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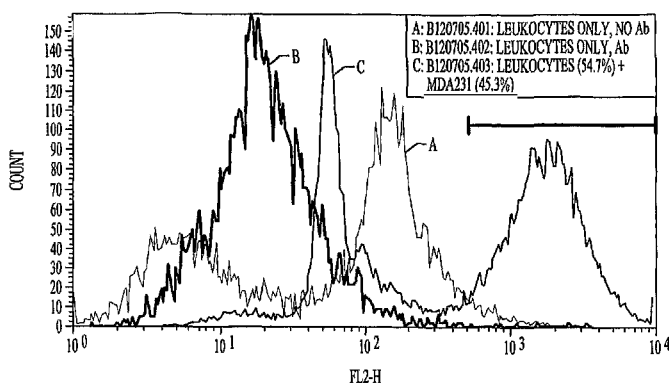
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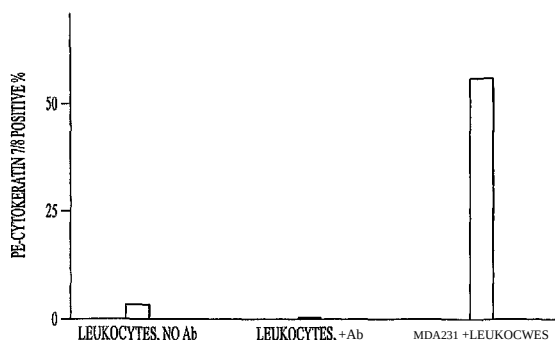
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(54) **Title:** METHOD TO DETECT BREAST CANCER CELLS



A

(57) **Abstract:** A quantitative method to detect variant and total peripheral -type benzodiazepine receptor expression is provided.



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METHOD TO DETECT BREAST CANCER CELLS

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Cross-Reference to Related Applications

This application claims the benefit of the filing date of U.S. application Serial No. 60/763,710, filed January 31, 2006, the disclosure of which is incorporated by reference herein.

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Background

Existing methods to detect breast carcinoma cells in blood are based on the identification and/or determination of expression levels of molecules specifically associated with distinct steps of tumor progression, being positively or negatively correlated with either tumor susceptibility, evaluated by analyzing
15 BRCA1 expression (Mark et al., 1996), the presence of abnormal levels or forms of p53, Bcl-2 or Ki67 (Makris et al., 1997), or the responsiveness to hormone therapy (estrogen receptor positive versus negative tumors) (Makris et al., 1997; Balte et al., 2004; Glinsky et al., 2004), or disease outcomes (HER2/neu expression; Ross et al., 2003). Although the recognition of these molecules has
20 improved tremendously the diagnosis, prognosis, and choice of therapies, none of these factors provides a means for early prediction of metastasis and/or disease recurrence (Coradini et al., 2004; Van Diest et al., 2004).

Several investigators have recently attempted to detect micrometastases in peripheral blood by measuring the expression of several proteins, such as
25 cytokeratin 19 and 20, epidermal growth factor receptor, epithelial glycoprotein-2, carcinoembryonic antigen, maspin, and stanniocalcin-I, as indicators of the presence of tumor cells of diverse origins (Stathopoulou et al., 2002; Stathopoulou et al., 2003; Gradilone et al., 2003). With the exception of cytokeratin 19, where there is some promising although controversial data
30 (Stathopoulou et al., 2002; Schroder et al., 2003; Ismail et al., 2003), the other markers tested failed to provide a consistent and sensitive tool that has a high level of confidence for the diagnosis and prognosis of cancer. Although progress has been made in this field, the absence of a clear marker for detection of

minimal amounts of tumor cells and the lack of a sensitive method to quantify those cells have been limiting factors in the development of such technology.

Thus, what is needed is a sensitive method to detect cancer cells, e.g., breast carcinoma cells.

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Summary of the Invention

The present invention provides a method to detect peripheral-type benzodiazepine receptor (PBR) normal ("wild type") and mutant ("variant") expression in cells in a physiological fluid sample from a mammal, e.g., a blood sample, which may be employed as a bioassay for cancer detection. In one embodiment, the method detects breast cancer cells in a blood sample. The physiologic fluid sample may optionally be subjected to methods that enrich for epithelial cells, which takes advantage of the epithelial nature of breast cancer cells. The enrichment (isolation) of epithelial cells from the sample may be by any suitable method. For instance, to enrich for epithelial cells in blood, a ligand for a cell surface marker on or intracellular marker in epithelial cells, such as cytokeratins, integrins, mucins, or ligands for a combination of epithelial cell markers, which marker(s) is/are absent from hematopoietic cells, may be employed, allowing for isolation of those epithelial cells from millions of blood cells. In another embodiment, a blood sample is contacted with a ligand for a cell surface marker on or intracellular marker in hematopoietic cells, or ligands for a combination of hematopoietic cell markers, which marker(s) is/are absent from epithelial cells. The complexes formed between the ligand and hematopoietic cells are separated from the sample, yielding a composition that is preferentially substantially-free of hematopoietic cells and enriched in nonhematopoietic cells. . In one embodiment, cells are fixed, e.g., fixed with ethanol, prior to or after contact with a ligand. In one embodiment, the level of both wild type and variant PBR mRNA species in cells in the isolated epithelial cells or composition that is substantially-free of hematopoietic cells is detected or determined, for example, using a very sensitive nucleic acid amplification method. The method permits the detection of variant PBR mRNA species carrying a single base mutation as compared to wild type PBR mRNA. As described below, in validation and standardization experiments using known

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numbers of breast cancer cells and normal blood cells mixed at different proportions, tumor cells were detected at a 1 in 2,000,000 ratio using this method.

In one embodiment, an antibody against cytokeratins 7/8 is employed to
5 isolate epithelial cells including breast tumor cells potentially present in blood. In one embodiment, cells that bind antibody are isolated by flow cytometry. RNA of cytokeratin-positive cells is optionally isolated, and analyzed for quantification of total PBR RNA and expression of missense polymorphism PBR variants (e.g., for two amino acid substitutions, Ala147Thr and
10 His162Arg).

The same method may also be employed in the detection, diagnosis and prognosis of several types of cancer such as those that express elevated levels of PBR as compared to their normal tissue counterparts, including glioma, colon carcinoma, and prostate and ovarian cancers. In one embodiment, the cancer is
15 lymphoma, prostate cancer, colon cancer, brain cancer, e.g., glioma, stomach carcinoma, lung carcinoma, ovarian carcinoma, pancreatic adenocarcinoma, endometrial carcinoma, renal cell adenocarcinoma, bone sarcoma or bone carcinoma

The invention thus provides clinicians with a new and practical
20 prognostic/diagnostic tool for breast and other cancers that allows an easy evaluation of both progression and outcome of the disease directly from blood samples. Indeed, PBR wild type and variant mRNA species represent sensitive biomarkers of an aggressive phenotype for human breast cancer. The detection of the cells in the peripheral blood from patients permits oncologists to closely
25 follow the outcome of treatment and to identify early on any recurrence of the disease, enabling them to better predict disease outcome and to choose the most appropriate treatment.

Accordingly, the invention provides a method to detect cancer cells in a sample. The method includes contacting a mammalian biological, e.g.,
30 physiological fluid, sample and a ligand specific for epithelial cells so as to yield a composition comprising complexes comprising epithelial cells and the ligand. The complexes are isolated, thereby isolating epithelial cells, and the level of PBR RNA expressed in the isolated epithelial cells is detected or determined.

Further provided is a method to detect cancer cells in a sample. The method includes contacting a mammalian biological, e.g., physiological fluid, sample and a ligand specific for hematopoietic cells, and separating the complexes having hematopoietic cells and ligand from non-complexed molecules. In one embodiment, an affinity column is employed and the flow-through fraction collected. The level of PBR RNA in that fraction is then detected or determined.

Also provided is a method to detect cancer cells in a physiological fluid sample. The method includes detecting or determining levels of PBR RNA in epithelial cells isolated from a mammalian blood sample.

Furthermore, a method of diagnosing metastatic breast cancer is provided. The method comprises detecting or determining the expression of PBR in epithelial cells in a mammalian physiological fluid sample.

The invention also provides a method to detect progression of disease or monitor therapy in a mammal. The method includes comparing PBR expression in cells in a physiological fluid sample of a mammal having a disease and optionally subjected to therapy for that disease from two or more periods of time. An increase in PBR expression over time in a mammal may be indicative of disease progression, e.g., cancer progression. A decrease in PBR expression over time in a mammal subjected to therapy may be indicative of efficacy.

Brief Description of the Figures

Figure 1 depicts Q-PCR standard curves of standard (wild type) and variant (mutant) PBR mRNA expression in breast cancer cells (BCC).

Figure 2 depicts a validation of the efficiency of PBR detection by Q-PCR. Top panels: cells, COS7 (kidney SV40-transformed simian) cells (498,800 cells), TM4 (mouse Sertoli) cells (2,333,800 cells), Hepal-6 (mouse hepatoma) cell (534,900 cells) or leukocytes isolated from 2 healthy volunteers (BD10204 and BD10196, about 10 million cells each) were mixed with either 10 μ l (55,440 cells) or 100 μ l (554,400 cells) of MDA231 BCC. Several samples were also prepared from MDA231 alone, corresponding to final values of MDA231 cells per well of either 944 (n1); 1,207 (n9) or 1,618 (n8). Bottom

panels: the indicated numbers of MDA231 cells were mixed with 1,010 non-BCC cells (TM4 + Hepal-6).

Figure 3 shows an immunoreaction of MDA231 cells with phycoerythrin-coupled anti-cytokeratin (CK) 7/8 antibody.

5 Figure 4 depicts a flow cytometry analysis of MDA231BCC mixed with non BCC cells using PE-anti-CK7/8 antibody.

Figure 5 shows a flow cytometry analysis of MDA231 BCC mixed with normal human blood cells using PE-anti-CK7/8 antibody.

Figure 6 depicts Q-PCR results for variant and total PBR expression in colon and breast cancer tissues. Frozen samples from tumoral biopsies from colon (CT) and breast (BR) tissues were analyzed. Results are the average $\pm$ sem of the relative expression levels of 5-6 samples per condition, each measured in triplicate.

15 Detailed Description of the Invention

Definitions

A "physiological fluid sample" includes blood, urine, cerebrospinal fluid, duct fluid, a bronchial aspirate, or any other physiological fluid that may contain cells.

20 A "blood sample" as used herein includes blood cells, such as lymphocytes or other white blood cells, or blood fractions, and cells removed from bone marrow.

"Providing a biological sample or physiological fluid sample" means to obtain a sample for use in the methods described in this invention. Most often, this will be done by removing a sample of cells (tissue biopsy) or physiological fluid from a patient, but can also be accomplished by using previously isolated cells.

A "biological sample" refers to a sample of material obtained from an organism, e.g., it can be a physiological sample, such as one from a human patient, a laboratory mammal such as a mouse, rat, pig, monkey or other member of the primate family, containing a cell or population of cells including a quantity of tissue or fluid from a mammal. Most often, the sample is removed from a mammal, but the term "biological sample" but may also refer to cells or

tissue analyzed *in vivo*, i.e., without removal from a mammal. For instance, the sample may be obtained by drawing a blood sample, sputum sample, spinal fluid sample, a urine sample, or providing a culture of such a sample. Ordinarily, the biological sample will contain hybridizable polynucleotides. These

5 polynucleotides may have been released from cells in the biological sample, using techniques such as sonic disruption or enzymatic or chemical lysis of cells to release polynucleotides so that they are available for amplification with one or more polynucleotide primers or hybridization with a polynucleotide probe.

A "control sample" refers to a sample of biological material
10 representative of a healthy, cancer-free mammal. The level of a target nucleic acid or other cellular molecule, e.g., protein, in a control sample is desirably typical of the general population of normal, cancer-free mammals. This sample can be removed from a patient expressly for use in the methods described in this invention, or can be any biological material representative of normal, cancer-free
15 mammals, including cancer-free biological material taken from a mammal with cancer elsewhere in its body. A control sample can also refer to an established level of a target that is representative of the cancer-free population, i.e., a level that has been previously established based on measurements from normal, cancer-free mammals.

20 As used herein, the term "isolated" refers to *in vitro* preparation, separation and/or purification of nucleic acid, ligand-receptor complexes, or a particular cell type, from other substances that it is normally associated with *in vitro* or other natural setting. Thus "isolated" cells may be separated from non-cellular material or from another type of cell, "isolated" nucleic acid maybe
25 separated from at least component that it is normally associated with in a cell, and "isolated" complexes of ligand-receptor may be separated from non complexed molecules or complexes formed with a different ligand.

"Detecting a level of a marker" refers to determining the expression level of a gene or genes encoding a target polypeptide. Gene expression can be
30 monitored in a variety of ways, including by detecting mRNA levels, protein levels, or protein activity, any of which can be measured using standard techniques. Detection can involve quantification of the level of, e.g., cDNA,

mRNA, protein, or enzyme activity, or, alternatively, can be a qualitative assessment of the level of a target.

To "compare" levels of markers means to detect marker levels in two samples and to determine whether the levels are equal or if one or the other is greater. A comparison can be done between quantified levels, allowing statistical
5 comparison between the two values, or in the absence of quantification, for example using qualitative methods of detection such as visual assessment by a human.

The phrase "detecting metastatic cancer" refers to the ascertainment of
10 the presence or absence of micrometastases of a certain cancer in a mammal at a site distant from the primary tumor. "Detecting a metastatic cancer" can also refer to obtaining indirect evidence regarding the likelihood of the presence of metastatic cancerous cells in the mammal.

A "ligand" is a molecule that specifically binds one or more other
15 molecules ("receptor"). For instance, an antibody binds an antigen, biotin binds streptavidin, a His tag binds metals, e.g., nickel, maltose binding protein binds maltose, and glutathione S transferase binds glutathione.

As used herein, the term "antibody" refers to a protein having one or more polypeptides substantially encoded by immunoglobulin genes or fragments
20 of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad of immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG,
25 IgM, IgA, IgD and IgE, respectively. An antibody may be labeled with a detectable moiety or may be attached to a solid support, e.g., a column, sepharose, a well of a tissue culture plate, or a magnetic bead.

The basic immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of
30 polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H)

refer to these light and heavy chains respectively. The variable region of an antibody binds a particular ligand, and the Fc portion binds to Fc receptors on certain cells.

Antibodies may exist as intact immunoglobulins, or as modifications in a variety of forms including, for example, FabFc₂, Fab, Fv, Fd, (Fab')₂, an Fv fragment containing only the light and heavy chain variable regions, a Fab or (Fab')₂ fragment containing the variable regions and parts of the constant regions, a single-chain antibody, e.g., scFv, complimentary determining region (CDR)-grafted antibodies and the like. The heavy and light chain of a Fv may be derived from the same antibody or different antibodies thereby producing a chimeric Fv region. The antibody may be of animal (especially mouse or rat) or human origin or may be chimeric or humanized. As used herein the term "antibody" includes these various forms.

Humanized antibodies are chimeric molecules of Igs, Ig chains or fragments thereof from two or more sources, one of which is a human source, which are further altered in primary sequence to reduce non-human Ig sequences and/or to increase sequences corresponding to those found in human antibodies, e.g., human Ig consensus sequences. Humanized antibodies include residues that form a complementary determining region (CDR) in the Fv region that are from a CDR of a non-human species such as mouse, rat or rabbit having desired properties, e.g., specificity and/or affinity for a particular antigen. In general, a humanized antibody includes substantially all of at least one, and typically two, variable domains, in which all or substantially all of the sequences in the CDR regions correspond to those of non-human Ig sequences and all or substantially all of the framework regions correspond to human Ig sequences, such as human Ig consensus sequences. Replacement of non-human residues to a corresponding human residue, human residues to a corresponding consensus residue, non-human residues to a corresponding consensus residue, or human residues to a corresponding non-human residue, are based on comparisons of human Ig sequences or comparisons of human Ig sequences with non-human Ig sequences, such as rat, mouse and monkey Ig sequences, using conserved residues between species for alignment but allowing for insertions and/or deletions. Methods for

humanizing non-human or chimeric antibodies and aligning antibody sequences are well known in the art.

A "nucleotide" is a subunit of a nucleic acid comprising a purine or pyrimidine base group, a 5-carbon sugar and a phosphate group. The 5-carbon
5 sugar found in RNA is ribose. In DNA, the 5-carbon sugar is 2'-deoxyribose. The term also includes analogs of such subunits, such as a methoxy group (MeO) at the 2' position of ribose.

An "oligonucleotide" is a polynucleotide having two or more nucleotide subunits covalently joined together. Oligonucleotides are generally about 10 to
10 about 100 nucleotides in length, or more preferably 10 to 50 nucleotides in length. The sugar groups of the nucleotide subunits may be ribose, deoxyribose, or modified derivatives thereof. The nucleotide subunits may be joined by linkages such as phosphodiester linkages, modified linkages or by non-nucleotide moieties that do not prevent hybridization of the oligonucleotide to its
15 complementary target nucleotide sequence. Modified linkages include those in which a standard phosphodiester linkage is replaced with a different linkage, such as a phosphorothioate linkage, a methylphosphonate linkage, or a neutral peptide linkage. Nitrogenous base analogs also may be components of oligonucleotides in accordance with the invention. Ordinarily, oligonucleotides
20 will be synthesized by organic chemical methods and will be single-stranded unless specified otherwise. Oligonucleotides can be labeled with a detectable moiety.

A "target nucleic acid" is a nucleic acid comprising a target nucleic acid sequence.

25 A "target nucleic acid sequence," "target nucleotide sequence" or "target sequence" is a specific deoxyribonucleotide or ribonucleotide sequence that can be hybridized by an oligonucleotide. In the methods of the invention, the target nucleic acid sequence is a PBR sequence. As used herein, a nucleic acid is a "PBR nucleic acid sequence" if the overall identity of the nucleic acid sequence
30 to the nucleic acid sequences encoding PBR is greater than about 80%, even more preferably greater than about 85% and most preferably greater than 90% relative to PBR sequences such as those having accession numbers NM009775, NM0073 11, NM00714, BC002055, AL 031778, which are incorporated by

reference herein, and the like, or amino acid sequences encoded thereby.

Sequence identity may be determined using standard techniques known in the art, including, but not limited to, the algorithm of Smith & Waterman, Adv. Appl. Math., 2:482 (1981); Needleman & Wunsch, J. Mol. Biol., 48:443 (1970);

- 5 and Pearson & Lipman, PNAS USA, 85:2444 (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.), the Best Fit sequence program described by Devereux et al., Nucl. Acid Res., 12:387 (1984), preferably using the default
- 10 settings, or by inspection. One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol., 35:35 1-360 (1987); the method is similar to that described by Higgins &
- 15 Sharp, CABIOS, 5:151(1989). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

- Another example of a useful algorithm is the BLAST algorithm, described in Altschul et al., J. Mol. Biol., 215, 403 (1990) and Karlin et al.,
- 20 PNAS USA, 90:5873-5787 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., Methods in Enzymology, 266:460 (1996). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span=1, overlap fraction=0.125, word
- 25 threshold (T)=1. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity. A % amino acid sequence identity value is determined by
- 30 the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

A "primer" is a single-stranded polyoligonucleotide that combines with a complementary single-stranded target to form a double-stranded hybrid, which primer in the presence of a polymerase and appropriate reagents and conditions, results in nucleic acid synthesis.

5 A "probe" is a single-stranded polynucleotide that combines with a complementary single-stranded target polynucleotide to form a double-stranded hybrid. A probe may be an oligonucleotide or a nucleotide polymer, and may contain a detectable moiety which can be attached to the end(s) of the probe or can be internal to the sequence of the probe. The nucleotides which combine
10 with the target polynucleotide need not be strictly contiguous as may be the case with a detectable moiety internal to the sequence of the probe.

A "detectable moiety" is a label molecule attached to, or synthesized as part of, a polynucleotide probe or a ligand. This moiety should be uniquely detectable and will allow the probe or ligand to be detected or isolated as a
15 result. By "labeled" herein is meant that a compound has at least one element, isotope, e.g., radioactive or heavy isotopes, or chemical compound, e.g., antibody, antigen or colored or fluorescent dye, attached thereto allowing for the detection and or isolation of the compound. For example, the label may be capable of producing, either directly or indirectly, a detectable signal. The
20 detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{36}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Detectable moieties thus include but are not limited to radioisotopes, colorimetric, fluorometric or chemiluminescent
25 molecules, enzymes, haptens, redox-active electron transfer moieties such as transition metal complexes, metal labels such as silver or gold particles, or unique oligonucleotide sequences.

A "hybrid" is the complex formed between two single-stranded polynucleotide sequences by Watson-Crick base pairings or non-canonical base
30 pairings between the complementary bases. By "nucleic acid hybrid" or "probe:target duplex" is meant a structure that is a double-stranded, hydrogen-bonded structure, preferably 10 to 100 nucleotides in length, more preferably 10 to 50 nucleotides in length. The structure is sufficiently stable to be detected by

means such as chemiluminescent or fluorescent light detection, colorimetry, autoradiography, electrochemical analysis, gel electrophoresis and the like. Such hybrids include RNA:RNA, RNA:DNA, or DNA:DNA duplex molecules.

"Hybridization" is the process by which two complementary strands of polynucleotide combine to form a stable double-stranded structure ("hybrid complementarity" is a property conferred by the base sequence of a single strand of DNA or RNA which may form a hybrid or double-stranded DNA:DNA, RNA:RNA or DNA:RNA through hydrogen bonding between Watson-Crick base pairs on the respective strands). Adenine (A) ordinarily complements thymine (T) or uracil (U), while guanine (G) ordinarily complements cytosine (C).

The term "stringency" is used to describe the temperature and solvent composition existing during hybridization and the subsequent processing steps. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two polynucleotide strands forming a hybrid. Stringency conditions are chosen to maximize the difference in stability between the hybrid formed with the target and the non-target polynucleotides.

The term "probe specificity" or "primer specificity" refers to a characteristic of a probe or primer which describes its ability to distinguish between target and non-target sequences. Probe or primer specificity is dependent on sequence and assay conditions and may be absolute (i.e., the primer or probe can distinguish between nucleic acid from target organisms and any non-target organisms), or it may be functional (i.e., the primer or probe can distinguish between the nucleic acid from a target organism and any other organism normally present in a particular sample).

"Polynucleotide" means either RNA or DNA, along with any synthetic nucleotide analogs or other molecules that may be present in the sequence and that do not prevent hybridization of the polynucleotide with a second molecule having a complementary sequence. The term includes polymers containing

analog of naturally occurring nucleotides and particularly includes analogs having a methoxy group at the 2' position of the ribose (MeO).

"T_m" refers to the temperature at which 50% of the probe or primer is converted from the hybridized to the unhybridized form.

5 One skilled in the art will understand that probes or primers that substantially correspond to a reference sequence or region can vary from that reference sequence or region and still hybridize to the same target nucleic acid sequence. Probes of the present invention substantially correspond to a nucleic acid sequence or region if the percentage of identical bases or the percentage of perfectly complementary bases between the probe and its target sequence is from 10 100% to 80% or from 0 base mismatches in a 10 nucleotide target sequence to 2 bases mismatched in a 10 nucleotide target sequence. In one embodiment, the percentage is from 100% to 85%. In another embodiment this percentage is from 90% to 100%; and in yet other embodiments, this percentage is from 95% 15 to 100%. Probes or primers that substantially correspond to a reference sequence or region include probes or primers having any additions or deletions which do not prevent the probe or primer from having its property, such as being able to preferentially hybridize under high stringency hybridization conditions to its target nucleic acid over non-target nucleic acids.

20 By "sufficiently complementary" or "substantially complementary" is meant nucleic acids having a sufficient amount of contiguous complementary nucleotides to form a hybrid that is stable for detection or to initiate nucleic acid synthesis.

By "anti-sense" is meant a nucleic acid molecule perfectly 25 complementary to a reference (i.e., sense) nucleic acid molecule.

"RNA and DNA equivalents" refer to RNA and DNA molecules having the same complementary base pair hybridization properties. RNA and DNA equivalents have different sugar groups (i.e., ribose versus deoxyribose), and may differ by the presence of uracil in RNA and thymine in DNA. The 30 difference between RNA and DNA equivalents do not contribute to differences in substantially corresponding nucleic acid sequences because the equivalents have the same degree of complementarity to a particular sequence.

As will be appreciated by those in the art, nucleic acids or other molecules, e.g., antibodies, can be attached or immobilized to a solid support in a wide variety of ways. By "immobilized" is meant the association or binding between the nucleic acid or ligand and the solid support is sufficient to be stable
5 under the conditions of binding, washing, and analysis. The binding can be covalent or non-covalent. By non-covalent binding herein is meant one or more of either electrostatic, hydrophilic, and hydrophobic interactions. By covalent binding is meant that the two moieties, e.g., the solid support and the antibody, are attached by at least one bond. Covalent bonds can be formed directly or can
10 be formed by a cross linker or by inclusion of a specific reactive group on either the solid support, the nucleic acid or other molecule, or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

By "substrate" or "solid support" is meant any material that can be
15 modified to contain discrete individual sites appropriate for the attachment or association of nucleic acid or other molecule, e.g., ligand for a specific cell type, and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or functionalized glass, plastics (including
20 acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, TeflonJ, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, etc. In general, the substrates allow optical detection and do not
25 appreciably fluoresce.

PBR and Cancer

PBR was originally described as a high affinity peripheral receptor for the benzodiazepine diazepam (Valium™), and was further shown to bind compounds such as the isoquinolines. PBR is present at low levels in most
30 tissues but at high levels in steroidogenic tissues, such as adrenal, testis, ovary, and glia (Li et al., 2001; Lacapere et al., 2001). PBR is primarily located in the outer mitochondrial membrane, is a functional component of the steroidogenic machinery, and its role appears to be at the level of cholesterol delivery from the

outer to the inner mitochondrial membrane, the rate-limiting step in steroidogenesis (Papadopoulos, 1993; Papadopoulos et al., 1990). Molecular modeling, crosslinking experiments, and *in vitro* reconstitution studies demonstrated that the 18 kDa PBR protein is a high affinity cholesterol-binding protein (Culty et al., 1999; Li et al., 2001; Lacapere et al., 2001). During studies on the structure and function of PBR, it was observed that the receptor is highly expressed in testicular, adrenocortical, and brain glial tumor cells and that PBR drug ligands, such as benzodiazepines, regulated their proliferation (Gamier et al., 1993). At the same time, PBR expression was shown to correlate with the high metastatic ability of gliomas and astrocytomas (Miettinen et al., 1995; Cornu et al., 1992). Moreover, PBR ligands were shown to affect the proliferation of various tumor cells such as glioma cells, astrocytomas and lymphoma cells (Ikezaki et al., 1990; Neary et al., 1995). Higher levels of PBR were also found in colonic adenocarcinoma and ovarian carcinoma as compared to normal tissue (Katz et al., 1990a; Katz et al., 1990b). Moreover, a 12-fold increase in PBR density as compared to normal tissue was found in human brain glioma or astrocytoma (Miettinen et al., 1995; Cornu et al., 1992). In that study, the authors suggested that studying PBR densities with positron or gamma-ray emitting PBR ligands may help for the clinical investigation and detection of human brain proliferative diseases. Indeed, the radiolabeled isoquinoline [¹¹C]PKI 1195 PBR ligand was used for imaging human gliomas in conjunction with positron emission tomography (Pappata et al., 1991). Recent studies have reported the use of antitumoral drugs acting via PBR for the treatment of pancreas and brain tumors (Ratcliffe et al., 1995; Kupczyk-Subotkowska et al., 1997). Moreover, PBR-negative Leydig tumor cells, generated by PBR gene disruption using homologous recombination, have a higher doubling time than control cells (Papadopoulos et al., 1997) suggesting a role of PBR in cell proliferation.

The examination of the expression, characteristics, localization, and function of PBR in a battery of human breast cancer cell lines differing in their invasive and chemotactic potential, as well as in several human tissue biopsies, revealed that the expression of PBR ligand binding and mRNA was dramatically increased in the highly aggressive cell lines, such as MDA-MB-231 (MDA-231),

relative to non-aggressive cell lines, such as MCF-7 (Hardwick et al., 1999). Pharmacological characterization of PBR in MDA-231 cells indicated that the receptor is similar to previously described human PBR. Addition of high affinity PBR drug ligands to MDA-231 cells increased the incorporation of
5 bromodeoxyuridine into the cells, in a dose-dependent biphasic manner, suggesting a role for PBR in the regulation of MDA-231 cell proliferation. However, high micromolar concentrations of these compounds inhibited cell proliferation and ultimately killed the cells (Hardwick et al., 1999). In these studies, PBR was localized at the perinuclear and nuclear area. Additional
10 studies demonstrated that, in agreement with its widespread mitochondrial localization, the perinuclear localization is in mitochondria. Despite all these similarities, nucleotide sequencing of the PBR in aggressive breast tumor cells (MDA-231 and HS-578-T) identified two DNA sequence variations, compared to PBR present in non-tumor cells, resulting in the replacement of an alanine
15 residue at position 147 with a threonine residue (Ala147Thr) and a histidine residue at position 162 with an arginine residue (His162Arg) (Hardwick et al., 2002). The genetic status of the PBR gene in PBR-enriched MDA-231 cells, and MCF-7 cells, which contains low levels of PBR was investigated using both DNA (Southern) blot and fluorescence *in situ* hybridization (FISH) analyses, and
20 revealed that the PBR gene is amplified by 4-fold in MDA-MB-231 relative to MCF-7 cells (Hardwick et al., 2001). These unexpected data suggested that PBR gene amplification may be an important indicator of breast cancer progression.

The role of PBR in breast cancer was further demonstrated in a series of experiments where a cell line expressing low levels of PBR and behaving as a
25 benign tumor cell line (tetracycline-repressible MCF-7 cell line: MCF-7 Tet-Off) was transfected with human PBR cDNA, to look at the consequences of increasing PBR in these cells. Consecutively, a cell line normally expressing high PBR levels (MDA-MB-231) was incubated with small interfering RNAs (siRNAs) targeting human PBR to suppress PBR expression. In both cases,
30 radioligand binding assays were performed to assess PBR activity, and protein and mRNA PBR levels were determined. As predicted, the increase of PBR in MCF-7 cells, which is normally non-aggressive and expresses extremely low PBR levels, induced both an increase in PBR ligand binding and cell

proliferation (data not shown). On the other hand, the transfection of MDA-MB-231 cells with PBR-siRNAs targeting different sites of PBR mRNA led to different levels of mRNA and protein knockdown that was associated with decreased cell proliferation, and with increased levels of the cyclin-dependent protein kinase inhibitor p21^{WAF/CIP1} which is known to be involved in tumor-suppression (data not shown). Collectively, these results indicate that PBR protein expression is directly involved in regulating cell proliferation in human breast cancer cells, probably by influencing a mechanism involved in cell cycle control. In conclusion, these data suggested that PBR expression is part of the sequence of events involved in human breast cancer cell proliferation and aggressive phenotype expression (Papadopoulos, 1997). These findings have been now confirmed by other research groups (Beinlich et al., 2002; Sanger et al., 2002; Galiegue et al., 2004).

Exemplary Methods of the Invention

The present invention provides novel methods for diagnosis and prognosis evaluation for cancers, in particular cancers associated with PBR expression and those that are optionally epithelial in nature, such as breast cancer, as well as methods for screening for compositions which modulate those cancers. The identification of sequences that are differentially expressed in cancer tissue versus normal tissue, as well as differential expression resulting in different prognostic outcomes, allows the use of this information in a number of ways. For example, the evaluation of a particular treatment regime may be evaluated: does a chemotherapeutic drug act to improve the long-term prognosis in a particular patient. Similarly, diagnosis may be done or confirmed by comparing PBR expression in a patient sample with the known expression profiles. Furthermore, PBR gene expression profiles allow screening of drug candidates with an eye to mimicking or altering a particular expression profile; for example, screening can be done for drugs that suppress the PBR expression profile or convert a poor prognosis profile to a better prognosis profile.

In one embodiment, the method includes positive or negative selection by either enriching for epithelial cells or removing hematopoietic cells. For instance, to enrich for epithelial cells, antibodies against cytokeratins, integrals or mucins either alone or as a cocktail may be employed. To remove

hematopoietic cells, hematopoietic cell ligands may be employed. For example, cells are passed through an affinity chromatography column that retains blood cells. Cells may be fresh (no fixation) or fixed, e.g., ethanol fixed, to preserve RNA integrity which may allow ligands such as antibodies to reach intracellular targets. Thus, compositions enriched for epithelial cells (enriched by positive or negative selection), or samples prior to enrichment, may optionally be treated with fixatives.

Once cells are contacted with an epithelial cell- or hematopoietic cell-specific ligand, ligand-receptor complexes are sorted or separated, for instance from noncomplexed molecules, by FACS, affinity column chromatography, by batch separation (pull down), e.g., using protein A/G-agarose or magnetic beads, or any other suitable means. For example, an antibody specific for epithelial cells maybe labeled with a metal and cells that bind the antibody separated from other cells using magnetic beads.

In one embodiment, cell sorting is performed by flow cytometry, using fluorescent-labeled antibodies, either on fresh (surface marker) or ethanol-fixed (intracellular marker) cells. For positive selection using FACS of epithelial cell markers such as cytokeratins, integrins, and mucins, a cocktail of antibodies specific for two or more epithelial cell markers coupled to a single type of fluorescence may be employed prior to quantification of and/or typing of PBR mRNAs, e.g., by Q-PCR. Alternatively, the initial enrichment step can be performed using a fluorescent PBR ligand (7-nitro-2, 1, 3-benzoxandiazol-4-yl derivative of 2-phenylindole-3-acetamide; compound 4), allowing for labeling of breast carcinoma cells at a much higher intensity signal than blood cells (which express much less PBR). The PBR-rich cell population may be sorted on FACS prior to quantification of and/or typing of PBR mRNAs, for instance, by Q-PCR. In another embodiment, cells are captured on 96 well plates using antibody-coated plates (for cell surface markers) or magnetic bead-coupled antibody (for intracellular markers). These approaches allow for epithelial-type cells to become anchored onto the wells and the hematopoietic cells or other contaminants washed away. Subsequent RNA isolation and/or Q-PCR may be conducted in the same plates or after transfer of an aliquot to another receptacle.

Oligonucleotide Primers and Probes

First, the stability of the oligonucleotide -target polynucleotide hybrid is chosen to be compatible with the assay conditions. This may be accomplished by avoiding long A and T rich sequences, by terminating the hybrids with G:C
5 base pairs and by designing the probe in such a way that the T_m will be appropriate for standard conditions to be employed in the assay (amplification or hybridization). The nucleotide sequence of the primer or probe should be chosen so that the length and % G and % C result in a probe having a T_m about 2 to 10°C higher than the temperature at which the final assay is performed. The
10 base composition of the primer or probe is significant because G:C base pairs exhibit greater thermal stability when compared with A:T base pairs. Thus, hybrids involving complementary polynucleotides having a high G:C content are generally stable at higher temperatures when compared with hybrids having a lower G:C content.

15 Second, the position at which the primer or probe binds its target polynucleotide is chosen to minimize the stability of hybrids formed between probe:non-target polynucleotides. This may be accomplished by minimizing the length of perfect complementarity with polynucleotides of non-target sequences, by avoiding G:C rich regions of homology with non-target sequences, and by
20 positioning the primer or probe to span as many destabilizing mismatches as possible. Whether a primer or probe sequence is useful for amplifying or detecting a specific gene depends largely on thermal stability differences between probe:target hybrids and probe:non-target hybrids. The differences in
25 T_m should be as large as possible to produce highly specific primers and probes.

The length of the target polynucleotide sequence and the corresponding length of the primer or probe sequence also are important factors to be considered when designing a primer or probe. While it is possible for polynucleotides that are not perfectly complementary to hybridize to each other, the longest stretch of perfectly homologous base sequence is ordinarily the
30 primary determinant of hybrid stability.

Third, regions which are known to form strong internal structures inhibitory to hybridization of a primer or probe are less preferred as targets.

Primers or probes having extensive self-complementarity also should be avoided.

Defined oligonucleotides that can be used to practice the invention can be produced by any of several well-known methods, including automated solid-phase chemical synthesis using phosphoramidite precursors. Other well-known methods for construction of synthetic oligonucleotides may, of course, be employed. All of the oligonucleotides of the present invention may be modified with chemical groups to enhance their performance. Backbone-modified oligonucleotides, such as those having phosphorothioate or methylphosphonate groups, are examples of analogs that can be used in conjunction with oligonucleotides of the present invention. These modifications render the oligonucleotides resistant to the nucleolytic activity of certain polymerases or to nuclease enzymes. Other analogs that can be incorporated into the structures of the oligonucleotides include peptide nucleic acids, or "PNAs." The PNAs are compounds comprising ligands linked to a peptide backbone rather than to a phosphodiester backbone. Representative ligands include either the four main naturally occurring DNA bases (i.e., thymine, cytosine, adenine or guanine) or other naturally occurring nucleobases (e.g., inosine, uracil, 5-methylcytosine or thiouracil) or artificial bases (e.g., bromothymine, azaadenines or azaguanines, etc.) attached to a peptide backbone through a suitable linker. PNAs are able to bind complementary ssDNA and RNA strands. Methods for making and using PNAs are disclosed in U.S. Patent No. 5,539,082. Another type of modification that can be used to make oligonucleotides having the sequences described herein involves the use of non-nucleotide linkers (e.g., see U.S. Patent No. 6,031,091) between nucleotides in the nucleic acid chain which do not interfere with hybridization or optionally elongation of a primer.

Yet other analogs include those which increase the binding affinity of a probe to a target nucleic acid and/or increase the rate of binding of the probe to the target nucleic acid relative to a probe without the analog. Such analogs include those with a modification (substitution) at the *T* position of a ribofuranosyl nucleotide. Analogues having a modification at the *T* position of the ribose are one embodiment. Other substitutions at the 2' position of the sugar are expected to have similar properties so long as the substitution is not so large as

to cause steric inhibition of hybridization. Thus, hybridization assay probes can be designed to contain modified nucleotides which, alone or in combination, may have the advantage of increasing the rate of target-specific hybridization.

Preferably, probes are labeled. Essentially any labeling and detection
5 system that can be used for monitoring specific nucleic acid hybridization can be used in conjunction with the probes disclosed herein when a labeled probe is desired. Included among the collection of useful labels are: radiolabels, enzymes, haptens, linked oligonucleotides, colorimetric, fluorometric, e.g., 6-carboxyfluorescein (FAM), carboxytetramethylrhodamine (TAMRA), or VIC
10 (Applied Biosystems), or chemiluminescent molecules, and redox-active moieties that are amenable to electrochemical detection methods. In one embodiment, probes are labeled at one end with a reporter dye and with a quencher at the other end, e.g., reporters including FAM, 6-tetrachlorofluorescein (TET), MAX (Synthegen), Cy5 (Synthegen), 6-carboxy-
15 X-rhodamine or 5(6)-carboxy-X-rhodamine (ROX), and TAMRA and quenchers including TAMRA, BHQ (Biosearch Technologies) and QSY (Molecular Probes). Standard isotopic labels that can be used to produce labeled oligonucleotides include ^3H , ^{35}S , ^{32}P , ^{125}I , ^{57}Co and ^{14}C . When using radiolabeled probes, hybrids can be detected by autoradiography, scintillation
20 counting or gamma counting.

Non-isotopic materials can also be used for labeling oligonucleotide probes. These non-isotopic labels can be positioned internally or at a terminus of the oligonucleotide probe. Modified nucleotides can be incorporated enzymatically or chemically with modifications of the probe being performed
25 during or after probe synthesis, for example, by the use of non-nucleotide linker groups. Non-isotopic labels include colorimetric molecules, fluorescent molecules, chemiluminescent molecules, enzymes, cofactors, enzyme substrates, haptens or other ligands. For instance, U.S. Patent No. 5,998,135 discloses yet another method that can be used for labeling and detecting probes using
30 fluorimetry to detect fluorescence emission from lanthanide metal labels disposed on probes, where the emission from these labels becomes enhanced when it is in close proximity to an energy transfer partner. Exemplary electrochemical labeling and detection approaches are disclosed in U.S. Patent

Nos. 5,591,578 and 5,770,369, and PCT/US98/12082, the disclosures of which are hereby incorporated by reference. Redox active moieties useful as electrochemical labels include transition metals such as Cd, Mg, Cu, Co, Pd, Zn, Fe and Ru. Indeed, any number of different non-isotopic labels can be used for preparing labeled oligonucleotides in accordance with the invention. For example, a probe may contain more than one label.

Alternative procedures for detecting particular genes can be carried out using either labeled probes or unlabeled probes. For example, hybridization assay methods that do not rely on the use of a labeled probe are disclosed in U.S. Patent No. 5,945,286 which describes immobilization of unlabeled oligonucleotide probe analogs made of peptide PNAs, and detectably labeled intercalating molecules which can bind double-stranded PNA probe/target nucleic acid duplexes. In these procedures, as well as in certain electrochemical detection procedures, such as those disclosed in PCT/US98/12082, PCT/US98/12430 and PCT/US97/20014, the oligonucleotide probe is not required to harbor a detectable label.

Amplification and Hybridization

Amplification or hybridization assays may be performed either in tubes or in microtitration plates having multiple wells. For assays in plates, the wells may be coated with the specific amplification primers or probes and/or control DNAs, and the detection of amplification products or the formation of hybrids may be automated. Hybridization assays may also be performed on a solid substrate.

Amplification

Cells may be subjected to conditions which release polynucleotides from the cells, thus forming an extract. For example, cells may be treated with detergents, base and/or heat denatured. If the base is employed, the mixture is then neutralized with an acidic composition. Then reagents are added to yield an amplification reaction (containing, for example, monovalent ions, detergent, dNTPS, primers, and a polymerase).

For DNA amplification by the widely used PCR (polymerase chain reaction) method, primer pairs may be derived from sequenced DNA fragments from clinical samples or from data bank sequences. Prior to synthesis, the

potential primer pairs may be analyzed by using the program Oligo™ 4.0 (National Biosciences) to verify that they are likely candidates for PCR amplifications. A select set of primers can then be tested in PCR or other amplification-based assays performed directly from a bacterial suspension or a
5 known standard to determine their specificity.

During DNA amplification by PCR, two oligonucleotide primers binding respectively to each strand of the denatured double-stranded target DNA are used to amplify exponentially *in vitro* the target DNA by successive thermal cycles allowing denaturation of the DNA, annealing of the primers and synthesis
10 of new targets at each cycle. An exemplary PCR protocol is as follows. A nucleic acid containing sample may be added directly to a 50 µL PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl₂, 0.4 µM of each of the two primers, 200 µM of each of the four dNTPs and 1.25 Units of Taq DNA polymerase (Perkin Elmer). PCR reactions are then subjected
15 to thermal cycling (3 minutes at 95°C followed by 30 cycles of 1 second at 95°C and 1 second at 55°C) using a Perkin Elmer 480™ thermal cycle and subsequently analyzed by standard ethidium bromide-stained agarose gel electrophoresis. It is clear that other methods for the detection of specific amplification products, which may be faster and more practical for routine
20 diagnosis, may be used. Such methods may be based on the detection of fluorescence after amplification (e.g. TaqMan™ system from Perkin Elmer or Amplisensor™ from Biotronics) or other labels such as biotin (SHARP Signal™ system, Digene Diagnostics), or liquid or solid phase hybridization with an oligonucleotide probe binding to internal sequences of the specific amplification
25 product, e.g., a labeled probe. Methods based on the detection of fluorescence are very rapid and quantitative, and can be automated. For instance, one of the amplification primers or an internal oligonucleotide probe specific to the amplicon(s) is coupled with the fluorochrome or with any other label. Moreover, methods based on the detection of fluorescence are particularly
30 suitable for diagnostic tests since they are rapid and flexible as fluorochromes emitting different wavelengths are available (Perkin Elmer). Further, a variety of fluorochromes emitting at different wavelengths, each coupled with a specific oligonucleotide linked to a fluorescence quencher which is degraded during

amplification, thereby releasing the fluorochrome (e.g., TaqManTM, Perkin Elmer), may be employed.

To assure PCR efficiency, glycerol or dimethyl sulfoxide (DMSO) or other related solvents, may be used to increase the sensitivity of the PCR and to overcome problems associated with the amplification of target with a high GC content or with strong secondary structures. The concentration ranges for glycerol and DMSO are 5 to 15% (v/v) and 3 to 10% (v/v), respectively. For the PCR reaction mixture, the concentration ranges for the amplification primers and the MgCl₂ maybe about 0.1 to 1.0 and 1.5 to 3.5 mM, respectively.

10 Modifications of the standard PCR protocol using external and nested primers (i.e., nested PCR) or using more than one primer pair (i.e., multiplex PCR) may also be used.

The person skilled in the art of DNA amplification knows the existence of other rapid amplification procedures which include linear amplification procedure, e.g., ligase chain reaction (LCR), transcription-based amplification systems (TAS), self-sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA) and branched DNA (bDNA). The scope of this invention is not limited to the use of amplification by PCR, but rather includes the use of any rapid nucleic acid amplification methods or any other procedures which may be used to increase rapidity and sensitivity of the tests. Any oligonucleotides suitable for the amplification of specific nucleic acid sequences by approaches other than PCR and within scope of this invention. Standard precautions to avoid false positive PCR results should be taken. Methods to inactivate PCR amplification products such as the inactivation by uracil-N-glycosylase may be used to control PCR carryover.

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Alternatively, amplification may be performed as described above but using a "hot start" protocol. In that case, an initial reaction mixture containing the target DNA, primers and dNTPs was heated to about 85°C prior to the addition of the other components of the PCR reaction mixture. The final concentration of all reagents was as described above. Subsequently, the PCR reactions were submitted to thermal cycling and analysis as described above.

30

Heat treatments of the lysates, prior to DNA amplification, using the thermocycler or a microwave oven may also be performed to increase the efficiency of cell lysis. PCR has the advantage of being compatible with crude DNA preparations. Thus, samples such as blood, cerebrospinal fluid and sera
5 may be used directly in PCR assays, e.g., after a brief heat treatment.

Hybridization

In hybridization experiments, oligonucleotides (of a size less than about 100 nucleotides) have some advantages over DNA fragment probes of greater than 100 nucleotides in length for the detection of bacteria such as ease of
10 preparation in large quantities, consistency in results from batch to batch and chemical stability. The oligonucleotide probes may be derived from either strand of the target duplex DNA. The probes may consist of the bases A, G, C, or T or analogs thereof. In one embodiment, the target DNA is denatured, fixed onto a solid support and hybridized with a DNA probe. Conditions for pre-
15 hybridization and hybridization can be as follows: (i) pre-hybridization in 1 M NaCl+10% dextran sulfate+1% SDS (sodium dodecyl sulfate)+1 μ g/ml salmon sperm DNA at 65°C for 15 minutes, (ii) hybridization in fresh pre-hybridization solution containing the labeled probe at 65°C overnight, and (iii) post-
hybridization including washing twice in 3 X SSC containing 1% SDS (1 X SSC
20 is 0.15 M NaCl, 0.015 M NaCitrate) and twice in 0.1 X SSC containing 0.1% SDS; all washes at 65°C for 15 minutes. For probes labeled with radioactive labels, the detection of hybrids is preferably by autoradiography. For non-
radioactive labels, such as probes having colorimetric, fluorescent or chemiluminescent labels, target DNA need not be fixed onto a solid support.

25 For example, stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate (SSC); 0.1% sodium lauryl sulfate (SDS) at 50°C, or (2) employ a denaturing agent such as formamide during hybridization, e.g., 50% formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1%
30 polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution,

sonicated salmon sperm DNA (50 μ g/ml), 0.1% sodium dodecylsulfate (SDS), and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1 X to 2 X SSC (20 X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to IX SSC at 55 to 60°C. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65°C for 15 minutes. Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3.

In one embodiment, more than one probe per sequence is used, with overlapping probes, probes to different portions of the target or probes to different strands of the target, being used. That is, two, three, four or more probes, may be used to build in a redundancy for a particular target.

The invention will be further described by the following non-limiting examples.

Example 1

Materials and Methods

Blood collection and treatment

Blood samples are collected from breast cancer patients in tubes containing ethylenediaminetetraacetate (EDTA) as anticoagulant, and put on ice immediately. Red blood cell lysis is performed on total human peripheral blood with 1x RBS lysis buffer (eBioscience, San Diego, CA) by adding 10 mL of lysis buffer per 1 mL of human blood, and incubating for 10 minutes at room temperature. The reaction is stopped by diluting the sample with 20-30 mL of 1x PBS, spinning the cells (300-400 g) at 4°C for 10 minutes, then resuspending the pellet in 1X PBS.

Human breast cancer cell enrichment by flow cytometry

Prior to sorting, cells submitted for flow cytometric analysis are fixed in 2 mL of 70% ethanol and stored at -20°C until needed. Cells are collected by centrifugation at 1500 *g* for 3 minutes, washed with 1 mL 1x PBS₅ and re-

5 centrifuged. An antibody specific for human cytokeratins 7 and 8 and conjugated to the fluorochrome phycoerythrin (PE-Ab) is then used to select cells expressing cytokeratin 7/8 and isolate them from leukocytes, following the manufacturer recommendations (BD Biosciences, San Jose, CA). Briefly, the cell pellet is resuspended with 20 µL of antibody and incubated at RT for 30

10 minutes, diluted with 1 mL 1x PBS, and incubated an additional 10 minutes to allow unbound antibody to diffuse out of the cells. Cells are then collected by centrifugation at 1500 *g* for 3 minutes. The supernatant is removed, and the pellet is resuspended in 200 µL 1x PBS for cell sorting on flow cytometry apparatus. Flow cytometry is performed on a FACS equipment, and cell

15 doublets are eliminated by performing side scatter and forward scatter profile. The fraction containing cytokeratin-positive cells is further processed for RNA analysis.

RNA isolation and reverse transcription

Total RNA is isolated from cytokeratin 7/8 positive-cell fractions

20 obtained by flow cytometry using RNeasy Mini kit according to the manufacturer's specifications, and using On-Column DNase I digestion with the RNase-free DNase set (QIAGEN) to remove genomic DNA contamination from the RNA sample. RNA concentrations are measured using a spectrophotometer (260/280 nm). Control blood samples are collected from healthy volunteers and

25 processed in the same fashion as described above, to be used as negative controls in the Q-PCR method described below. TaqMan reverse transcription reagents are from Applied Biosystems (Foster City, CA). For reverse transcription, 1 µg total RNA was incubated in 1x TaqMan RT buffer, 5.5 mM MgCl₂, 500 µM dNTP, 2.5 µM random hexamers, 1.25 to 3.125 U/µL MultiScribe™ reverse

30 transcriptase, 0.4 U/µl RNase inhibitor, in a total volume of 50 µL. The reaction mixture is sequentially incubated at 25°C for 10 minutes, either 37°C or for 60 minutes or 48°C for 30 minutes, and 95°C for 5 minutes. The resulting cDNA is

then processed for amplification using specific primer sets designed according to *Primer Express* guidelines.

Real-time Quantitative PCR TO-PCR)

Q-PCR is performed using the ABI PRISM 7700 Sequence Detection
 5 System (Applied Biosystems). Following reverse-transcription, the resulting cDNA is processed for amplification of the PBR gene using specific primers and probes for both standard and variant (missense polymorphism) PBRs. Each sample is run in triplicate. For PBR Q-PCR, 2 µL cDNA, 12.5 µL TaqMan Universal PCR Master Mix (2X) (Applied Biosystems), 450 nM forward primer,
 10 450 nM reverse primer, 100 nM PBR147 probe#1 and #2, or 100 nM PBR1 62 probe#1 and #2, and nuclease-free water are added to a final volume of 25 µL. The reaction mixture is incubated in the ABI PRISM 7700 sequence detector (Applied Biosystems) at 95°C for 10 minutes, and 92°C(15 seconds)/60°C (1 minute) for 40 cycles, successively.

15 The following forward and reverse primers (5'-3') are used for detecting the DNA sequence variant G/ACG at codon 147: PBR 490F5'-CGCCTGCTCT ACCCCT ACCT-3¹(SEQ ID NO:1), PBR 557R5'-TCCCGCCATACGCAGTAGTT-S¹(SEQ ID NO:2), PBR1 47 probe#1 5'-FAMTMdye-labeled -CCTTCGCGACCACA-MGBNFQ-S¹(SEQ ID NO:3),
 20 PBR147 probe#2 5'-VIC[®]-dye-labeled -TGGCCTTCACGACC- MGBNFQ-3' (SEQ ID NO:4).

The following forward and reverse primers (5'-3') are used for detecting the DNA sequence variant CG/AG at codon 162: PBR 546 F 5'-CGTATGGCGGGACAACCAT-3' (SEQ ID NO:5), and PBR 660 R 5'-
 25 CCACCACATCAC AAGCGTGAT-3¹(SEQ ID NO:6), PBR1 62 probe#1 5'-FAMTMdye-labeled-TGGCAGCCGCGGT- MGBNFQ-3' (SEQ ID NO:7), and PBR1 62 probe#2 5'-VIC[®]-dye-labeled-CTGGC AGCTGCGGT- MGBNFQ-3' (SEQ ID NO:8).

MGB (Minor Groove Binder) attachment probe enables the use of shorter
 30 fluorescent probes and results in improved mismatch discrimination. The MGB probe shows better performance both in producing higher signal when the probe matches the template and in producing negligible signal when there is single base mismatch between probe and template. A non-fluorescent quencher (NFQ)

is used to reduce the complexity of the calculation and improve the determination precision of reporter dye contributions. The main steps in the reaction sequence are polymerization, strand displacement and cleavage. A fluorescent reporter (R) and a quencher (Q) are attached to the fluorogenic probe. When both reporter and quencher are attached to the probe, reporter dye emission is quenched. During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe. Once separated from the quencher, the reporter dye fluoresces. This results in an increase in fluorescence intensity proportional to the accumulation of PCR product. The presence of a mismatch between probe and target destabilized probe binding during strand displacement, reduces the efficiency of probe cleavage. ROX, a rhodamine derivative, is present in the buffer solution as a passive reference label.

The amount of PBR mutation mRNA expression is normalized to the endogenous reference (18S rRNA) which is run separately. The following forward and reverse primers (5'-3') are used for 18S Q-PCR: 5' CCAGTAAGTGCGGGTCAT 3' (SEQ ID NO:9) and 5' CCAATCGGTAGTAGCGACGG 3' (SEQ ID NO: 10) for 18S rRNA. For 18S reference, the forward/reverse primers final concentration are 250 nM, 2 µl cDNA is used as template, 10 µL SYBR® Green PCR Master Mix(Applied Biosystems), nuclease-free water are added to a final volume of 20 µL. Each sample is run in triplicate. The reaction mixture is incubated in the ABI PRISM 7700 sequence detector (Applied Biosystems) at 50°C for 2 minutes, 95°C for 10 minutes, and 95°C(15 seconds)/60°C (1 minute) for 40 cycles, successively. Direct detection of the PCR products is achieved by measuring the increase in fluorescence caused by the binding of SYBR Green I Dye to double-stranded (ds) DNA. The comparative Ct method is used to analyze the 18S data.

Q-PCR Standard Curve

A standard curve is established using serial dilutions of the MDA-MB-231 human breast cancer cells (that express high levels of wild type and variant PBR), mixed with blood cells from healthy volunteers. The standard curve is calculated using linear regression analysis and has been shown to display a linear relationship between Ct values and the logarithm of the initial number of positive MDA-MB-231. The dynamic range of the standard curve spanned at least six

orders of magnitude. The amount of product in a particular sample is determined by interpolation from a standard curve of Ct values generated from MDA-MB-231 cells dilution series. In all runs, a standard curve is obtained using the same samples of RNA from dilutions of MDA-MB-231 cells in healthy donor

5 leukocytes ($1:10$; $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, respectively), with a total of 1×10^6 cells. Results obtained with patient blood are compared to those obtained for the standard curve (representing a known number of MDA-MB-231 cells in a total number of 1×10^6 leukocytes).

Results

10 Wild type and variant forms of PBR have been shown to be highly expressed in aggressive human breast tumor cells (Hardwick et al., 1999; Hardwick et al., 2001; Hardwick et al., 2002; Han et al., 2003), and the abundance of PBR in malignant breast tumor biopsies as compared to benign tumor and normal tissues was found to be due to PBR gene amplification and
15 increased gene transcription (Hardwick et al., 1999). Moreover, nucleotide sequencing identified two missense polymorphisms, Alal46Thr and Hisl62Arg in PBR in breast tumor cells and in biopsies of breast tumors (Hardwick et al., 1999). By contrast, human blood cells were found to express only wild type PBR at very low levels and did not express variant PBR forms. To determine
20 whether the expression levels of PBR wild type and variant forms in blood samples could be a sensitive way to detect the presence of breast carcinoma cells or other cancers in peripheral blood, a PBR Q-PCR based assay was employed.

Figure 1 is a Q-PCR standard curve of standard and variant PBR mRNA levels in MDA231 cells. PBR standard curves were calculated using linear
25 regression analysis of Q-PCR reactions performed on serial dilutions of MDA231 human BCC. The standard curves displayed a linear relationship between Ct values and the logarithm of the initial number of positive MDA231 cells. The data also showed that one can detect the presence of one BCC cell per well using this sensitive method.

30 To validate the efficiency of PBR detection by Q-PCR, levels of wild type and variant PBR expression in MDA231 BCC mixed with various cell lines or leukocytes from healthy volunteers, were determined by Q-PCR. Cells from various sources were mixed with MDA231 cells. RNA was isolated from the

cell mixtures and extracts corresponding to cell numbers ranging from 750 to 1,629 cells per well were analyzed by Q-PCR. The results showed that the levels of variant PBR measured were proportional to the number of BCC cells present in the samples, confirming it as a reliable BCC marker (Figure 2).

- 5 Moreover, variant PBR determination was found to be a good parameter to use independently of the levels of standard PBR present in the non-BCC cells.

The efficiency of anti-CK7/8 antibody recognition of MDA231 cells was first tested by performing immunohistological reactions of these BCC either alone or mixed with blood cells or MA10 mouse Leydig cells using a CK7/8
10 antibody coupled to the fluorochrome phycoerythrin (Figure 3). The results showed that MDA231 cells were positively stained by the antibody, while cells from hematopoietic or mesenchymal origin were negative, confirming the usefulness of this antibody as an epithelial (BCC) cell enrichment tool.

MDA231 cells were mixed at a ratio of 0.2 to 100% with 1.6 million
15 MA-10 Leydig cells. The mixture (1.6 to 5 million cells per sample) was incubated with PE-anti-CK7/8 antibody and fluorescent-positive cells were sorted on a FACS apparatus. Results (Figure 4) show that the number of CK-positive cells sorted by FACS was proportional to the number of MDA231 present in the original sample, thus confirming the efficiency of the method in
20 sorting BCC from non-BCC cells.

In another flow cytometry analysis, MDA231 cells were mixed at a ratio of 45:55 with leukocytes from healthy volunteers. The mixture was incubated with PE-anti-CK7/8 antibody and fluorescent-positive cells were sorted on FACS apparatus. Data showed that the number of CK-positive cells correlated
25 with the number of BCC and that FACS sorted efficiently epithelial and non-epithelial cells in separate fractions (Figure 5).

Summary

The method described herein employs the epithelial nature of breast tumor cells to increase their proportion in a sample of patient blood prior to
30 measurement of PBR wild type and variant mRNAs as a way to enhance the sensitivity of the method and allow the detection of one tumor cell in 2 million blood cells. Any ligand which preferentially bind epithelial cells over hematopoietic cells, e.g., a cytokeratin 7/8 antibody, can be used to enrich for

(isolate) breast tumor cells from the blood cells. Cells which bind ligand can be isolated by any means, for instance, by flow cytometry. Once isolated, those cells are screened for PBR species as tumor cell markers. This approach is possible because hematopoietic cells contain much lower levels of wild type PBR than breast cancer cells, and none or undetectable levels of variant PBR. Using this approach, it was found that the presence of a few circulating aggressive breast tumor cells amongst millions of normal hematopoietic cells could be detected using relatively simple techniques.

Example 2

Methods

Total RNA was extracted from the tissues and contaminating genomic DNA removed, and cDNA was synthesized using random hexamers and TaqMan reverse transcription reagents and reverse transcriptase. Q-PCR was performed on an ABI PRISM 7700 Sequence Detection System, using two TaqMan MGB probes for detecting alleles and specific primers and probes for both standard and variant (missense polymorphism) PBR. The amounts of standard and variant PBR mRNAs were normalized to an endogenous reference (18S rRNA) which was run separately. The primers and probes for detecting the variant G/ACG at codon 147 were: forward primer 5'-CGCCTGCTCTACCCCT ACCT-3' (SEQ ID NO: 11); reverse primer 5'-TCCCGCCATACGCAGTAGTT-S¹(SEQ ID NO:12); probe#1 5'- FAMTMdye-labeled -CCTTCGCGACCACA-MGBNFQ-3'; PBR147 probe#2 5'- VIC[®]dye-labeled -TGGCCTTCACGACC- MGBNFQ-3'. The primers and probes for detecting the variant CG/AG at codon 162 were: forward primer 5'-CGTATGGCGGGACAACCAT-3' (SEQ ID NO: 13); reverse primer 5'-CCACCACATCACAAGCGTGAT-3' (SEQ ID NO:14); probe#1 5-FAMTMdye-labeled-TGGCAGCCGCCGT-MGBNFQ-3'; probe#2 5'-VIC[®]-dye-labeled-CTGGCAGCTGCCGT-MGBNFQ-3'.

Results

RNA was extracted from frozen samples from six colon tumor biopsies and five breast tumor biopsies and analyzed for the presence and relative amount of wild-type and variant codon 147 PBR mRNA (Figure 6). Generally, PBR

expression was elevated in the majority of the colon tumor biopsy samples and all of the breast tumor biopsy samples. Variant PBR RNA was expressed in all colon tumor samples and highly expressed in 2/6 colon tumor samples.

Moreover, variant PBR RNA was expressed in all breast tumor samples and
 5 highly expressed in 1/5 breast tumor samples. In 2 of the 3 samples where variant PBR RNA was highly expressed, the relative expression of variant PBR was greater than that of wild-type PBR.

References

- 10 Balfe P, McCann A, McGoldrick A, McAllister K, Kennedy M, Dervan P, Kerin MJ. Estrogen receptor alpha and beta profiling in human breast cancer. *Eur J Surg Oncol* 2004; 30:469-474.
- Beinlich, A.; Strohmeier, R.; Kaufmann, M.; Kuhl, H Relation of cell proliferation to expression of peripheral benzodiazepine receptors in
 15 human breast cancer cell lines. *Biochem Pharmacol.* 2002;60, 397-402.
- Canat X, Carayon P, Bouaboula M, Cahard D, Shire D, Roque C, Le Fur G, Casellas P Distribution profile and properties of peripheral-type benzodiazepine receptors on human hemopoietic cells. *Life Sci* 1993; 52:107-118.
- 20 Clarke GD, Ryan PJ Tranquillizers Can Block Mitogenesis in 3T3 Cells and Induce Differentiation in Friend Cells. *Nature* 1980; 287:160-161.
- Coradini D, Daidone MG. Biomolecular prognostic factors in breast cancer. *Curr Opin Obstet Gynecol.* 2004; 16:49-55.
- Cornu P., Benavides J Scatton B, Hauw JJ, Philippon J Increase in α_3
 25 (Peripheral-type Benzodiazepine) Binding Site Densities in Different Types of Human Brain Tumors: A Quantitative Autoradiography Study. *Acta Neurochir.* 1992; 119: 146-152.
- Culty M, Li H, Boujrad N, Bernassau JM, Reversat JL, Amri H, Vidic B, Papadopoulos V In vitro studies on the role of the peripheral
 30 benzodiazepine receptor in steroidogenesis. *J Steroid Biochem Mol Biol.* 1999;69:123-130.

- Galiegue S, Casellas P, Kramar A, Tinel N, Simony-Lafontaine J.
Immunohistochemical assessment of the peripheral benzodiazepine
receptor in breast cancer and its relationship with survival. *CHn Cancer
Res.* 2004; 10:2058-2064.
- 5 Gamier M, Boujrad N, Oke BO, Brown AS, Riond J, Ferrara P, Shoyab M,
Suarez-Quian CA, Papadopoulos V Diazepam Binding Inhibitor is a
Paracrine/Autocrine Regulator of Leydig Cell Proliferation and
Steroidogenesis: Action Via Peripheral-type Benzodiazepine Receptor
and Independent Mechanisms. *Endocrinology* 1993;132:444-458.
- 10 Glinsky GV, Higashiyama T, Glinskii AB. Classification of human breast cancer
using gene expression profiling as a component of the survival predictor
algorithm. *Clin Cancer Res.* 2004; 10:2272-2283.
- Gradilone A, Gazzaniga P, Silvestri I, Gandini O, Trasatti L, Lauro S, Frati L,
15 Agliano AM Detection of CK19, CK20 and EGFR mRNAs in peripheral
blood of carcinoma patients: correlation with clinical state of disease.
Oncol Rep. 2003; 10:217-222.
- Han Z, Slack SR, Li W, Papadopoulos V Expression of peripheral
benzodiazepine receptor (PBR) in human tumors: relationship to breast,
colon and prostate tumor progression. *J Rec Res Signal Transd.*
20 2003;23:225-238.
- Hardwick M, Fertikh D, Culty M, Li H, Vidic B, Papadopoulos V Peripheral-
type benzodiazepine receptor (PBR) in human breast cancer: correlation
of breast cancer cell aggressive phenotype with PBR expression, nuclear
localization, and PBR-mediated cell proliferation and nuclear transport of
25 cholesterol. *Cancer Res.* 1999;59:831-842.
- Hardwick M, Rone J, Barlow K, Haddad B, Papadopoulos V Peripheral-type
benzodiazepine receptor (PBR) gene amplification in MDA-231
aggressive breast cancer cells. *Cancer Genetics Cytogen.* 2002; 139:48-
51.
- 30 Hardwick M, Rone J, Han Z, Haddad B, Papadopoulos V Peripheral-type
benzodiazepine receptor levels correlate with the ability of human breast

- cancer MDA-MB-23 1 cell line ability to grow in SCID mice. *Int J Cancer* 2001;94:322-327.
- Ikezaki K, Black KL Stimulation of Cell Growth and DNA Synthesis by Peripheral Benzodiazepine. *Cancer Letters* 1990;49:1 15-120.
- 5 Tsmail MS, Wynendaele W, Aerts JL, Paridaens R, Van Mellaert L, Anne J, Gaafar R, Shakankiry N, Khaled HM, Christiaens MR, Omar S, Vandekerckhove P, Van Oosterom AT (2003) Real-time quantitative RT-PCR and detection of tumour cell dissemination in breast cancer patients: plasmid versus cell line dilutions. *Ann Oncol*, 14:1241-1245.
- 10 Katz Y, Ben-Baruch G, Kllog Y, Menczer J, Gavish M Increased Density of Peripheral Benzodiazepine Binding Sites in Ovarian Carcinomas as Compared with Benign Ovarian Tumours and Normal Ovaries. *Clinical ScL* 1990;78:155-15S.
- Katz Y, Eitan A, Gavish M Increase in Peripheral Benzodiazepine Binding Sites
15 in Colonic Adenocarcinoma. *Oncology* 1990;47:139-142.
- Kupczyk-Subotkowska L, Siahaan TJ, Basile AS, Friedman HS, Higgins PE, Song D, Gallo JM Modulation of mephalan resistance in glioma cells with a peripheral benzodiazepine receptor ligand-mephalan conjugate. *J Med Chem.* 1997;40: 1726- 1730.
- 20 Lacapere JJ, Delavoie F, Li H; Peranzi G, Maccario J, Papadopoulos V, Vidic B Structural and functional study of reconstituted peripheral benzodiazepine receptor (PBR). *Biochem Biophys Res Commun.* 2001;284:536-641.
- Li H, Yao Z, Degenhardt B, Teper G, Papadopoulos V Cholesterol binding at
25 the cholesterol recognition/interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT -CRAC peptide. *Proc. Natl. Acad. Sc. USA* 2001;98:1267-1272.
- Makris A, Allred DC, Powles TJ, Dowsett M, Fernando IN, Trott PA, Ashley
30 SE, Ormerod MG, Titley JC, Osborne CK Cytological evaluation of

- biological prognostic markers from primary breast carcinomas. *Breast Cancer Res Treat.* 1997;44:65-74.
- Mark HF, McGowan KJD. Issues in the genetic assessment of predispositions for familial breast and ovarian cancer. *Cytobios.* 1996; 87:229-235.
- 5 Miettinen H, Kononen J, Haapasalo H, Helen P, Sallinen P, Harjuntausta T, Helin H, Alho H Expression of Peripheral-type Benzodiazepine Receptor and Diazepam Binding Inhibitor in Human Astrocytomas: Relationship to Cell Proliferation. *Cancer Res.* 1995;55: 2691-2695.
- 10 Neary JT, Jorgensen SL, Oracion AM, Bruce JH, Norenberg MD Inhibition of Growth Factor-induced DNA Synthesis in Astrocytes by Ligands of Peripheral-type Benzodiazepine Receptors. *Brain Res.* 1995;675:27-30.
- Papadopoulos V Peripheral-type Benzodiazepine/Diazepam binding Inhibitor Receptor: Biological Role in Steroidogenic Cell Function. *Endocrine Rev.* 1993; 14: 222-240.
- 15 Papadopoulos V, Amri H, Li H, Boujrad N, Vidic B, Gamier M Targeted Disruption of the Peripheral-type Benzodiazepine Receptor Gene Inhibits Steroidogenesis in the R2C Leydig Tumor Cell Line. *J Biol Chem.* 1997;272:32 129-32 135.
- 20 Papadopoulos V, Mukhin AG, Costa E, Krueger KE The Peripheral-type Benzodiazepine Receptor is Functionally Linked to Leydig Cell Steroidogenesis. *J Biol Chem.* 1990;265: 3772-3779.
- Papadopoulos V. Peripheral benzodiazepine receptor: structure and function in health and disease. *Ann Pharm Fr.* 2003;61:30-50.
- 25 Pappata S, Cornu P, Samson Y, Prenant C, Benavides J, Scatton B, Crouzel C, Hauw JJ, Syrota A PET Study of Carbon-11 PK 11195 Binding to Peripheral-type Benzodiazepine Sites in Glioblastoma: A Case Report. *J Nuclear Med.* 1991 ;32:1608-1610.
- 30 Ratcliffe SL, Matthews EK Modification of the photodynamic action of delta-aminolaevulinic acid (ALA) on rat pancreatoma cells by mitochondrial benzodiazepine receptor ligands. *BrJ Cancer.* 1995;71 :300-305.

Ross JS, Gray GS. Targeted therapy for cancer: the HER-2/neu and Herceptin story. *Clin Leadersh Manag Rev.* 2003; 17:333-340.

5 Sanger N, Strohmeier R, Kaufmann M, Kuhl H Cell cycle-related expression and ligand binding of peripheral benzodiazepine receptor in human breast cancer cell lines. *Eur J Cancer* 2002;36:2157-2163.

Schroder CP, Rutgers MH, de Jong S, Tiebosch AT, Wesseling J, VeenstraR, de Vries J, Hoekstra HJ, de Leij LF, de Vries EG (2003) Detection of micrometastatic breast cancer by means of real time quantitative RT-PCR and immunostaining in perioperative blood samples and sentinel nodes.
10 *Int J Cancer*, 106:611-618.

Stathopoulou A, Mavroudis D, Perraki M, Apostolaki S, Vlachonikolis I, Lianidou E, Georgoulas V Molecular detection of cancer cells in the peripheral blood of patients with breast cancer: comparison of CK-19, CEA and maspin as detection markers. *Anticancer Res.* 2003;23: 1883-
15 1890.

Stathopoulou A, Vlachonikolis I, Mavroudis D, Perraki M, Kouroussis Ch, Apostolaki S, Malamos N, Kakolyris S, Kotsakis A, Xenidis N, Reppa D, Georgoulas V Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of
20 their prognostic significance. *J CHn Oncol.* 2002; 20:3404-3412.

van Diest PJ, van der Wall E, Baak JP. Prognostic value of proliferation in invasive breast cancer: a review. *J Clin Pathol.* 2004; 57:675-681.

25 All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from
30 the basic principles of the invention.

WHAT IS CLAIMED IS:

1. A method to detect breast carcinoma cells in a sample, comprising
 - a) contacting a mammalian physiological fluid sample and one or more ligands specific for epithelial cells so as to yield a composition comprising complexes comprising epithelial cells and the one or more ligands;
 - b) isolating the complexes, thereby isolating the epithelial cells; and
 - c) detecting or determining the level of peripheral benzodiazepine receptor (PBR) RNA expressed in the isolated epithelial cells.
2. The method of claim 1 wherein the sample is a human sample.
3. The method of claim 1 or 2 wherein the ligand is an antibody.
4. The method of claim 1 or 2 wherein the ligand binds cytokeratin, integrin or mucin.
5. The method of claim 4 wherein the cytokeratin is cytokeratin 7/8.
6. The method of any of claims 1 to 5 wherein the complexes are isolated by flow cytometry, affinity chromatography or immunoprecipitation.
7. The method of any of claims 1 to 5 wherein the complexes are isolated by contacting the composition with an antibody that binds the ligand.
8. The method of any of claims 1 to 7 wherein the ligand is labeled.
9. The method of claim 8 wherein the label is a fluorophore or a magnetic bead.
10. The method of any of claims 1 to 7 wherein the ligand is attached to a solid support.

11. The method of any of claims 1 to 10 wherein the level of PBR RNA is detected or determined by a nucleic acid amplification reaction.
12. The method of claim 11 wherein the amplification reaction includes at least one PBR-specific primer.
13. The method of claim 11 wherein the amplification reaction includes at least two PBR-specific primers.
14. The method of claim 13 wherein the primers have SEQ ID NO:1 and SEQ ID NO:2.
15. The method of claim 13 wherein the primers have SEQ ID NO:5 and SEQ ID NO:6.
16. The method of claim 11 wherein the level of PBR RNA is detected or determined with a labeled probe.
17. The method of claim 16 wherein the probe comprises a fluorophore and a quencher.
18. The method of claim 16 wherein the probe comprises SEQ ID NO:3 or SEQ ID NO:4.
19. The method of claim 16 wherein the probe comprises SEQ ID NO:7 or SEQ ID NO:8.
20. The method of any of claims 1 to 19 wherein RNA is isolated from the isolated epithelial cells prior to detecting or determining the level of PBR RNA.

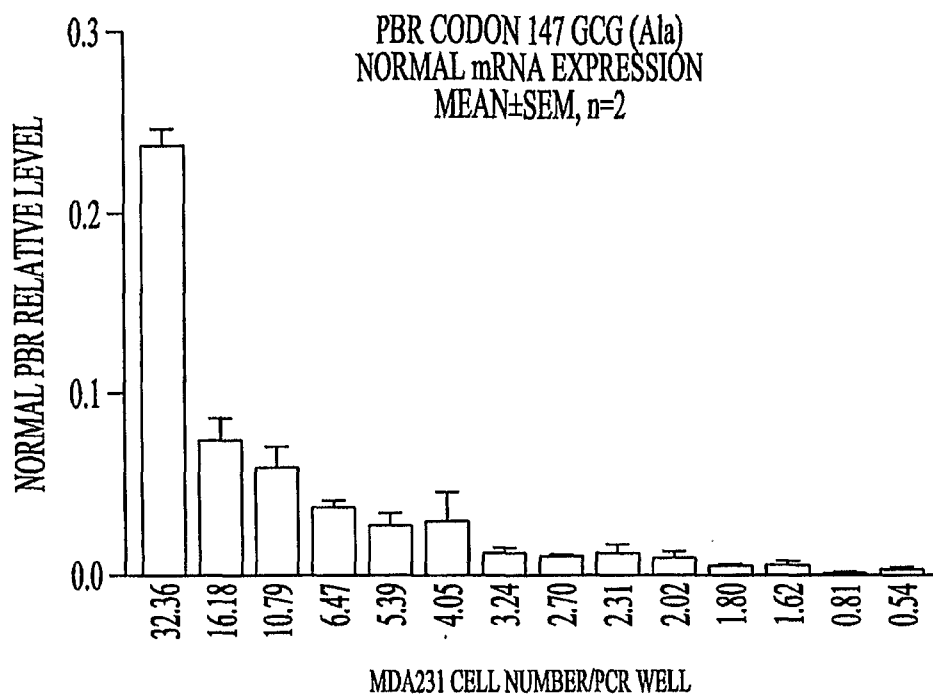
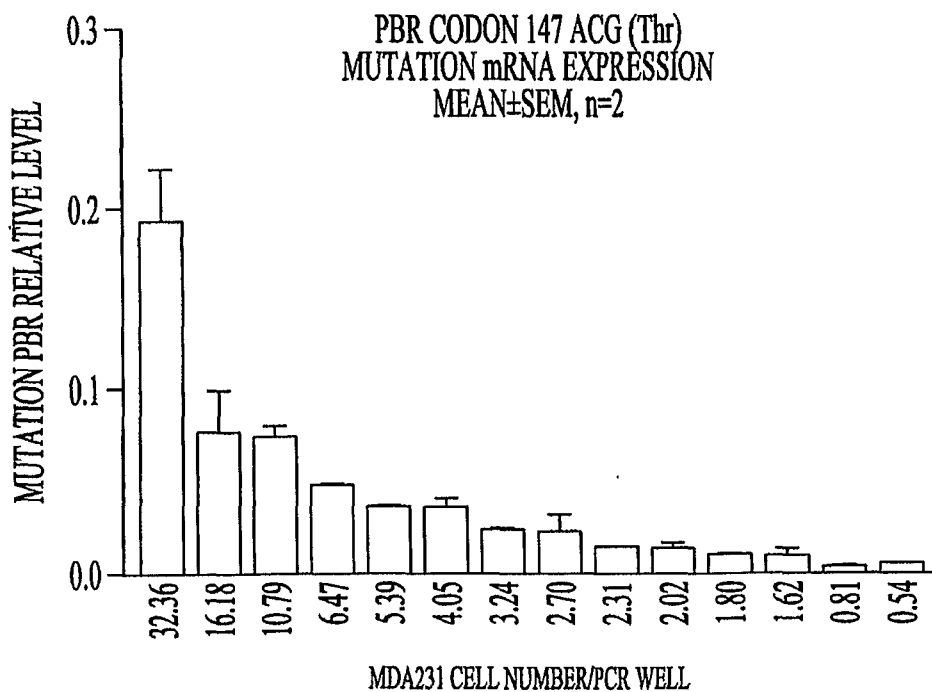
21. The method of any of claims 1 to 19 wherein the level of variant PBR RNA is detected or determined.
22. The method of claim 21 wherein the variant PBR RNA encodes a PBR with a substitution at residue 147.
23. The method of claim 21 wherein the variant PBR RNA encodes a PBR with a substitution at residue 162.
24. The method of any of claims 1 to 23 wherein the level of wild type and variant PBR RNA is detected or determined.
25. The method of any of claims 1 to 24 which is capable of detecting one ~~breast~~ carcinoma cell in a physiological fluid sample of at least 1×10^6 cells.
26. The method of any of claims 1 to 25 wherein the sample is a blood sample.
27. A method to detect breast carcinoma cells in a sample, comprising:
 - a) separating epithelial cells from hematopoietic cells in a mammalian physiological fluid sample by contacting the sample and a ligand specific for hematopoietic cells; and
 - b) detecting or determining the level of PBR RNA in the separated epithelial cells.
28. The method of claim 27 wherein the sample is a blood sample.
29. The method of any of claims 27 to 28 wherein the sample is a human sample.
30. The method of any of claims 27 to 29 wherein the ligand is an antibody.

31. The method of any of claims 27 to 30 wherein affinity chromatography or immunoprecipitation is employed to separate the epithelial and hematopoietic cells.
32. The method of any of claims 27 to 30 wherein the ligand is attached to a solid support.
33. The method of any of claims 27 to 32 wherein the level of PBR RNA is detected or determined by a nucleic acid amplification reaction.
34. The method of claim 33 wherein the amplification reaction includes at least one PBR-specific primer.
35. The method of claim 33 wherein the amplification reaction includes at least two PBR-specific primers.
36. The method of claim 35 wherein the primers have SEQ ID NO:1 and SEQ ID NO:2.
37. The method of claim 35 wherein the primers have SEQ ID NO:5 and SEQ ID NO:6.
38. The method of claim 33 wherein the level of PBR RNA is detected or determined with a labeled probe.
39. The method of claim 38 wherein the probe comprises a fluorophore and a quencher.
40. The method of claim 38 wherein the probe comprises SEQ ID NO:3 or SEQ ID NO:4.
41. The method of claim 38 wherein the probe comprises SEQ ID NO:7 or SEQ ID NO:8.

42. The method of any of claims 27 to 41 wherein KNA is isolated from the composition prior to detecting or determining the level of PBR RNA.
43. The method of any of claims 27 to 42 wherein the level of variant PBR RNA is detected or determined.
44. The method of claim 43 wherein the variant PBR RNA encodes a PBR with a substitution at residue 147.
45. The method of claim 43 wherein the variant PBR RNA encodes a PBR with a substitution at residue 162.
46. The method of claim 43 wherein the level of wild type and variant PBR RNA is detected or determined.
47. A method to detect breast carcinoma cells in a sample, comprising:
detecting or determining levels of PBR RNA in epithelial cells isolated from a mammalian blood sample.
48. A method to detect the ratio of variant PBR to total PBR expression in a biological sample, comprising:
 - a) detecting relative mRNA expression of variant PBR and total PBR in a sample suspected of having cancer cells, wherein mRNA expression for each of variant PBR and total PBR is detected using distinct amplification primers and distinct labeled probes; and
 - b) determining whether the sample has an increased relative expression of variant PBR.
49. The method of claim 48 wherein the probes are labeled with distinct fluorophores.

50. The method of claim 49 wherein each probe further comprises a quencher of the fluorophore.

1/7

*FIG. 1A**FIG. 1B*

2/7

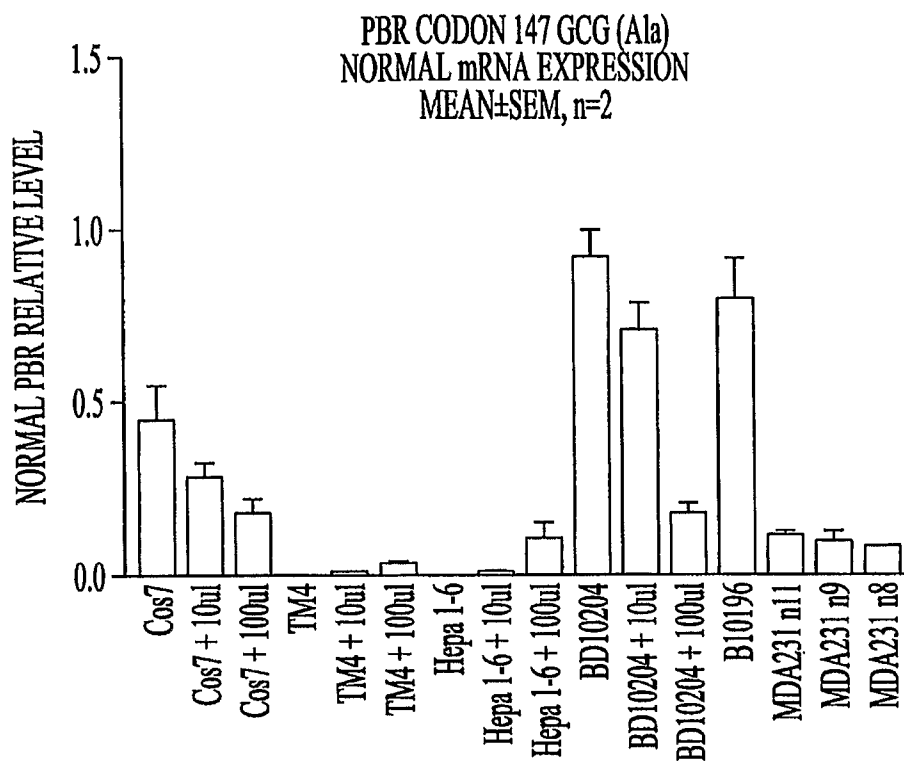


FIG. 2A

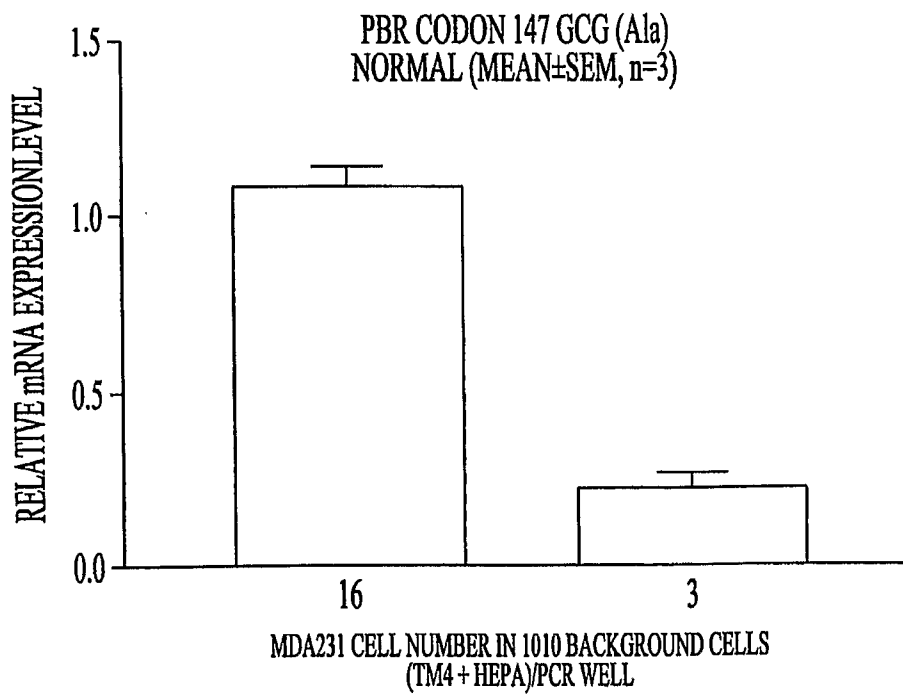


FIG. 2B

3/7

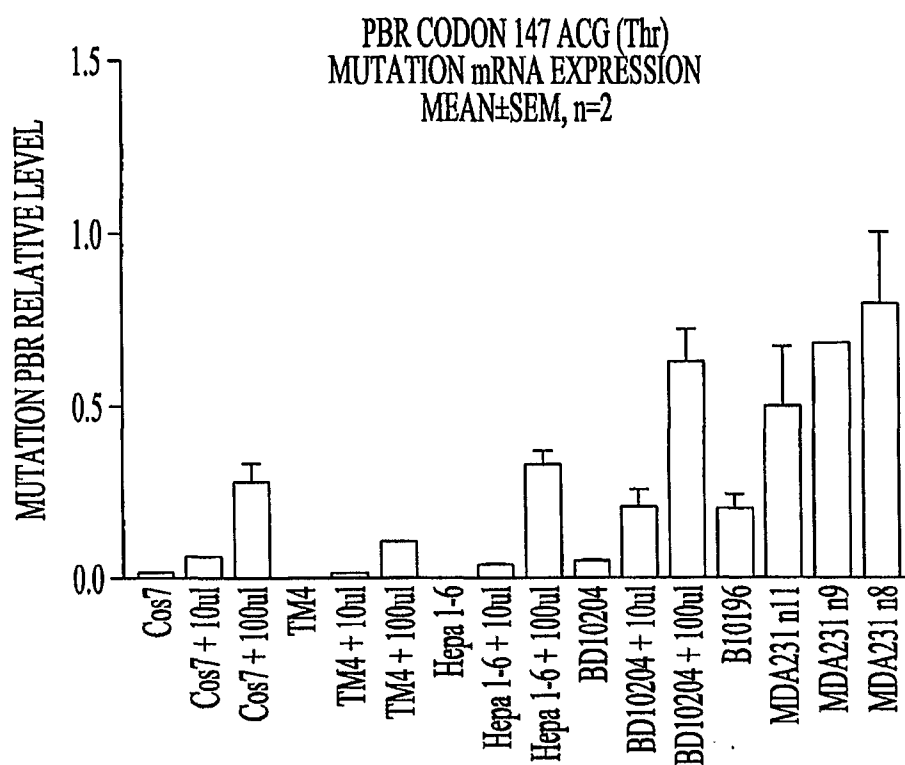


FIG. 2C

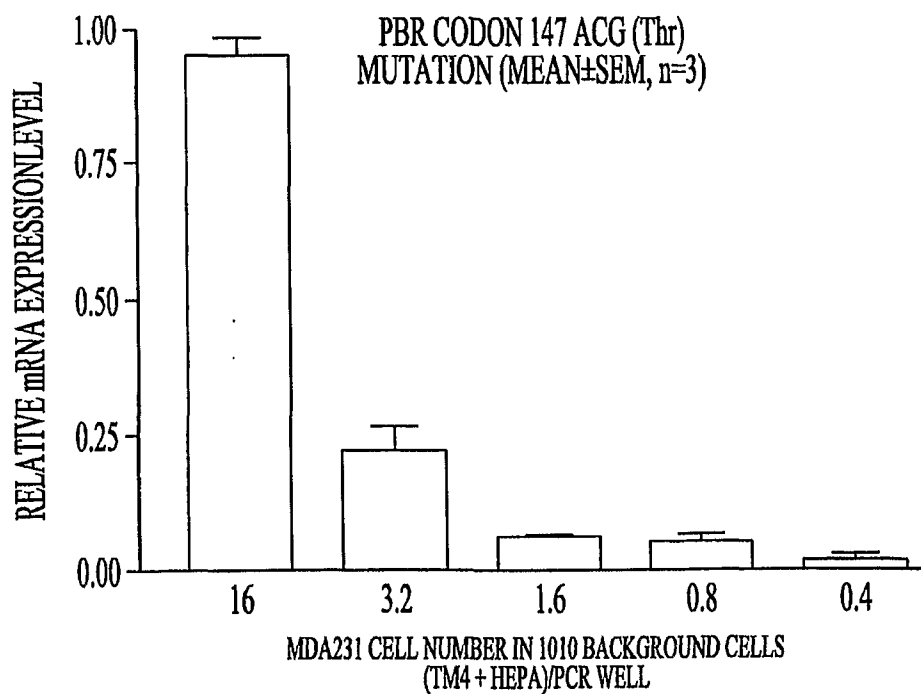


FIG. 2D

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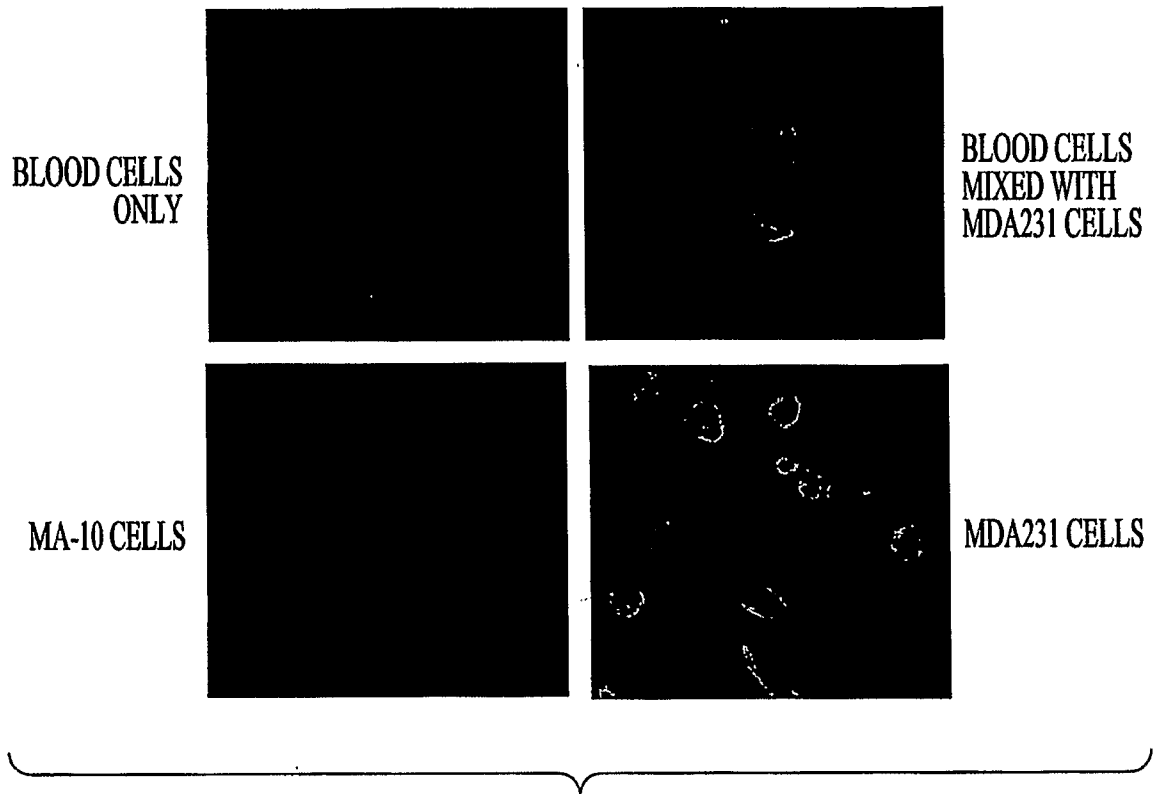


FIG. 3

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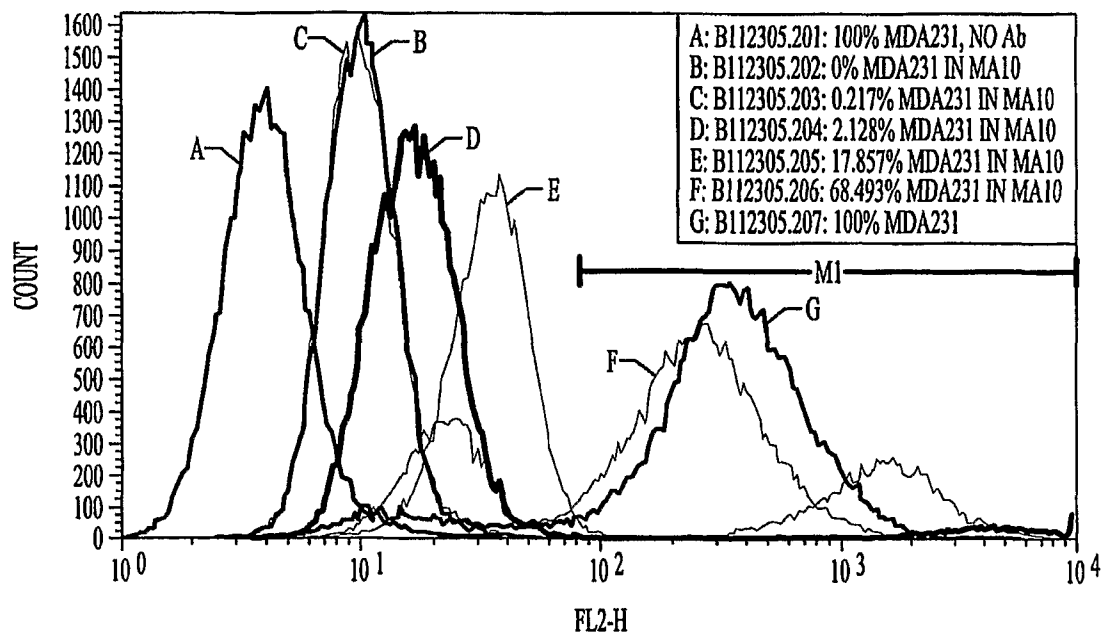


FIG. 4A

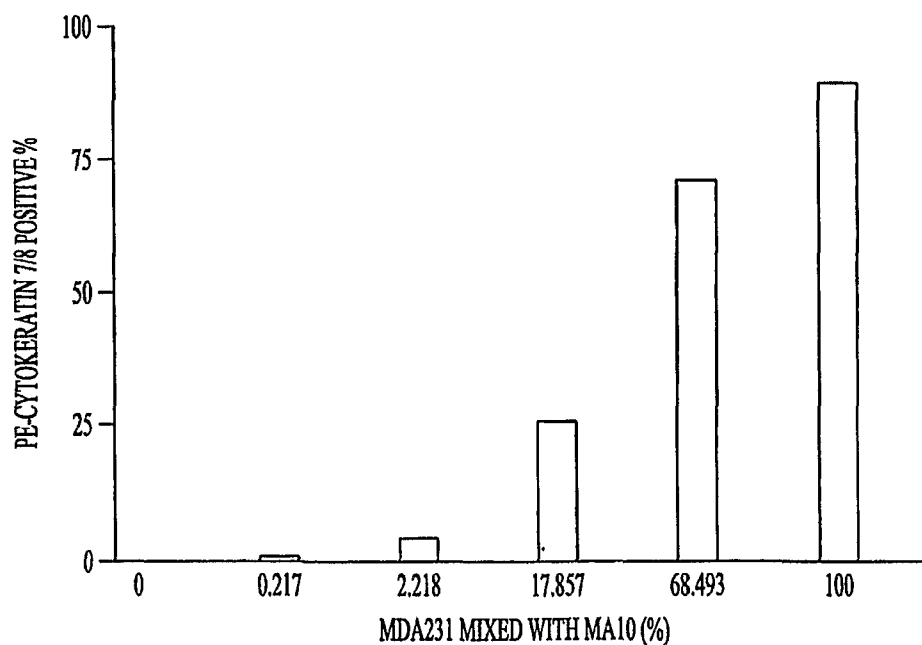


FIG. 4B

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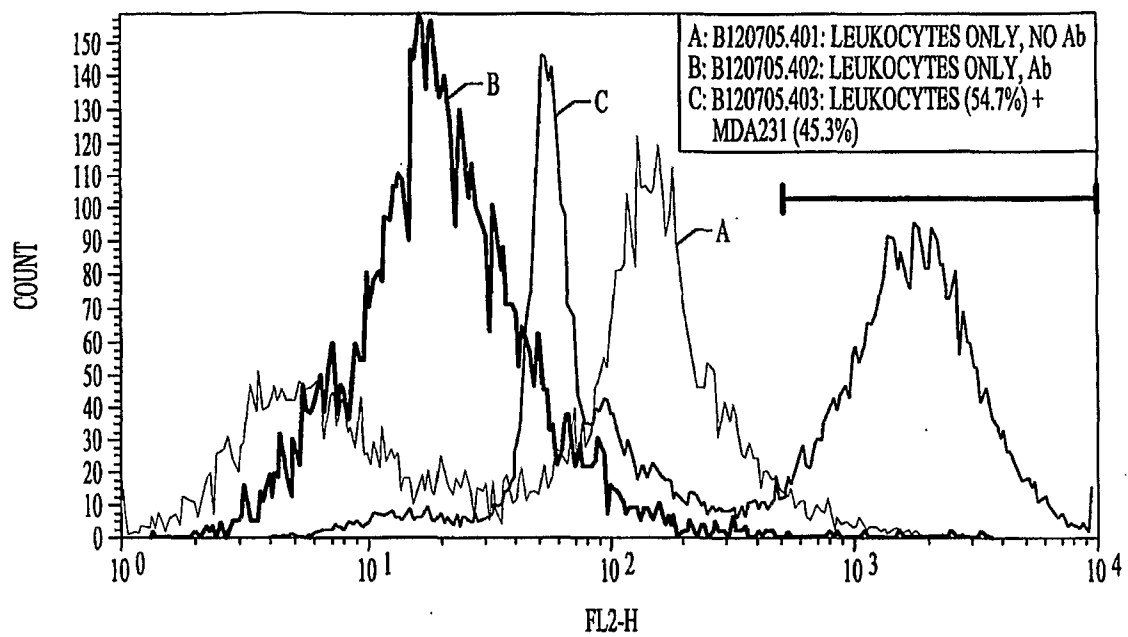


FIG. 5A

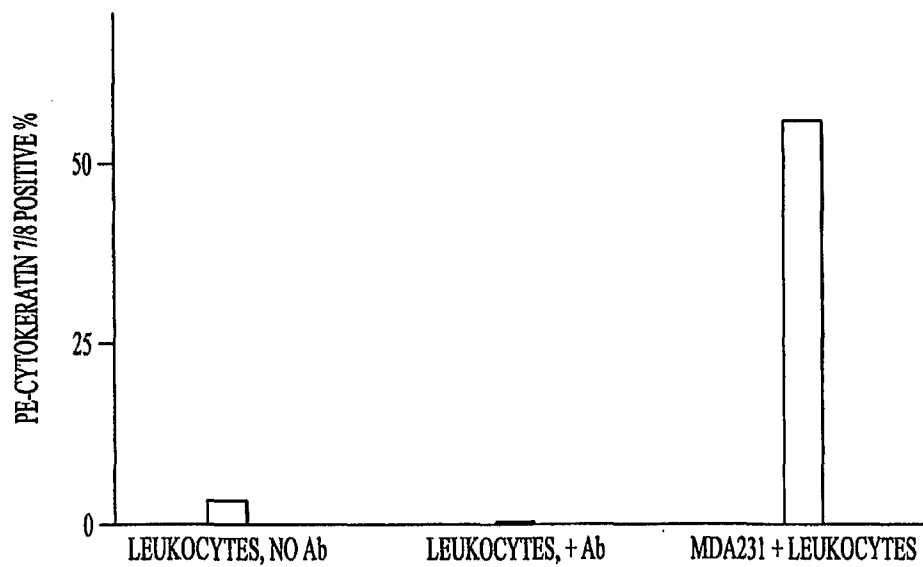
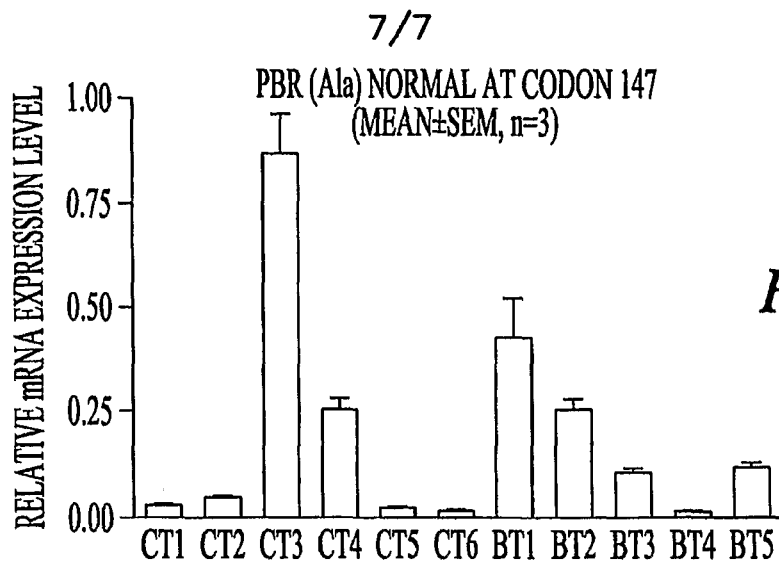
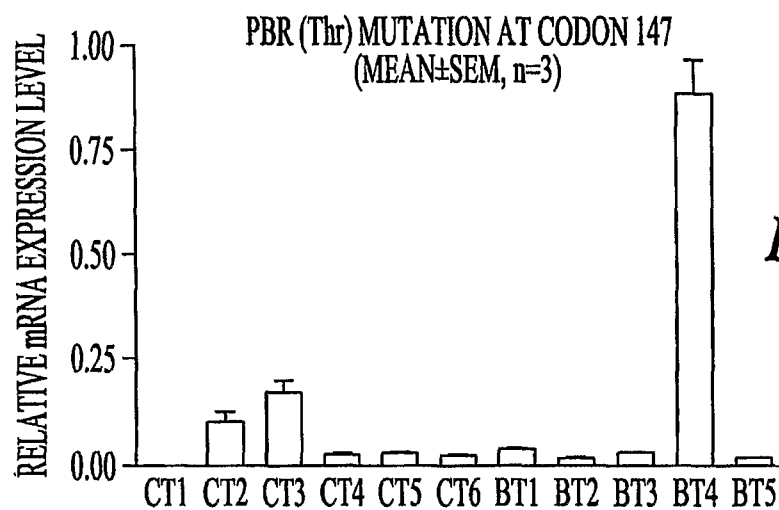
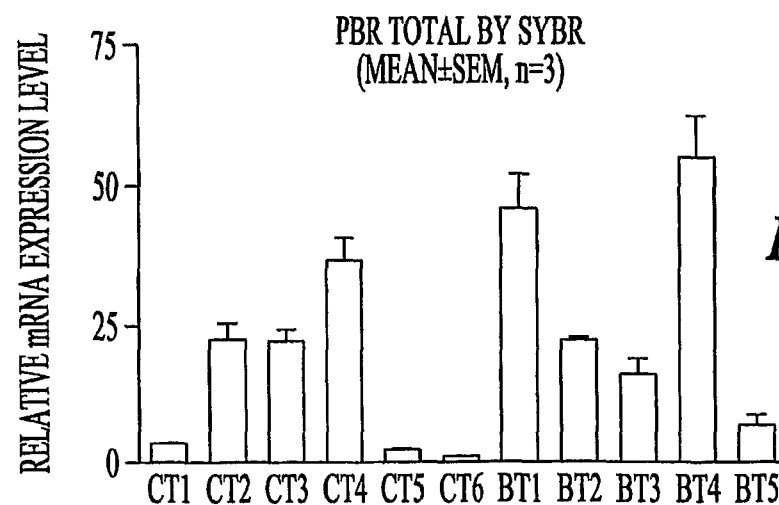


FIG. 5B

*FIG. 6A**FIG. 6B**FIG. 6C*