrice Yin and Dr. Ken Lloyd, MSKCC, New York, NY) for 30 minutes on ice. Cells in Eppendorf tubes were also surface stained with 1 μ g/tube of non-specific isotype matched control mouse antibodies (13C4 for IgG1 and 4E11 for IgG2b monoclonals obtained from MSKCC Monoclonal Core Facility) and incubated on ice for 30 minutes. All cells were washed three times with FACS buffer. Cells were incubated with 1 μ g/tube of second antibody Goat anti-mouse IgG1-PE or IgG2b-PE for 30 minutes on ice and then washed three times with FACS buffer. The cells were analyzed by a FACScalibur™ machine at the MSKCC Flow Cytometry Core Facility.

Western Blot Analysis:

Stable cell lines were cultured in 10 cm dishes in their respective culture media and incubated with 5% CO₂ at 37°C for 3 days. They were washed twice with ice cold PBS to remove the serum-containing media. Adherent cells were scraped with 1-2 ml of ice cold PBS, and the cells were spun down in an Eppendorf tube at 4°C in an Eppendorf centrifuge. Supernatant was discarded, and the cells were lysed with 0.2 ml of modified Ripa lysis buffer (20 mM Tris-HCL; pH 7.4; 150 mM NaCl; 1% NP-40; 1 mM Na₃VO₄; 1 mM PMSF; 1 mM DTT; 10 μ g/ml leupeptin; and 10 μ g/ml aprotinin) for 30 minutes on ice and spun at 4°C for 10 minutes. The soluble solution was separated into a tube and the debris pellet was discarded. Protein concentration was measured using the Bio-Rad Protein Assay (BioRad Laboratories, Hercules, CA). Equal amounts of proteins (GST-MUC16-CD-fusion protein or stable cell line extracts) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane using a BioRad transfer apparatus in a cold room at 4°C. The membranes were blocked with 3% bovine serum albumin (BSA) in PBS with 0.1% TWEEN® 20 (PBST) at 4°C overnight. Membranes were probed with primary antibody (1:1000

dilution) for 1 hr at room temperature and then washed three times with PBST. Then the membranes were stained with corresponding second antibody, anti-Mouse IgG Horse Radish Peroxidase (HRP) linked whole antibody from sheep (GE Healthcare, UK) (1:5000 dilution), for 1 hr at room temperature. Membranes were washed three times with PBST and developed with a Western Lightning®
 5 chemiluminescence reagent (ECL, Perkin Elmer, Waltham, MA) for 1-5 minutes at room temperature, and the signals were developed on Kodak® BioMax® Film.

Binding and internalization studies with monoclonal antibodies and OVCAR3 and SKOV3 stable transfectants:

Purified monoclonal antibodies were labeled with ^{131}I using the iodogen method and purified by
 10 size exclusion chromatography (22). Saturation binding studies were performed with radiolabeled antibodies using substrates of intact OVCAR-3 cells. Briefly, 10 test solutions were prepared (in triplicate) and they contained increasing amounts of the radioiodinated antibodies, 3-500 000 cells in a total volume of 500 μL of PBS (0.2 % BSA; pH 7.4). The cells were isolated by rapid filtration through a glass fiber membrane and washed with ice cold tris buffered saline. Cells were counted in a gamma
 15 counter with standards of total activity added. For each concentration of radiolabeled antibody, non-specific binding was determined in the presence of 100 nM of the unmodified antibody. The data were analyzed with a least squares regression method (Origin, Microcal, Software Inc., Northampton, MA) to determine the K_d and B_{max} values, and a Scatchard transformation was performed.

Antibody cell internalization studies were performed with ^{131}I -4H11 and ^{131}I -OC125
 20 monoclonal antibodies and SKOV3-phrGFP- $\Delta\text{MUC16}^{\text{c334}}$ stable transfected cells. Briefly, radiolabeled antibody (370 MBq/mg, 100 kcpm) in 2 mL of medium was added to SKOV3 cells plated in a 6-well plate. The plates were incubated at 37°C for up to 24 hours. At various time points, the medium was removed from three wells and the cells washed with 2 x 2 mL PBS. Cell surface bound activity was then stripped and collected with 2 x 2 mL of an ice cold acid wash (100 mM acetic acid 100 mM
 25 glycine; pH 3.0). The cells were then dissolved with 2 x 1 ml 1 M NaOH and collected. At the end of the study all samples were counted with a gamma counter together with standards, representing the initial amount of radioactivity added. All the media samples were analyzed by ITLC-SG with mobile phases of 5% TCA to determine unbound ^{131}I .

Tissue microarray (TMA):

30 Tissue microarrays were either constructed within our institution or bought from a commercial laboratory if not available internally. Briefly, core-needle biopsies of pre-existing paraffin-embedded tissue were obtained from the so-called donor blocks and then relocated into a recipient paraffin-arrayed “master” block by using the techniques by Kononen et al. and subsequently modified by Hedvat et al

(23-24). A manually operated Tissue Arrayer MTA-1 from Beecher Instruments Inc. (Sun Prairie, WI) was used to produce sample circular spots (cores) that measured 0.6 to 1.0 mm in diameter. The cores were arrayed 0.3 to 0.4 mm apart from each other. A layer of control tissues was strategically laid around the actual tissue microarrays in order to avoid edging effects. The specific composition of each tissue microarray is delineated below. Slides of tissue microarrays for ovarian cancer, prostate cancer, adenocarcinoma of the lung, mucinous neoplasms of the pancreas, and invasive ductal and invasive lobular breast carcinoma were prepared by cutting 4 μ m sections from formalin-fixed paraffin-embedded tissue. Normal adult and fetal tissue microarrays were obtained from a commercial source (Biomax, US). OVCAR3 cells were used as positive controls.

10 **Immunohistochemistry:**

Immunohistochemistry was performed on the tissue microarrays with both standard OC125 (Ventana, Tuscon, AZ) and the novel monoclonal antibodies. Sections of the tissue microarrays were cut at 4 microns, placed on Superfrost™/Plus microscope slides (Fisher brand) and baked in a 60° oven for at least 60 minutes. The slides were then deparaffinized and hydrated to distilled water, soaked in citrate buffer at pH 6.00 for 30 minutes at 97° C, washed in running water for 2-5 minutes, incubated for 5 minutes in 3% hydrogen peroxide diluted in distilled water. Slides were washed in distilled water for 1 minute, transferred to a bath of phosphate buffered saline (PBS), pH 7.2, for two changes of 5 minutes each and placed in 0.05% BSA diluted in PBS for a minimum of 1 minute. After drying around tissue sections, normal serum was applied at a 1:20 dilution in 2% BSA/PBS and incubated for a minimum of 10 minutes at room temperature in a humidity chamber. The serum was then suctioned off without allowing the sections to dry, and approximately 150 λ of novel antibody at a dilution of 1:1000 was placed on the tissue. The slide was incubated overnight (approximately 15-18 hours) at 4° C in a humidity chamber. Primary antibody was washed off using three changes of PBS for 10 minutes each. Secondary antibody, biotinylated α -

mouse from Vector laboratories (Burlingame, Ca), was applied at 1:500 dilution in 1% BSA/PBS and incubated for 45-60 minutes at room temperature in humidity chamber. The antibody was washed off again using three changes of PBS as above. Slides were then transferred to a bath of diaminobenzidine (DAB), diluted in PBS for 5-15 minutes. The slides were then washed in tap
5 water for 1 minute, counterstained using Harris modified hematoxylin (Fisher), decolorized with 1% acid alcohol and blue in ammonia water, dehydrated with 3 changes each of 95% ethanol, 100% ethanol and xylene for 2 minutes each and coverslipped with permanent mounting medium.

Immunohistochemistry scoring:

10 Commercially available antibodies, such as OC125 and M11, target complex glycosylation-dependent epitopes. Our hypothesis is that glycosylation may be tissue specific; therefore, it was important to examine the utility of the peptide-directed antibodies in paraffin-fixed tissues and survey the prevalence of MUC16 expression. The three candidate antibodies, 4H11, 9C9 and 4A5, were characterized using OVCAR3 cell line pellets. Of the three, the 4H11 antibody showed the
15 strongest, most diffuse and consistent staining pattern at multiple dilutions, with the least amount of background staining and, therefore, was optimized for use in human tissues in the pathology core facility.

Using 4H11, the inventors stained and scored positivity using tissue microarrays from high-stage, high-grade ovarian serous carcinomas (Figure 2), these tumors being the most common type
20 of ovarian cancer, representing approximately 80-85% of all ovarian carcinomas in Western industrialized nations (25). To test the specificity of the novel antibody, the inventors also stained tissue microarrays of cancers of the prostate, lung, breast, and pancreas and compared their staining intensities with that of OC125 monoclonal antibody (Figure 6A-D). To determine whether there would be any cross-reactivity with normal human tissues, the antibodies were also tested on normal
25 human adult and fetal TMAs.

All of the stained sections were reviewed by a reference pathologist (KJP). A subset of cores for which there was equivocal staining was also independently scored by a second pathologist (RAS) to ensure consistency in scoring methods. Only cytoplasmic and/or membranous staining was considered positive. If a portion of the cell showed membranous staining, that was considered
30 partial staining. A scoring system was devised to provide a semiquantitative assessment of staining distribution and intensity in individual cores. At the same time, it was designed to be useful for comparing the staining distribution and intensity between OC125 and the novel antibodies. The score incorporated the percentage of cells, the intensity and pattern of the staining according to the following standards: score 0: no staining; score 1: <5% strong or weak; score 2: 5-50% strong or

weak; score 3: 51-75% strong or 51-100% weak; score 4: 76-99% strong; and score 5: 100% strong staining (Figure 3). The pathologist first reviewed all tissue microarrays stained with OC125 and scored each core. Then the same cores stained with the novel antibodies were scored 1 to several days after OC125 without reference to the previous results. Direct comparison of the scoring
5 between the stains for each core was made only after all of the scoring was completed. The same process was used for all non-ovarian tissue microarrays. After comparison, core staining was determined to be concordant, equivocal, or discordant based on the point differentials. Concordant cores differed by 0 to 1 point, equivocal cores differed by 2 points, and discordant cores differed by 3 to 5 points. The one exception to this rule was when the difference of 1 point was between a score
10 of 0 and 1, in which case, the differences were considered equivocal. This was in order to truly separate negative cases from even focally positive ones.

EXAMPLE 2

Generation and characterization of anti-MUC16 monoclonal antibodies

15 MUC16-directed monoclonal antibodies were isolated by ELISA-based screening using both the individual peptides and recombinant GST-ΔMUC16^{c114} protein followed by sequential subcloning for single cell clones.

Table 1A

5

42

Table 2: Antibodies specific for exemplary portions of MUC16

1. Muc16 Polypeptide 1:

5 14394 14410 (MUC16 sequence)
 NFSPLARRVDRVAIYEE (SEQ ID NO:01) 17 aa

Mouse monoclonals which are specific to this peptide are:

10 9B11.20.16 (IgG1)
 10A2 (IgG1, IgM)
 2F4 (IgG1, IgM)
 23D3 (IgG1, IgG2b)
 30B1 (IgG1)
 31B2 (IgM)

15

2. Muc16 Polypeptide 2:

20 14425 14442 (MUC16 sequence)
 TLDRSSVLVDGYSPNRNE (SEQ ID NO:02) 18 aa

Mouse monoclonals which are specific to this peptide are:

25 4H11.2.5 (IgG2b) 13H1 (IgG1) 29G9 (IgG1)
 9C9.21.5.13 (IgG2b) 28F8 (IgG1, IgM) 23G12 (IgG1, IgG2a)
 9C7.6 (IgG1) 11B6 (IgM) 25G4 (IgG1, IgM)
 5C2.17 (IgG1) 4C7 (IgG1) 26B2 (IgG1, IgG2b, IgM)
 4A5.37 (IgG1) 4A2 (IgG1) 25H3 (IgG1, IgM)
 28F7.18.10 (IgG1)

30

3. Muc16 Polypeptide 3 (SEQ ID NO:03)

35 14472 14492 (MUC16 sequence)
 CGVLVTTRRRKKEGEYNVQQQ 21 aa

Mouse monoclonals which are specific to this peptide are:

40 31A3.5.1 (IgG1) 19D1 (IgG2b) 10F6 (IgG1)
 22E10 (IgG2b) 22F1 (IgG2b, IgM) 3H8 (IgG1, IgM)
 22F11 (IgM) 4D7 (IgG3) 24G12 (IgG1, IgM)
 19G4 (IgG1, IgM) 9A5 (IgM)
 4C2 (IgG1, IgM) 31C8 (IgG2b)
 27G4 (IgM) 6H2 (IgG1, IgM)

45 14452 14475
 FWAVILIGLAGLLGLITCLICGVL (SEQ ID NO:14) is Transmembrane region 24 aa

4. Muc16 Polypeptide 4 (SEQ ID NO:15) containing a cysteine loop polypeptide (SEQ ID NO:19):

14367

14398 (MUC16 sequence)

KSYFSDCQVSTFRSVPNRHHTGVDSL CNFSPL (SEQ ID NO:15)

32 aa

5

Mouse monoclonals which are specific to this peptide are:

24B3 (IgM)

9C7 (IgM)

4F12	IgM kappa
6H6	IgM kappa
25C2	IgM kappa
6E8	IgM kappa
2A3	IgM, IgG1, IgG2b, kappa
2G4	IgM, IgG1, kappa
4C8	IgM, kappa
2A6	IgG1 kappa
24G12	IgG1 kappa
15D5	IgG1 kappa
6E2	IgM, IgG1, IgG3, IgG2a, kappa
7E6	IgM, kappa, lambda
7G11	IgM kappa
20C3	IgG1, IgG2b
9A3	IgM kappa
15B6	IgM kappa
19D3	IgM kappa
5H8	IgM, IgG1, IgG2b, kappa
24A12	IgM kappa
10	
2D10	IgG3, IgM kappa
5B2	IgM, IgG3, IgG2b, IgG2a, IgG1, kappa
8B6	IgG2a, IgG3, kappa
5A11	IgM, kappa
7D11	light kappa only
9F10	IgM, kappa
15D10	IgM, kappa
18D2	IgM, kappa
13A11	IgM, kappa

1A9	IgM, kappa
3B2	IgM, kappa
24F6	IgM, kappa
24E4	IgM, kappa
5A1	IgG2a, IgM, kappa
7B9	IgM, kappa
22F4	IgM, kappa

The identified monoclonal antibodies are listed in Table 1A and Table 2. Each of the selected monoclonal antibodies was reactive against GST- Δ MUC16^{c114}. The commercial MUC16-directed antibodies (OC125, M11, or VK8) did not bind to GST- Δ MUC16^{c114} in ELISA or Western blotting. The clones were tested in FACS against OVCAR3 ovarian cancer cells and in Western blot analysis against GST- Δ MUC16^{c114} (Table 1B), and selected purified monoclonal antibodies were isolated.

The inventors used the OVCAR3 wild type and the SKOV3 cells transduced with phrGFP- Δ MUC16^{c114} to characterize the selected antibodies by FACS analysis. All of the selected monoclonal antibodies bound to both cell lines while commercial VK8, M11 and OC125 antibodies bound to the OVCAR3 cells but not to the SKOV3-phrGFP- Δ MUC16^{c114} cell line. The antibodies against Polypeptide 3 required permeabilization since it is an internal epitope (Figure 7).

Western blot analysis using the GST- Δ MUC16^{c114} purified protein showed strong binding with 4H11 and 9C9 antibodies (Figure 4A), while the other selected antibodies showed less binding. The SKOV3-phrGFP- Δ MUC16^{c114} transfectant was also positive by Western blot analysis using 4H11 and 9C9 antibodies (Figure 4B). As before, the commercial antibodies did not interact with the GST- Δ MUC16^{c114} purified protein or cell lysates of the SKOV3-phrGFP- Δ MUC16^{c114} cell line.

The binding of six monoclonal antibodies against OVCAR3 MUC16 were examined in affinity binding studies. Three antibodies—9C7, 5C2 and 28F7—showed only modest levels of binding compared to the nonspecific binding of these antibodies to the OVCAR3 cells, which carry large numbers of MUC16 binding sites. In contrast, 4H11, 9C9, and 4A5 monoclonal antibodies showed highly specific binding affinity, as shown in Figure 5A, with binding affinities of 6.8-8.6 nM against the cell surface epitopes of OVCAR3 cells. The inventors also examined the internalization of antibody bound to cell surface MUC16 protein. The inventors examined internalization in the transfected SKOV3-phrGFP- Δ MUC16^{c334} cell line which bears the carboxy

terminus of MUC16, including the 4H11 epitope and a single degenerate tandem repeat sequence to interact with the OC125 antibody. The commercial antibodies OC125, M11, and VK8 all bind to the cell surface of this transduced cell line. The ¹³¹I-labeled 4H11 showed rapid internalization at a high level, whereas ¹³¹I-labeled OC125 antibody was internalized at a much lower rate (Figure 5B).

5

EXAMPLE 3

Immunohistochemistry results:

Given their highly specific binding affinities, the antibodies 9C9, 4A5, and 4H11 were characterized for utility in immunohistochemistry using OVCAR3 cell lines. Of the three, the 4H11 antibody was selected to be optimized for use in human tissues based on its robust, sensitive and specific staining pattern as compared to the other two antibodies.

10

A. Ovary

Two high-stage, high-grade ovarian serous carcinoma tissue microarray slides composed of 419 cores, representing primary, metastatic and recurrent tumors from 40 patients were stained with both OC125 and 4H11 monoclonal antibodies (Figure 2). The OC125 tissue microarrays showed 279 (66%) cores with 3-5 staining, 99 (24%) with 1-2 staining, and 41 (10%) with no staining. The 4H11 tissue microarrays showed 236 (56%) with 3-5 staining, 91 (22%) with 1-2 staining, and 92 (22%) with no staining. The two antibodies were concordant in 233 (56%) cores, equivocal in 161 (38%), and discordant in 25 (6%). Of the 25 discordant cores, 12 (48% of discordant cases, 3% of all cases) showed greater 4H11 positivity than OC125. Nine were discordant by a difference of 4 points, and 3 were discordant by a difference of 5 points. There was a total of 186 discordant and equivocal cores together, 48 (26%) of which showed greater staining with 4H11 than OC125. The staining pattern of both 4H11 and OC125 was cytoplasmic and membranous, although the membranous pattern of OC125 was stronger and better defined than 4H11 in the majority of cases. Discordant cases demonstrated higher levels of 4H11 than other cases.

20

25

B. Breast Cancer

A variety of other tissues were also examined for 4H11 staining to test the antibody's specificity. Of the 50 cores of invasive ductal carcinomas of the breast (number of patients unavailable), only 2 (4%) showed a score of 4 or greater 4H11 staining and none had scores of 3-5 for OC125 staining. The staining pattern with OC125 was mostly apical/luminal with some granular cytoplasmic staining. Some tumors with intracytoplasmic lumina also picked up the OC125 stain. 4H11 showed a more diffuse cytoplasmic blush without membranous accentuation.

30

In contrast, the invasive lobular breast carcinoma tissue microarray (composed of 179 cores with viable tumor, number of patients unavailable) had frequent MUC16 staining with 4H11. In this tissue microarray, 168 cores (94%) showed no staining for OC125, 5 (3%) showed 1-2 staining, and only 6 (3%) showed a staining intensity of 3. 4H11 staining was different in its distribution pattern, with 49 (27%) showing no staining, 81 (45%) showing 1-2 staining, and 49 (27%) showing 3-4 staining. Neither OC125 nor 4H11 had cores with a staining intensity of 5. The staining pattern was of cytoplasmic, luminal/membranous, or intraluminal for both OC125 and 4H11. The intraluminal pattern was strong and intense for both stains and highlighted the intracytoplasmic lumen that is commonly present in lobular carcinomas. The concordance rates were 34% concordant, 43% equivocal, and 23% discordant. Of the equivocal and discordant cases, there was none in which the OC125 was greater than the 4H11. All 42 discordant cases and 76 of 77 equivocal cases had 4H11 greater than OC125. There was also focal luminal staining with 4H11 in benign breast ducts and lobular carcinoma in situ.

15 C. Lung, pancreatic and prostatic adenocarcinomas

Tumors from other organs were not reactive with either antibody. The lung adenocarcinoma TMA had 237 cores from 86 patients containing viable tumor. In the pancreatic TMA there were 92 cores from 21 patients containing pancreatic mucinous tumors, including intraductal papillary mucinous neoplasms (IPMN) and invasive ductal carcinomas. In the prostate cancer TMA there were 169 cores (number of patients not available). None of these cancer tissue microarrays had significant binding to either OC125 or 4H11. This information is summarized in Table 3.

Table 3. Staining intensity of OC125 as compared to 4H11 in tissue microarrays

Site	OC125 vs. 4H11 staining intensity score (%)					
	0		1-2		3-5	
	OC125	4H11	OC125	4H11	OC125	4H11
Ovary high grade serous	10	28	24	22	66	56
Breast invasive ductal	68	78	32	18	0	4
Breast invasive lobular	94	27	3	45	3	27
Lung adenocarcinoma	63	77	24	18	13	5
Pancreas mucinous neoplasms	98	88	2	10	0	2
Prostate adenocarcinoma	0	0	0	0	0	0

Score 0: 0% staining; 1: <5% strong or weak; 2: 5-50% strong or weak; 3: 51-75% strong or 51-100% weak; 4: 76-99% strong; 5: 100% strong

D. Normal Tissues

There was no staining with OC125 or 4H11 in normal adult colon, rectum, ectocervix, small intestine, ovary, liver, pancreatic ducts, spleen, kidney, and skin. OC125 and 4H11 both stained endocervical glands (OC125 luminal, 4H11 weak cytoplasmic), esophageal glands (luminal),
5 bronchial epithelium (OC125 luminal, 4H11 intracytoplasmic granules), and thymic corpuscles (cytoplasmic). 4H11 demonstrated weak to moderate staining of the gastric glands, particularly at the crypts, with an intracytoplasmic granular pattern. Other organs that showed punctuate intracytoplasmic staining with 4H11 only were prostate, seminiferous tubules of the testes, and the islet cells of the pancreas. The staining in the pancreatic islets cells was particularly strong and
10 consistent. There was also nonspecific staining of liver, kidney and brain with 4H11. There were no cases that stained with OC125 and not 4H11.

Similarly, there was no staining with either OC125 or 4H11 in fetal heart, gallbladder, colon, small intestine, liver, rectum, adrenal, thyroid, spleen, skin, bone, epididymis, brain, lung, muscle, smooth muscle, kidney, eye, umbilical cord, and placenta. OC125 only stained thymic
15 corpuscles in a pattern similar to that in adult tissue. 4H11 stained both fetal pancreatic endocrine cells and endocervical glands in a similar pattern to that of their adult counterparts. Islet cells showed a granular cytoplasmic pattern, and endocervical glands showed a linear luminal pattern, which was more similar to the OC125 pattern in the adult tissue.

20 EXAMPLE 4

Successful eradication of established peritoneal ovarian tumors in SCID-Beige mice following adoptive transfer of T cells genetically targeted to the MUC16 antigen.

Purpose: Most patients diagnosed with ovarian cancer will ultimately die from their disease. For this reason, novel approaches to the treatment of this malignancy are needed.
25 Adoptive transfer of a patients own T cells, genetically modified *ex vivo* through the introduction of a gene encoding an chimeric antigen receptor (CAR), an artificial T cell receptor, targeted to a tumor associated antigen, is a novel and promising approach to cancer therapy applicable to the treatment of ovarian cancer.

Experimental design: We have generated several CARs targeted to the retained
30 extracellular domain of MUC16, termed MUC-CD, an antigen highly expressed on a majority of ovarian carcinomas. We investigate the *in vitro* biology of human T cells retrovirally transduced to express these CARs by co-culture assays on artificial antigen presenting cells (AAPCs) generated from NIH3T3 fibroblasts genetically modified to express the target MUC-CD antigen, as well as by cytotoxicity assays utilizing the human OV-CAR3(MUC-CD) ovarian tumor cell line and primary

patient tumor cells. Finally, we assess the *in vivo* anti-tumor efficacy of MUC-CD targeted T cells in a SCID-Beige orthotopic, xenogeneic OV-CAR3(MUC-CD) murine tumor model.

Exemplary sequences used in this work are in Figure 17-19.

Results: CAR modified MUC-CD targeted T cells derived from both healthy donors and ovarian cancer patients exhibited efficient *in vitro* cytolytic activity against both human ovarian cell lines as well as primary ovarian carcinoma cells. MUC-CD targeted T cells may be further expanded *ex vivo* through multiple cycles of co-culture on 3T3(MUC-CD/B7.1) AAPCs. Expanded MUC-CD targeted T cells infused into SCID-Beige mice bearing intraperitoneal human OV-CAR3(MUC-CD) tumors either delayed progression or fully eradicated tumor even in the setting of advanced disease.

Conclusion: These promising pre-clinical studies justify further investigation of MUC-CD targeted T cells as a potential therapeutic approach in the clinical setting treating patients with high risk MUC-16⁺ ovarian carcinomas.

15 INTRODUCTION

Ovarian cancer is the sixth most common cancer worldwide and the seventh leading cause of cancer-related deaths in women (1, 2). Despite multimodality therapy with surgery and chemotherapy, most patients with ovarian carcinomas have a poor prognosis. For this reason, alternative approaches to treating this disease are urgently needed.

20 Infusion of a patient's own T cells genetically targeted *ex vivo* to antigens expressed on the surface of tumor cells is a promising novel approach to the adoptive immunotherapy of cancer, and one which has only recently been explored in earnest in the clinical setting. T cells may be genetically modified to target tumor associated antigens through the retroviral introduction of genes encoding artificial T cell receptors termed chimeric antigen receptors (CARs). Genetic engineering
25 of T cells to express artificial T cell receptors that direct cytotoxicity toward a tumor cell presents a means to enhance immune recognition and elimination of cancer cells. CARs are most commonly composed of a single chain fragment length antibody (scFv), derived from a murine monoclonal antibody targeting a given tumor associated antigen, fused to a transmembrane domain (typically CD8, CD28, OX-40, and 4-1BB), fused to the TCR ζ chain cytoplasmic signaling domain (3-13).
30 When used to reprogram T-cell specificity, these fusion receptors permit recognition of native antigen. When expressed by the T cells, the resulting construct, upon engagement with the targeted antigen, induces T cell activation, proliferation, and lysis of targeted cells. These fusion receptors transduce a functional antigen-dependent co-stimulatory signal in primary T cells, permitting sustained T-cell proliferation when both endogenous TCR and a chimeric receptor for stimulatory

signaling are engaged. To date, preclinical studies utilizing CAR-modified T cells have demonstrated promising results in a wide variety of malignancies (3, 4, 11, 14-18). More recently this approach been investigated clinically in the form of phase I trials (6, 19-21). These genetic approaches offer a means to enhance immune recognition and elimination of cancer cells.

5 Ovarian carcinomas appear to be relatively immunogenic tumors capable of inducing an endogenous immune response based on the fact that long-term prognosis of patients is markedly influenced by the degree and quality of the endogenous immune response to the tumor. Specifically, it has been well documented that the presence of endogenous effector T cells within the ovarian cancer tumor microenvironment directly correlates to prolonged patient survival (22-
10 25). In contrast, increasing numbers of immune suppressive $CD4^{+} CD25^{hi}$ regulatory T cells (Tregs) within the tumor, which in turn presumably abrogate the anti-tumor activity of infiltrating effector T cells, correlates with shorter patient survival (26-29). In fact, it appears that it is the ratio of Tregs to effector T cells within the tumor microenvironment which ultimately dictates whether the endogenous immune response to the cancer is of benefit or detriment to the patient (24, 28). In
15 this setting, the ability to generate and subsequently expand a population of tumor targeted effector T cells *ex vivo* which are subsequently infused back into the patient, may in turn skew the Treg to effector T cell ratio to one more favorable to eradicating the disease.

Mucins are important biomolecules for cellular homeostasis and protection of epithelial surfaces. Changes to expression of mucins in ovarian cancer might be exploited in diagnosis,
20 prognosis and treatment (1). MUC16 is one such mucin which is over expressed on most ovarian carcinomas and is an established surrogate serum marker (CA-125) for the detection and progression of ovarian cancers (30-33). MUC16 is a high-glycosylated mucin composed of a large cleaved and released domain, termed CA-125, consisting of multiple repeat sequences, and a retained domain (MUC-CD) which includes a residual non-repeating extracellular fragment, a
25 transmembrane domain, and a cytoplasmic tail (34). Since the antigen is otherwise only expressed at low levels in the uterus, endometrium, fallopian tubes, ovaries, and serosa of the abdominal and thoracic cavities, MUC16 is a potentially attractive target for immune-based therapies.

However, the fact that most of the extracellular domain of MUC16 is cleaved and secreted limits the utility of MUC16 as a target antigen on ovarian carcinomas. In fact, to date, all reported
30 MAbs to MUC16 bind to epitopes present on the large secreted CA-125 fraction of the glycoprotein, with none known to bind to the retained extra-cellular fraction (MUC-CD) of the antigen (35-37). Since the MUC-CD fraction of the antigen is retained on cell surface, generating T cells specific to this portion of MUC16 may largely overcome the limitation of MUC16 as a target for adoptive cellular immunotherapy. To this end, we have previously generated a series of murine

MAbs specific to the retained MUC-CD extracellular domain (38). Utilizing a hybridoma which expresses one such MAb, 4H11, we have successfully constructed several CARs specific to the MUC-CD antigen. This invention provides a nucleic acid encoding a chimeric T cell receptor, composed of, at least a zeta chain, a signaling region and a binding element that specifically interacts with a selected target as well as the chimeric T cell receptor comprising a zeta chain portion, a signaling region and a binding element.

In this report, we demonstrate highly efficient retroviral transduction of these MUC-CD targeted CARs into human T cells with resulting T cells able to specifically target and lyse MUC-CD⁺ tumor cells *in vitro*. Furthermore, we demonstrate efficient MUC-CD targeted T cell expansion *in vitro* through repeated co-culture on NIH (3T3) fibroblasts genetically modified to express MUC-CD and the co-stimulatory ligand B7.1 (CD80). Successful expansion of modified T cells allowed us to subsequently generate sufficient T cell numbers to conduct *in vivo* studies in immune compromised SCID-Beige mice bearing established intraperitoneal MUC-CD⁺ human ovarian tumors. Significantly, in these studies we demonstrate marked anti-tumor efficacy of MUC-CD targeted T cells, both following direct intraperitoneal as well as intravenous injection when compared to either untreated mice, or mice treated with T cells bearing a CAR targeted to an irrelevant antigen. In addition, we demonstrate significant cytotoxicity of 4H11-28z⁺ patient's T cells and healthy donor's T cells targeting primary ascites-derived ovarian carcinoma cells from cancer patients.

To our knowledge this is the first report wherein T cells genetically targeted to the MUC16 antigen demonstrate marked anti-tumor efficacy against MUC16⁺ tumors either *in vitro* or *in vivo*. These data serve as a rationale for proposing future clinical trials utilizing this approach in patients with high risk ovarian carcinomas.

MATERIALS AND METHODS

Cell lines and T cells

The OV-CAR3 tumor cell line was cultured in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated FBS, nonessential amino acids, HEPES buffer, pyruvate, and BME (Invitrogen). The PG13 and gpg29 retroviral producer cell lines were cultured in DMEM (Invitrogen) supplemented with 10% FCS, and NIH-3T3 artificial antigen-presenting cells (AAPC), described previously (3), were cultured in DMEM supplemented with 10% heat-inactivated donor calf serum. T cells were obtained from peripheral blood of healthy donors under IRB approved protocol #95-054, in BD Vacutainer® CPT tubes (Becton Dickinson, Franklin Lakes, NJ) as per the manufacturer's instructions. All media were supplemented with 2 mmol/L L-glutamine (Invitrogen),

100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). T cells were cultured RPMI 1640 media as above supplemented with 20 IU/ml IL-2 (Novartis Pharmaceuticals, East Hanover, NJ) and where indicated, medium was supplemented with 10 ng/mL interleukin 15 (R&D Systems, Minneapolis, MN).

5 *Isolation of patients ascites-derived cancer cells*

Primary human ascites-derived cancer cells were obtained from ovarian cancer patients undergoing surgery for newly diagnosed advanced serous ovarian carcinoma under IRB approved protocol #97-134. The tumor cells were isolated from ascitic fluid of patients by centrifugation at 600g for 10 min at room temperature. Cells were washed once with 1x PBS and cultured in RPMI 1640 media supplemented with 10% FBS for future analysis.

Generation of the MUC-CD targeted 4H11z and 4H11-28z CARs

The heavy and light chain variable regions of the 4H11 monoclonal antibody were derived from the hybridoma cell line 4H11. Utilizing cDNA generated from 4H11 RNA we isolated the V_H coding region by RACE PCR utilizing modified primers as described elsewhere (39, 40). The V_L chain variable region was cloned by standard PCR utilizing modified primers as described by Orlandi et al (41, 42). The resulting V_H and V_L fragments were subcloned into the TopoTA PCR 2.1 cloning vector (Invitrogen) and sequenced. The V_H and V_L fragments were subsequently ligated to a (Gly₄Ser)₃ spacer domain, generating the 4H11 scFv and fused to the human CD8 leader peptide (CD8L) by overlapping PCR (9, 41). In order to construct the MUC-CD targeted 4H11 CARs, the coding region of the CD8L-4H11 scFv was fused to the human CD8 hinge and transmembrane domains (to generate the 4H11z CAR), or alternatively to the CD28 transmembrane and cytoplasmic signaling domains (to generate the 4H11-28z CAR), fused to the T cell receptor CD3-ζ signaling domain (3, 9, 43). The resulting CAR constructs were subsequently sub-cloned into the modified MMLV retroviral vector SFG (44). VSV-G pseudotyped retroviral supernatants derived from transduced gpg29 fibroblasts were used to construct stable PG13 gibbon ape leukemia virus (GaLV) envelope-pseudotyped retroviral producing cell lines (41).

Retroviral gene transfer

Isolated healthy donor peripheral blood mononuclear cells (PBMCs) were activated with phytohemagglutinin (PHA) at 2µg/ml (Sigma, St. Louis, MO) and retrovirally transduced on retronectin coated non-tissue culture plates (45). Briefly, six-well non-tissue culture plates (BD Biosciences, San Jose, CA) were coated with RetroNectin® (RN) (Takara Biomedicals, Otsu, Japan)

as per manufacturer's instructions. Forty-eight hours after PHA activation, aliquots of 1×10^6 T cells in 1 ml of supplemented RPMI medium were placed in each well of the RN-coated plates, along with 1 ml of SFG retroviral supernatant. T cells were centrifuged daily for 3 consecutive days with fresh retroviral supernatant added daily at 2000g at 30°C for 1hr (45). Gene transfer was assessed on day 7 by FACS.

In order to generate the relevant NIH-3T3 murine fibroblast artificial antigen presenting cells, a MUC-CD construct encoding the retained extracellular, transmembrane and cytoplasmic domains of the MUC-16 antigen was initially subcloned into SFG retroviral vector, SFG(MUC-CD). 3T3(MUC-CD) AAPCs were generated by retroviral transduction of SFG(MUC-CD) into wild-type NIH-3T3 fibroblasts, while 3T3(MUC-CD/B7.1) AAPCs were generated by retroviral transduction of previously established 3T3(B7.1) fibroblasts (41, 46). Highly enriched cell lines were isolated by FACS.

To generate the OV-CAR3(MUC-CD) and OV-CAR3(MUC-CD/GFP-FFLuc) cell lines, we retrovirally transduced the WT OV-CAR3 human ovarian cancer cell line with SFG(GFP-FFLuc) as described previously (47) and/or SFG(MUC-CD) VSV-G pseudotyped retroviral supernatants derived from gpg29 fibroblasts as described elsewhere (44). Resulting tumor cells were sorted by FACS for either MUC-CD expression alone for the OVCAR3(MUC-CD) cell line, or dual MUC-CD and GFP expression for the OVCAR3(MUC-CD/GFP-FFLuc) cell line. MUC-CD expression by FACS was assessed using the 4H11 MAbs.

In vitro analyses of CAR⁺ human T cells

To assess *in vitro* expansion and cytokine release upon stimulation, transduced T cells were co-cultured for 7 days after retroviral transduction in 6-well tissue culture plates (BD Biosciences) on confluent NIH 3T3 AAPCs in RPMI medium supplemented with 10% FBS in the absence of supplemented cytokines. In order to generate sufficient numbers of CAR-modified T cells for *in vivo* studies, transduced T cells were co-cultured on B7.1⁺ AAPCs (3T3(MUC-CD/B7.1)) in RPMI medium supplemented with 20 IU IL-2/mL and 10 ng/mL IL-15 as described previously (3, 43). Patients T cells were activated and expanded with human CD3/CD28 beads (DYNAL®, Invitrogen, Carlsbad, CA) following manufacturer's recommendations.

Western Blot analysis of CAR expression

Western blot analysis of T-cell lysates under reducing conditions with 0.1 mol/L DTT (Sigma) was performed as previously described (46). Briefly, transduced T cells were washed in PBS and resuspended in radioimmunoprecipitation assay (RIPA) buffer (Boston BioProducts,

Worcester, MA) with mini complete protease inhibitor as per the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Resulting proteins were separated on 12% SDS-PAGE mini gels (Bio-Rad, Hercules, CA) after the addition of 6X reducing loading buffer (Boston BioProducts, Worcester, MA) and heating at 100°C for 10 min. Separated proteins were subsequently transferred to Immobilon
 5 membranes and probed using an anti-human CD3ζ chain monoclonal antibody (BD Biosciences). Antibody binding was detected by probing the blot with goat anti-mouse horse radish peroxidase-conjugated antibody followed by luminescent detection using Western Lighting® Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA) as per the manufacturer's instructions.

Cytotoxicity assays

10 *In vitro* modified T cell cytotoxicity was assessed using the DELFIA® EuTDA assay (PerkinElmer LAS, Inc, Boston, MA) following manufacturer's recommendations. Cytotoxicity was assessed at 2 hours at effector T cell to target OV-CAR3(MUC-CD) or primary tumor cells (E:T) at indicated ratios. Effector T cells in these assays represent the number of CD8⁺ CAR⁺ T cells.

Cytokine detection assays

15 Cytokine assays were performed as per manufacturer's specifications using a multiplex Human Cytokine Detection assay to detect IL-2 and IFNγ (Millipore Corporation, Billerica, MA) utilizing the Luminex IS100 system. Cytokine concentrations were assessed using IS 2.3 software (Luminex Corp., Austin, TX).

In vivo SCID-Beige mouse tumor models

20 In all *in vivo* studies, 8-12 week-old FOX CHASE C.B.-17 (SCID-Beige mice) (Taconic, Hudson, NY) were initially injected ip with either 3 x 10⁶ OV-CAR3(MUC-CD), or for bioluminescent imaging (BLI) studies 3 x 10⁶ OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells. Subsequently, 3x10⁷ CAR⁺ T cells were injected ip or iv on day 1 or 7 following tumor injection as indicated. Mice were monitored for distress as assessed by increasing abdominal girth, ruffled fur, and decreased response to
 25 stimuli. Distressed mice were euthanized. All murine studies were done in context of an Institutional Animal Care and Use Committee-approved protocol (#00-05-065).

Bioluminescent imaging (BLI) of OVCAR3(MUC-CD/GFP-FFLuc) tumor cells in SCID-Beige mice

BLI was performed using Xenogen IVIS imaging system with Living Image software
 30 (Xenogen; Alameda, CA). Briefly, OVCAR3(MUC-CD/GFP-FFLuc) tumor bearing mice were injected

by ip with D-luciferin (150 mg/kg; Xenogen) suspended in 200µl PBS and imaged under 2% isoflurane anesthesia after 10 min. Image acquisition was done on a 25-cm field of view at medium binning level for 0.5-min exposure time (3, 43).

Flow cytometry

- 5 All flow cytometric analyses of T cells and tumor cells was performed using a FACScan™ cytometer with Cellquest software (BD Biosciences). T cells were analyzed using CAR-specific polyclonal goat Alexa Fluor 647 antibody (Molecular probes, Eugene, OR) phycoerythrin-labeled anti-human CD4, CD8, B7.1 (Caltag Laboratories, Burlingame, CA), B7.2 (Invitrogen, Camarillo, CA), 4-1BBL, and OX40 antibodies (Ansell Corporation, Bayport, MN). 3T3(MUC-CD) and OV-
10 CAR3(MUC-CD) cells were stained with Alexa Fluor® 647 labeled 4H11 antibody (generated and labeled in the MSKCC monoclonal antibody core facility).

CFSE labeling of CARP T cells

- CAR⁺ T cells were stained with CFSE using the CellTrace™ CFSE cell proliferation kit following manufacturer's recommendations (Molecular Probes, Eugene, OR). Proliferation of CFSE
15 labeled T cells was analyzed by FACS. For detection of CFSE labeling T cells *in vivo*, ovarian tumors were macerated through 40 µm cell strainer (BD Falcon, Franklin Lakes, NJ) and washed twice with 2% FBS/PBS before antibody staining and FACS analysis.

Statistics

- Survival data assessed by log-rank analysis using GraphPad Prism software (GraphPad Prism software,
20 San Diego, CA). Cytokine data were analyzed by Student's one-tailed t-test.

RESULTS

- We have constructed SFG retroviral vectors encoding first (4H11z) and second generation (4H11-28z) CARs targeted to the MUC-CD antigen using the 4H11 hybridoma which generates a MAbs specific to the MUC-CD antigen (Figure 11A). We confirmed expression of appropriately sized CAR
25 proteins by Western blot analysis of resulting PG-13 retroviral producer cells (SFG-4H11z and SFG-4H11-28z) probed with a ζ-chain specific antibody (data not shown).
In order to assess the function of the first generation 4H11z CAR, healthy donor T cells isolated from peripheral blood were retrovirally transduced to express the 4H11z and control 19z1

CARs (Figure 11B). Function of the 4H11z CAR was assessed by proliferation of 4H11z transduced T cells following co-culture on 3T3(MUC-CD/B7.1) AAPCs. Results demonstrate specific proliferation of 4H11z transduced T cells, when compared to 19z1 modified T cells (Figure 11C). These data are consistent 4H11z CAR mediated specific binding to the MUC-CD antigen and subsequent T cell activation.

Since most malignancies fail to express co-stimulatory ligands, we further modified the 4H11z CAR to express the CD28 transmembrane and cytoplasmic co-stimulatory signaling domains, constructing the second generation 4H11-28z CAR (Figure 11A). To assess whether the 4H11-28z CAR, when expressed by human T cells, was capable of generating both a primary activating signal (termed "signal 1") through the ζ chain, as well as a co-stimulatory signal (termed "signal 2") through the CD28 cytoplasmic domain, which in turn allows for efficient T cell proliferation in the absence of exogenous co-stimulatory ligands, we compared T cell proliferation following co-culture on either 3T3(MUC-CD) or 3T3(MUC-CD/B7.1) AAPCs in the absence of exogenous cytokines. As expected, the second generation 4H11-28z⁺ T cells markedly expanded when compared to 4H11z⁺ T cells upon co-culture with 3T3(MUC-CD) AAPCs. In contrast, both 4H11z⁺ and 4H11-28z⁺ T cells expanded similarly on 3T3(MUC-CD/B7.1) AAPCs (Figure 12A). Co-stimulation mediated by the 4H11-28z CAR was further verified by analysis of day 2 tissue culture supernatants from co-culture experiments on 3T3(MUC-CD) AAPCs demonstrating enhanced IL-2 secretion, a cytokine typically secreted in the context of T cell co-stimulation, when compared to control 19z1⁺ and 19-28z⁺ T cells and first generation 4H11z⁺ T cells (Figure 12B). Secretion of IFN γ was comparable between 4H11z⁺ and 4H11-28z⁺ activated T cells.

We next assessed the ability of MUC-CD targeted T cells to expand following weekly re-stimulations through co-culture on 3T3(MUC-CD/B7.1) AAPCs in the context of exogenous IL-2 and IL-15 (3). Both 4H11z⁺ and 4H11-28z⁺ T cells expanded greater than 2 logs over 3 weeks (Figure 12C). T cells transduced with the 4H11-28z were further analyzed by FACS for CAR expression 7 days after initial activation on AAPCs and following two subsequent co-stimulations on AAPCs demonstrating an expected enrichment of the CAR⁺ T cell fraction (Figure 12D). Similar data was generated with expanded 4H11z⁺ T cells (data not shown).

In vitro cytotoxicity and proliferation of MUC-CD targeted T cells following co-culture with OV-CAR3(MUC-CD) and freshly isolated ascites derived ovarian tumor cells.

In order to assess the ability of 4H11z⁺ and 4H11-28z⁺ T cells to target and lyse human ovarian carcinoma tumors, we utilized the human OV-CAR3 cell line which was genetically modified to express the MUC-CD antigen thereby better reflecting the majority of clinical ovarian

tumor samples which express the 4H11-targeted MUC-CD antigen (48). We initially verified specific lysis by MUC-CD targeted T cells demonstrating similar significant cytotoxic activity of 4H11z and 4H11-28z CAR modified T cells targeting OV-CAR3(MUC-CD) tumor cells when compared control T cells expressing the irrelevant first and second generation CD19-targeted 19z1 and 1928z CARs (Figure 13A). Healthy donor T cells modified to express the 4H11-28z CAR similarly exhibited lysis of freshly isolated ascites derived MUC-CD⁺ ovarian carcinoma cells when compared to 19-28z transduced T cells (Figure 13B). Moreover, patient's peripheral blood T cells modified to express the 4H11-28z CAR similarly lysed autologous primary MUC-CD⁺ tumor cells derived from the same ascites sample when compared to T cells modified to express the control 19-28z CAR (Figure 13C).

We further assessed the ability of 4H11z⁺ and 4H11-28z⁺ T cells from healthy donors to proliferate following co-culture on OV-CAR3(MUC-CD) as assessed by FACS of CFSE labeled T cells, as well as absolute T cells numbers over 7 days following co-culture with tumor (Figures 13D and E). Surprisingly, we found that both 4H11z⁺ and 4H11-28z⁺ T cells expanded equally well following co-culture with OV-CAR3(MUC-CD) tumor cells suggesting the ability of this tumor cell line to co-stimulate T cells through expression of a co-stimulatory ligand. To address this possibility, we conducted further FACS analyses of OV-CAR3(MUC-CD) tumor cells demonstrating expression of the co-stimulatory 4-1BBL ligand (Figure 13F), but not the B7.1, B7.2, or OX-40L co-stimulatory ligands (data not shown).

20

In vivo anti-tumor activity of MUC-CD targeted T cells in SCID-Beige mice.

To assess the *in vivo* anti-tumor activity of 4H11z⁺ and 4H11-28z⁺ T cells, we next generated an orthotopic xenotransplant ovarian cancer tumor model by ip injection of OV-CAR3(MUC-CD) tumor cells into SCID-Beige mice. If left untreated, these mice developed marked ascites and multiple nodular peritoneal tumors by 3 weeks following tumor cell injection (Figure 14A). All untreated tumor bearing mice had to be euthanized by 7 weeks following tumor cell injection due to evidence of distress.

To assess the *in vivo* anti-tumor efficacy of MUC-CD-targeted T cells, SCID-Beige mice were injected ip with OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells on day 1 followed by ip injection of 4H11z⁺ or 4H11-28z⁺ T cells on day 2. For negative controls, tumor bearing mice were either untreated or treated with T cells modified to express the irrelevant CD19-targeted CAR. Collectively, we found that 27% of all mice treated with MUC-CD targeted T cells (3/11 mice) remained alive without clinical evidence of disease 120 days out from tumor injection with no statistically significant difference in survival when comparing the 4H11z⁺ and 4H11-28z⁺ T cell

treated cohorts (Figure 14B). In contrast, both MUC-CD-targeted T cell treated cohorts demonstrated statistically significant enhanced survival when compared to untreated and 19z1⁺ T cell treated control cohorts.

To assess whether systemically infused MUC-CD-targeted T cells successfully traffic to ip tumors, we next compared ip to iv infusion of 4H11-28z⁺ T cells in SCID-Beige mice bearing ip OV-CAR3(MUC-CD/GFP-FFLuc) tumors. Both ip and iv 4H11-28z⁺ T cell treated mice exhibited statistically enhanced survival when compared to untreated or 19-28z⁺ T cell treated control cohorts as assessed by overall survival (Figure 15A) as well as by BLI of tumor progression (Figure 15B). Furthermore, we found overall survival between the ip and iv treated groups to be statistically equivalent by log rank analysis. These data imply successful trafficking of iv infused 4H11-28z⁺ T cells to peritoneal tumors. We further confirmed trafficking of iv infused CFSE labeled 4H11-28z⁺ T cells to the peritoneum by FACS analysis of single cell suspensions of macerated OV-CAR3(MUC-CD) tumors (Figure 15C).

In vivo anti-tumor activity of MUC-CD targeted T cells in SCID-Beige mice bearing well established OV-CAR3(MUC-CD/GFP-FFLuc) tumors.

To further assess whether 4H11-28z⁺ T cells were able to eradicate more clinically relevant tumor burdens, we next treated SCID-Beige mice bearing well established ip OV-CAR3(MUC-CD/GFP-FFLuc) tumor injected 7 days prior to adoptive T cell therapy. Once more, we found that therapy with MUC-CD targeted T cells markedly eradicated BLI evident disease in all treated mice (Figure 16A) with 5 of 8 treated mice eventually developing relapsed progressive disease, and 3 mice remaining disease free as assessed by BLI imaging (not shown) out to 120 days post-tumor cell infusion (Figure 16B). These data demonstrate potent *in vivo* anti-tumor activity mediated by MUC-CD targeted T cells even in the setting of advanced disease.

DISCUSSION

Based on extensive analyses of patient tumor samples, ovarian carcinomas appear to be relatively immunogenic tumors. Specifically, researchers have found there to be a direct correlation between prognosis following surgery and chemotherapy and the quantity of tumor infiltrating effector T cells (TILs) in pretreatment tumor samples (25, 49, 50). Furthermore, others have described an inverse correlation between prognosis following therapy and pre-treatment levels of Tregs within the tumor, which in turn presumably inhibit the anti-tumor function of tumor specific effector TILs (26, 28, 51). Both of these findings imply a role for an endogenous effector T cell response to tumor in controlling disease progression both prior to and following initial

therapy and strongly support the contention that ovarian carcinomas may be susceptible to killing by adoptive infusion of autologous T cells targeted to ovarian tumor cell antigens.

While endogenous effector TILs are one source for presumably tumor specific T cells, an alternative approach to adoptive T cell therapy is to isolate autologous peripheral blood T cells which in turn may be genetically modified *ex vivo* to target tumor cell antigens. One such genetic approach is to retrovirally transduce patient T cells with CARs targeted to surface exposed antigens either unique to or over-expressed by the tumor. To this end, promising preclinical studies utilizing this approach in other malignancies have recently been translated into the clinical setting (6, 16, 19, 52). Similarly, we have previously generated CARs targeted to the CD19 antigen expressed on normal B cells as well as most B cell malignancies and are currently conducting clinical trials treating patients with relapsed B cell chronic lymphocytic leukemia and acute lymphoblastic leukemias with autologous T cell modified to express a CD19 specific CAR (53).

Application of this approach to ovarian carcinomas requires the identification to suitable target antigens expressed on the tumor cell surface. Significantly, other investigators have studied this approach in both the pre-clinical and clinical setting (4, 11, 54-57). Specifically, several groups have demonstrated significant anti-tumor responses to subcutaneous human ovarian carcinoma cell line tumors in immune compromised mice following intratumoral and/or intravenous infusion of T cells expressing CARs specific to the mesothelin and Lewis-Y antigens overexpressed on these tumor cell lines (56, 58, 59). Furthermore, Kershaw et al recently published the results of a phase I clinical trial treating patients with relapsed ovarian carcinomas with autologous T cells modified to express a CAR specific to the alpha-folate receptor (6). The authors of this study found that therapy with targeted T cells was well tolerated, but noted a lack of anti-tumor response in these studies related to poor persistence of modified T cells over time as well as a yet undefined T cell inhibitory factor in the serum of several treated patients.

In our studies, we have chosen to target the MUC-16 glycoprotein which is over-expressed on a majority of ovarian carcinomas (1, 30, 32, 33). The utility of MUC-16 as a target antigen for adoptive T cell therapy is compromised by the fact that most of the extracellular portion of this molecule is cleaved by the tumor cell, secreted, and may be detected in the serum as the CA-125 tumor marker. However, following cleavage of this secreted fraction of MUC-16, there remains a residual extracellular fraction of the glycoprotein, termed MUC-CD, which is retained on the tumor surface and is therefore an attractive target for immune-based therapies. To this end, we utilized a series of murine hybridomas generated to the MUC-CD antigen to construct CARs specific to MUC-CD. Of these CARs, we identified a CAR generated from the 4H11 murine hybridoma termed 4H11z, which, when expressed in human T cells, following co-culture on 3T3(MUC-

CD/B7.1) AAPCs, resulted in rapid destruction of AAPC monolayers as well as marked modified T cell expansion. Significantly, the antigen to the 4H11 antibody is highly expressed on a majority of pre-treatment ovarian carcinoma surgical tumor samples obtained from patients treated at our institution as assessed by immuno-histochemistry (48).

5 Optimal T cell activation requires both a primary T cell receptor mediated signal, "signal 1," along with a co-stimulatory "signal 2." Classically, this co-stimulatory signal may be provided by ligation of either B7.1 (CD80) or B7.2 (CD86) on the target cell with the T cell co-stimulatory receptor CD28. Alternatively, co-stimulation may be generated by ligation of 4-1BBL or OX-40L on the target cell with the respective 4-1BB or OX40 co-stimulatory receptors on the T cell (12, 60,
10 61). Since most tumor cells fail to express co-stimulatory ligands, we and others have previously demonstrated that second generation CARs further incorporating the cytoplasmic signaling domains the co-stimulatory receptors CD28, 4-1BB, and/or OX40 resulted in CARs capable of providing both signal 1 and signal 2 to the T cell upon binding to cognate antigen in the absence of exogenous co-stimulatory ligands (7-10, 12, 13, 15, 16, 62-65). To this end, we constructed a
15 second generation CAR from the 4H11z CAR incorporating the transmembrane and cytoplasmic signaling domain of CD28 as described elsewhere (3, 9, 43). Consistent with previous studies, we found that T cells transduced to express the resulting 4H11-28z CAR, but not the first generation 4H11z CAR, efficiently expanded upon co-culture with 3T3(MUC-CD) fibroblasts in the absence of exogenous co-stimulation consistent with the ability of the 4H11-28z CAR to deliver both signal
20 1 and signal 2 to the T cell. This conclusion is further supported by the finding that 4H11-28z⁺ T cells secreted significantly higher levels of IL-2, a cytokine indicative of T cell co-stimulation, upon co-culture on 3T3(MUC-CD) fibroblasts when compared to T cells transduced to express the first generation 4H11z CAR.

We next assessed the ability of 4H11z⁺ and 4H11-28z⁺ T cells to target and lyse human
25 ovarian carcinoma tumor cells. To this end, we initially utilized the OV-CAR3 human ovarian cancer cell line. Since the OV-CAR3 tumor cell line binds the 4H11 antibody weakly, we further genetically modified the cell line to express MUC-CD (OV-CAR3(MUC-CD)) to better mimic the clinical setting wherein a majority of clinical ovarian carcinoma tumor specimens highly express the 4H11 MUC-CD antigen (48). We demonstrated that human T cells modified to express either
30 4H11z or 4H11-28z eradicated OV-CAR3(MUC-CD) tumor cells *in vitro*, and surprisingly observed that both 4H11z⁺ and 4H11-28z⁺ T cells expanded following co-culture with tumor *in vitro*. To define the etiology of this unanticipated 4H11z⁺ T cell expansion, we further assessed whether OV-CAR3(MUC-CD) tumor cells expressed co-stimulatory ligands, and found that this tumor cell line expressed 4-1BBL, consistent with our experimental findings as well as with

previously published reports demonstrating 4-1BBL expression by a variety of carcinoma cell lines (66-68). In order to further validate the clinical relevance of these findings, we subsequently demonstrated specific *in vitro* lysis of primary ascites-derived tumor cells isolated from untreated ovarian carcinoma patients by both healthy donor allogeneic 4H11-28z⁺ T cells as well as more
5 significantly autologous 4H11-28z⁺ patient peripheral blood T cells. These data strongly support the contention that treatment with autologous 4H11-based CAR⁺ T cells have promise in future clinical applications.

In order to assess the *in vivo* relevance of our *in vitro* findings, we next generated a murine orthotopic OV-CAR3(MUC-CD) tumor model in SCID-Beige mice. We injected mice i.p. with
10 OV-CAR3(MUC-CD) tumor cells and the following day infused 4H11z⁺, 4H11-28z⁺, and control 19z1⁺ T cells i.p. This treatment approach resulted in a significant but similar delay to tumor progression and long-term survival in both the 4H11z⁺ and 4H11-28z⁺ T cell treated cohorts when compared to untreated mice or mice treated with control T cells targeted to the irrelevant CD19 antigen. We next compared ip to iv treatment with 4H11-28z⁺ T cells of orthotopic OV-
15 CAR3(MUC-CD/GFP-FFLuc) bearing mice, and found similar statistically significant survivals of mice over time with either direct ip infusion of T cells or systemic iv infusion of targeted T cells. Significantly, iv treated mice by day 1 following treatment, exhibited successful trafficking of targeted T cells to the peritoneum. These data suggests that adoptive therapy with targeted T cells may be equally efficacious following either a direct infusion into the peritoneum or through
20 systemic iv infusion. These findings further support the future clinical potential of this approach in treating patients both with local relapse of disease as well as metastatic relapse to sites outside of the peritoneum.

Finally, we assessed the ability of 4H11-28z⁺ T cells to eradicate more established disease by delaying modified T cell ip infusion by 7 days, when mice had greater established tumor
25 burdens as assessed by bioluminescent imaging. This experimental setting better reflects the initial clinical setting wherein this adoptive T cell approach would be utilized. Significantly, despite the setting of markedly established disease, 4H11-28z⁺ T cells retained the ability to lyse larger tumor burdens, delay relapse of tumor, and in a significant percentage of mice, fully eradicate disease.

In the studies presented here, we have consistently utilized mixed populations of CD4⁺ and
30 CD8⁺ CAR⁺ T cells to assess both *in vitro* and *in vivo* anti-tumor activity. To this end, ongoing studies will address the role of isolated CD4⁺ and CD8⁺ CAR⁺ T cell subsets in the successful eradication of disease in this SCID-Beige OV-CAR3(MUC-CD) tumor model. The results of these studies may have implications to translating this therapeutic approach to the clinical setting. Furthermore, we acknowledge the limitations associated with the presented SCID-Beige tumor

model. Namely, this is a xenotransplant model in an immune compromised mouse. To this end, ongoing studies in our laboratory are focused on generating a more clinically relevant syngeneic immune competent tumor model to better define the biology and anti-tumor efficacy of MUC-CD targeted CAR-modified T cells in the context of an intact immune system.

5 In conclusion, herein we present the first published data demonstrating the feasibility of targeting MUC-16, an antigen over-expressed on a majority of ovarian carcinomas, through adoptive therapy with genetically modified T cells targeted to the retained MUC-CD portion of the MUC-16 antigen. Further, this report is the first to demonstrate efficient targeting of T cells in an orthotopic, clinically relevant, murine model of ovarian cancer, demonstrating efficacy both by ip
10 and iv infusion of modified T cells. Finally, these data support the further translation of this approach to the clinical setting in the form of a phase I clinical trial in patients with persistent or relapsed ovarian carcinomas following initial therapy with surgery and chemotherapy. [0]

EXAMPLE 5

15 **Raising Mouse MUC16 monoclonal antibodies in mice and hamsters.**

We selected 3 different regions of mouse MUC16 genome for which monoclonal antibodies were generated in mouse and hamster. The selected regions of the mouse MUC16 are Peptide 1 (SEQ ID NO:21, ecto region of cytoplasmic domain), Peptide 2 (SEQ ID NO:22, first cysteine loop) and Peptide 3 (SEQ ID NO:23, second cysteine loop) (Figure 20A) and its comparison with human
20 MUC16 is shown in Figure 20B. A cysteine was added to the peptide sequence at the N terminus of Peptide 1 (SEQ ID NO:21) and Peptide 3 (SEQ ID NO:23) for better conjugation with KLH. Individual peptides were conjugated to KLH using Promega kit. These 3 conjugated peptides were pooled and immunized into 5 mice and 4 hamsters. 5 immunizations with a 3 week interval for each immunization were administered. Sera from these animals were tested by ELISA for their
25 specific reactivity with individual peptides (SEQ ID NO:21, 22 and 23). Positive selected animals were allowed to rest for a month and then i.v. boosted with pooled peptides immunogen (SEQ ID NO:21, 22 and 23) and harvested the spleens after 4 days. Splenocytes were mixed with hybridoma partners and plated into microtiter plates at various clonal densities. Plates were cultured at 37°C 5% CO₂ for 10 days and then selected the clones. Supernatants from these selected clones were
30 tested by ELISA for their specific reactivity with individual peptides (SEQ ID NO:21, 22 and 23). Positive clonal sups were tested by FACS, western blot and imaging using 2 mouse cell lines (ID8 and BR5-FVBI) and a human cell line (OVCAR-3).

Table 4 shows the summary of mouse and hamster monoclonal antibodies against mouse MUC16 peptide antigens Peptide 1 (SEQ ID NO:21), Peptide 2 (SEQ ID NO:22), and Peptide 3 (SEQ ID NO:23). A very strong antigenic response was seen with Peptide 1 (SEQ ID NO:21).

5 Table 4

Mouse MUC16		Mouse mAbs	Frozen Mouse mAb
Peptide 1		46	16 (3-IgG1; 8-IgG2b; 1-IgM; 4-Unkown isotype)
Peptide 2		0	0
Peptide 3		6	6 (4-IgG1; 2-IgM)
Peptide 1,2,3		0	0
Peptide 1,2		0	0
Peptide 2,3		0	0
No Peptide		0	0

Animals not iv
boosted with
peptide 2

Mouse MUC16		Hamster mAbs	Frozen Hamster mAb
Peptide 1		69	21
Peptide 2		6	6
Peptide 3		7	7
Peptide 1,2,3		2	1
Peptide 1,2		1	1
Peptide 2,3		1	0
No Peptide		10	2

- 5 Details of mouse and hamster mAbs against Peptide 1 (SEQ ID NO:21), Peptide 2 (SEQ ID NO:22), and Peptide 3 (SEQ ID NO:23) are listed in Table 5 and Table 6 respectively.

Table 5:

Table 3.

isotype	PEPTIDE	Fusion Well	Cloned	Clones			
-	1	01D01	no success				
-	1	09F07					
IgG 1	1	16A09					
-	1	21A07					
-	1	24G10					
IgG 1	1	10C04	yes	10C4-3H5	10C4-1F2	10C4-2H8	10C4-1G7
IgG 1	1	17F02	yes	17F2-3G5	17F2-3F6	17F2-2F9	17F2-1E11
IgG 2b	1	01A08	yes				
IgG 2b	1	01F08					
IgG 2b	1	12B10		12B10-3F7	12B10-3G10	12B10-2F6	12B10-2F10
IgG 2b	1	17H10					
IgG 2b	1	18D05					
IgG 2b	1	23B12					
IgG 2b	1	25E09		25E9-3	25E9-5	25E9-13	25E9-16
IgM	1	16F12					
IgG 1	2	04A06	no success				
IgG 1	2	05D01	no success				
IgG 1	2	21B08	yes				
IgG 1	2	21E01	yes	21E1-1E3	21E1-1G9	21E1-2G7	21E1-3G12
IgM	2	08A02					
IgM	2	13E05					

Table 6:

Hamster mAb	Peptide	Cloned			
01H03					
02F02	1				
04E 4					
04G07	1				
04H01	3	4H1-2E1	4H1-2E3	4H1-3E1	4H1-3H3
06A08	1				
06F02	1				
07F08	3				
07H05	2				
09A05					
09E 1	3				
09F08	1				
09H10					
10G06	1				
10H11	1				
11B10	1				
12F09	2				
15A08	1	15A8-2E2	15A8-2E10	15A8-2E11	15A8-3D2
15H08	3				
19B05	1				
21H04	3				
22B05	2	22B5-1F6	22B5-3G9	22B5-2G8	22B5-3F11
22D11	3				
23G12	1				
25E 8	1				
27H09	3				
28B12	1&2&3				
28C12	2				
30H02	1				
31A11	2				
31C01	2				
33H06	1&2				
34F10	1				
34H05	1				
36C01	1				
36C11					
36E 4	1				
37E 10	1				
10H11	1				

Hamster antibody 22B05 recognizes mouse (SEQ ID NO:22) and also the corresponding human sequence (SEQ ID NO:15).

Western blot analysis using mouse ID8 and BR5-FVB1 cell extracts were also performed for all the selected monoclonal antibodies as shown in Figure 21 and Figure 22 respectively.

5 Among the mouse MUC16 monoclonal antibodies, we selected 12B10-3G10 subclone mouse mAb for further screening. Similarly, hamster monoclonal antibodies, 15A8-2E10, 22B5-2G8 and 4H1-2E1 subclones were selected for further screening.

10 Immunohistochemical analysis was performed with paraffin and cryosections of ID8 (mouse), OVCAR-3 (human), BR5-FVB1 (mouse) cell lines and 13.5 days of Embryo. Paraffin or cryosections were probed with mouse 12B10 mAb, hamster 15A8, hamster 22B5 and hamster 4E1 mAbs to see the early development of mouse MUC16 (Figure 23)

12B10-3G10 sub clone were further analyzed for single chain Fv fragments. Figure 24 shows 12B10-3G10 V_H and V_L DNA and Amino Acids sequences. Bioreactive supernatants and purified 12B10-3G10 were generated for animal studies and other characterization studies. FACS
15 analysis was performed with purified 12B10-3G10 on ID8, OVCAR3 and BR5-FVB1 cells showing over 90% positivity to both mouse and human MUC16 ecto-domain fragment (Figure 25).

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The scope of the invention as defined by the attached claims should not be limited by the specific embodiments set forth in the examples, but should be given the broadest interpretation consistent with the specification as a whole.

SEQUENCE LISTING IN ELECTRONIC FORMAT

This description contains a sequence listing in electronic form in ASCII text format. A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

We claim:

1. An isolated antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is:

CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

2. The antibody or fragment thereof of claim 1, wherein the antibody or fragment thereof is, in the alternative: a monoclonal antibody, a chimeric antibody, a recombinant antibody, an antigen-binding fragment of a recombinant antibody, a humanized antibody or an antibody displayed upon the surface of a phage.

3. The antibody or antigen-binding fragment thereof, of claim 1, wherein the antibody is a monoclonal antibody.

4. The antibody or antigen-binding fragment thereof, of claim 1, wherein the antibody is a chimeric antibody.

5. The antibody or antigen-binding fragment thereof, of claim 1, wherein the antibody is a humanized antibody.

6. The antibody, or antigen-binding fragment thereof, of claim 5, wherein substantially all of the framework domain residues of the humanized antibody are those of a human immunoglobulin sequence, and wherein one or more residues of the framework domain are replaced by corresponding nonhuman residues.

7. The antibody, or antigen-binding fragment thereof, of any one of claims 1 to 6, wherein the antigen-binding fragment thereof is a Fab fragment, a F(ab')₂ fragment, or a Fv fragment.

8. The antibody, or antigen-binding fragment thereof, of any one of claims 1 to 7, wherein the antibody lacks specific binding to a glycosylated MUC16 extracellular domain.

9. The antibody, or antigen-binding fragment thereof, of any one of claims 1 to 8, wherein the antibody, or antigen-binding fragment thereof, is covalently linked to a cytotoxic agent or a prodrug of a cytotoxic agent.

10. The antibody, or antigen-binding fragment thereof, of any one of claims 1 to 9, wherein the antibody internalizes into a cell.

11. A composition comprising (a) an antibody, or antigen-binding fragment thereof, of any one of claims 1 to 10, and (b) a pharmaceutically acceptable carrier.

12. A hybridoma cell that produces an antibody as defined in any one of claims 1 to 8.

13. Use of the antibody or antigen-binding fragment thereof, of any one of claims 1 to 10, for identifying a subject as having a cancer in which MUC16 is expressed.

14. The use of claim 13, wherein the antibody is for administration to the subject to determine the presence and location of the antibody in the subject, wherein the antibody is labeled.

15. The use of claim 13 or 14, wherein the cancer is selected from the group consisting of ovarian cancer and breast cancer.

16. The antibody of any one of claims 1 to 10, for use in identifying a subject as having a cancer in which MUC16 is expressed.

17. The antibody for use of claim 16, wherein the antibody is for administration to the subject to determine the presence and location of the antibody in the subject, wherein the antibody is labeled.

18. The antibody for use of claim 16 or 17, wherein the cancer is selected from the group consisting of ovarian cancer and breast cancer.

19. An *ex vivo* method for identifying a subject as having a cancer in which MUC16 is expressed, wherein said method comprises:

(a) contacting a sample obtained from the subject with the antibody of any one of claims 1 to 10; and

(b) determining whether the antibody has an increased level of binding to the sample as compared to a control sample lacking the cancer in which MUC16 is expressed.

20. The *ex vivo* method of claim 19, wherein the cancer is selected from the group consisting of ovarian cancer and breast cancer.

21. The *ex vivo* method of claim 19 or 20, further comprising detecting an altered level of binding of the antibody to the sample compared to a control sample.

22. The *ex vivo* method of claim 19, 20 or 21, wherein the detecting is selected from the group consisting of immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, immunoprecipitation, and radiographic imaging.

23. An isolated polynucleotide comprising a nucleotide sequence that encodes at least one of a variable heavy (VH) chain sequence and the variable light (VL) chain sequence of an antibody that specifically binds to a MUC16 polypeptide, wherein the MUC16 polypeptide is:

CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

24. Use of a polypeptide of the sequence:

CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3);

for producing an antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof.

25. Use of an antibody or antigen-binding fragment thereof, as defined in any one of claims 1 to 10, for reducing one or more symptoms of a cancer.

26. Use of an antibody or antigen-binding fragment thereof, as defined in any one of claims 1 to 10, for formulating a medicament for reducing one or more symptoms of a cancer.

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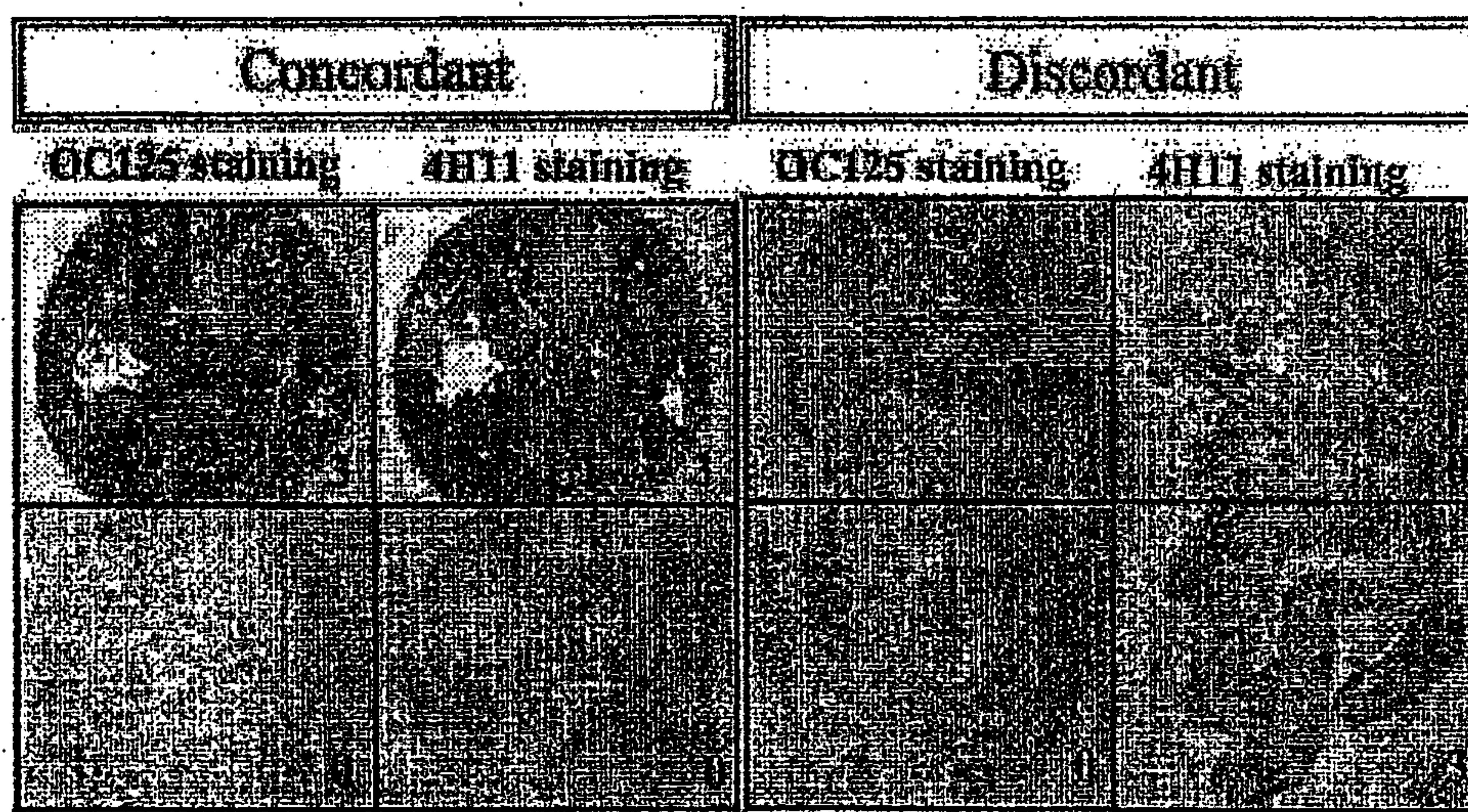
27. The use according to claim 25 or 26, wherein the cancer is, in the alternative, ovarian cancer or breast cancer.

Peptide 1 near Cleavage Site:
NFSPLARRVDRVAIYEE (SEQ ID NO:01)

Peptide 2 before Transmembrane:
TLDRSSVLVDGYSPNRNE (SEQ ID NO:02)

Peptide 3 inside Transmembrane:
CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03)

FIGURE 1



Tissue microarrays were scored from 0 to 3 in one-half (+) increments for cytoplasmic and/or membranous staining

0 – no staining

1 – strong or weak <5%

1+ – strong or weak 5-50%

2 – strong 51-75% or weak 51-100%

2+ – strong (76-99%)

3 – strong (100%)

Stain score

	0	1-1+	2-3
n=518			
OC125	60 (11%)	122 (24%)	336 (65%)
4H11	129 (25%)	116 (22%)	273 (53%)

Concordance between OC125 and 4H11 staining was based on the amount of difference between the scores

Concordant = 0 - ½ difference

Partially concordant = 1-1 ½ difference

Discordant = 2-3 difference

Of the 39 discordant cases,

OC125 > 4H11 in 59%

4H11 > OC125 in 41%

Overall 3-4% of all ovarian serous carcinomas expressed 4H11 but not OC125

OC125/4H11 (n=518)	
Concordant	286 (55%)
Partially concordant	193 (37%)
Discordant	39 (8%)
OC125>4H11	23 (59%)
4H11>OC125	16 (41%)

FIGURE 2

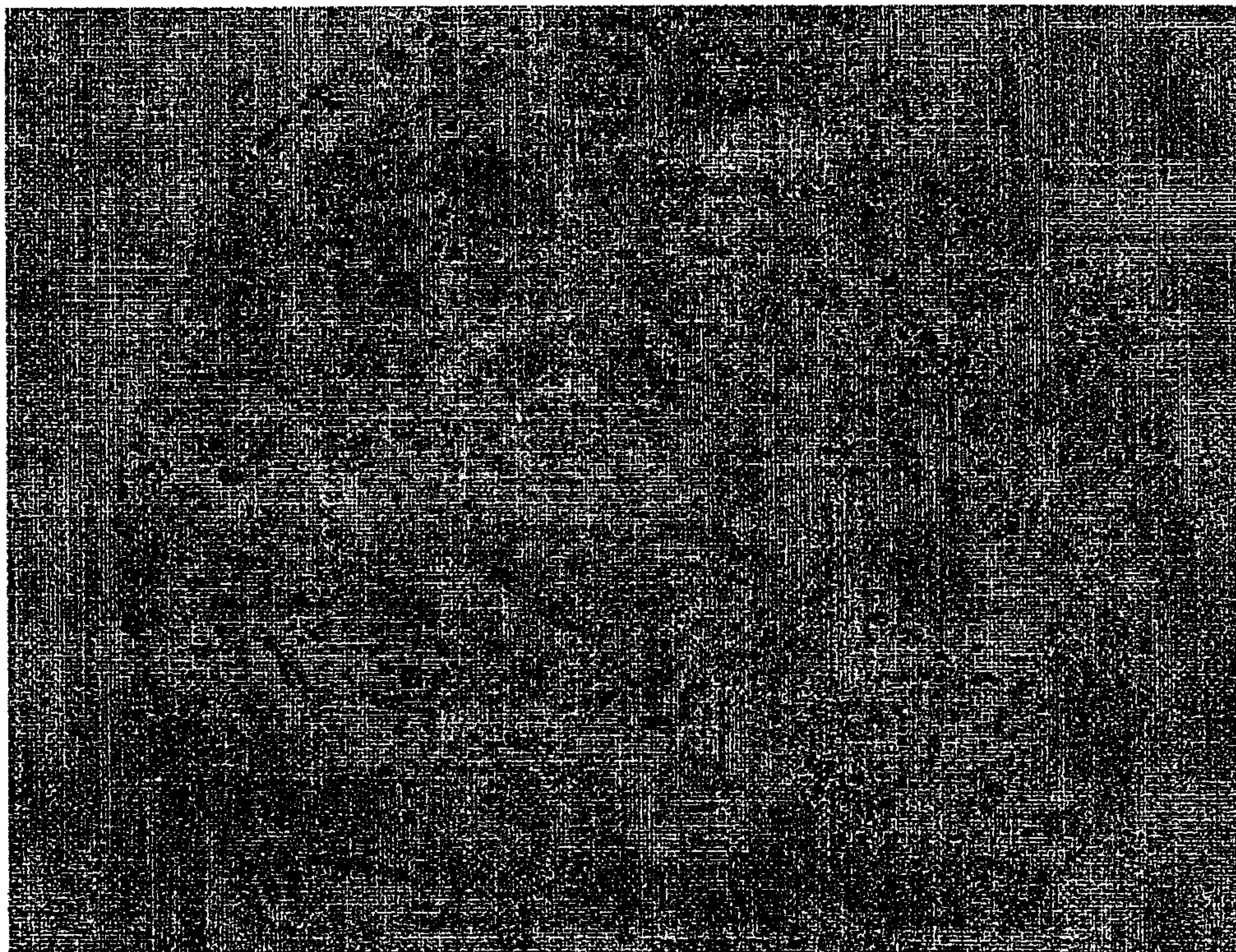


FIGURE 3A

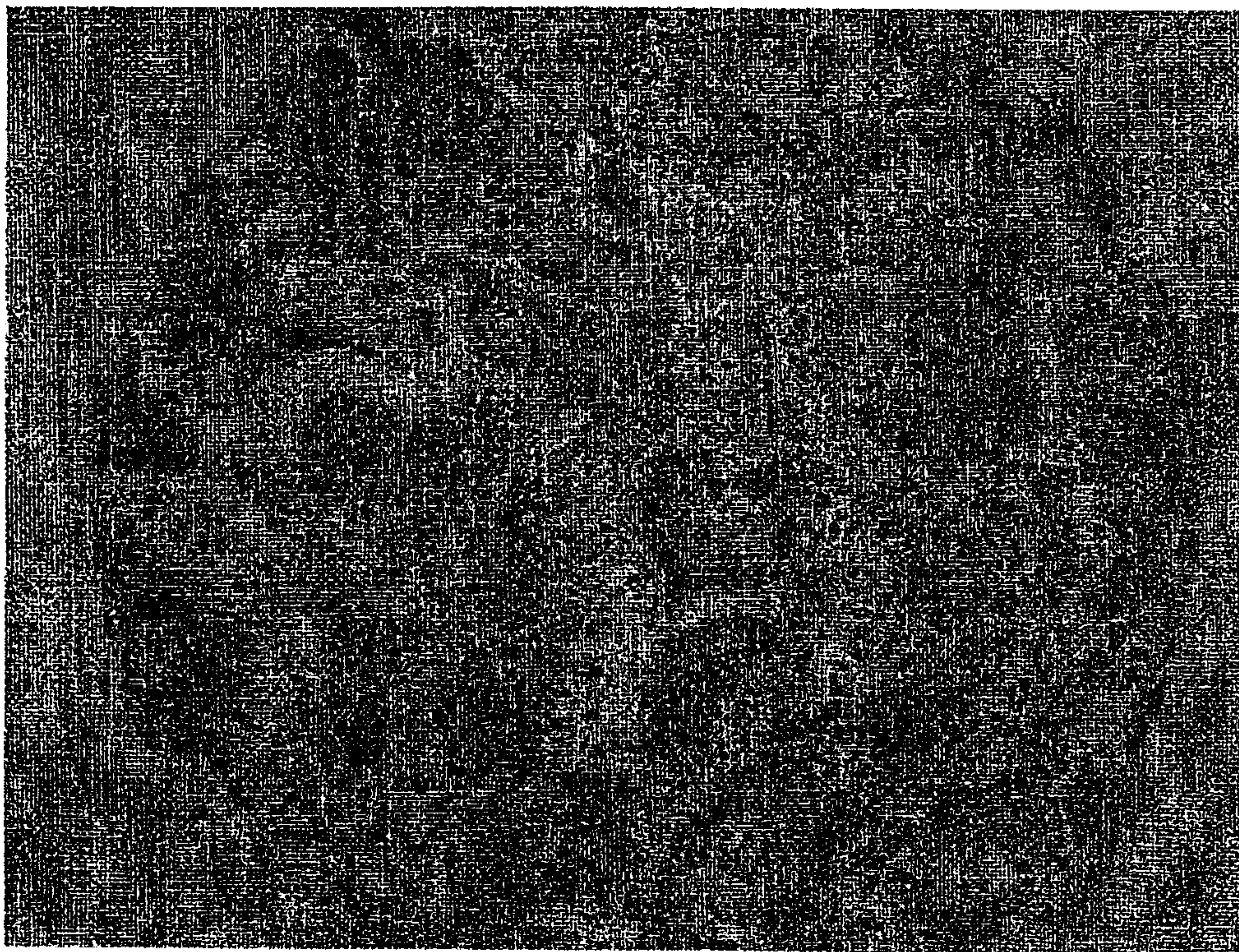


FIGURE 3B



FIGURE 3C



FIGURE 3D



FIGURE 3E



FIGURE 3F

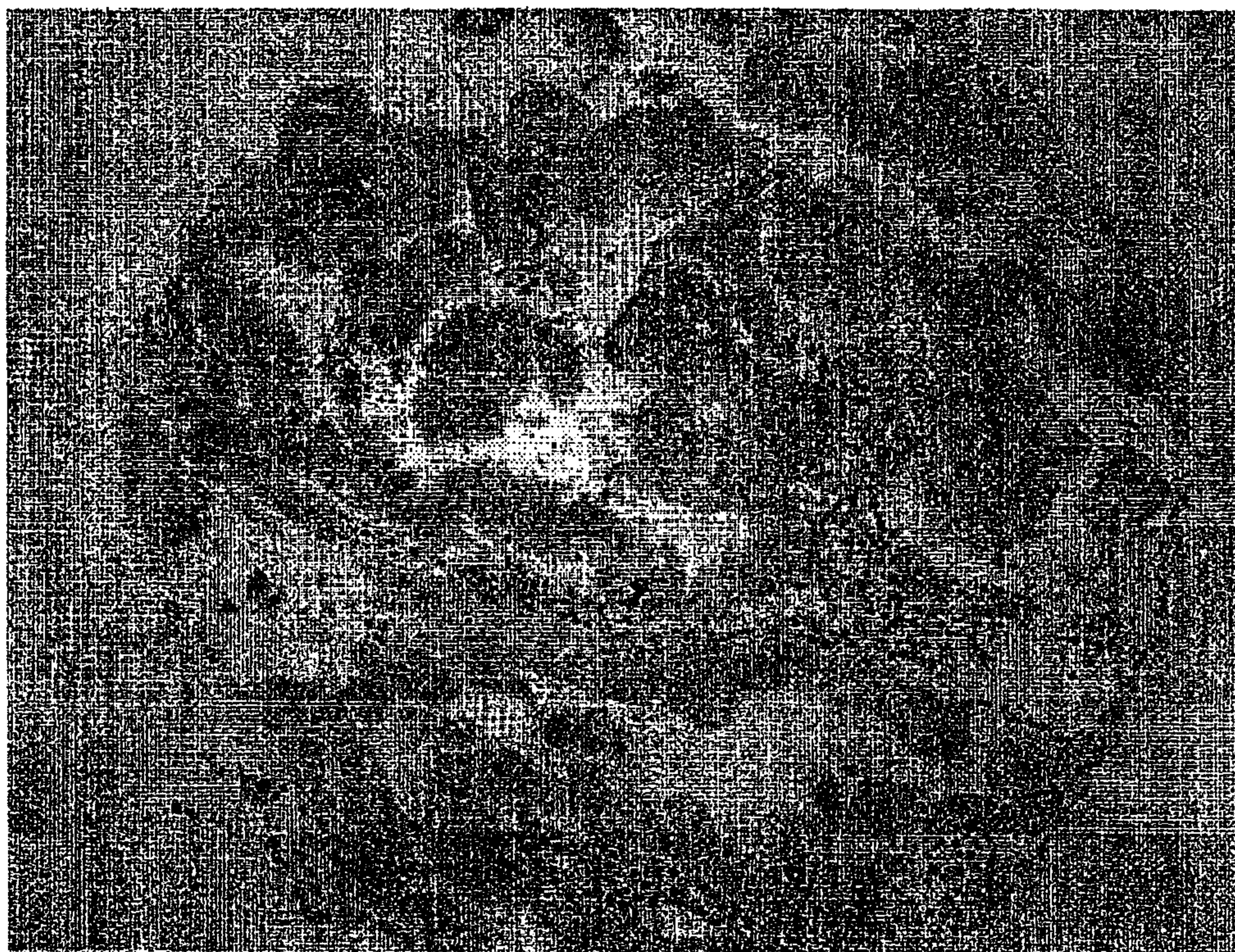


FIGURE 3G



FIGURE 3H



FIGURE 3I

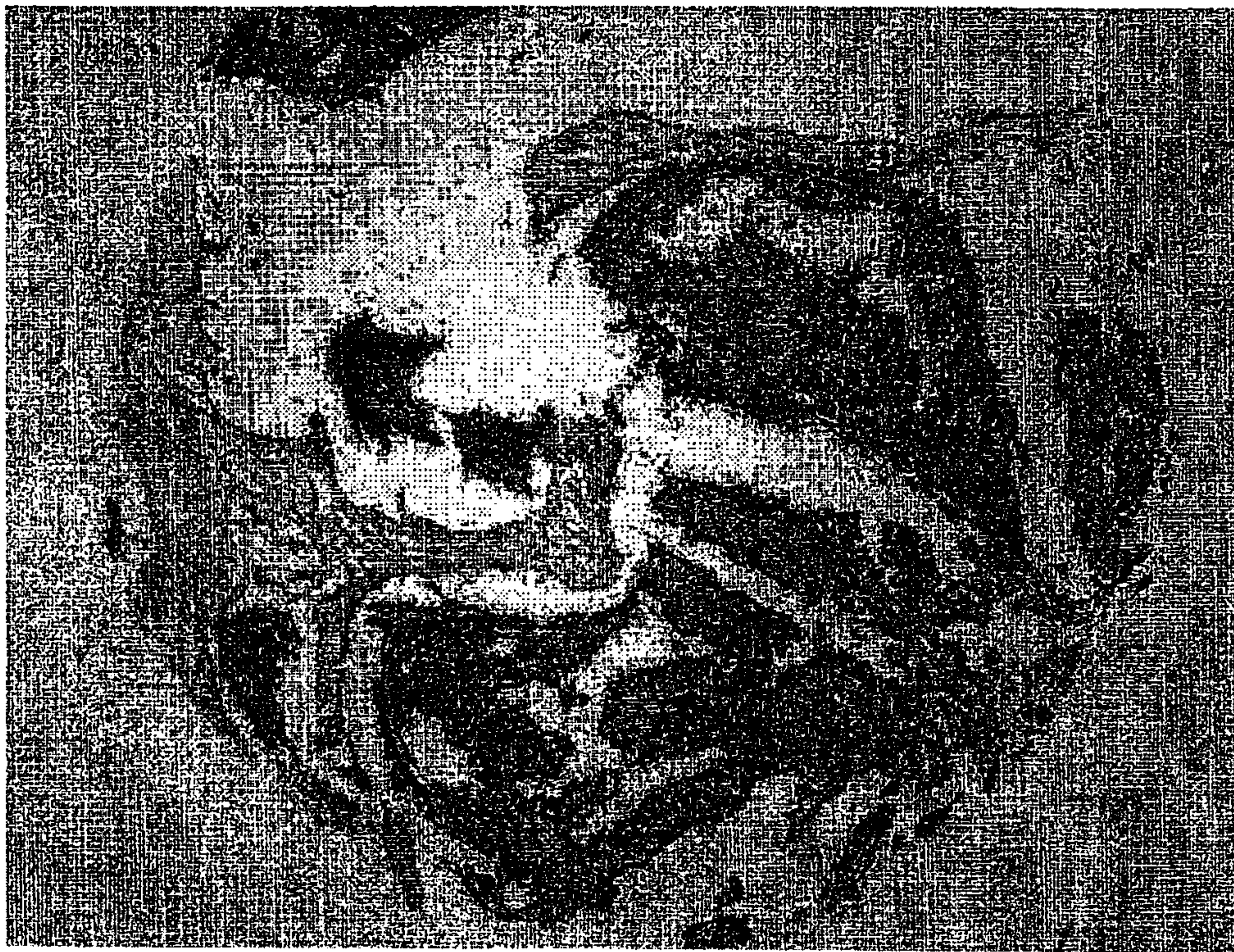


FIGURE 3J



FIGURE 3K

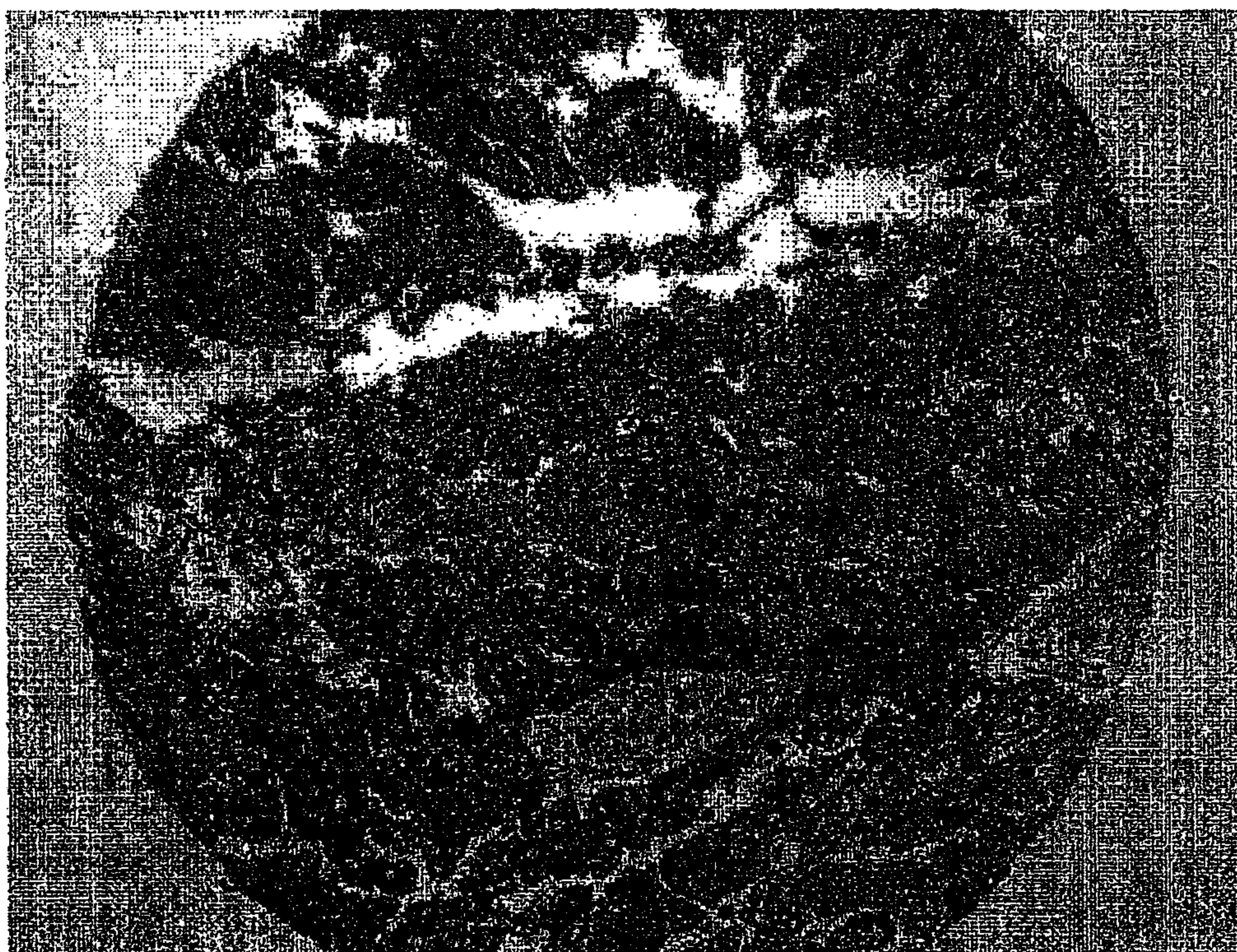
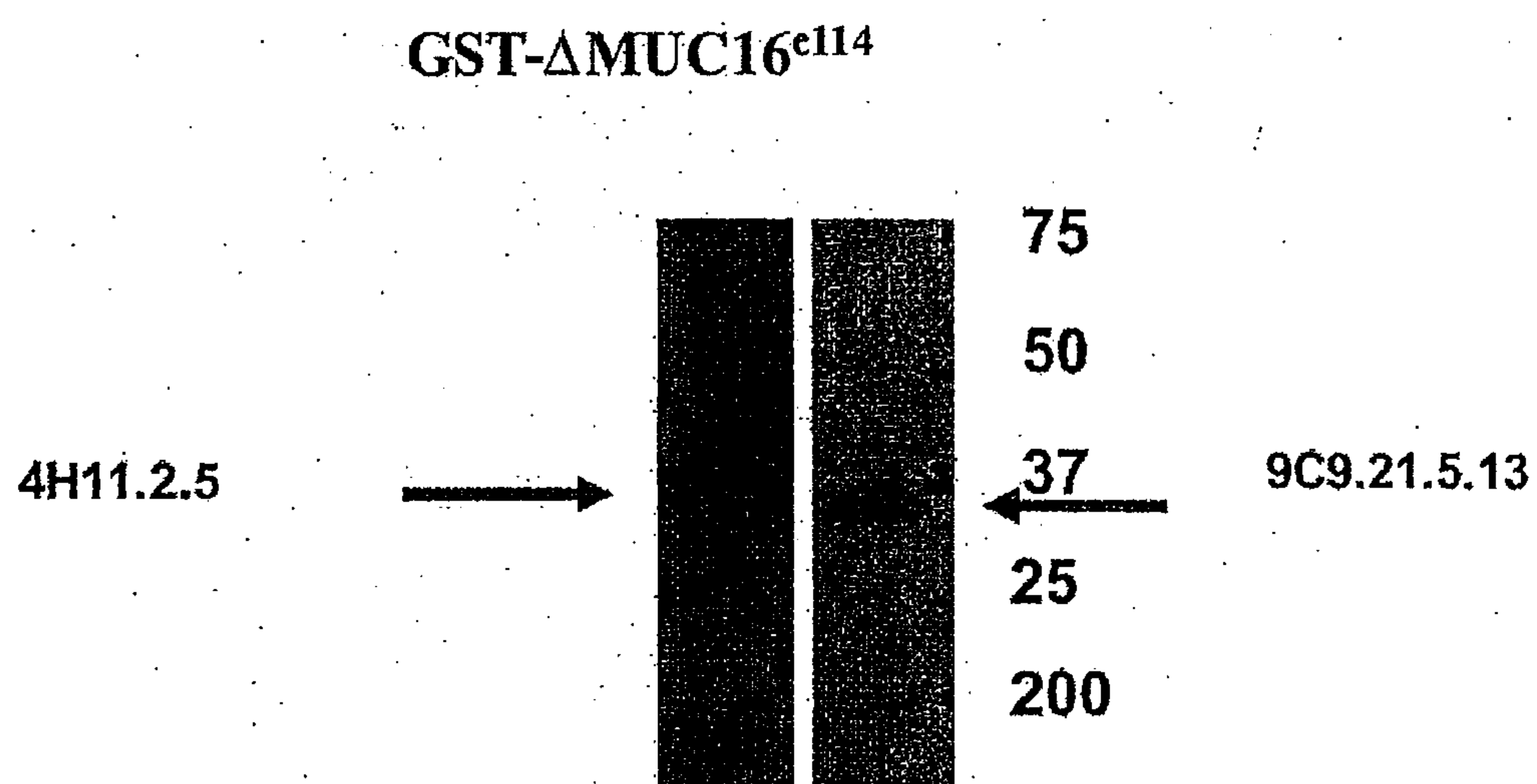


FIGURE 3L

**FIGURE 4A**

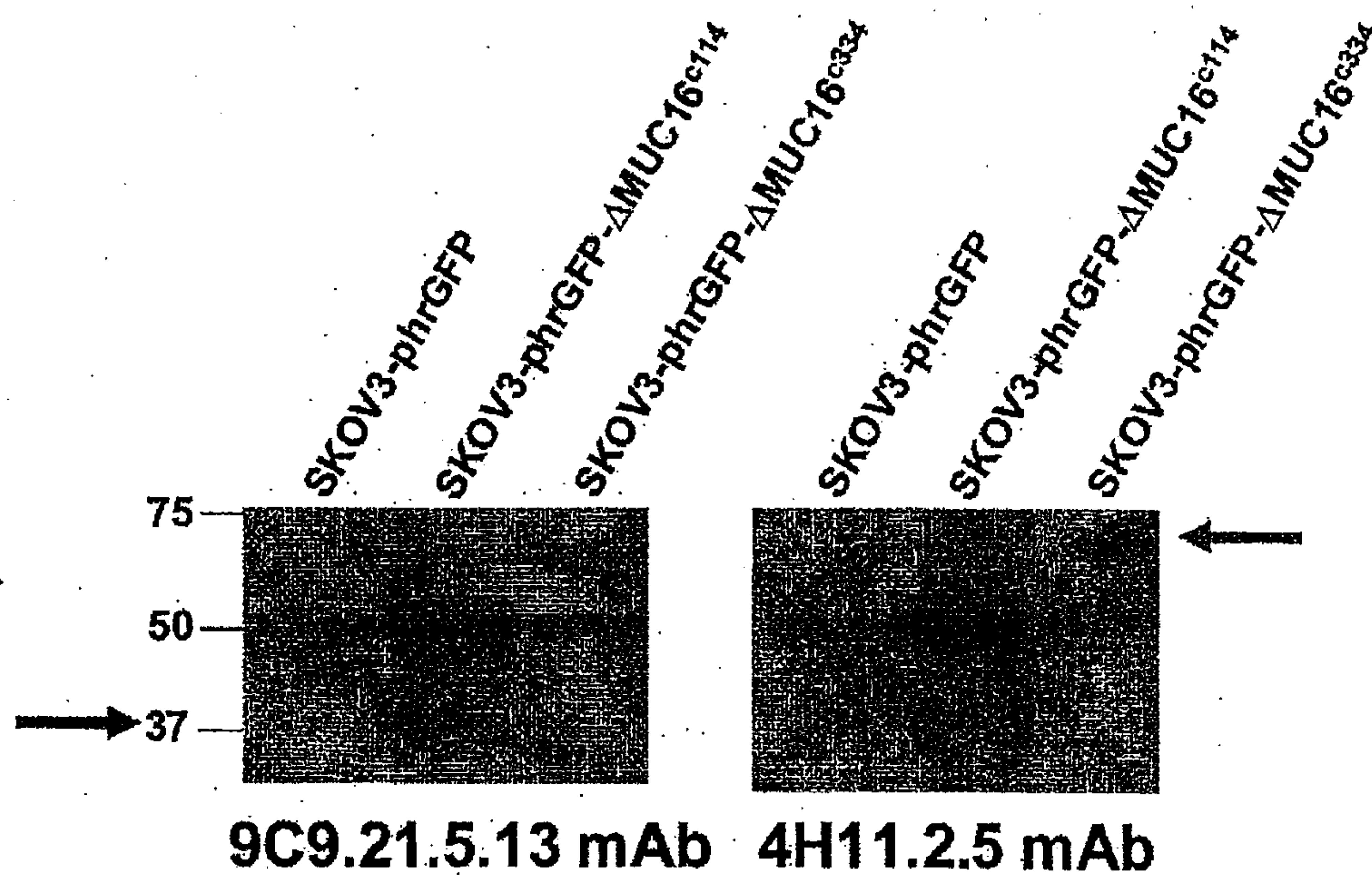
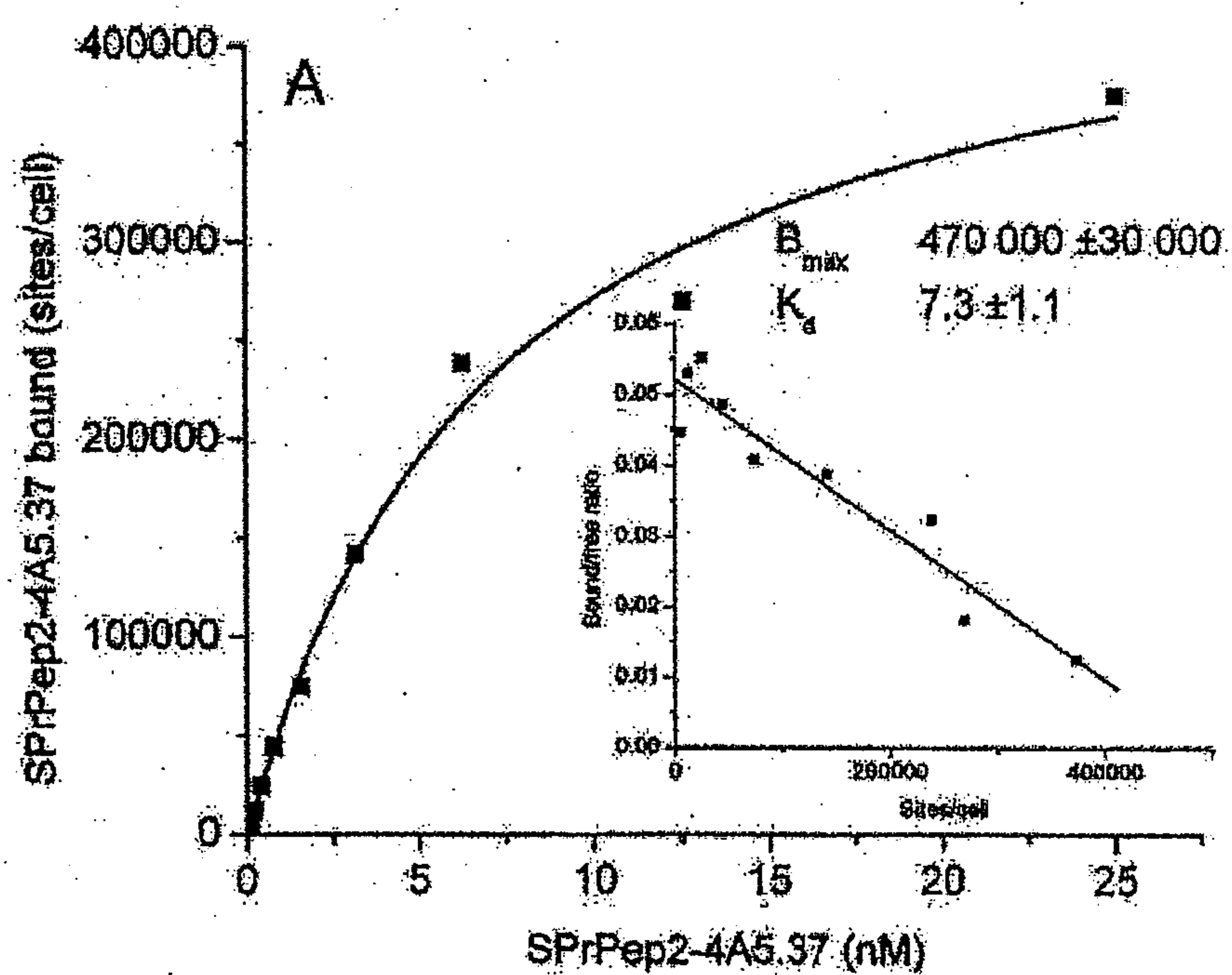


FIGURE 4B

**FIGURE 5A, PANEL A**

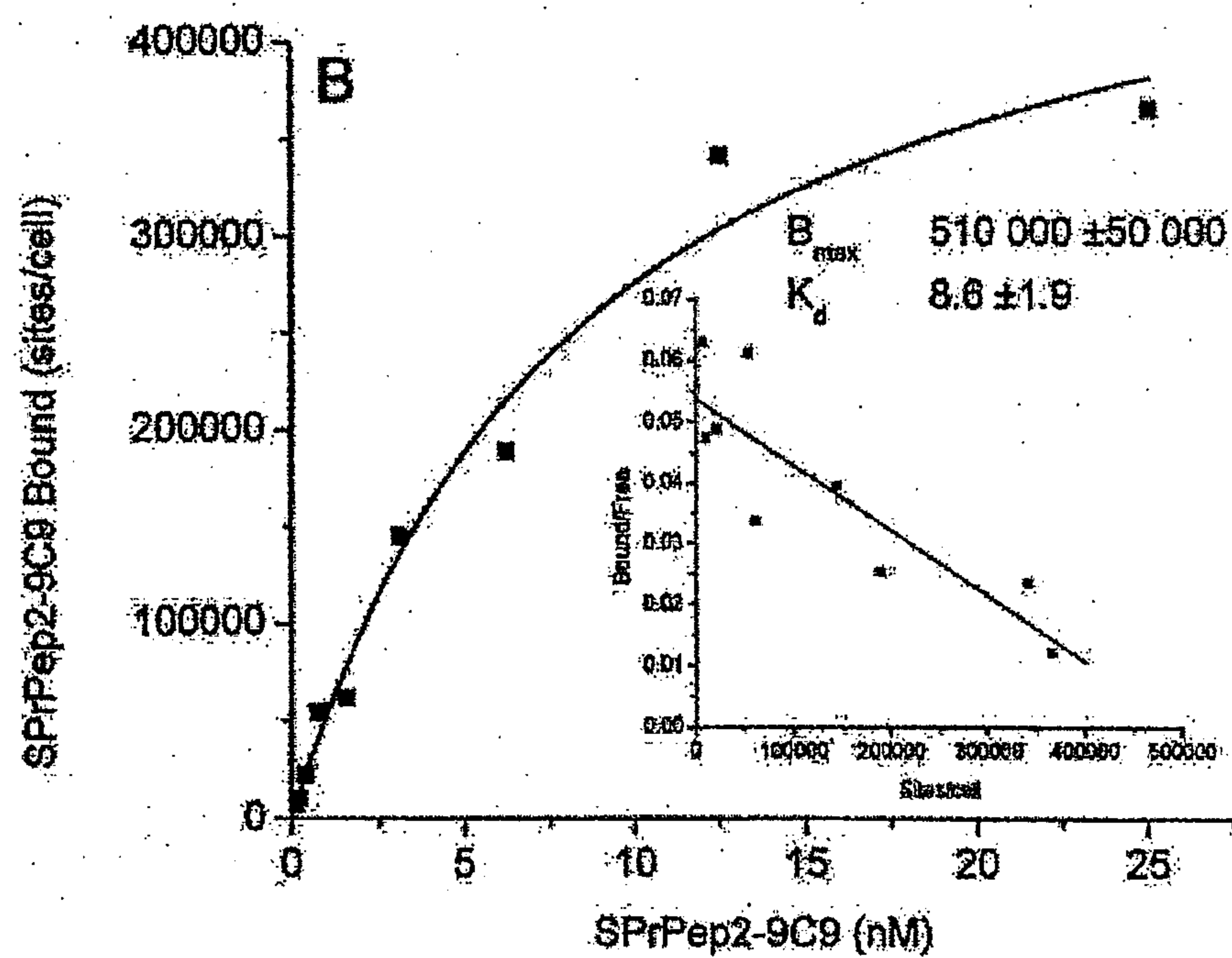


FIGURE 5A, PANEL B

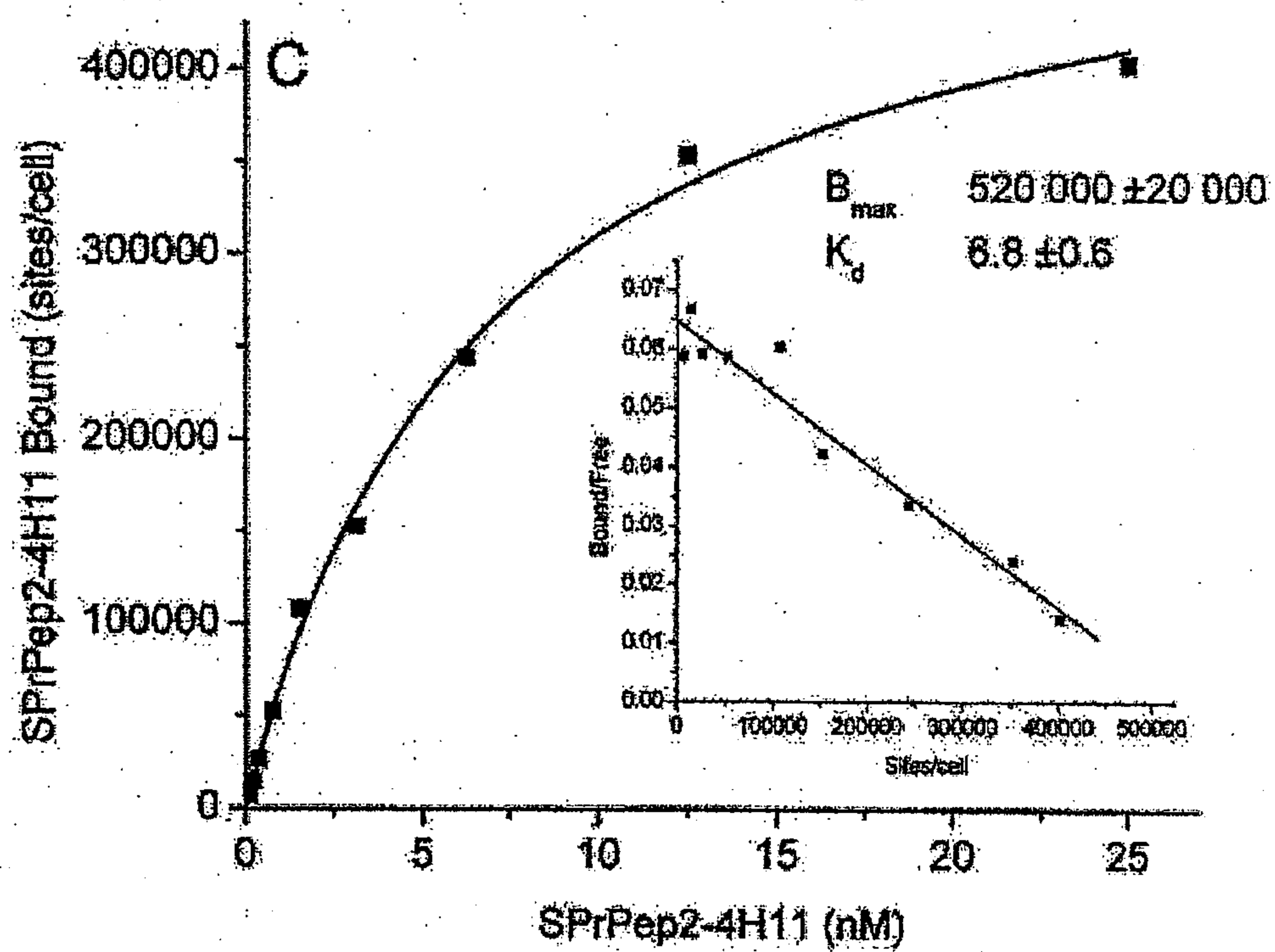


FIGURE 5A, PANEL C

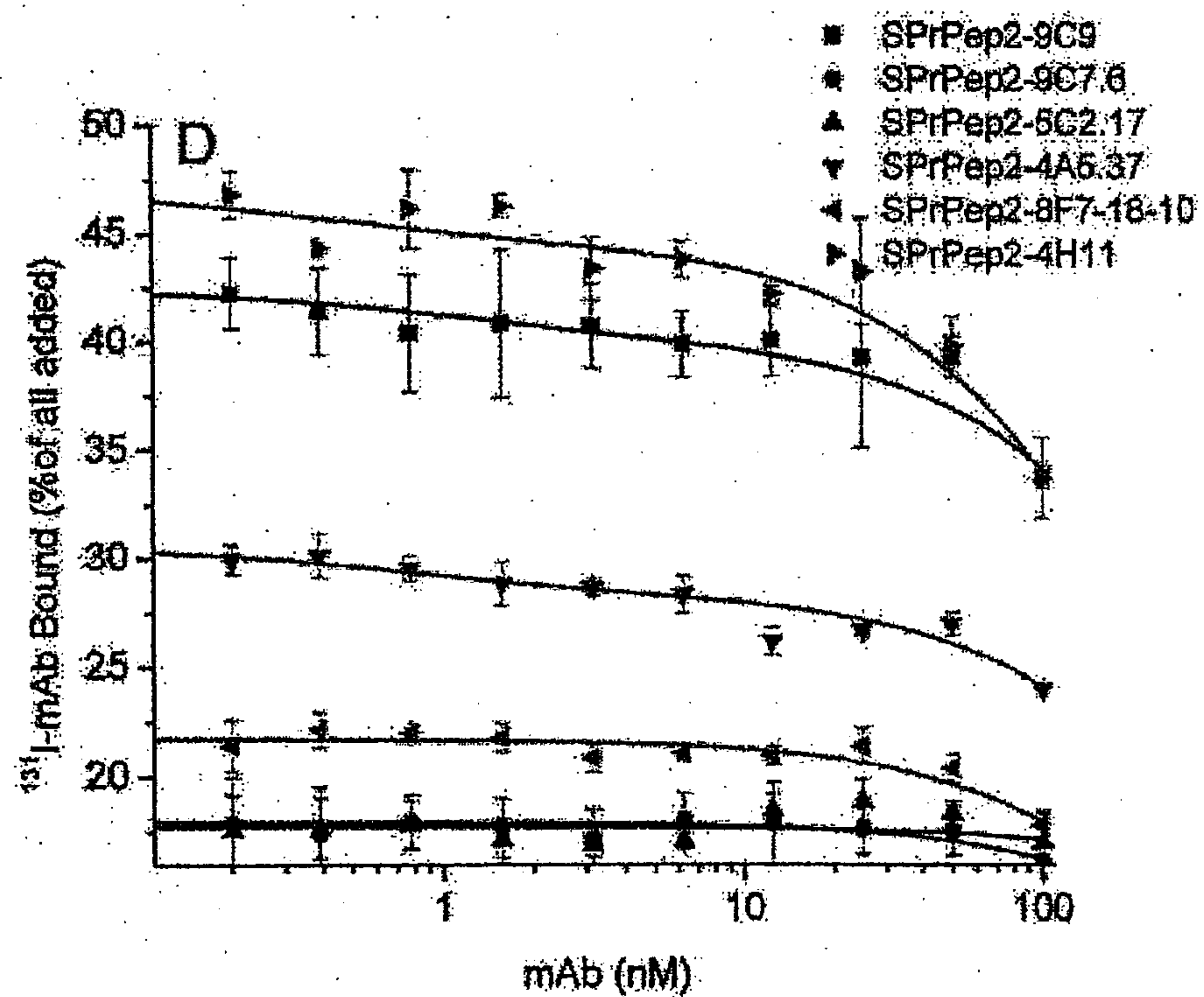


FIGURE 5A, PANEL D

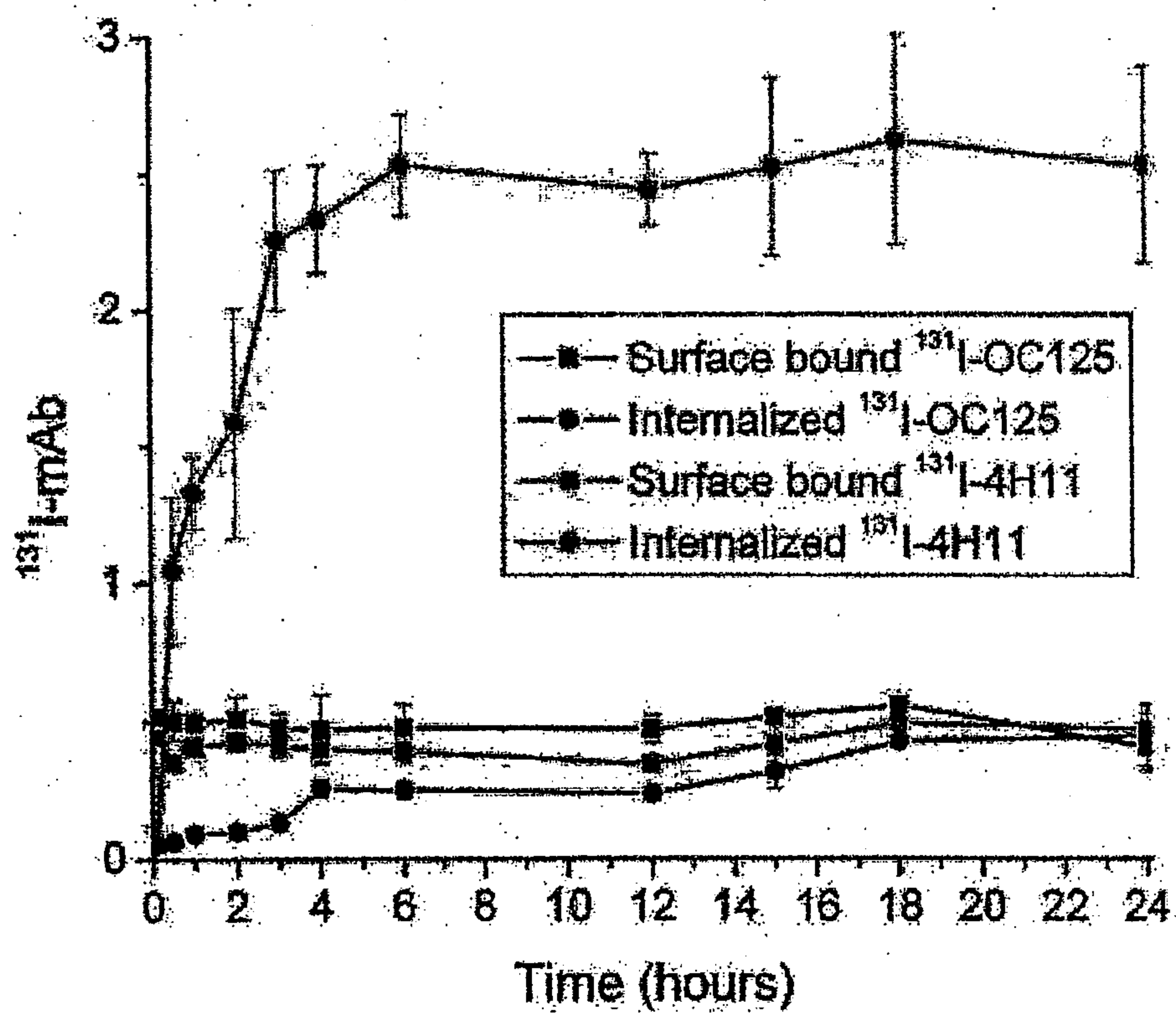


FIGURE 5B

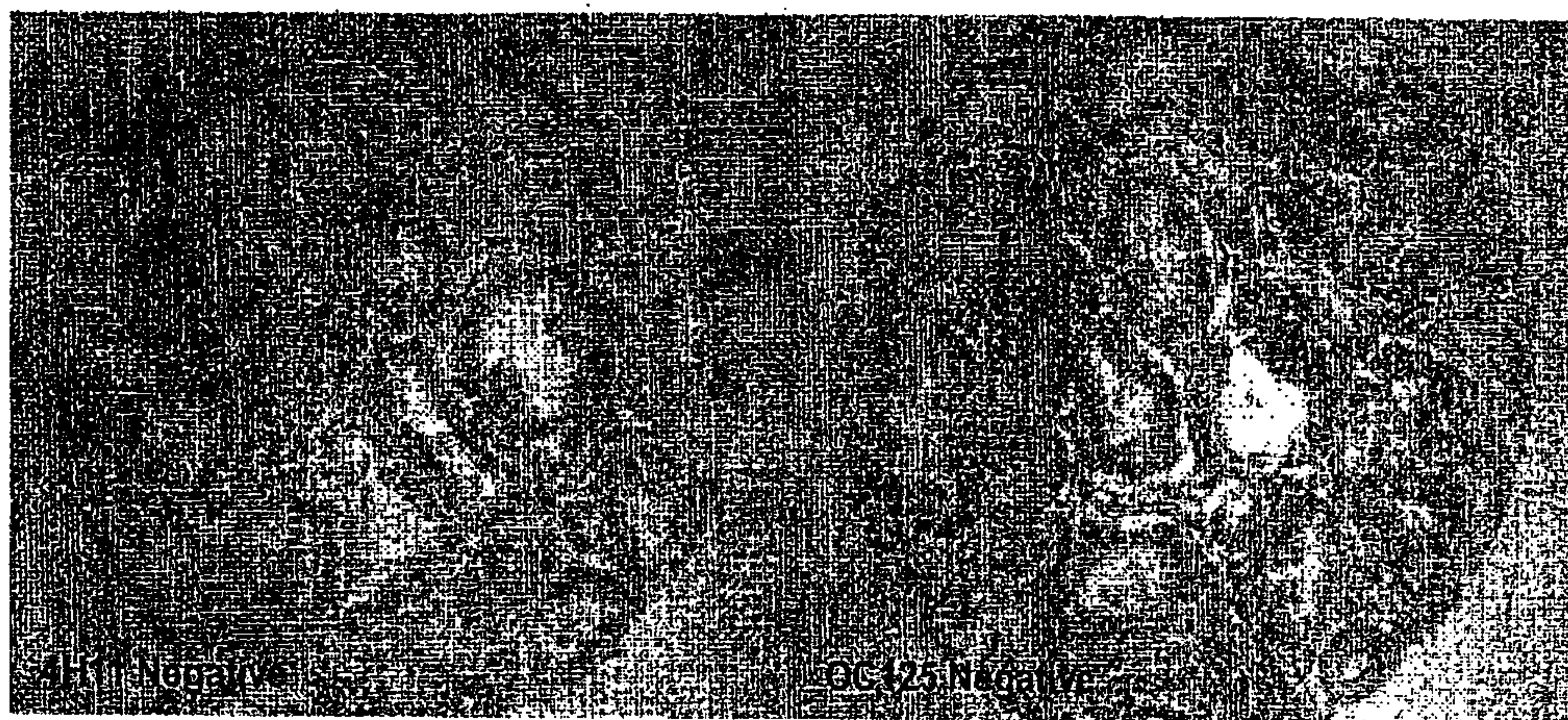


FIGURE 6A



FIGURE 6B

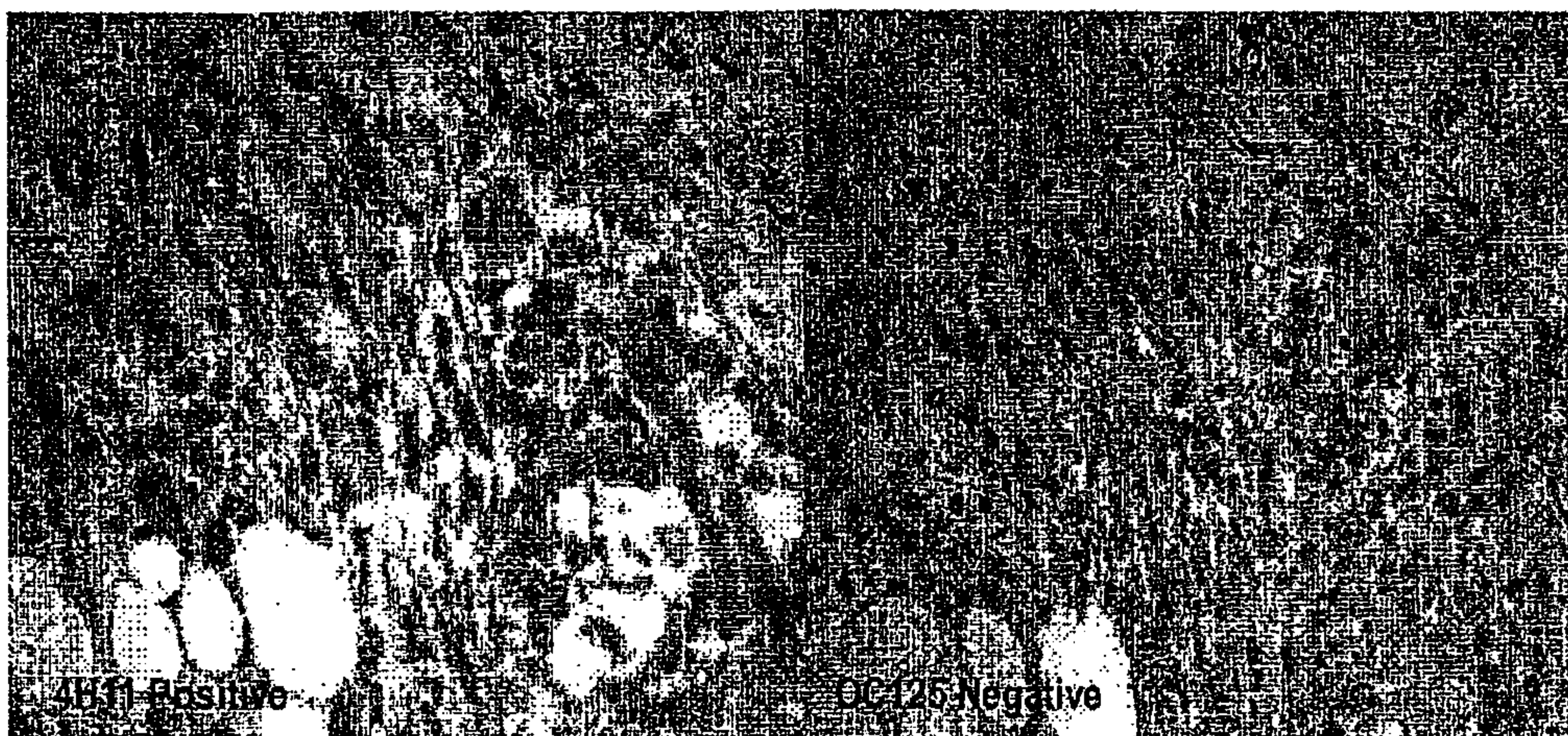


FIGURE 6C



FIGURE 6D

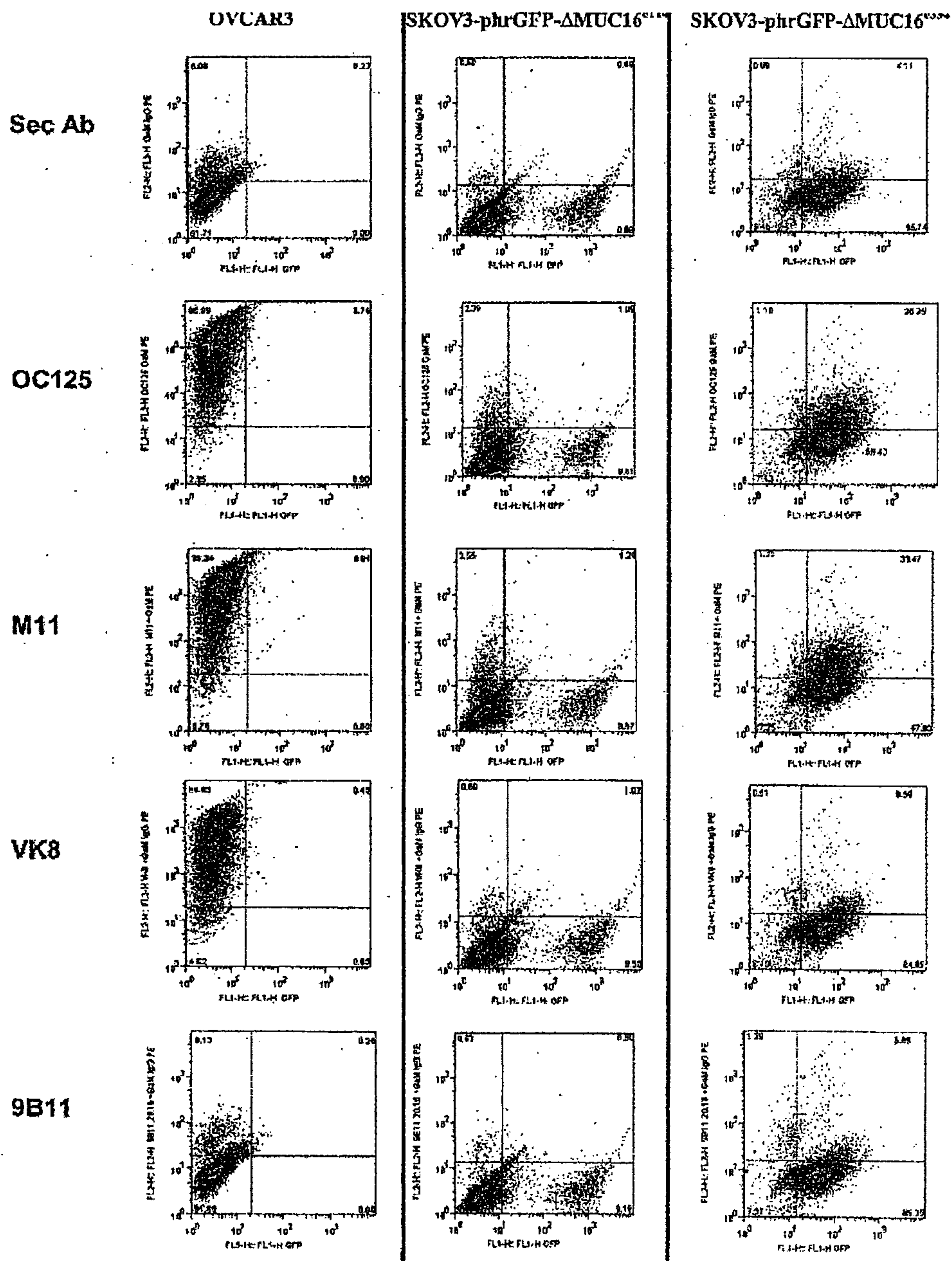


FIGURE 7, PAGE 1 OF 2

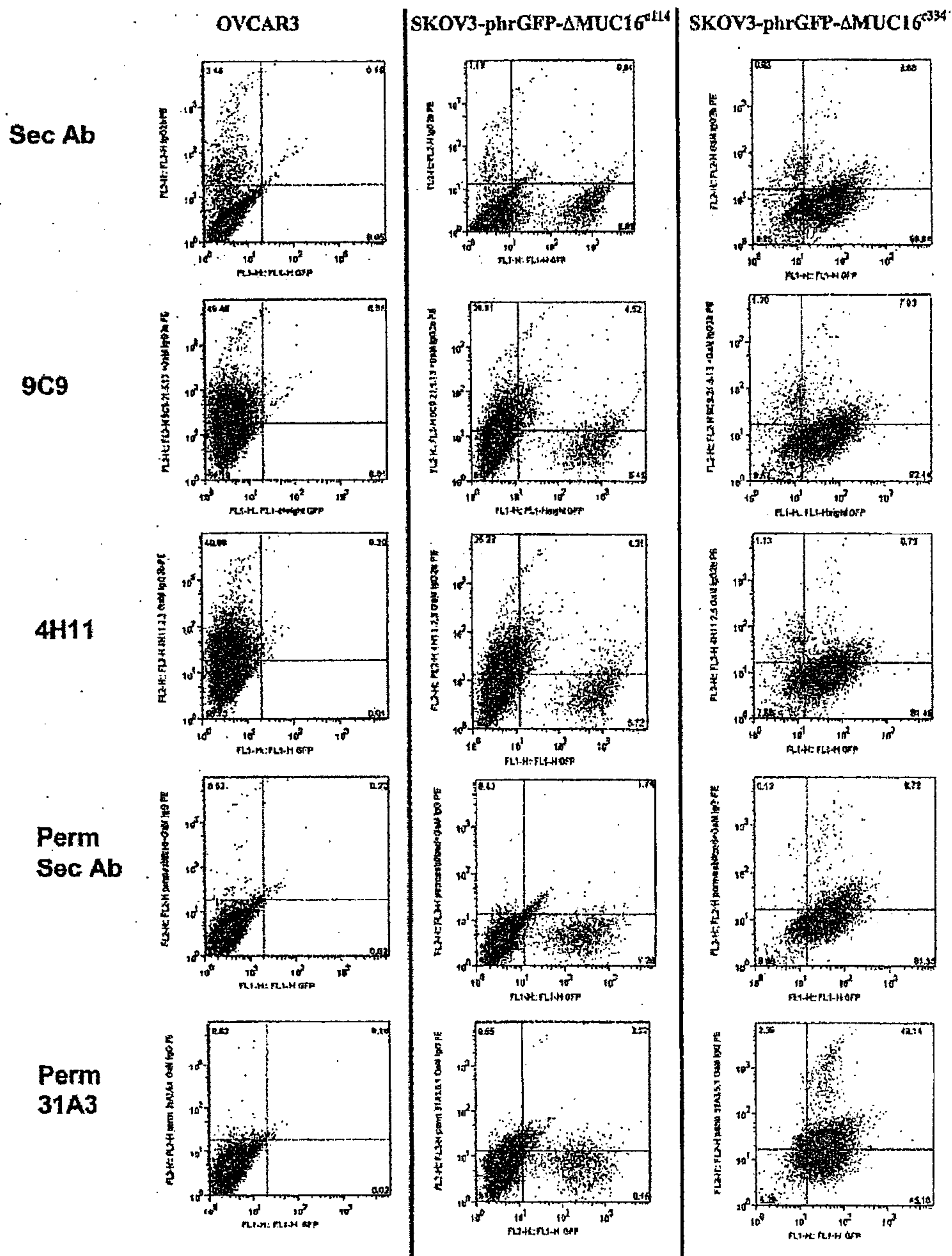


FIGURE 7, PAGE 2 OF 2

(A) 4A5 VH (SEQ ID NO:04)

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tttcagaaactatgccatgtcctgggttcgcctgagtcgggagatgaggctggagtggtcgcaaccattagcagtgctg
gtggttacatcttctattctgacagtgtgcaggagcattcaccatttccagagacaatgccaagaacaccctccacttg
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tgctatggactactggggccaagggaaccacgggtcacctctcctca

(B) 4A5 VL (SEQ ID NO:05)

gacattgagctcaccagctctccatcctccctggctgtgtcagcaggagagaaggctcactatgagctgcaaatccagtca
gagctctgctcaacagtagaaccgaaagaaccagttggcttggtaccagcaaaaaaccaggacagctctcctgaactgctga
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atcagcagtggtgcaggctgaagacctggcagtttattactgccagcaatcttataatctactcacgttcggctcctgggac
caagctggagatcaaacgg

(C) 4H11 VH (SEQ ID NO:06)

gtgaagctgcaggagtcagggggaggcttcgtgaagcctggagggccctcaaaagtctcctgtgcagcctctggattcac
tttcagtagctatgccatgtcctgggttcgcctgagtcgggagatgaggctggagtggtcgcaaccattagcagtgctg
gtggttacatcttctattctgacagtgtgcaggagcattcaccatttccagagacaatgccaagaacaccctgcacctg
caaatgggcagctctgaggtctggggacacggccatgtattactgtgcaaggcagggatttggttaactacggtgattacta
tgctatggactactggggccaagggaaccacgggtcacctctcctca

(D) 4H11 VL (SEQ ID NO:07)

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tctactgggcatccactaggcaatctggagtcctgatcgcttcacaggcagtggtctgggacagatttcactctcacc
atcagcagtggtgcaggctgaagacctggcagtttattactgccagcaatcttataatctactcacgttcggctcctgggac
caagctggaggtcaaacgg

(E) 9B11 VH (SEQ ID NO:08)

gtgaagctggaggagtcagggggagacttggtgaagcctggagggccctgaaactctcctgtgcagctctctggattcac
tttcagtagccattccatgtcttggttcgtcagactccagagaagaggctagagtggtcgcatccgtgagtagtggtg
gtaggatctactattcggacagtggtgaagggccgattcacctgcaccagagaaaatgacaggaacaccctgtatttgta
atgagtagtctgaggtctgaggacacggccatgtattattgtggaagaggacaggtattttatgctttggacaattgggg
ccaagggaaccacgggtcacctctcctca

(F) 9B11 VL.A (SEQ ID NO:09)

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gagctctgctcaacagtagaaccgaaagaaccagttggcttggtaccagcaaaaaaccaggacagctctcctgaactgctga
tctactgggcatccactaggcaatctggagtcctgatcgcttcacaggcagtggtctgggacagatttcactctcacc
atcagcagtggtgcaggctgaagacctggcagtttattactgccagcaatcttataatctactcacgttcggctcctgggac
caagctggaggtcaaacgg

(G) 9B11 VL.B (SEQ ID NO:10)

gacattgagctcaccagctctccaaagctcctgatctacaagggtttccaaccgattttctgggggtcccagacagggttcag
tggcagtggtcagggacagatttcacactcaagatcagcagagtgaggctgaggatctgggagtttattactgcttcc
aagggtcacatgttccgtggacgttcgggtggagggaccaagctggagatcaaacgg

FIGURE 8 (1 of 2)

(H) 24B3-VH (SEQ ID NO:11)

GAGGTGAAGCTGGAGGAGTCAGGACCTGAACTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGCAAGGCTTCTGGTTA
CTCATTTACTGGCTACTTTATGAACTGGGTGAAGCAGACCCATGGAAAGAGCCTTGAGTGGATTGGACGTATTAATCCTT
ACAATGGTGCTACTTTCTACAATCAGAAGTTCACGGGCAAGGCCACAATGACTGTAGACAAATCCTCTACCACAGCCCAC
ATGGAGCTCCTGAGCCTGACATCTGAGGACTCTGCAGTCTATTATTGTGGAAAGGGGAATTACTACGGCCCCCTTTGATTA
CTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA

(I) 24B3-VL (SEQ ID NO:12)

GACATTGAGCTCACCCAGTCTCCATCTTATCTTGCTGCATCTCCTGAAGAAACCATTACTATTAATTGCAGGGCAAGTAA
GAGCATTAGCAAATATTTAGCCTGGTATCAAAAGAAACCTGGGAAACTAATAAGCTTCTTATCTACTCTGGATCCACTT
TGCAATCTGGAATTCCATCAAGGTTTCAGTGGCAGTGGATCTGGTACAGATTTCACTCTCACCATCAGTAGCCTGGAGCCT
GAAGATTTTGCAATGTATTACTGTCAACAGCATAATGAATACCCGTGGACGTTTCGGTGGAGGGACCAAGCTGGAGATCAA
ACGGGCGGCCGCA

FIGURE 8 (2 of 2)

(A) Homo sapiens MUCIN-16 (GenBank NP_078966) (SEQ ID NO:13)

1 mlkpsglpgs ssptrlmtg srstkatpem dsgrtgatls pktstgaivv tehtlpfts
61 dktlasptss vvgrrttqslg vmssalpest srgmthsegr tspslspqvn gtpsrnypa
121 smvsglsspr trtsstegnf tkeastytlt vettsgpvte kytvptetst tegdstetp
181 dtryipvkit spmktfadst askenapvsm tpaettvtds htpgrtnpsf gtlyssfld
241 spkgtpnsrg etslelilst tgypfsspep gsaghsrist saplssasv ldnkisets
301 fsgqsltspl spgvpearas tmpnsaipfs mtlanaetsa ervrstissl gtpsistkq
361 aetiltfhaf aetmdipsth iaktlasewl gspgtlpgts tsalttttsp tllvseetn
421 hhstsgkete gtlntsmtp ltsapgeese mtatlvptlg fttldskirs psqvssshp
481 relrttgsts grqssstaah gssdilratt sstskasswt sestaqqfse pqhtqvwet
541 psmkterppa stsvaapitt svpsvsgft tlktsstkgi wleetsadtl igestagpt
601 hqfavptgis mtggsstrgs qgtthlltra tassetsadl tlatngvpvs vspavskta
661 gssppggtkp sytmvssvip etsslqssaf regtslgltp lntrhpfssp epdsaghtk
721 stsipllssa svledkvsat stfshhkats sittgtpeis tktkpssavl ssmtlsnaa
781 spervrnats plthpspsge etagsvltls tsaettdspn ihptgtltse ssespstls
841 psavgvkttf ssstpsthl ftsgeeteets npsvsqpets vsrvrttlas tsvptpvfp
901 mdtwptrsaq fssshlvsel ratsstsvtn stgsalpkis hltgtatmsq tnrdtfnfs
961 apqsttwpet sprfktglps atttvstsat slsatvmvsk ftspatssme atsirepst
1021 ilttettngp gsmavastni pigkgyiteg rldtshlpig ttassetsmd ftmakesvs
1081 svspsgsmda agsstpgrts qfvdtdfddv yhltsreiti prdgtssalt pqmtathpp
1141 pdpgsarstw lgilssspss ptpkvtsmsst fstqrvttsm imdtvetsrw nmpnlpstt
1201 ltpsniptsg aigkstlvpl dtpspatsle aseggltpls typestntps ihlgahass
1261 spstikltma svvkpgsytp ltfpsiethi hvstarmays sgsspemtap getntgstw
1321 pttyitttdp kdtssaqvst phsvrtlrtt enhpktesat paaysgspki ssspnltsp
1381 tkawtitdtt ehstqlhytk laekssgfet qsapgpvsvv iptsptigss tleltsdvp
1441 eplvlapseq ttitlpmatw ltslteema stdldissps spmstfaifp pmstpshef
1501 kseadtsair ntdsttldqh lgirslgrtg dltdvpitpl tttwtsvieh stqaqdtls
1561 tmspthvtqs lkdqtsipas aspshltevy pelgtqgrss seattfwkps tdtlsreie
1621 gptniqstpp mdntttgsss sgvtlgiah lpgtsspaet stnmalerrs statvsmag
1681 mgllvtsapg rsisqslgrv ssvlseste gvtddsskgss prlntqgnta lssslpsy
1741 egsgmstsis ltsspttpdv efiggstfwt kevttvmtsd iskssartes ssatlmsta
1801 gstentgkek lrtasmdlps ptpsmevtpw isltlsnapn ttdsldlshg vhtssagtl
1861 tdrslntgvt rasrlengsd tsskslsmgn sthtsmtyte ksevsssihp rpetsapga
1921 ttltstpgnr aisltlpfss ipveevistg itsgpdinsa pmthspitpp tivwtstgt
1981 eqstqplhav ssekvsvtq stpyvnsvav saapthensv ssgsstsspy ssaslesld
2041 tisrrnaitw wlwdlttslp tttwpstsls ealssghsgv snpsstttef plfsaasts
2101 akqrnpetet hgpqntaast lntdassvtg lsetpvgasi ssevplpmi tsvsdvsgl
2161 sestansplg tassagtklt rtislptses lvsfrmnkdp wtvsiplgsh pttntetsi
2221 vnsagppglv tvasdvidtp sdgaesiptv sfspspdtev ttishfpekt thsfrtiss
2281 theltsrvtv ipgdwmssam stkptgasps itlgerrtit saapttspiv ltsaftets
2341 vsldnettkv tsdildarkt nelpsdssss sdlnstias stmdvktas isptsisgm
2401 asspslflss drpqvptstt etntatpsv ssntysldgg snvggtpstl ppftithpv
2461 tssallawsr pvrftstmvs tdtasgenpt ssnsvvtsvp apgtwtsvgs ttdlpamgf
2521 ktspageahs llastiepat aftphlsaav vtgssatsea slttsseska ihsspqtpt

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2581 ptsganwets atpesllvvt etsdttltsk ilvtdtilfs tvstppskfp stgtlsgas
 2641 ptllpdtpai pltateptss latsfdstpl vtiasdslgt vpettltmse tsngdalvl
 2701 tvsnpdrsip gitiggtves plhpsstsp kivaprntty egsitvalst lpagttgsl
 2761 fsqssenset talvdssagl erasvmltt gsqgmassgg irsgsthstg tktfsslpl
 2821 mnpgevtams eittnrlltat qstapkgipv kptsaesgll tpvsasssps kafasltta
 2881 ptwgipqstl tfefsevpsl dtksaslptp gqslntipds dastasssps kspeknpra
 2941 mmtstkaisa ssfqstgfte tpegsaspsm agheprvpts gtgdpryase smsypdpsk
 3001 ssamtstsla sklttlfstg qaarsgssss pislsteket sflsptasts rktslflgp
 3061 marqpnilvh lqtsaltlsp tstlnmsqee ppeltssqti aeeegttaet qtlfttpse
 3121 ptsllpvssp teptarrkss petwassiv paktslvett dgtlvttikm ssqaaqgns
 3181 wpapaeetgs spagtspgsp emsttlkims skepsispei rstvrnspwk tpettvpme
 3241 tvepvtlqst alsgstsis hlptgttspt ksptenmlat ervslspsp eawtnlysg
 3301 pggtrqslat mssvslespt arsitgtgqq sspelvsktt gmefsmwhgs tggttgdth
 3361 slstssnile dpvtspnsvs sltdkshkt etwvsttaip stvltnnkima aeqqtsrsv
 3421 eaysstssws dqtsgsditl gaspdvntnl yitstaqtts lvslpsgdqg itsltnpsg
 3481 ktssassvts psigletlra nvsavksdia ptaghlsqts spaevsildv ttaptpgis
 3541 tittmgtnsi stttnpevg mstmdstpat errttstehp stwsstaasd swtvtdmts
 3601 lkvarspgti stmhttsfla ssteldsmst phgritvigt slvtpssdas avktetsts
 3661 rtlspsdtt stpistfsrv qrmsisvpdi lstswtpsst eaedvpvsmv stdhastkt
 3721 pntplstflf dslstldwdt grslssatat tsapqgattp geltletmis patsqlpfs
 3781 ghitsavtpa amarssgvtf srpdptska eqtstqlptt tsahpgqvpr saattldvi
 3841 htaktpdatf qrqggtaltt earatsdwn ekekstpsap witemmnsvs edtikevts
 3901 ssvlrtnltnl dinlesgtts spswksspye riapsestd keaihpstnt vettgwvts
 3961 ehashstipa hsasskltsp vttstrega ivsmstttwp estrartepn sfltielrd
 4021 spymdtsst qtsiisspgs taitkgprte itsskriess flaqsrmrdd spseaitrl
 4081 nfpamtesgg milamqtsp gatslsaptl dtsataswtg tplattqrft ysekttlfs
 4141 gpedtsqpsp psveetssss slvpihatts psnilltsqg hpsstppvt svflsetsg
 4201 gkttmdsris lepgtslppn lssstageals tyeasrdka ihhsadtavt nmeatssey
 4261 pipghstkpsk atsplvtshi mgditsstsv fgssetteie tvssvngglq erstsqvas
 4321 atetstvith vssgdatthv tktqatfssg tsissphqfi tstntftdvs tnpstslim
 4381 essgvttittg tgptgaatqg pylltdstmp yltetplavt pdfmqsekt liskgpkdv
 4441 wtspssvaet sypssltplf vttippatst lggqhtsspv satsvltsgl vkttdmlnt
 4501 mepvtnspqn lnnpsneila tlaattdiet ihpsinkavt nmgtassahv lhstlpvss
 4561 pstatpmvp assmgdals isipgsettd iegeptsslt agrkenstlq emnsttesn
 4621 ilsnvsvgai teatkmevps fdatfiptpa qstkfpdifs vassrlsnsp pmtisthmt
 4681 tqtgssgats kiplaldtst letsagtpsv vtegfahski ttamnndvkd vsqtnppfq
 4741 easspsqap vlvttlpssv aftpqwhsts spvsmssvlt sslvktagkv dtsletvts
 4801 pqsmntldd isvtsaattd ietthpsint vvtnvgttgs afeshstvsa ypepskvts
 4861 nvtstmedt tisrsipks ktrtetett ssltpklret sisgeitsst etstvpkye
 4921 tgattevsrt dvtsssstf pgpdqstvl distetntrl stspimtesa eitittqtg
 4981 hgatsqdtft mdpsnttpqa gihsamthgf sqldvttlms ripqdvswts ppsvdkts
 5041 ssflsspamt tpslisstlp edklsspmts lltsglvkit dilrtrlepvt ssllpnfss
 5101 sdkilatskd skdtkeifps inteetnvka nnsgheshsp aladsetpka ttqmvtitt
 5161 gdpapstmp vhgsssettni kreptyfltp rlretstsqe ssfptdtsfl lskvptgti

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5221 evsstgvnss skistpdhdk stvppdtftg eiprvftssi ktksaemtitt tqasppesa
5281 hstlpldtst tlsqggthst vtqgfpvsev ttlmgmppgn vswmttppve etssvsslm
5341 spamtspspv sstspqsips splpvtalpt svlvtttdvl gttspevts sppnlssit
5401 erpatykda hteaamhst ntavtnvgtg sgghksqssv ladsetskat plmsttstl
5461 dtstvststpn isqtnqigte ptaslsprlr esstsektss ttetntafsy vptgaitqa
5521 rteisssrts isdldrptia pdistgmitr lftspimtkas aemtvtqtgt tpgatsggi
5581 pwdtsttlfq ggthstvsqg fphseittlr srtpgdvswm ttpvveetss gfsmlmspm
5641 spspvssstsp esipssplpv talltsvlvt ttnvlgttsp epvtssppnl ssptqerlt
5701 ykdahteam hasmhtntav anvgtsisgh esqssvpads htskatspmg itfamgds
5761 ststpaffet riqtestssl ipglrdtrts eeintvtets tvlsevpttt ttevsrtev
5821 tssrttisgp dhskmspyis tetitrlstf pfvtgstema itnqtgpgit isqatltd
5881 sstaswegth spvtqrfphs eettmsrst kgvswqssps veetsspspp vplpaitsh
5941 slysavsgss ptsalpvtsl ltsgrrktid mldthselvt sslpsassfs geiltseas
6001 ntetihfsen taetnmgttn smhklhssvs ihsqpsgthp pkvtgsmmed aivststpg
6061 petknvdrds tspltpelke dstalvmnst tesntvfssv sldaatevsr aevtyydp
6121 mpasaqstks pdispeasss hnsppltis thktiatqtg psgvtslgql tldtstiat
6181 agtpsartqd fvdsettsvm nndlnvlt spfsaeans lssqapllvt tpspvtst
6241 gehstsslvs vtsvptptla kitdmdtnle pvtrspqnlr ntlatseatt dthtmhpsi
6301 tavanvgts spnefyftvs pdsdpykats avvitstsgd sivstsmprs samkkiese
6361 tfslifrlre tstsqkigss sdtstvfda ftaattevsr teltsssrts iggtektptm
6421 pdtstrsvtm lstfagltks eertiatqtg phratsqgtl twdtsittsq agthsamth
6481 fsqldlstlt srpeyisgt sppsvektss sssllslpai tpspvpptl pesrpsppv
6541 ltslptsglv kttdmlasva slppnlgst hkipttsedi kdekmpst niavtnvgt
6601 tsekesyssv payseppkv spmvtsfnir dtivstsmg sseitrieme stfslahgl
6661 gtstsqdpiv steksavlhk lttgatetsr tevassrrts ipgpdhstes pdistevip
6721 lpislgites snmtiitrtg pplgstsggt ftldtpttss ragthsmatq efphsemtt
6781 mnkdpeilsw tippsiekts fssslmpspa mtspvssstl pktihttpsp mtalltpsl
6841 mtdtlgtsp epttssppnl sstsheilt dedttaieam hpststaate vettssghg
6901 qssvladsek tkatapmddt stmghttvt smsvssettk ikrestyslt pglretsis
6961 nasfstddsi vlsevpgtt aevsrtevt sgrtsipggs qstvlpeist rtmtrlfas
7021 tmtesaemti ptqtgpgst sqdtltldts ttksqakths tltqrfphse mttlmsrgp
7081 dmswqsspsl enpslpsll slpattspvp isstlpvtis ssplpvtall tsspvtttd
7141 lhtspelvt sppklshtsd erlttgkdt nteavhpstn taasnveips sghepsa
7201 adsetskats pmfitstqed ttvaistphf letsriqkes isslsplkre tgssvetss
7261 ietsavlsev sigatteisr tevtssrts isgsaestml peisttrkii kfptspila
7321 ssemtiktgt sppgstsest ftldtsttps lvithstmtq rlpheittl vsrgagdv
7381 psslpveets ppssqlslsa mispspvst lpasshssa svtslltpg vkttevl
7441 aepetsspps lsstsveila tsevttdtek ihpfsntav kvgtsssghe spssvlpds
7501 ttkatsamgt isimgdtsvs ttpalsntr kigsepasll ttrretsts eetlatea
7561 tvlskvstga ttevsrteai sfsrtmsgp eqstmsqdis igtiprisas svltesakm
7621 ittqtgpses tlestlnlnt attpswveth siviqgfphp emttsmgrgp ggvswwpspp
7681 vketsppssp lsipavtsph pvsttflahi ppslpvtsl ltsgpatttd ilgtstepg
7741 sssslstts herlttykdt ahteavhpst ntggtnvatt ssgyksqssv ladsspmct
7801 stmgdtsvlt stpafletrr iqtelasslt pglressgse gtssgskmst vlsvkvtga

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7861 teiskedvts ipgpaqstis pdistrtrvsw fstspvmtes aeitmnhths plgattqgt
 7921 tldtssttsl tmthstisqg fshsqmstlm rrgpedvswm sppllektrp sfslmsspa
 7981 tspspvstsl pesissplp vtllltsgla kttldmlhkss epvtnspanl sstsveila
 8041 sevttdekt hpssnrtvtd vgtsssghes tsfvladsqt skvtspmvit stmedtsvs
 8101 stpgffetsr iqteptssl lglrktssse gtlatemst vlsqvptgat aevsrtev
 8161 sartsisgfa qltvspetst etitrlptss imtesaemmi ktqtdppgst pesthtvdi
 8221 ttpnwveths tvtqrfshse mttlvrsrpg dmlwpsqssv eetssassll slpattsp
 8281 vsstlvedfp saslpvtsll npglvitttr mgisrepqts stsnlsstsh erlttledt
 8341 dtedmqpsth tavnrvrtsi sghesqssvl sdsetpkats pmgttytmge tsvsistsd
 8401 fetsriqiep tssltsglre tssserissa tegstvlsev psgattevsr tevissrgt
 8461 msgpdqftis pdisteaitr lstspimtes aesaitietg spgatsegtl tldtstttf
 8521 sgthstaspg fshsemttlm srtpgdvwp slpsveeass vssslsspa tstsffstl
 8581 esissphpv talltlgpkv ttdmlrtsse petssppnls stsaailats evtkdreki
 8641 psantpvvnv gtviykhls ssvladlvt kptspmatss tlgntsvsts tpaipetmm
 8701 qptssltsgl reistsqets satersasls gmpgtatkv srtealslgr tstpgpaqs
 8761 ispeistet tristplttt gsaemtltk tghsgassqg tftldtssra swpgthsa
 8821 hrsphsgmtt pmsrgpedvs wpsrpsvekt sppsslvsl avtspsplys tpsesshss
 8881 lrvtslftpv mmkttldt slepvtssp smnitsdesl atskatmete aiqlsenta
 8941 tqmgtisarq efysypglp epskvtspvv tsstikdivs ttipasseit riemestst
 9001 tptpretsts qeihsatkps tvpykaltsa tiedsmqvm sssrgpspdq stmsqdist
 9061 vitrlstspi ktestemtit tqtgspgats rgtltldtst tfmsgthsta sqgfshsqm
 9121 almsrtpgdv pwlshpsvee assasfsls pvmtssspvs stlpdsihss slpvtallt
 9181 glvkttellg tssepetsp pnlsstsaie laitevttdt eklemtnvvt sgythesps
 9241 vladsvttka tssmgitypt gdnvltstp afsdtsriqt ksklsltpgl metsiseet
 9301 satekstvl svptgattev srteaissr tsipgpaqst mssdtmeti tristpltr
 9361 estdmaitpk tgpsgatsqg tftldsssta swpgthsaat qrfpqsvvt pmsrgpedv
 9421 wpsplsvekn sppsslvss svtspsplys tpsgsshss vpvtslftsi mmkatdmd
 9481 slepettsap nmnitsdesl aaskattete aihvfentaa shvettsate elyssspgfi
 9541 eptkvispvv tssirdnmv sttmppgssi trieiesmss ltpglretrt sqditsste
 9601 stvlykmpsg atpevsrtev mpssrtsipg paqstmsldi sdevvtrlst spimtesae
 9661 tittqtgysl atsqvtlplg tsmtflsgth stmsqglshs emtnlmsrgp eslswtspr
 9721 vettrsssl tslplttsls pvsstlldss psslpvtsl ilpglvkte vldtssepki
 9781 ssspnlssts veipatseim tdekihpss ntavakvrt ssvheshssv ladsettiti
 9841 psmgitsavd dtvftsnpa fsetrripte ptfsltpgfr etstseetts itetsavly
 9901 vptsattevs mteimssnri hipdsdqstm spdiitevit rlssssmmse stgmtittql
 9961 sspgataqst ltlatttapl arthstvprr flhsemttlm srspenpswk sslfvekt
 10021 sssllslpvt tspsvsstlp qsipsssfsv tslltpgmvk ttdtstepgt slspnlsgt
 10081 veilaasevt tdekihpss smavtnvgtt ssghelyssv sihsepskat ypvgtppsm
 10141 etsistsmpa nfettgfeae pfshltsgfr ktnmsldtss vtptntpssp gsthllqssl
 10201 tdftssakts spdwpasqy teipvdiitp fnaspsites tgitsfpesr ftmsvtestl
 10261 hlstdllpsa etistgtvmp slseamtsfa ttgvpraisg sgspfsrtes gpgdatlst
 10321 aeslpsstpv pfssstfttt dsstipalhe itsssatpyr vdtslgtess ttegrlvmv
 10381 tldtsaqpgr tssspildtr mtesvelgtv tsayqvpsls trltrtdgim ehitkipne
 10441 ahrgtirpvk gpqtstspas pkglhtggtk rmettttalk ttttalktts ratltsvyl

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10501 ptlgtltpln asmqmastip temmittpyv fpdvpettss latslgaets talprttpe
10561 fnresettas lvrsrgaers pviqtladvss sepdttaswv ihpaetiptv skttpnffh
10621 eldtvsstat shgadvssai ptnispseld altplvtisg tdtsttfptl tksphetet
10681 ttwlthpaet sstiprtipn fshhesdatp siatspgaet ssaipimtv ssgaedlvts
10741 vtssgtdrnm tiptltlspg epktiaslvt hpeagtssai ptstispavs rlvtsmvt s
10801 aaktsttnra ltntspgepat tvslvthpaq tsptvpwttt iffhsksdtt psmttshga
10861 sssavptptv stevpgvvtv lvtssravis ttipiltlsp gepettpsma tshgeeass
10921 iptptvspgv pgvvtslvts sravtsttip iltfslgepe ttpsmatshg teagsavpt
10981 lpevpqmvts lvassravts ttltltlsp gepettpsma tshgaeasst vptvspevp
11041 vvtslvtsss gvnstsiptl ilspgelett psmatshgae assavptptv spgvsgvvt
11101 lvtssravts ttipiltlss sepettpsma tshgveassa vltvspevp mvtslvtss
11161 avtsttiptl tissdepett tsvthseak misaiptlav sptvqglvts lvtssgset
11221 afsnlvass qpetidswva hpgteassvv ptltvstgep ftntslvthp aessstlpr
11281 tsrfshseld tmpstvtsp aesssaistt ispgipgvt slvtssgrdi satfptvpe
11341 pheseatasw vthpavtst vprttpnys hsepdttpsia tspgaeatsd fptitvspd
11401 pdmvtsgvts sgttdtsitip tltlssgepe tttstfityse thtssaiptl pvspgaskm
11461 tsvlvisgtd stttfptlte tpyepettai qlihpaetnt mvprttpkfs hksdttlp
11521 aitspgpeas savstttisp dmsdlvtslv pssgtdtst fptlsetpye pettatwlt
11581 paetsttvsg tipnfshrgs dtapsmvtsp gvdtrsgvpt ttippispgv vtsqvtssa
11641 dtstaipilt pspgepetta ssathpgtgt gftvpirtvp ssepdtmasw vthppgtst
11701 vsrttssfsh sspdatpvma tsprteassa vlttispgap emvtsgitss gaatsttv
11761 lthspgmpet tallsthprt etsktfpast vfpqvsetta sltirpgaet stalptgtt
11821 slftllvtgt srvdlsptas pgvsaktapl sthpgtetst miptstlslg llettglla
11881 sssaetstst ltlvtspavs glssasitt dkpqvtswnt etspsvtsvg ppefsrtvt
11941 ttmtlipsem ptppktshge gvspttilrt tmveatnlat tgssptvakt tttfntlag
12001 lftplttpgm stlasesvts rtsynhrswi sttssynrry wtpatstpv stfsggist
12061 sipsstaavt pfmvpftlnf titnlqyeed mrhpgsrkfn aterelqgl kplfrnssl
12121 ylysgcrlas lrpekdsat avdaicthrp dpedlgldre rlywelsnlt ngigelgpy
12181 ldrnslyvng fthrssmptt stpgtstvdv gtsptsssp spttagpllm pftlnftit
12241 lqyeedmrst gsrkntmes vlqglkplf kntsvgplys gcrlllrpe kdgaatgvd
12301 icthrlpkp pglntrelyw elsklndie elgpytldr slyvngfthq ssvststtp
12361 tstvdrtsg tpsslsspti maagpllvf tlnftitnlq ygedmghpgs rkfntterv
12421 qglgpfikn tsvgplysg rltslrsek gaatgvdaic ihhldpkspg lnrerlywe
12481 sqltngikel gpytldrns lyvngfthrts vptsstpgts tvdlgtsgtp fslpspata
12541 pllvftlnf titnlqyeed mhrpgsrkfn ttervltll gpmfkntsvg llysgcrlt
12601 lrsekdgat gvdaicthrl dpkspgvdr glywelsql ngikelgpyt ldrnslyvn
12661 fthwipvpts stpgtstvd gsgtpsslp pttagpllv ftlnftitnl kyedmhcp
12721 srkntterv lqslgpmfk ntsgvplys crltllrsek dgaatgvdaic thrlpkp
12781 gvdreqlywe lsqltngike lgytldrns lyvngfthqt sapntstpgt stvdltsg
12841 psslpstsa gpplvpftln ftitnlqye dmhpgsrkf nttervltll lqslgpmfknt
12901 gllysgcrlt llrpekngaa tgmdaicshr ldpkspglr eqlywelsql thgikelgp
12961 tldrnslyvn gfthrsvap tstpgtstvd lgtsgtpssl psptavpl vpftlnfti
13021 nlqygedmrh pgsrkntte rvlqglgpl fknssvgply sgcrllslrsk ekdgaatgv
13081 aicthhlnpq spgldreqly wqlsqmtngi kelgpytldr nslyvngfth rsglttst

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13141 wtstvdldgts gtpspvpspt ttgpllvpft lnftitnlqy eenmghpgsr kfnitesvl
 13201 gllkplfkst svgplysgcr ltllrpekdg vatrvaict hrpdkipgl drqglywel
 13261 qlthsitelg pytlrdslly vngftqrssv pttstpgtft vqpetsetps slpgptatg
 13321 vllpftlnft itnlqyeedm rrpgsrkfnt tervlqgllm plfkntsvss lysgcrltl
 13381 rpekdgatr vdavcthrpd pkspgldrer lywklsqllth gitelgpytl drhslyvng
 13441 thqssmtttr tpdsttmhla tsrtpaslsg pmtasppllvl ftinftitnl ryeenmhph
 13501 srkfntterv lqgllrpvfk ntsvgplysg crltlllrpkk dgaatkvdai ctyrpdps
 13561 gldreqlywe lsqllthsite lgpytlrds lyvngftqrs svpttsipgt ptvdlgtsg
 13621 pvskpgpsaa spllvltln ftitnlryee nmghpgsrkf nttervlqgl lrslfksts
 13681 gplysgcrlt llrpekdgta tgvdaicth pdpkprldr eqlywelsql thnitelgp
 13741 aldndslfvn gfthrsvst tstpgtptvy lgasktpasi fgpsaashll ilftlnfti
 13801 nlryeenmwp gsrkfntter vlqgllrplf kntsvgplys gcrltllrpe kdgeatgvd
 13861 icthrpdtg pgldreqlyl elsqllthsit elgpytlrdr slyvngfthr ssvpttstg
 13921 vseepftlnf tinnlrymad mgqpgslkfn itdnvmqhl splfqrsslg arytgcervi
 13981 lrsvkngaet rvdllctylq plsgpglpik qvfhelssqgt hgitrlgpys ldkdslyln
 14041 ynepgpdepp ttpkpattfl pplseattam gyhlktltln ftisnlqysp dmkgksatf
 14101 stegvlqhl rplfqkssmg pfylgcqlis lrpekdgat gvdttctyhp dpvgpgldi
 14161 glywelsqlt hgvtqlgyf ldrdsifing yapqnlsirg eyqinfhivn wlnsnpdpt
 14221 seyitllrldi qdkvttlykg sqlhdtfrfc lvtnltdsv lvtvkalfss nldpslveq
 14281 fldktlnasf hwlgstyqlv dihvtemess vyqptsssst qhfylntit nlpysqdk
 14341 pgttnyqrnk rniedalnql frnssiksyf sdcqvstfrs vprhhtgvd slcnfspla
 14401 rvdrvaiyee flrmtrngtq lqnftldrss vlvdgyspnr nepltgnsdl pfwavilig
 14461 agllgvitcl icgvlvttrr rkkegeynvq qqcpgyyqsh ldledlg

(B) Peptide 1

14394 14410
 nfsplar rvdrvaiyee (SEQ ID NO:01)

(C) Peptide 2

14425 14442
 tldrss vlvdgyspnr ne (SEQ ID NO:02)

(D) Peptide 3

14472 14492
cgvlttrr rkkegeynvq qq (SEQ ID NO:03)

(E) Transmembrane Region:

14452 14475
fwaviligl agllgvitcl icgvl (SEQ ID NO:14)

(F) Peptide containing the cysteine loop peptide:

14367 14398
 ksyf sdcqvstfrs vprhhtgvd slcnfspl (SEQ ID NO:15)

FIGURE 9 (page 6 of 6)

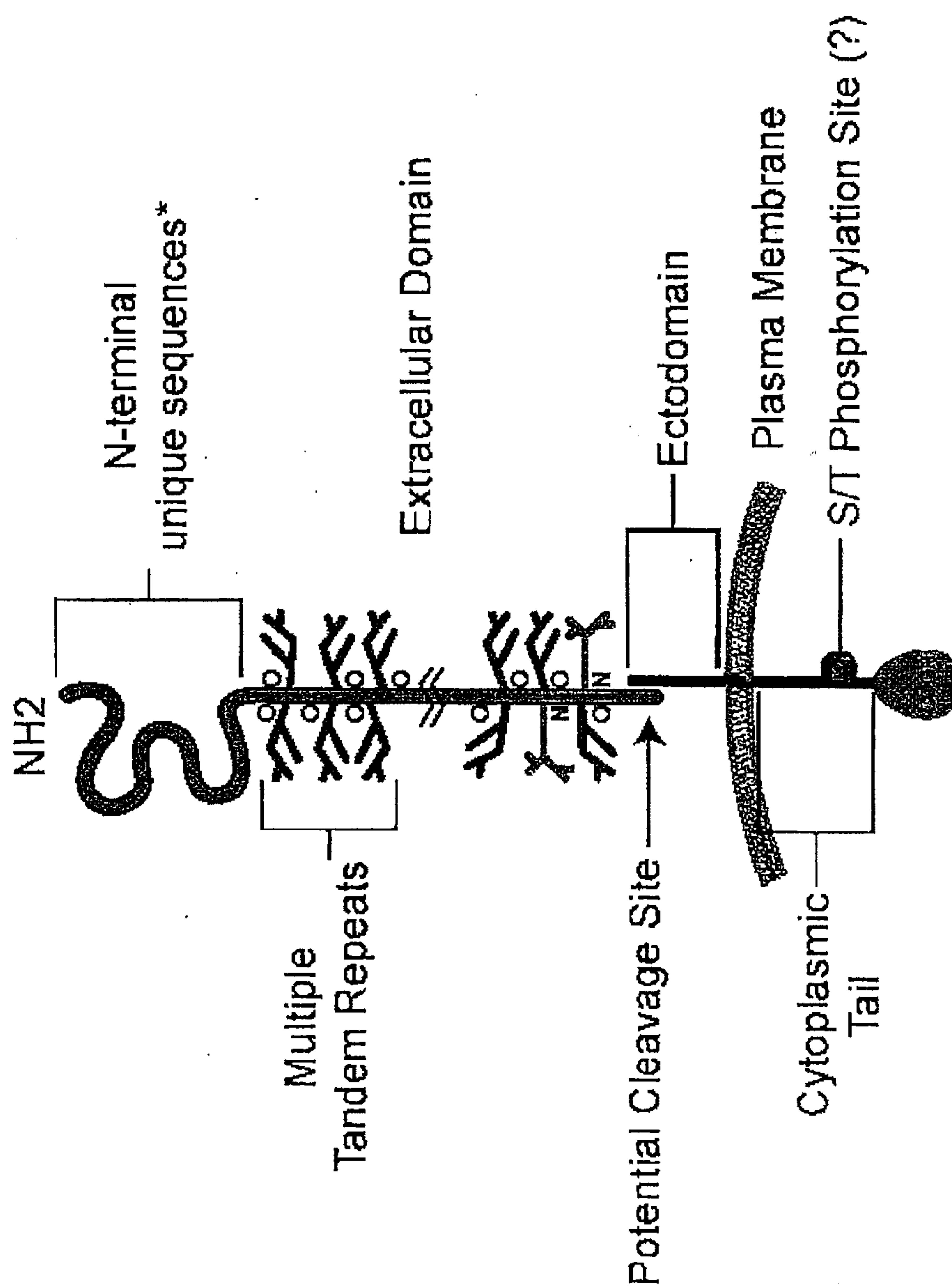


Figure 10

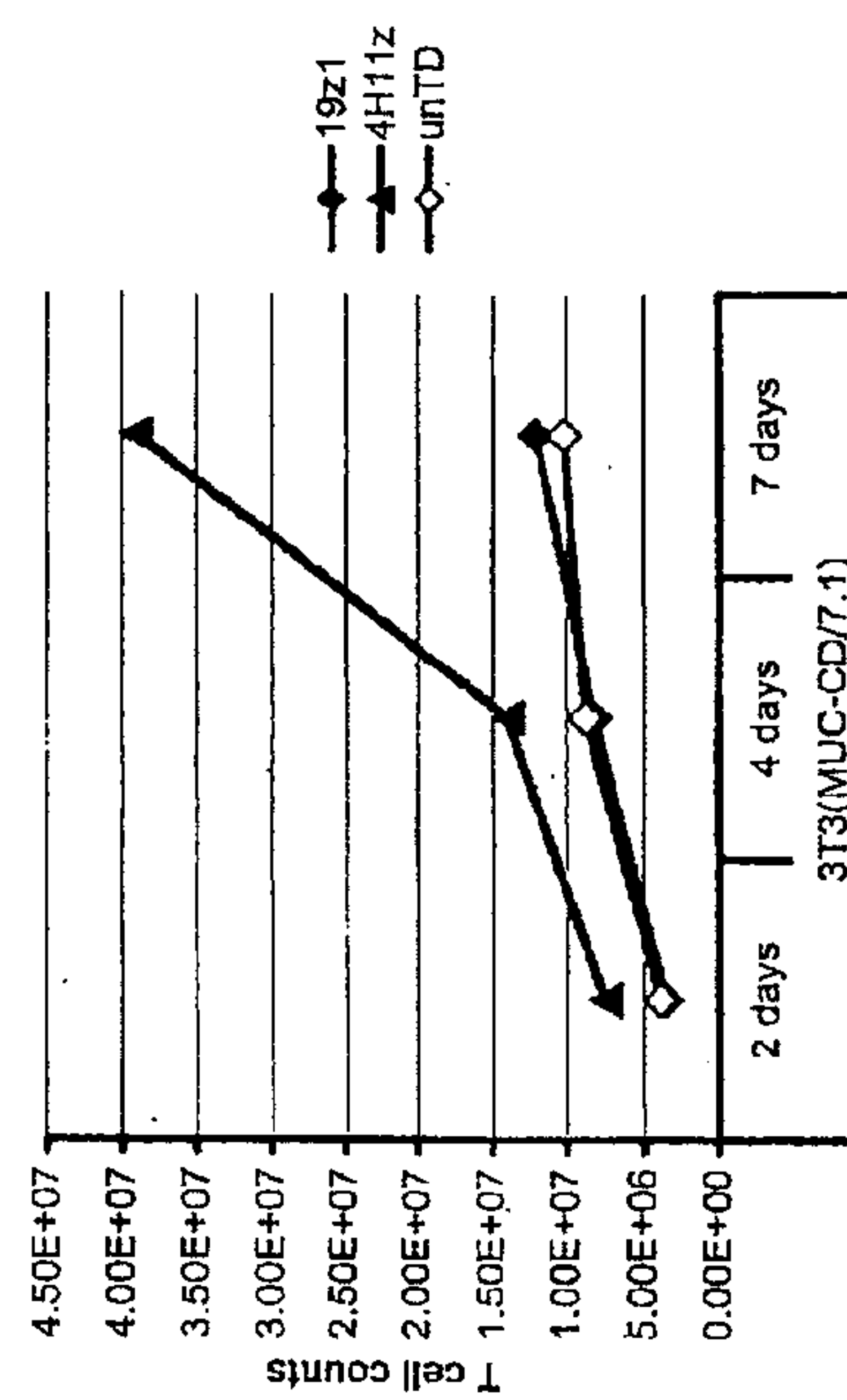
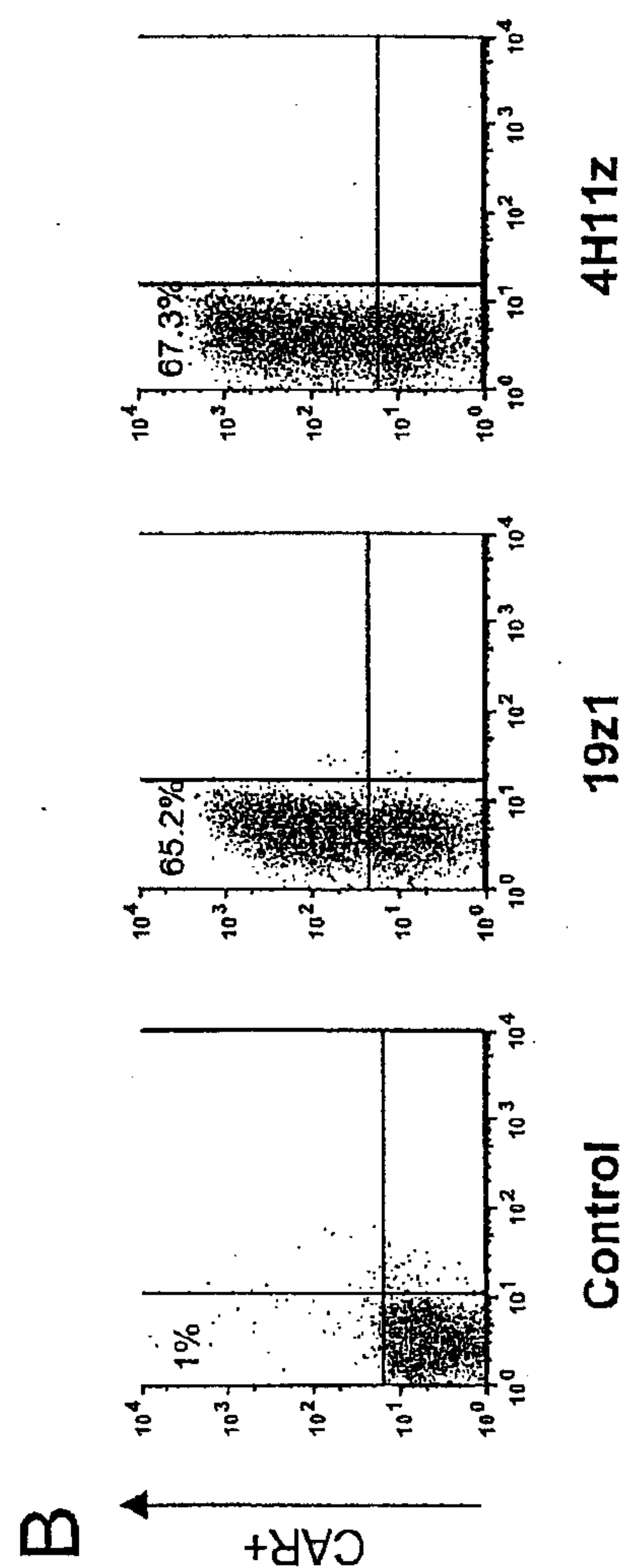
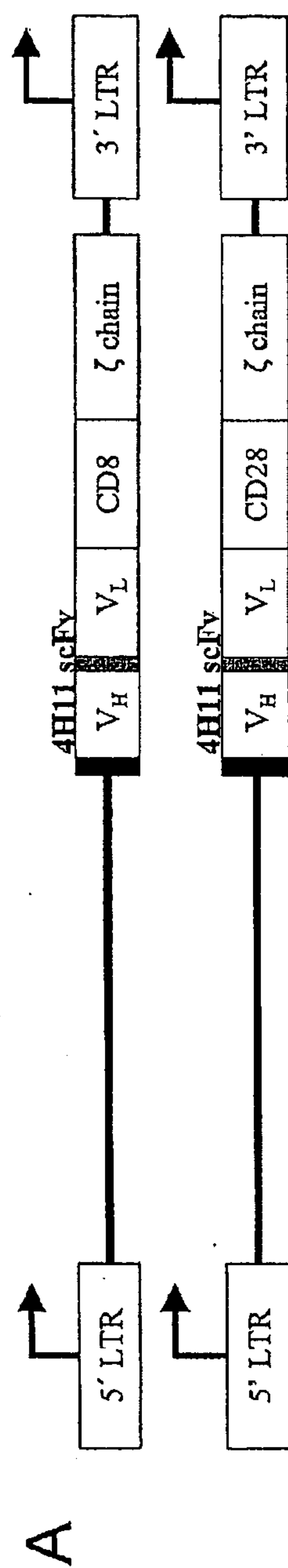
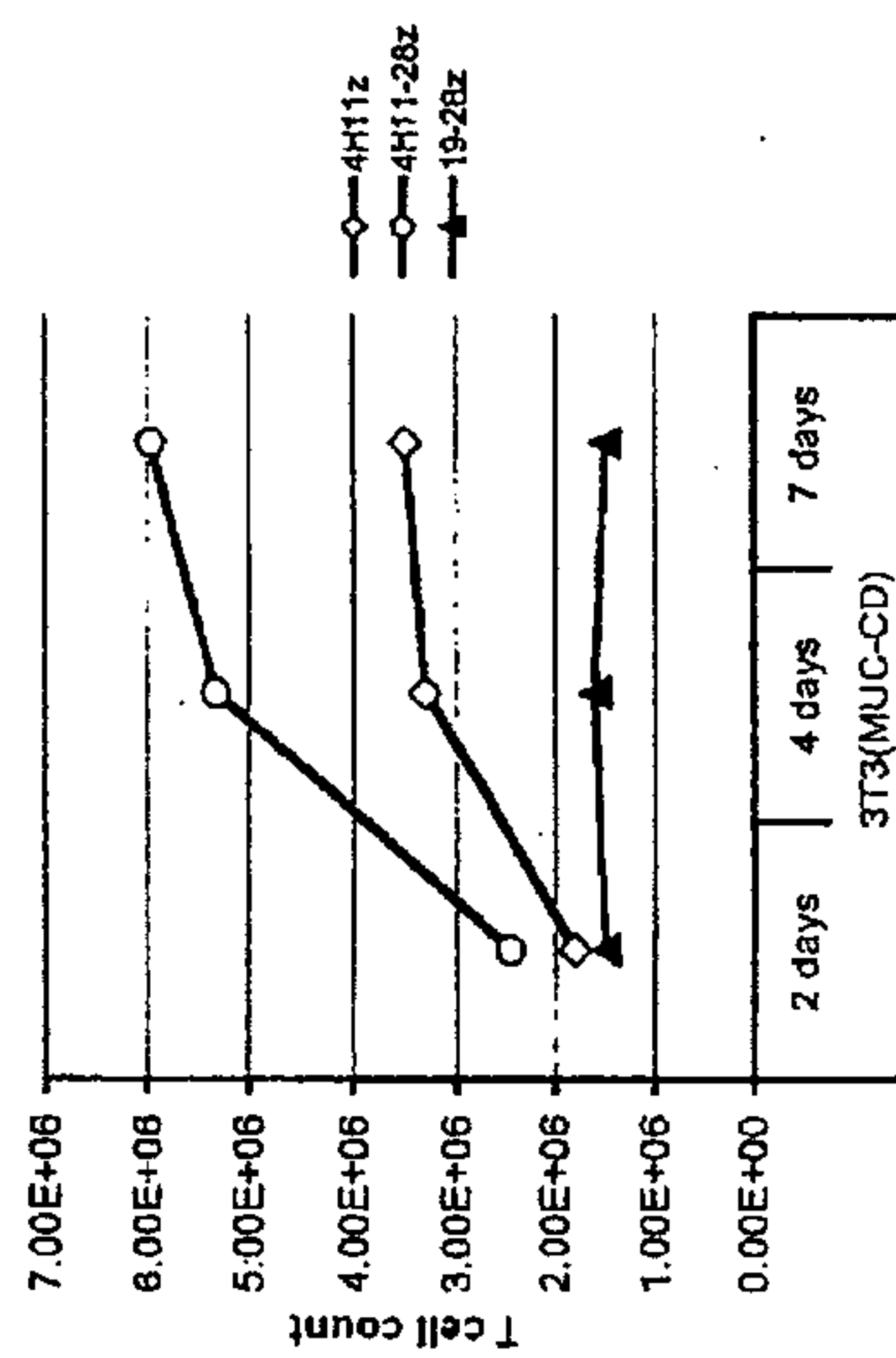
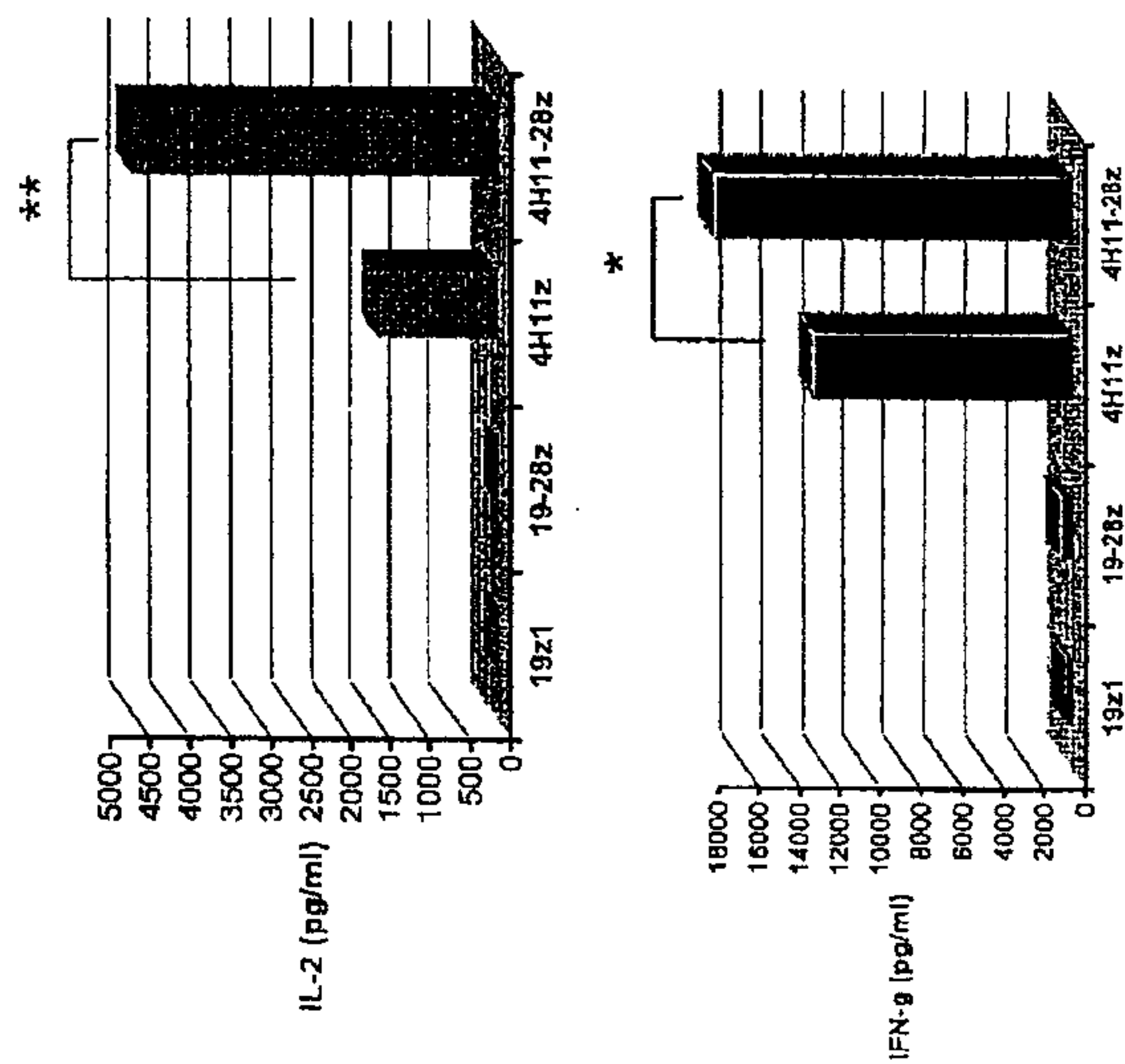


Figure 11

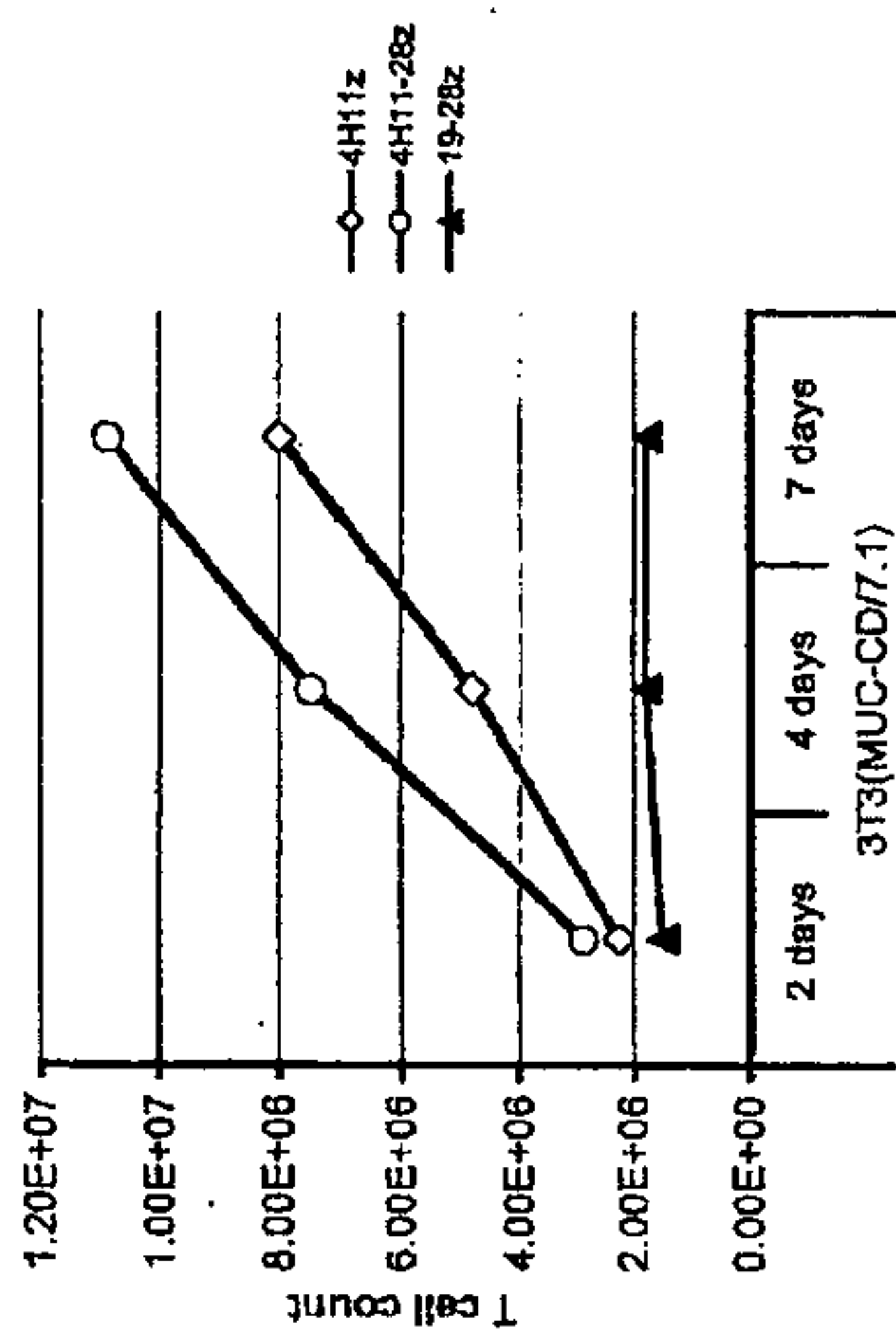
A



B



C



D

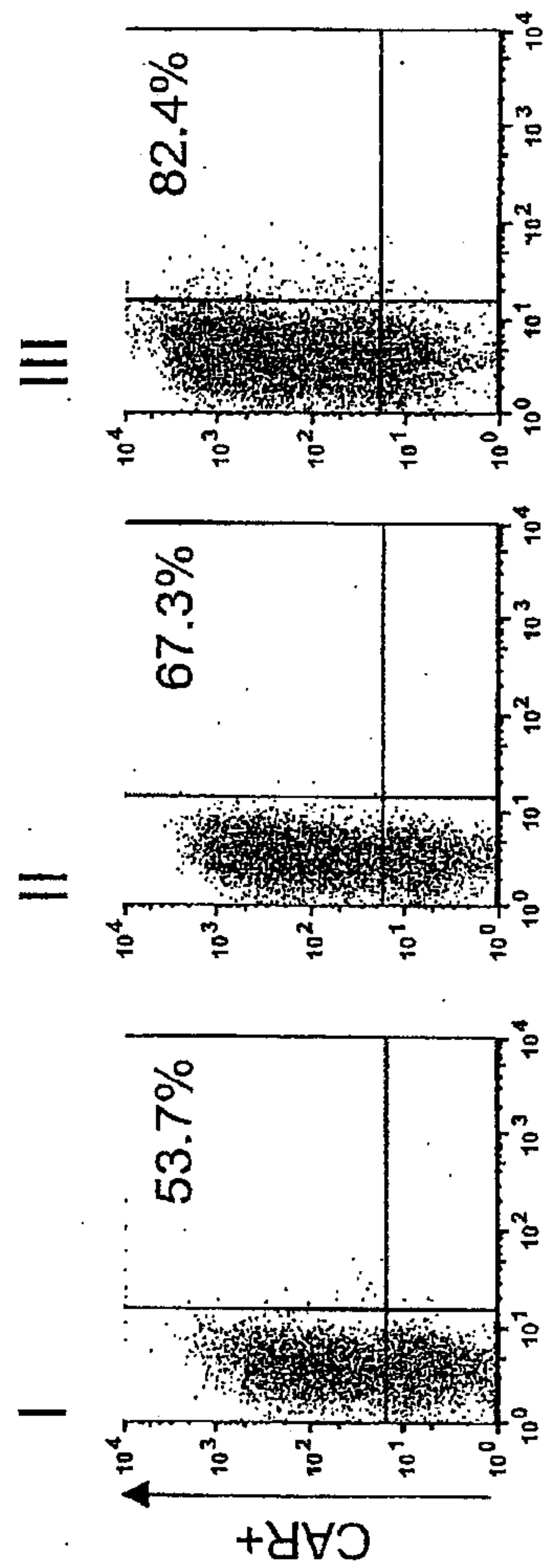


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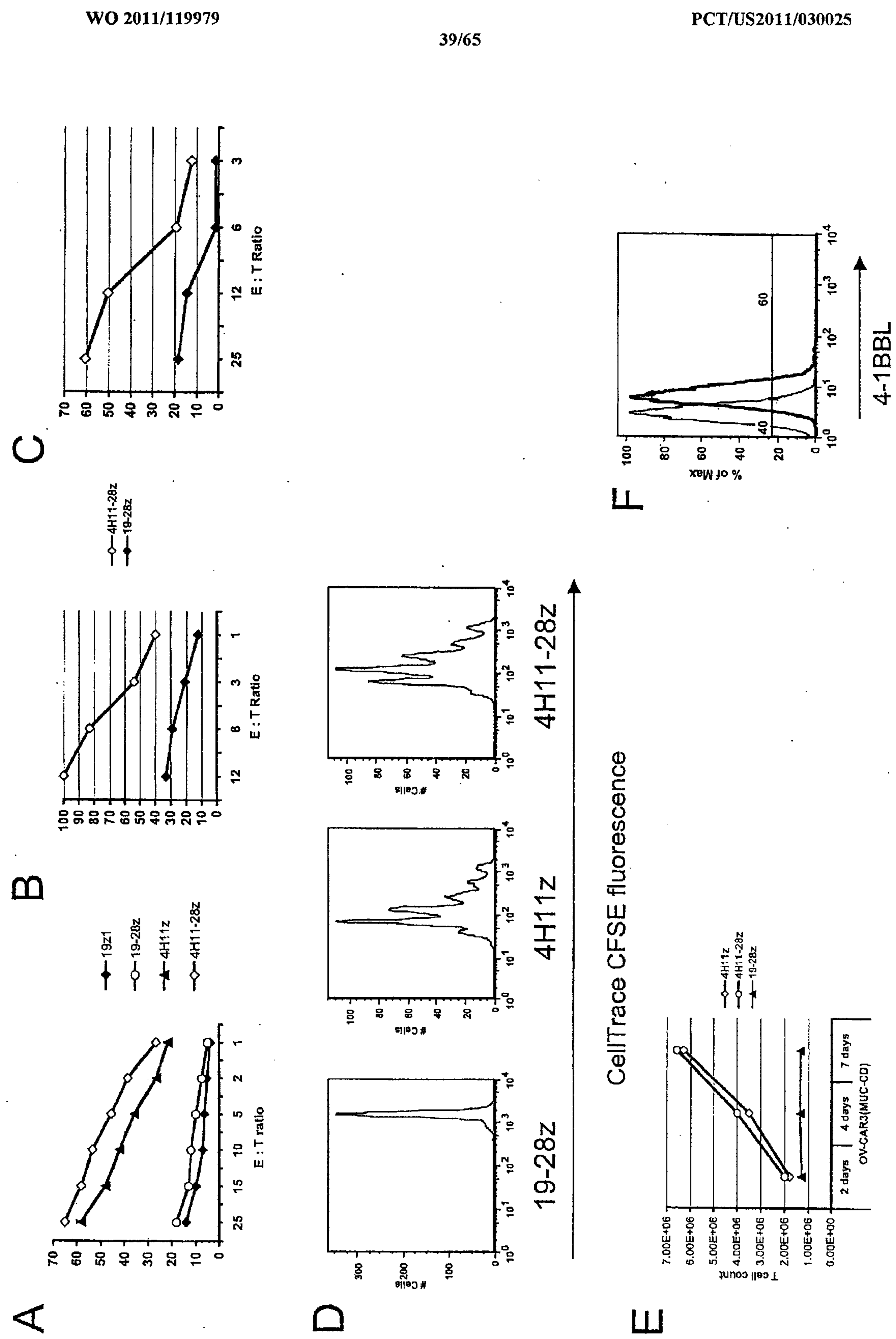


Figure 13

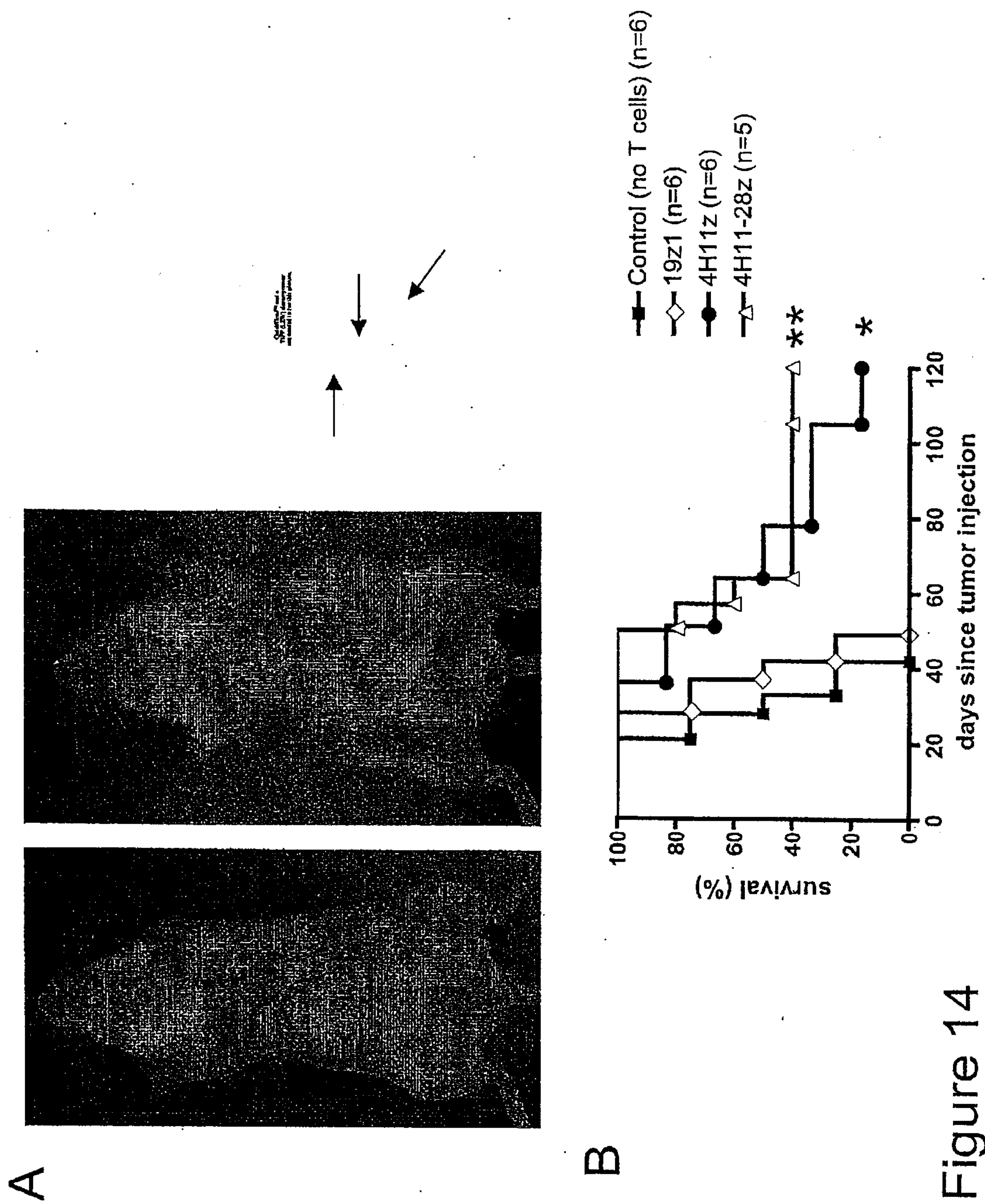


Figure 14

A

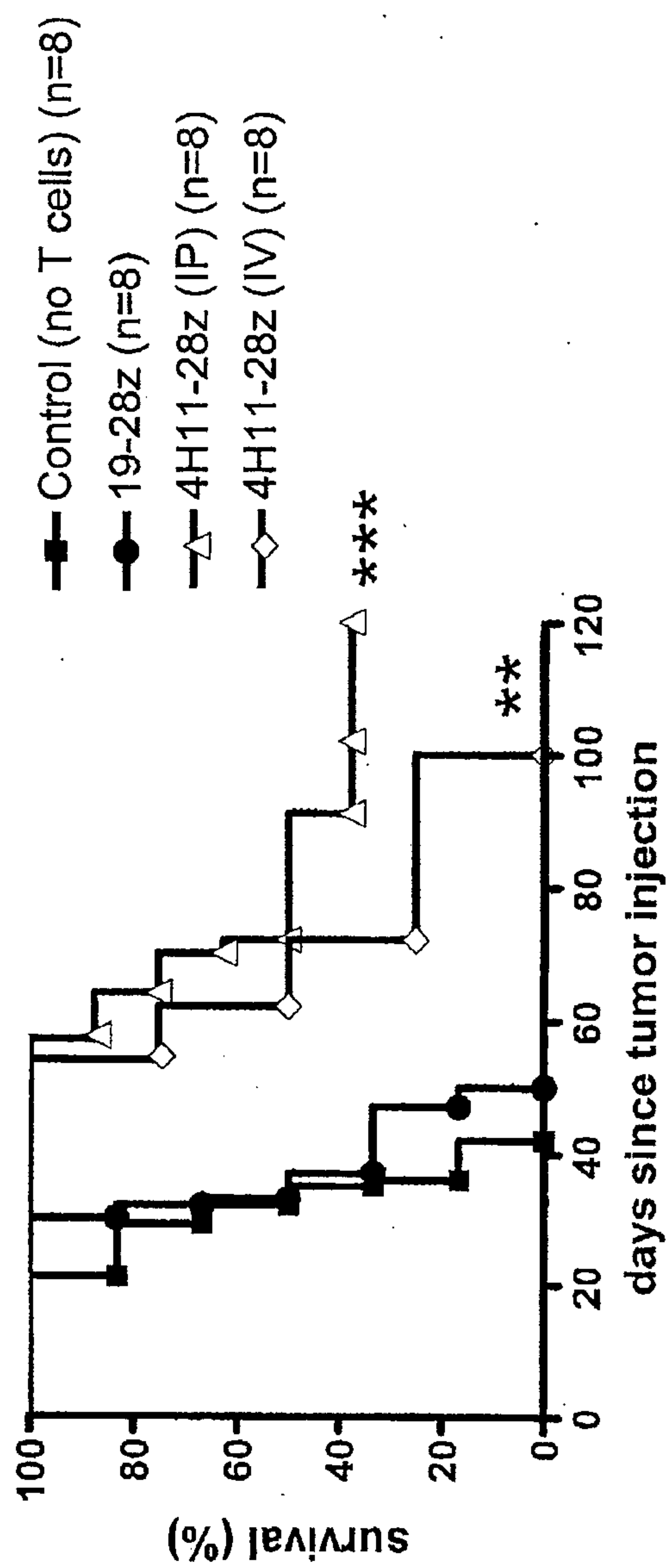
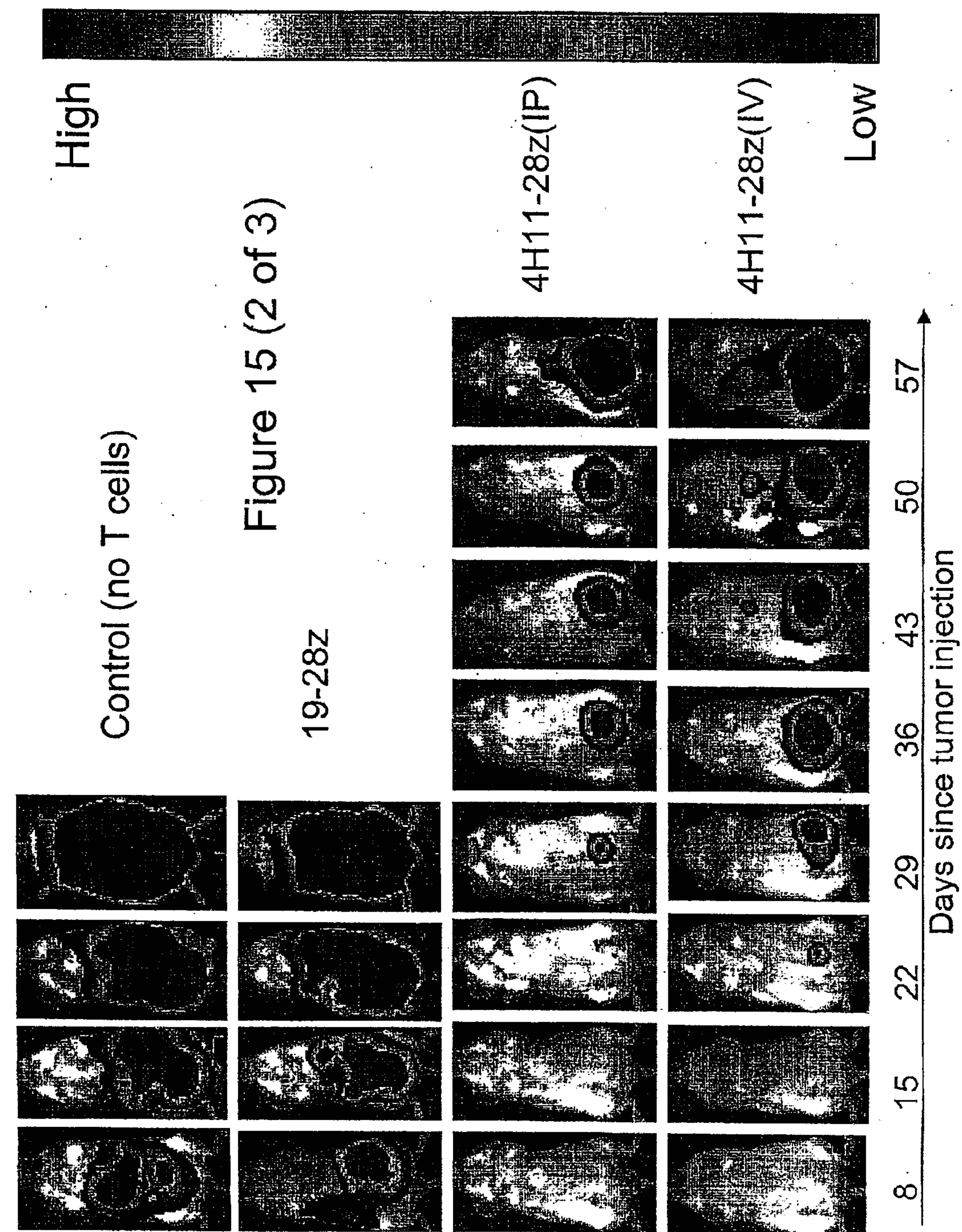


Figure 15 (1 of 3)



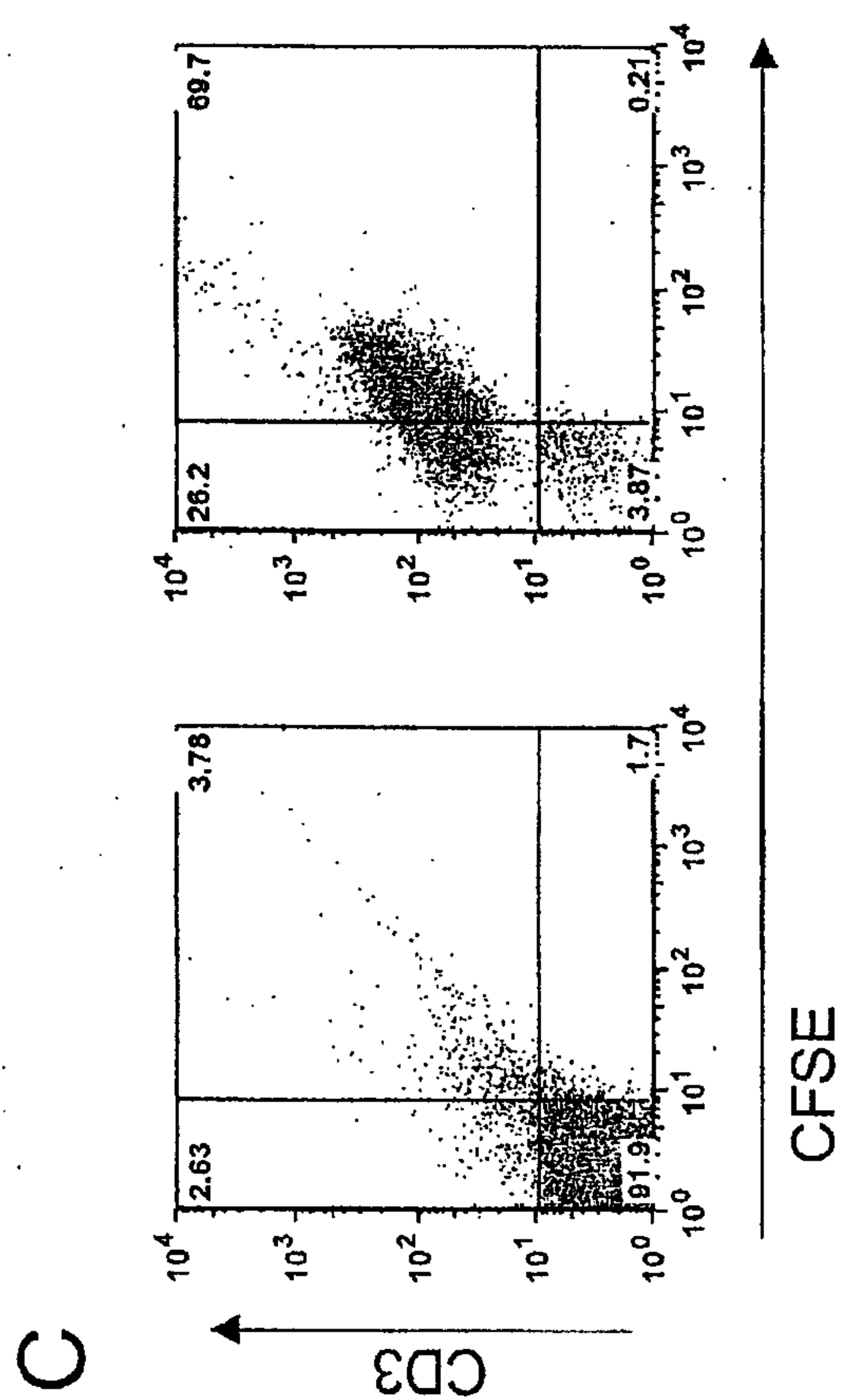
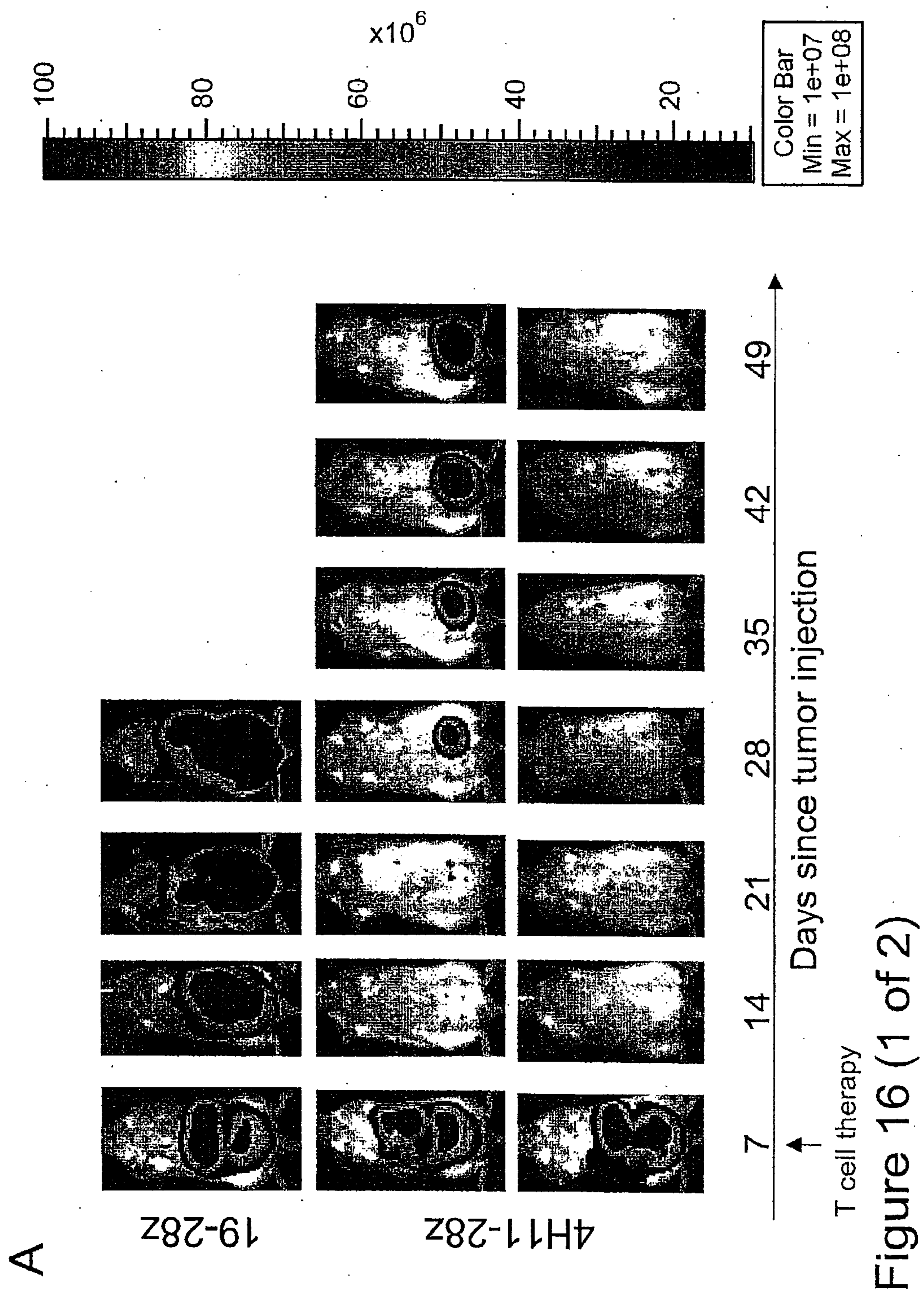


Figure 15 (3 of 3)



B

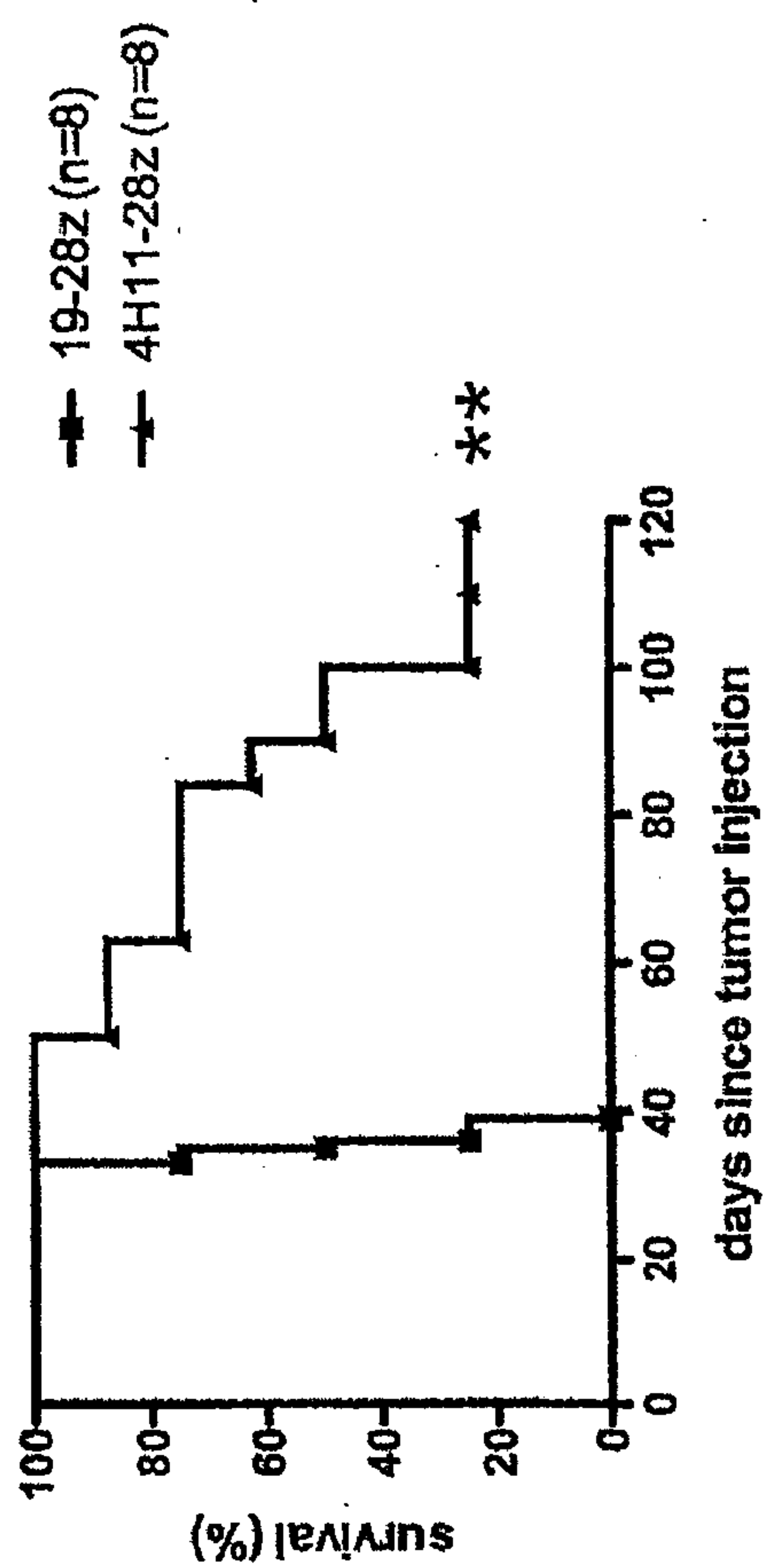


Figure 16 (2 of 2)

CD8 leader sequence

ATGGCTC TCCAGTGAC TGCCCTACTG CTTCCCCTAG CGCTTCTCCT GCATGCAGAG (SEQ ID NO:32)

CD3 zeta chain intracellular domain

AGAGT GAAGTTCAGC AGGAGCGCAG AGCCCCCGC GTACCAGCAG GGCCAGAACC AGCTCTATAA
 CGAGCTCAAT CTAGGACGAA GAGAGGAGTA CGATGTTTTG GACAAGAGAC GTGGCCGGGA CCCTGAGATG
 GGGGGAAAGC CGAGAAGGAA GAACCCTCAG GAAGGCCTGT ACAATGAACT GCAGAAAGAT AAGATGGCGG
 AGGCCTACAG TGAGATTGGG ATGAAAGGCG AGCGCCGGAG GGGCAAGGGG CACGATGGCC TTTACCAGGG
 TCTCAGTACA GCCACCAAGG ACACCTACGA CGCCCTTCAC ATGCAGGCCC
 TGCCCCCTCG (SEQ ID NO:33)

(G4S)3 serine-glycine linker

GGTG GAGGTGGATC AGGTGGAGGT GGATCTGGTGGAGGTGGATC T (SEQ ID NO:34)

CD8 transmembrane domain

GCGGCCGCAC CCACCACGAC GCCAGCGCCG CGACCACCAA CCCC GGCGCC CACGATCGCG TCGCAGCCCC
 TGTCCCTGCG CCCAGAGGCG TGCCGGCCAG CGCGGGGGG CGCAGTGCAC ACGAGGGGGC TGGACTTCGC
 CTGTGATATCTACATCTGGG CGCCCTTGGC CGGGACTTGT GGGGTCCTTC TCCTGTCACT GGTATCACC
 CTTTACTGCA ACCAC (SEQ ID NO:35)

CD28 transmembrane + intracellular domains (-STOP)

CAA TTGAAGTTAT GTATCCTCCT CTTACCTAG ACAATGAGAA GAGCAATGGA ACCATTATCC
 ATGTGAAAGG GAAACACCTT TGTCCAAGTC CCCTATTTCC CGGACCTTCT AAGCCCTTTT GGGTGCTGGT
 GGTGGTTGGT GGAGTCCTGG CTTGCTATAG CTTGCTAGTA ACAGTGGCCT TTATTATTTT CTGGGTGAGG
 AGTAAGAGGA GCAGGCTCCT (SEQ ID NO:36)

FIGURE 17

HamHI

1 GGATCCGGAT TAGTCCAATT TGTTAAAGAC AGGATATCAG TGGTCCAGGC TCTAGTTTGG ACTCAACAAT ATCACCAGCT GAAGCCTATA GAGTACGAGC
CCTAGGCGTA ATCAGGTTAA ACAATTTCTG TCCTATATGC ACCAGTCCG AGATCAAAAC TGAGTTGTTA TAGTGTCGA CTCTGCTCG CTCTGCTCG
CATAGATATA ATAAAGATTT TATTATATGC TCTAGTATGC AGGAGGATAG AAGACCCCA GGTATAGGT TGGCAGCTA GCTTAGTAA CGCATTTTG
GTATCTATTT TATTCTTCTA ATAAATCAG AGGTCTTTT CCCCCCTTAC TTTCTGGGGT CGATATCCAA ACCGTTGAT CGAATTCATT CGGTAAAC
CAAGGCATGG AAAATACAT AACTGAGAT AGAGAGTTC AGATCAAGT CAGGAACAGA TGGATATGCG GAATATGCG CAACACAGAT ATCTGTGTA
GTTCGGTACC TTTTATGTA TTGACTCTTA TCTCTCTAAG TCTAGTTCCA TCTCTGCT ACCTTGCTA CTATATCCG GTTGTCTTA TAGACACCAT
AGCAGTTCTT GCGCCGCTC AGGCGCTAGA ACAGTGAATA CAGCTGAATA TGGGCCAAAC AGGATATCTG TGGTAAGCAG TTCTGCCCC GGTCAAGGC
TCGTCAAGGA CGGGCCCGAG TCCCGTTCT TGTCTACCTT GTCTACTTAT ACCCGTTTG TCTATAGAC ACCATTCTG AAGACGGGG CCGAGTCCCG
CAAGAACAGA TGGTCCCGAG ATGCGTCTCA GCGCTCAGCA GTTCTTAGAG AACCATCAGA TGTTCACAG GTGCCCAAG GACCTGAAT GACCTGTGC
GTTCTGTCT ACCAGGGTC TACGGGCTC AGTTCGCTC TCGCTCTGT TCGCGGCTT CCGTCCCG GTCTCCCT ACAGAGTCC CAGGGGTC CTGACTTA CTGGACACG
CTTATTTGAA CTAACTAATC AGTTCGCTC TCGCTCTGT TCGCGGCTT CCGTCCCG GTCTCCCT ACAGAGTCC CAGGGGTC CTGACTTA CTGGACACG
GAATAAATT GATGGTTAG TCAAGCGAG AGCGGCGTA CCAATAACCC GACGAGGGC TCGATTAAT TCTCGGGT TTCTGGGTG AGCCCGCGG
AGTCTCCGA TTGACTGAT TCGCTGAT GCGCGGTA CCGGTGTAT CCGGTGTAT CCGGTGTAT CCGGTGTAT CCGGTGTAT CCGGTGTAT CCGGTGTAT
TCAGGAGGCT AACTGACTA GCGGGGCTA GCGGGGCTA GCGGGGCTA GCGGGGCTA GCGGGGCTA GCGGGGCTA GCGGGGCTA GCGGGGCTA GCGGGGCTA
CTCTGAGTGA TTGACTACC GTCACTACC GTCACTACC GTCACTACC GTCACTACC GTCACTACC GTCACTACC GTCACTACC GTCACTACC GTCACTACC
GAGACTCACT AACTGATGG CAGTCCGCT CAGTCCGCT CAGTCCGCT CAGTCCGCT CAGTCCGCT CAGTCCGCT CAGTCCGCT CAGTCCGCT CAGTCCGCT
ATAGTACTTA AAGTTACAT GGTCTCTTG AATAAATC AATAAATC AATAAATC AATAAATC AATAAATC AATAAATC AATAAATC AATAAATC
TATCAGTAT TTCAATGTA CCGAAGGAC TTTATTTGA TTTATTTGA TTTATTTGA TTTATTTGA TTTATTTGA TTTATTTGA TTTATTTGA TTTATTTGA
CTACTTTTC TTTATTTT TTTTGTCTC TTTTGTCTC TTTTGTCTC TTTTGTCTC TTTTGTCTC TTTTGTCTC TTTTGTCTC TTTTGTCTC TTTTGTCTC
GATGAAAAG AAATATATA AAACAGGAT CTAGACTAT AGTACTCTG TAACCCAGG TCGACTCTG AATGGTCC ATTTGGTCC ATTTGGTCC ATTTGGTCC
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TACCCACACA CACTTACACA CACTTACACA CACTTACACA CACTTACACA CACTTACACA CACTTACACA CACTTACACA CACTTACACA CACTTACACA
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CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA
EcoRI

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AAAAACTCTG TCTCAGAAAG TGAAATCGAAC CTTAAGTGAC CGGCAGCAAA ATGTTGCAAG ACTGACCTT TTGGGACCGC AATGGGTTGA ATTAGCGGAA
GCAGACATC CCGCTTTCCG CAGTGGGCT AATAGCGAAG AGGCCCGAC CAGTGGGCT TCCCAACAGT TCCGCAAGCT TCCGCAAGCT TCCGCAAGCT TCCGCAAGCT
CGTCGTATG GGGGAAAGG GTGACCGCA TTATCGCTC TCCGGGCGTG GCTAGCGGGA AGGTTGTCA AGGTTGTCA AGGTTGTCA AGGTTGTCA AGGTTGTCA
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ACGCCATAA AGAGGAATGC GTAGACACGC CATAAAGTGT GCGGTATACC ACGTGAGT CATGTTAGAC GAGACTACG CGTATCAAT CGGTCCGGG
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AGGTTTTCAC CGTCATCAC CGTCATCAC CGTCATCAC CGTCATCAC CGTCATCAC CGTCATCAC CGTCATCAC CGTCATCAC CGTCATCAC CGTCATCAC
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TCAGGTGCA CTTTTCGGG GAATGTGGG GAATGTGGG GAATGTGGG GAATGTGGG GAATGTGGG GAATGTGGG GAATGTGGG GAATGTGGG GAATGTGGG
AGTCCAGCT GAAAGGCC TTTACACCG CTTTGGGAT AAACAAATAA AAGATTTAT GTAGTTTAT ACATAGGCA CATCTCTGT ATTGGGACTA
AATGCTTCA ATATATTGA AAGGAGGA GTATGATAT TCAACATTC CGTCTCCCT TTTTGGGCA TTTTGGGCTT TTTTGGGCTT TTTTGGGCTT
TTTACGAGT TATTATTA TTTTCTTCT CATATCATA AGTTGTAAG GCACAGCGGG AATAAGGGA AAAAGGCCGT AAAAGGCCGT AAAAGGCCGT AAAAGGCCGT

Figure 18 (1 of 5)

2101 TCACCCAGAA ACGCTGGTGA AAGTAAAGAA TGCTGAAGAT CAGTTGGGTG CACGAGTGGG TTACATCGAA CTGGATCTCA ACAGCGGTAA GATCCTTGAG
 2201 AGTGGGTCTT TGGGACCACT TTCAATTTCT ACAGCTCTTA GTCAACCCAC GTGCTCACCC AATGATAGCTT GACCTAGAGT TGTCGCCATT CTAGGAACCTC
 2301 TCAAAAGCGG GGGTCTCTGC AAAAGGTAC TACTCTGAAA AATTTCAAGA CAGTACACCG CGCCTAATAT GGCNTAACT GCGGCCCTTT CAGCAACTCG
 2401 GTCGCCCAT ACACATATCT TGGTATGATC CTACACAGTC ACAGAAAGC AGCTTAACGA TGGCCTAATA TGGCCTAATA ACCTGTTGTA CCCCCTAGTA
 2501 TGCCATAACC ATGAGTGATA ACATGCGGC CAACCTACTT CTGACAAAGA TCGGAGGAGC TGTCTTTTCT AGCCTCTCTG GAGTGGTGTG TGAACAGCTT
 2601 ACGGTATGG TACTCACTAT TGTGAGCGCG GTTGAAGAA GACTGTTGCT AGCCTCTCTG GAGTGGTGTG TGAACAGCTT TGAACAGCTT TGAACAGCTT
 2701 GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG CCATACCAAA CAGACAGGCT GAGTGGTGTG TGAACAGCTT TGAACAGCTT TGAACAGCTT
 2801 CATTGAGCGG AACTAGCAAC CCTTGGCTTC GACTTACTTC GGTATGTTT GGTATGTTT GGTATGTTT GGTATGTTT GGTATGTTT GGTATGTTT
 2901 AACTATTAAC TGGCGAATA CTTACTCTAG CTTCCCGCA ACAATTAATA GACTGGATGG AGCGGATTA TGAACAGCTT TGAACAGCTT TGAACAGCTT
 3001 TTGATTAATG ACCGCTTGAT GAATGAGATC GAAGGCGCTT TGTAAATAT CTGACCTTAC TCGGCTTAC CTGACCTTAC CTGACCTTAC CTGACCTTAC
 3101 AGGCCGACCG ACCAAATAAC GACTATTAAG ACCTCGSCCA CTGCGACCCA GAGCGCCATA GTAACTGCTT TGAACAGCTT TGAACAGCTT TGAACAGCTT
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 3801 CAAATACTGT CTTCTAGTG TAGCCGATAG TAGCCGATAG TAGCCGATAG TAGCCGATAG TAGCCGATAG TAGCCGATAG TAGCCGATAG TAGCCGATAG
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 4001 TGCTGCCAAT GGCATAAGT CGTCTCTTAC CGGTCTTAC TCAAGACGAT TCAAGACGAT TCAAGACGAT TCAAGACGAT TCAAGACGAT TCAAGACGAT
 4101 ACAGCGGTCA CCGCTATTTA GCACAGATG GCGCAACTG AGTCTGCTA TCAATGCTG TCAATGCTG TCAATGCTG TCAATGCTG TCAATGCTG
 4201 CAGCCCAAGT TGAAGATGAG GACTTACAC GACTTACAC GACTTACAC GACTTACAC GACTTACAC GACTTACAC GACTTACAC GACTTACAC
 4301 GTCGGGTGCA ACCTGCTTGG TGGATGATG TGGATGATG TGGATGATG TGGATGATG TGGATGATG TGGATGATG TGGATGATG TGGATGATG

Figure 18 (2 of 5)

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TTATGCCAGT CATATTAAAT ACTAGTCAAT TAGTTGATTT TTATTTTGA CATATACATG TGAATGAAG ACCCCACCTG TAGGTTTGGC AAGCTAGCTT
AATACGGTCA GTATAATTAA TGATCAGTAA ATCAACTAAA AATAAANAAT GTATATGTAC ACTTACTTTC TGCGGTGGAC ATCCAAACCG TTCGATCGAA
AAGTACGCC ATTTGCAAG GCATGCAAG TATCTGCAAG TATCTGCAAG TATCTGCAAG TATCTGCAAG TATCTGCAAG TATCTGCAAG TATCTGCAAG
TTCAATTGCG TAAACGTTT CGTACCTTTT TATGATTTGA CTCTTATCTT TTAAGTCTA GTTCCAGTCC TTGCTACCTT TGCTGACTTA TACCCGGTTT
CAGGATATCT GTGGTAAGCA GTTCTGCCC GTTCTGCCC GCGCTCAGG CCAAGAACAG ATGGAACAGC TGAATATGG CCAACACAGG TATCTGTGGT AAGCAGTTCC
GTCTTATAGA CACCATTCGT CAAGGACCGG GCGGAGTCCC GGTCTTGTG TACTTGTGCT ACTTATACCC GGTGTTGCT ATAGACACCA TTCGTCAAGG
TGCCCGCGCT CAGGCGCAAG AACAGATGGT CCCAGATGTC GGTCCAGCCC TCAGCAGTTT CTAGAGAAC ACCAGATGTT TCCAGGTTGC CCAAGGACC
ACGGGGCCGA GTCCCGGTTT TTGCTACCA GGGGTCTACG CAGGTCGGG AGTCTCTGGA GATCTCTGGA TAGTCTACAA AGTCCCCAGG GGGTCTCTGG
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CCTCACTCG GCGCCAGTC CTCCGTTGA CTGAGTGGC GGTGATCCAT AAGCTCTT AAACCTCTT CAGTGTGCT CCGACTTGT GTCTGCTGT
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TCCTTGGGAG GGTCTCTCT GAGTGATTA CTACCCGTC CTACCCGTC GCGTCTCCG GGTCTGAGG GATCGGGAG ACCCTGCCC AGGACCCACC
AGGAACCCCT CCAGAGGAGA CTCACTAAT GATGGGAGT GGTCTGTCG TGTCTGTCG AATGCTAGT GTCTATGCT GATTTATGC GCTGCTGCTG TACTAGTTA
GACCCACCAC CCGGAGGTA GCTGGCCAGC AACTTATCT TGTCTGTCG AATGCTAGT GTCTATGCT GATTTATGC GCTGCTGCTG TACTAGTTA
CTGGTGGTG GCGCTCCAT CAGGAGGTC TGAATAGC ACAGACGTC TAACAGATCA CAGTACTGA CTAATATAC GCGACGCTG CATGATCAAT
GCTAACTAG TCTGTATCT GCGGACCGT GGTGAACTG ACAGTCTCG AACACCCGCG CCGAACCCTG GCGACGCTG CAGGACTTC GGGGCGCTT
CGATTGATCG AGACATAGC CCGCTGGCA CCACTTGA CCGTCTGAG TGTGAGCGG TGTGAGCGG TGTGAGCGG TGTGAGCGG TGTGAGCGG
TTTGTGGCC GACCTGATC CTAAATCCG GATCGTTAG GACTCTTGG TGACCCGCC TTGAGGAGG GATATGAGG TCTGTAGGA GACGAGAAC
AAACACCGG CTGGACTCAG GATTTAGG GATTTAGG CTGAAATC ACCTGCGGG AATCTCTCC CTATACACCA AGACCTCTT CTGCTCTTGG
TAAACAGTT CCGGCTCCG TCTGATCTG TGTGATCTG TGTGATCTG TGTGATCTG TGTGATCTG TGTGATCTG TGTGATCTG TGTGATCTG
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TCTGACTGTG TTTCTGTAT TGTCTGAT TGTCTGAT TGTCTGAT TGTCTGAT TGTCTGAT TGTCTGAT TGTCTGAT TGTCTGAT
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ATCGCTCACA ACCAGTCGGT AGATGTCAG AGATGTCAG AGATGTCAG AGATGTCAG AGATGTCAG AGATGTCAG AGATGTCAG AGATGTCAG
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CCTTTAACCG AGACTCTATC ACCAGTTA AGATCAAGT TCTAGTTCA GAAAGTGA GAAAGTGA GAAAGTGA GAAAGTGA GAAAGTGA
GAAATGCG TCTGAGTAG TGGTCCAT TGTGAGTAG TGTGAGTAG TGTGAGTAG TGTGAGTAG TGTGAGTAG TGTGAGTAG TGTGAGTAG
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CGTTCGACC CCGCTCGAT CTCCCTTAT CCGCTCGAT CTCCCTTAT CCGCTCGAT CTCCCTTAT CCGCTCGAT CTCCCTTAT CCGCTCGAT
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4501

4601

4701

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5001

5101

5201

5301

5401

5501

5601

5701

5801

5901

Figure 18 (3 of 5)

```

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6101 ACCTCTGGCG GCAGCTTACC AAGAACAACT GACCCGACCG GTGGTACCTC ACCCTTACCG AGTCGGCGAC ACAGTGTGGG TCCGCCCACA CCAGACTAAG
TGGAGACCCG CGTCGGATCG TTCTTGTTGA CCTGGCTGGC CACCATGGAG TGGGAATGGC TCAGCCGCTG TGTACACACC AGCGCGCTGT GGTCTGATTC
PmlI -----
6201 AACCTAGAAC CTGGCTGGA AGGACCTTAC ACAGTCTCTG TGACCACCCC CACCGCCCTC AAGTAGACG GCATCGCAGC TTGGATACAC GCCGCCACG
TTGGATCTTG GAGCGACCTT TCCTGGAATG TGTGAGGACG ACTGCTGGGG GTGGCGGGAG TTTCATCTGC CGTAGCGTGC AACCTATGTG CGCGGGGTGC
CD8-Leader
-----
6301 TGAAGGCTGC CGACCCCGGG GGTGACCAAT CCTCTAGACT GCCATGGCTC TCCAGTGC TGCCTACTG CTTCCCTTAG CGCTTCTCTT GCATGCAGAG
ACTTCCGACG GCTGGGGCCC CCACCTGGTA GGAGATCTGA CGGTACCGAG AGGCTCAGT ACGGATGAC GAAGGGGATC GCGAAGAGGA CGTACGTCTC
VH
-----
6401 GTGAAGCTGC AGGAGTCAGG GCGAGGCTTC GTGAAGCTG GAGGTCCCT CAAAGTCTCC TGTGCAGCCT CTGGATTAC TTTTCACTAGC TATGCCATGT
CACTTCGACG TCCTCAGTCC CCCTCCGAAG CACTTCGGAC CACTTCGGAC CTCCAGGGA GTTTCAGAGG ACACGTGGA GACCTAAGTG AAAGTCATCG ATACGGTACA
VH
-----
6501 CCTGGGTTCC CTTGAGTCCG GACATGAGC TGGAGTGGGT CGCAACCAAT AGCAGTCTG GTGGTTACAT CTTCTATTCT GACAGTGTGC AGGACGAT
GGACCCAAAG GGAATCAGGC CTCTACTCCG ACCTCACCAC GCGTTGGTAA TCCTCAGGAC CACCAATGTA GAAGATAAGA CTGTACACAG TCCCTGCTAA
VH
-----
6601 CACCATTTCC AGAGACATG CCAAGAACAC CTTGCACCTG CAAATGGGCA GTCTGAGTC TGGGGACAG GCAATGTATT ACTGTGCAAG GCAGGATTT
GTGGTAAGG TCTCTGTTAC GGTCTTGTG GGAGCTGGAC GTTTACCCGT CAGACTCCAG ACCCTGTGTC CGGTACATAA TGACAGTTT CGTCCCTAAA
(G4S) 3 Serine-glycine linker
-----
6701 GGTAACTAG GTGATTAATA TGGATGGAC TACTGGGGC AAGGGACCC GGTCAACGTC TCTCAGGT GAGGTGGATC AGGTGGAGGT GGATCTGGTG
CCATTGATGC CACTAATGAT ACGATACCTG ATGACCCCGG TTCCCTGGTG CCAGTGGCAG AGGAGTCCAG CTCACCTAG TCCACCTCCA CCTAGACCCAC
VL
-----
(G4S) 3 Serine-glycine linker
-----
6801 GAGGTGGATC TGACATTGAG CTCACCCAGT CTCATCCTC CTGGCTGTG TCAGCAGGAG AGAAGGTAC TATGAGCTGC AATCCAGTC AGAGTCTGCT
CTCCACCTAG ACTGTAACTC GAGTGGGTCA GAGGTAGGAG GGACCGACAC AGTCTCTCTC TCTTCCAGTG ATACTCGAGG TTTAGGTGAG TCTCAGACGA
VL
-----
6901 CACAGTAGA ACCCGAAGA ACCAGTTGGC TTGGTACCAG CAAAACCCAG GACAGTCTCC TGAAGTCTG ATCTACTGGG CATCCACTAG GCAATCTGGA
GTTGTCACTT TGGGCTTTCT TGGTCAACCG AACCATGGTC GTTTTGGTGC CTGTGAGGAG ACTTGACGAC TAGATGACCC GTAGGTGATC CGTTAGACCT
VL

```

Figure 18 (4 of 5)

7001

GTCCCTGATC GCTTCACAGG CAGTGGATCT GGCACAGATT TCACTCTCAC CATCAGCACT GTGCAGGCTG AAGACTGGC AGTTATTAC TGCCAGCAAT
CAGGCACTAG CGAAGTGTC GTACCTAGA CCTGTCTAA ACTCAGAGTG GTAGTGTCA CAGTCCGAC TTCTGGACCG TCAATAATG ACGTCTGTTA
VL

CD8 transmembrane domain

NotI

7101
CTTATAATCT ACTCACGTTT GGTCTCTGGA CCAAGCTGGA GATCAAAAGG GCGGCCGCAC CCACACAGAC GCCAGCGCGG CGACCAACAA CCGCGGCGCG
GAATATTAGA TGAGTGCAAG CCAGGACCTT GGTTCGACCT CTAGTTTCCC GCGCGGCGTG GTGTGTGCTG CCGTCCGCGC GCTGTGCTT GCGGCGCGCG

Cd8 transmembrane domain

7201
CAGGATCGCG TCGCAGCCCG TGTCCCTGCG CCACAGGCG TCGCGGCGCG GCGAGTGAC AGAGGCGCG TGGACTTCC CTGTGATATC
GTGCTAGCGC AGCTCGCGG ACAGGAGCG GGTCTCCG CCGGCCCGG GGTCCAGTG TGCTCCCGG ACCTGAAGCG GACACTATAG

Cd8 transmembrane domain

7301
TACATCTGGG CGCCCTTGGC CGGCACTTGT GGGTCTCTT TCCTCTCACT GGTATCACC CTTTACTGCA ACCACAGACT GAAGTTCAGC AGGAGCGCAG
ATGTAGACCC GCGGAACCG GCGCTGACA CCGCAGGAG AGGACAGTGA CCAATAGTGG GAATGACGT TGGTGTCTCA CTTCAAGTGC TCCTCGCGTC

CD3 zeta chain intracellular domain

7401
AGCCCCCGG GTACACAGG GGCAGAAC AGCTCTATA CGAGCTCAAT CTAGGACGAA GAGAGGAGTA CGATGTTTG GACAAGAGC GTGGCGCGGA
TCGGGGGGCG CATGTCTGTC CCGTCTTGG TCGAGATATT GCTCGAGTTA GATCCTGCTT CTCTCCTCAT GTACAAAAC CTGTCTCTG CACCGGCGCT

CD3 zeta chain intracellular domain

7501
CCCTGAGATG GGGGGAAGC CGAGAAGGA GAACCTCAG GAAGGCTCT ACAATGAACT GCAGAAAGAT AAGATGCGG AGGCCTACAG TGAGATTGGG
GGGACTCTAC CCCCCCTTCC GCTCTTCTT CTTGGGAGTC CTTCCGGACA TGTACTTGA CGTCTTTCTA TTCTACCGCC TCCGGATGC ACTCTAACCC

CD3 zeta chain intracellular domain

7601
ATGAAAGCG AGCGCCGAG GGGCAAGGG CACGATGGC TTTACCAAGG TCTCAGTACA GGCACCAAG ACACCTAGA CCGCTTTCAC ATGCAGGCCC
TACTTTCCG TCGCGGCTC CCGTTCCG GTGCTACCG AAATGCTCC CCGTGTCTC AGATCAATG CCGTGTCTC TGTGGATGCT GCGGGAAGTG TACGTCCGGG
CD3 zeta chain intracellular domain
XhoI

7701
TGCCCCCTCG CTAACAGCA CTCGAG
ACGGGGGAGC GATTGTCGT GAGCTC

Figure 18 top strand: SEQ ID NO:37
Figure 19 bottom strand: SEQ ID NO:38

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BamHI

1 GGATCCGGAT TAGTCCAAAT TGTAAAGAC AGGATATCAG TGGTCCAGGC TCTAGTTTGT ACTCAACAT ATCACCAGCT GAAGCCTATA GAGTACGAGC
CCTAGGCCCTA ATCAGGTCTG TCCATATAGT ACCAGTCCG AGATCAAAAC TGAATGTTA TAGTGGTGA CTTCGGATAT CTCATGCTCG
101 CATAGATATA ATAAAGAT TATATAGT TCCAGAAAAG GGGGGGAAAG AAAGACCCCA CCGTAGGTT TGGCAAGCTA GCTTAGTAA CGCCATTTG
GTATCTATTT TATTTTCTAA AATTAATCAG AGGTCTTTT CCCCCTTTAC TTTCTGGGT GGCATCCAA ACCGTCGAT GCGTAAAC
201 CAAGGCATGG AAAATATAT AACTAGAT AGAGAAGT TCTAGTTTCA TCTAGTTTCA GTCTTGTCT ACCTGTCTA CTATATACCAT
GTTCCGTACC TTTTATGTA TTGACTCTTA TGTCTTCAAG TCTAGTTTCA GTCTTGTCT ACCTGTCTA CTATATACCAT TAGACACCAT
301 AGCAGTTCCT GCGCCGCTC AGGCGCAAG ACAGATGGA TGGGCAAAAC AGGATATCTG TGGTAAAGCAG TTCTGCCCCC GGTCAAGGC
TCGTCAAGGA GGGGGCCGAG TCCCGGTTCT TGTCTACCTT GTCGACTTAT ACCGGTTTG TCCATATAGT ACCATTCGTC AAGGACGGG CCGAGTCCCG
401 CAAGAACAGA TGTCCCCAG ATGCGGTCCA GCGCTCAGCA GTTCTTAGAG AACATCAGA TGTTCCTCAG GTGCCCCAAG GACCTGAAT GACCTGTGC
GTTCTGTCT ACCAGGGTC TAGGCCAGT CCGGAGTCT CAAAGATCTC TGGTAGTCT ACAAGGTCC CACGGGTTC CTGACTTTA CTGGACACG
501 CTTATTGAA CTAACCAATC AGTTCGCTC TCGCGGCTT TCGCGGCTT CCGTCCCG AGCTCAATA AAGAGCCAC AACCCCTCAC TCGGGGCGGC
GAATAAATTT GATTGGTTAG TCAAGCGAAG AGCGCGGCA GACGAGGGG TCGAGTTAT TCTCGGGT TGGGAGTG AGCCCCCGG
601 AGTCTCCGA TTGACTGAT CCGCGGCTT CCGCGGCTT CCGCGGCTT CCGCGGCTT CCGCGGCTT CCGCGGCTT CCGCGGCTT CCGCGGCTT
TCAGGAGGCT AACTGACTCA GCGGCGGCTT GCGGCGGCTT GCGGCGGCTT GCGGCGGCTT GCGGCGGCTT GCGGCGGCTT GCGGCGGCTT
701 CTCTGAGTGA TTGACTACCC GTGAGGCGG GTCTTTTACA CATGACAGAT GTATCAAAAT TAAATTTGTT TTTTCTTA AGTATTACA TTAATGCGC
GAGACTCAT AACTGATGGG CAGTCGCGCC CAGAAAGTGT GTAGTCTGA CATAGTTTA AATAACCA AAAAAAAT TCATATATGT AATTACCGG
801 ATAGTACTTA AAGTTACAT GCGTCTCTTG AATAAATAT GAGTATCTA GATGTGTCA TAAATTTT TAAATTTT AATGATATCT CATTGGCTTT
TATCATGAAT TTCAATGTA CCGAAGGAC TTTTATTTT TGTGTTTGT TGTGTTTGT TGTGTTTGT TGTGTTTGT TGTGTTTGT TGTGTTTGT
901 GATGAAAAAG AAAATATAA AAGGAGGCTT AAGGAGGCTT AAGGAGGCTT AAGGAGGCTT AAGGAGGCTT AAGGAGGCTT AAGGAGGCTT
ATCTTACACT ATAGTTCAAG CTAGACTATT AGCTACTCTG TACCCAGGG TGACCTGTA GTCAATGTA GCTGCTGT TTAGGCTTCC CACATCTAAG
1001 TAGGATGTA TATCAAGTTC GATCTGATA TCGATGAGT ATGCGGCTT CAGTACCCAT CAGACGACA AATCGAAG GTGTAGATTC
1101 ATTACAGGTA TGAGCTATCA TTTTGGTAT AAAACCATTA TAATACTA TAATACTA TAATACTA TAATACTA TAATACTA TAATACTA
TAAATGCTAT ACTGATATGT GTATGATGT GTATGATGT GTATGATGT GTATGATGT GTATGATGT GTATGATGT GTATGATGT
1201 ATGGGTGTGT GTGATATGT GTGATATGT GTGATATGT GTGATATGT GTGATATGT GTGATATGT GTGATATGT GTGATATGT
TACCCACACA CACTTACACA CATACACACA CATACACACA CATACACACA CATACACACA CATACACACA CATACACACA CATACACACA
1301 GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA CACACACACA
EcoRI

1401 TTTTGGAGAC AGAGTCTTTC ACTTAGCTTG GAATTCAGTG GCGGTGCTTT TACAAGCTCG TGAATGGGA AACCTGGCG TTACCCAAT TAATCGCCTT
AAAACTCTG TCTCAGAAAG TGAATCGAAC CTTAAGTGAC CGGCAGCAAA ATGTTGAGC ACTGACCTT TGGGACCGC AATGGTTGA ATTACCGAA
1501 GCAGCACATC CCGCTTTTCC CAGCTGCGGT AATAGCGAAG AGGCGCGCAC CGATCGCCT TCCCAACAGT TCGGACGCT GAATGGGAA TGGCGCCTGA
CGTCTGTAG GGGGAAAGCG GTGACCGGCA TTATCGCTTC TCCGGGCTG GCTAGCGGA AGGTTGTCA ACCTGCTGA CTTACCGCTT ACCGCGGACT
1601 TCGGTATTT TCTCTTACG CATCTGTGG GTATTTCACA CCGCATATG TGCATCTCA GTCAATCTG CTCTGATGCC GCATGCTAA CCGTGGCGG
ACGCCATAA AGAGGAATGC GTAGACACGC CATAAGTGT GCGGTATACC ACCTGAGAT CATGTTAGAC GAGACTACG CGTATCAAT CCGTGGCGG
1701 ACACCCGCA ACACCCGCTG ACACCCGCTG ACACCCGCTG ACACCCGCTG ACACCCGCTG ACACCCGCTG ACACCCGCTG ACACCCGCTG
TGTGGCGGT TGTGGCGGT TGTGGCGGT TGTGGCGGT TGTGGCGGT TGTGGCGGT TGTGGCGGT TGTGGCGGT TGTGGCGGT
1801 AGGTTTTCAG CGTCATCACC GAAAGCGCG ATGACGAAAG GCGCTCTGA TACGCTTAT TTTATAGTT AATATCTCA TTATATAGT TTCTTAGAC
TCCAAAGTG GCAGTAGTG CTTTGGCGG AAATGCGCG GAAACCCCTA TTTGTTTAT TTTCTAATA AATATCTCA TTATATAGT TTCTTAGAC
1901 TCAGGTGGCA CTTTTCGGG AATGCGCG CTTTACGCG CTTTACGCG CTTTACGCG CTTTACGCG CTTTACGCG CTTTACGCG
AGTCCACCGT GAAAGCCCTT TTTACGCG CTTTACGCG CTTTACGCG CTTTACGCG CTTTACGCG CTTTACGCG CTTTACGCG
2001 AATGCTTCA ATATATATGA AAAAGGAGA GTATGAGTAT TCAACATTC CCGTCTGCG TTTTCTCTT TTTTCTCTT CTTTCTCTT
TTTACGAAGT TATTATACT TTTCTCTT CATACTATA AGTTGTAAG GCACAGCGG AATAAGGAA AAAAGCGGT AAAAGCGG GACAAAAAG

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2101 TCACCCAGAA ACGCTGGTGA AAGTAAGA TGCTGAAGAT CAGTTGGTGG CACGAGTGGG TTACATCGAA CTGGATCTCA ACAGCGGTAA GATCCTTGAG
 2201 AGTGGGTCTT TCGACCACTT TTCAATTTCT ACACCTTTCTA GTCAACCCAC GTGCTCACCC AATGTAGCTT GACCTAGAGT TGTGCGCAAT CTAGGACTC
 2301 TCAAAAGCGG GGGTCTTCTG AAGAGTAC TACTCTGTGA AATTTCAAGA CGATACACCG CGCCATAATA GGGCATAACT GCGGCCCTT CTGTTGAGC
 2401 GTGCGCGCAT ACATATTTCT CAGANTGAT TGGTTGAGTA CTCACCACTC ACAGAAAGC ATCTTACGGA TGGCATGACA GTAAAGAGAT TATGCACTGC
 2501 CAGCGGCGTA TGTGATAAGA GTCTTACTGA ACCAATCTAT GAGTGGTCTAG TGTCTTTTTC TAGAATGCTT ACCGTACTGT CATCTCTTA ATACGTGAG
 2601 TGCCATAACC ATGAGTGATA ACATGCGGC CAATTTACTT CTGACAACGA TCGGAGGACC GAAAGAGCTA ACCGCTTTT TGCACAACAT GGGGATCAT
 2701 ACGGTATGG TACTCACTAT TGTGACGCG GTTGAATGGA CTGAATGAA GATGTTGCT AGCTCTCTG CTCTCTGAT TGGCGAATA AATGTTGTA CCCCTAGTA
 2801 CATFGAGCG AACTAGCAAC CTTAGCTCAG CTTCCCGCA ACAATTAATA GACTGATGG AGCGGATAA AGTTGACGA CCACCTCTGC GCTCGGCCCT
 2901 AACTATTAAC TGGCGAATA CTTACTCTAG CTTCCCGCA ACAATTAATA GACTGATGG AGCGGATAA AGTTGACGA CCACCTCTGC GCTCGGCCCT
 3001 TTGATAATTG ACCGCTTGTG GAACCGGAG CTGAATGAA GATGTTGCT AGCTCTCTG CTCTCTGAT TGGCGAATA AATGTTGTA CCCCTAGTA
 3101 TCCGGCTGGC TGGTTTATTG CTGATAATC TGGAGCGGT GAGCGGCTAT CATTCAGCA CTGGGGCCAG ATGTAAGCC TACCATTGCG GAGGGCATAG
 3201 AGCCGACCG ACCAATAAC GACTATTTAG AACTCGGCA CTTCCCGCA ACAATTAATA GACTGATGG AGCGGATAA AGTTGACGA CCACCTCTGC GCTCGGCCCT
 3301 GTAGTTATCT ACACGACGGG GAGTCAGGA ACTATGATG AACGAATAG ACAGATCGCT GAGTGGTGGT CTGCGCTAT CATTCAGCA CTGGGGCCAG ATGTAAGCC
 3401 CATCAATAGA TGTGCTGCC CTGATGCCGT TGATACCTAC TTGCTTTATC TGTCTAGCGA CTCTATCCAC GGAGTGACTA ATTCGTAACC ATGACAGTC
 3501 ACCAAGTTTA CTCATATATA CTTTAAATCT TCAATTTTAA TTTAAAGGA TCTAGGTGA GATCCTTTT GATATCTCA TGACCAAAAT
 3601 TGGTTCAAT GAGTATATAT GAATCTAAC TAAATTTTGA AGTATTTCT AGATCCACT CTAGGATAA CTATTAGAT ACTGTTTAA
 3701 CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAAGGATC TTCTGAGAT TCTTGTTC TCGCGTAAAT CTGCTGCTTG TCGCTGCTTG
 3801 GGAATTTGCA CTCAAAAGCA AGGTGACTCG CAGTCTGGGG CATCTTTCT AGTTTCTAG AAGACTCTA AGAATAAAG ACBGCATTA GACGACGAA
 3901 CAAACAAA AACCACCGCT ACCAGCGTGC CAAACAAAG CCTAGTCT CGATCAAGA GCTACCACT CTTTTCGGA AAGTAAGCT TCCAGCAGA GCGCAGATAC
 4001 GTTGTGTTT TGGTGGCGA TGGCTAGTG TAGCGTACT TAGCGTACT TCTGTAGCA CCGCTACTG CCGCTACTG CCGCTACTG CCGCTACTG
 4101 CAATACTGT CTTCTAGTG ATCGGATCA ATCGGTTGAT CAGGTTGAT GAACTGAT GAGATGAT GAGATGAT GAGATGAT GAGATGAT
 4201 TGTGCTGAGT GCGGTAAGT CGTCTTAC CCGGTTGAG TCAAGAGCT TCAAGAGCT TCAAGAGCT TCAAGAGCT TCAAGAGCT TCAAGAGCT
 TACGAGCTCA CCGTATTTCA GCACGATAG GCGCAACCTG AGTTCTGCTA TCAATGGCT ATTCGCGTC GCCAGCCCGA CTTGCCCCC AAGCACCTGT
 CAGCCAGCT TGGAGCGAAC GACTACGAC GACTAGAT AACTAGAT AACTAGAT AACTAGAT AACTAGAT AACTAGAT AACTAGAT
 GTCGGCTGA ACTCGCTT TGTGATGAGT CTGATGAGT CTGATGAGT CTGATGAGT CTGATGAGT CTGATGAGT CTGATGAGT CTGATGAGT
 ATCCGCTAG CCGTATTTCT TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC
 TAGGCCATT CCGATTTTCT GATGCTGCTC AGGGGGGGG AGCTATGGA AAGCGGCG CCAACGCGG CCGCTGCTC TCGCTGCTC
 TGAATCTGCA GCTAAACA CTACGAGCAG TCCCGCGCG CCGTATCTG TGGATAACCG TATTACCGG TTTGCGGCT GATGAGTCTA
 TTTGCTCACA TGTCTTTCT TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC
 AAACGAGTGT ACAAGAAAGG ACGAATAGG GGAATAAGC ACCTATGAG CCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC
 AGCGCAGCGA GTCAGTGAGC GAGGAAGCG AAGAGCGCC AATACGGA CCGCTGCTC TCGCTGCTC TCGCTGCTC TCGCTGCTC
 TCGCTGCTC CAGTCACTCG CTCTCTGCG TTTCTGCGG TTTCTGCGG TTTCTGCGG TTTCTGCGG TTTCTGCGG TTTCTGCGG
 GTTCTCCGAC TGGAAAGCGG GCGATGAGC GCGATGAGC GCGATGAGC GCGATGAGC GCGATGAGC GCGATGAGC GCGATGAGC
 CAAAGGCTG ACCTTTGCG CCGTATGAG GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA
 ATGTTGCTG GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA GATGAGTGA
 TACAACACAC CTTAACAATC GCTATGAT GCTATGAT GCTATGAT GCTATGAT GCTATGAT GCTATGAT GCTATGAT
 AAATCAAT ATATAAGCA TTTGATGAT TTTGATGAT TTTGATGAT TTTGATGAT TTTGATGAT TTTGATGAT TTTGATGAT
 TTTGATGAT TATAATTTCT AAATCAAT TTTGATGAT TTTGATGAT TTTGATGAT TTTGATGAT TTTGATGAT TTTGATGAT
 AATGACAGCA TGTGTTTAT TCAATGCTG AATGATGCT AATGATGCT AATGATGCT AATGATGCT AATGATGCT AATGATGCT
 TTACGCTGCT ACAAATAA AGTATTTCCA AGTATTTCCA AGTATTTCCA AGTATTTCCA AGTATTTCCA AGTATTTCCA AGTATTTCCA

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4301 AATTACTTAG AGTTTCTGTC ATTAAGGTTT CCTTCTCTAG TTGACACAT AATGCGCTG CTGAGCAAGC CAGTTTGCAT CTGTACGAT CAATTTCCCA
4401 TTAATGATC TCAAGACAG TAATTCGAA GGAAGGATC AACTGTGTA TTTACGCGAC GACTCGTTCG GTCAACGTA GACAGTCTTA GTTAAAGGT
4501 TTATGCCAGT CATATTAAT ACTAGTCAAT TAGTTGATTT TTAATTTGA CATATACATG TGAATGAAG ACCCCACTG TAGTTTGGC AGCTAGCTT
4601 AATACGGTCA GTATTAATAA TGAATTAAT ATCAATTAAT AATAAATAT GTATATACAT ACTTACTTC TGGGTGAGC ATCCAAACCG TTGATCGAA
4701 AAGTAACGCC ATTTTGAAG GCATGGAAG ATCAATTAAT GAGATAGAA AATTCAGAT TATGATTTGA CACTTATCTT TTCAAGTCTA GTTCAGTTC TGTCTACTT TGTCTACTT
4801 TTCAATTTGGG TAAACGTTT CATTCTTTT TATGATTTGA CACTTATCTT TTCAAGTCTA GTTCAGTTC TGTCTACTT TGTCTACTT TGTCTACTT
4901 CAGGATATCT GTGTAAGCA GTTCTCTGCC GTTCTCTGCC CCAAGAACAG ATGGAACAGC TGAATATGCG CCAACAGGA TATCTGTGT AAGCAGTTCC
5001 GTCTATAGA CACCAATCTG CAAGGACGGG GCTTCTTTCG TACTTGTTCG ACTTATACCC GGTTCGTCT ATAGACACCA TTCTCAAGG
5101 TGCCCGGCT CAGGCGCAAG AACAGATGGT CCCAGATGC GGTCCAGGCC TCAGCAGTTT CTAGAGAAC ATCAGATGTT TCCAGGTGC CCAAGGACC
5201 ACGGCGCGA GTCCCGGTTT TTGTCTACCA GGGGTCTACG CCAGGTCTGG AGTCTCTTGG TAGTCTACAA AGTCTCTACG GGTCTCTGG
5301 TGAATGACC CTGTGCTTA TTGAACTAA CCAATCAGTT CGCTTCTGCC TTCTGTCTCG GCGTATATG TCCCGAGCT CAATAAAGA GCCACAAAC
5401 ACTTTACTGG GACACGGAAT AACTTGAAT GGTATGTCAA GCGAAGAGCG AAGCAAGCG CGCENATAG AGGGTCTGA GTTATTTCT CGGTGTGTG
5501 CTTCACTCGG GCGGCCAGTC CTCCGATTA CTGATTCGG CCGTATCCCG TGTATCCAT AAACCTCTT GAGTTTGCAT GGCTGAGT GTCTCGCTG
5601 GGAGTGAGC CCGCGGTICAG GAGGTAACT GAGTCAAGG CCGTATCCCG TGTATCCAT AAACCTCTT GAGTTTGCAT GGCTGAGT GTCTCGCTG
5701 TCCCTGGAG GGTCTCTCT CTCACTAAT GATGAGTGA CTACCCCTCA GCGGGGTCT TTTCTGTCTG TGTCTGTCTG TGTCTGTCTG TGTCTGTCTG
5801 AGGAACCTC CCAAGAGAGA CTCACTAAT GATGAGTGA CTACCCCTCA GCGGGGTCT TTTCTGTCTG TGTCTGTCTG TGTCTGTCTG TGTCTGTCTG
5901 GACCCACAC CCGGAGGTAA GCTGGGCTAG GATGAGTGA CTACCCCTCA GCGGGGTCT TTTCTGTCTG TGTCTGTCTG TGTCTGTCTG TGTCTGTCTG
6001 CTGGGTGGT GCCCTCCAT CTGAGTCTG GATGAGTGA CTACCCCTCA GCGGGGTCT TTTCTGTCTG TGTCTGTCTG TGTCTGTCTG TGTCTGTCTG
6101 GCTAACTAG TCTGTATCTG CCGGACCTCT GATGAGTGA CTACCCCTCA GCGGGGTCT TTTCTGTCTG TGTCTGTCTG TGTCTGTCTG TGTCTGTCTG
6201 CGATTGATCG AGACTAGATC GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG
6301 TTTGTGGCC GACTGATCAG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG
6401 AAACACCGG CTGGACTCAG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG
6501 TAAACAGTT CCCGCTCTG TCTGATCTG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG GATTTAGGG
6601 ATTTGTCAA GGGCGGAGG AGACTAATA AGACTAATA AGACTAATA AGACTAATA AGACTAATA AGACTAATA AGACTAATA AGACTAATA
6701 TCTGACTGT TTTCTGTAT TGTCTGAAA TATGAGGCTG TATGAGGCTG TATGAGGCTG TATGAGGCTG TATGAGGCTG TATGAGGCTG
6801 AGACTGACAC AAGACATTA ACAGACTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT
6901 ATCGCTCACA ACCAGTCTG AGACTTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT AGACTTTT
7001 TAGCGAGTGT TGGTACGCA TCTAGATTC TCTCTGCAA CCAATGGA CCAATGGA CCAATGGA CCAATGGA CCAATGGA
7101 CCTTTAACCG AGACCTCAT TCTGAGTAG TGGGTCCAAT TCTGAGTGA CCAATGGA CCAATGGA CCAATGGA CCAATGGA CCAATGGA
7201 GGAATTTGG TCTGAGTAG TGGGTCCAAT TCTGAGTGA CCAATGGA CCAATGGA CCAATGGA CCAATGGA CCAATGGA
7301 CTGAGCTTTT GACCCCTCT CCGGTGCAA CCGGTGCAA CCGGTGCAA CCGGTGCAA CCGGTGCAA CCGGTGCAA CCGGTGCAA
7401 GAACCGAAA CTGGGGGAG GAGCCAGTT GAGCCAGTT GAGCCAGTT GAGCCAGTT GAGCCAGTT GAGCCAGTT GAGCCAGTT
7501 CGTTGACCC CGCTCTGAT CCGCTCTAT CCGCTCTAT CCGCTCTAT CCGCTCTAT CCGCTCTAT CCGCTCTAT CCGCTCTAT
7601 GCAAGCTGG GCGAGCTAG GAGGAAATA GAGGAAATA GAGGAAATA GAGGAAATA GAGGAAATA GAGGAAATA GAGGAAATA
7701 TGTAAACTT CCGTACCTT GACATGACA GAGTACTTA GAGTACTTA GAGTACTTA GAGTACTTA GAGTACTTA GAGTACTTA
7801 AACATTTGAA GGGACTGGG CTGACTGTT CTGACTGTT CTGACTGTT CTGACTGTT CTGACTGTT CTGACTGTT CTGACTGTT CTGACTGTT

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6101  ACCTCTGGCG GCAGCCTACC AAGAACAACCT GGACCGACCG GTGGTACTTC ACCCTTACCG AGTCGGGCGAC ACAGTGTGGG TCCGCCGACA CCAGACTAAG
      TGGAGACCGC CGTCGGGATGG TTCTTGTTGA CCTGGCTGGC CACCATGGAG TGGGATGGC TCAGCCGCTG TGTACACACC AGGCGGCTGT GGTCTGATTC
      PmlI
6201  AACCTAGAAC CTGGCTGGAA AGGACCTTAC ACAGTCCTGC TGACCACCCG CACCGCCCTC AAGTAGACG GCATCGCAGC TTGGATACAC GCCGCCACG
      TTGGATCTTG GAGCGACCTT TCCTGGAATG TGTGAGGAG ACTGGTGGG GTGGCGGAG TTTCATCTGC CGTAGCGTGC AACCTATGTG CGCGGGGTGC
      VH
      CD8-Leader
      PmlI
      NcoI
6301  TGAAGGCTGC CGACCCCGGG GGTGGACCAT CCTCTAGACT GCCATGGCTC TCCAGTGAC TCCCTACTG CTTCCCTAG CGCTTCTCT GCATGCAGAG
      ACTTCCGACG GCTGGGGCCC CCACCTGGTA GGAGATCTGA CGGTACCGAG AGGTCACTG ACGGATGAC GAAGGGGATC GCGAAGAGGA CGTACGTCTC
      VH
6401  GTGAGCTGC AGGATCAGG GGGAGGCTTC GTGAAGCCTG GAGGTCCCT CAAGTCTCC TGTGAGCCT CTGGATCAC TTTCAGTAGC TATGCCATGT
      CACTTCGACG TCCTCAGTCC CCGTCCGAG CACTTCGGAC CTCCGAGGA GTTTCAGAG ACACGTGCGA GACCTAAGTG AAGTCAATCG ATACGGTACA
      VH
6501  CCTGGGTTGC CTGAGTCCG GAGATGAGGC TGGAGTGGGT CGCAACCATT AGCAGTGCTG GTGGTTACAT CTTCTATTCT GACAGTGTGC AGGACCATTT
      GGACCCGAGG GGACTCAGGC CTCTACTCCG ACCTCACCCA GCGTTGGTAA TCCTCAGAC CACCAATGTA GAAGATAAGA CTGTACACAG TCCCTGCTAA
      VH
6601  CACCAATTTCC AGAGACAATG CCAAGAACAC CTGACACCTG CAATGGGCA GTCTGAGTC TGGGGACAG GCCATGTATT ACTGTGCAAG GCAGGGATTT
      GTGGTAAAGG TCTCTGTTAC GGTCTGTTG GACGTGACG GTTTACCCGT CAGACTCCAG ACCCTGTGC CGGTACATAA TGACACGTTT CGTCCCTAAA
      (GAS)3 Glycine-Serine Linker
      VH
6701  GGTAAGTACG GTGATTACTA TGTATGAC TACTGGGCC TACTGGGCC AAGGACCCG GGTACCCGT TCCTCAGGTG GAGGTGGATC AGGTGGAGT GGATCTGGTG
      CCAATTGATG CACTAATGAT ACGATACCTG ATGACCCCGG TTCCCTGGTG CCAAGTGGCAG AGGAGTCCAC CTCACCTAG TCCACCTCCA CCTAGACCCAC
      VL

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Figure 19 (4 of 6)

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(G4S)3 Glycine-Serine linker
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6801  GAGGTGGATC TGACATTGAG CTCACCCAGT CTCGATCTCTC CCGTGGTGTG TCAGCAGGAG AGAAGGTGAC TATAGCTGC AATCCAGTC AGAGTCTGCT
      CTCCACCTAG ACTGTAACTC GAGTGGGTCA GAGTAGGAG GGACCGACAG AGTCGTCTCTC TCTCCAGTG ATACTCGAGG TTTAGGTGAG TCTCAGACGA
      VL
6901  CAACAGTAGA ACCCGAAGA ACCAGTTGGC TTGTACACAG CAAAACACAG GACAGTCTCC TGAAGTCTG ATCTACTGGG CATCCACTAG GCAATCTGGA
      GTTGTCACTT TGGGCTTTCT TGGTCAACCG AACATGGTC GTTTTGTGTC CTGTCAAGAG ACTTGACGAC TAGATGACCC GTAGTGATC CGTTAGACCT
      VL
7001  GTCCCTGATC GCTTCACAGG CAGTGGATCT GGCACAGATT TCACTCTCAC CATCAGCAGT GTGCAGGCTG AAGACCTGGC AGTTTATTAC TGCACGCAAT
      CAGGACTAG CGAAGTGTCC GTACACTAGA CCGTGTCTAA ACTCAGAGTG GTAGTCTGTA CACCTCGGAC TTCTGGACCG TCAATAATG ACGTCTGTTA
      VL
      CD28 transmembrane + intracellular domains (-STOP)
      -----
      NotI
7101  CTTATATCT ACTCAGCTTC GGTCTTGGG CCAAGCTGGA GATCAACGG GCGCGGCAA TTGAAGTAT GTATCTCTCT CTTACCTAG ACAAAGAGAA
      GAATATTAGA TGAGTGCAG CCAGCACCTT GGTTCGACCT CTAGTTGCC GCGCGGCTT AACTTCAATA CATAGGAGGA GGAATGGATC TGTACTCTT
      CD28 transmembrane + intracellular domains (-STOP)
7201  GAGCAATGGA ACCATTATCC ATGTGAAGG GAAACACCTT TGTCCAAGTC CCTATTTC CCGACCTTCT AAGCCCTTT GGTGCTGCTT GGTGGTTGCT
      CTCGTTACCT TGGTAATAGG TACACTTTCC CTTTGTGGA ACAGGTTTCA GGNATAAGG GCTTGAAGA TTGGGGAAG CCCACGACCA CCACCAACCA
      CD28 transmembrane + intracellular domains (-STOP)
7301  GGAGTCTGG CTTGCTATAG CTTGCTATGA ACAGTGGCCT TTATTATTTT CTGGTGAGG AGTAACAGGA CCAGGCTCT GCACAGTGAC TACATGAACA
      CCTCAGGACC GAACGATATC GAACGATCAT TGTACACCGA AATAATAAA GACCCACTCC TCAATCTCT CGTCCGAGGA CGTGCTACTG ATGTACTTGT
      CD28 transmembrane + intracellular domains (-STOP)
      -----
      CD3 zeta chain intracellular domain
7401  TGAATCCCG CCGCCCGGG CCGACCGCA AGCAATACCA GCGCTATGCC CCACACGGC ACTTCCGAGC CTATGCTCC AGAGTGAAT TCAGCAGGAG
      ACTGAGGGC GCGCGGGCC GGTGGGCGT TCGTATGCT CCGGATACCG GGTGGTGGC TGAAGCGTCC GATAGCGAGG TCTCACTTCA AGTCGTCTCT
      CD3 zeta chain intracellular domain
7501  CCGAGAGCC CCGCGTACC AGCAGGCGCA GAACGAGTC TATACGAGC TCAATCTAGG ACGAAGAGG GATACGATG TTTTGGACAA GAGACGTGGC
      GCGTCTCGG GCGCGCATGG TCGTCCCGGT CTTGCTCGAG ATATTGCTC AGTTAGATCC TGCTTCTCT CTCATGCTAC AAAACCTGTT CTCTGACCG
      CD3 zeta chain intracellular domain
7601  CCGGACCTG AGATGGGGG AAGCCGAGA AGGAGAAC CCACGAGG CCGTACCAAT GAATGACGA AAGATAAGAT GCGGAGGCC TACAGTGAGA
      GCGCTGGAC TCTACCCCC TTTCGGCTCT TCCTCTTGG GAGTCTTCC GGACATGTTA CTTGAGCTCT TTCTATTCTA CCGCTCCGG ATGTCACTCT
      CD3 zeta chain intracellular domain

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Figure 19 (5 of 6)

7701

TTGGGATGAA AGGCGAGCGC CGGAGGGCCA AGGGGACGGA TGGCCTTTAC CAGGCTCTCA GTACAGCCAC CAAGGACACC TAGGAGGCCC TTCACATGCA
AACCTACTT TCCGCTCGCG GCCTCCCGGT TCCCGGTGCT ACCGGAATG GTCCAGAGT CATGTCGGTG GTTCCTGTTG ATGCTGCGGG AAGTGTACGT
CD3 zeta chain intracellular domain

7801

XhoI

GGCCCTGCCC CCTCGCTAAC AGCCACTCGA G
CCGGGACGGG GGAGCGATTG TCGGTGAGCT C
Figure 19 top strand: SEQ ID NO:39
Figure 19 bottom strand: SEQ ID NO:40

Figure 19 (6 of 6)

1. Mouse MUC16-CD Peptide 1 (SEQ ID NO:21):

TLDRKSVFVDGYSQNRDD

19 AA

2. Mouse 1st Cysteine Loop peptide 2 (SEQ ID NO:22):

KSYFSDQVLAFRSVSNNNNHTGVDSLNFSP

33 AA

3. Mouse 2nd Cysteine Loop peptide 3 (SEQ ID NO:23):

SLYSNRLASLRPKKNGTATGVNAISYHQ

32 AA

Figure 20B

mouse complete 8244
human complete 14167

mouse complete 8304
human complete 14227

mouse complete 8364
human complete 14287

mouse complete 8424
human complete 14343

mouse complete 8484
human complete 14402

mouse complete 8544
human complete 14462

mouse complete
human complete

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Figure 21

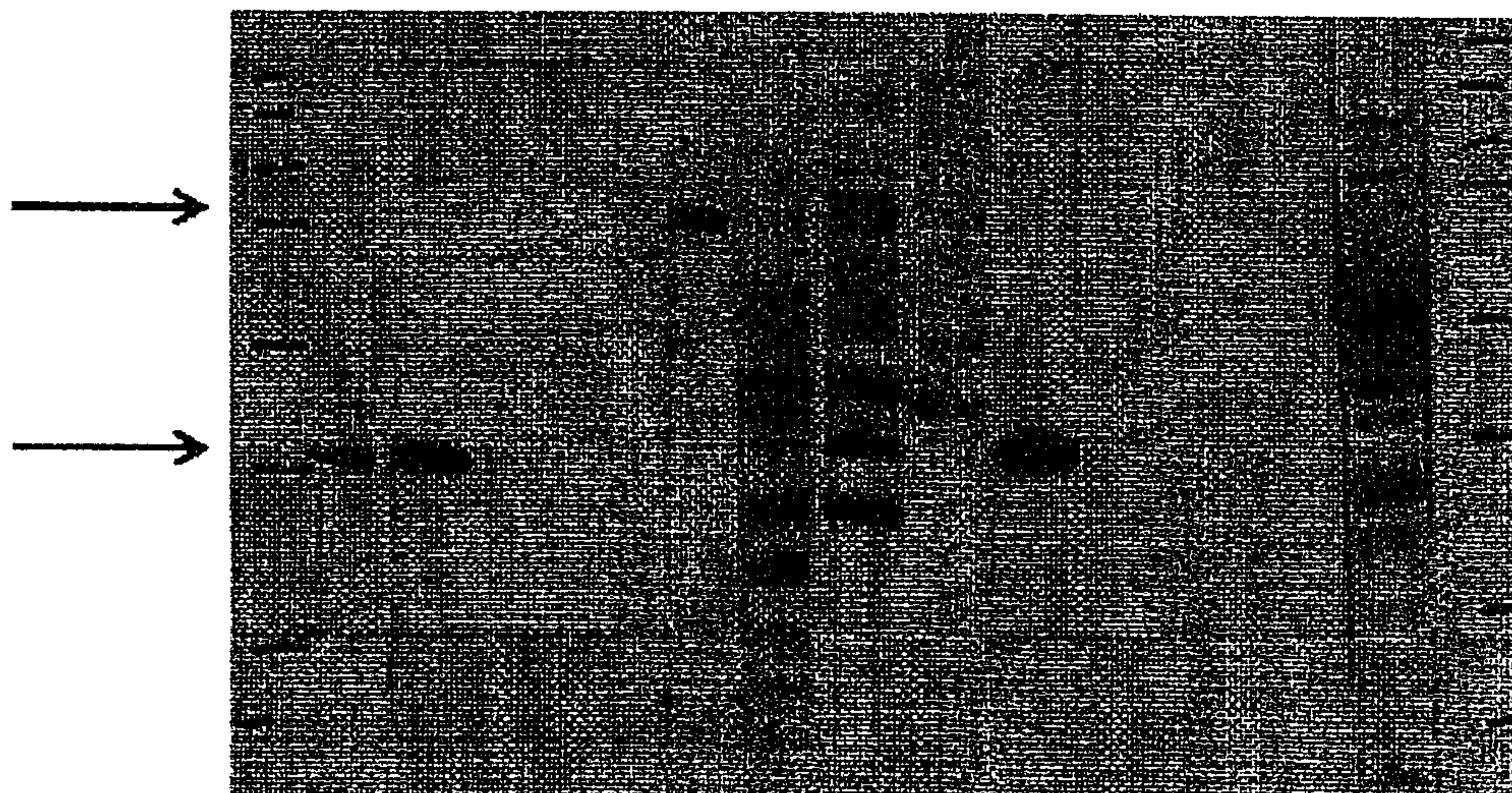
Mouse MUC16 CD Peptide 1

1D1	9F7	16A9	21A7	24G10	10C4	17F2	1A8	1F8	12B10	17H10	18D5	23B12
1	2	3	4	5	6	7	8	9	10	11	12	13



Mouse MUC16 CL Peptide 3

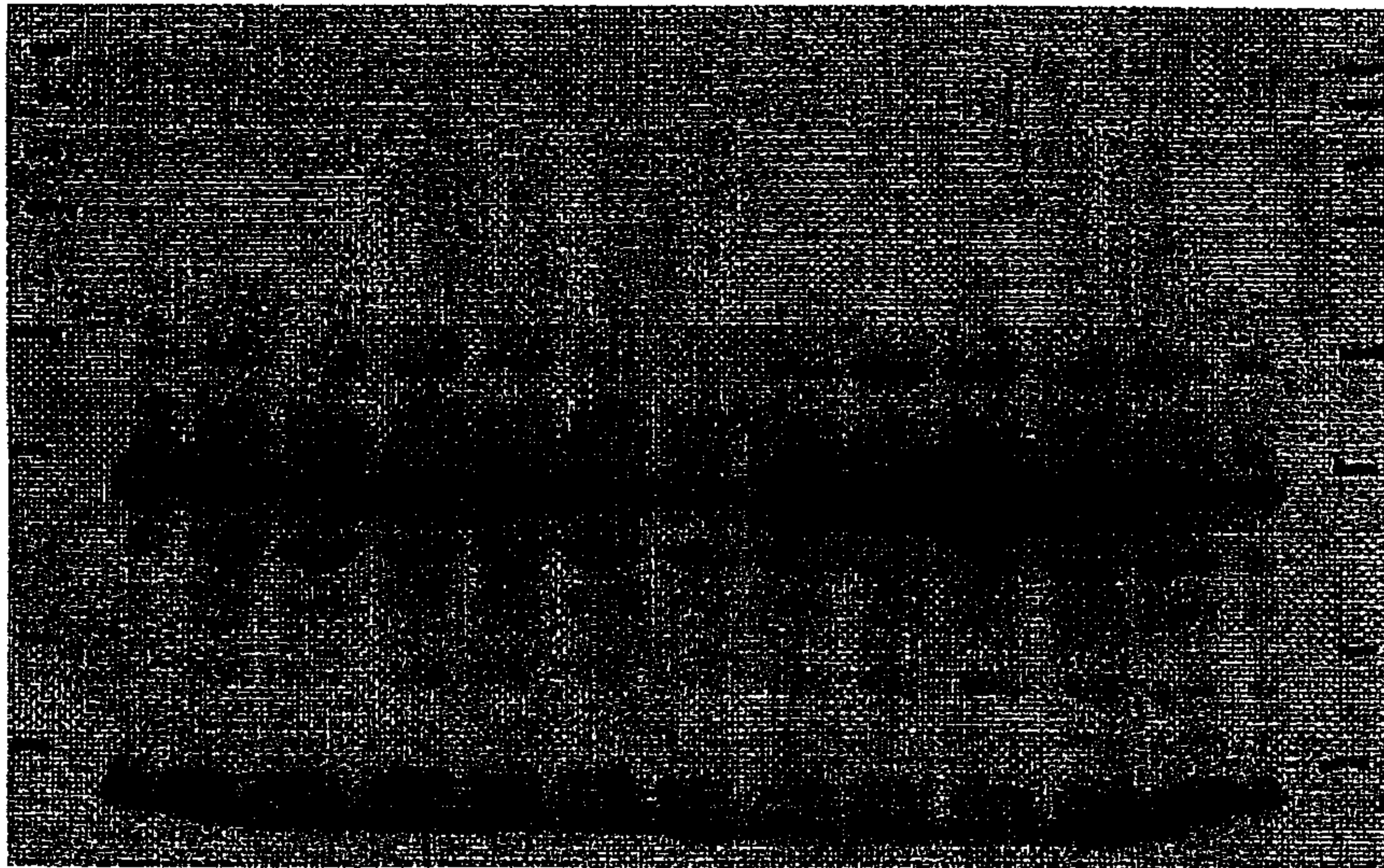
25E9	16F12	4A6	5D1	21B8	21E1	8A2	13E5	23G4	21D3	FB	XX	4H11hu
14	15	16	17	18	19	20	21	22	23	24	25	26



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Figure 22

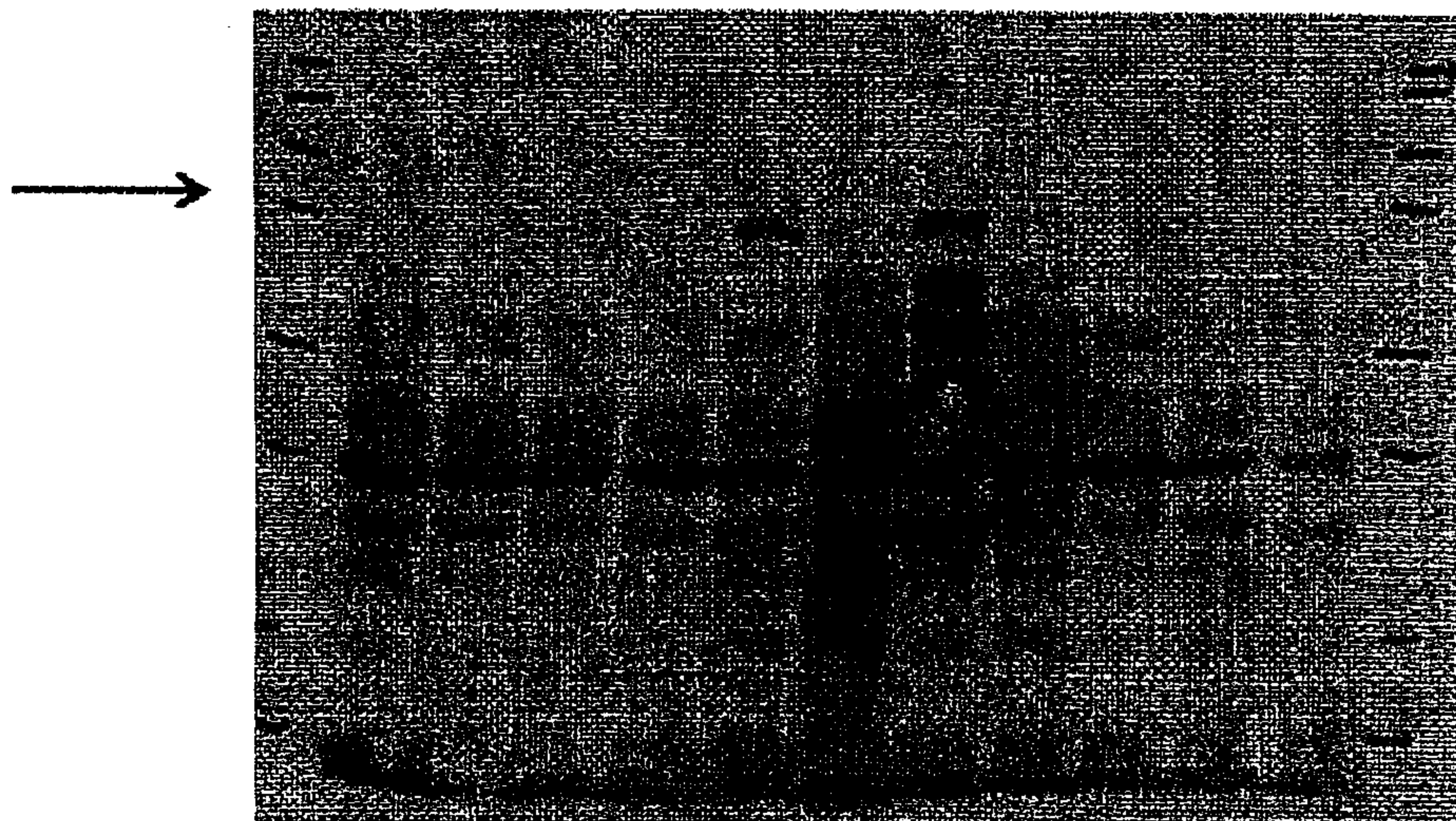
Mouse MUC16 CD Peptide 1

ID1	9F7	16A9	21A7	24G10	10C4	17F2	1A8	1F8	12B10	17H10	18D5	23B12
1	2	3	4	5	6	7	8	9	10	11	12	13

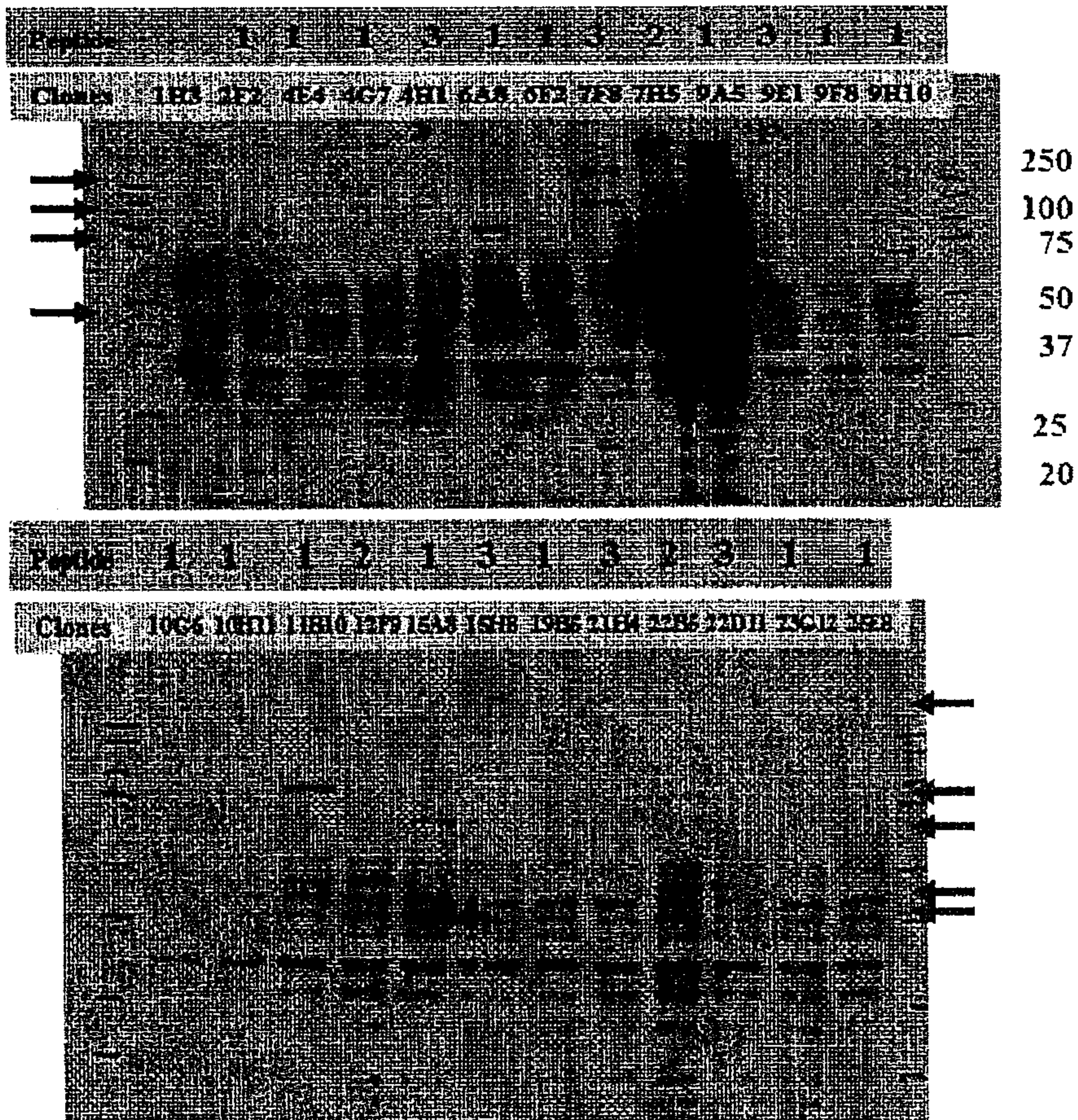


Mouse MUC16 CL Peptide 3

25E9	16F12	4A6	5D1	21B3	21E1	8A2	13E5	23G4	21D3	FB
14	15	16	17	18	19	20	21	22	23	24



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Figure 23



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Peptide 3 1 2 3 2 1 2 2 1 2 1 1 1 1 1 1

Clones 27H9 28B12 29C12 30H2 31A11 31C13 32B5 34F10 34H5 36C13 36C11 36E4 37E10 FB



Peptide 1

Clones 12 10H11



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Figure 24

A. Nucleotide sequence encoding 12B10.3G10-V_H (SEQ ID NO:26)

GAGGTGAAGCTGGAGGAGTCAGGTGGAGGATTGGTGACGCCTAAAGGATCATTGAAACTCTCATGTGCCGCCTCT
GGTTTCACCTTCAATACCTATGCCGTGCACCTGGGTCCGCCAGGCTCCAGGAAAGGGTATGGAATGGGTTCCTCGC
ATAAGAAGTAAAGTGGAAATTATGCAACATATTATGGCGATTTCAGTGAAAGACAGATTACCATCTCCAGAAAT
GATTACAGAGCATGCTCTATCTGCAATGAACAACCTGAAAAGTGGAGACACAGCCATATATTACTGTGTGAGA
GCGGGTAACAACGGGGCCTTTCCTTACTGGGGCCAAGGGAGCACGGTCACCGTCTCCTCA

B. 12B10.3G10-V_H Amino Acid sequence (SEQ ID NO:27)

EVKLEESGGGLVQPKGSLKLSAASGFTFNTYAVHWVRQAPGKGMWVVARIRSKSGNYAT
YYADSVKDRFTISRNDQSMLYLQMNNLKTEDTAIYYCVRAGNNGAFPYWGQGTTVTVSS

C. Nucleotide sequence encoding 12B10.3G10-V_L (SEQ ID NO:28)

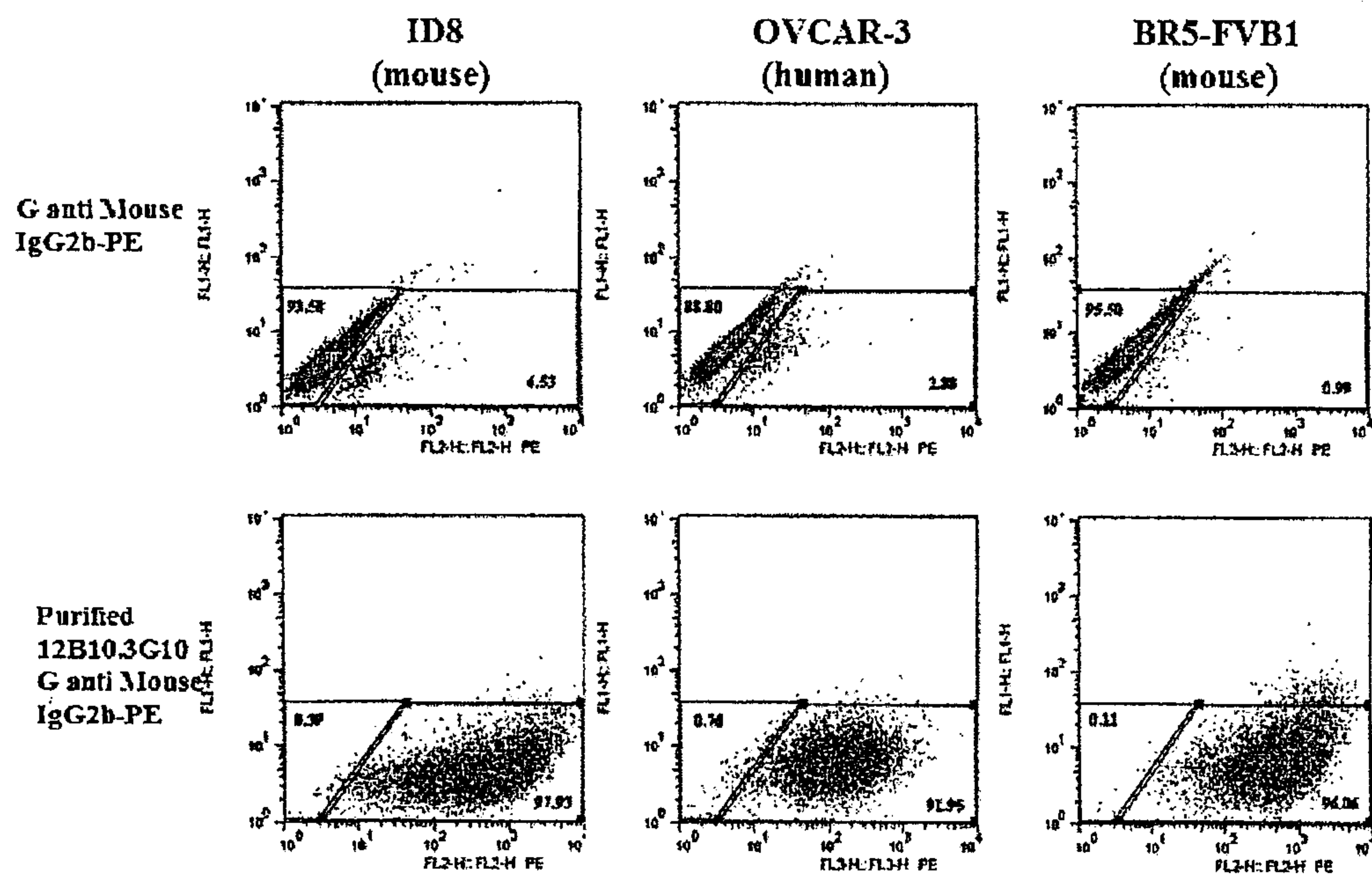
Note the VL has an optional *NotI* site added by the primer for cloning.

GACATTGAGCTCACCCAGTCTCCATCCTCACTGTCTGCATCTCTGGGAGGGAGAGTCACCATCACTTGCAAGGCT
AGCCAAGATATTAAGAAGTATATAGCTTGGTACCAACACAAAGCCTGGAAAACTCCTCGACTACTCATACTTTC
ACATCTACATTAACAGACAGGCATCCCATCAAGGTTTCAGTGGACGTGGGTCTGGGAGAGACTATTCCCTTCAGCATC
AGCAACCTGGAGTCTGAAGATATTGCAACTTATTATTGTCTACAGTATGATAGTCTGTACACGTTGGGAGGGGGG
ACCAAGCTGGAGATCAAACGGGCGGGCGCA

D. 12B10.3G10-V_L Amino Acid sequence (SEQ ID NO:29)

DIELTQSPSSLASLGGRVTTICKASQDIKKYIAWYQHKPGKTPRLLIHFTSTLQTGPS
RFSGRGSGRDYSFISISNLESEDIATYYCLQYDSLTYFGGGTKLEIKRAAA

Figure 25



Peptide 1 near Cleavage Site:

NFSPLARRVDRVAIYEE (SEQ ID NO:01)

Peptide 2 before Transmembrane:

TLDRSSVLVDGYSPNRNE (SEQ ID NO:02)

Peptide 3 inside Transmembrane:

CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03)