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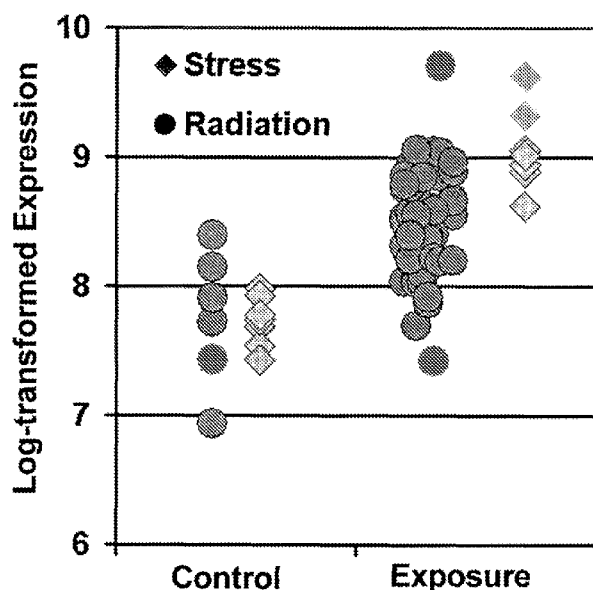
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(54) Title: METHODS AND COMPOSITIONS FOR MEASURING RADIATION EXPOSURE IN A SUBJECT

FIGURE 1A

(57) Abstract: The present disclosure provides a method for assessing exposure to ionizing radiation comprising the steps of (a) measuring the RNA expression level of certain biomarker genes in a biological sample; (b) correcting the RNA expression level of the measured biomarker in step (a) to a reference standard or threshold value, and (c) comparing the RNA expression level of the biomarker measured in step (a) and corrected in accordance with step (b) to the corrected expression of a calibration sample or threshold value, wherein a difference in expression level between the biomarker in the biological sample and the calibration sample indicates that the source from which the biological sample is obtained was exposed to ionizing radiation. The method can further comprise classifying the source of the biological sample as either exposed or not exposed to ionizing radiation. Kits for assessing exposure to ionizing radiation are also described.



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METHODS AND COMPOSITIONS FOR MEASURING RADIATION EXPOSURE IN A SUBJECT

CROSS-REFERENCE TO RELATED APPLICATION

5 The present application claims benefit to US Provisional Application No.
61/488,631, filed May 20, 2011, which is incorporated herein by reference.

STATEMENT REGARDING SEQUENCE LISTING

The sequence listing associated with this application is provided in text format in lieu of a paper copy and is hereby incorporated by reference into the specification. The name of the text file containing the sequence listing is: 39420_SEQ_Final_2012_05-08.txt. The file is 46KB; was created on May 8, 2012; and is being submitted via EFS-Web with the filing of the specification.

STATEMENT OF GOVERNMENT LICENSE RIGHTS

This invention was made with government support under Grant No. U19-AI06777
15 awarded by the National Institutes of Health. The government has certain rights in the
invention.

FIELD OF THE INVENTION

This invention relates to methods, reagents, and kits for use in assessing the exposure to ionizing radiation in a biological sample.

20 BACKGROUND

In the event of a large-scale radiological event, it will be imperative to have a quick and reliable screening assay that can differentiate between victims who have and have not been exposed to ionizing radiation (IR). The ability to rapidly stratify victims based on IR exposure will help to reduce anxiety and panic, and ensure that medical interventions can be administered appropriately and in a time-efficient manner.

Although there is debate about what treatments are appropriate for specific dose levels, the Strategic National Stockpile (SNS) Radiation Working Group recently proposed a working guideline for patients in the event of a nuclear detonation. The SNS Group broke subjects into four major categories: normal care (1-3 Gy), critical care (3-5 Gy), intensive care (5-10 Gy) and expectant care (≥ 10 Gy) (Waselenko, J.K. et al., *Ann Intern Med* 140:1037-1051 (2004)). Identification of dosage ranges of exposure is important to facilitate appropriate treatment, which varies depending on dosage. The

patients could then be treated according to the assessed exposure. For example, as described in a recent report, specific therapeutic guidelines have been recommended for antibiotics, cytokines, and transplantation in the event of radiologic event; patients exposed to >2 Gy would receive antibiotics, and patients exposed to >3 Gy would receive
5 cytokine support. Transplantation would be reserved for patients exposed to 7-10 Gy (Weisdorf D., et al., *Biol Blood Marrow Transplant* 12:672-82 (2006)).

Physical signs (e.g., burns) or symptoms (e.g., nausea) may be helpful in the dose assessment of radiation exposures. However, accurate measurements of these clinical parameters can be difficult, and recently it has been suggested that clinical findings
10 should be combined with dosimetry tests (e.g., chromosomal aberration assays) in order to provide a more predictive assessment tool for discriminating between radiation exposure levels. Chromosomal aberration (CA) assays for dicentric abnormalities are currently considered by some to be the gold standard for determining previous radiation exposure (see, e.g., Coleman, C.N., *Radiat Res* 159:812-834 (2003)). However, these CA
15 assays are time-intensive, requiring 48 to 72 hours to perform, labor-intensive, requiring technicians to individually inspect hundreds of cells, and lack specificity at very low and high doses (Amundson, S.A. et al., *Mol Diagn* 1:211-219 (2001); Blakely, W.F. et al., *Health Phys* 89:494-504 (2005)). These issues make current CA assays impracticable in the event of a large-scale terrorist attack using radioactive materials where thousands of
20 individuals would need rapid exposure assessments.

Thus, there is a need for radiation exposure screening and dosimetry assays that have specificity at high and low doses, are capable of use in a high-throughput, automated setting or in the field, and are cost-efficient and capable of being stockpiled for rapid response and risk-stratification of mammalian subjects within 24-48 hours after radiation
25 exposure.

SUMMARY

In one aspect, the present invention provides a method for assessing exposure to ionizing radiation. The method comprises (a) measuring the RNA expression level of at least one biomarker gene selected from the group consisting of AEN, APRT, CDKN1A,
30 DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC and ZMAT3 in a biological sample; (b) correcting the RNA expression level of the biomarker measured in step (a) to a reference standard or threshold value, and (c) comparing the RNA expression level of the biomarker measured in step (a) and corrected in accordance with step (b) to the corrected

expression of a calibrator sample or threshold value, wherein a difference in expression level between the biomarker in the biological sample and the calibrator sample indicates that the source from which the biological sample was obtained was exposed to ionizing radiation. In some embodiments, the biological sample is obtained from a mammalian subject, such as a human. In some embodiments, the subject is assessed in a time period of from 30 minutes after initial exposure to 14 days after the end of the potential exposure to ionizing radiation. In some embodiments, the method further comprises classifying the source of the biological sample as either exposed or not exposed to ionizing radiation. In some embodiments, the method further comprises classifying the source of the biological sample as either exposed to a low dose of ionizing radiation of 1-2 Gy, or exposed to a higher dose of ≥ 2 Gy of ionizing radiation.

In another aspect, the present invention provides a kit for assessing exposure to ionizing radiation in a biological sample. The kits according to this aspect of the invention comprise: (a) at least one reagent for measuring the RNA expression level of at least one biomarker gene selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC and ZMAT3 and (b) written instructions for the use of the reagents and interpretation of the results with regard to comparison to a threshold value or calibrator for determining whether a biological sample has been exposed to ionizing radiation. In some embodiments, the kit further comprises at least one reagent for measuring the RNA expression level of at least one endogenous control gene that is not affected by exposure to radiation, such as GUSB.

The kits may be utilized in accordance with the methods of the invention, and the methods of the invention may be practiced with the kits of the invention.

DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1A graphically illustrates the log-transformed expression levels of RAB13 in human peripheral blood treated *ex vivo* under control conditions, 24 hours after exposure to various doses of radiation (0.15, 2, 4, 6, 9, and 12 Gy), and after exposure to blunt trauma. As shown in FIGURE 1A, RAB13 gene expression was found to be induced by both trauma and by exposure to radiation, therefore this gene was excluded

from the list of potential biomarkers for detecting radiation exposure, as described in Example 1;

FIGURE 1B graphically illustrates the log-transformed expression levels of AEN in human peripheral blood treated *ex vivo* under control conditions, after exposure to various doses of radiation (0.15, 2, 4, 6, 9, and 12 Gy), and after exposure to blunt trauma. As shown in FIGURE 1B, AEN expression is induced by radiation exposure, but not by exposure to blunt trauma, therefore this gene was selected as a candidate biomarker for detecting radiation exposure, as described in Example 1;

FIGURE 1C is a Venn diagram showing the overlap of genes identified by microarray analyses of human peripheral blood treated *ex vivo* to have significant radiation-induced expression changes at different time points after exposure to radiation. As shown in FIGURE 1C, these analyses identified 1642, 1277 and 1198 genes with significant radiation-specific expression changes observed at 3, 8 and 24 hours, respectively, after human peripheral blood was exposed *ex vivo* to radiation. As further shown in FIGURE 1C, 414 genes displayed similar expression changes at all three time points. Of these 414 genes, a set of candidate markers was chosen for further analysis, as described in Example 1;

FIGURE 1D graphically illustrates the log-transformed expression values of GUSB (beta-glucuronidase) which will be used as the reference standard for quantitative normalization of RNA quality. The expression of GUSB is stable across lymphocytes and whole blood cells exposed to various doses of radiation (sham, 0.15, 2, 4, 6, 9 and 12 Gy), as described in Example 1;

FIGURE 2A shows the peripheral blood collection schedule for study #2, the canine single dose total body irradiation (TBI) model, with the time of peripheral blood collection after radiation exposure shown by the arrows labeled "PB" along the bottom of the chart, and the total dose shown in each box within the chart, and the cumulative dose for each study shown in the box on the far right side of the chart, as described in Example 2;

FIGURE 2B shows the peripheral blood collection schedule for study #3, the human patients undergoing multiple fractionated TBI doses over various time periods ranging from one to four days. Fractionated doses are shown in the boxes within the chart, with the total cumulative dose for each patient (study) shown in the box at the far right of the chart. The time of peripheral blood collection after the first radiation

exposure is shown by the arrows labeled "PB" along the bottom of the chart, as described in Example 2;

FIGURE 3A graphically illustrates the validated genes in CD3⁺ lymphocytes from *ex vivo* irradiated peripheral blood. RNA was extracted from positively selected
5 CD3⁺ lymphocytes isolated from mononuclear cells (MNC) exposed to sham radiation (no IR) and 2, 6 and 12 Gy of radiation (IR) from 5 healthy donors. Error bars represent standard deviations among the 5 donors, as described in Example 2;

FIGURE 3B graphically illustrates an example of radiation-induced expression changes for FDXR (y-axis) in CD3⁺ lymphocytes and plasma obtained from *ex vivo*
10 irradiated peripheral blood of 5 healthy donors. RNA extracted from samples harvested 24 hours after exposure to 0 (sham), 2, 6 and 12 Gy. The results in FIGURE 3B demonstrate that FDXR is an informative biomarker for measuring radiation-induced expression changes in subcompartments of blood, such as lymphocytes and plasma, as described in Example 2;

FIGURE 4A graphically illustrates the Receiver Operating Characteristic (ROC)
15 curves for AEN (solid line), APRT (dashed line) and the computed DS₁ (dotted line) in the human TBI model (training set, N=16). As shown in FIGURE 4A, the computed DS₁ was shown to have >95% sensitivity and specificity for identifying humans exposed to at least 1.5 Gy of ionizing radiation, as described in Example 3;

FIGURE 4B graphically illustrates DS₁ dosimetry score (y-axis) in the training set
20 of samples collected from humans exposed to variable doses of radiation (x-axis). As shown in FIGURE 4B, the DS₁ algorithm is informative across a range of radiation doses from 1.5 Gy up to 12 Gy, as described in Example 3;

FIGURE 4C graphically illustrates the Receiver Operating Characteristic (ROC)
25 curves for AEN (solid line), APRT (dashed line) and the computed DS₁ (dotted line) dosimetry score in the canine TBI model (training set, N=18). As shown in FIGURE 4C, the DS₁ algorithm was found to have > 95% sensitivity and specificity in identifying canines that were exposed to at least 2 Gy of radiation. Furthermore, FIGURE 4C demonstrates the robustness of the DS₁ that was generated using human *in vivo* model
30 (training set, FIGURES 4A and 4B) and validated in the canine *in vivo* model (testing set FIGURES 4C and 4D), as described in Example 3;

FIGURE 4D graphically illustrates the DS₁ dosimetry score (y-axis) in the testing set of samples collected from canines exposed to a single dose of either 2, 6, or 10 Gy of

radiation (x-axis) at either 24, 48 or 68 hours after exposure. As shown in FIGURE 4D, the DS₁ algorithm is informative across a range of radiation doses and across a long time interval, as described in Example 3; and

FIGURE 5 graphically illustrates the Receiver Operating Characteristic (ROC) curves for AEN (solid line), APRT (dashed line) and the computed DS₂ (dotted line) in the human TBI model (training set, N=16). As shown in FIGURE 5, the computed DS₂ was found to have >95% sensitivity and specificity for discriminating between individuals exposed to IR doses <2 Gy and ≥2 Gy, as described in Example 4.

DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. The following definitions are provided in order to provide clarity with respect to terms as they are used in the specification and claims to describe various embodiments of the present invention.

As used herein, the term "exposure to ionizing radiation" refers to exposure to subatomic particles or electromagnetic waves with sufficient energy to remove electrons from atoms. Examples of ionizing subatomic particles include alpha particles, beta particles and neutrons. Electromagnetic waves with shorter wave lengths (higher frequencies) possess higher energy and are more likely to be ionizing. Examples of high energy, or high frequency, ionizing electromagnetic waves include ultraviolet (UV) rays, X-rays and gamma-rays. Exposure to ionizing radiation is commonly known to cause damage to living tissue, including breaks in DNA molecules.

As used herein, the term "procedure to diagnose or treat a medical condition" refers to any medical procedure to assess the presence, progression, or resolution of a medical disease in a subject, or to any medical procedure to cure, facilitate the resolution of, or ameliorate the harmful effects of a medical disease.

As used herein, the term "about" refers to plus or minus ten percent (10%) of the referenced value.

As used herein, "oligonucleotide sequences that are complementary to one or more of the genes described herein" refers to oligonucleotides that are capable of hybridizing under stringent conditions to at least part of the nucleotide sequence of said genes. Such hybridizable oligonucleotides will typically exhibit at least about 75% sequence identity at the nucleotide level to said genes, preferably about 80% or 85%

sequence identity, or more preferably about 90%, 95%, 96%, 97%, 98% or 99% sequence identity to said genes.

As used herein, the phrase "hybridizing specifically to" refers to the binding, duplexing or hybridizing of a molecule substantially to or only to a particular nucleotide
5 sequence or sequences under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

As used herein, the term "biomarker" means any gene, i.e., transcript, protein, or an expressed sequence tag (EST) derived from that gene, the expression or level of which changes between certain conditions. Where the expression of the gene correlates with a
10 certain condition, the gene is a marker for that condition. Sets of gene expression markers are often referred to as a "signature."

As used herein, the term "biomarker-derived polynucleotides" means the RNA transcribed from a marker gene, any cDNA or cRNA produced therefrom, and any nucleic acid derived therefrom, such as a synthetic nucleic acid having a sequence
15 derived from the gene corresponding to the marker gene.

A gene biomarker is "informative" for a condition, phenotype, genotype or clinical characteristic if the expression of the gene marker is correlated or anti-correlated with the condition, phenotype, genotype or clinical characteristic to a greater degree than would be expected by chance.

As used herein, the term "signature" refers to a set of one or more differentially
20 expressed genes that are statistically significant and characteristic of the biological differences between two or more cell samples, e.g., normal and diseased cells, cell samples from different cell types or tissue, or cells exposed to an agent or not. A signature may be expressed as a number of individual unique probes complementary to
25 signature genes whose expression is detected when a cRNA product is used in microarray analysis or in a PCR reaction. A signature may be exemplified by a particular set of biomarkers.

As used herein, the terms "measuring expression levels," "obtaining expression level," and "detecting an expression level" and the like, include methods that quantify a
30 gene expression level of, for example, a transcript of a gene, or a protein encoded by a gene, as well as methods that determine whether a gene of interest is expressed at all. Thus, an assay which provides a "yes" or "no" result without necessarily providing quantification of an amount of expression is an assay that "measures expression" as that

term is used herein. Alternatively, a measured or obtained expression level may be expressed as any quantitative value, for example, a fold-change in expression, up or down, relative to a control gene or relative to the same gene in another sample, or a log ratio of expression, or any visual representation thereof, where a color intensity is representative of the amount of gene expression detected. Exemplary methods for detecting the level of expression of a gene include, but are not limited to, Northern blotting, dot or slot blots, reporter gene matrix (see for example, U.S. Patent No. 5,569,588) nuclease protection, RT-PCR, microarray profiling, differential display, 2D gel electrophoresis, SELDI-TOF, ICAT, enzyme assay, antibody assay, and the like.

The present invention is based, at least in part, on the discovery by the present inventors of a set of radiation exposure biomarkers that can be used individually or in combination in accordance with the methods, reagents, kits and devices of the invention for carrying out a diagnostic assay to assess the exposure to ionizing radiation in a biological sample of interest. As described in EXAMPLES 1-4, the inventors have performed extensive *ex vivo* and *in vivo* studies to identify and validate biomarkers for use in a radiation exposure assay. As described in EXAMPLE 1, the approach for selecting the radiation exposure biomarkers entailed (1) identifying genes with radiation exposure responses in human peripheral blood (PB) using *ex vivo* models and (2) eliminating the genes that displayed stress-induced expression changes in non-irradiated individuals who sustained trauma. As described in EXAMPLE 2, the candidate biomarkers were validated using real-time quantitative RT/PCR (qRT/PCR) in peripheral blood samples from an *ex vivo* human radiation model (in subpopulations of cells and in plasma) as well as from human and canine subjects undergoing total body irradiation (TBI) as part of transplant conditioning.

The expression of one or more of the validated radiation exposure biomarkers set forth in TABLE 3 may be measured in a biological sample in accordance with the methods, kits and devices as described herein. In preferred embodiments, the kits and devices can be stockpiled and distributed for use under emergency conditions to detect radiation exposure in the event of a real or suspected nuclear or radiological event.

As described in EXAMPLES 3 and 4, statistical tools were employed to develop computational algorithms to identify and differentiate subjects that were exposed to any level of radiation (dosimetry score 1, DS₁ EXAMPLE 3) and further stratify exposed

individuals into those who received less than 2 Gy of radiation from those who received higher doses of radiation (dosimetry score 2, DS₂, EXAMPLE 4).

In accordance with the foregoing, in one aspect, the present invention provides a method for assessing exposure to ionizing radiation. The method according to this aspect of the invention comprises (a) measuring the RNA expression level of at least one radiation exposure biomarker gene selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC, and ZMAT3 in a biological sample; (b) correcting the RNA expression level of the biomarker(s) measured in step (a) to a reference standard (e.g., GUSB) or a threshold value, and (c) comparing the RNA expression level of the biomarker measured in step (a) and corrected in accordance with step (b) to the corrected expression of a calibrator sample or a threshold value, wherein a difference in expression level between the biomarker(s) and the reference standard indicates that the source from which the biological sample was obtained was exposed to ionizing radiation.

The methods of the invention can be used to assess exposure to radiation in any biological sample that contains RNA, such as a biological fluid or a biological tissue. Examples of biological fluids include whole blood, bone marrow aspirate, plasma, serum, saliva, and urine, and populations of cells obtained therefrom. Examples of biological tissues include organs, tumors, lymph nodes, arteries and individual cells, such as white blood cells, mononuclear cells, subpopulations of blood cells (e.g., lymphocytes, monocytes, granulocytes), including cells grown in culture.

In one embodiment of the present invention, a biological sample obtained from the subject comprising one or more cells from the subject to be tested are obtained and RNA is extracted from the cells. In some embodiments, the biological sample is obtained from a mammalian subject, such as a human, dog, cat, mouse, rat, horse, and the like. In some embodiments, a cell sample obtained from a subject is enriched for a desired cell type, such as lymphocytes, prior to RNA extraction.

RNA may be extracted from the biological sample by a variety of methods, for example, guanidium thiocyanate lysis followed by CsCl centrifugation (Chirgwin, et al., *Biochemistry* 18:5294-5299, 1979), or by preparation of a cell lysate using Trizol™ reagent (Invitrogen). RNA from single cells may be obtained as described in methods for preparing cDNA libraries from single cells (see, e.g., Dulac, *Curr. Top. Dev. Biol.* 36:245-258, 1998; Jena, et al., *J. Immunol. Methods* 190:199-213, 1996). Methods of

RNA extraction are well known in the art, and commercially available RNA extraction kits are suitable for use in accordance with the methods of the invention.

In some embodiments, the biological sample is assessed for radiation exposure within a time period ranging from 30 minutes after initial potential exposure to ionizing radiation up to 14 days after the end of the potential exposure (such as from 2 hours to 72 hours, such as from 4 hours to 24 hours) to ionizing radiation, such as from a nuclear accident or attack, or after a diagnostic test or therapeutic treatment (e.g., cancer treatment).

In some embodiments, the method is capable of providing a binary distinction as to whether the source of the biological sample was exposed or not exposed to radiation. The biomarkers presented in TABLES 1-4 can be used for such assays. In some embodiments of the method, AEN and APRT expression corrected for the expression of GUSB reference standard and normalized relative to a calibrator sample (e.g., pooled RNA from peripheral blood of healthy donors) is used to compute a Dosimetry Score 1:

$$[DS_1 = 3.4041 (\text{AEN}) - 18.6343 (\text{APRT})]$$

DS_1 can be used to differentiate subjects exposed to ionizing radiation (such as, for example, a dose over 1.5 Gy or over 2 Gy) from those who have not been exposed to radiation, as described in EXAMPLE 3.

In some embodiments, the method is capable of determining the dose of radiation to which the source of the biological sample (e.g., human subject) was exposed and classifying the subject as either exposed to doses less than 2 Gy of ionizing radiation, or exposed to a dose greater than 2 Gy of ionizing radiation. The same biomarkers presented in TABLES 1-4 can be employed in such assays. In one embodiment of the method, AEN and APRT expression corrected for the expression of GUSB reference standard and normalized relative to a calibrator sample (e.g., pooled RNA from peripheral blood of healthy donors) is used to compute a Dosimetry Score 2:

$$[DS_2 = 0.6572 (\text{AEN}) - 11.3785 (\text{APRT})]$$

DS_2 can be used to differentiate subjects exposed to less than 2 Gy of ionizing radiation from those who have been exposed to greater than 2 Gy of radiation, as described in EXAMPLE 4.

In some embodiments, the method comprises measuring the RNA expression level of at least two biomarkers, wherein at least one biomarker gene is selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1,

XPC, and ZMAT3, and optionally measuring the RNA expression level of a cell surface marker, such as CD3D, as set forth in TABLE 3. RNA based CD3D expression corresponds to the flow cytometric expression of this lymphocyte marker, which can be used as a surrogate marker for the proportion of lymphocytes within the tested population of cells. Other suitable cell surface markers for use in this embodiment of the method include: CD3G, CD4 and CD8.

In the practice of this aspect of the invention, the expression level of at least one biomarker gene selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC, and ZMAT3 is determined and compared to a reference standard control, such as GUSB, as set forth in TABLE 3. In some embodiments, the method further comprises normalizing the expression value of the biomarker(s) in the test sample to the expression value of the biomarker(s) in a calibrator sample (e.g., a pool of RNA from the peripheral blood of healthy donors). The fold difference (increase or decrease) between the expression level measured in the test biological sample and the calibrator provides an indication of whether the source of the biological sample was exposed to ionizing radiation.

The calibrator may be chosen based on the circumstances of the analysis. In one embodiment, the calibrator is a biological sample obtained from one or more control subjects that have not been exposed to ionizing radiation. In another embodiment, the calibrator is a sample containing known concentrations of one or more biomarkers, or gene products thereof. In other embodiments, the calibrator may be obtained by determining the average or median expression value for one or more biomarker gene expression products from a survey of subjects, which can be controlled for various factors such as exposure to IR, age, disease status, treatment history, source cell type, and/or sex. In one embodiment, the calibrator comprises transcript levels that are computed relative to a calibrator comprised of RNA extracted from peripheral blood mononuclear cells from donors with known status of exposure to ionizing radiation (i.e., non-exposed, exposed, dosage of exposure). In some embodiments, the calibrator is a predetermined threshold value based on analysis of one or more control subjects.

In some embodiments, a significant difference in expression level comprises a statistically significant increase, or decrease, in the RNA expression level of one or more of the biomarkers set forth in TABLES 1-4, as compared to the expression level determined for the comparison group or the calibrator. In some embodiments, a significant

difference in expression level comprises an increase, or decrease, of about 2, 5, 10, 20, 100, or 1000-fold or more of the level of RNA expression of the biomarkers set forth in TABLES 1-4, as compared to the expression level determined for the comparison group or the calibrator.

5 Measuring the RNA expression level of one or more biomarkers

In accordance with various embodiments of the invention, the level of expression (increase or decrease) of specific biomarker genes can be computed by determining the amount of mRNA, or polynucleotides derived therefrom, present in a biological sample. Any method for determining RNA levels can be used. For example, RNA may be
10 isolated from a sample and separated on an agarose gel. The separated RNA is then transferred to a solid support, such as a filter. Nucleic acid probes representing one or more biomarkers are then hybridized to the filter by northern hybridization, and the amount of biomarker-derived RNA is determined. Such determination can be visual, or machine-aided, for example, by use of a densitometer.

15 Another method of determining RNA levels is by use of a dot-blot or a slot-blot. In this method, RNA from a sample, or nucleic acid derived therefrom, is labeled. The RNA or nucleic acid derived therefrom is then hybridized to a filter containing oligonucleotides derived from one or more biomarker genes, wherein the oligonucleotides are placed upon the filter at discrete, easily-identifiable locations.
20 Hybridization, or lack thereof, of the labeled RNA to the filter-bound oligonucleotides is determined visually or by densitometer. Polynucleotides can be labeled using a radiolabel or a fluorescent (i.e., visible) label.

Again by way of example, ArrayPlate™ kits (sold by High Throughput Genomics, Inc., 6296 E. Grant Road, Tucson, Arizona 85712) can be used to measure
25 gene expression. In brief, the ArrayPlate™ mRNA assay combines a nuclease protection assay with array detection. Cells in microplate wells are subjected to a nuclease protection assay. Cells are lysed in the presence of probes that bind targeted mRNA species. Upon addition of S1 nuclease, excess probes and unhybridized mRNA are degraded, so that only mRNA:probe duplexes remain. Alkaline hydrolysis destroys the
30 mRNA component of the duplexes, leaving probes intact. After the addition of a neutralization solution, the contents of the processed cell culture plate are transferred to another ArrayPlate™ called a programmed ArrayPlate™. ArrayPlates™ contain a 16-element array at the bottom of each well. Each array element comprises a

position-specific anchor oligonucleotide that remains the same from one assay to the next. The binding specificity of each of the 16 anchors is modified with an oligonucleotide, called a programming linker oligonucleotide, which is complementary at one end to an anchor and at the other end to a nuclease protection probe. During a hybridization reaction, probes transferred from the culture plate are captured by an immobilized programming linker. Captured probes are labeled by hybridization with a detection linker oligonucleotide, which is in turn labeled with a detection conjugate that incorporates peroxidase. The enzyme is supplied with a chemiluminescent substrate, and the enzyme-produced light is captured in a digital image. Light intensity at an array element is a measure of the amount of corresponding target mRNA present in the original cells. The ArrayPlateTM technology is described in Martel, R.R., et al., *Assay and Drug Development Technologies 1(1)*:61-71, 2002, which publication is incorporated herein by reference.

By way of further example, DNA microarrays can be used to measure gene expression. In brief, a DNA microarray, also referred to as a DNA chip, is a microscopic array of DNA fragments, such as synthetic oligonucleotides, disposed in a defined pattern on a solid support, wherein they are amenable to analysis by standard hybridization methods (see Schena, *BioEssays 18*:427, 1996). Exemplary microarrays and methods for their manufacture and use are set forth in T.R. Hughes et al., *Nature Biotechnology 19*:342-347, April 2001, which is hereby incorporated herein by reference.

Briefly, a microarray containing a multiplicity of polynucleotide probes corresponding to the coding regions of the biomarker genes can be used to provide simultaneous determination of the expression levels of a plurality of genes in a transformed cell sample or reference standard sample. Detectably labeled polynucleotides representing the nucleotide sequences in mRNA transcripts present in a cell sample (e.g., fluorescently labeled cDNA synthesized from total cell mRNA) are applied to a microarray under defined conditions to permit specific hybridizations between the labeled cDNA and the polynucleotide probes adhering to a defined position on the array. After rinsing off unbound cDNA, the fluorescent intensities remaining at each spot on the array indicate the relative abundance of the gene product, and thus, gene expression level. The intensity can be compared against a similar assay that generated similarly labeled cDNA from a reference standard. Alternatively, the microarray may be simultaneously probed with cDNA from multiple sources, each probe population being

labeled with distinctly detectable probes. For instance, cDNA from the experimental cell sample and the control reference sample can be labeled with two different fluorophores and hybridized simultaneously on the same array. This scheme permits a direct comparison between the cell states, and variations due to minor differences in experimental conditions (e.g., hybridization conditions) will not affect subsequent analyses.

In another example, reverse transcription followed by PCR (referred to as RT-PCR) can be used to measure gene expression. RT-PCR involves the PCR amplification of a reverse transcription product, and can be used, for example, to amplify very small amounts of any kind of RNA (e.g., mRNA, rRNA, tRNA). RT-PCR is described, for example, in Chapters 6 and 8 of *The Polymerase Chain Reaction*, Mullis, K.B., et al., Eds., Birkhauser, 1994, the cited chapters of which publication are incorporated herein by reference. In practice, a gene expression-based assay examining a small number of genes (i.e., about 1 to 3000 genes) can be performed with relatively little effort using existing quantitative real-time PCR technology familiar to clinical laboratories. Quantitative real-time PCR measures PCR product accumulation through a dual-labeled fluorogenic probe or other suitable detection chemistry. A variety of normalization methods may be used, such as an internal competitor for each target sequence, a normalization gene contained within the sample, or a housekeeping gene. Sufficient RNA for real time PCR can be isolated from a subject. Quantitative thermal cyclers may now be used with microfluidics cards preloaded with reagents making routine clinical use of multigene expression-based assays a realistic goal.

The gene biomarker expression signatures or subset of genes selected from these signatures, which are assayed according to the present invention, are typically in the form of total RNA or mRNA or reverse transcribed total RNA or mRNA. General methods for total and mRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al., *Current Protocols of Molecular Biology*, John Wiley and Sons (1997). RNA isolation can also be performed using purification kit, buffer set, and proteases from commercial manufacturers, such as Qiagen (Valencia, CA), Invitrogen (Carlsbad CA), Ambion (Austin, TX), or other sources, according to the manufacturer's instructions.

Taqman quantitative real-time PCR can be performed using commercially available PCR reagents (e.g., Applied Biosystems, Foster City, CA) and equipment, such

as ABI Prism 7900HT Sequence Detection System (Applied Biosystems) according the manufacturer's instructions. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera, and computer. The system amplifies the template in a 96-well or 384-well format on a thermocycler. During amplification, laser-induced fluorescent
5 signal is collected in real-time through fiber-optic cables for all wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data. Commercially available reagents from other manufacturers (i.e., Thermo Fisher Scientific, Roche Applied Biosciences) and other real-time equipment (e.g., ViiA Real-Time PCR system by Applied Biosystems, CFX Real-time PCR detection system
10 from Bio-Rad, LightCycler by Roche Applied Science) can be used for quantitative real-time PCR.

Based upon the biomarker gene sets provided in various embodiments of the present invention, a real-time PCR Taqman assay can be used to make gene expression measurements and perform the classification and sorting methods described herein. As is
15 apparent to a person of skill in the art, a wide variety of oligonucleotide primers and probes that are complementary to or hybridize to the expression products of the radiation detection biomarkers listed in TABLE 3 may be selected based upon the biomarker sequences set forth in the Sequence Listing.

The measuring of the RNA expression of the biomarkers of the invention, can be
20 done by using those polynucleotides which are specific and/or selective for the RNA products of the invention to quantitate the expression of the RNA product. In a specific embodiment of the invention, the polynucleotides which are specific to and/or selective for the RNA products are probes or primers. In one embodiment, these polynucleotides are in the form of nucleic acid probes which can be spotted onto an array to measure
25 RNA from the sample of an individual to be measured. In another embodiment, commercial arrays can be used to measure the expression of the RNA product. In yet another embodiment, the polynucleotides which are specific and/or selective for the RNA products of the invention are used in the form of probes and primers in techniques such as quantitative real-time RT PCR, using for example, SYBR®Green, or using TaqMan® or
30 Molecular Beacon techniques, where the polynucleotides used are used in the form of a forward primer, a reverse primer, a TaqMan labeled probe or a Molecular Beacon labeled probe.

In some embodiments, the nucleic acids derived from the biological sample may be preferentially amplified by use of appropriate primers such that only the genes to be analyzed are amplified to reduce background signals from other genes expressed in the cell. Alternatively, the nucleic acids from the sample may be globally amplified before
5 hybridization to the immobilized polynucleotides. RNA, or the cDNA counterpart thereof, may be directly labeled and used, without amplification, by methods known in the art. These examples are not intended to be limiting; other methods of determining RNA abundance are known in the art.

In another aspect, the invention provides a kit for use in the practice of the
10 methods described herein. The kit comprises (a) at least one reagent specific for measuring the RNA expression level of at least one biomarker gene selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC, and ZMAT3 in a biological sample and (b) written instructions for the use of the reagents and interpretation of the results with regard to comparison to a threshold value or
15 calibrator for determining whether a biological sample has been exposed to ionizing radiation. In some embodiments, the kit further comprises at least one reagent for measuring the RNA expression level of at least one endogenous control gene that is not affected by exposure to radiation, such as GUSB.

In some embodiments, the kit includes one or more reagents to facilitate the
20 specific amplification of sequences corresponding to the biomarkers set forth in TABLE 3 using the polymerase chain reaction (PCR). In accordance with such embodiments, the kit comprises one or more oligonucleotide primers that specifically anneal to a template sequence unique to one or more biomarker product(s) to specifically amplify sequences corresponding to the one or more biomarker product(s) described in
25 TABLE 3. As such, in one embodiment, the kit comprises at least one PCR primer capable of annealing to a sequence corresponding to AEN (SEQ ID NO:1), APRT (SEQ ID NO:2), CDKN1A (SEQ ID NO:3), DDB2 (SEQ ID NO:4), FDXR (SEQ ID NO:5), PCNA (SEQ ID NO:6), RPS27L (SEQ ID NO:7), TRIAP1 (SEQ ID NO:8), XPC (SEQ ID NO:9) and ZMAT3 (SEQ ID NO:10), GUSB (SEQ ID NO:11); and CD3D (SEQ ID
30 NO:12), to allow detection of the expression products derived therefrom.

In some embodiments, the kit comprises detection reagents for measuring the RNA expression level of at least one biomarker gene from TABLE 3, wherein at least one biomarker gene is AEN (SEQ ID NO:1), APRT (SEQ ID NO:2), CDKN1A (SEQ ID

NO:3), DDB2 (SEQ ID NO:4), FDXR (SEQ ID NO:5), PCNA (SEQ ID NO:6), RPS27L (SEQ ID NO:7), TRIAP1 (SEQ ID NO:8), XPC (SEQ ID NO:9), ZMAT3 (SEQ ID NO:10), GUSB (SEQ ID NO:11); and CD3D (SEQ ID NO:12).

In some embodiments, the kit comprises detection reagents for measuring the
5 RNA expression level of at least two biomarker genes selected from the group consisting of AEN (SEQ ID NO:1), APRT (SEQ ID NO:2), CDKN1A (SEQ ID NO:3), DDB2 (SEQ ID NO:4), FDXR (SEQ ID NO:5), PCNA (SEQ ID NO:6), RPS27L (SEQ ID NO:7), TRIAP1 (SEQ ID NO:8), XPC (SEQ ID NO:9) and ZMAT3 (SEQ ID NO:10).

In some embodiments, the kit comprises detection reagents for measuring the
10 RNA expression level of at least one biomarker gene selected from the group consisting of AEN (SEQ ID NO:1), APRT (SEQ ID NO:2), CDKN1A (SEQ ID NO:3), DDB2 (SEQ ID NO:4), FDXR (SEQ ID NO:5), PCNA (SEQ ID NO:6), RPS27L (SEQ ID NO:7), TRIAP1 (SEQ ID NO:8), XPC (SEQ ID NO:9) and ZMAT3 (SEQ ID NO:10) and detection reagents for measuring the expression level of at least one control gene,
15 e.g., GUSB (SEQ ID NO:11) or a surrogate marker for the proportion of lymphocytes within the tested population of cells, e.g., CD3D (SEQ ID NO:12).

In some embodiments, the kit comprises detection reagents for measuring the RNA expression level of AEN (SEQ ID NO:1) and APRT (SEQ ID NO:2).

In some embodiments, the kit further comprises one or more PCR primer sets
20 and/or probes to amplify one or more biomarkers, for example, as set forth in TABLE 3.

In some embodiments, the kit comprises PCR primers to facilitate the specific amplification of sequences corresponding to the biomarkers AEN (SEQ ID NO:1) and APRT (SEQ ID NO:2), such as at least one primer selected from the group consisting of SEQ ID NO:13-15, and at least one primer selected from the group consisting of SEQ ID
25 NO:16-18.

In some embodiments, the kit further comprises PCR primers to facilitate the specific amplification of sequences corresponding to at least one of the control markers GUSB (SEQ ID NO:11), (e.g., SEQ ID NO:43-45), and/or a surrogate marker for lymphocytes CD3D (SEQ ID NO:12) (e.g., SEQ ID NO:46-48).

30 The reagents for the kits of the invention may deploy any method well known in the art to assay expression of transcripts and/or proteins with known sequences, including, but not limited to, Northern blot, PCR, quantitative RT-PCR, or hybridization to a microarray containing biomarker-specific probes. In some embodiments, the kit

comprises at least one reagent for detecting a biomarker that is disposed in or on a substrate suitable for high-throughput analysis, such as a microarray.

In some embodiments, the kit includes additional reagents to facilitate the quantification of the one or more amplified biomarker derived nucleic acid molecule during the PCR cycles. Reagents and amplification parameters for PCR are well-known in the art. As is well known, the thermocycling profile in PCR includes an annealing step to allow the formation of stable hydrogen bonds between single stranded oligonucleotide primers or probes and the single stranded nucleic acid template. The standard reaction temperatures for annealing typically range from about 50°C to about 70°C and persist from about 15 seconds to at least a minute.

In some embodiments, the kit comprises a detection reagent such as a fluorescent dye that provides a signal when intercalated with double stranded DNA. In some embodiments, the kit comprises a probe that provides a detectable signal when annealed to a sequence in the target sequence. It will be apparent to one skilled in the art that the probe must be specifically designed to anneal to a sequence that is between the sequences corresponding to the forward and reverse primers used to amplify the target sequence. In these embodiments, starting samples with more template, i.e., gene transcripts, will exhibit stronger signals at earlier cycles of the assay, and can be thus quantified against a reference standard based on cycle threshold (Ct).

In some embodiments, the kit further comprises written instructions for use of the reagents and interpretations of the results. In some embodiments, the kit is in the format of a point of care assay.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention. All literature citations are expressly incorporated by reference.

EXAMPLES

Background/Rationale:

The Examples describe the development of two distinct assays used to screen for two categories of candidate biomarkers for radiation exposure detection.

The first category of biomarkers is suitable for use in an initial screening assay to determine if a subject has been exposed to radiation (yes, no, or inconclusive for radiation exposure). The screening assay will enable differentiation of individuals who have and have not been exposed to radiation. Examples of individuals who may benefit from this

initial screening assay include, but are not limited to, survivors of accidental or intentional small- and large-scale nuclear detonations, "dirty" bombs, military field exposure, military and civilian nuclear accidents, and any individual who is concerned about radiation exposure. Such an initial screening assay will allow for a rapid triage of survivors into those who need additional testing and/or immediate therapy and those who have not received a significant radiation exposure.

The second category of biomarkers is suitable for use in a comprehensive dosimetry assay that discriminates between different levels of radiation exposure (up to 12+ Gy). The comprehensive radiation dosimetry assay allows for the estimation of the dose of radiation exposure in a mammalian subject, such as a human subject. Examples of individuals who may benefit from this comprehensive dosimetry assay include, but are not limited to, survivors of accidental or intentional small- and large-scale nuclear detonations, "dirty" bombs, military field exposure, military and civilian nuclear accidents, and any individual who is concerned about radiation exposure. The comprehensive dosimetry assay enables responders to triage the exposed survivors for appropriate medical interventions based on the doses of radiation exposure, such that those exposed to lower doses would receive less aggressive treatment, while those who received ablative doses of radiation would be considered for transplantation. In addition, the comprehensive dosimetry assay can be employed in a clinical setting and benefit patients receiving radiation therapy as part of their medical treatment. For example, the comprehensive dosimetry assay can be used to estimate radiation toxicity and sensitivity of neoplasms, allowing for a more personalized approach to therapy. The comprehensive dosimetry assay can also be used in a research setting to assess the physiological levels of radiation exposure in large animal models other than non-human primate models.

As described below, the radiation exposure biomarker genes for use in the initial screening and comprehensive dosimetry assays were chosen such that they, either alone or in combination, can be used to reproducibly and reliably identify exposed human and large animal subjects and estimate the level of radiation absorbed after an exposure. Unlike the dicentric chromosome aberration assay, which is the current assay available for detecting radiation exposure, the screening and dosimetry assays of the present invention are quicker, less labor intensive, and easier to interpret, thus promoting faster integration in a clinical setting. In addition, the screening and dosimetry assays of the

invention provide a very robust estimate of radiation exposure and/or level of exposure following a significant time delay (i.e., up to 14 days after the end of exposure).

EXAMPLE 1

This Example describes the identification of candidate biomarker genes with robust, significant, and specific radiation-induced RNA expression changes in human peripheral blood.

Methods:

Ex vivo irradiation of whole blood cells (total leukocytes)

For microarray assessment of radiation-induced expression changes, whole blood samples (N = 10 donors) were obtained from healthy human donors and were exposed to ionizing radiation using a GammaCell 1000 Elite Irradiator (MDS Nordion, Kanata, Ontario, Canada) at an average rate of 7.6 Gy per minute at the following doses: 0 (sham), 0.15, 2, 4, 6, 9 and 12 Gy. Post irradiation, the samples were kept at 37°C, 5% CO₂ and harvested 3, 8 and 24 hours after radiation exposure as described in Pogossova-Agadjanyan et al., *Radiat Res.* 175(2):172-184 (2011), hereby incorporated herein by reference.

White blood cells (granulocytes and mononuclear cells, WBCs) were separated using RBC lysis reagent (5-Prime/Fisher Scientific, Waltham, MA). RNA was extracted using Trizol reagent (Invitrogen) per manufacturer's recommendations and assessed for quality as described in Stirewalt, D.L. et al., *Genes Chromosome Cancer* 47:8-20 (2008).

DNA Microarrays

Five micrograms of total RNA was labeled using the Eukaryotic Target Labeling protocol, fragmented, and hybridized (15 µg of biotin-labeled and fragmented antisense cRNA) onto HG-U133A arrays (N=175) according to the Affymetrix protocol (Affymetrix, Santa Clara, CA). DAT, CEL, and CHP files were generated, and results were screened for quality as previously described in Stirewalt, D.L. et al., *Genes Chromosome Cancer* 47:8-20 (2008); Pogossova-Agadjanyan et al., *Radiat Res.* 175(2):172-184 (2011); and Brazma, A. et al., *Nat Genet* 29:365-371 (2001), hereby incorporated herein by reference.

Generation of Expression Values and Normalization

Image (DAT), cell intensity (CEL), and chip (CHP) files were generated using MAS 5.0 software (Affymetrix). Individual arrays were screened for quality, such that any array with a 3'/5' GAPDH or β-actin ratio >1.5 or background >100 was eliminated

from further analysis. The scaling factor of all arrays was within 3-fold differences and had similar average intensities. Expression values for individual probe sets were generated from CEL files robust multi-array average (RMA), which generates background-adjusted, quantile-normalized log-transformed values, as described in
 5 Irizarry, R.A., et al., *Biostatistics* 4:249-264 (2003), and Bolstad, B.M., et al., *Bioinformatics* 19:185-193 (2003), both of which are hereby incorporated herein by reference. These log₂-transformed expression values were imported into GenePlus™ software (Enodar Biologic, Seattle, WA), which can perform cluster (hierarchical and K-means), two group, time course (one and two arm), and the multivariable regression
 10 analyses (<http://enodar.com>).

Generalized Regression Model Framework for Expression Profile Analyses.

A regression-based statistical framework was used to analyze the gene expression arrays, as described in Xu, X.L., et al., *Hum Mol Genet* 11:1977-1985 (2002); Zhao, L.P., et al., *PNAS* 98:531-5636 (2001); Thomas, J.G., et al., *Genome Res* 11:1227-1236 (2001),
 15 hereby incorporated herein by reference. This framework, which is implemented in GenePlus software, uses estimating equation, as described in Liang, K. and S.L. Zeger, *Biometrika* 73:13-22 (1986). The advantage of this regression approach is that multiple covariates (e.g., radiation dose, time post exposure, age) can be included in the model to assess their impact on gene expression simultaneously.

20 In this model, gene expression from individual arrays are treated as a vector of outcomes, and radiation exposure dosage (X^d), time post exposure (X^t), and age of the subjects (X^a) as covariates. Let Y_{jk} denote gene expression for j th gene in the k th array. Therefore, the generalized regression model is as follows:

$$Y_{jk} = \alpha_j + \beta_j^a X_k^a + \beta_j^{d1} X_k^d + \beta_j^{d2} (X_k^d)^2 + \beta_j^{t1} X_k^t + \beta_j^{t2} (X_k^t)^2 + \beta_j^{dt} X_k^d X_k^t + \varepsilon_{jk},$$

25 where $X_k^d, (X_k^d)^2$ are the linear and quadratic terms for covariate X_k^d , and similarly, $X_k^t, (X_k^t)^2$ for covariate X_k^t , and $X_k^a, (X_k^a)^2$ for the covariates X_k^a . $X_k^d X_k^t$ is the cross product term for assessing interaction between the two covariates. α_j is the intercept and β_j are the vectors of gene-specific regression coefficients quantifying associations of interest. Lastly, ε_{jk} is the residual reflecting variation coming from sources other than
 30 the ones identified by known covariates. This model can be simplified based on the number of variables.

Identification of Candidate Biomarkers.

Dose and temporal patterns of gene expression responses after irradiation were identified using the general regression model described above under *Generalized Regression Model Framework for Expression Profile Analyses*. The microarray data was examined for both binary (yes, no) and linear radiation induced expression changes. For binary analysis, the expression profiles of sham-irradiated samples were compared to irradiated samples for each of the three time points. For linear dose analyses, dose was treated as a continuous independent variable for each of the three time points. Furthermore, multivariable regression analysis was also performed to incorporate gender, age, time, dose and dose*time interaction. Results from multivariable regression analyses were used to confirm the linear regression results and to identify other covariates that may influence dose responses. Genes (e.g., XPC) displaying linear dose responses were identified using a simplified version of general regression model. In the simplified regression model, gene expression was assumed to be linear with respect to both dose and time covariates as is the case of first degree approximation.

Therefore, the simplified regression model was $Y_{jk} = \alpha_j + \beta_j^a X_k^a + \beta_j^{d1} X_k^d + \beta_j^{t1} X_k^t + \beta_j^{dt} X_k^d X_k^t + \varepsilon_{jk}$. Coefficient β_j^{dt} in the simplified regression model was then be used to select genes displaying a linear dose response.

Binary Analyses:

Statistical inference was tested by examining the null hypothesis. Since the properties of the random variations are usually unknown, robust standard errors for each gene's parameter were calculated using generalized estimating equation method as described in Liang, K. and S.L. Zeger, *Biometrika* 73:13-22 (1986). For example, when testing parameter β_j^{dt} , the null hypothesis H_0 will be: $\beta_j^{dt}=0, j=1,2,...,J$. H_0 will be tested by calculating the Z-scores: $\beta_j^{dt}/SE(\hat{\beta}_j^{dt})$ where $SE(\hat{\beta}_j^{dt})$ is the standard error for β_j^{dt} . Z-score was transformed via an asymptotic distribution into a p-value, which is statistically significant at the predefined threshold of p-value ≤ 0.05 . This statistical cut-off is similar or more stringent than those that have previously been used to assess biological expression changes (see Xu, X.L., et al., *Hum Mol Genet* 11:1977-1985 (2002); Zhao, L.P., et al., *PNAS* 98:5631-5636 (2001)). To overcome the well recognized "multiple comparison" phenomena, the p-value was calculated for each gene using a modified Bonferroni's correction (Hochberg, Y. et al., *Stat Med* 9:811-818 (1990)).

Screening candidate biomarkers for stress-induced expression changes.

Candidate biomarkers that showed radiation-induced expression changes were identified as described above, which were then filtered for stress-related changes, such that genes displaying both radiation and stress-related changes were excluded as potential
5 biomarker candidates. Stress-related expression changes were determined by examining expression profiles of total leukocytes, monocytes and lymphocytes from 7 severely injured trauma patients and 7 matched healthy control subjects (Laudanski et al., *Proc. Natl. Acad. Sci. USA* 103:15564-15569 (2006)).

Results:

10 FIGURE 1A graphically illustrates the log-transformed expression levels of RAB13 in human peripheral blood treated *ex vivo* under control conditions, 24 hours after exposure to various doses of radiation (0.15, 2, 4, 6, 9 and 12 Gy), and after exposure to blunt trauma. As shown in FIGURE 1A, RAB13 gene expression was found to be induced by both trauma and by exposure to radiation, therefore this gene was excluded
15 from the list of potential biomarkers for detecting radiation exposure.

FIGURE 1B graphically illustrates the log-transformed expression levels of AEN in human peripheral blood treated *ex vivo* under control conditions, after exposure to various doses of radiation (0.15, 2, 4, 6, 9 and 12 Gy), and after exposure to blunt trauma. As shown in FIGURE 1B, AEN expression is induced by radiation exposure, but not by
20 exposure to stress, therefore this gene was selected as a candidate biomarker for detecting radiation exposure.

FIGURE 1C is a Venn diagram showing the overlap of genes identified by microarray analysis of human peripheral blood treated *ex vivo* to have significant radiation-induced expression changes at different time points after exposure to radiation.
25 As shown in FIGURE 1C, this analysis identified 1642, 1277 and 1198 genes with significant radiation-specific expression changes observed at 3, 8 and 24 hours, respectively, after human peripheral blood was exposed *ex vivo* to radiation. As further shown in FIGURE 1C, 414 genes displayed similar expression changes at all three time points. Of these 414 genes, a set of candidate biomarkers was chosen for further analysis.

30 FIGURE 1D graphically illustrates the log-transformed expression values of GUSB (beta-glucuronidase) which was used as the reference standard for quantitative normalization of RNA quality. The expression of GUSB is stable across lymphocytes

and whole blood cells exposed to various doses of radiation (sham, 0.15, 2, 4, 6, 9 and 12 Gy).

Table 1 shows the results of the microarray analyses of radiation-induced expression changes and expression changes induced by trauma-related stress in whole blood for a set of candidate biomarkers.

TABLE 1: Results of Microarray analysis of radiation-induced expression changes in whole blood for binary and linear dose analyses and trauma-induced changes in leukocytes, lymphocytes and monocytes for the same targets.

Gene ID	Radiation								Trauma
	Binary				Linear				Leukocyte/ Lymphocyte/ Monocyte
	8 hr		24 hr		8 hr		24hr		
	Z-score	NFD	Z-score	NFD	Z-score	NFD	Z-score	NFD	
AEN	7.86	<0.0001	5.76	0.0087	5.00	0.1237	5.12	0.0908	-/-/-
APRT	NS	NS	NS	NS	NS	NS	-4.78	0.2970	-/-/-
CDKN1A	7.23	<0.0001	6.44	0.0007	9.41	<0.0001	7.82	<0.0001	-/-/-
DDB2	18.51	<0.0001	14.40	<0.0001	6.27	0.0011	5.41	0.0319	-/-/-
FDXR	19.37	<0.0001	11.78	<0.0001	6.33	0.0009	7.11	0.0001	-/-/-
PCNA	16.64	<0.0001	14.95	<0.0001	8.53	<0.0001	4.81	0.2733	-/-/-
RPS27L	17.88	<0.0001	19.53	<0.0001	NS	NS	NS	NS	-/-/-
TRIAP1	7.14	<0.0001	5.26	0.0530	NS	NS	NS	NS	-/-/-
XPC	13.02	<0.0001	9.70	<0.0001	NS	NS	NS	NS	-/-/-
ZMAT3	6.12	0.0019	6.79	0.0002	NS	NS	NS	NS	-/-/-

NS: not statistically significant

The results shown in TABLE 1 of the microarray data analyses (N = 10 donors, doses examined: sham-irradiated, 0.15, 2, 4, 6, 9, 12 Gy) demonstrate the robustness of radiation-induced changes of RNA-based biomarkers at 8 and 24 hours in *ex vivo* irradiated whole blood cells (total leukocytes). Z-scores provide both significance and directionality for expression changes. A positive Z-score indicates an increase in expression in the irradiated cells and a negative Z-score indicates a decrease in expression in the irradiated cells. Number of False Discovery (NFD) provides a statistical assessment for the expression change, such that an expression change is deemed to be

statistically significant if NFD is less than 1. Statistical assessment of expression changes for the genes are provided for both the screening assay (binary, none versus any radiation, columns 2-5), and the comprehensive dosimetry assay (linear), which provides the ability to discriminate between different doses of radiation (columns 6-9). The microarray assessment of stress response (physical trauma) in total leukocytes, monocytes and lymphocytes is shown in the last column.

TABLE 2: Candidate Biomarkers for Detecting Radiation Exposure

Biomarker: Gene Name	Radiation-induced Response detected in Whole blood/lymphocytes*	Genbank Number	SEQ ID NO:
AEN (Apoptosis-enhancing nuclease)	both	NM_022767	1
APRT (adenine phosphoribosyltransferase)	both	NM_000485	2
CDKN1A (cyclin-dependent kinase inhibitor 1A (p21, Cip1))	both	NM_000389	3
DDB2 (damage-specific DNA binding protein 2)	both	NM_000107	4
FDXR (ferredoxin reductase nuclear gene encoding mitochondrial protein)	both	NM_004110	5
PCNA (proliferating cell nuclear antigen)	both	NM_002592	6
RPS27L (ribosomal protein S27-like)	both	NM_015920	7
TRIAP1 (TP53 regulated inhibitor of apoptosis 1)	both	NM_016399	8
XPC (xeroderma pigmentosum, complementation group C)	both	NM_004628	9
ZMAT3 (zinc finger, matrin type 3)	both	NM_022470	10

*lymphocyte data obtained from expanded naïve T cells exposed to IR *ex vivo*.

Control genes used as a reference standard include the following:

GUSB (glucuronidase, beta), NM_000181 (SEQ ID NO:11). As shown in FIGURE 1D, we determined that expression of GUSB is not impacted by radiation. Furthermore, it was determined that GUSB expression level is similar to the candidate genes under control conditions, making it an optimal control for RNA integrity and comparative PCR cycle threshold (Ct) computations.

Other endogenous control genes can be used as a reference standard in place of GUSB, such as, but not limited to, B2M (beta-2 microglobulin, NM_004048.2), GAPDH (glyceraldehyde-3-phosphate dehydrogenase, NM_002046.3), ACTB (actin beta, NM_001101.2), HPRT1 (hypoxanthine phosphoribosyltransferase, NM_000194.1, PGK1 (phosphoglycerate kinase 1, NM_000291.2), PPIA (cyclophilin A, NM_021130.3), RPLP0 (large ribosomal protein, NM_001002.3), TBP (TATA-box binding protein, M55654.1), or TFRC (transferring receptor, CD71, NM_003234.1), as accessed in Genbank on 4/25/2011, each Genbank sequence referenced above is hereby incorporated herein by reference.

In summary, we have identified 10 biomarkers for the detection of radiation exposure assessment that are highly robust and specific. In addition, we have determined that GUSB is a optimal endogenous control gene to use for quantitative assays as the means to correct for RNA integrity.

EXAMPLE 2

This Example describes the validation studies that were carried out on the candidate biomarkers identified in Example 1 in *ex vivo* and *in vivo* canine and human models.

Methods:

The candidate biomarkers, shown above in TABLE 2, that were identified using the methods described in Example 1 were further evaluated using real-time quantitative RT/PCR (qRT/PCR) in peripheral blood samples in the following four independent studies:

1. Peripheral blood from normal human donors (N=5) irradiated *ex vivo* using a GammaCell 1000 Elite Irradiator (MDS Nordion, Kanata, Ontario, Canada) at an average rate of 7.6 Gy per minute at the following doses (0) sham, 0.15, 2, 4, 6, 9 and 12 Gy. Post irradiation, the samples were kept at 37°C, 5% CO₂ for 24 hours. During

collection, plasma was collected from each sample and RNA was extracted from plasma as previously described (Arroyo et al., *Proc. Natl. Acad. Sci. U.S.A.*, epub 2011 Mar. 7; Mitchell et al., *Proc. Natl. Acad. Sci. U.S.A.*, 105(30):10513-10518 (2008)). White blood cells (granulocytes and mononuclear cells) were separated using RBC lysis reagent
 5 (5-Prime/Fisher Scientific, Waltham, MA). Mononuclear cells (MNCs) were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) per manufacturers' recommendations. A fraction of MNCs was positively selected for lymphocytes *via* magnetically labeled CD3 beads (Miltenyi) using manufacturer's recommendation and as described in Pogossova-Agadjanyan et al., *Radiat. Res.* 175(2):172-184 (2011),
 10 incorporated herein by reference. In summary, the RNA from following fractions was obtained: WBC, MNC, CD3+ lymphocytes, and plasma;

2. Peripheral blood from canines (N=18) exposed to a single fraction of total body irradiation (TBI), carried out as shown in FIGURE 2A; and
3. Peripheral blood from human patients (N=16) undergoing total body
 15 irradiation (TBI) as conditioning for transplant, carried out as shown in FIGURE 2B.

FIGURE 2A shows the peripheral blood collection schedule for study #2, the canine single dose TBI model, with the time of peripheral blood collection after radiation exposure shown by the arrows labeled "PB" along the bottom of the chart, and the total dose shown in each box within the chart, and the cumulative dose for each study shown
 20 in the box on the far right side of the chart.

FIGURE 2B shows the peripheral blood collection schedule for study #3, the human patients undergoing multiple fractionated TBI doses over various time periods ranging from one to four days. Each dose is shown in the boxes within the chart, with the total cumulative dose for each patient (study) shown in the box at the far right of the
 25 chart. The time of peripheral blood collection after the first radiation exposure is shown by the arrows labeled "PB" along the bottom of the chart.

Collection of Peripheral Blood

Paxgene blood RNA vacutainers were used for blood collection in the *in vivo* validation studies. The Paxgene system rapidly stabilizes RNA at the time the sample is
 30 collected, which is an important factor considering the fact that cells in peripheral blood may continue to undergo radiation-induced transcriptional changes after collection.

qRT/PCT Assays

The expression of the candidate biomarker genes shown below in TABLE 5 was determined using real-time quantitative RT/PCR in peripheral blood samples in the three independent study populations described above, using the reagents (sequence-specific primers and probes, TABLE 4) similar to those shown in TABLE 3. Validation studies were completed on the ABI 7900HT Fast Real-time PCR system. GUSB was used as a reference standard control because it was determined that expression of GUSB is not impacted by radiation, as described in Example 1.

The sequence-specific primers and probe mixes substantially similar to the ones shown in TABLE 3 for each candidate biomarker were purchased from Applied Biosystems (TABLE 4) for each target shown in TABLE 2, and for CD3D (CD3D molecule, delta of CD3-TCR complex, NM_000732.4 (SEQ ID NO:12)). These assays utilize universal standard Taqman design guidelines as described in Stirewalt, D.L. et al., *Leuk Res* 25:1085-1088 (2001), incorporated herein by reference.

Quantitative RT/PCR was performed as previously described in Pogossova-Agadjanyan et al., *Radiat. Res.* 175(2):172-184 (2011). Briefly, RNA was reverse-transcribed with oligodT primer using AMV-RT according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA). cDNA generated from this transcription step was diluted down to 2.5 ng/μL RNA equivalent with molecular biology grade water and 10 ng was used in each quantitative PCR reaction. GUSB was used as a reference standard to control for RNA quality as described above in Example 1. The gene expression ratio was computed relative to the pool of RNA from the peripheral blood of 7 healthy donors (the calibrator) using the $2^{-\Delta\Delta C_t}$ method as described in Livak et al. (*Methods* 24:402-408 (2001)), hereby incorporated herein by reference. All assays were performed in triplicate with appropriate negative and positive controls.

TABLE 3: Reagents for Biomarker Assays

Target Gene	Forward Primer	Reverse Primer	Probe
AEN (SEQ ID NO:1)	CCTGCAGCTGCTGC ACAA (SEQ ID NO:13)	GGCTGTCGTGGCATC TTCTAC (SEQ ID NO:14)	ATCCAGGTGGGCCA GCACGG (SEQ ID NO:15)

Target Gene	Forward Primer	Reverse Primer	Probe
APRT (SEQ ID NO:2)	GAAGGCTGAGCTG GAGATTCA (SEQ ID NO:16)	GCTCACAGGCAGCGT TCA (SEQ ID NO:17)	TGATCTGCTGGCCAC TGGTGGAAACCATGA AC (SEQ ID NO:18)
CDKN1A (SEQ ID NO:3)	GAGACTCTCAGGG TCGAAAACG (SEQ ID NO:19)	GATTAGGGCTTCCTC TTGGAGAAG (SEQ ID NO:20)	CAGACCAGCATGAC AGATTTCTACCACTC CA (SEQ ID NO:21)
DDB2 (SEQ ID NO:4)	TCAGTTCGCTTAAT GAATTCAATCC (SEQ ID NO:22)	CTCCTGGCTCCAGAT GAGAATG (SEQ ID NO:23)	CGCTGGCCTCTGCAA TGGGTTACC (SEQ ID NO:24)
FDXR (SEQ ID NO:5)	GTGCCCTTTGACTC CAAGCTT (SEQ ID NO:25)	TGACACCTGTAGGTC CTCTCTTCAC (SEQ ID NO:26)	ATGTGCCAGGCCTCT ACTGCAGCG (SEQ ID NO:27)
PCNA (SEQ ID NO:6)	CAAGTGGAGAACT TGGAAATGGA (SEQ ID NO:28)	GAAGTTCAGGTACCT CAGTGCAAA (SEQ ID NO:29)	CGATAAAGAGGAGG AAGCTGTTACCATAG AGATGAATG (SEQ ID NO:30)
RPS27L (SEQ ID NO:7)	CAGTGTTGTGCCAG CCTACAG (SEQ ID NO:31)	AATTCAGGAAGCTGT TTGAATCATT (SEQ ID NO:32)	CCAGACTCACAGAA GGGTGTTCAATTTAGA AGAAAG (SEQ ID NO:33)
TRIAP1 (SEQ ID NO:8)	CGTGCACCGACCTC TTCAA (SEQ ID NO:34)	CCCATGAACTCCAGT CCTTCA (SEQ ID NO:35)	CCAGCAGTGTGTTCA GAAAGCAATAAAGG AG (SEQ ID NO:36)
XPC (SEQ ID NO:9)	TGGCCAAAGGTCT GCTCATC (SEQ ID NO:37)	GAGAGTCCACCTCCT GCATCTG (SEQ ID NO:38)	CCCAAGAGTGAGGC AGCAGCTCCC (SEQ ID NO:39)
ZMAT3 (SEQ ID NO:10)	AGTTTAAAGATGATG CCTAACAGGAGAAA (SEQ ID NO:40)	GCCAGATCACGTGG AATTCTCT (SEQ ID NO:41)	CAGTACAGAATAATT CAGCAGGTCCTTACT TCAATCCC (SEQ ID NO:42)

Target Gene	Forward Primer	Reverse Primer	Probe
Control: GUSB (SEQ ID NO:11)	CGTGGTTGGAGAG CTCATTG (SEQ ID NO:43)	TTGTCTCTGCCGAGT GAAGATC (SEQ ID NO:44)	ATGACTGAACAGTCA CCGACGAGAGTGC (SEQ ID NO:45)
control: CD3D (SEQ ID NO:12)	GACATGAGACTGG AAGGCTGTCT (SEQ ID NO:46)	TCCAAGGTGGCTGTA CTGCAT (SEQ ID NO:47)	CCAGGTCTATCAGCC CCTCCGAGATC (SEQ ID NO:48)

TABLE 4: Applied Biosystems Assays used for Validation Studies

Target	Human Assay ID	Canine Assay ID
AEN	HS00901422_m1	Cf02651003_m1
APRT	HS00975725_m1	Cf02644988_g1
CDKN1A	HS00355782_m1	Cf02625099_m1
DDB2	HS00172068_m1	Cf02689329_m1
FDXR	HS00244586_m1	Cf02697391_m1
PCNA	HS00427214_g1	Cf02634116_g1
RPS27L	HS00274853_m1	Cf02662197_m1
TRIAP1	HS00429934_g1	Cf02710496_g1
XPC	HS00190295_m1	Cf02639629_m1
ZMAT3	HS00975725_m1	Cf02652248_m1
GUSB	4333767F	Cf02622816_m1
CD3D	HS00174158_m1	Cf02644399_m1

Statistical Analysis

- 5 Student's *t* test with one-tailed distribution and two-sample unequal variance was used to determine statistical significance of candidates tested for the screening assay (binary). For comprehensive dosimetry assay (linear), a regression line was fitted using the radiation doses (x-axis) and expression level as measured by quantitative RT/PCR (y-axis). A 95% confidence interval was constructed for the fitted line, and from the
- 10 confidence interval, the ability to discriminate between different doses was determined

for the genes. For all statistical analyses, p-values ≤ 0.05 were considered to be statistically significant.

Results:

The results of the qRT/PCR analysis for study #1 (human *ex vivo*), study #2
5 (canine *in vivo*) and study #3 (human *in vivo*) are shown below in TABLE 5. p-values for the screening (binary) assay and R^2 and p-values for the comprehensive dosimetry (linear) assay were computed from the qRT/PCR data.

TABLE 5: qRT/PCR Validation of Candidate Biomarkers in peripheral blood
10 from *ex vivo* human model (N=5) and *in vivo* canine (N=18) and human (N=16) models. Peripheral blood for the *in vivo* models was collected according to the dosage/sample collection schedule shown in FIGURE 2A (canine) and FIGURE 2B (human).

Gene ID	Human <i>ex vivo</i>			Canine <i>in vivo</i>			Human <i>in vivo</i>		
	binary	linear		binary	linear		binary	linear	
	p-value	p-value	R^2	p-value	p-value	R^2	p-value	p-value	R^2
AEN	<0.0001	0.0399	0.2142	<0.0001	NS	0.0302	<0.0001	<0.0001	0.23
APRT	NS	NS	0.0084	<0.0001	<0.0001	0.4732	NS	<0.0001	0.30
CDKN1A	<0.0001	NS	0.1096	<0.0001	0.0004	0.1703	<0.0001	<0.0001	0.54
DDB2	<0.0001	0.0009	0.5546	NS	NS	0.5106	<0.0001	<0.0001	0.46
FDXR	0.0002	0.0054	0.4357	NS	NS	0.6101	<0.0001	<0.0001	0.57
PCNA	<0.0001	0.0009	0.5546	<0.0001	<0.0001	0.4801	<0.0001	0.0323	0.16
RPS27L	<0.0001	0.0008	0.4708	<0.0001	<0.0001	0.2678	0.0092	0.0002	0.18
TRIAP1	<0.0001	0.0005	0.5018	<0.0001	NS	0.0230	<0.0001	0.0010	0.35
XPC	<0.0001	0.0069	0.4168	<0.0001	<0.0001	0.2212	<0.0001	<0.0001	0.58
ZMAT3	0.0001	0.0137	0.2928	<0.0001	NS	0.0008	<0.0001	0.0002	0.34

As shown above in TABLE 5, nine of the ten candidate biomarkers tested were
15 validated in the *ex vivo* human model. Eight of the ten candidate biomarkers examined in the *in vivo* canine TBI model were validated up to 68 hours after IR exposure. All ten candidate biomarkers were validated in the peripheral blood of human patients exposed to TBI.

FIGURE 3A graphically illustrates the validated genes in CD3+ lymphocytes from *ex vivo* irradiated peripheral blood. RNA was extracted from positively selected CD3+ lymphocytes isolated from mononuclear cells (MNCs) from 5 healthy donors exposed to sham radiation (no IR) and 2, 6, and 12 GY (IR). Error bars represent standard deviations among the 5 donors.

FIGURE 3B graphically illustrates an example of radiation-induced expression changes for FDXR (y-axis) in CD3+ lymphocytes and plasma obtained from *ex vivo* irradiated peripheral blood of 5 healthy donors. RNA extracted from samples harvested 24 hours after exposure to 0 (sham), 2, 6, and 12 Gy. The results in FIGURE 3B demonstrate that FDXR is an informative biomarker for measuring radiation-induced expression changes in subcomponents of blood, such as lymphocytes and plasma.

In summary, the results described in this Example demonstrate that many of the radiation dosimetry biomarkers (TABLES 1-4) identified by microarray analyses using *ex vivo* irradiated white blood cells (Example 1) have been independently validated in *ex vivo* irradiated WBC, lymphocytes and plasma, and in peripheral blood samples collected from canines and humans exposed to TBI (*in vivo* models).

EXAMPLE 3

This Example demonstrates the generation of a dosimetry score algorithm from data produced in validation studies presented in Example 2 of biomarkers identified in Example 1. These validated biomarkers were examined individually, or in combination, to generate an assay to reliably and reproducibly differentiate between subjects who have and have not been exposed to ionizing radiation.

Methods:

Stepwise regression procedure (SAS PROC REG) was used to identify the most predictive biomarker or a combination of biomarkers that would reliably and reproducibly differentiate between subjects who have and have not been exposed to ionizing radiation. The data used for this training set was obtained from human patients (N=16) receiving TBI as part of their conditioning (see FIGURE 2B for radiation and sample collection schedules). The significance cut off for entry into the dosimetry score model was $P < 0.01$. (See Pogossova-Agadjanyan et al., *Radiat Res* 175(2):172-184 (2011)). The gene with the smallest P value entered the model first and then additional genes were added based on their previous significance. Genes were removed from the

model if they did not significantly contribute to the dosimetry estimation. The dosimetry score model was validated in an independent set of peripheral samples obtained from the *in vivo* canine model described in Example 2 and illustrated in FIGURE 2A.

Results:

5 Two biomarkers, AEN and APRT, identified as described in Example 1, and validated as described in Example 2, were selected by SAS PROC REG as the candidates with the most informative and reliable radiation-induced expression changes, individually and in combination. Using AEN and APRT expression fold difference in the human TBI model (training set), we have developed a dosimetry score (DS_1) algorithm that can
10 differentiate between no radiation exposure and radiation exposure greater than 1.5 Gy.

$$DS_1 = 3.4041 (\text{AEN}) - 18.6343 (\text{APRT}),$$

where the (GENE ID) refers to the expression fold difference of that particular biomarker corrected for GUSB and normalized relative to the expression of the given biomarker in the calibrator sample (i.e., the pooled RNA from 7 healthy donors, as described in
15 Example 2).

FIGURE 4A graphically illustrates the Receiver Operating Characteristic (ROC) curves for AEN (solid line), APRT (dashed line) and the computed DS_1 (dotted line) in the human TBI model (training set, N=16). As shown in FIGURE 4A, the computed DS_1 was shown to have >95% sensitivity and specificity for identifying humans exposed to at
20 least 1.5 Gy.

FIGURE 4B graphically illustrates the DS_1 dosimetry score (y-axis) in the training set of samples collected from humans exposed to variable doses of radiation (x-axis). As shown in FIGURE 4B, the DS_1 algorithm is informative across a range of radiation doses from 1.5 Gy up to 12 Gy.

25 FIGURE 4C graphically illustrates the Receiver Operator Characteristic (ROC) curves for AEN (solid line), APRT (dashed line), and the computed DS_1 (dotted line) in the canine TBI model (testing set, N=18). As shown in FIGURE 4C, the DS_1 algorithm was found to have > 95% sensitivity and specificity in identifying canines that were exposed to at least 2 Gy of radiation. Furthermore, FIGURE 4C demonstrates the
30 robustness of the DS_1 that was generated using the human *in vivo* model (training set, FIGURES 4A and 4B) and validated in the canine *in vivo* model (testing set FIGURES 4C and 4D).

FIGURE 4D graphically illustrates the DS₁ dosimetry score (y-axis) in the testing set of samples collected from canines exposed to a single dose of either 2, 6, or 10 Gy of radiation (x-axis) at either 24, 48 or 68 hours after exposure. As shown in FIGURE 4D, the DS₁ algorithm is informative across a range of radiation doses and across a long time interval.

In summary, these results demonstrate how robust the DS₁ algorithm is that utilizes a combination of expression of two biomarkers, AEN and APRT, for identifying mammals, including humans, that were exposed to IR over a range of doses and duration after initial exposure and illustrates the applicability of the canine model for dosimetry studies.

EXAMPLE 4

This Example demonstrates the generation of a different dosimetry algorithm from data produced in validation studies presented in Example 2 of biomarkers identified in Example 1. These validated biomarkers were examined individually, or in combination to generate an assay to reliably differentiate between subjects who have been exposed to a radiation dose <2 Gy and those who have been exposed to radiation doses greater than 2 Gy.

Methods:

Stepwise regression procedure (SAS PROC REG) was used to identify the most predictive biomarker or a combination of biomarkers that would reliably and reproducibly differentiate between subjects who have been exposed to less than 2 Gy of ionizing radiation and those who were exposed to doses greater than 2 Gy. The data and procedures used for this training set were the same as used to generate the DS₁ algorithm described in Example 3.

Results:

The same two biomarkers used for DS₁ algorithm described in Example 3, AEN and APRT, identified as described in Example 1, and validated as described in Example 2, were selected by SAS PROC REG as the candidates with the most informative and reliable radiation-induced expression changes, individually and in combination, that can differentiate between subjects exposed to less than 2 Gy of ionizing radiation and those who received more than 2 Gy of IR. Using AEN and APRT expression fold difference in the human TBI model (training set), we have developed

another dosimetry score (DS₂) algorithm that can differentiate between subjects who were exposed to less than 2 Gy from those who were exposed to greater than or equal to 2 Gy of ionizing radiation.

$$DS_2 = 0.6572 (\text{AEN}) - 11.3785 (\text{APRT}),$$

- 5 where the (GENE ID) refers to the expression fold difference of that particular biomarker corrected for GUSB and normalized relative to the expression of the given biomarker in the calibrator sample (i.e., pooled RNA from 7 healthy donors as described in Example 2).

FIGURE 5 graphically illustrates the Receiver Operating Characteristic (ROC)
10 curves for AEN (solid line), APRT (dashed line) and the computed DS₂ (dotted line) in the human TBI model (training set, N=16). As shown in FIGURE 5, the computed DS₂ was found to have >95% sensitivity and specificity for discriminating between individuals exposed to IR doses <2 Gy and ≥ least 2 Gy.

In summary, these results demonstrate how robust the DS₂ algorithm is that
15 utilizes a combination of expression of two biomarkers, AEN and APRT, for identifying samples from human patients exposed to IR over a range of doses.

Discussion:

RNA-based assays include global expression platforms, such as DNA microarrays and more focused expression assessment via quantitative RT-PCR (qRT-PCR). Previous
20 studies have found that DNA microarrays can be employed to identify and quantify thousands of radiation-induced expression changes within a single sample. Most microarray investigations have examined radiation-induced expression changes in malignant cell lines, immortalized human lymphoblastoid cells and heterogeneous populations of primary cells. However, these studies are limited by biological differences
25 between immortalized and non-immortalized cells and the limited range of radiation doses previously studied. In contrast to these previous studies, the examples described herein examined hematopoietic cells across an expansive range of radiation doses to identify a set of biomarkers useful to detect radiation exposure in mammalian subjects, such as humans, and the use of this set of biomarkers to assess whether or not the subject
30 was exposed to radiation, and to discriminate between subjects that were exposed to a low dose or a high dose of radiation.

While illustrative embodiments have been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

CLAIMS

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for assessing exposure to ionizing radiation comprising:
 - (a) measuring the RNA expression level of at least one biomarker gene selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC and ZMAT3 in a biological sample;
 - (b) correcting the RNA expression level of the biomarker measured in step (a) to a reference standard or threshold value; and
 - (c) comparing the RNA expression level of the biomarker measured in step (a) and corrected in accordance with step (b) to the corrected expression of a calibrator sample or threshold value, wherein a difference in expression level between the biomarker in the biological sample and the calibrator sample indicates that the source from which the biological sample was obtained was exposed to ionizing radiation.
2. The method of Claim 1, wherein the biological sample is obtained from a mammalian subject.
3. The method of Claim 2, wherein the subject is a human.
4. The method of Claim 1, wherein the biological sample is obtained from cultured mammalian cells.
5. The method of Claim 2, wherein the subject is assessed in a time period of from 30 minutes after initial exposure to 14 days after the end of the potential exposure to ionizing radiation.
6. The method of Claim 5, wherein the ionizing radiation exposure is the result of a nuclear attack or accident.
7. The method of Claim 5, wherein exposure to ionizing radiation is the result of a procedure to diagnose or treat a medical condition.

8. The method of Claim 1, wherein the biological sample is selected from the group consisting of a biological fluid, a tissue sample or a population of cells obtained therefrom.

9. The method of Claim 8, wherein the biological fluid is selected from the group consisting of whole blood, bone marrow aspirate, serum, plasma, saliva and urine, or a population of cells obtained therefrom.

10. The method of Claim 8, wherein the tissue sample is selected from the group consisting of organs, tumors, lymph nodes and arteries, or a population of cells obtained therefrom.

11. The method of Claim 1, wherein the reference standard according to step (b) comprises GUSB expression.

12. The method of Claim 1, further comprising classifying the source of the biological sample as either exposed or not exposed to ionizing radiation.

13. The method of Claim 1, wherein the method is capable of determining the dose of radiation to which the source of the biological sample was exposed.

14. The method of Claim 13, further comprising classifying the source of the biological sample as either exposed to a low dose of ionizing radiation of 1-2 Gy, or exposed to a higher dose of ≥ 2 Gy of ionizing radiation.

15. The method of Claim 14, further comprising administering treatment to the source of the biological sample classified as exposed to a higher dose of ionizing radiation.

16. The method of Claim 1, wherein the at least one biomarker is AEN.

17. The method of Claim 1, wherein the at least one biomarker is APRT.

18. The method of Claim 1, comprising measuring the RNA expression level of at least two biomarkers, wherein at least one biomarker is selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC and ZMAT3 and at least one additional biomarker set forth in TABLE 3.

19. The method of Claim 18, comprising measuring the RNA expression level of AEN and APRT.

20. The method of Claim 18, comprising measuring the RNA expression levels of at least one biomarker gene selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC and ZMAT3 and correcting the expression of the given biomarker by the expression of a lymphocyte marker.

21. The method of Claim 20, wherein the lymphocyte marker is selected from the group consisting of CD3D, CD3G, CD4 and CD8.

22. A kit for assessing exposure to ionizing radiation in a biological sample, the kit comprising:

(a) at least one reagent for measuring the RNA expression level of at least one biomarker gene selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC and ZMAT3; and

(b) written instructions for the use of the reagents and interpretation of the results with regard to comparison to a threshold value or calibrator for determining whether a biological sample has been exposed to ionizing radiation.

23. The kit of Claim 22, further comprising a reference standard, wherein the reference standard is the expression value of a gene that is not affected by exposure to radiation.

24. The kit of Claim 22, wherein the at least one reagent is an oligonucleotide that hybridizes under stringent conditions to the gene expression product of the at least one biomarker.

25. The kit of Claim 22, wherein the at least one biomarker gene is AEN.

26. The kit of Claim 22, wherein the at least one biomarker gene is APRT.

27. The kit of Claim 22, comprising at least one reagent for detecting a first biomarker selected from the group consisting of AEN, APRT, CDKN1A, DDB2, FDXR, PCNA, RPS27L, TRIAP1, XPC and ZMAT3 and at least one reagent for detecting a second biomarker selected from the biomarkers set forth in TABLE 3.

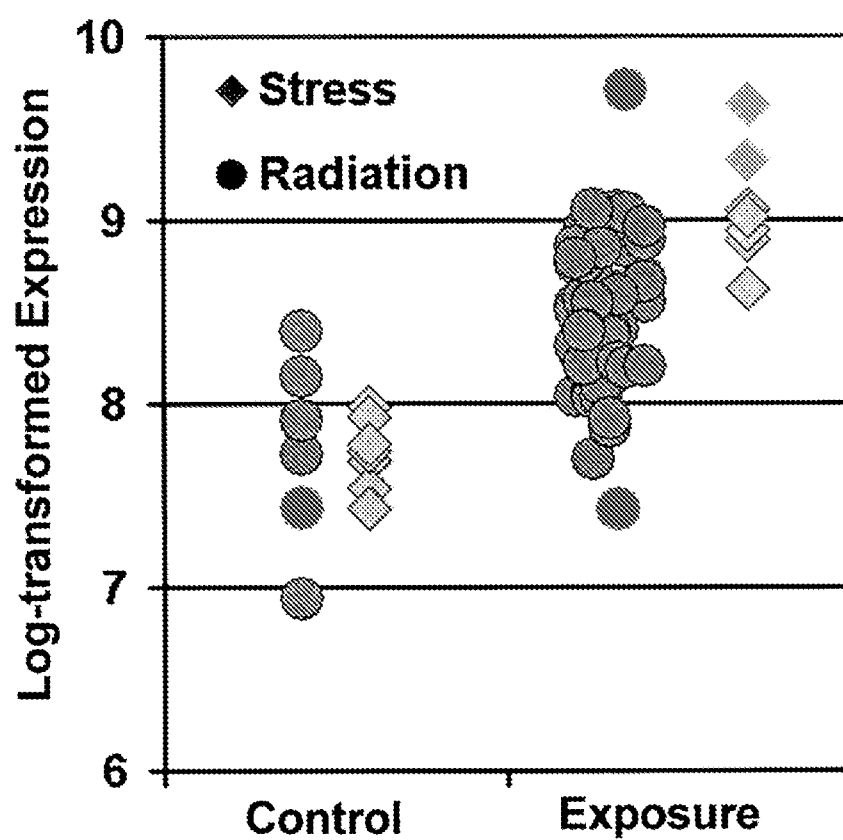
28. The kit of Claim 27, wherein the first biomarker is AEN and the second biomarker is APRT.

29. The kit of Claim 22, further comprising at least one reagent for RNA extraction from a biological sample.

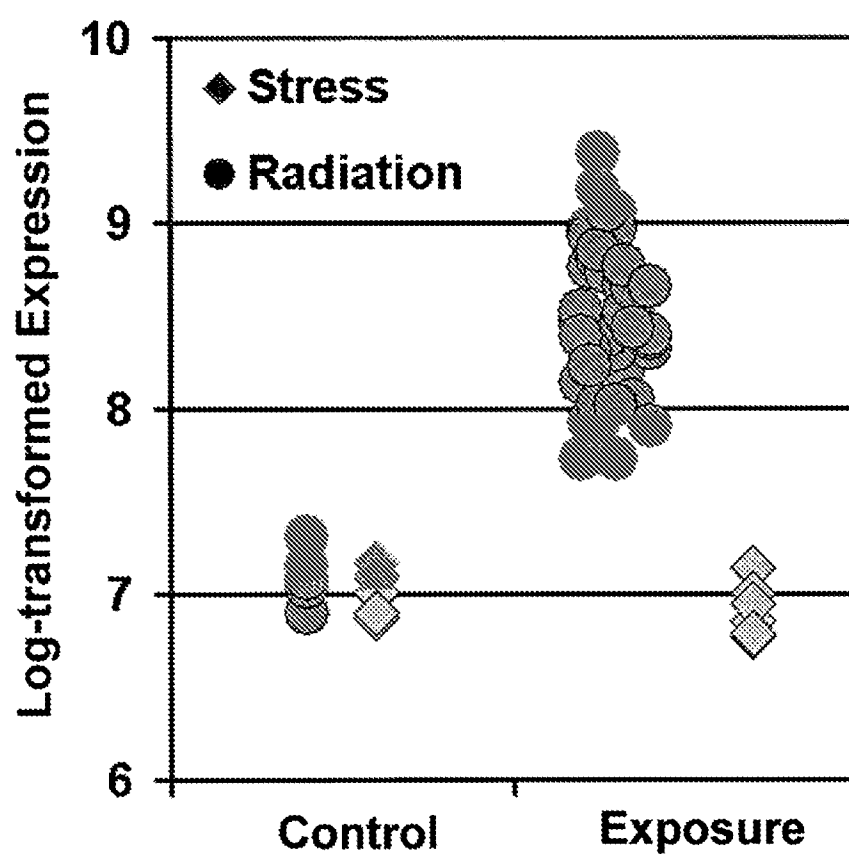
30. The kit of Claim 22, further comprising at least one reagent for amplification of at least a portion of the expression product of the biomarker.

31. The kit of Claim 22, wherein the at least one reagent is disposed in or on a substrate suitable for high-throughput analysis.

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FIGURE 1A

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FIGURE 1B

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FIGURE 1C

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FIGURE 1D
GUSB Microarray Expression Data

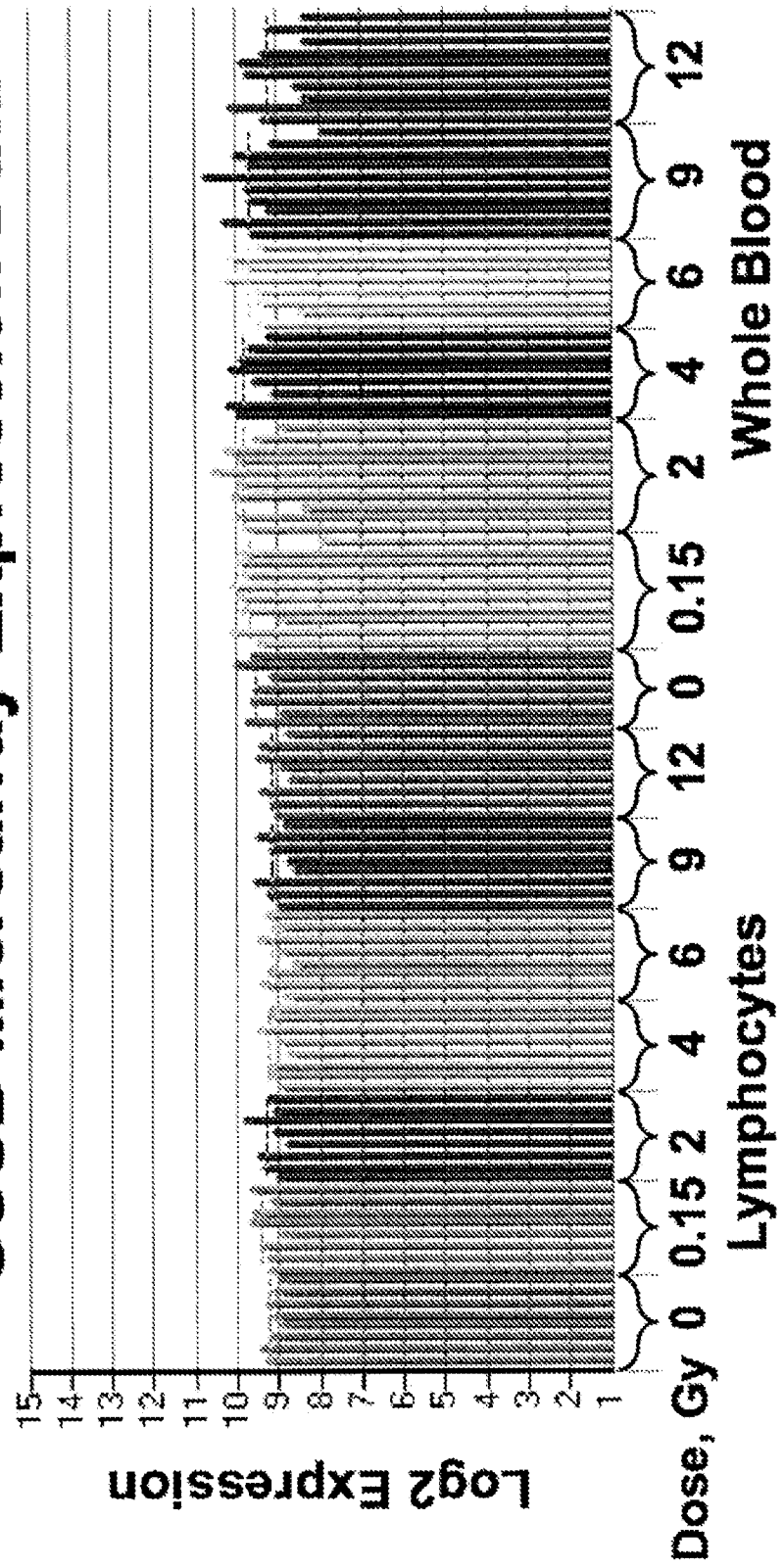
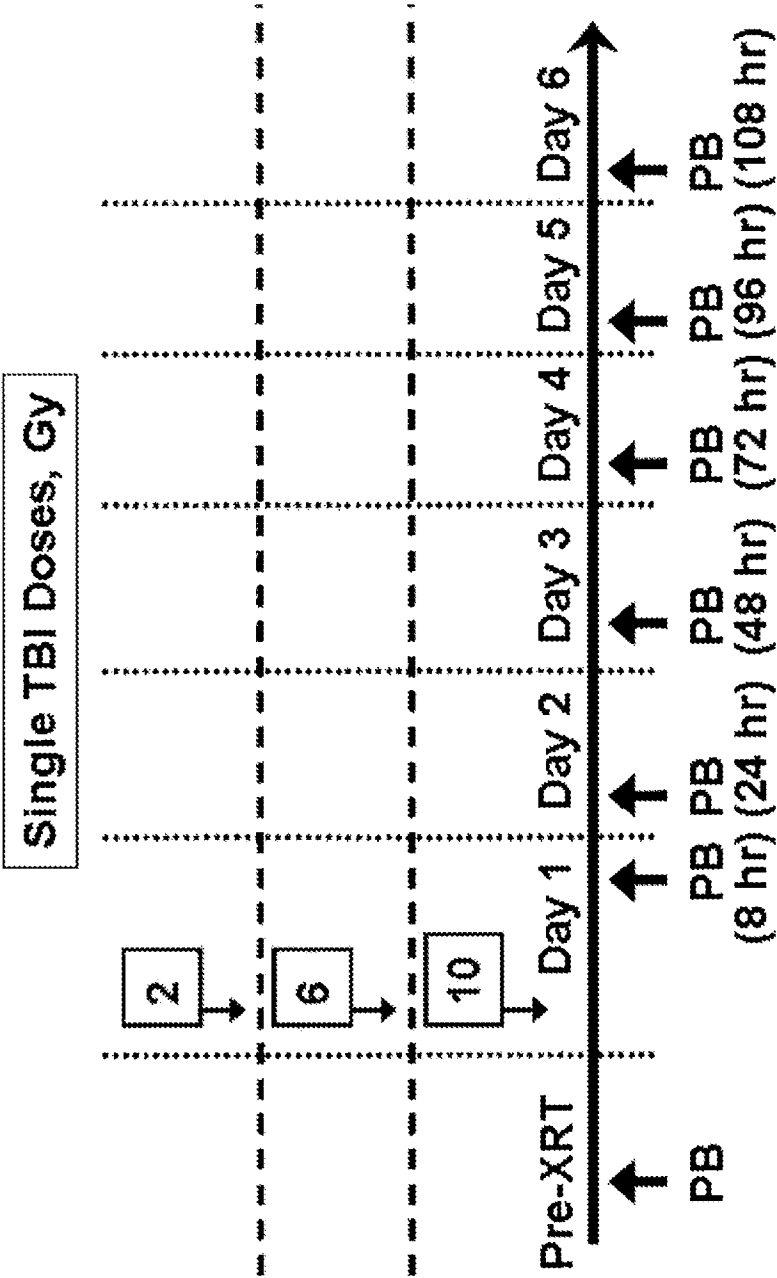
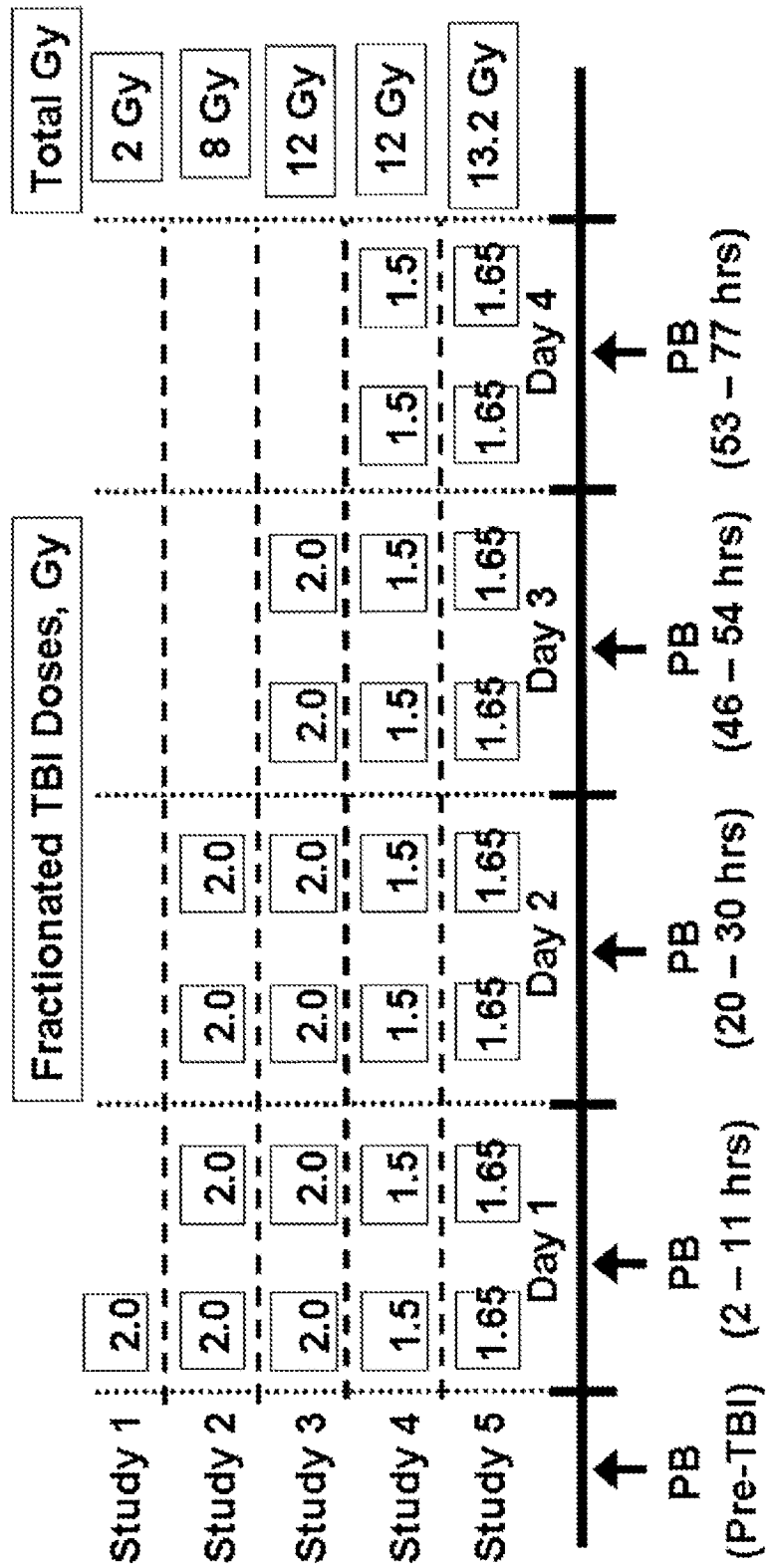


FIGURE 2A

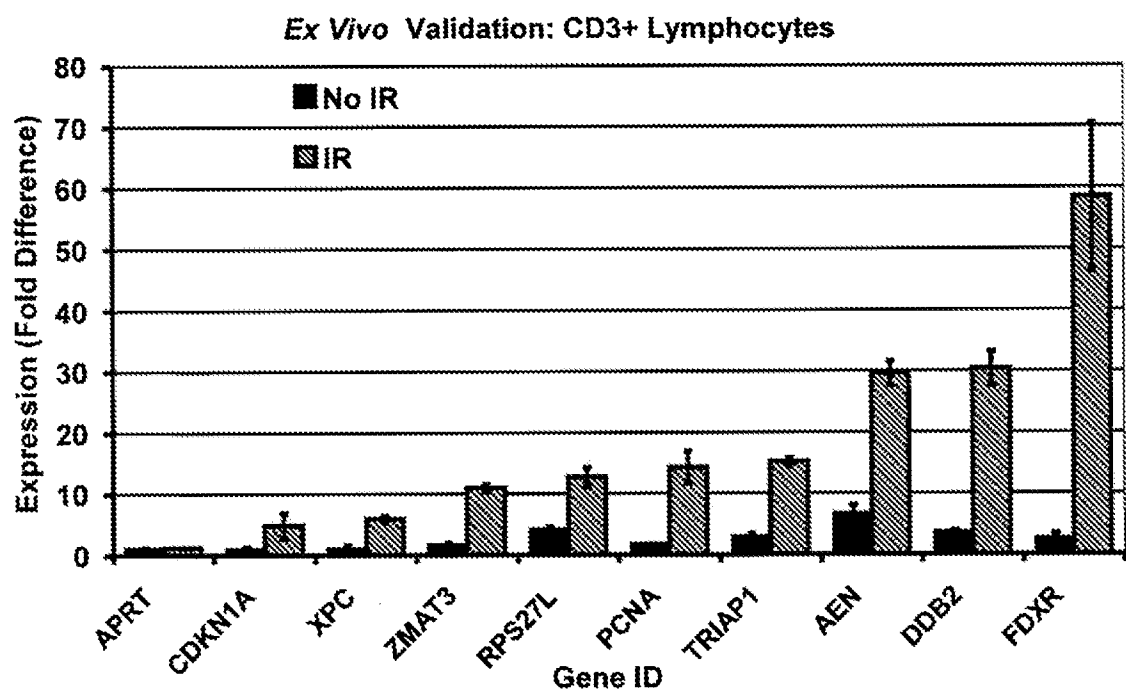


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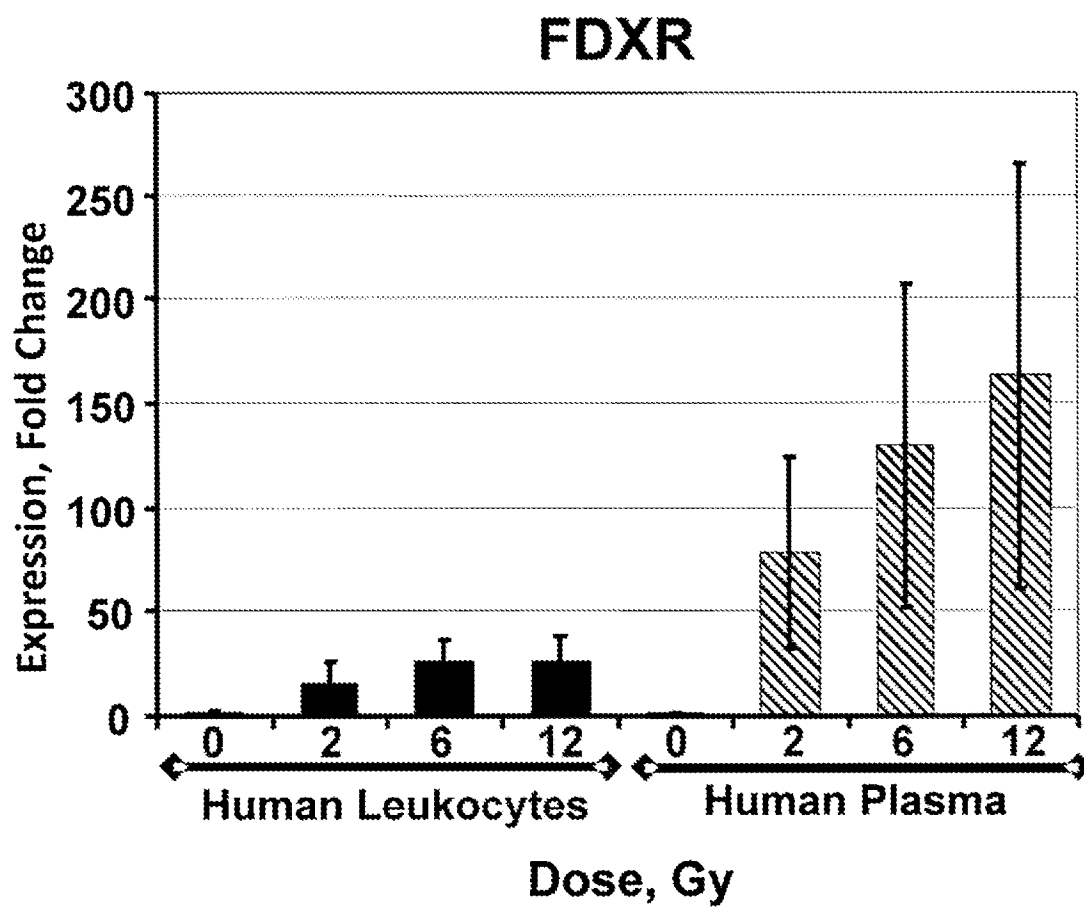
FIGURE 2B



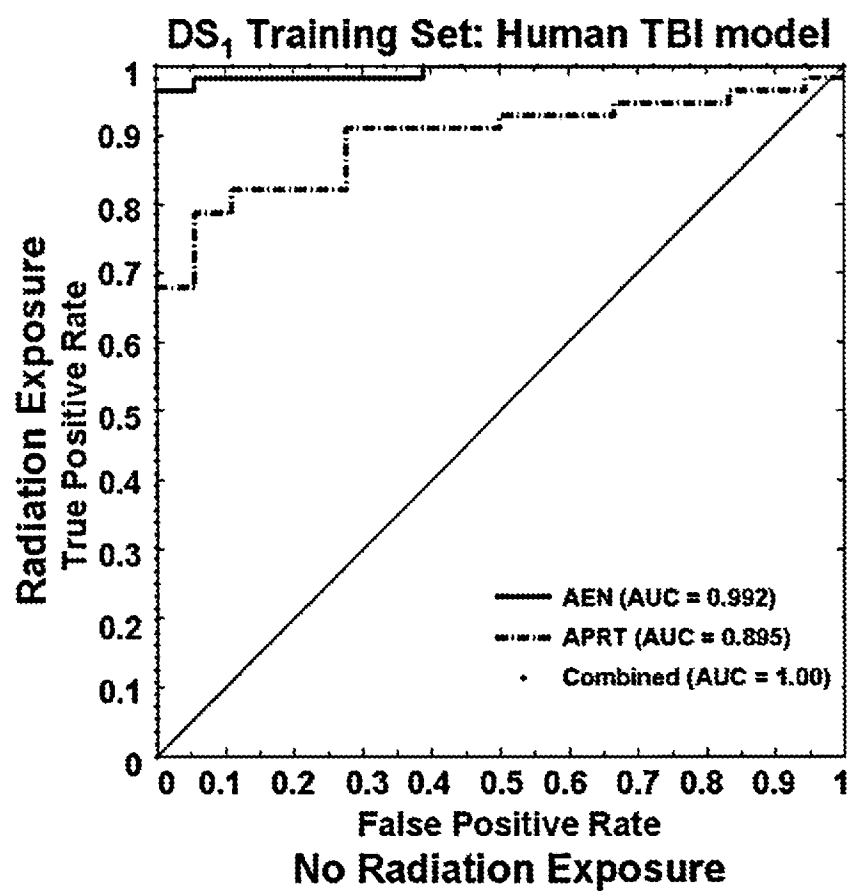
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FIGURE 3A

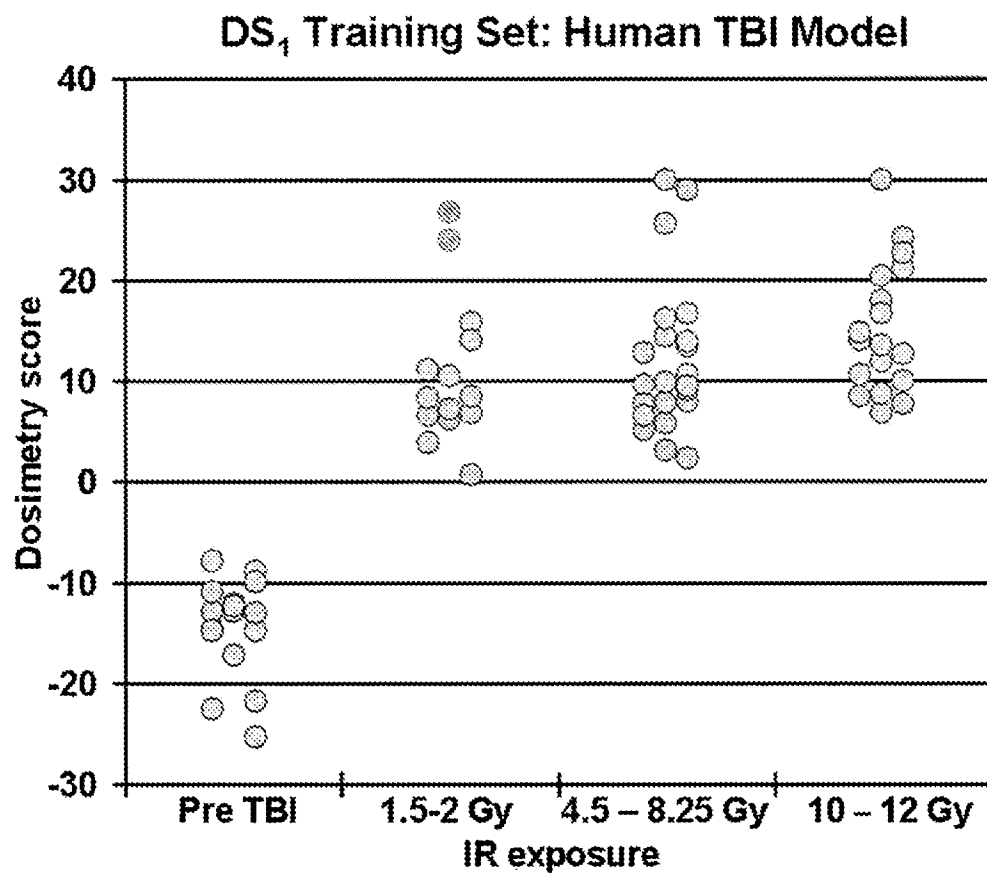
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FIGURE 3B

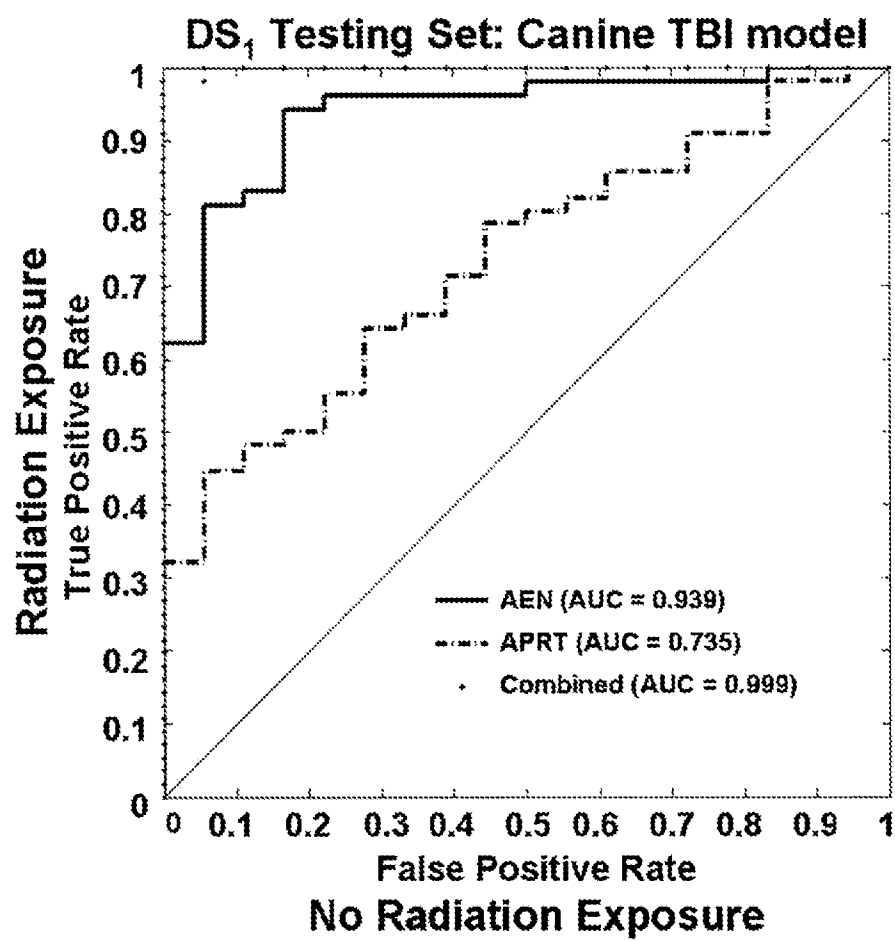
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FIGURE 4A

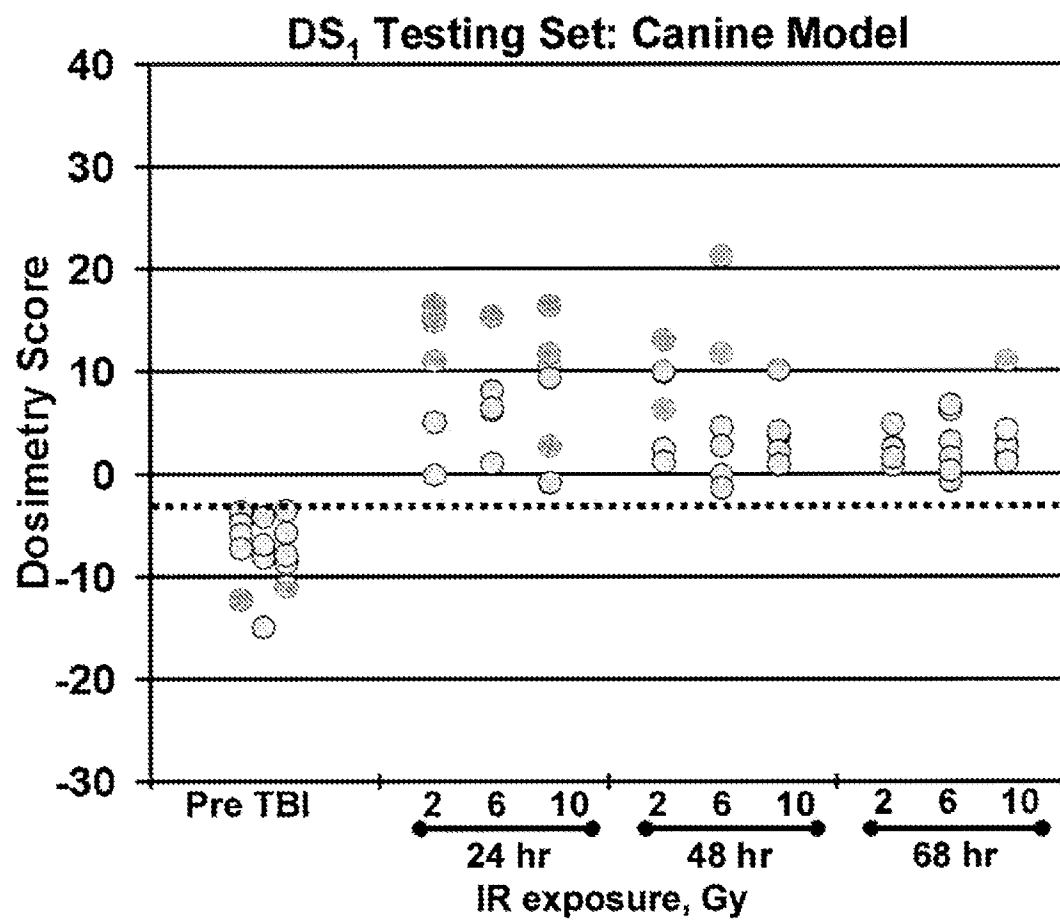
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FIGURE 4B

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FIGURE 4C

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FIGURE 4D

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FIGURE 5