(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 May 2007 (03.05.2007)

PCT

(10) International Publication Number
WO 2007/051077 A2

(51) International Patent Classification:
G01N 33/574 (2006.01)

(21) International Application Number:
PCT/US2006/042689

(22) International Filing Date: 30 October 2006 (30.10.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/731,210 28 October 2005 (28.10.2005) US

(71) Applicant *(for all designated States except US)*: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 5th Floor, Oakland, California 94607 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KIPPS, Thomas, J.** [US/US]; 131755 Mendiola, San Diego, California 92103 (US). **FUKUDA, Tetsuya** [JP/JP]; 1-25-31 Koyamadai Sakae-ku, Yokohama, Kanagawa, 247-0002 (JP). **ENDO, Tomoyuki** [JP/US]; 8465 Regents Road, #207, San Diego, California 92122 (US).

(74) Agents: **BUCKLEY, Kevin, W.** et al.; Sonnenschein Nath & Rosenthal LLP, P.O. Box 061080, Wacker Drive Station - Sears Tower, Chicago, Illinois 60606-1080 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

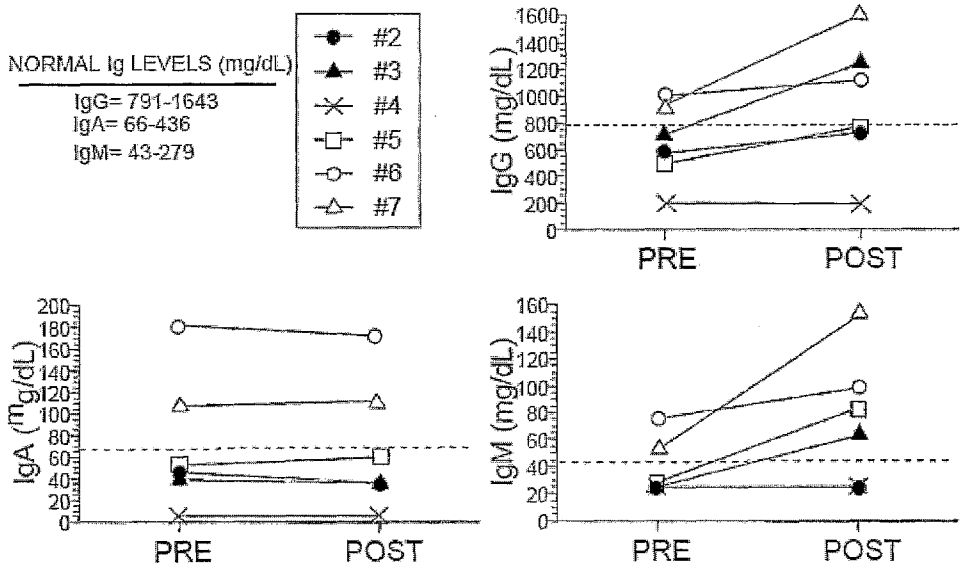
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

[Continued on next page]

(54) **Title:** METHODS AND COMPOUNDS FOR LYMPHOMA CELL DETECTION AND ISOLATION



(57) **Abstract:** What is provided are compositions comprising a purified, isolated antibody directed against ROR1, wherein the antibody binds ROR1 with moderate to high affinity. In addition, methods are provided for detecting and isolating an amount of ROR1 in a subject sample. Additionally, a diagnostic method is provided for evaluating the appearance, status, course, or treatment of lymphocytic leukemia in a subject. A kit is also provided to detect the presence of ROR1 protein comprising the antibody above. Also provided are vaccines and related methods for protecting a subject against diseases that involve expression of ROR1. Humanized antibodies and ROR1 antibody precipitates are also provided.

WO 2007/051077 A2

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS AND COMPOUNDS FOR LYMPHOMA CELL DETECTION AND ISOLATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional Patent Application No. 60/731,210, filed October 28, 2005.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made in part with Government support under National Institutes of Health Grant 5P01 CA81543. The Government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ON A COMPACT DISC

[0003] Not Applicable.

FIELD OF THE INVENTION

[0004] The present invention generally relates to antibodies directed against antigens specific for chronic lymphocytic leukemia.

BACKGROUND

[0005] ROR1 is an embryonic protein that is expressed uniquely on lymphoma cells, including in chronic lymphocytic reukemia (CLL), small lymphocytic lymphoma, marginal cell B-Cell lymphoma, Burkett's Lymphoma, and other cancers (e.g., breast cancers), but not on normal adult tissues and cells. Anti-ROR1 antibodies raised against ROR1 peptide are commercially available, but monoclonal anti-ROR1 antibodies that react with the native ROR1 protein have not been made or isolated. In addition, no anti-ROR1 antibodies capable of detecting cell-surface expression of ROR1 for flow cytometric analysis have been made or isolated. What is needed, therefore, is an antibody that can react with native ROR1 protein.

SUMMARY OF THE INVENTION

[0006] Among the various aspects of the present invention is the provision of an antibody directed to a surface receptor tyrosine kinase protein expressed on cells found in samples of subjects with a lymphoma, including CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, Burkett's Lymphoma, but not in blood or splenic lymphocytes of nonleukemic patients or normal adults

[0007] Briefly, therefore, the present invention is directed to an antibody useful for

differentiation between lymphoma cells and normal lymphocytes as well as immunotherapy against lymphoma and determination of response to lymphoma therapy.

[0008] The present teachings include compositions that include a purified, isolated antibody that binds specifically to ROR1 receptor protein.

[0009] The present teachings include methods for an immunoassay that detects ROR1 in a sample from a subject by contacting the sample with an ROR1-specific antibody and detecting immunoreactivity between the antibody and ROR1 in the sample.

[0010] In accordance with a further aspect, a lymphoma is diagnosed in a subject by detecting the presence or quantity of ROR1 protein in a sample derived from the subject.

[0011] In accordance with yet another aspect, a lymphoma is treated in a subject by administering to the subject in need of such therapy a therapeutically effective amount of an ROR1 receptor agonist.

[0012] In accordance with yet another aspect, the appearance, status, course, or treatment of lymphocytic leukemia in a subject is evaluated by contacting a biological sample obtained from the subject with an anti-ROR1 antibody and detecting immunoreactivity between the antibody and ROR1 to determine presence or quantity of ROR1 in the sample.

[0013] In accordance with yet another aspect, also provided is a vaccine composition comprising a polynucleotide encoding ROR1 protein or a fragment or variant thereof, and a pharmaceutically acceptable carrier or diluent.

[0014] In accordance with yet another aspect, also provided is a vaccine composition comprising ROR1 protein or a fragment or variant thereof, and a pharmaceutically acceptable carrier or diluent.

[0015] In accordance with yet another aspect, also provided is a method for protecting against the occurrence of diseases involving expression of ROR1 in a subject, the method comprising administering to the subject in need thereof a polynucleotide encoding ROR1 protein or a fragment or variant thereof in an amount effective to induce a protective or therapeutic immune response against ROR1, and a pharmaceutically acceptable carrier or diluent.

[0016] In accordance with yet another aspect, also provided is a method for protecting against the occurrence of diseases involving expression of ROR1 in a subject, the method comprising administering to the subject in need thereof ROR1 protein or a fragment or variant thereof in an amount effective to induce a protective or therapeutic immune response against ROR1 in the subject, and a pharmaceutically acceptable carrier or diluent.

[0017] In accordance with yet another aspect, a humanized ROR1 antibody is provided. In another aspect, a precipitate comprising a ROR1 antibody bound with a ROR1 protein, fragment or variant is provided. The ROR1 antibody can be conjugated to a

magnetic bead.

[0018] Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Those of skill in the art will understand that the drawings, described below, are for illustrative purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0020] Figure 1 shows change of serum antibody after Ad-CD154 therapy.

[0021] Figure 1A is a series of scatter and line plots showing total levels of IgG, IgA, and IgM. IgG, IgA, IgM blood concentrations, measured just prior to initiating Ad-CD154 therapy (PRE) and 2-4 week following the final treatment time point (POST). The dashed bar in each line graph indicates the minimum normal Ig concentration. The concentration range of normal Ig levels is shown to the left of legend.

[0022] Figure 1B is a series of scatter and line plots showing antibody response to recombinant Ad-CD154. Anti-adenovirus antibodies were analyzed by an ELISA assay. Serial dilutions of patient serum before (dotted line) and after (filled line) treatment were incubated in 96 well plates coated with Ad-CD154. Bound adenovirus-specific antibody was then detected using AP-conjugated antibody specific for human Ig.

[0023] Figure 1C is a series of bar graphs showing change of antibody response against Adenovirus in serial samples. Anti-adenovirus antibodies were analyzed by an ELISA using anti-isotype specific secondary antibodies conjugated AP. The bar graphs represent the mean increase in adenovirus-specific antibody over the baseline pre-treatment antibody levels. IgE and IgG4 levels for all patients were below the assay detection limits (data not shown).

[0024] Figure 1D is a series of scatter and line plots showing anti-tetanus-toxin antibody response before and after Ad-CD154 treatment. ELISA assay was performed with purified tetanus toxin and sera from patients. Bound tetanus-specific antibody was detected using AP conjugated goat anti-human Ig antibody.

[0025] Figure 2 is a series of histograms showing antibody production against surface molecules on CLL B cells by Ad-CD154 therapy. Antibody bound on CD19+ CD3- cells were detected by goat anti-human antibody.

[0026] Figure 2A is a series of histograms showing diluted serum from patient before (open histograms) or after (shaded histograms) treatment was incubated with PBMC from CLL patient.

[0027] Figure 2 B is a series of histograms showing diluted serum from patient

before (open histograms) or after (shaded histograms) treatment was incubated with PBMC from a healthy donor.

[0028] Figure 3 is an immunoblot of Immune Precipitates of Lysates with 4A5 Probed With Rabbit anti-ROR1 Raised Against Ror1 Peptides.

[0029] Figure 4 is a series of images depicting gels that show expression of ROR1 in CLL B cells.

[0030] Figure 4A are gel images of an immunoblot analysis of ROR1 protein. Total cell lysates of PBMC from CLL patients or healthy donor and those of splenocytes from CLL patients or idiopathic thrombocytopenia purpura patient were analyzed by immunoblot using rabbit anti-ROR1 antibody (Cell signaling).

[0031] Figure 4B are gel images showing ROR1 expression in B cell lines. Immunoblot analysis of total cell lysates of B cell lines was performed.

[0032] Figure 4C shows production of mouse anti-ROR1 sera. CHO cells stained with PKH26 and were mixed with CHO transfected ROR1 cDNA (CHO-ROR1). Sera collected from mice before and after immunization with ROR1 cDNA were incubated with mixed CHO cells. Bound antibodies were detected flow cytometry.

[0033] Figure 4D is a series of histograms showing flow cytometric analysis of expression of ROR1 on cell surface of CLL. PBMC from CLL patients and healthy donor were incubated antisera before (open histograms) and after (shaded histograms) DNA immunization.

[0034] Figure 5 is a series of histograms showing production of anti-ROR1 antibody detected by flow cytometric analysis.

[0035] Figure 5A is a series of histograms where CHO (open histograms) or CHO-ROR1 (shaded histograms) was incubated with serum from patients before (pre) or after (post) therapy. Histograms indicated the bound human Ig detected by PE labeled goat anti-human Ig.

[0036] Figure 5 B shows results where CHO stained with PKH26 were mixed and incubated with serum from patient. APC conjugated anti-human Ig antibody was used for detection.

[0037] Figure 6 shows production of anti-ROR1 antibody detected by ELISA.

[0038] Figure 6A is a series of gel images showing production of recombinant ROR1 protein. *ROR1* extracellular region was fused with rabbit IgG Fc region in frame (*ROR1rIg*). Fused cDNA were transfected into CHO cells and secreted recombinant protein was immuneabsorbed using protein A sepharose. Absorbed protein was immunoblotted with goat anti-ROR1 antibody (R&D) or goat anti-rabbit Ig antibody. KSHV K8.1 protein fused with rabbit Fc region was also used for control. The purified recombinant ROR1 was

visualized with GelCode blue stain reagent (Pierce) staining after SDS-PAGE.

[0039] Figure 6B is a series of line and scatter plots showing antibody reaction to ROR1 detected by ELISA. Diluted sera were reacted with coated ROR1rIg and bound antibody was detected by goat anti-human Ig antibody conjugated with HRP.

[0040] Figure 6C is a series of line and scatter plots showing antibody reaction to rabbit IgG detected by ELISA. Diluted sera were reacted with coated rabbit IgG and bound antibody was detected by goat anti-human Ig antibody conjugated with HRP.

[0041] Figure 7 shows ROR1 and Wnt5a activated NF-κB reporter expression.

[0042] Figure 7A is a series of bar graphs showing the effect of ROR1 on LEF/TCF1, NF-AT, and AP-1 activity. HEK293 cells were transfected with indicated reporter construct and β-galactosidase vector along with expression vector of ROR1 and Wnt5a.

[0043] Figure 7B is a series of bar graphs showing the effect of ROR1 on NF-κB activity. HEK293 cells were transfected with NF-κB reporter construct and β-galactosidase vector along with expression vector of ROR, Wnt5a, Wnt3, Wnt5b and Wnt16.

[0044] Figure 7C is a series of gel images showing *in vitro* binding of ROR1 and Wnt5a. Conditioned medium of transfectant with *Wnt5a* tagged with HA was incubated with ROR1rIg or rabbit IgG. Immunoprecipitation and immunoblotting were done with indicated materials.

[0045] Figure 8 is a series of histograms showing gated CLL patients and CD19+ and CD19+CD5+ cells.

[0046] Figure 9 is a series of histograms showing gated normal patients and CD19+ and CD19+CD5+ cells.

[0047] Figure 10 is a series of histograms showing gated "exceptional" normal patients and CD19+ and CD19+CD5+ cells.

[0048] Figure 11 is a series of histograms showing gated CLL patients and CD19+ and CD19+CD5+ cells.

[0049] Figure 12 depicts the expression of 4A5 versus normals versus CLLs and the gating effect.

[0050] Figure 13 is a series of histograms showing different levels of 4A5 expression on titrated CLL cells.

[0051] Figure 14 is a series of histograms showing different levels of 4A5 expression and that such cells can be purified using magnetic beads and methods provided herein.

DETAILED DESCRIPTION OF THE INVENTION

[0052] As noted above, the instant invention provides new and useful antibodies

directed against ROR1 protein. Full length ROR1, a surface receptor tyrosine kinase, is found in samples of subjects with CLL, but not in blood or splenic lymphocytes of nonleukemic patients or normal adults. The invention also provides diagnostic and therapeutic antibodies, including monoclonal antibodies, and related compositions and methods for use in the diagnosis, management and treatment of disease. The ROR1 antibody described herein is more sensitive and more specific to lymphoma cells than using a combination of several cell surface markers that cannot exclude a small fraction of normal lymphocytes.

[0053] Applicants have discovered expression of full-length ROR1 in CLL samples, but not other tissues, including blood or splenic lymphocytes of non-leukemic patients or normal adult donors, and also generated mouse anti-sera against full-length human ROR1. Fukuda et al., Blood: ASH Annual Meeting Abstracts 2004 104, Abstract 772 (2004) (incorporated herein by reference in its entirety).

[0054] ROR1 Antibody

[0055] Certain embodiments comprise immunopeptides directed against ROR1 protein. The immunoglobulin peptides, or antibodies, described herein are shown to bind to the ROR1 protein. The ROR1 binding activity is specific; the observed binding of antibody to ROR1 is not substantially blocked by non-specific reagents. These ROR1 specific antibodies can be used to differentiate between lymphoma cells (including lymphoma cells derived from CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, Burkett's Lymphoma) and normal lymphocytes. The ROR1 specific antibodies can also be used in immunotherapy against a lymphoma and to determine the response after therapy for a lymphoma.

[0056] Such immunopeptides can be raised in a variety of means known to the art. For example, and as shown in the examples, Ad-CD154 therapy induces humoral immunity against CLL, thus allowing the derivation of immunoglobulin peptides specific against ROR1. The inventors have discovered that tandem injections of Ad-CD154 induces antibody production against a novel cell surface TAA of CLL B cells, orphan tyrosine kinase receptor ROR1.

[0057] As used herein, the term antibody encompasses all types of antibodies, e.g., polyclonal, monoclonal, and those produced by the phage display methodology. Particularly preferred antibodies of the invention are antibodies which have a relatively high degree of affinity for ROR1. In certain embodiments, the antibodies exhibit an affinity for ROR1 of about $Kd<10^{-8}$ M.

[0058] Substantially purified generally refers to a composition which is essentially

free of other cellular components with which the antibodies are associated in a non-purified, e.g., native state or environment. Purified antibody is generally in a homogeneous state, although it can be in either in a dry state or in an aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography.

[0059] Substantially purified ROR1-specific antibody will usually comprise more than 80% of all macromolecular species present in a preparation prior to admixture or formulation of the antibody with a pharmaceutical carrier, excipient, adjuvant, buffer, absorption enhancing agent, stabilizer, preservative, adjuvant or other co-ingredient. More typically, the antibody is purified to represent greater than 90% of all proteins present in a purified preparation. In specific embodiments, the antibody is purified to greater than 95% purity or may be essentially homogeneous wherein other macromolecular species are not detectable by conventional techniques.

[0060] Immunoglobulin peptides include, for example, polyclonal antibodies, monoclonal antibodies, and antibody fragments. The following describes generation of immunoglobulin peptides, specifically ROR1 antibodies, via methods that can be used by those skilled in the art to make other suitable Immunoglobulin peptides having similar affinity and specificity which are functionally equivalent to those used in the examples.

[0061] Polyclonal Antibodies

[0062] Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Briefly, ROR1 antigen is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous injections, with an adjuvant such as Freund's complete or incomplete adjuvant. Following several booster immunizations, samples of serum are collected and tested for reactivity to ROR1. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to ROR1, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

[0063] Monoclonal Antibodies

[0064] Monoclonal antibody (MAb) technology can be used to obtain MAbs to ROR1. Briefly, hybridomas are produced using spleen cells from mice immunized with ROR1 antigens. The spleen cells of each immunized mouse are fused with mouse myeloma Sp 2/0 cells, for example using the polyethylene glycol fusion method of Galfre, G. and

Milstein, C., Methods Enzymol., 73:3-46 (1981). Growth of hybridomas, selection in HAT medium, cloning and screening of clones against antigens are carried out using standard methodology (Galfre, G. and Milstein, C., Methods Enzymol., 73:3-46 (1981)).

[0065] HAT-selected clones are injected into mice to produce large quantities of MAb in ascites as described by Galfre, G. and Milstein, C., Methods Enzymol., 73:3-46 (1981), which can be purified using protein A column chromatography (BioRad, Hercules, Calif.). MAbs are selected on the basis of their (a) specificity for ROR1, (b) high binding affinity, (c) isotype, and (d) stability.

[0066] MAbs can be screened or tested for ROR1 specificity using any of a variety of standard techniques, including Western Blotting (Koren, E. et al., Biochim. Biophys. Acta 876:91-100 (1986)) and enzyme-linked immunosorbent assay (ELISA) (Koren, E. et al., Biochim. Biophys. Acta 876:91-100 (1986)).

[0067] Humanized Antibodies

[0068] Humanized forms of mouse antibodies can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques (see, e.g., Queen et al., Proc. Natl. Acad. Sci. USA 86:10029-10033, 1989 and WO 90/07861, each incorporated by reference). Human antibodies can be obtained using phage-display methods (see, e.g., Dower et al., WO 91/17271; McCafferty et al., WO 92/01047). In these methods, libraries of phage are produced in which members display different antibodies on their outersurfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity may be selected by affinity enrichment. Human antibodies may be selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody.

[0069] Antibody Fragments

[0070] It may be desirable to produce and use functional fragments of a MAb for a particular application. The well-known basic structure of a typical IgG molecule is a symmetrical tetrameric Y-shaped molecule of approximately 150,000 to 200,000 daltons consisting of two identical light polypeptide chains (containing about 220 amino acids) and two identical heavy polypeptide chains (containing about 440 amino acids). Heavy chains are linked to one another through at least one disulfide bond. Each light chain is linked to a contiguous heavy chain by a disulfide linkage. An antigen-binding site or domain is located in each arm of the Y-shaped antibody molecule and is formed between the amino terminal regions of each pair of disulfide linked light and heavy chains. These amino terminal regions of the light and heavy chains consist of approximately their first 110 amino terminal amino

acids and are known as the variable regions of the light and heavy chains. In addition, within the variable regions of the light and heavy chains there are hypervariable regions which contain stretches of amino acid sequences, known as complementarity determining regions (CDRs). CDRs are responsible for the antibody's specificity for one particular site on an antigen molecule called an epitope. Thus, the typical IgG molecule is divalent in that it can bind two antigen molecules because each antigen-binding site is able to bind the specific epitope of each antigen molecule. The carboxy terminal regions of light and heavy chains are similar or identical to those of other antibody molecules and are called constant regions. The amino acid sequence of the constant region of the heavy chains of a particular antibody defines what class of antibody it is, for example, IgG, IgD, IgE, IgA or IgM. Some classes of antibodies contain two or more identical antibodies associated with each other in multivalent antigen-binding arrangements.

[0071] Fab and $F(ab')_2$ fragments of MAbs that bind ROR1 can be used in place of whole MAbs. Because Fab and $F(ab')_2$ fragments are smaller than intact antibody molecules, more antigen-binding domains are available than when whole antibody molecules are used. Proteolytic cleavage of a typical IgG molecule with papain is known to produce two separate antigen binding fragments called Fab fragments which contain an intact light chain linked to an amino terminal portion of the contiguous heavy chain via by disulfide linkage. The remaining portion of the papain-digested immunoglobin molecule is known as the Fc fragment and consists of the carboxy terminal portions of the antibody left intact and linked together via disulfide bonds. If an antibody is digested with pepsin, a fragment known as an $F(ab')_2$ fragment is produced which lacks the Fc region but contains both antigen-binding domains held together by disulfide bonds between contiguous light and heavy chains (as Fab fragments) and also disulfide linkages between the remaining portions of the contiguous heavy chains (Handbook of Experimental Immunology. Vol 1: Immunochemistry, Weir, D. M., Editor, Blackwell Scientific Publications, Oxford (1986)).

[0072] Recombinant DNA methods have been developed which permit the production and selection of recombinant immunoglobulin peptides which are single chain antigen-binding polypeptides known as single chain Fv fragments (ScFvs or ScFv antibodies). Further, ScFvs can be dimerized to produce a diabody. ScFvs bind a specific epitope of interest and can be produced using any of a variety of recombinant bacterial phage-based methods, for example as described in Lowman et al. (1991) Biochemistry, 30, 10832-10838; Clackson et al. (1991) Nature 352, 624-628; and Cwirla et al. (1990) Proc. Natl. Acad. Sci. USA 87, 6378-6382. These methods are usually based on producing genetically altered filamentous phage, such as recombinant M13 or fd phages, which display on the surface of the phage particle a recombinant fusion protein containing the antigen-

binding ScFv antibody as the amino terminal region of the fusion protein and the minor phage coat protein g3p as the carboxy terminal region of the fusion protein. Such recombinant phages can be readily grown and isolated using well-known phage methods. Furthermore, the intact phage particles can usually be screened directly for the presence (display) of an antigen-binding ScFv on their surface without the necessity of isolating the ScFv away from the phage particle.

[0073] To produce an ScFv, standard reverse transcriptase protocols are used to first produce cDNA from mRNA isolated from a hybridoma that produces an MAb for ROR1 antigen. The cDNA molecules encoding the variable regions of the heavy and light chains of the MAb can then be amplified by standard polymerase chain reaction (PCR) methodology using a set of primers for mouse immunoglobulin heavy and light variable regions (Clackson (1991) Nature 352, 624-628). The amplified cDNAs encoding MAb heavy and light chain variable regions are then linked together with a linker oligonucleotide in order to generate a recombinant ScFv DNA molecule. The ScFv DNA is ligated into a filamentous phage plasmid designed to fuse the amplified cDNA sequences into the 5' region of the phage gene encoding the minor coat protein called g3p. *Escherichia coli* bacterial cells are than transformed with the recombinant phage plasmids, and filamentous phage grown and harvested. The desired recombinant phages display antigen-binding domains fused to the amino terminal region of the minor coat protein. Such "display phages" can then be passed over immobilized antigen, for example, using the method known as "panning", see Parmley and Smith (1989) Adv. Exp. Med. Biol. 251, 215-218; Cwirla et al. (1990) Proc. Natl. Acad. Sci. USA 87, 6378-6382, to adsorb those phage particles containing ScFv antibody proteins that are capable of binding antigen. The antigen-binding phage particles can then be amplified by standard phage infection methods, and the amplified recombinant phage population again selected for antigen-binding ability. Such successive rounds of selection for antigen-binding ability, followed by amplification, select for enhanced antigen-binding ability in the ScFvs displayed on recombinant phages. Selection for increased antigen-binding ability may be made by adjusting the conditions under which binding takes place to require a tighter binding activity. Another method to select for enhanced antigen-binding activity is to alter nucleotide sequences within the cDNA encoding the binding domain of the ScFv and subject recombinant phage populations to successive rounds of selection for antigen-binding activity and amplification (see Lowman et al. (1991) Biochemistry 30, 10832-10838; and Cwirla et al. (1990) Proc. Natl. Acad. Sci. USA 87, 6378-6382).

[0074] Once an ScFv is selected, the recombinant ROR1 antibody can be produced in a free form using an appropriate vector in conjunction with *E. coli* strain HB2151. These bacteria actually secrete ScFv in a soluble form, free of phage components

(Hoogenboom et al. (1991) Nucl. Acids Res. 19, 4133-4137). The purification of soluble ScFv from the HB2151 bacteria culture medium can be accomplished by affinity chromatography using antigen molecules immobilized on a solid support such as AFFIGEL™ (BioRad, Hercules, Calif.).

[0075] Other developments in the recombinant antibody technology demonstrate possibilities for further improvements such as increased avidity of binding by polymerization of ScFvs into dimers and tetramers (*see* Holliger et al. (1993) Proc. Natl. Acad. Sci. USA 90, 6444-6448).

[0076] Because ScFvs are even smaller molecules than Fab or $F(ab')_2$ fragments, they can be used to attain even higher densities of antigen binding sites per unit of surface area when immobilized on a solid support material than possible using whole antibodies, $F(ab')_2$, or Fab fragments. Furthermore, recombinant antibody technology offers a more stable genetic source of antibodies, as compared with hybridomas. Recombinant antibodies can also be produced more quickly and economically using standard bacterial phage production methods.

[0077] Recombinant Antibody Production

[0078] To produce antibodies described herein recombinantly, nucleic acids encoding light and heavy chain variable regions, optionally linked to constant regions, are inserted into expression vectors. The light and heavy chains can be cloned in the same or different expression vectors. For example, the heavy and light chains of SEQ ID NOs: 1-5 can be used according to the present invention. The teachings of U.S. Patent No. 6,287,569 to Kipps et al., incorporated herein by reference in its entirety, and the methods provided herein can readily be adapted by those of skill in the art to create the vaccines of the present invention. The DNA segments encoding antibody chains are operably linked to control sequences in the expression vector(s) that ensure the expression of antibody chains. Such control sequences include a signal sequence, a promoter, an enhancer, and a transcription termination sequence. In one embodiment, the

[0079] Expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosome. E. coli is one procaryotic host particularly useful for expressing antibodies of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which typically contain expression control sequences compatible with the host cell (e.g., an origin of replication) and regulatory sequences such as a lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter

system, or a promoter system from phage lambda. Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired. Mammalian tissue cell culture can also be used to express and produce the antibodies of the present invention (see, e.g., Winnacker, From Genes to Clones VCH Publishers, N.Y., 1987). Eukaryotic cells are preferred, because a number of suitable host cell lines capable of secreting intact antibodies have been developed. Preferred suitable host cells for expressing nucleic acids encoding the immunoglobulins of the invention include: monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line; baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary-cells (CHO); mouse sertoli cells; monkey kidney cells (CV1 ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL 1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); and TRI cells.

[0080] The vectors containing the polynucleotide sequences of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell. Calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation can be used for other cellular hosts (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 2nd ed., 1989). When heavy and light chains are cloned on separate expression vectors, the vectors are co-transfected to obtain expression and assembly of intact immunoglobulins. After introduction of recombinant DNA, cell lines expressing immunoglobulin products are cell selected. Cell lines capable of stable expression are preferred (i.e., undiminished levels of expression after fifty passages of the cell line).

[0081] Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, e.g., Scopes, Protein Purification, Springer-Verlag, N.Y., 1982). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred.

[0082] Labeled Antibody

[0083] A labeled antibody or a detectably labeled antibody is generally an antibody (or antibody fragment which retains binding specificity), having an attached detectable label. The detectable label is normally attached by chemical conjugation, but where the label is a polypeptide, it could alternatively be attached by genetic engineering techniques. Methods for production of detectably labeled proteins are well known in the art. Detectable labels known in the art include radioisotopes, fluorophores, paramagnetic labels, enzymes (e.g., horseradish peroxidase), or other moieties or compounds which either emit a detectable signal (e.g., radioactivity, fluorescence, color) or emit a detectable signal after exposure of the label to its substrate. Various detectable label/substrate pairs (e.g., horseradish peroxidase/diaminobenzidine, avidin/streptavidin, luciferase/luciferin), methods for labeling antibodies, and methods for using labeled antibodies are well known in the art (see, for example, Harlow and Lane, eds., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, pyridoxal, and fluorescein, which can react with specific antihapten antibodies.

[0084] Diagnosis of Lymphoma

[0085] The ROR1 antibodies described herein can be used to differentiate between lymphoma cells and normal lymphocytes and, thus, can be used to detect and/or diagnose disease in subjects.

[0086] The methods for detecting such disease generally include contacting a sample from a subject having, or at risk of having, a lymphoma with a reagent that detects ROR1, and detecting the reaction of the reagent. Within these methods, detection of a reaction is indicative of the presence and/or quantity of ROR1 in the sample. The reaction of the reagent with the sample is then compared to a control. Any biological sample which may contain a detectable amount of ROR1 can be used. Examples of biological samples of use with the invention are blood, serum, plasma, urine, mucous, feces, cerebrospinal fluid, pleural fluid, ascites, and sputum samples. Tissue or cell samples can also be used with the subject invention. These samples can be obtained by many methods such as cellular aspiration, or by surgical removal of a biopsy sample. The level of ROR1 in the sample can be compared with the level in a sample not affected by the targeted disorder or condition. Control samples not affected by a targeted disease processes can be taken from the same subject, or can be from a normal control subject not affected by the disease process, or can be from a cell line.

[0087] Contacting the sample and anti-ROR1 antibody generally includes incubation under conditions which allow contact in solution and/or solid phase between the reagent and sample. Detection can be performed by any means suitable to identify the interaction of the reagent with ROR1. In one embodiment, when the reagent is an antibody, the antibody can be detectably labeled. Detectable labels are well known in the art, and include radioisotopes, fluorophores, paramagnetic labels, enzymes (e.g., horseradish peroxidase), or other moieties or compounds which either emit a detectable signal (e.g., radioactivity, fluorescence, color) or emit a detectable signal after exposure of the label to its substrate. Alternatively, when the reagent is an antibody, detection can be performed using a second antibody which is detectably labeled which recognizes the antibody that binds ROR1. The antibody may also be biotinylated, and a second avidinated label used to determine the presence of the biotinylated reagent which detects ROR1.

[0088] The antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. The antibodies employed in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can effectively employ antibodies of the invention are, competitive and non-competitive immunoassays, in either a direct or indirect format. Examples of such immunoassays include a radioimmunoassay (RIA), and a sandwich (immunometric) assay. Those of skill in the art will readily discern additional immunoassay formats useful within the invention.

[0089] Other immunoassays for use within the invention include "forward" assays for the detection of a protein in which a first anti-protein antibody (e.g., an anti-ROR1 antibody) bound to a solid phase support is contacted with the test sample. After a suitable incubation period, the solid phase support is washed to remove unbound protein. A second, distinct anti-protein antibody is then added which is specific for a portion of the specific protein not recognized by the first antibody. The second antibody is preferably detectable. After a second incubation period to permit the detectable antibody to complex with the specific protein bound to the solid phase support through the first antibody, the solid phase support is washed a second time to remove the unbound detectable antibody. Alternatively, the second antibody may not be detectable. In this case, a third detectable antibody, which binds the second antibody is added to the system. This type of "forward sandwich" assay may be a simple yes/no assay to determine whether binding has occurred or may be made quantitative by comparing the amount of detectable antibody with that obtained in a control.

[0090] Other types of immunometric assays are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step wherein the first antibody bound to the solid phase support, the second, detectable antibody and the test

sample are added at the same time. After the incubation is completed, the solid phase support is washed to remove unbound proteins. The presence of detectable antibody associated with the solid support is then determined as it would be in a conventional "forward sandwich" assay. The simultaneous assay may also be adapted in a similar manner for the detection of antibodies in a test sample. The "reverse" assay comprises the stepwise addition of a solution of detectable antibody to the test sample followed by an incubation period and the addition of antibody bound to a solid phase support after an additional incubation period. The solid phase support is washed in conventional fashion to remove unbound protein/antibody complexes and unreacted detectable antibody. The determination of detectable antibody associated with the solid phase support is then determined as in the "simultaneous" and "forward" assays. The reverse assay may also be adapted in a similar manner for the detection of antibodies in a test sample.

[0091] The antibody component of immunometric assays described herein may be added to a solid phase support capable of immobilizing proteins. By "solid phase support" or "support" is intended any material capable of binding proteins. Well-known solid phase supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses (including nitrocellulose sheets and filters), polyacrylamides, agaroses, and magnetite. The nature of the support can be either soluble to some extent or insoluble for the purposes of the present invention. The support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will know many other suitable "solid phase supports" for binding proteins or will be able to ascertain the same by use of routine experimentation. A preferred solid phase support is a 96-well microtiter plate. For immunoassay and immunodiagnostic purposes, the antibodies of the invention can be bound to many different carriers, both soluble and insoluble, and can be used to detect the presence of an antigen comprising ROR1 (or fragments, derivatives, conjugates, homologues, or variants thereof). Those skilled in the art will discern other suitable carriers for binding antibodies useful within the invention. In addition, there are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds, as described above.

[0092] In using the antibodies described herein for the in vitro or in vivo detection of ROR1, the detectably labeled antibody is provided in an amount which is diagnostically effective. Thus, an amount of detectably labeled antibody is contacted or administered in

sufficient quantity to enable detection of ROR1 in the subject sample to be assayed.

[0093] Within more detailed diagnostic methods of the invention, in vivo immunodiagnostic tools are provided, as exemplified by immunoscintigraphic methods and compositions. Immunoscintigraphy (IS) is discussed in detail in P. Lechner et al., Dis Colon Rectum 1993;36:930-935 and F. L. Moffet et al., J Clin Oncol 14:2295-2305 (1966). IS (or radioscintigraphy) employs radioactive-labeled antibody, typically Fab' fragments (Goldenberg et al.; Eur J Nucl Med 1989;15:426), to recognize defined epitopes of targeted proteins. Fab' fragments of the antibodies provided herein, comprising immunoglobulins of the IgGI fraction that have their Fc portions removed, are highly capable of targeting epitopes on proCPR, activated CPR, and/or inactivated CPR in a test sample or subject. Because these Fab' fragments have minimal antigenity, they cause neither human antimouse antibody response, nor any allergic reactions of unpredictable nature. The smaller molecular weight of Fab' fragments compared with intact antibody allows the fragment to leave the intravascular space and target a broader array of in vivo compartments for diagnostic purposes.

[0094] For radioscintigraphy, an anti-ROR1 radioactive monoclonal antibody is typically injected into a patient for identifying, measuring, and/or localizing ROR1 in the subject, (see, e.g., Delaloye et al., Seminars in Nuclear Medicine 25(2):144-164, 1995). In radioimaging with monoclonal antibodies, a chemically modified (chelate) form of the monoclonal antibody is typically prepared and stored as a relatively stable product. To be used clinically, however, the monoclonal antibody sample must be mixed with a radioactive metal, such as ^{99}Tc, then purified to remove excess, unbound radioactive metal, and then administered to a patient within 6 hours, (see, e.g., Eckelman et al., Nuc. Med. Biol. 16: 171-176, 1989). Radioisotopes, for example ^{99}Tc, an isotope with a short physical half-life and high photon abundance, can be administered at high doses and allow early imaging with a gamma camera. This is very suitable for use in conjunction with Fab' fragments, the half-lives of which are also short.

[0095] Monitoring of a Lymphoma and Lymphoma Therapy

[0096] Further, the anti-ROR1 antibodies described herein can be used in vitro and in vivo to monitor the appearance, status, course, or treatment of a lymphoma in a subject. For example, by measuring an increase or decrease in the amount of ROR1 in a subject (optionally in comparison to control levels in a normal subject or sample), the appearance, status, course, or treatment of the lymphoma or condition in the subject number can be observed or evaluated. Based on these and comparable diagnostic methods, it is further possible to determine whether a particular therapeutic regimen, such as a treatment regimen

employing antibodies of the invention directed against the lymphoma is effective. Methods of detecting and/or quantifying levels of ROR1 and corresponding lymphoma disease state are as described above.

[0097] Therapeutic Treatment of Lymphoma

[0098] ROR1 agonists can be employed as therapeutic or prophylactic pharmacological agents in any subject in which it is desirable to administer, in vitro, ex vivo, or in vivo the subject agonists that bind ROR1. Typical subjects for treatment or management according to the methods herein are subjects presenting with a lymphoma. The agonists described herein specifically recognize ROR1 protein, found in lymphoma samples but not expressed in cells of normal adults, and therefore can be used for detecting and/or neutralizing these biomolecules, and/or blocking their interactions with other biomolecules, in vitro or in vivo. Examples of such ROR1 agonists include antibodies, small molecule inhibitors, antisense RNA, and siRNA.

[0099] While under no obligation to provide a mechanism of action, it is thought that ROR1 can serve as a receptor for Wnt5a to trigger NF-kappa B pathway, which pathway is implicated in oncogenesis. See e.g. Example 12. Thus, ROR1 gene, which plays a role in disease pathogenesis and/or progression, encodes a protein that can be targeted by immune therapy for patients with lymphoma.

[0100] Antibodies

[0101] In certain therapeutic embodiments, the selected antibody will typically be an aniti-ROR1 antibody, which may be administered alone, or in combination with, or conjugated to, one or more combinatorial therapeutic agents. When the antibodies described herein are administered alone as therapeutic agents, they may exert a beneficial effect in the subject by a variety of mechanisms. In certain embodiments, monoclonal antibodies that specifically bind ROR1 are purified and administered to a patient to neutralize one or more forms of ROR1, to block one or more activities of ROR1, or to block or inhibit an interaction of one or more forms of ROR1 with another biomolecule.

[0102] The immunotherapeutic reagents of the invention may include humanized antibodies, and can be combined for therapeutic use with additional active or inert ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, and optionally with adjunctive or combinatorially active agents such as anti-inflammatory ant anti-fibrinolytic drugs.

[0103] In other embodiments, therapeutic antibodies described herein are coordinately administered with, co-formulated with, or coupled to (e.g., covalently bonded) a

combinatorial therapeutic agent, for example a radionuclide, a differentiation inducer, a drug, or a toxin. Various known radionuclides can be employed, including ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, and ^{211}At. Useful drugs for use in such combinatorial treatment formulations and methods include methotrexate, and pyrimidine and purine analogs. Suitable differentiation inducers include phorbol esters and butyric acid. Suitable toxins include ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein. These combinatorial therapeutic agents can be coupled to an anti-ROR1 antibody either directly or indirectly (e.g., via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (e.g., a halide) on the other. Alternatively, it may be desirable to couple a combinatorial therapeutic agent and an antibody via a linker group as a spacer to distance an antibody from the combinatorial therapeutic agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. It will be further evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, Ill.), may be employed as a linker group. Coupling may be affected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues.

[0104] Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates described herein, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (e.g., U.S. Pat. No. 4,489,710, to Spitler), by irradiation of a photolabile bond (e.g., U.S. Pat. No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (e.g., U.S. Pat. No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (e.g., U.S. Pat. No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (e.g., U.S. Pat. No. 4,569,789, to Blattler et al.) It may also be desirable to couple more than one agent to an anti-ROR1 antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers which provide multiple sites for

attachment can be used. Alternatively, a carrier can be used.

[0105] A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration is intravenous, intramuscular, or subcutaneous.

[0106] It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon such factors as the antibody used, the antigen density, and the rate of clearance of the antibody. A safe and effective amount of an anti-ROR1 agent is, for example, that amount that would cause the desired therapeutic effect in a patient while minimizing undesired side effects. Generally, a therapeutically effective amount is that sufficient to promote production of one or more cytokines and/or to cause complement-mediated or antibody-dependent cellular cytotoxicity. The dosage regimen will be determined by skilled clinicians, based on factors such as the exact nature of the condition being treated, the severity of the condition, the age and general physical condition of the patient, and so on.

[0107] siRNA

[0108] In certain therapeutic embodiments, the ROR1 agonist is siRNA. The levels of ROR1 can be down-regulated by RNA interference by administering to the patient a therapeutically effective amount of small interfering RNAs (siRNA) specific for ROR1. siRNA specific for ROR1 can be produced commercially from a variety of sources, such as Ambion (Austin, TX. The siRNA can be administered to the subject by any means suitable for delivering the siRNA to the blood. For example, the siRNA can be administered by gene gun, electroporation, or by other suitable parenteral or enteral administration routes, such as intravitreous injection.

[0109] RNA interference is the process by which double stranded RNA (dsRNA) specifically suppresses the expression of a gene bearing its complementary sequence. Suppression of the ROR1 gene inhibits the production of the ROR1 protein. Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. First, the dsRNAs get processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation step). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effecter step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand. Preferably, the siRNA comprises short double-stranded RNA from about 17 nucleotides to about 29 nucleotides in length,

preferably from about 19 to about 25 nucleotides in length, that are targeted to the target mRNA.

[0110] As an example, an effective amount of the siRNA can be an amount sufficient to cause RNAi-mediated degradation of the target ROR1 mRNA, or an amount sufficient to inhibit the progression of a lymphoma in a subject. One skilled in the art can readily determine an effective amount of the siRNA of the invention to be administered to a given subject by taking into account factors such as the size and weight of the subject; the extent of the neovascularization or disease penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of siRNA comprises an intercellular concentration of from about 1 nanomolar (nM) to about 100 nM, preferably from about 2 nM to about 50 nM, more preferably from about 2.5 nM to about 10 nM. It is contemplated that greater or lesser amounts of siRNA can be administered.

[0111] The siRNA can be targeted to any stretch of approximately 19-25 contiguous nucleotides in any of the ROR1 mRNA target sequences. Target sequences can be selected from, for example, the sequence of ROR1, Genebank accession number: NM_005012. Searches of the human genome database (BLAST) can be carried out to ensure that selected siRNA sequence will not target other gene transcripts. Techniques for selecting target sequences for siRNA are given, for example, in Elbashir et al. ((2001) Nature 411, 494–498). Thus, the sense strand of the present siRNA comprises a nucleotide sequence identical to any contiguous stretch of about 19 to about 25 nucleotides in the target mRNA of ROR1. Generally, a target sequence on the target mRNA can be selected from a given cDNA sequence corresponding to the target mRNA, preferably beginning 50 to 100 nt downstream (i.e., in the 3' direction) from the start codon. The target sequence can, however, be located in the 5' or 3' untranslated regions, or in the region nearby the start codon.

[0112] Antisense

[0113] In certain therapeutic embodiments, the ROR1 agonist is an antisense oligonucelotide. The levels of ROR1 can be down-regulated by administering to the patient a therapeutically effective amount of an antisense oligonucleotide specific for ROR1 mRNA. The antisense oligonucleotide specific for ROR1 mRNA may span the region adjacent to the initiation site of ROR1 translation.

[0114] An effective amount of the antisense oligonucleotide specific for ROR1 mRNA as isolated in a purified form may is generally that amount capable of inhibiting the production of ROR1 or reducing the amount produced or the rate of production of ROR1

such that a reduction in symptoms of lymphoma occurs. Antisense oligonucleotides can be administered via intravitreous injection at a concentration of about 10 μg/day to about 3 mg/day. For example, administered dosage can be about 30 μg/day to about 300 μg/day. As another example, IL-10 antisense oligonucleotide can be administered at about 100 μg/day. Administration of antisense oligonucleotides can occur as a single event or over a time course of treatment. For example, IL-10 antisense oligonucleotides can be injected daily, weekly, bi-weekly, or monthly. Time course of treatment can be from about a week to about a year or more. In one example, IL-10 antisense oligonucleotides are injected daily for one month. In another example, antisense oligonucleotides are injected weekly for about 10 weeks. In a further example, IL-10 antisense oligonucleotides are injected every 6 weeks for 48 weeks.

[0115] Vaccines

[0116] As will be clear from the description herein of anti-ROR1 antibody, the present invention also provides for use of ROR1 in vaccines against diseases, such as a lymphoma, e.g., CLL, that involve the expression of ROR1. Because normal adult tissues do not appear to express ROR1, it represents a tumor-specific antigen that can be targeted in active immune therapy. For example, the levels of ROR1 can be down-regulated by administering to the patient a therapeutically effective amount of an ROR1 polynucleotide or polypeptide that produces in animals a protective or therapeutic immune response against ROR1 and the effects of its expression. The vaccines can include polynucleotides or polypeptides. Methods of using such polynucleotides and/or polypeptides include use in vaccines and for generating antibodies against the polypeptides, such as those expressed by the polynucleotides. The polynucleotides can be an *ror1* gene, or a variant or fragment thereof. The polypeptides can be an ROR1 protein, or a variant or fragment thereof. In certain aspects, the *ror1* polynucleotide fragment can be a fragment comprising a fragment of the *ror1* gene. Such polynucleotide fragments can be comprised by a vector. A cell can be transformed and/or transfected by such polynucleotides and vectors and in certain aspects, the polynucleotides and vectors can express polypeptides of the invention. Typically the vaccine composition includes a pharmaceutically acceptable carrier or diluent. The teachings of U.S. Patent No. 6,287,569 to Kipps et al., incorporated herein by reference in its entirety, can readily be adapted by those of skill in the art to create the vaccines of the present invention.

[0117] When describing a vaccine, a "polynucleotide variant" refers to any degenerate nucleotide sequence. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference

polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. For example, a variant polynucleotide consisting of 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, and 99% to the polynucleotide consisting of ibpA. A "polynucleotide fragment" of a *ibpA* polynucleotide is a portion of a *ibpA* polynucleotide that is less than full-length and comprises at least a minimum length capable of hybridizing specifically with a native *ibpA* polynucleotide under stringent hybridization conditions. The length of such a fragment is preferably at least 15 nucleotides, more preferably at least 20 nucleotides, and most preferably at least 30 nucleotides of a native *ibpA* polynucleotide sequence. A "polypeptide variant" refers to a polypeptide of differs in amino acid sequence from the ibpA polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. Finally, a "polypeptide fragment" refers to any polypeptide of a portion of a ibpA polypeptide that is less than full-length (*e.g.*, a polypeptide consisting of 5, 10, 15, 20, 30, 40, 50, 75, 100 or more amino acids of a native ibpA protein), and preferably retains at least one functional activity of a native ibpA protein.

[00100] DNA vaccines for ROR1

[00101] The constructs will be used for development of a DNA vaccine. The constructs encode the tumor specific antigen ROR1. Two different construct were developed. They differ in one amino acid -- one construct contains methionine M and the other and arginine R. The latter construct is expected to cause rapid degradation of the protein and thus a more predominant cellular immune response. Both constructs contain a sequence from the ubiquitine gene (SEQ ID NO: 6), followed by methionine or arginine sequence, followed by a LacI sequence (SEQ ID NO 7), and finally followed by the ROR1 cDNA sequence (SEQ ID NO: 8).

[0118] Many embodiments of the invention are provided through well known protocols established in the art. For example, the following references provide multiple protocols which may be adapted for use with anti-ROR1 antibody: Vernon, S.K., Lawrence, W.C., Long, C.A., Cohen, G.H., and Rubin, B.A. Herpesvirus vaccine development: Studies of virus morphological components. In New Trends and Developments in Vaccines, ed. by A. Voller and H. Friedman. Chapter 13, pp. 179-210. MTP Press, Ltd., Lancaster (1978); Sambrook et al. (1989) Molecular Cloning--A Laboratory Manual (2nd ed.) Vol. 1-3, Cold

Spring Harbor Laboratory, Cold Spring Harbor Press, N.Y., ("Sambrook"); and Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (e.g., current through 1999, e.g., at least through supplement 37) ("Ausubel")), each of which are incorporated herein by reference in its entirety. With respect to vaccine technologies, U.S. Patent Application Nos. 20040253240 and 20030124141, are incorporated herein by reference in their entirety. These references also provide one of skill in the art instructions how to make and use the polynucleotides and polypeptides of the present invention for active and passive vaccines. Those of skill in the art will readily recognize how to adapt the disclosures of these references to the present polynucleotides and polypeptides of the present invention.

[0119] Kit

[0120] In carrying out various assay, diagnostic, and therapeutic methods of the invention, it is desirable to prepare in advance kits comprises a combination of an anti-ROR1 antibody described herein with other materials. For example, in the case of sandwich enzyme immunoassays, kits of the invention may contain a monoclonal antibody that specifically binds ROR1 optionally linked to an appropriate carrier, a freeze-dried preparation or a solution of an enzyme-labeled monoclonal antibody which can bind to the same antigen together with the monoclonal antibody or of a polyclonal antibody labeled with the enzyme in the same manner, a standard solution of purified ROR1, a buffer solution, a washing solution, pipettes, a reaction container and the like. In addition, the kits optionally include labeling and/or instructional materials providing directions (*i.e.*, protocols) for the practice of the methods described herein. While the instructional materials typically comprise written or printed materials, they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

[0121] Discussion

[0122] While a B cell itself expresses MHC class I and II molecules on its surface, a lymphoma cell, including a B-CLL cell, usually has low potential to activate immune system behaving as stealth APC. The signal from CD40 by means of CD154 induces the increase of expression of co-stimulatory molecules and the cellular immunity against CLL cells *in vitro*. Katoet al., J Clin Invest 101, 1133-41 (1998). Autologous CLL cells transduced Ad-

CD154 also induce the cytotoxic activity of isolated T cells against CLL cells *in vivo*. Wierda et al., Blood 96, 2917-24 (2000). Th1 dominant T cell immunity have also observed (unpublished data). In addition, humoral immunity is induced against CLL by Ad-CD154 therapy. Although viral infection can induce autoantibody response (Ludewig et al., J Exp Med 200, 637-46 (2004)) induction of IgM rheumatoid factor could be detected in some cases (Figure 6c), the anti-ROR1 antibody of the present invention does not bind non-specifically as an autoantibody because anti-RBC antibody or symptoms associated autoantibody could not be detected (data not shown). The difference between the response of anti-adenovirus and anti-tetanus toxin indicates that this therapy induced the immune response more strongly against infused CLL cells transduced with CD154 than non-specific immune-activation. Because IgG reaction of anti-adenovirus were increased with repeated infusion in compared with IgM reaction (Figure 1c), this Ad-CD154 therapy may induce the humoral immunity from the pool of naïve B cells rather than memory B cell populations.

[0123] In at least six (6) patients, anti-ROR1 antibody could not be detected before Ad-CD154 therapy. ROR1 was not identified by SEREX using sera from untreated CLL patients. Krackhardt et al., Blood 100, 2123-31 (2002). Because it is likely that all secondary lymphatic organs in patients contain an abundant number of lymphoma cells, the normal B cells bearing anti-ROR Ig might be eliminated or be forced to be anergic by surrounding ROR1 molecules without co-stimulatory signals. It is also likely that a certain amount of anti-ROR1 antibody was trapped by ROR1 protein on lymphoma cell surfaces as implied by indirect Coombs tests which often show negative in patients with autoimmune hemolytic anemia.

[0124] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

[0125] Examples

[0126] The following non-limiting examples are provided to further illustrate the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and

scope of the invention.

[0127] Example 1: Demonstration of Production of Anti-Adenovirus Antibody

[0128] Chronic lymphocytic leukemia (CLL) CLL cells were transduced with replication-defective adenovirus encoding CD154 (Ad-CD154). The seven patients of the study all had progressive intermediate or high-risk CLL by the modified Rai criteria. All patients had performance status of 0 to 2, life expectancy of more than 3 months, and normal renal, hepatic, and pulmonary function on study entry. Ad-CD154 were prepared and transduced into CLL cells as described in Wierda et al. (2000) Blood 96, 2917-24; and Cantwell et al. (1996) Blood 88, 4676-83. 3-6 x 108 transduced CLL cells were injected biweekly 5 times in 6 patients except for one patient. Sera, collected sequentially during the studies from these 6 patients, was examined. Before treatment 4 out of 6 patients had hypogammaglobulinemia and residual 2 patients also have relatively low titers of immunoglobulins. After completion of therapy total IgG and IgM were slightly increased. (IgG; 656 ± 297 to 940 ± 487 p=0.04, IgA; 72 ± 63 to 69 ± 61 p=0.4, IgM; 38 ± 21 to 74 ± 48 p=0.07) (Figure 1a).

[0129] The antibody response was measured against the recombinant adenovirus used to transduce the CLL cells. Five of six patients had a vigorous polyclonal antibody response to adenovirus antigens following treatment (Figure 1b). This response initially involved antibodies of the IgM class, and then subsequently antibodies of the IgG and IgA classes, but not IgE (Figure 1c and not shown). On average, 50-fold, 60-fold, or nearly 1,000-fold increases in the titers of IgM, IgA, or IgG anti-adenovirus antibodies were observed, respectively. The IgG response involved antibodies of IgG1 and IgG3 isotypes (Figure 1c), which primarily are observed in Th1-type immune responses. Moreover, no significant increases were observed in anti-adenovirus antibodies of the IgG4 isotype (data not shown), which typically are observed in Th2-type immune responses. In compared with this vigorous response, the increases in the titers of anti-tetanus toxin antibodies were not obvious unless patients received subsequent booster immunizations with tetanus toxoid. In addition, development of autoantibodies to red cells, platelets, or the human CD154 molecule following treatment was not observed (data not shown).

[0130] Example 2: Flow cytometry Analysis of Anti-CLL activities

[0131] Anti-CLL activities were determined by flow cytometry. Peripheral blood mononuclear cells (PBMC) from IgG negative CLL case or healthy donor were incubated with one-fifth diluted serum from the patient or healthy donor, and bound IgG was detected by mouse anti-human IgG antibody (Pharmingn). B cells (CD19+CD3-) were gated using

anti-CD19 antibodies conjugated APC and anti-CD3 antibody conjugated with FITC.

[0132] Example 3: ROR1 Anti-Sera Production

[0133] Anti-ROR1 mouse sera by means of DNA vaccination with ROR1 expression vector. Eight-week old Balb/c female mice were injected intradermally with 100μg of ROR1cDNA (Origene) with 50μg of GM-CSF and CD154 expression vector as adjuvants. After 3 courses of injection, sera was collected from the mice. Chinese hamster ovary cell (CHO) with or without transfection with ROR1 cDNA cloned into pcDNA3 vector by lipofectamine 2000 (Invitrogen) was used to determine the titer of anti-ROR1 antibody in serum. Bound antibody from immunized mice was detected by flow cytometry using anti-mouse antibody with fluorescence (Pharmingen). To distinguish the untransfected CHO from transfectants in the mixture, it was stained with PKH26 (Sigma) according to the manufacture's protocol. Anti-ROR1 activity was determined by incubating CHO transfectants and serum from patient followed by detection with anti-human Ig labeled with fluorescence (Southern Biotech).

[0134] Example 4: ELISA

[0135] To produce recombinant ROR1 protein, its extracellular region was cloned into the pcDNA3-zeocin vector encoding rabbit IgG Fc region in frame. Stable CHO transfectant (CHO-ROR1rIg) was made with this vector, and was adapted to suspension culture using IMGX II medium (HyClone). Suspended CHO-ROR1rIg was cultured in ProCHO-5 medium, and recombinant ROR1rIg was purified using protein A sepharose (Pierce).

[0136] 5 μg/ml protein or 10^8/ml adenovirus was absorbed 96 well plate overnight at 4°C. After washing and blocking with 2% BSA/PBS, serum dilutions were added and incubated for 1 hour at room temperature. Goat anti-human Ig, IgG, IgA, IgM, IgG1, IgG2, IgG3 and IgG4 conjugated with horseradish peroxidase (HRP) or alkaline phosphatase (AP) (Southern Biotechnology, Birmingham, AL) were used as secondary antibody. TMB (KPL, Gaithersburg, MD) or pNPP (Sigma) was used for substrate for HRP and AP respectively. All experiments were done duplicate and were shown the average.

[0137] Example 5:Analysis of microarray data

[0138] Gene expression profiles of normal human tissues were obtained from the data series GSE803 of Gene Expression Omnibus (GEO) database. The gene set of CLL signature genes were made according to the published papers Klein et al. (2001) J Exp Med 194, 1625-38; Rosenwald et al. (2001) J Exp Med 194, 1639-47. The data were clustered

and visualized with GeneSpring software (Silicon Genetics).

[0139] Example 6: Immunoblotting

[0140] Total cell lysates were made by incubation cells in a lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM glycerophosphate, 1 mM sodium orthovanadate, with complete protease inhibitor mix (Roche). Cell lysates were separated 7.5% or 5-15% gradient SDS-PAGE and blotted on Immobilon-P membrane (Millipore). For immunoblot, rabbit (Cell signaling) or goat (R&D) anti-ROR1 antibodies were used followed with anti-rabbit or anti-goat antibodies conjugated with HRP (Santa Cruz). Conditioned medium of culture with CHO with or without transfection with HA-tagged Wnt5a cDNA (Upstate) was incubated with 1μg of ROR1rIg or rabbit IgG followed by immunoprecipitation with anti-HA matrix (Roche) or protein A/G agarose (SantaCruz). Bound proteins were immunoblotted with anti-HA (Roche) or anti-rabbit Ig antibody.

[0141] Example 7: Reporter assay

[0142] Reporter assay was performed as described in Lu et al. (2004) Proc Natl Acad Sci U S A 101, 3118-23. Briefly, HEK293 cells were transfected in 12-well plates by using FuGENE (Roche, Mannheim, Germany), and 0.5 μg of reporter plasmid, 0.1–0.2 μg of the control plasmid pCMXβ-gal, 100–200 ng of the various expression plasmids, and carrier DNA pBluescriptKSII, for a total of 1 μg per well. The luciferase values were normalized for variations in transfection efficiency by using the β-galactosidase internal control, and are expressed as fold stimulation of luciferase activity, compared with the designated control cultures. All of the transfection results are representative of a minimum of three independent transfections.

[0143] Example 8: Induction of Humoral Immunity Against CLL Cell

[0144] The production of anti-adenovirus antibody supports the induction of humoral immunity against the CLL cell itself. Allogeneic CLL cells were incubated with serum from patient before and after treatment. Antibody binding was checked by flow cytometry.

[0145] Results showed that the sera from 3 patients after Ad-CD154 therapy had the reactivity against CLL B cells compared with the sera before therapy (Figure 2a). The shift of the histograms were reproducible with another 3 CLL B cells, and it was not detectable against B cells from healthy donors (Figure 2b). This data suggests a TAA(s) may exist on the surface of CLL cells in a hidden fashion from surveillance of immunity, becoming immunogenic after CLL received the immune-costimulatory molecules.

[0146] The microarray analyses of CLL samples identified the relatively small number of genes that are differentially expressed in CLL cells in compared with normal B cell subsets and another types of B cell malignancies. Klein et al. (2001) J Exp Med 194, 1625-38; Rosenwald et al. (2001) J Exp Med 194, 1639-47. These CLL signature genes are candidates for TAAs of CLL. The expressions of these genes were examined in normal human tissues because where there is an abundant expression in normal tissue, antibody production against such a gene cannot occurr *in vivo*. The expression profiles of CLL signature genes in normal adult tissues wad determined (data not shown). Genes that had low expressions in all tissues were spotlighted. Attention was directed to receptor tyrosine kinase ROR1 gene, because it is a probable cell surface molecule and is expressed mainly in developing cells. Yoda et al. (2003) J Recept Signal Transduct Res 23, 1-15; Al-Shawi et al. (2001) Dev Genes Evol 211, 161-71; Matsuda et al.(2001) Mech Dev 105, 153-6 (2001).

[0147] Example 9: Immunoblot

[0148] Fig. 3 depicts an immunoblot demonstrating that the anti-Ror1 mAb (designated 4A5) can immune precipitate the Ror1 protein from cells made to express human Ror1 (e.g. Chinese Hamster Ovary (CHO)) cells or chronic lymphocytic leukemia (CLL) cells. Prior antibodies to Ror1 were not mAbs, were generated against peptides to Ror1, are of low affinity, and cannot immune precipitate the Ror1 protein. As such, the 4A5 mAb can be used to detect and/or isolate the Ror1 protein, which could have diagnostic, treatment, and/or investigative value.

[0149] Example 10: ROR1 Expression in CLL B Cells

[0150] To confirm the ROR1 protein expression in CLL B cells, immunoblot analysis, as described above, using anti-ROR1 antibody was performed. Results showed that the bands at the level of 128 kD were detected in peripheral blood or splenocytes from CLL patients (Figure 4a). The size is compatible with the reported murine ROR1 and bigger than deduced size of 101kD from amino acid sequence without putative leader sequence probably due to the glycosylation29. This band could be detected neither in samples of peripheral blood from healthy donor nor splenocytes from idiopathic thrombocytopenia purpura patient. ROR1 protein was detectable also in some Burkitt's B cell lines at the same molecular weight (Figure 4b).

[0151] Example 11: Cell Surface Localization of ROR1 Protein in CLL B Cells

[0152] Cell surface localization of ROR1 protein in CLL B cells was confirmed via flow cytometry. Anti-ROR1 mouse sera, produced by means of DNA vaccination with ROR1

expression vector, as described above, was reacted with CHO transfected with ROR1 (CHO-ROR1) but not with CHO parental cell (Figure 4c). Using this anti-serum, ROR1 expression was detected on cell surface of all CLL samples examined (n=8) but not on PBMC from healthy donors (n=3) (Figure 4d).

[0153] Example 12: Induction of Anti-ROR1 Antibody by Ad-CD154 Therapy

[0154] To confirm that the antibody against ROR1 is included in the antibodies against CLL cells induced by Ad-CD154 therapy, the sera from patients was reacted with CHO and CHO-ROR1 shown as Figure 4c. Results showed that although serum from healthy donor or patient before treatment contained same reactivity against CHO and CHO-ROR1, sera from patient after Ad-CD154 therapy contained more Ig reacted with CHO-ROR1 than with CHO (Figure 5a and b).

[0155] Further verification of the induction of anti-ROR1 antibody by Ad-CD154 therapy was established with an ELISA assay using the recombinant extracellular domain of ROR1 fused with rabbit IgG Fc (Figure 6a). Results showed that anti-ROR1 antibody was clearly identified in 4 patients (#2,5,6,7) after Ad-CD154 therapy. The remaining one patient (#3) also had a weak anti-ROR1 reaction although one patient (#4) did not get anti-ROR1 antibody by this therapy. This #4 patient was profound hypogammaglobulinemia and was totally unresponsive to this therapy with no decrease of white blood cell count (data not shown). Thus, all responsive patient to Ad-CD154 had induction of anti-ROR1 antibody after completion of therapy. In these patients, anti-ROR1 antibody was not obvious before Ad-CD154 therapy. Although three patients (#5,6,7) had some reactivity also against rabbit IgG, this reactivity was also detected before therapy (Figure 6c). Collectively ROR1 was expressed on CLL B cells restrictedly and could induce humoral immunity by means of immune-gene therapy.

[0156] Example 13: ROR1 Activation of Intracellular Machinery Associated with Development and Progression of CLL

[0157] To demonstrate that ROR1 can activate intracellular machinery associated with development or progression of CLL, the influence of exogenous ROR1 expression on the reporter gene regulating various transcription factors in HEK293 cells was examined. Various Wnt family members were co-transfected, as ROR1 has a cystein-rich domain, which is shared between frizzled receptors and can bind with Wnt family members.

[0158] Results showed that the expression of ROR1 with any Wnt factor did not activate T-cell transcription factor (TCF) (Figure 7a, data not shown), suggesting that ROR1 does not signal via the canonical Wnt-signaling pathway. ROR1 could not activate nuclear

factors of activated T cells (NFAT), or AP-1 dependent gene expression (Figure 7a). However, it was observed that co-expression of ROR1 in HEK293 cells with Wnt5a, but not with any other Wnt factor, induced activation of NF-κB (Figure 7b). Induction of NF-κB was dose dependent on expression of ROR1 and Wnt5a, but independent of expression of LPR5/6 that ordinarily serve as co-receptors for the frizzled family of Wnt receptors (data not shown). Recombinant extracellular region of ROR1 could bind with Wnt5a *in vitro* (Figure 7c). This data suggests non-canonical Wnt member, Wnt5a may be the ligand of ROR1 and induce the activation signaling in cells.

[0159] Example 14: Lymphoma Cell Isolation and Purification

[0160] Staining of CLL cells from patients #1, 2, or 3 with 4A5 mAb

[0161] As depicted in Fig. 8, The number of the CLL patient is indicated at the left-hand margin. Each panel depicts the staining of CLL with Alexa-647-conjugated 4A5 mAb (blue histogram) versus an Alexa-647-conjugated isotype control mAb (red histograms). In the first column is the staining of total peripheral blood mononuclear cells, in the middle column is the staining of the CD19+ (total B cells), and in the far right column is the staining of cells that express both CD19 and CD5 (CLL cells), as indicated at the bottom of each column.

[0162] Staining of cells from normal donors #1, 2, or 3 with 4A5 mAb

[0163] As depicted in Fig. 9, The number of the normal donor (NORM) is indicated at the left-hand margin. Each panel depicts the staining of cells with Alexa-647-conjugated 4A5 mAb (blue histogram) versus an Alexa-647-conjugated isotype control mAb (red histograms). In the first column is the staining of total peripheral blood mononuclear cells, in the middle column is the staining of the CD19+ (total B cells), and in the far right column is the staining of cells that express both CD19 and CD5, as indicated at the bottom of each column.

[0164] Staining of cells from an exceptional normal donors

[0165] As depicted in Fig. 10, Recent studies indicate that close to 4% of adults over the age of 40 might have low numbers of cells similar to CLL cells in the peripheral blood. Moreover, over 11% of normal donors who have first degree relatives with CLL might have such cells in the peripheral blood. In this slide, we see that anti-Ror1 mAb 4A5 can detect an occasional normal donor with Ror1 positive cells. Each panel depicts the staining of cells with Alexa-647-conjugated 4A5 mAb (blue histogram) versus an Alexa-647-conjugated isotype control mAb (red histograms). In the first column is the staining of total

peripheral blood mononuclear cells, in the middle column is the staining of the CD19+ (total B cells), and in the far right column is the staining of cells that express both CD19 and CD5, as indicated at the bottom of each column. As noted from this figure, the Ror1 positive cells co-express CD5 and CD19, a phenotype common with CLL cells.

[0166] Staining of CLL cells in the marrow

[0167] As depicted in Fig. 11, The number of the CLL patient is indicated at the left-hand margin. Each panel depicts the staining of cells with Alexa-647-conjugated 4A5 mAb (blue histogram) versus an Alexa-647-conjugated isotype control mAb (red histograms). In the first column is the staining of total marrow mononuclear cells, in the middle column is the staining of the CD19+ (total B cells), and in the far right column is the staining of cells that express both CD19 and CD5 (CLL cells), as indicated at the bottom of each column.

[0168] Staining of CLL cells in the marrow

[0169] As depicted in Fig. 12, The proportion of cells that express Ror1, as detected by the mAb 4A5. Each dot represents the proportion of cells from a single donor. The percent of cells scoring positive is indicated by the y-axis. The left hand panel provides the percent lymphocytes (as per light scatter) that stain with 4A5 mAb. The right panel provides the percent of CD5+CD19+ B cells that stain with 4A5. The left panel provides the percent of lymphocytes that stain with 4A5 in samples obtained from the blood normal donors (far left), the marrow of patients with CLL (middle), or blood of patients with CLL (far right).

[0170] Example 15: Magnetic Bead Detection and Isolation of Lymphoma Cells

[0171] Lymphoma cells can be isolated and purified using the following procecure:

1. Stain CLL cells with PKH67
2. Titrate CLL cells in normal PBMCs (10% to 0.1%)
3. Stain cells with:

a.Iso-Alexa647 , CD5,CD19

b.4A5-Alexa647, CD5,CD19

4. Incubate for 20 min on ice followed by a wash 2X with PBS-0.5%BSA
5. Add magnetic beads(Miltenyi)to cells; Incubate 15 min on ice; Wash 1X with PBS-0.5%BSA)
6. Add column to magnet; Wash 1X with 3ml PBS-0.5%BSA
7. Add this mixture to pre washed column; Wash unbound cells 3X with 3ml

PBS-0.5%BSA; (unbound fraction=4A5 NEG)

8. Remove column from magnet; Add 5ml PBS-0.5%BSA; (bound fraction=4A5 POS)

[0172] Detection of CLL cells admixed with normal lymphocytes

[0173] As depicted in Fig. 13, Detection of 4A5+ CLL cells admixed with the lymphocytes from normal donors. CLL cells were first stained with PKH67, which labeled them bright green (as observed on the x axis), allowing for their detection after being admixed with normal lymphocytes. The stained CLL cells were mixed with the lymphocytes of a normal donor and then the mixture was stained with an Alexa-647-conjugated isotype control mAb (ISO) Alexa-647-conjugated 4A5, allowing for detection of the red fluorescence seen on the y-axis. Each panel represents a different mixture of cells stained with either the isotype control mAb or 4A5, as indicated in the key, which refers to the number in each panel of the figure. Those samples stained with the isotype control mAb are indicated by the term "Iso", those samples stained with 4A5 are indicated. The percent preceding the CLL is the percent at which the CLL cells are represented in the mixture. As seen from this figure, the 4A5 mAb does not stain normal lymphocytes, allowing for detection of minute proportions of CLL cells that are labeled green.

[0174] Isolation of CLL cells admixed with normal lymphocytes

[0175] As depicted in Fig. 14, Isolation of 4A5+ CLL cells admixed with the lymphocytes from normal donors. CLL cells were stained, mixed with normal lymphocytes at various ratios, and then stained with fluorochrome-conjugated 4A5 mAb, as in Slide #6. Each panel represents analyses of cells isolated from different mixtures of CLL cells with normal lymphocytes, as indicated in the key, which refers to the number in each panel of the figure. The percent preceding the CLL is the percent at which the CLL cells are represented in the mixture. As seen from this figure, the 4A5 mAb does not stain normal lymphocytes, allowing for detection of minute proportions of CLL cells that are labeled green. As can be seen in these panels, this method can isolate fairly pure populations of CLL cells from mixtures of CLL cells with normal lymphocytes in which the CLL cells constitute only a small fraction of the total cells.

CLAIMS

What is claimed is:

1. A composition comprising a purified, isolated antibody directed against ROR1, wherein the antibody binds ROR1 with moderate to high affinity.

2. A composition according to claim 1, wherein the antibody is an anti-ROR1 polyclonal antibody, a monoclonal antibody, or a functional antibody fragment.

3. A composition according to claim 2, wherein the antibody comprises a sequence expressed by a heavy chain sequence and at least one light chain sequence of SEQ ID NOs: 1-5.

4. A composition according to claim 2, wherein the antibody is a polyclonal antibody.

5. A composition according to claim 2, wherein the antibody is a monoclonal antibody.

6. A composition according to claim 2, wherein the antibody is a functional antibody fragment.

7. A composition according to claim 1, wherein the antibody is selected from the group consisting of whole antibody, humanized antibody, chimeric antibody, Fab fragment, Fab' fragment, $F(ab')_2$ fragment, single chain Fv fragment and diabody.

8. A composition according to claim 1, wherein the antibody has an affinity to binding ROR1 with a dissociation constant of below a Kd value selected from the group consisting of 10^{-6} mol/l, 10^{-7} mol/l, and 10^{-8} mol/l.

9. A composition according to claim 1, wherein the antibody is a detectably labeled antibody.

10. A composition according to claim 1, further comprising a pharmaceutically acceptable agent.

11. A method for detecting an amount of ROR1 in a subject sample, the method comprising:

(a) contacting the subject sample with the anti-ROR1 antibody of claim 1; and

(b) detecting immunoreactivity between the anti-ROR1 antibody and ROR1 in the sample.

12. A method according to claim 11, wherein the antibody is covalently attached to a detectable label.

13. A method according to claim 11, wherein the immunoreactivity detection is provided by immunoperoxidase staining, immunofluorescence, immunoelectronmicroscopy, or ELISA.

14. A method according to claim 11, further comprising

(c) correlating binding of the antibody to a standardized antibody binding profile, wherein such correlation provides quantitative value for total CPR in the sample.

15. A method for detecting a lymphoma, the method comprising:

detecting the presence or quantity of ROR1 protein in a subject sample.

16. A method according to claim 15, wherein the lymphoma is selected from the group consisting of CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, and Burkett's Lymphoma.

17. A method according to claim 15, wherein detection of ROR1 comprises the method of claim 10.

18. A method for treating a lymphoma in a subject, the method comprising:

administering to the subject in need thereof a therapeutically effective amount of an ROR1 receptor agonist.

19. A method according to claim 18, wherein the lymphoma is selected from the group consisting of CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, and Burkett's Lymphoma.

20. A method according to claim 18, wherein the ROR1 receptor agonist is the anti-ROR1 antibody of claim 1.

21. A method according to claim 20, wherein the antibody is administered in an amount of (i) about 0.05 mg to about 2.5 mg; (ii) about 0.1 mg to about 1 mg; or (iii) about 0.3 mg to about 0.5 mg.

22. A method according to claim 20, wherein the anti-ROR1 antibody is a polyclonal antibody, a monoclonal antibody, or a functional antibody fragment.

23. A method according to claim 20, wherein the anti-ROR1 antibody is selected from the group consisting of whole antibody, humanized antibody, chimeric antibody, Fab fragment, Fab' fragment, $F(ab')_2$ fragment, single chain Fv fragment and diabody.

24. A method according to claim 18, wherein the ROR1 receptor agonist is an antisense inhibitor of ROR1.

25. A method according to claim 24, wherein the ROR1 antisense inhibitor is administered in an amount of (i) about 10 μg/day to about 3 mg/day; (ii) about 30 μg/day to about 300 μg/day; or (iii) about 100 μg/day.

26. A method according to claim 18, wherein the ROR1 receptor agonist is administered by injection, inhalation, orally, liposome, or retroviral vector.

27. A diagnostic method for evaluating the appearance, status, course, or treatment of lymphocytic leukemia in a subject, the method comprising:

(a) contacting a subject sample with the anti-ROR1 antibody of claim 1; and

(b) detecting immunoreactivity between the anti-ROR1 antibody and ROR1 to determine presence or quantity of ROR1 in the sample.

28. A method according to claim 27, wherein the antibody specifically binds to ROR1.

29. A method according to claim 27, wherein a diagnostic criterion or value is determined based on an increase or decrease in an amount of ROR1 in the subject compared to a control level(s) of ROR1 in a normal subject or sample.

30. A method according to claim 27, wherein immunoreactivity detection is provided by immunoperoxidase staining, immunofluorescence, immunoelectronmicroscopy, or ELISA.

31. A method according to claim 27, wherein the antibody is an anti-ROR1 polyclonal antibody, a monoclonal antibody, or a functional antibody fragment.

32. A method according to claim 31, wherein the antibody is selected from the group consisting of whole antibody, humanized antibody, chimeric antibody, Fab fragment, Fab' fragment, $F(ab')_2$ fragment, single chain Fv fragment and diabody.

33. A kit to detect the presence of ROR1 protein comprising the antibody of claim 1.

34. A vaccine composition comprising a polynucleotide encoding ROR1 protein or a fragment or variant thereof, and a pharmaceutically acceptable carrier or diluent.

35. A vaccine composition comprising ROR1 protein or a fragment or variant thereof, and a pharmaceutically acceptable carrier or diluent.

36. A method for protecting against the occurrence of diseases involving expression of ROR1 in a subject, the method comprising:

administering to the subject in need thereof a polynucleotide encoding ROR1 protein or a fragment or variant thereof in an amount effective to induce a protective or therapeutic immune response against ROR1 in the subject, and a pharmaceutically acceptable carrier or diluent.

37. A method for protecting against the occurrence diseases involving expression of ROR1 in a subject, the method comprising:

administering to the subject in need thereof ROR1 protein or a fragment or variant thereof in an amount effective to induce a protective or therapeutic immune response against ROR1 in the subject, and a pharmaceutically acceptable carrier or diluent.

38. A method for identifying or isolating ROR1 protein in a sample, the method comprising:

(a) contacting the sample with the anti-ROR1 antibody of claim 1; and

(b) detecting immunoreactivity between the anti-ROR1 antibody and ROR1 to determine presence or quantity of ROR1 in the sample.

39. A method according to claim 38, wherein the ROR1 antibody is conjugated to a magnetic bead.

40. A method for detecting minimal residual disease, the method comprising:

(a) contacting the sample with the anti-ROR1 antibody of claim 1; and

(b) detecting immunoreactivity between the anti-ROR1 antibody and ROR1 to determine presence or quantity of ROR1 in the sample.

41. A method according to claim 40, wherein the ROR1 antibody is conjugated to a magnetic bead.

42. A humanized ROR1 antibody.

43. A precipitate comprising an ROR1 antibody and a polypeptide selected from the group consisting of a ROR1 protein, ROR1 polypeptide fragment and ROR1 polypeptide variant.

Figure 1a

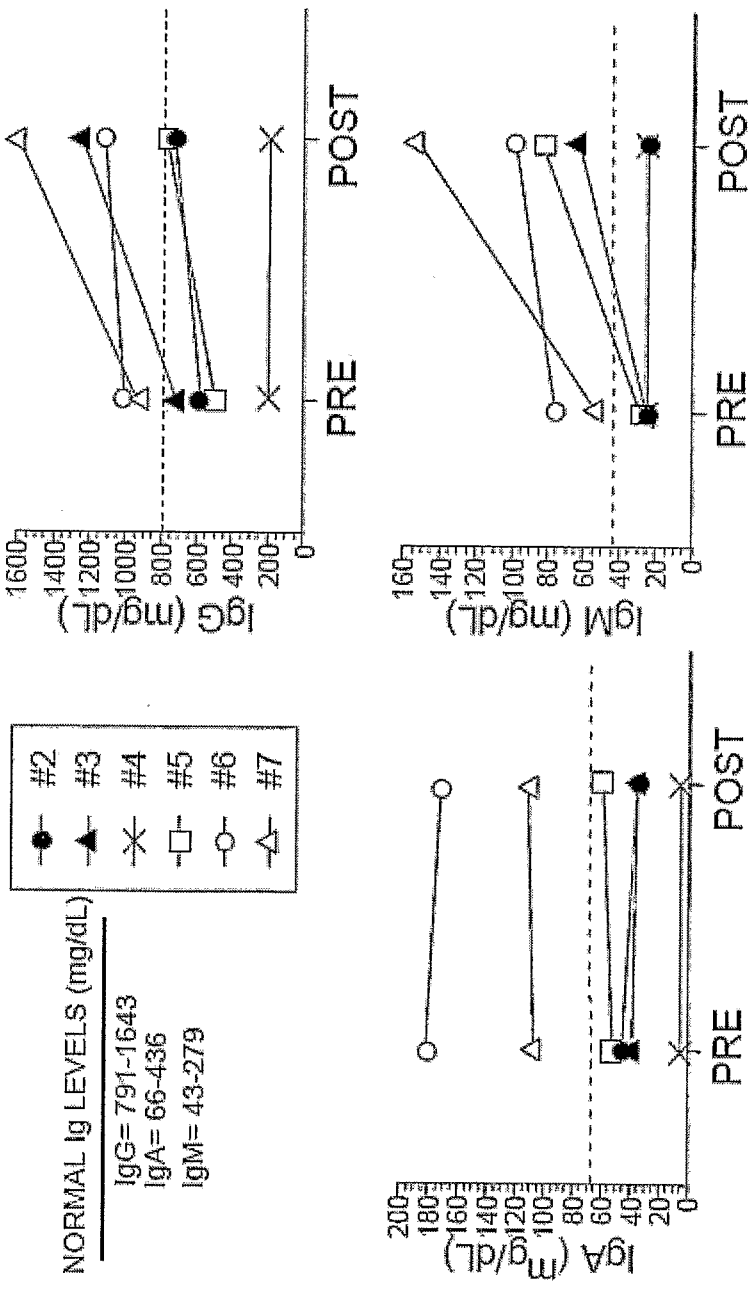

IgG (mg/dL)
IgM (mg/dL)
IgA (mg/dL)
PRE
POST
#2
#3
#4
#5
#6
#7
NORMAL Ig LEVELS (mg/dL)
IgG= 791-1643
IgA= 66-436
IgM= 43-279

Figure 1b

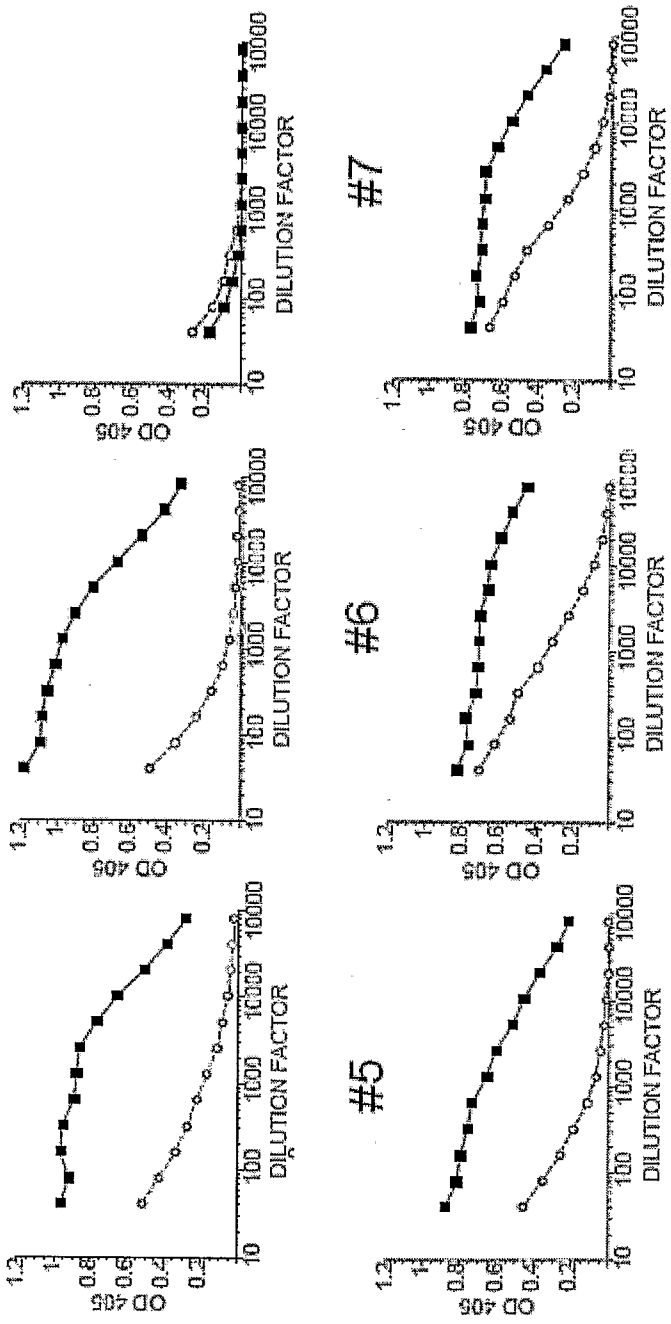


Figure 1c

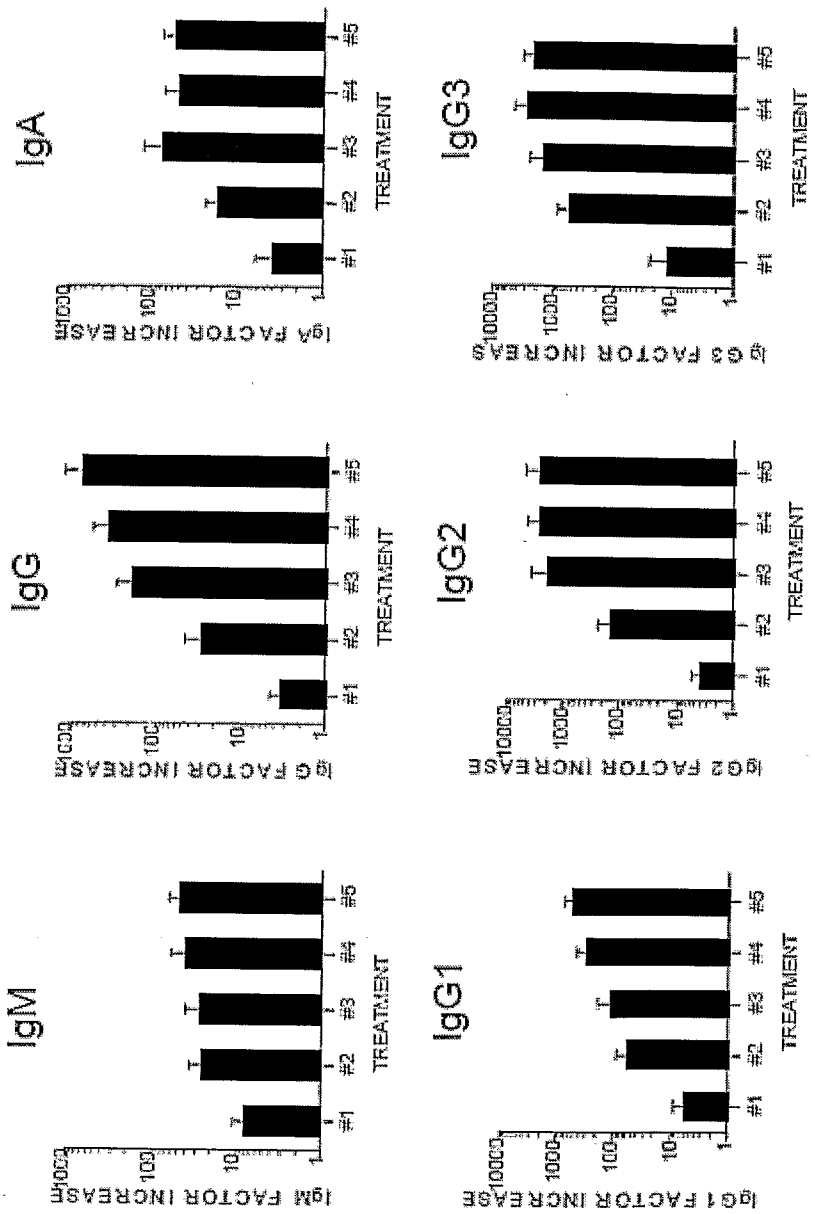


Figure 1d

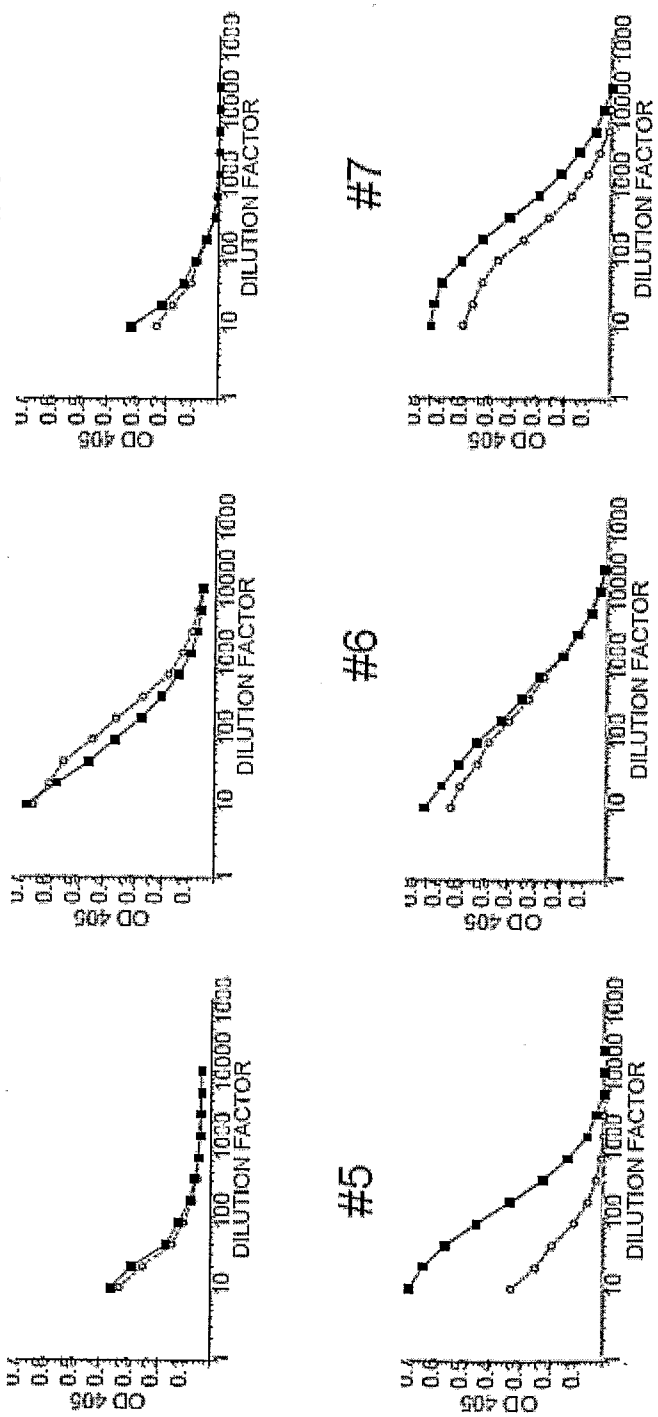
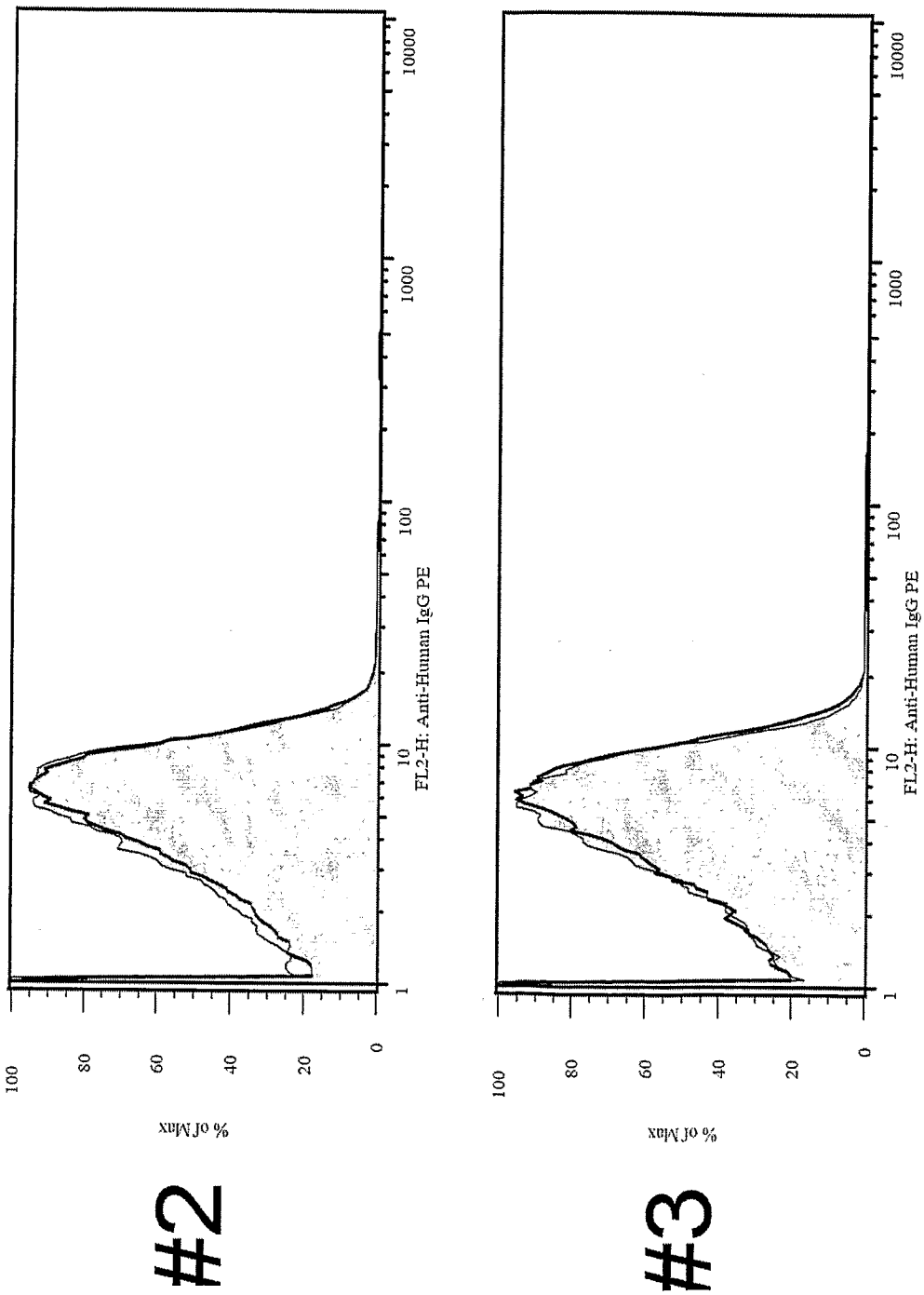


Figure 2a


#2
#3
% of Max
FL2-H: Anti-Human IgG PE
0
20
40
60
80
100
1
10
100
1000
10000

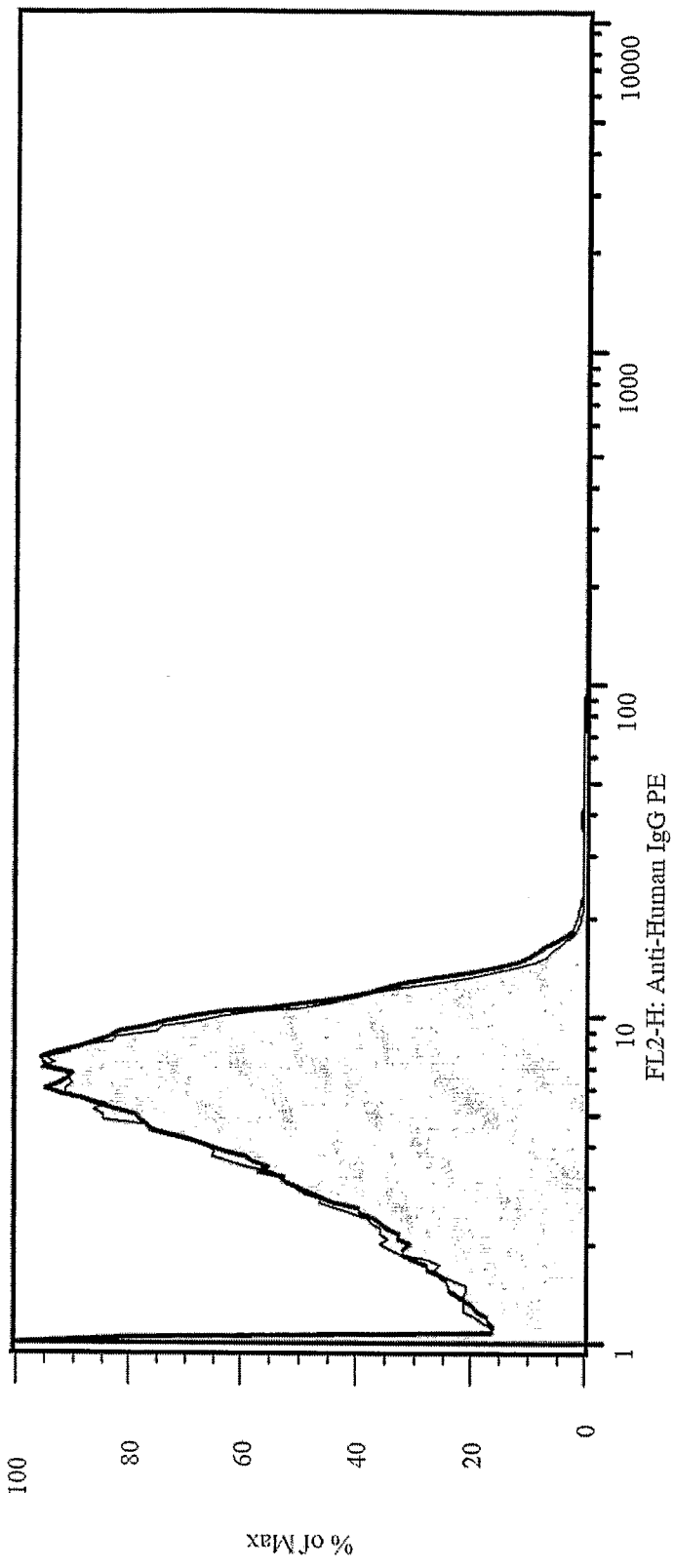
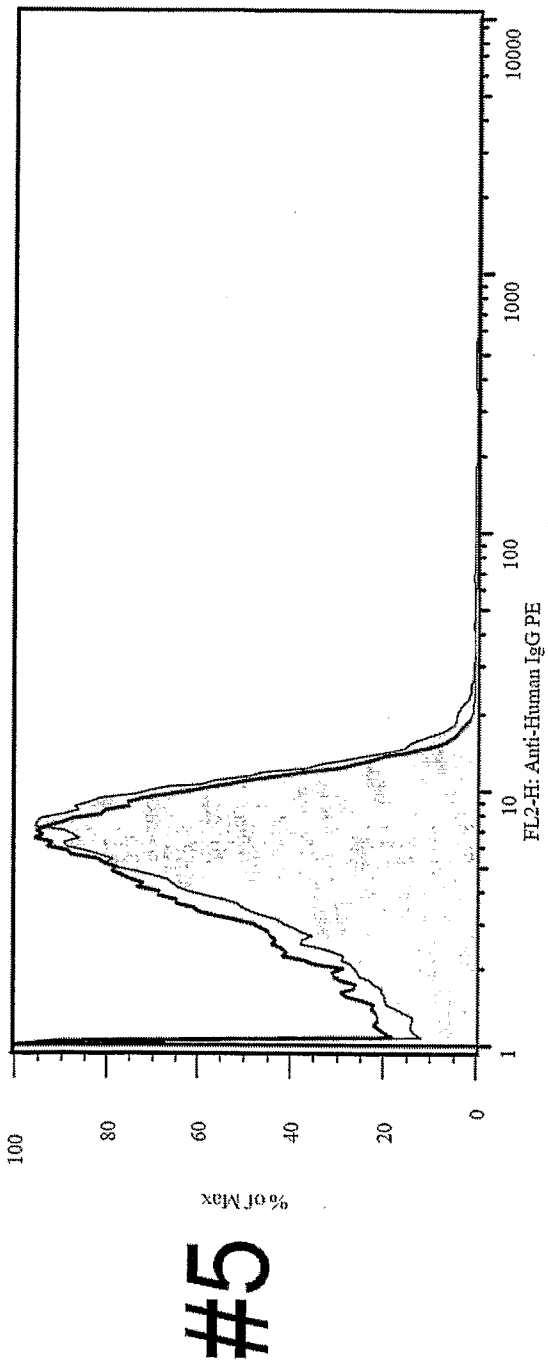
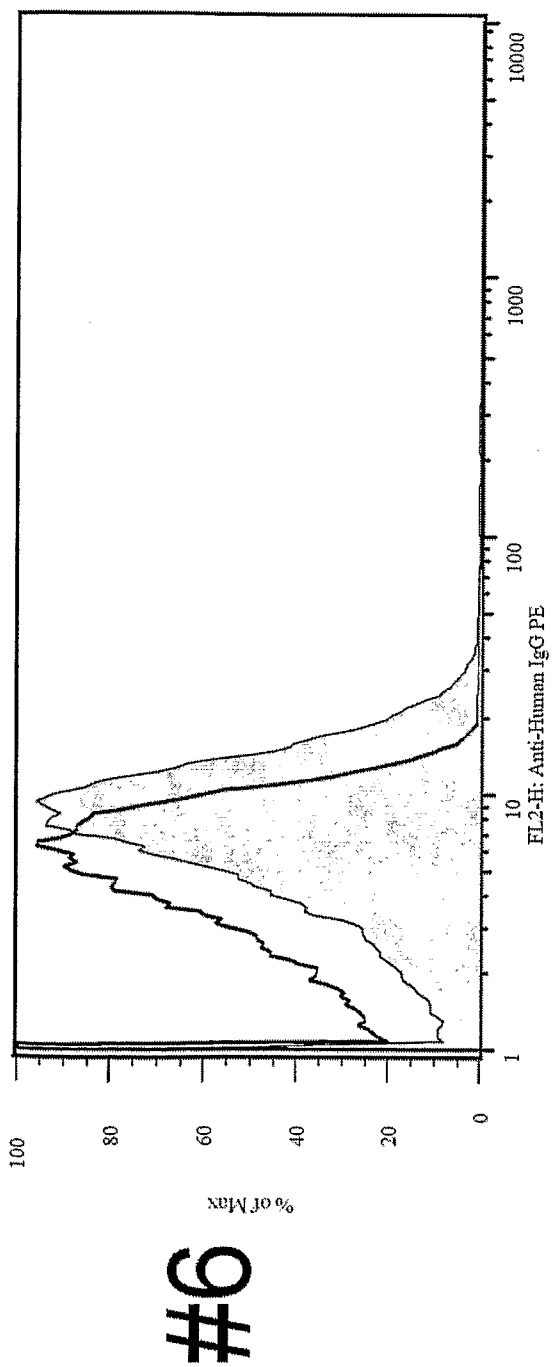


Figure 2A

cont.

Figure 2A
cont.





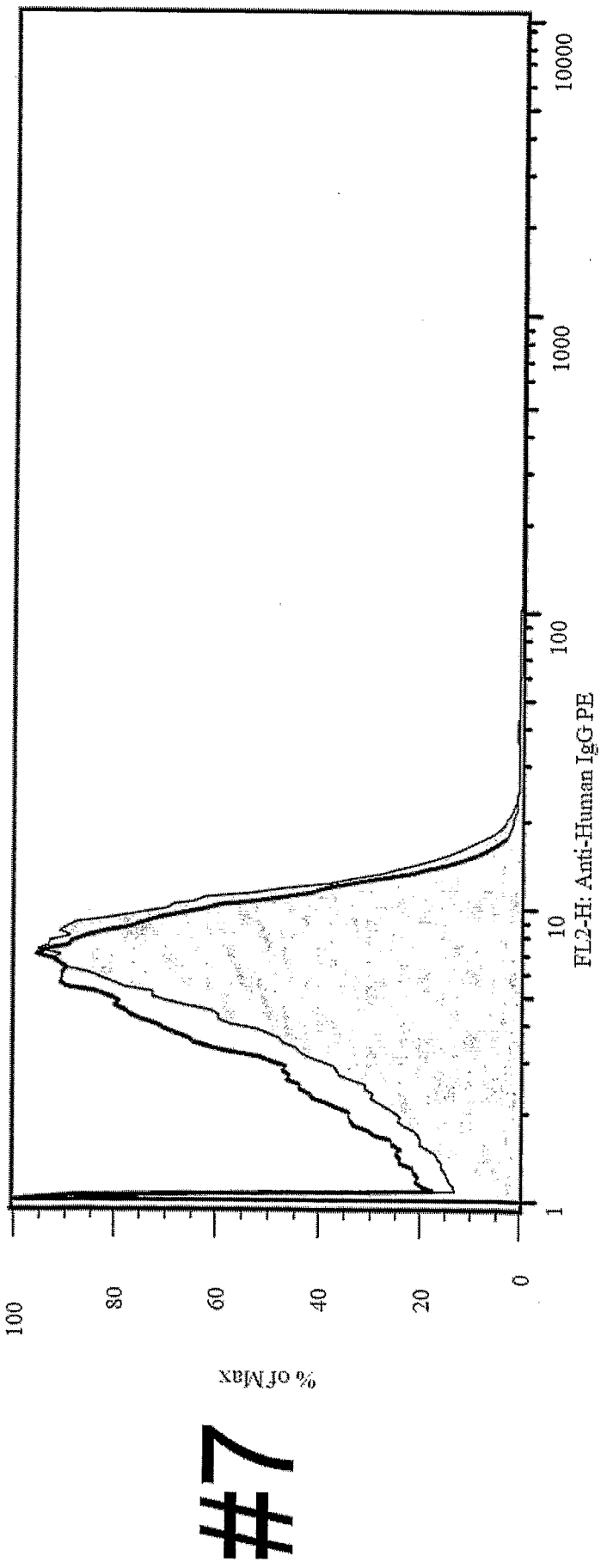
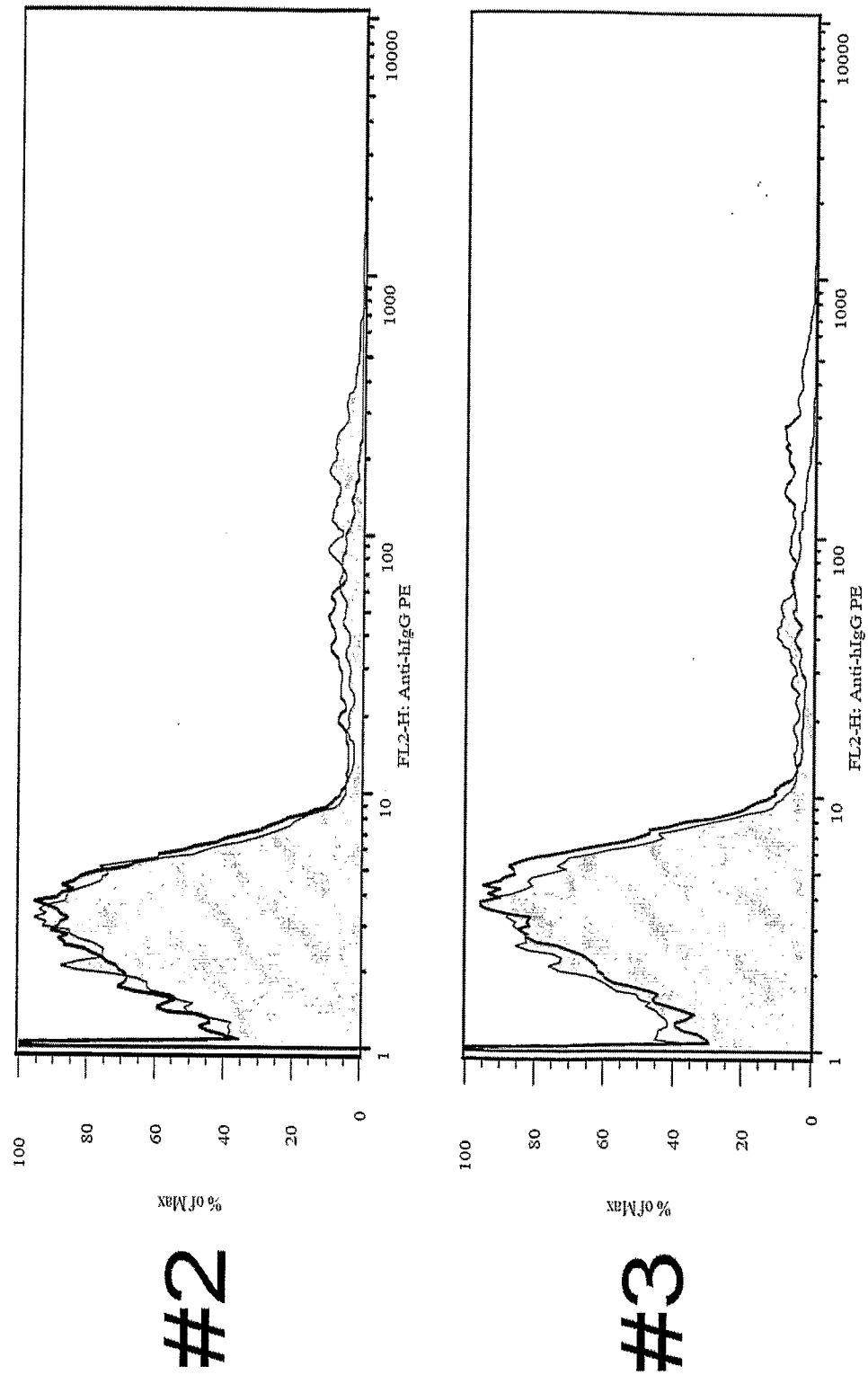


Figure 2A
cont.

Figure 2b



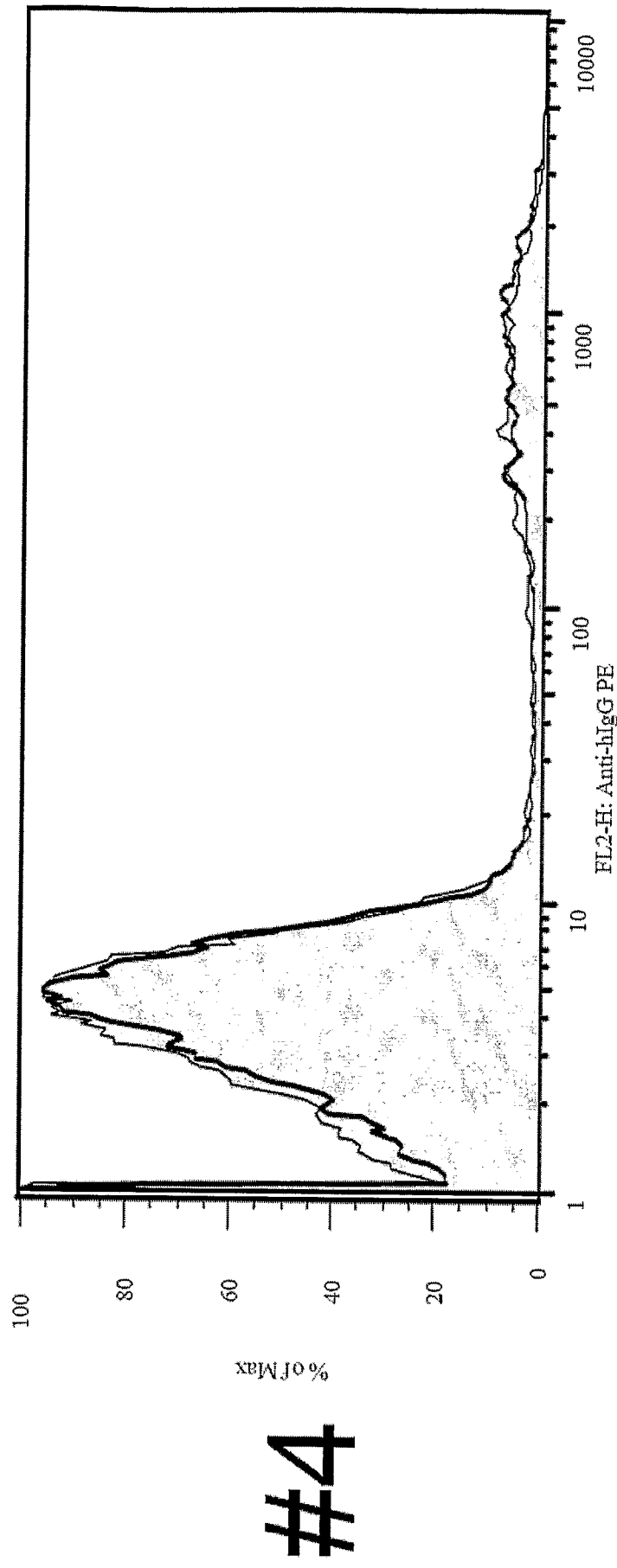


Figure 2B
cont.

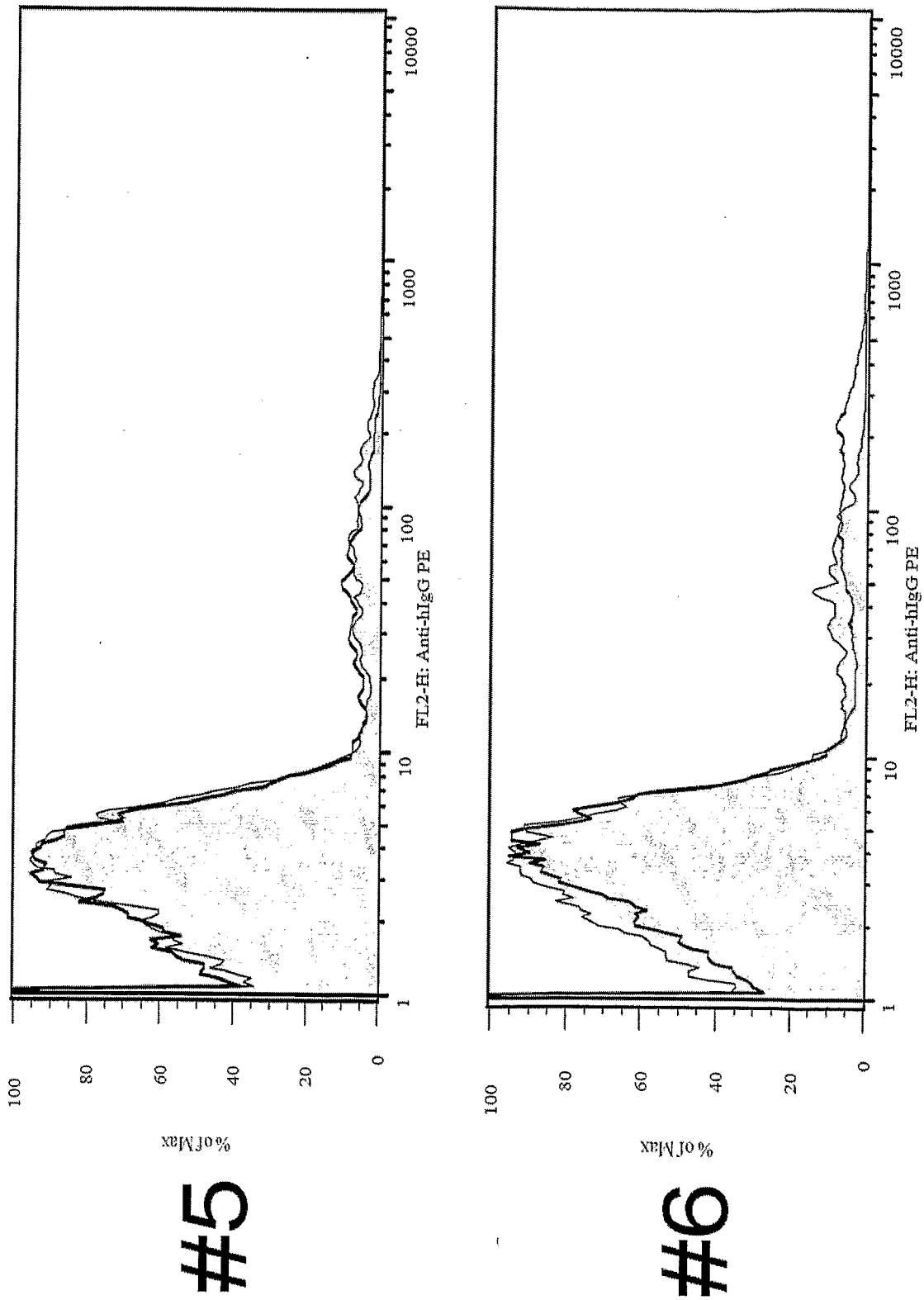


Figure 2B

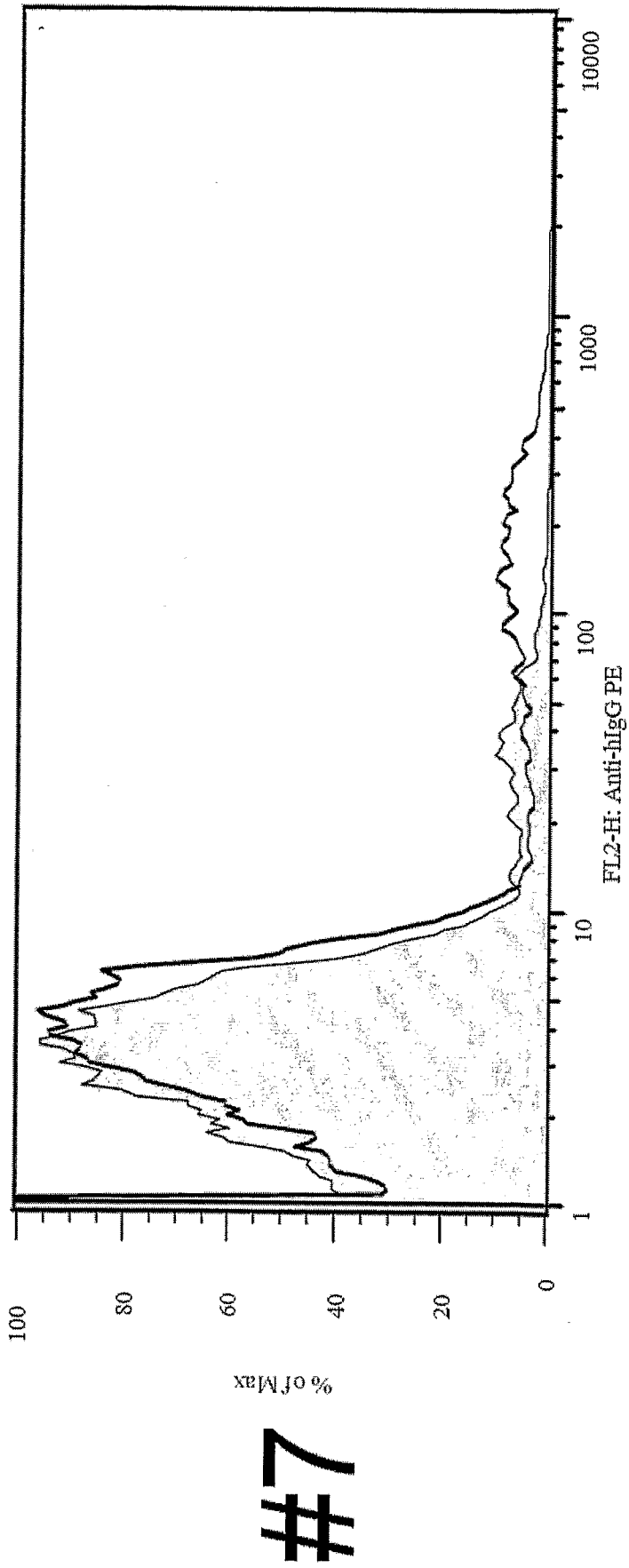
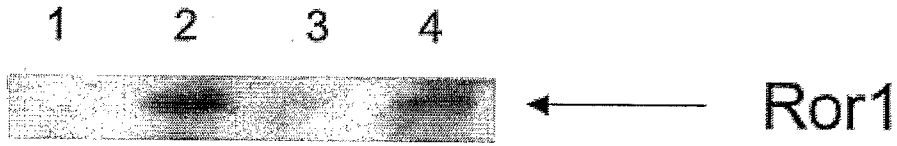


Figure 2B

Figure 3



1: 300 µg of CHO cell lysate
2: 300 µg of CHO-ROR1 cell lysate
3: 300 µg of CLL cell lysate
4: 600 µg of CLL cell lysate

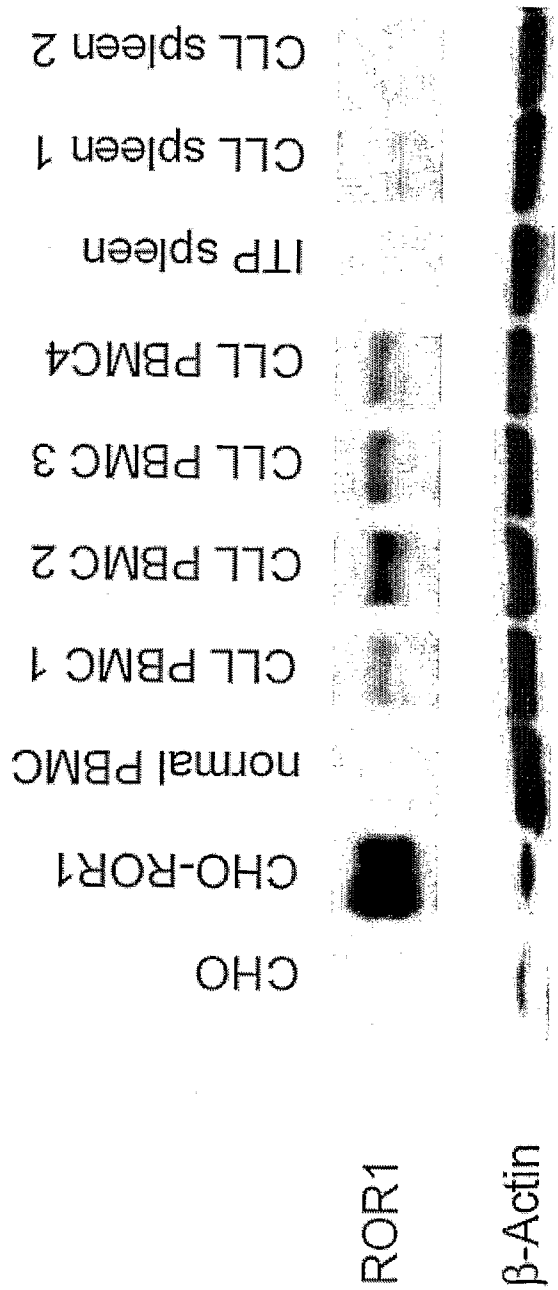

CHO
CHO-ROR1
normal PBMC
CLL PBMC 1
CLL PBMC 2
CLL PBMC 3
CLL PBMC4
ITP spleen
CLL spleen 1
CLL spleen 2
ROR1
β-Actin

Figure 4a

Figure 4b

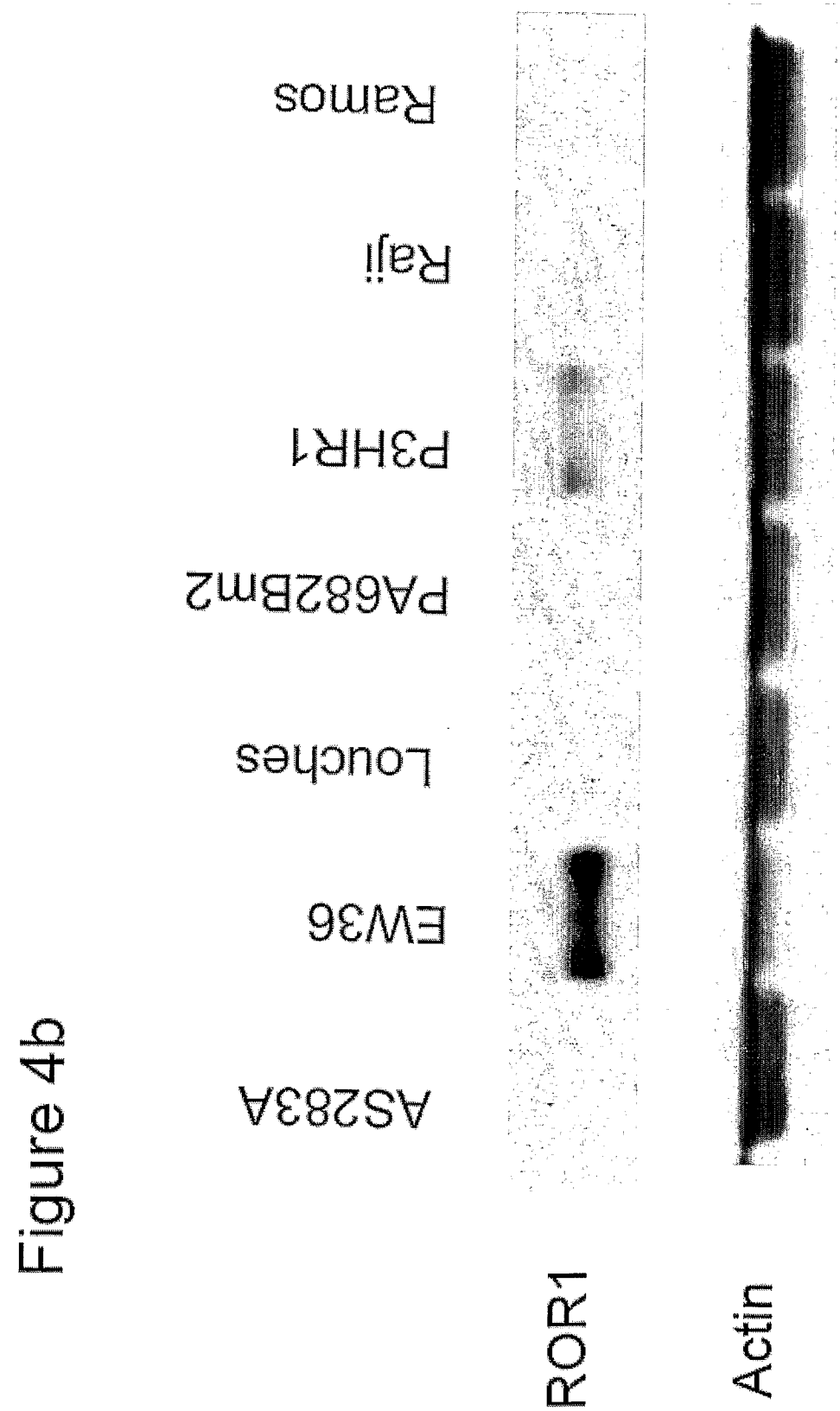


Figure 4c

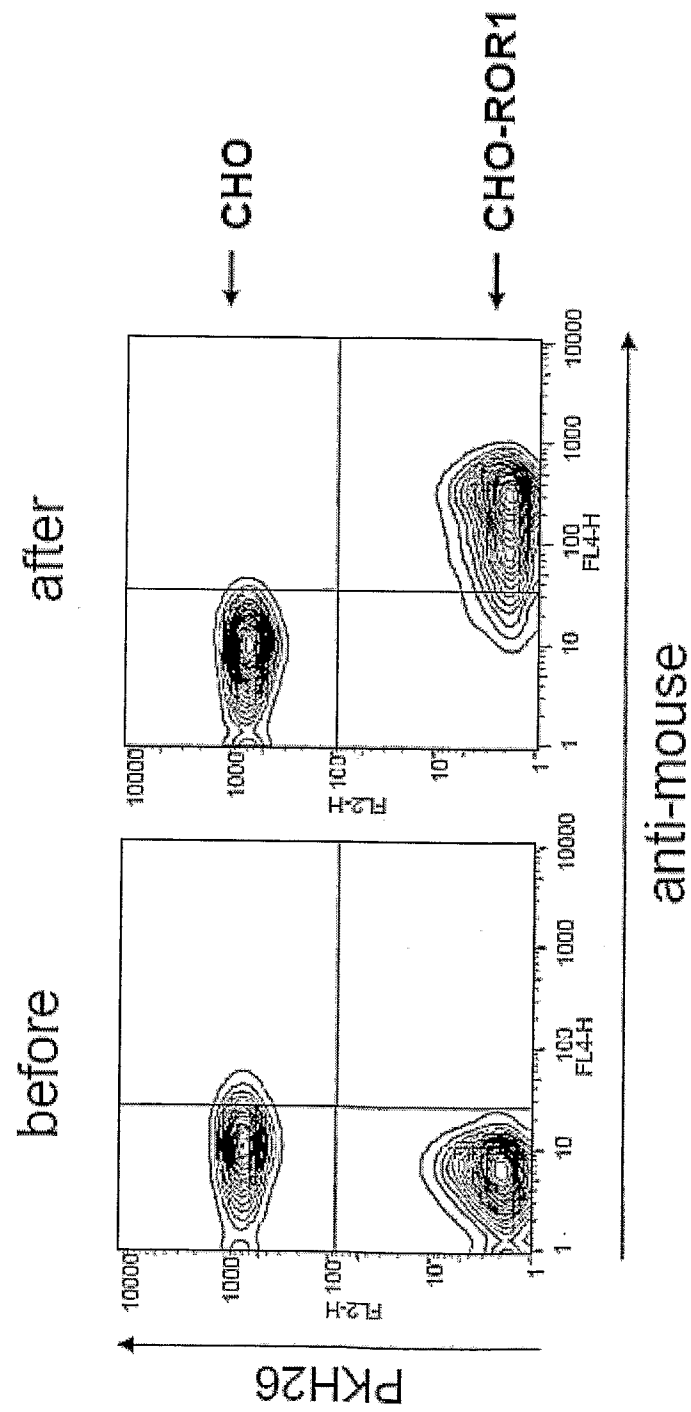
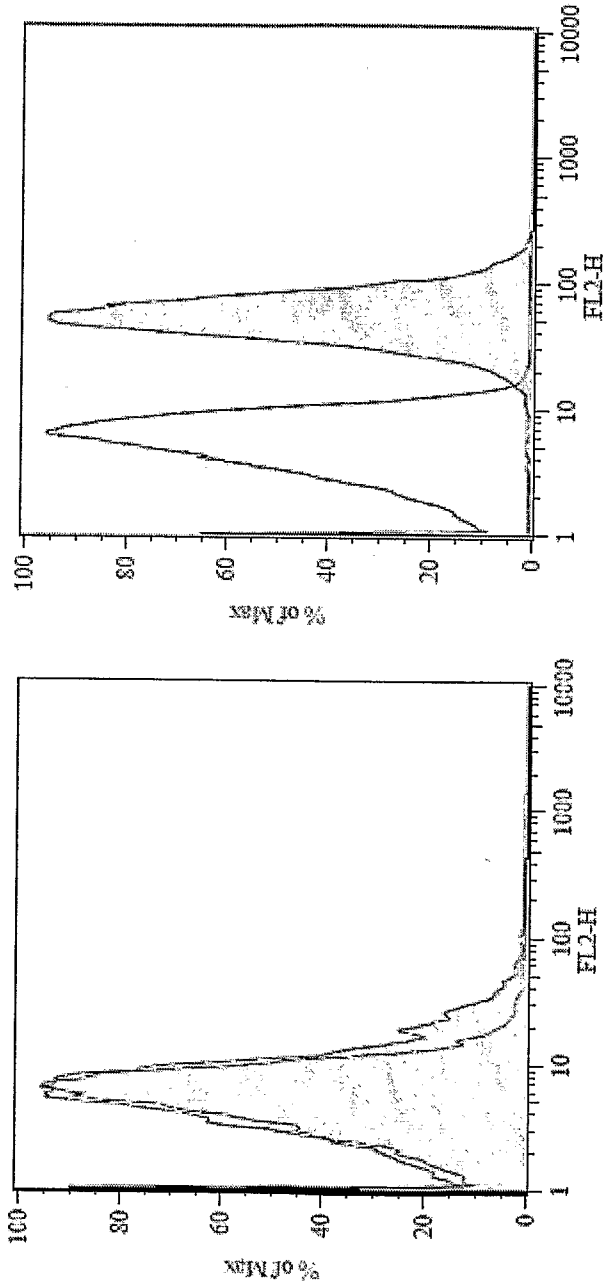
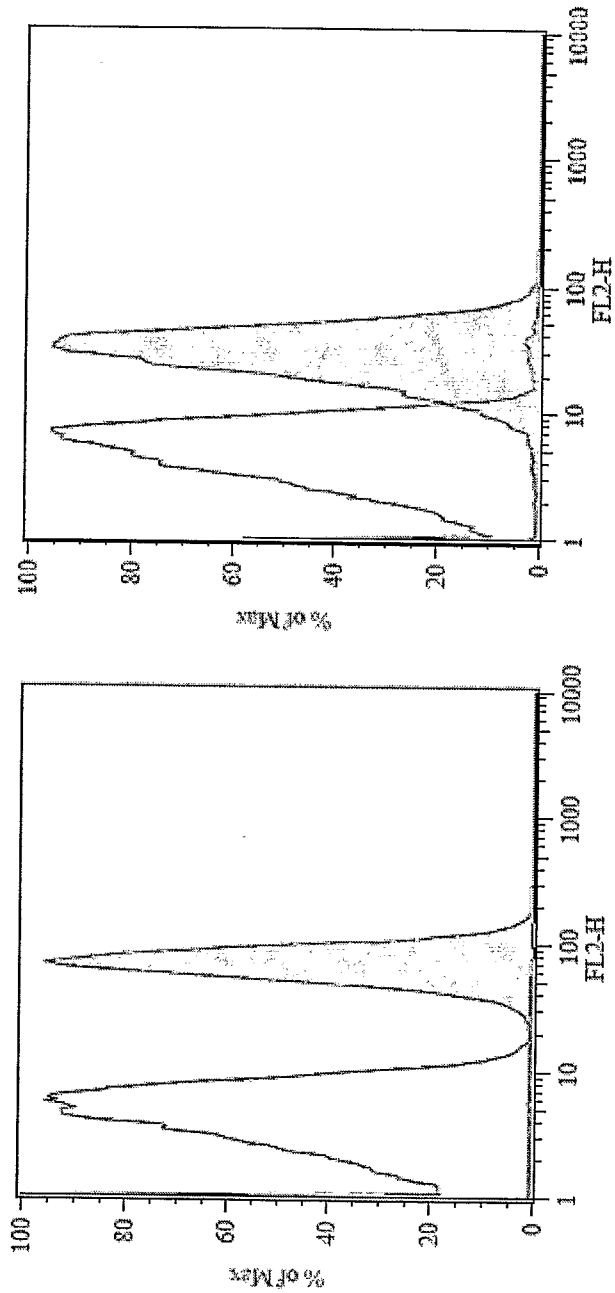

CHO
CHO-ROR1
after
before
anti-mouse
PKH26
FL2-H
FL4-H
10000
1000
100
10
1

Figure 4d

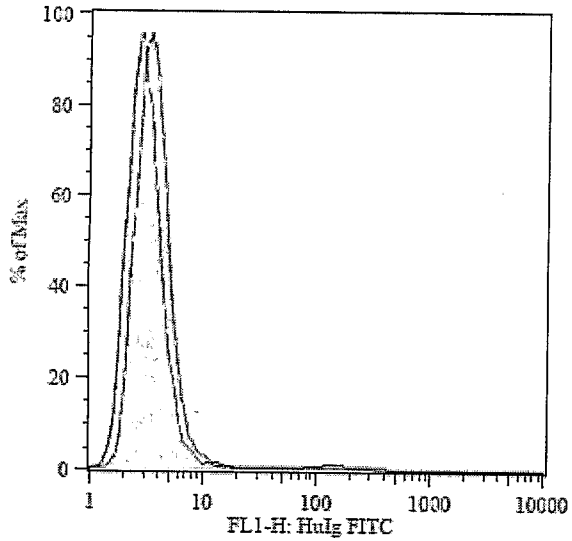




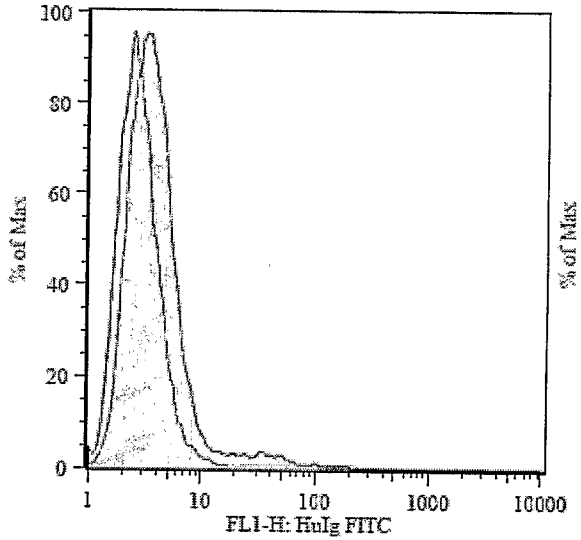
CLL #2 CLL #3

Figure 4D

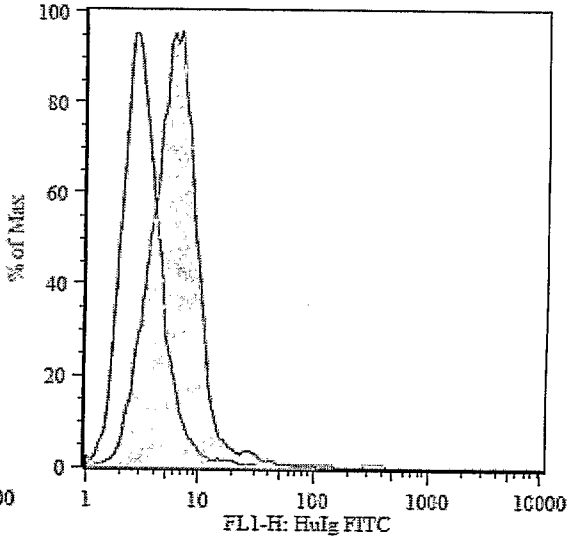
Figure 5a



healthy donor



#2 pre



#2 post

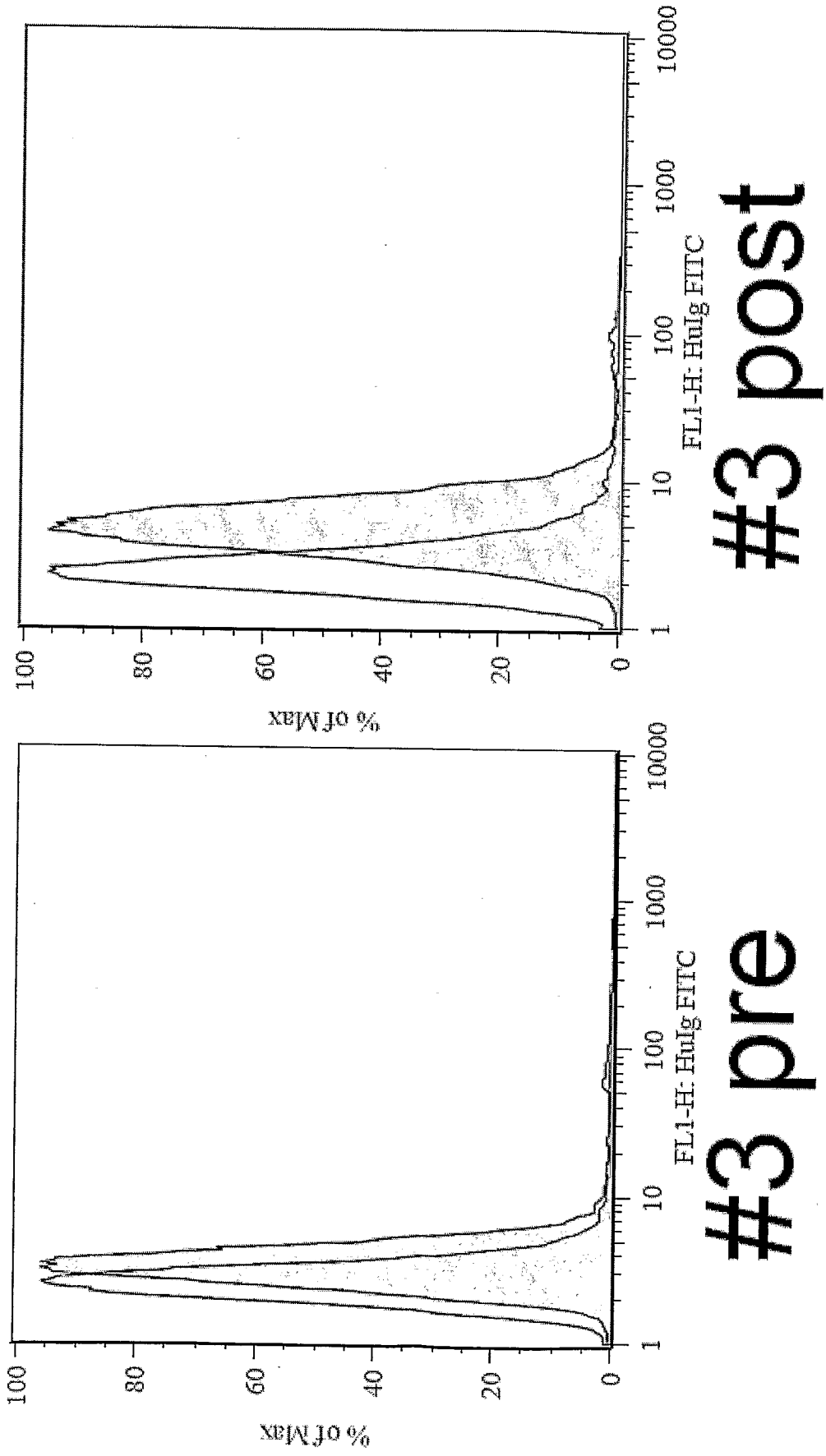


Figure 5A

Figure 5A

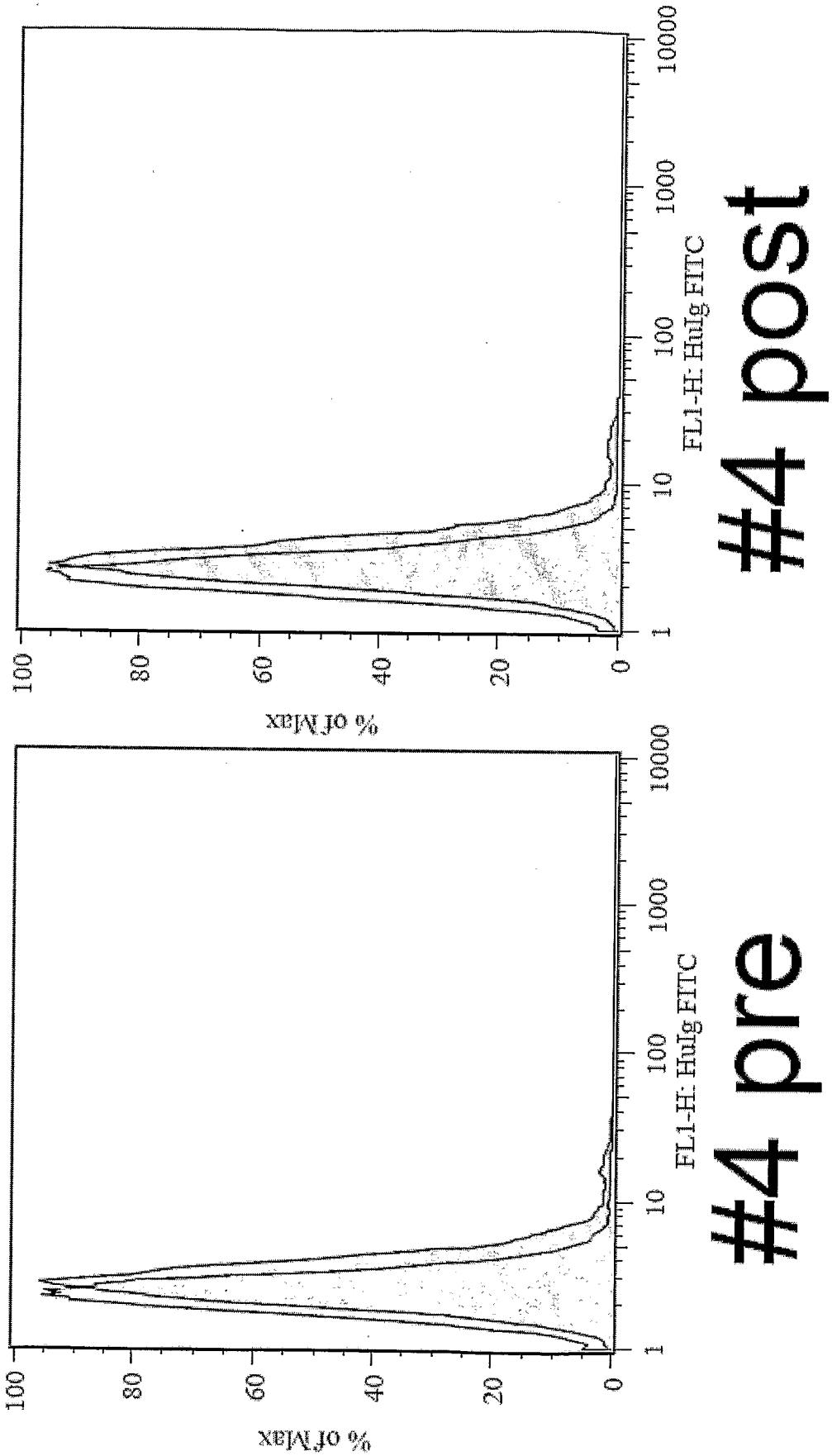


Figure 5A

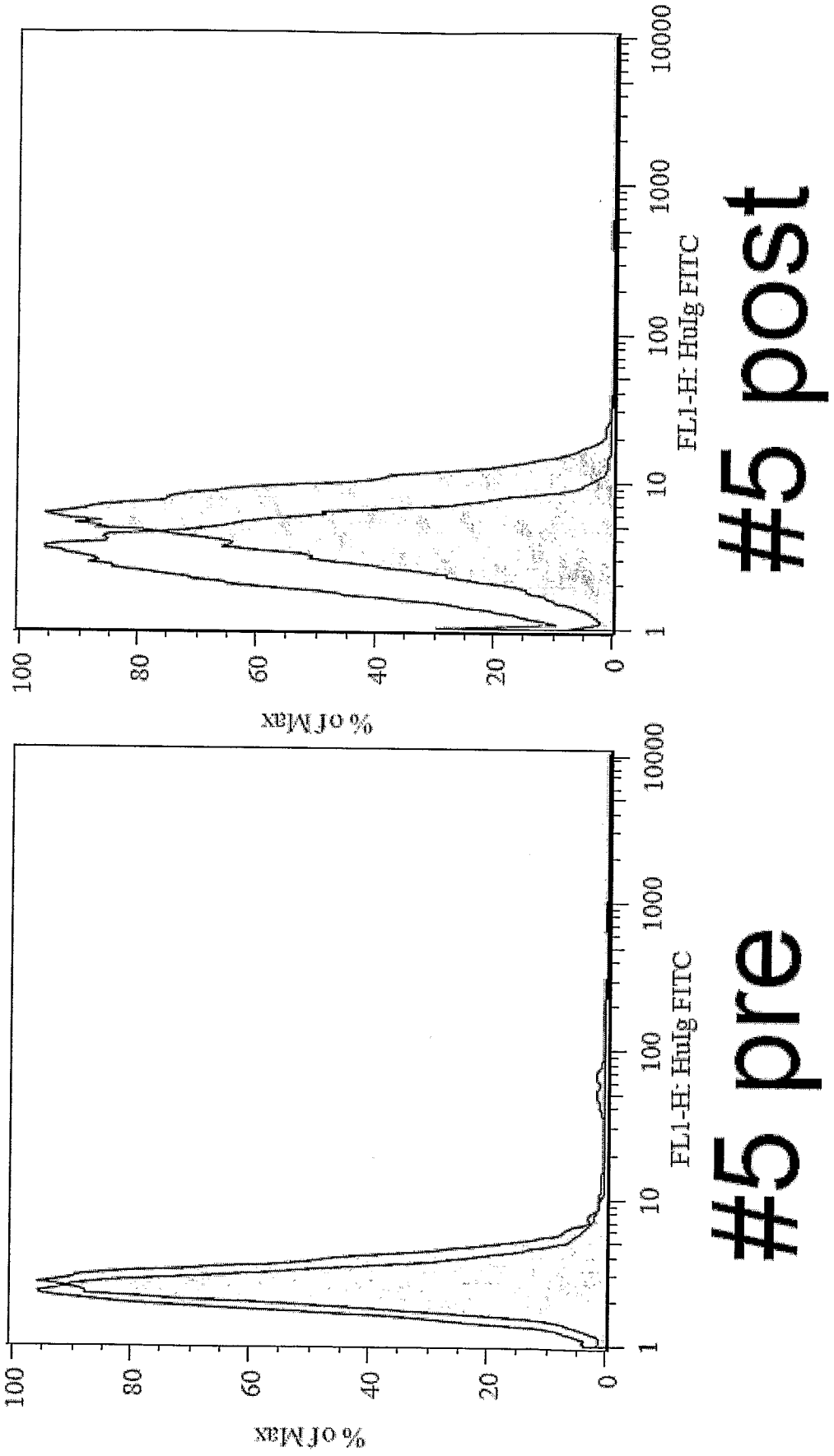


Figure 5A

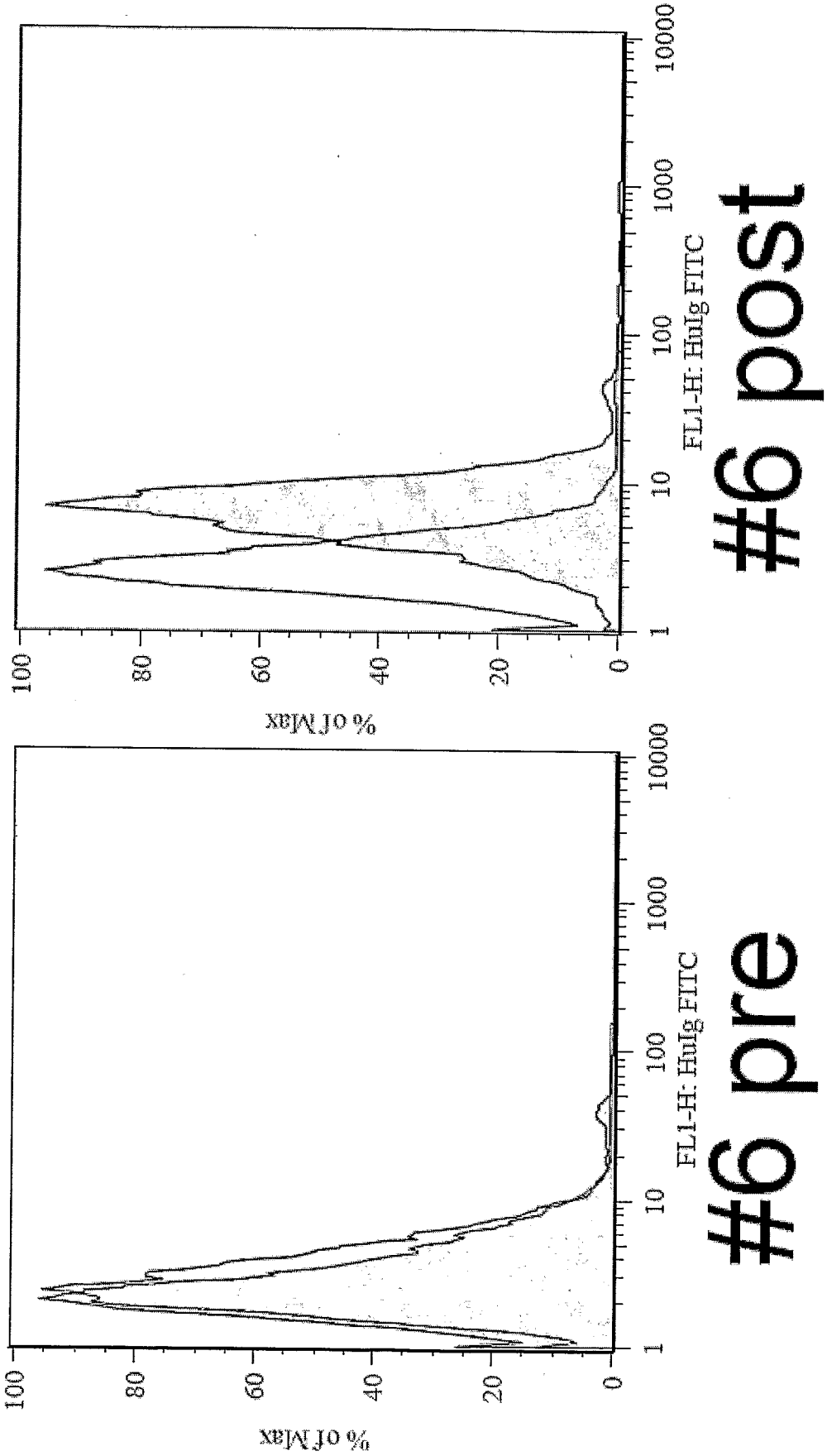


Figure 5A

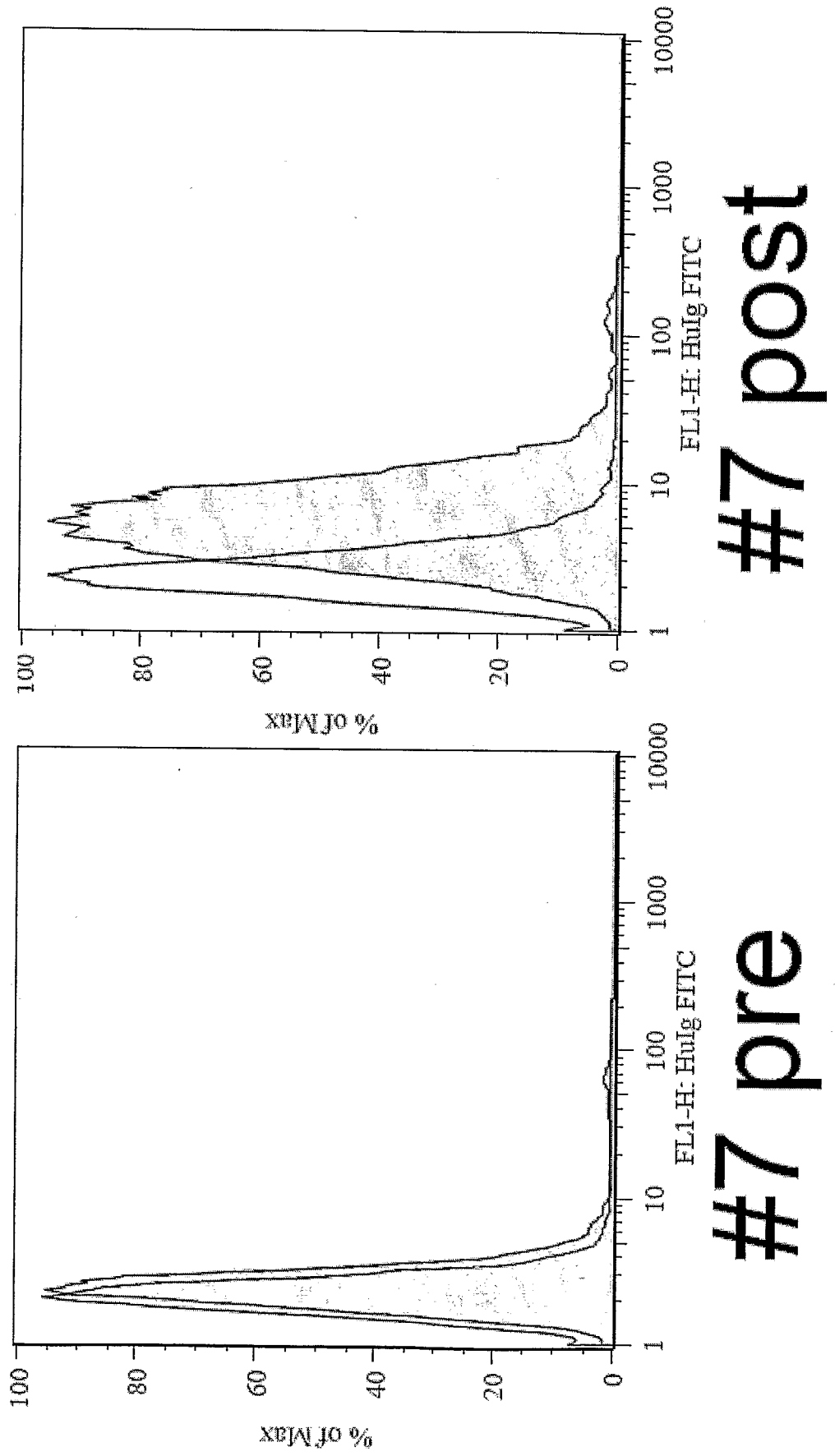
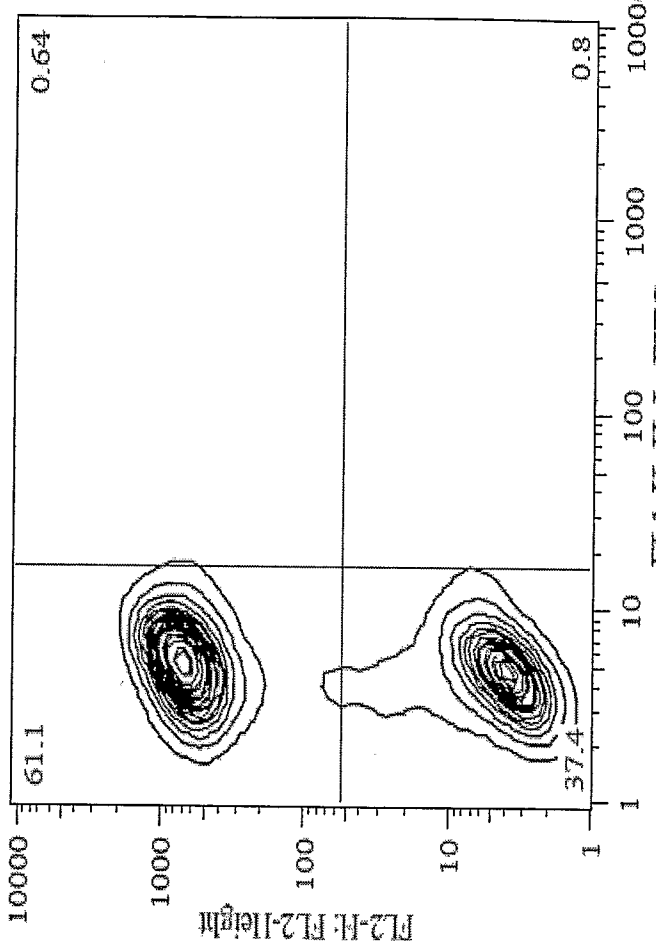


Figure 5b



healthy donor

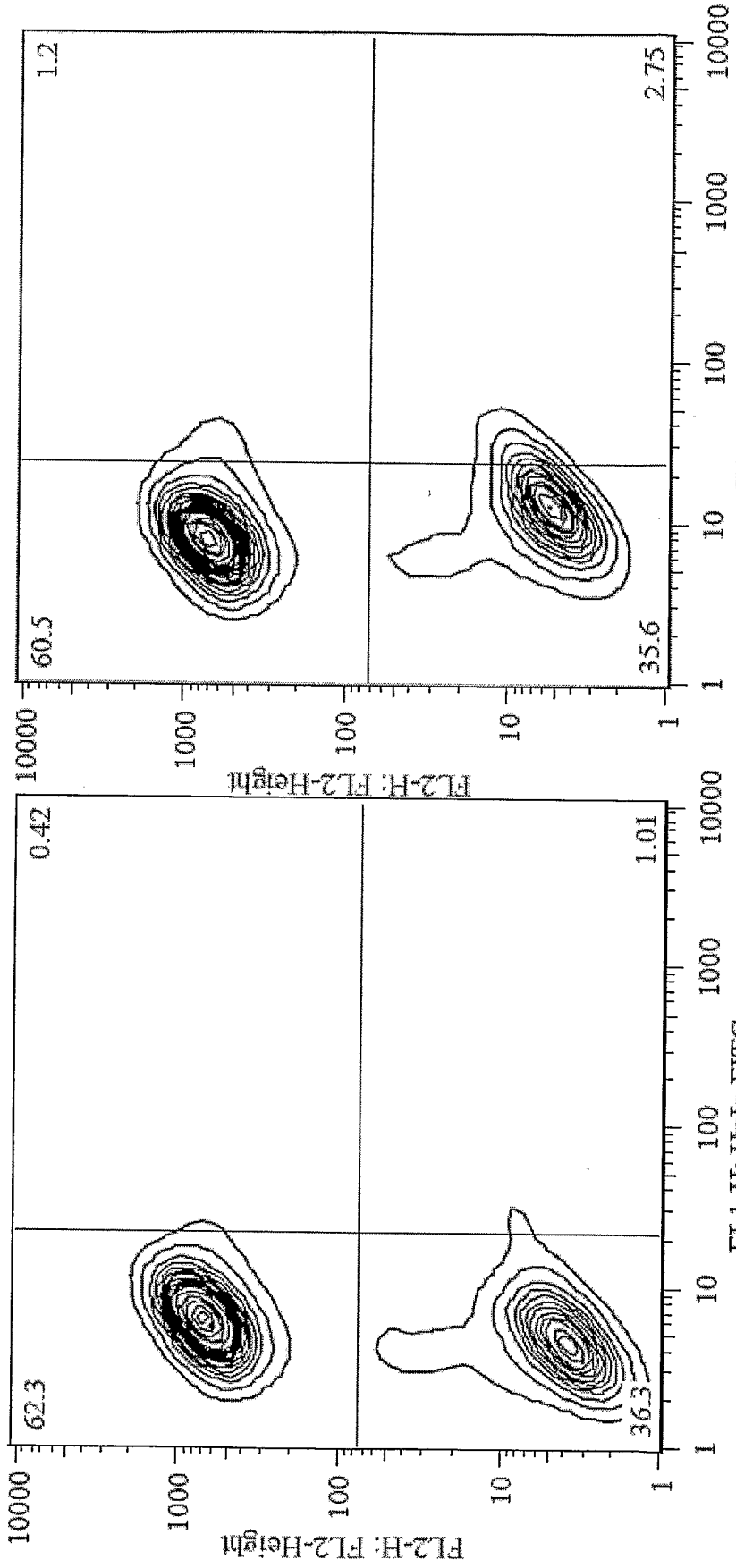


Figure 5B

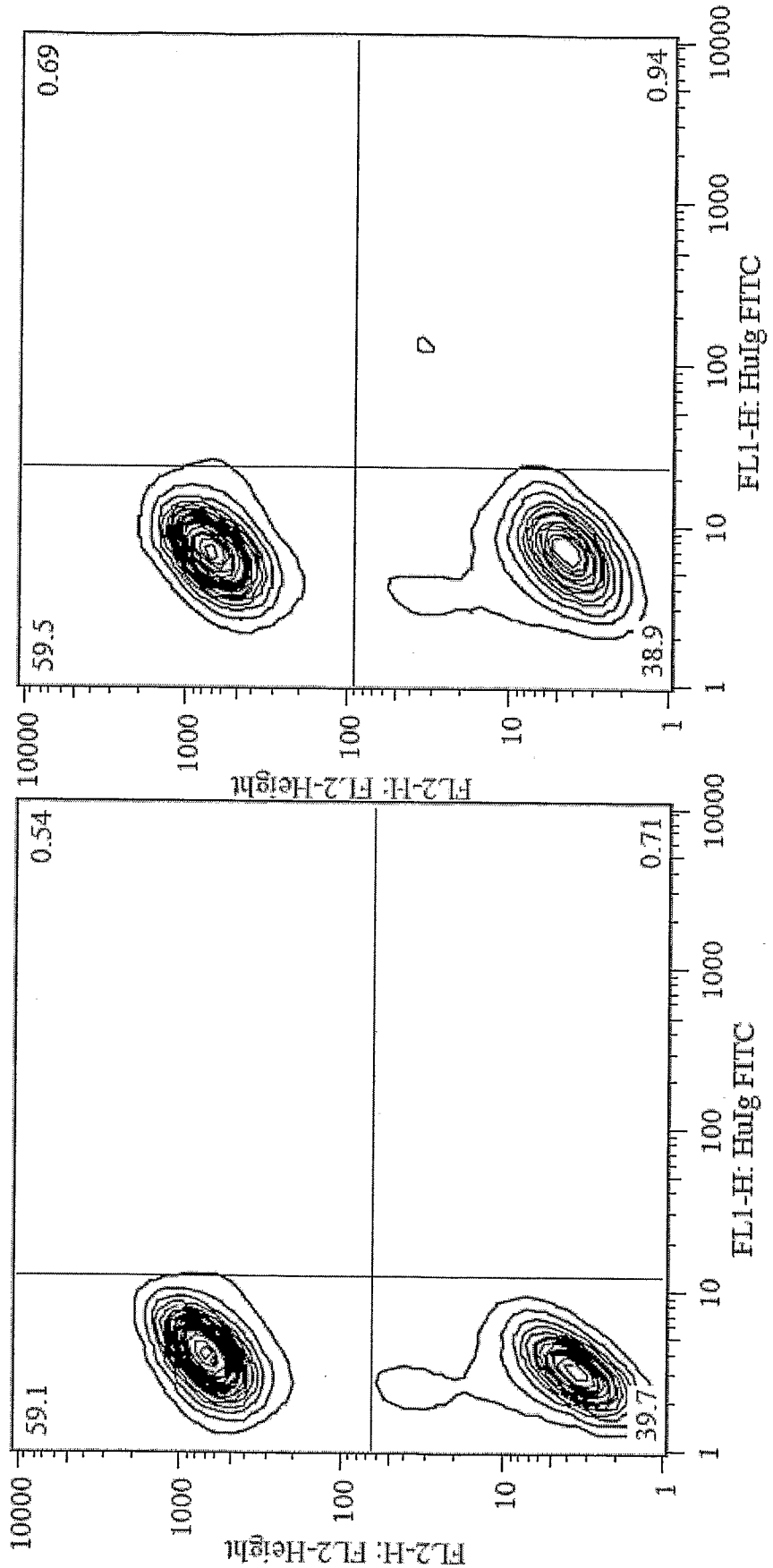


Figure 5B

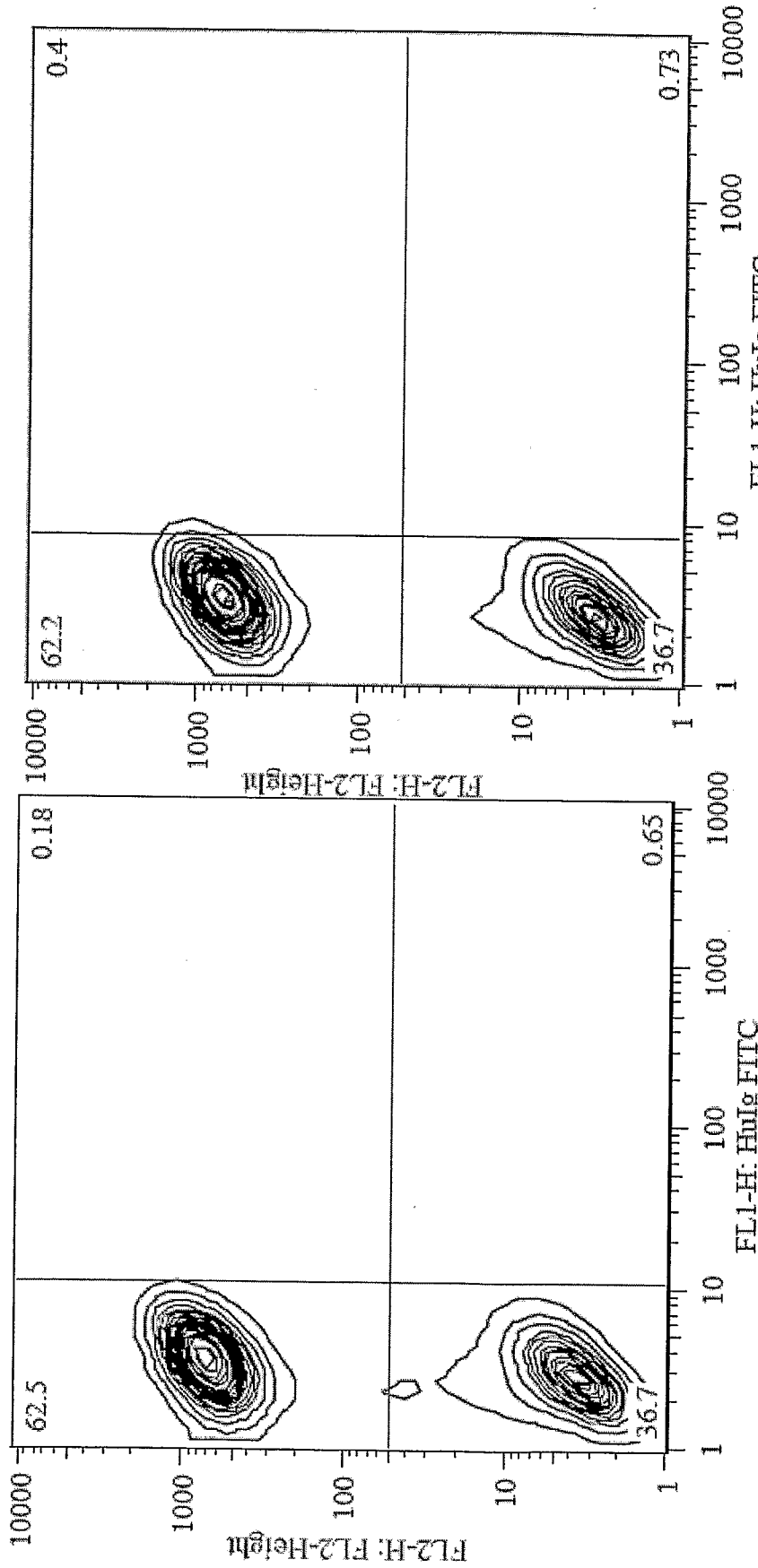


Figure 5B

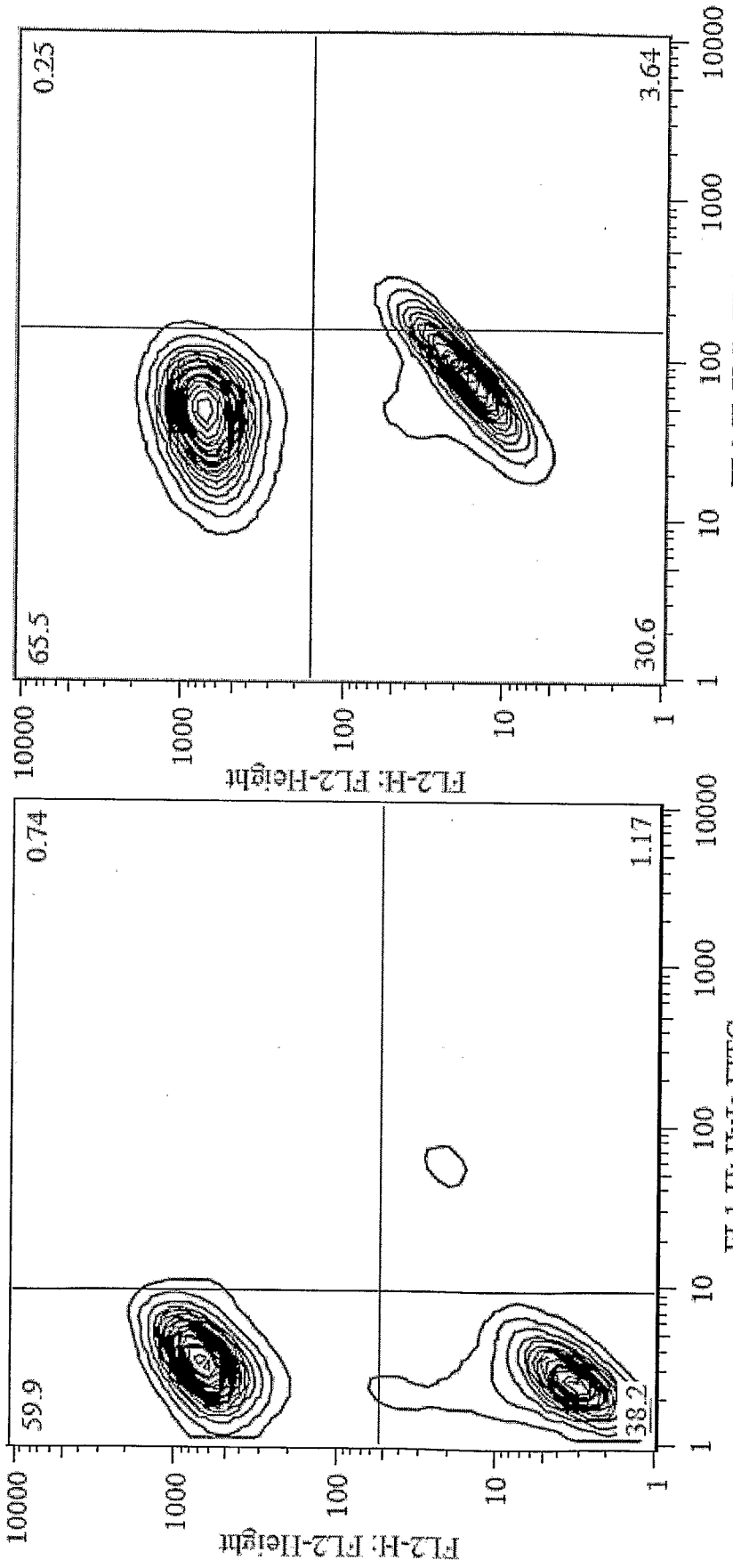


Figure 5B

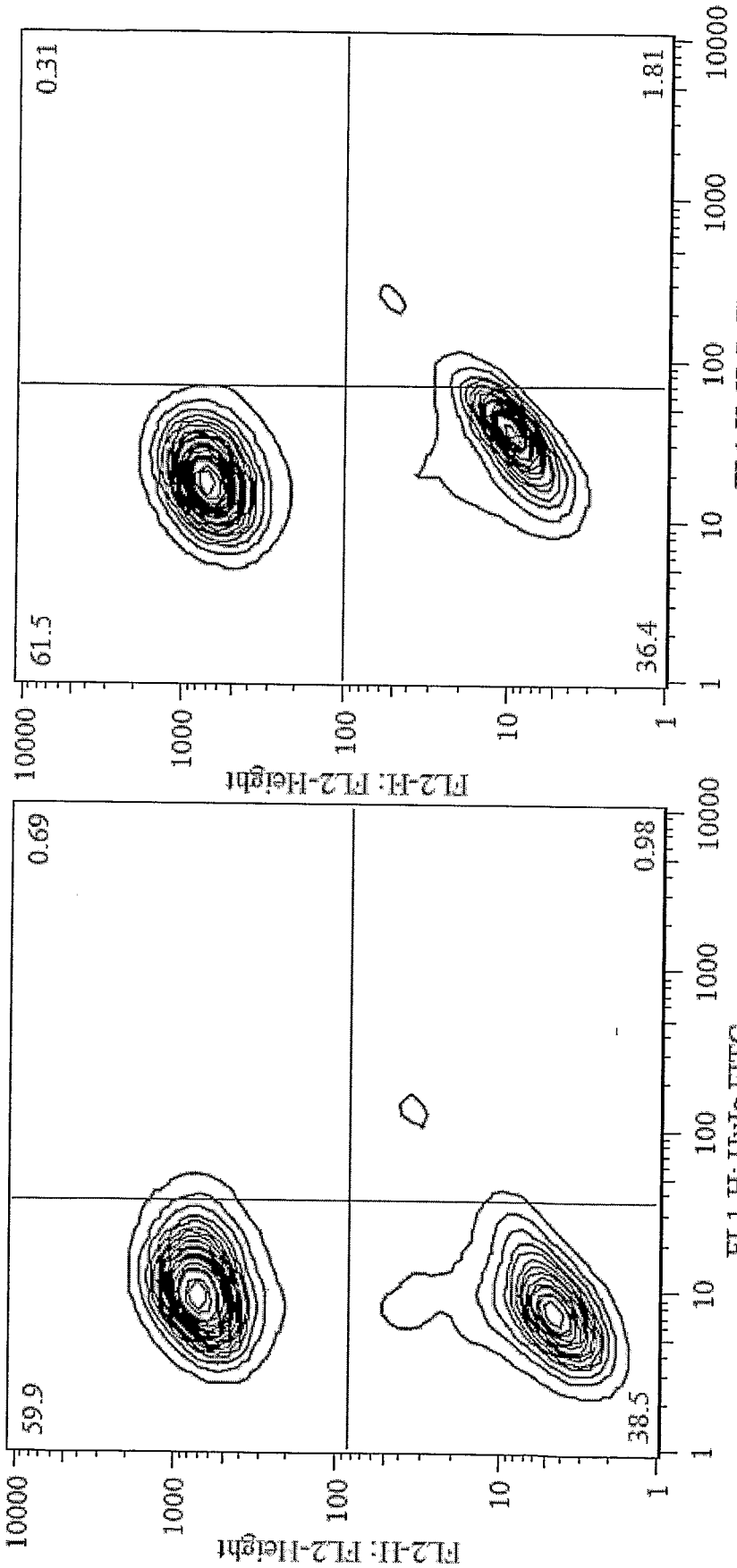


Figure 5B

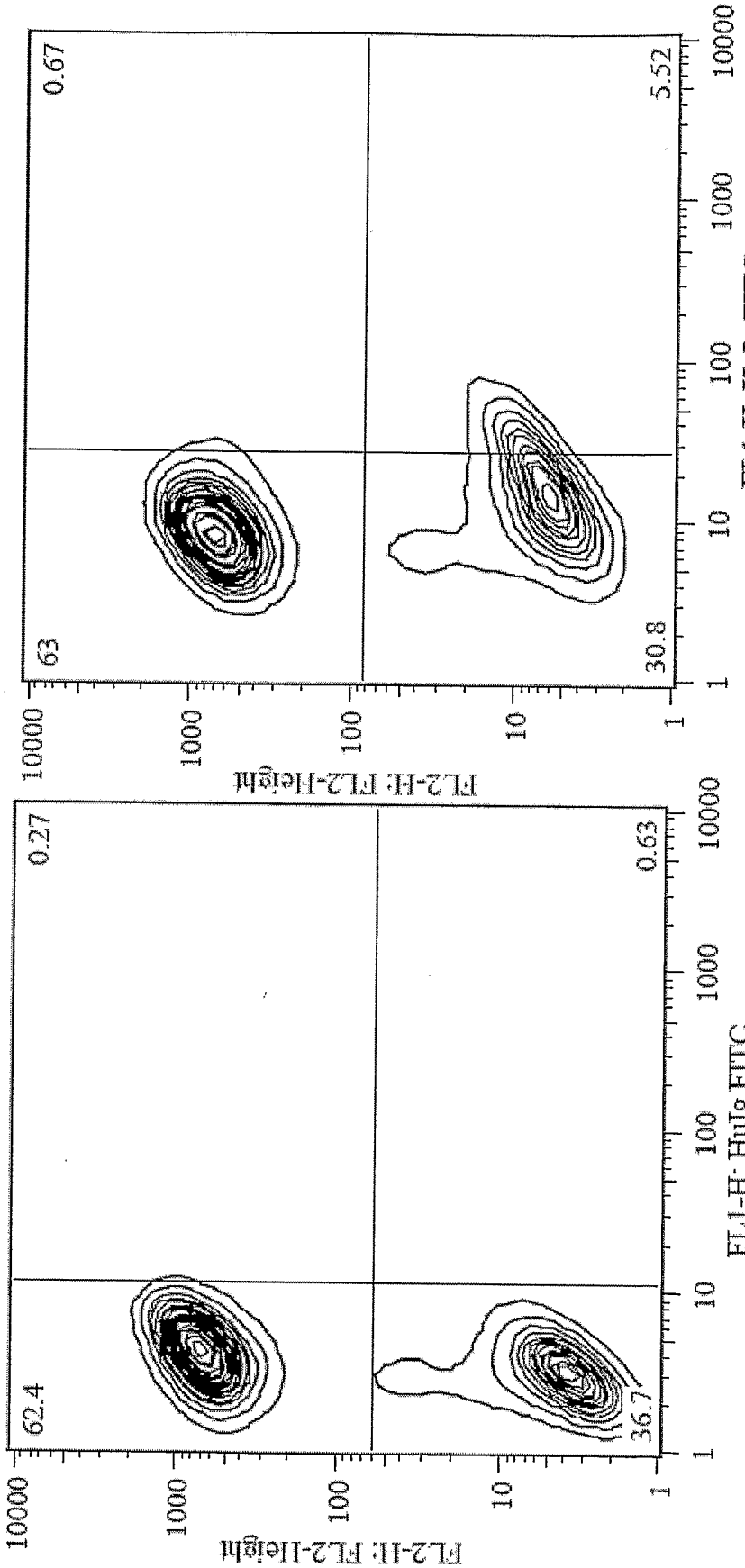


Figure 5B

Figure 6a

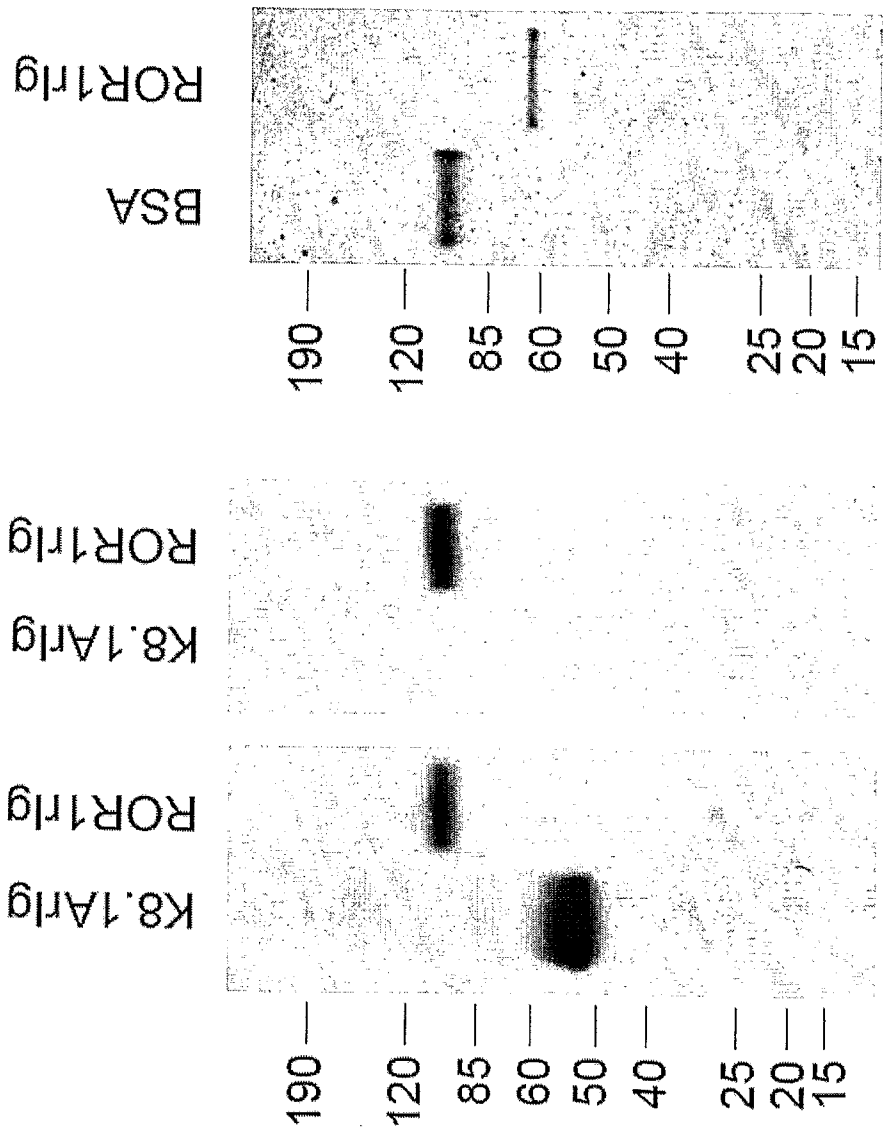


Figure 6b

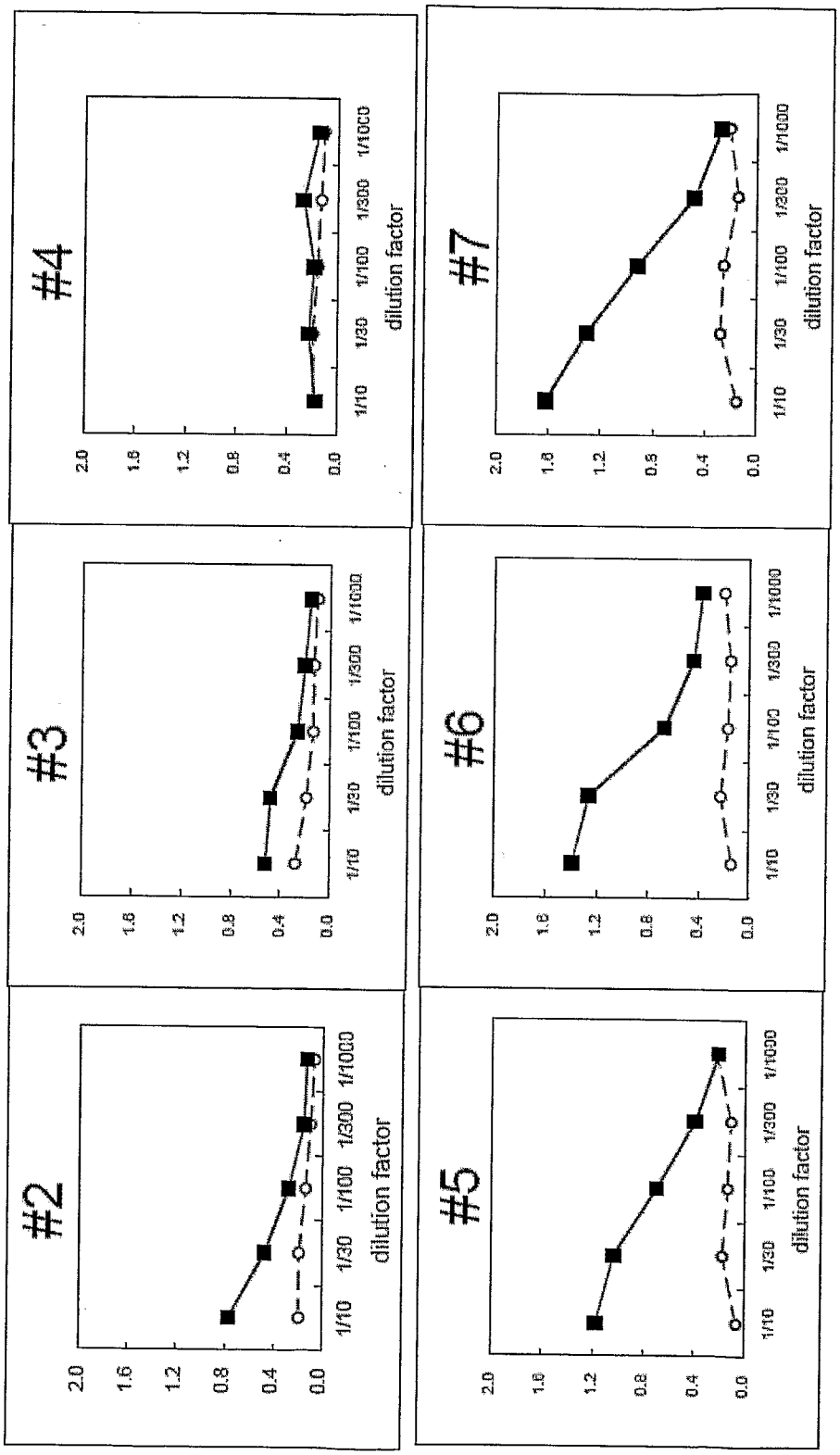


Figure 6c

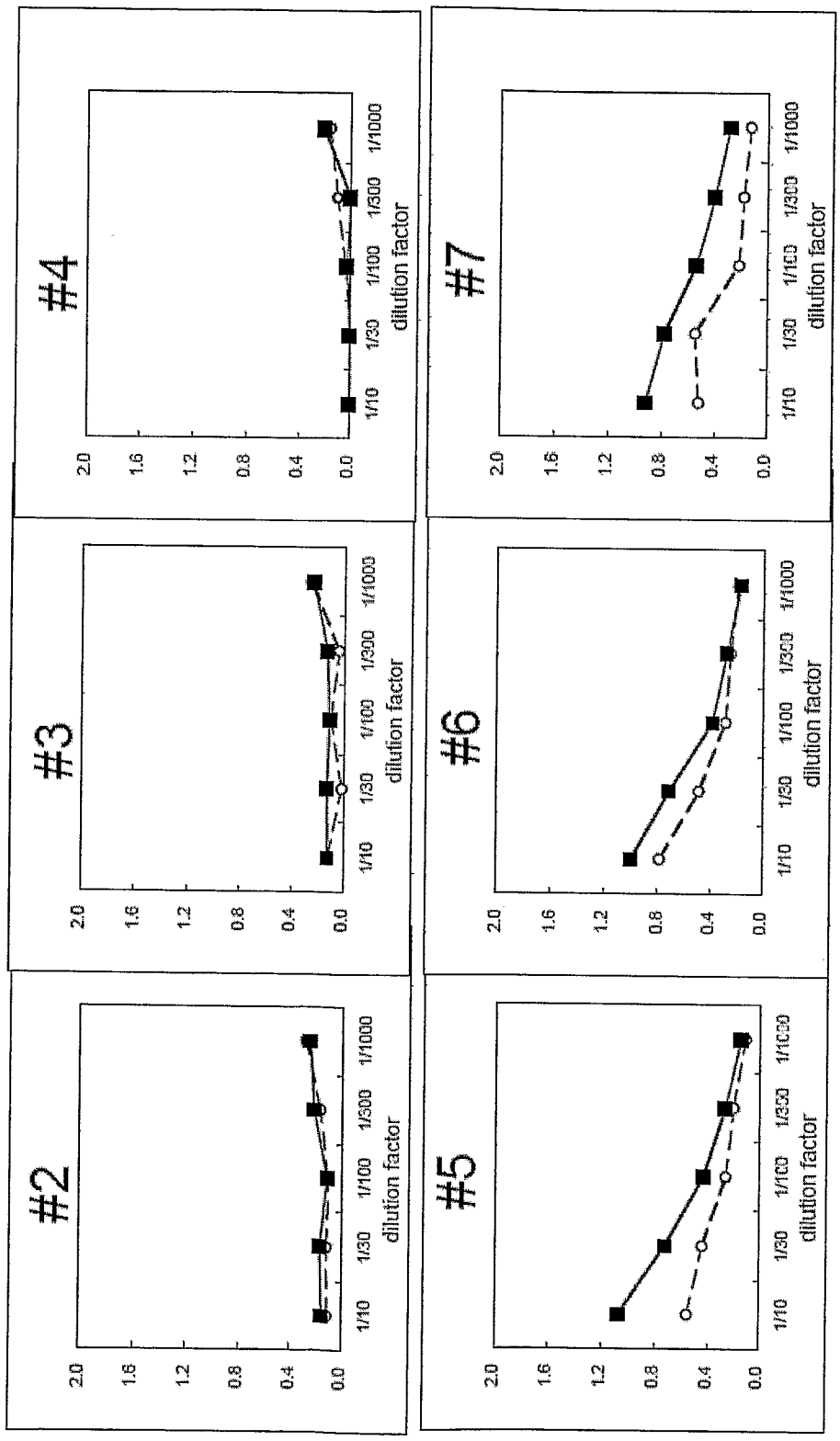
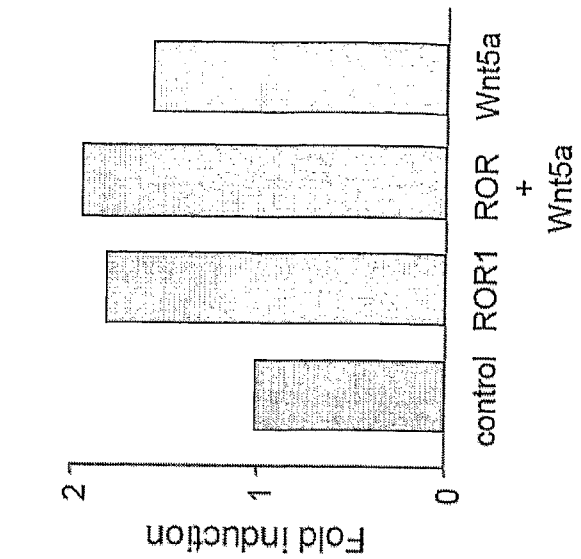
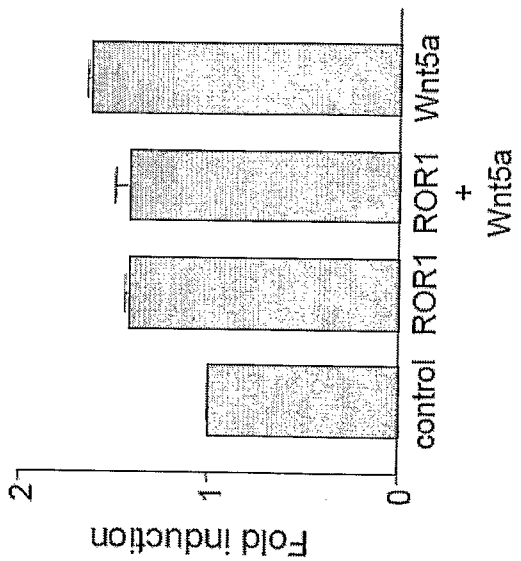


Figure 7a





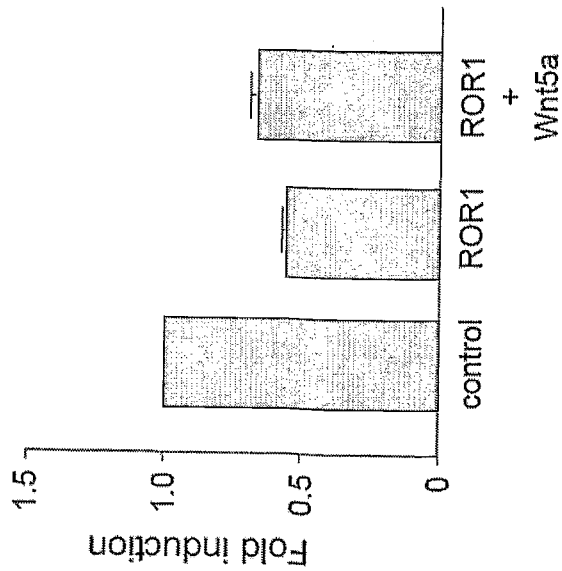


Figure 7b

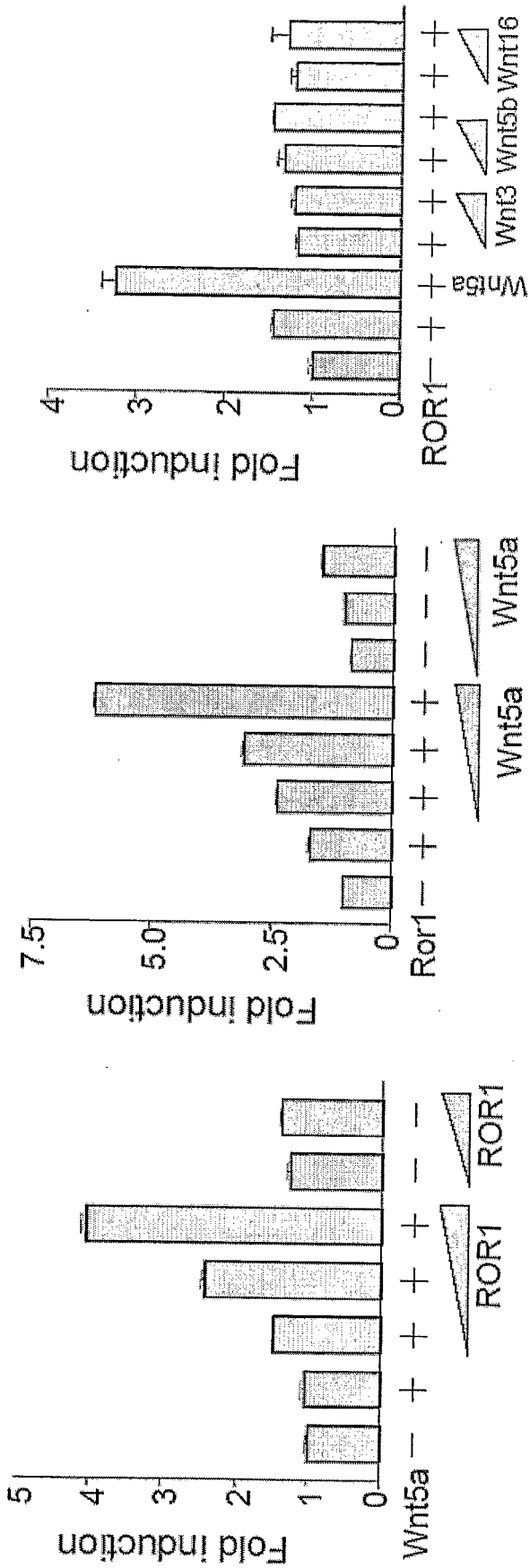


Figure 7c

rabbit IgG	+	+		
ROR1rIg			+	+
HA-Wnt5a	+		+	
pcDNA3		+		+

IP; antiHA
blot; anti-rabbit Ig

IP; proteinA/G
blot; anti-rabbit Ig

IP; anti-HA
blot; anti-HA

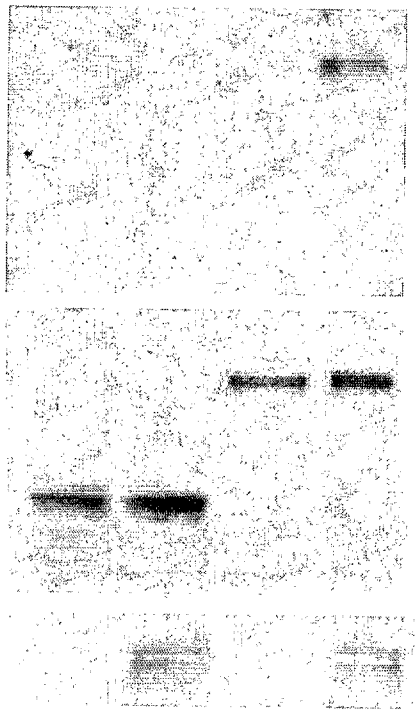
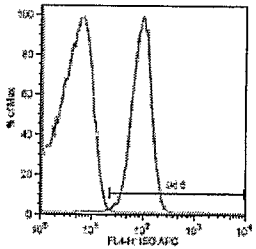
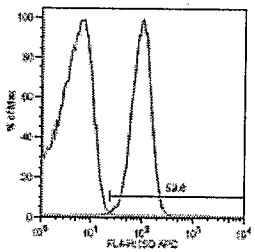


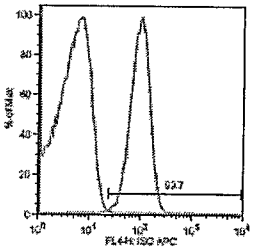
Figure 8

4A5 expression on CLLs (PBMCs)

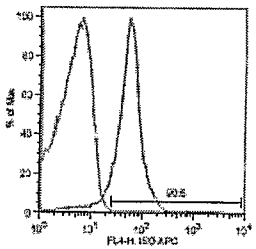
CLL #1

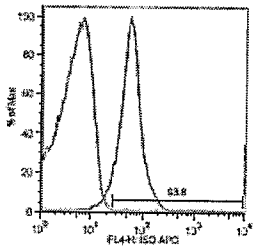


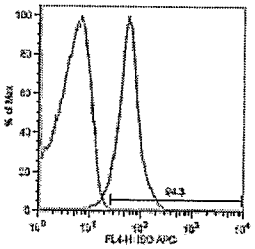




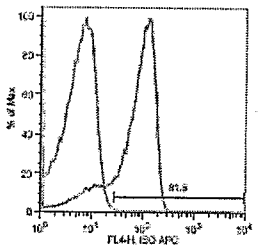
CLL #2

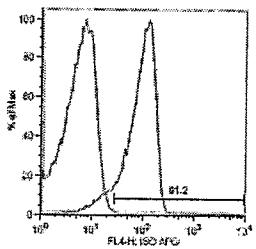


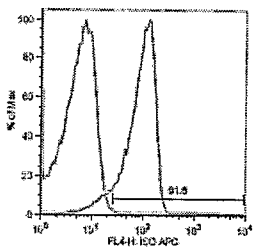




CLL #3

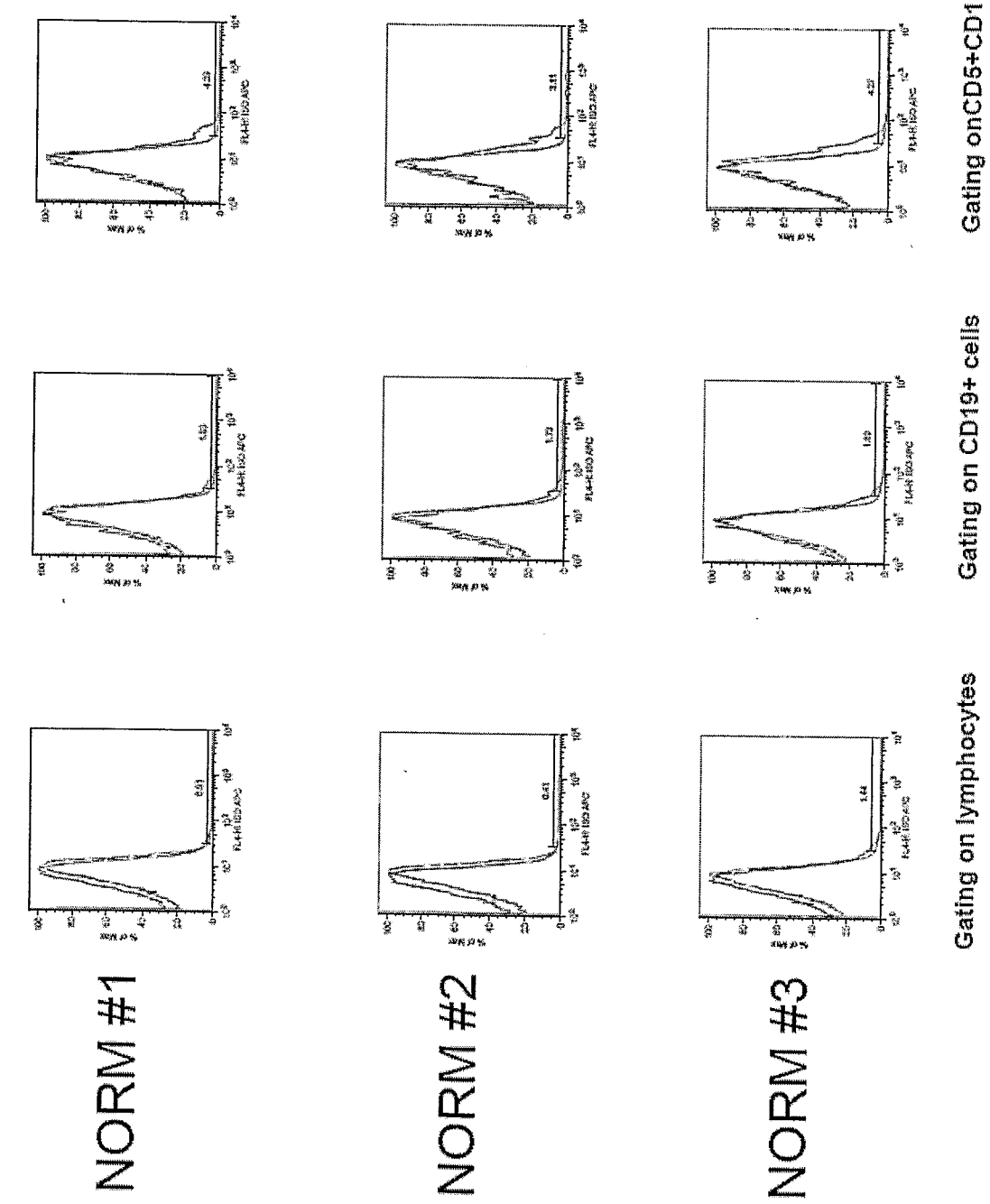






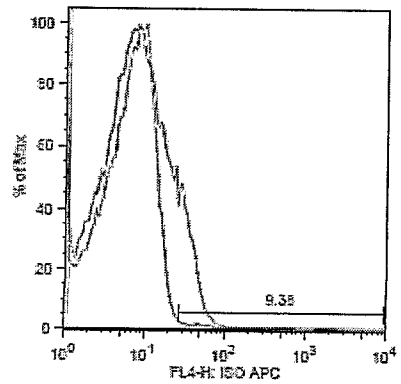
Gating on lymphocytes Gating on CD19+ cells Gating onCD5+CD19+cells

Figure 9

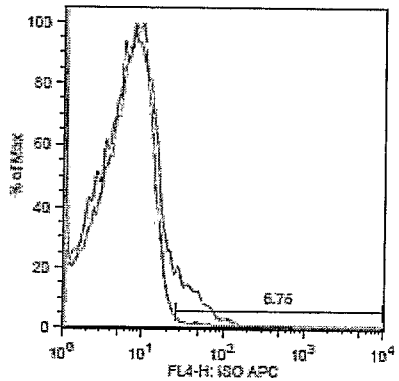


4A5 expression on an "exceptional" Normal (PBMCs)

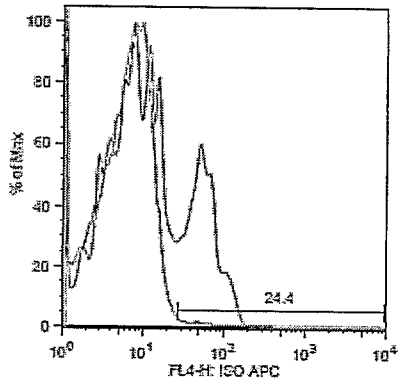
(Example of early detection of leukemic clone in a healthy individual)



Gating on lymphocytes



Gating on CD19+ cells



Gating onCD5+CD19+cells

Figure 10

Figure 11

4A5 expression on CLLs (BM)

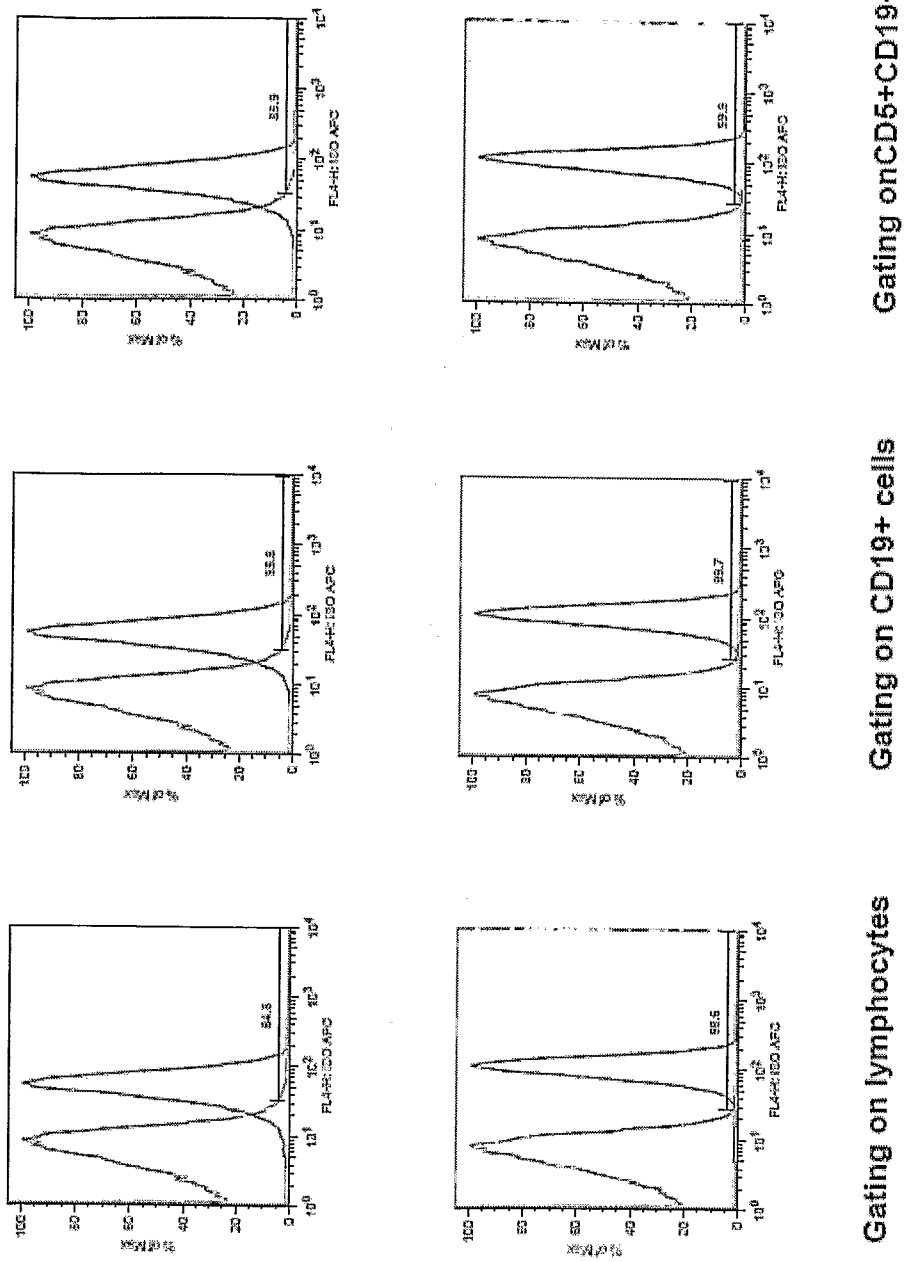
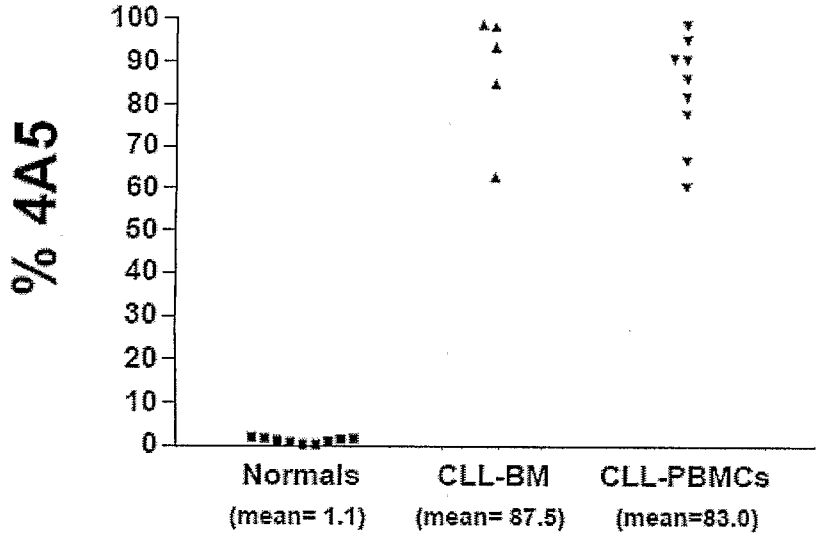
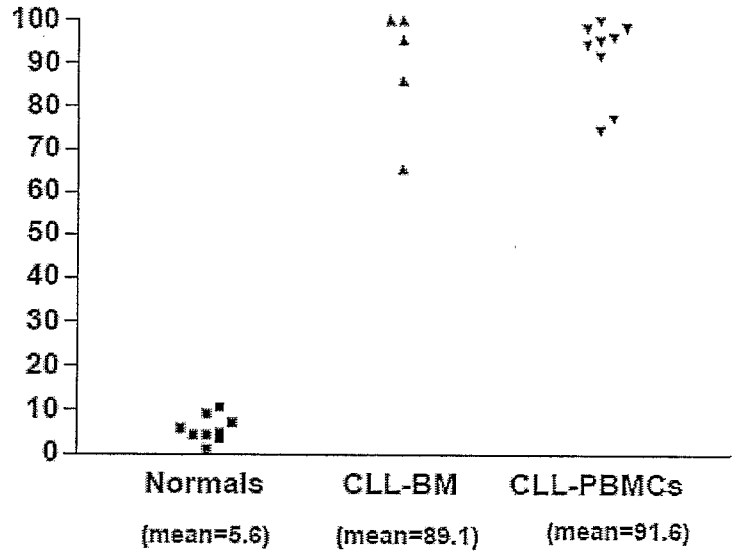


Figure 12

Expression of 4A5 on Normals vs CLLs

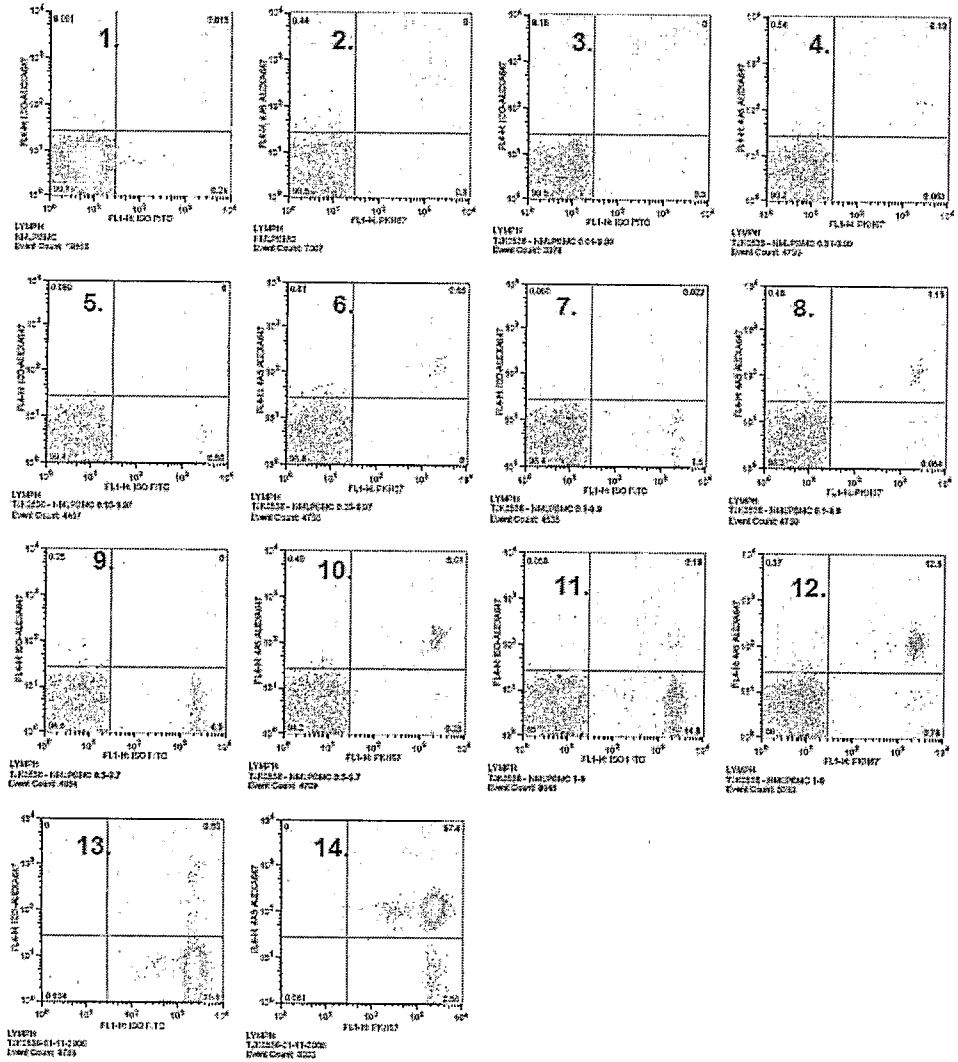


Gating on lymphocytes



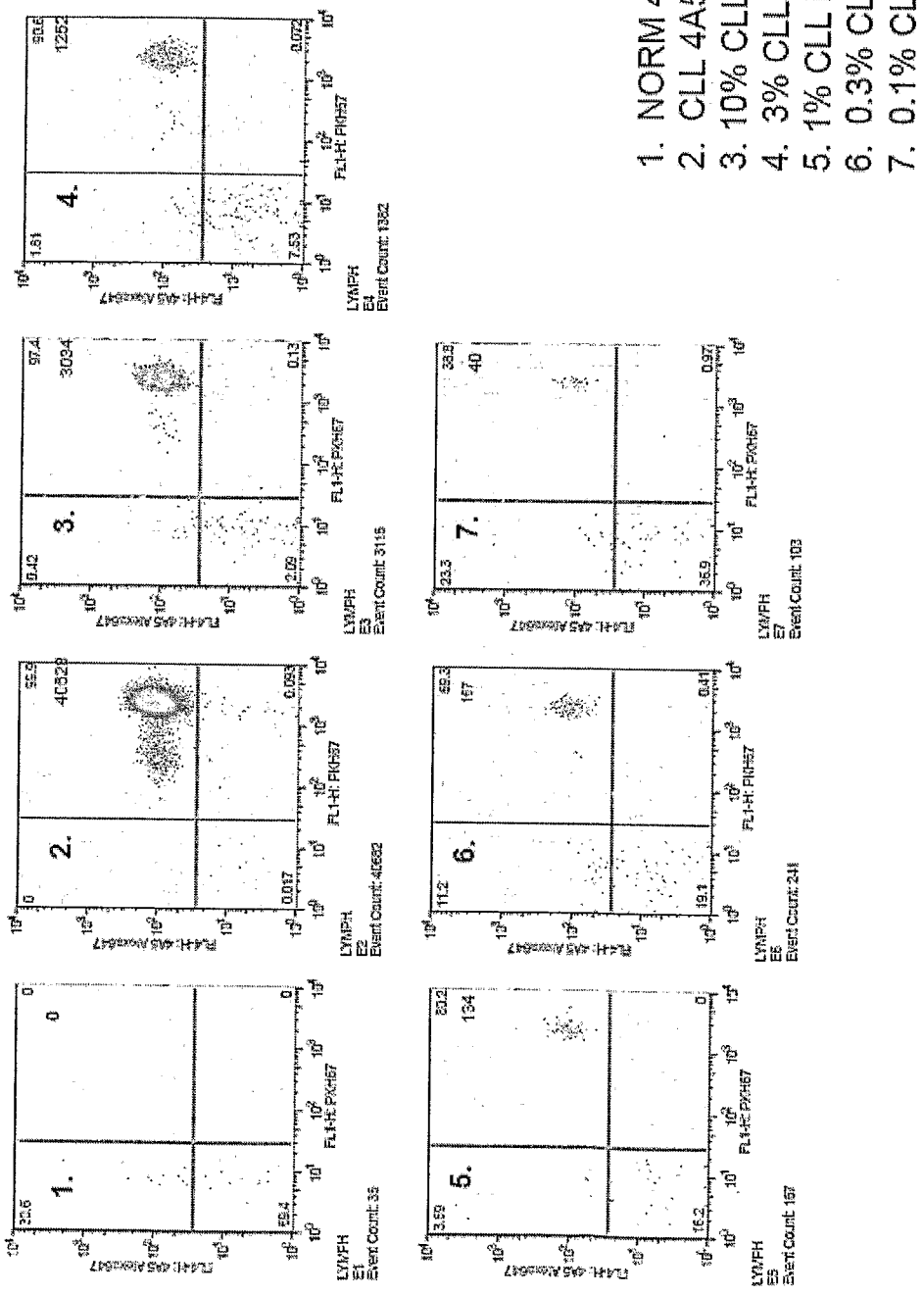
Gating on CD5+CD19+ cells

Figure 13 4A5 expression on titrated CLLcells



1. NORM Iso
2. NORM 4A5
3. 0.1%CLL in NORM Iso
4. 0.1%CLL in NORM 4A5
5. 0.3%CLL in NORM Iso
6. 0.3%CLL in NORM 4A5
7. 1%CLL in NORM Iso
8. 1%CLL in NORM 4A5
9. 3%CLL in NORM Iso
10. 3%CLL in NORM 4A5
11. 10%CLL in NORM Iso
12. 10%CLL in NORM 4A5
13. CLL Iso
14. CLL 4A5

Figure 14 Magnetic Beads Purification



1. NORM 4A5
2. CLL 4A5
3. 10% CLL in NORM 4A5
4. 3% CLL in NORM 4A5
5. 1% CLL in NORM 4A5
6. 0.3% CLL in NORM 4A5
7. 0.1% CLL in NORM 4A5

60021010-0070.ST25.txt
SEQUENCE LISTING

<110> The Regents of the University of California
Kipps, Thomas J
Fukuda, Tetsuya
Endo, Tomoyuki

<120> METHODS AND COMPOUNDS FOR LYMPHOMA CELL DETECTION AND ISOLATION

<130> 60021010-0070

<150> 60/731,210
<151> 2005-10-28

<160> 8

<170> PatentIn version 3.3

<210> 1
<211> 292
<212> DNA
<213> Human

<220>
<221> misc_feature
<222> (169)..(169)
<223> n is a, c, g, or t

<400> 1
gaagtgaaac tggtggagtc tgggggaggc ttagtgaagc ctggagggtc cctgaaactc 60

tcctgtgcag cctctggatt cactttcagt agctatgcca tgtcttgggt tcgccagatt 120

ccagagaaga ggctggagtg ggtcgcatcc attagtcgtg gtggtaccna cctactatcc 180

agacagtgtg aagggccgat tcaccatctc cagagataat gtcaggaaca tcctgtacct 240

gcaaatgagc agtctgaggt ctgaggacac ggccatgtat tactgtggaa ga 292

<210> 2
<211> 15
<212> DNA
<213> Human

<400> 2
tatgattacg acggg 15

<210> 3
<211> 51
<212> DNA
<213> Human

<400> 3
tactatgcaa tggactactg gggtcaagga acctcagtca ccgtctcctc a 51

<210> 4
<211> 284
<212> DNA
<213> Human

<400> 4
gacatcaaga tgacccagtc tccatcttcc atgtatgcat ctctaggaga gagagtcact 60

atcacttgca aggcgagtcc ggacattaat agctatttaa gctggttcca gcagaaacca 120

gggaaatctc ctaagaccct gatctatcgt gcaaacagat tggttgatgg ggtcccatca 180

aggttcagtg gcggtggatc tgggcaagat tattctctca ccatcaacag cctggagtat 240

gaagatatgg gaatttatta ttgtctacag tatgatgaat ttcc 284

<210> 5
<211> 38
<212> DNA
<213> Human

<400> 5
gtacacgttc ggaggggggga ccaagctgga aatgaaac 38

<210> 6
<211> 221
<212> DNA
<213> DNA

<220>
<221> misc_feature
<222> (2)..(2)
<223> n is a, c, g, or t

<400> 6
antttcgtca gactttgacc ggtaaaacca taacattgga agttgaatct tccgatacca 60

tcgacaacgt taagtcgaaa attcaagaca aggaaggtat ccctccagat caacaaagat 120

tgatctttgc cggtaagcag ctagaagacg gtagaacgct gtctgattac aacattcaga 180

aggagtccac cttacatctt gtgctaaggc taagaggtgg t 221

<210> 7
<211> 141
<212> DNA
<213> Human

<400> 7
cacggatccg gagcttggct gttgcccgtc tcactggtga aaagaaaaac caccctggcg 60

cccaatacgc aaaccgcctc tccccgcgcg ttggccgatt cattaatgca gctggcacga 120

caggtttccc gaagcggccg c 141

<210> 8
<211> 2814
<212> DNA
<213> Human

<400> 8
atgcaccggc cgcgccgccg cgggacgcgc ccgccgctcc tggcgctgct ggccgcgctg 60

ctgctggccg cacgcggggc tgctgcccaa gaaacagagc tgtcagtcag tgctgaatta 120
gtgcctacct catcatggaa catctcaagt gaactcaaca aagattctta cctgaccctt 180
gatgaaccaa tgaataacat caccacgtct ctgggccaga cagcagaact gcactgcaaa 240
gtctctggga atccacctcc caccatccgc tggttcaaaa atgatgctcc tgtggtccag 300
gagccccgga ggctctcctt tcggtccacc atctatggct ctcggctgcg gattagaaac 360
ctcgacacca cagacacagg ctacttccag tgcgtggcaa caaacggcaa ggaggtggtt 420
tcttccactg gagtcttgtt tgtcaagttt ggccccctc ccactgcaag tccaggatac 480
tcagatgagt atgaagaaga tggattctgt cagccataca gagggattgc atgtgcaaga 540
tttattggca accgcaccgt ctatatggag tctttgcaca tgcaagggga aatagaaaat 600
cagatcacag ctgccttcac tatgattggc acttccagtc acttatctga taagtgttct 660
cagttcgcca ttccttccct gtgccactat gccttcccgt actgcgatga aacttcatcc 720
gtcccaaagc cccgtgactt gtgtcgcgat gaatgtgaaa tcctggagaa tgtcctgtgt 780
caaacagagt acatttttgc aagatcaaat cccatgattc tgatgaggct gaaactgcca 840
aactgtgaag atctccccca gccagagagc ccagaagctg cgaactgtat ccggattgga 900
attcccatgg cagatcctat aaataaaaat cacaagtgtt ataacagcac aggtgtggac 960
taccggggga ccgtcagtgt gaccaaatca gggcgccagt gccagccatg gaattcccag 1020
tatccccaca cacacacttt caccgccctt cgtttcccag agctgaatgg aggccattcc 1080
tactgccgca acccagggaa tcaaaaggaa gctccctggt gcttcacctt ggatgaaaac 1140
tttaagtctg atctgtgtga catcccagct tgcgattcaa aggattccaa ggagaagaat 1200
aaaatggaaa tcctgtacat actagtgcca agtgtggcca ttcccctggc cattgcttta 1260
ctcttcttct tcatttgcgt ctgtcggaat aaccagaagt catcgtcggc accagtccag 1320
aggcaaccaa aacacgtcag aggtcaaaat gtggagatgt caatgctgaa tgcatataaa 1380
cccaagagca aggctaaaga gctacctctt tctgctgtac gctttatgga agaattgggt 1440
gagtgtgcct ttggaaaaat ctataaaggc catctctatc tcccaggcat ggaccatgct 1500
cagctggttg ctatcaagac cttgaaagac tataacaacc cccagcaatg gatggaattt 1560
caacaagaag cctccctaat ggcagaactg caccacccca atattgtctg ccttctaggt 1620
gccgtcactc aggaacaacc tgtgtgcatg ctttttgagt atattaatca gggggatctc 1680
catgagttcc tcatcatgag atccccacac tctgatgttg gctgcagcag tgatgaagat 1740
gggactgtga aatccagcct ggaccacgga gattttctgc acattgcaat tcagattgca 1800
gctggcatgg aatacctgtc tagtcacttc tttgtccaca aggaccttgc agctcgcaat 1860
attttaatcg gagagcaact tcatgtaaag atttcagact tggggctttc cagagaaatt 1920

tactccgctg attactacag ggtccagagt aagtccttgc tgcccattcg ctggatgccc 1980

cctgaagcca tcatgtatgg caaattctct tctgattcag atatctggtc ctttggggtt 2040

gtcttgtggg agattttcag ttttggactc cagccatatt atggattcag taaccaggaa 2100

gtgattgaga tggtgagaaa acggcagctc ttaccatgct ctgaagactg cccacccaga 2160

atgtacagcc tcatgacaga gtgctggaat gagattcctt ctaggagacc aagatttaaa 2220

gatattcacg tccggcttcg gtcctgggag ggactctcaa gtcacacaag ctctactact 2280

ccttcagggg gaaatgccac cacacagaca acctccctca gtgccagccc agtgagtaat 2340

ctcagtaacc ccagatatcc taattacatg ttcccgagcc agggtattac accacagggc 2400

cagattgctg gtttcattgg cccgccaata cctcagaacc agcgattcat tcccatcaat 2460

ggatacccaa tacctcctgg atatgcagcg tttccagctg cccactacca gccaacaggt 2520

cctcccagag tgattcagca ctgcccacct cccaagagtc ggtccccaag cagtgccagt 2580

gggtcgacta gcactggcca tgtgactagc ttgccctcat caggatccaa tcaggaagca 2640

aatattcctt tactaccaca catgtcaatt ccaaatcatc ctggtggaat gggtatcacc 2700

gtttttggca acaaatctca aaaaccctac aaaattgact caaagcaagc atctttacta 2760

ggagacgcca atattcatgg acacaccgaa tctatgattt ctgcagaact gtaa 2814