



RESEARCH ARTICLE

# Rapid Triage of Radiation Exposure Using a 4-Gene Real Time PCR Test

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**PUBLISHED**  
30 November 2024

**CITATION**  
Terbrueggen, RH., Dixon, S., et al., 2024. Rapid Triage of Radiation Exposure Using a 4-Gene Real Time PCR Test. Medical Research Archives, [online] 12(11). <https://doi.org/10.18103/mra.v12i11.6114>

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**DOI**  
<https://doi.org/10.18103/mra.v12i11.6114>

**ISSN:** 2375-1924

## ABSTRACT

The ability to distinguish the concerned public from the significantly exposed is of critical importance for mounting an effective response to a mass scale nuclear incident. Here we describe the development and performance of a radiation biodosimetry blood test with a sensitivity of 99.0% and specificity of 96.5% for the qualitative determination of irradiated vs non-irradiated. Similar performance is observed in human and non-human primate (rhesus macaque) blood samples for in vivo total body irradiation as well as ex vivo blood irradiation. The test is based on the relative expression of 3 radiation responsive mRNA biomarkers relative to a normalizer mRNA using a pre-established cut-off. Blood samples must be collected into an RNA stabilizing blood collection device and sample testing is compatible with the real time PCR infrastructure used worldwide for COVID-19 pandemic response.

## Introduction

Following a mass scale nuclear event caused by the detonation of a nuclear device or a nuclear reactor meltdown, hundreds to hundreds of thousands of individuals will require medical management<sup>1</sup>. Emergency responders will start with treating individuals with obvious physical injury, however many with radiation injury will not display physical symptoms until several days to a week post-incident<sup>2,3</sup>. Diagnostic tests that allow for the identification of those with radiation injury while calming the worried are key to providing an effective response<sup>4</sup>.

Many radiation response scenarios envision using a qualitative triage test that initially separates the significantly exposed from the worried well, and then using a quantitative biodosimetry test to help guide treatment decisions<sup>5</sup>. The REDI-Dx Biodosimetry Quantitative Test System recently became the first biodosimetry test to receive CE-IVD regulatory authorization<sup>6</sup>. REDI-Dx Quantitative is an 18-plex gene expression assay that uses capillary electrophoresis for readout and is intended for use in a hospital setting. Other gene expression biodosimetry tests based on real time PCR<sup>7,8,9</sup> or microarrays<sup>10,11,12</sup> have been described<sup>13</sup>.

Here we described the development of a high throughput, real time PCR biodosimetry test suitable for initial triage of a mass scale nuclear event. The test is designed for qualitative (irradiated versus non-irradiated) assessment using real time PCR.

## Materials and Methods

### Sample Collection and Stabilization

All human samples were collected from subjects providing appropriately documented, HIPAA compliant informed consent under clinical research protocols reviewed and approved by an accredited Institutional Review Board (IRB) and implemented in accordance with the ICH Harmonized Guidelines for Good Clinical Practice (GCP), applicable regulations (including CFR Title 21). Human TBI samples were obtained from Duke University and City of Hope Cancer Center. Ex Vivo Irradiated Blood Samples were obtained from the University of Arizona. Subject enrollment for human demographic samples occurred at various clinical sites, which were managed by Access Biologicals (Vista, California) and Cureline (Brisbane, California). Samples with potentially confounding medical conditions were obtained from Cureline (Brisbane, California and Serologix (New Hope, Pennsylvania). Burn and Trauma samples were obtained from Maricopa Integrated Health System (Phoenix, Arizona). For Burn victims, samples were assigned to Total Body Surface Area (TBSA) categories of <10%, 10-20% and >21%. Trauma patients were assigned to Injury Severity Scale (ISS) categories of 10-14, 15-24 and >25. Influenza samples were obtained from Duke University. Inclusion criteria for all conditions: ≥18 years of age; Negative testing for Hepatitis B/C, HIV-1 and HIV-2 in clinical history, with the exception of samples being collected due to HIV status. Donors were excluded if they had received chemotherapy or a blood transfusion within the last 6 months, had X-rays, CT scans,

mammography, or radioactive dyes for scans or heart evaluations within the last 3 months.

Human samples were collected with DxCollect® Blood Collection tubes (BCT- DxTerity Diagnostics) using standard phlebotomy techniques. These tubes contain RNA stabilization buffer, which lyses the cells and stabilizes the RNA.

Samples from single dose in-vivo irradiated Non-Human Primates (Rhesus Macaque) were provided by Duke University, which were obtained from studies performed in compliance with protocols approved by an accredited IACUC and under the Biomedical Advanced Research and Development Authority (BARDA), Office of the Assistant Secretary for Preparedness and Response, Office of the Secretary, Department of Health Human Services Contract No: HHSO100201000001C and HHSO100201600034C. For additional details on the irradiation protocol refer to Iversen, et al 2018<sup>14</sup>.

Samples from Total Body Irradiated (TBI) Patients were obtained from City of Hope (Duarte, California), UCLA (Los Angeles, CA) and Duke University (Durham, NC) and collected per IRB approved protocols. Inclusion criteria were for planned fractionated TBI as part of clinical care, for malignant and non-malignant diseases treated with hematopoietic stem cell transplant, Subjects were excluded if they had received chemotherapy within 21 days, had concurrent chemotherapy with fludarabine, cytokine inhibitor or cytokine-inducer therapy within 30 days either prior to or during the irradiation regimen. Patients receiving granulocyte colony-stimulating factor within 30 days prior to TBI, were also excluded.

### Human Ex Vivo Irradiation

Human whole blood was collected from consented healthy donor into 5ml sodium citrate vacutainers by venipuncture. After mixing by inversion, the blood was irradiated using a Varian 21EX linear accelerator within 15 minutes of collection. Samples were irradiated at 212 MU/min.

After irradiation, 2.5 mL of blood was mixed by inversion with 2.5 ml of pre-warmed to 37°C RPI-1640 media in a 15 ml falcon plastic tube. Samples were incubated at 37°C in a CO<sub>2</sub> incubator for 24 hours. The culture samples were then mixed with an equal volume of DxCollect® RNA stabilization buffer.

### Human Total Body Irradiation

Peripheral blood was collected from adult patients undergoing TBI-based myeloablative allogeneic or autologous stem-cell transplantation. Samples were collected prior to irradiation at the beginning of each day from patients receiving either 150 cGy (Duke University) or 120 cGy (City of Hope). TBI patient blood samples with cumulative doses of 300 cGy, 600 cGy, 900 cGy and 1050 cGy (Duke University) or 360 cGy, 720 cGy and 1080 cGy (City of Hope).

### Total RNA Isolation

Total RNA was isolated from DxCollect® stabilized blood samples by magnetic bead capture and elution using a

proprietary automated method similar to automated processing using Beckman Coulter Agencourt RNAdvance Blood kits. Briefly, 350 microliters of stabilized blood were mixed with 265 microliters of lysis buffer and 20 microliters of protein kinase reagent in a deep well plate. After vortexing, the plate is incubated at 55°C for 15 minutes. Then 5ul of Sera-Mag Speedbeads (GE Life Sciences) in 350 microliters of ethanol were added. The RNA isolation and elution was then performed using an Auto-Pure 96 instrument (Hangzhou Allsheng) with final elution in 50 microliters of nuclease free water.

**Reverse Transcriptase qPCR**

In a 96-well plate, 6.25 microliters of TaqPath 1-Step Multiplex Master Mix (No Rx), 1.25 microliters of Primer mix, 7.5 microliters of nuclease-free water, and 10.0 microliters of isolated total RNA from bead isolation procedure were mixed together. RT-qPCR was performed using ABI ViiA7 real time PCR machine with reporter dyes of FAM, VIC, Cy5 and TEXAS RED. After 2 minutes at 25°C for UNG incubation, 10 min at 53°C for reverse transcriptase, 2 min at 95°C for Taq activation and 45 cycles of cycling between 95°C for 3 sec and

60°C for 30 seconds. Ct Values were determined using QuantStudio V1.3 software.

**Data Analysis**

The Ct values observed for BAX, CDKN1A and DDB2 were normalized relative to MRPS5. A weighted averaged metagene was calculated and fold change relative to the averaged metagene for unirradiated was determined. The cutoff for exposed versus unexposed is based on 3 standard deviations of the metagene for 149 healthy human samples, corresponding to a delta Ct of -0.76, corresponding to a 1.7-fold change.

**Results**

**Testing of Ex Vivo Irradiated Human Blood Samples**

Total RNA isolated from human blood samples that had been ex vivo irradiated at 0, 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 Gy. The samples were tested using the 4-gene real time PCR assay. A total of 138 samples were tested. The observed fold changes of the BAX, CDKN1A, DDB2 metagene as a function of dose are shown in table 1.

Dose (Gy)	Samples	Metagene Fold Change
0.0	18	1
0.05	4	1.2
0.1	4	1.4
0.25	4	1.9
0.5	18	3.4
1.0	18	3.6
1.5	18	4.1
2.0	18	4.7
3.0	18	5.5
4.0	18	5.7

**Table 1.** Summary of fold change observed for the metagene as a function of dose for ex vivo irradiated human blood samples.

**Testing of In Vivo, Total Body Irradiated (TBI) Non-Human Primates (NHPs)**

NHP samples irradiated from 0.5 to 10.8 Gy were tested. The blood samples were collected from 24 hours to 168 hours (7 Days) post-exposure following

irradiation from 0.5 Gy to 10.8 Gy. The metagene fold changes as a function of dose and time are shown in Table 2. The results are based on a total of 461 NHP samples.

Time (Hours)	0.5 Gy	1 Gy	2 Gy	4 Gy	6 Gy	8 Gy	10.8 Gy
24	2.6	5.3	5.2	5.7	7.9	5.9	5.1
72	2.4	4.1	8.3	9.4	11.3	8.5	12.4
120	2.0	3.0	6.5	7.9	11.8	13.0	16.2
168	1.9	1.9	3.4	6.7	8.8	8.1	9.5

**Table 2.** Calculated fold change for the metagene as a function of time post irradiation (hours) and absorbed dose for NHP single dose TBI samples.

**Testing of Human TBI Fractionated Samples**

Blood samples from Human Cancer Patients undergoing Total Body Irradiation were tested. The total fractionated dose received varied from 1.5 Gy to 13.2 Gy and a

cumulative time post irradiation varied from 5.5 hour to 96 hours. Observed fold change as a function of time post irradiation and dose is shown in Table 3.

Dose (Gy)	Time Post Irradiation (Hr)	Samples	Fold Change
0	0	23	1.0
1.5	5.5	41	5.0
2.4	24	3	3.8
3	24	7	5.4
3.6	24	12	4.0
6	48	11	5.5
7.2	48	12	4.8
9	72	5	7.1
9.6	72	3	6.9
10.5	72	6	6.7
10.8	72	8	6.5
13.2	96	5	8.9

**Table 3.** Calculated fold change for the metagene as a function of time post irradiation (hours) and absorbed dose for human fractionated dose TBI samples.

**Potentially Confounding Human Samples**

Isolated total RNA from 22 different potentially confounding human conditions were tested including burn, trauma, flu, bacterial infection, and post-treatment with

granulocyte colony stimulating factor (G-CSF). 355 of the 360 (98.6%) of the samples tested were correctly called unexposed (Table 4).

Confounding Condition	Samples	Accuracy (percent called unirradiated)
Burn	36	97.2%
Trauma	19	100%
Influenza	50	96%
Granulocyte Colony-Stimulating Factor (G-CSF)	13	100%
Moderate to Severe Infection	36	94.4%
Anti-Emetic	25	100%
Anti-Diarrheal	10	100%
Aspirin	15	100%
Ibuprofen	10	100%
Methamphetamines	14	100%
Steroidal anti-inflammatories	15	100%
Inflammatory Bowel Disease (IBD)	13	100%
Arthritis	11	100%
Cardiovascular Disease	14	100%
Immunocompromised	15	100%
Lipemia	12	100%
Obesity/High BMI	11	100%
Pregnancy	11	100%
Lactation	10	100%
Smoker	10	100%
Type II Diabetes	10	100%
Total	<b>360</b>	<b>98.6%</b>

**Table 4.** Accuracy of prediction of blood samples from humans suffering from conditions likely to be present post-nuclear incident (burn, trauma, influenza and infection), likely treatments (G-CSF, anti-emetic, anti-diarrheal, aspirin, ibuprofen, steroidal anti-inflammatories) and chronic human conditions.

**Calculation of Assay Performance: Sensitivity and Specificity**

Assay Performance was assessed based on a delta Ct cutoff of -0.77Ct for the normalized metagene. A total of 1486 in vivo irradiated, ex vivo irradiated and non-

irradiated human samples were used to assess performance (Table 5). The actual versus predicted irradiation status is shown in Table 6, and the sensitivity, specificity, positive predictive value, negative predictive value and accuracy are shown in Table 7.

Species	Irradiation Type	N Rows
Human	Ex Vivo	150
Human	In Vivo Fractionated Dose	142
Human	Healthy-N/A	304
Human	Confounders-N/A	360
NHP	Ex Vivo	19
NHP	In Vivo Fractionated Dose	50
NHP	In Vivo Single Dose	461
<b>Total</b>		<b>1486</b>

**Table 5.** List of samples used to assess performance of radiation biodosimetry for qualitative assessment of irradiated vs non-irradiated.

Irradiation Status	Predicted	
	Irradiated	Non-Irradiated
Irradiated	613	6
Non-Irradiated	30	837

**Table 6.** Actual vs Predicted Radiation Status for 1486 blood samples based on a metagene cutoff of -0.77 (1.7-fold increase) in gene expression.

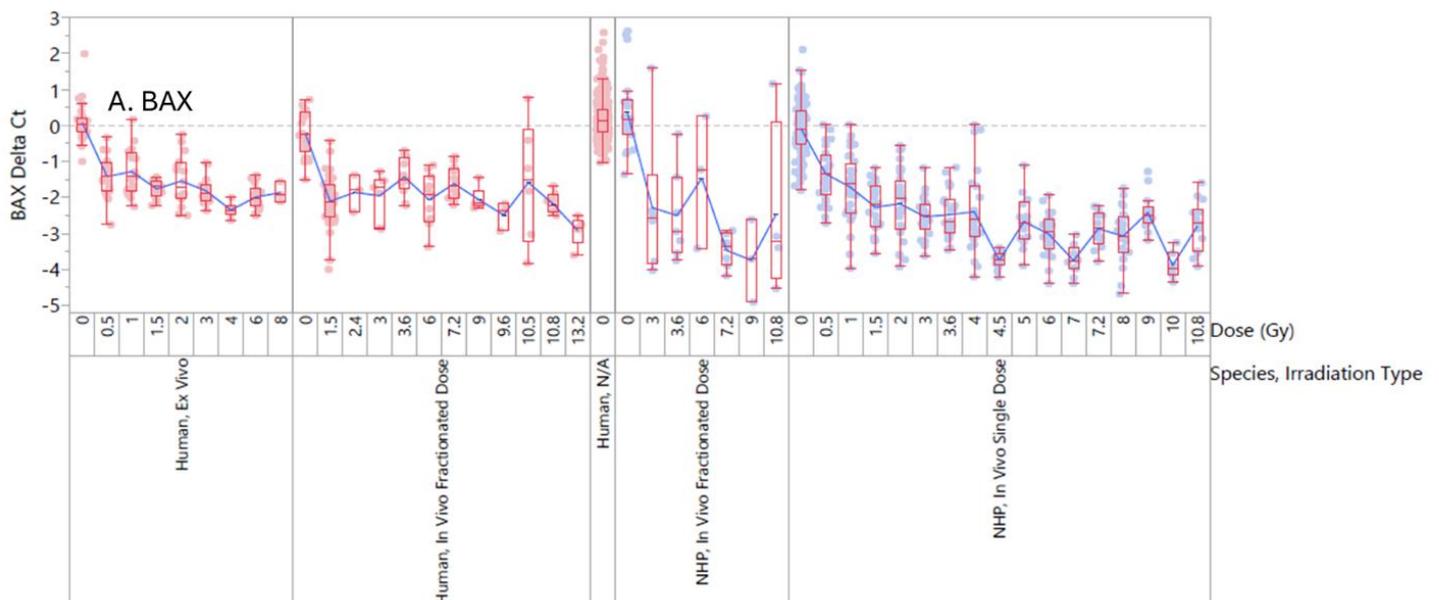
### Discussion

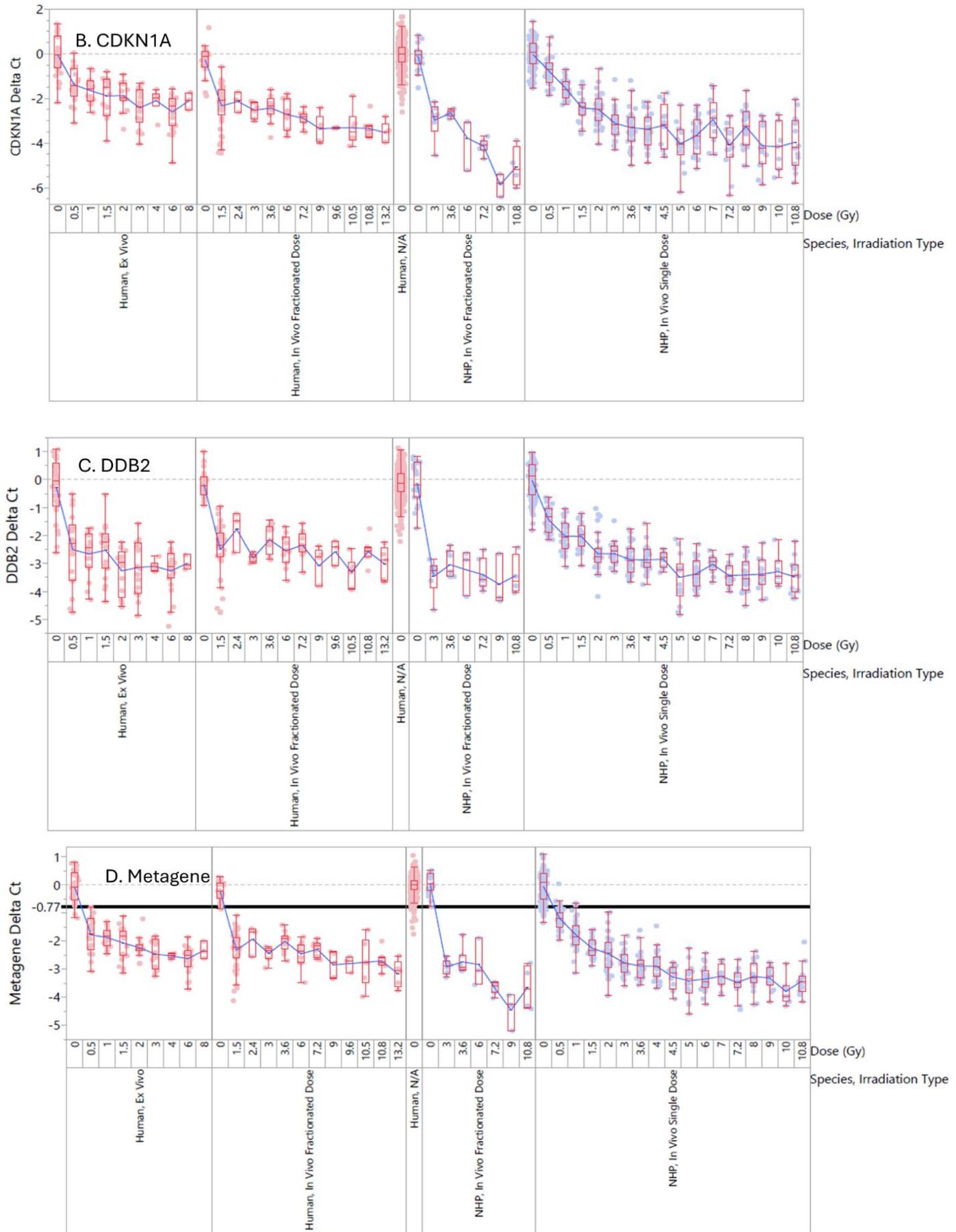
Genomic and proteomic technologies and diagnostic tests offer great promise for improving preparedness and response to a nuclear incident<sup>8-10</sup>, however the lack of human samples for new test development is a major barrier to promise fulfilment<sup>4,13</sup>. While Non-Human Primates (NHPs) are the preferred model system for test development<sup>15,16</sup>, the expense and humane considerations limit their availability. Many test developers are forced to rely upon less reliable surrogates such as mouse models<sup>17</sup> and ex vivo irradiated human blood<sup>18,19</sup>.

Here we used upon a combination of total body irradiated (TBI) single dose NHPs, TBI fractionated dose NHPs, ex vivo irradiated human blood, human fractionated dose TBI cancer patients, and ex vivo irradiated human blood to demonstrate test performance. The NHP samples enable evaluation for up

to 7 days post-exposure, the ex vivo irradiated samples provide evaluation at low dose without unnecessary use of NHPs, and the Human TBI samples provide verification of the NHP results.

The mRNA biomarkers used in the biodosimetry test were selected based on being highly responsive to radiation and showing comparable response in all model systems<sup>18,19</sup>. The highly radiation responsive gene FXDR<sup>20,21</sup> was excluded because of very different response in humans and NHPs. Figure 1 shows the MRPS5 normalized Ct response for BAX, CDKN1A and DDB2 as well as a metagene composed of the weighted combination of the three. The individual datasets were adjusted such that the means of the 0 Gy samples were set to delta Ct of 0. The metagene cutoff line for irradiated vs non-irradiated corresponds to 3 standard deviations of the unirradiated (0 Gy) Human Demographic samples which equals -0.77 Ct or ~1.7-fold increase in expression.





**Figure 1.** Plots of normalized, zero-centered Ct values for human and NHP systems as a function of dose. In vivo NHP samples include multiple timepoints (24, 48, 72, 96, 120 and 168 hours) post-irradiation. A.) BAX B.) CDKN1A C.) DDB2 and D.) Metagene

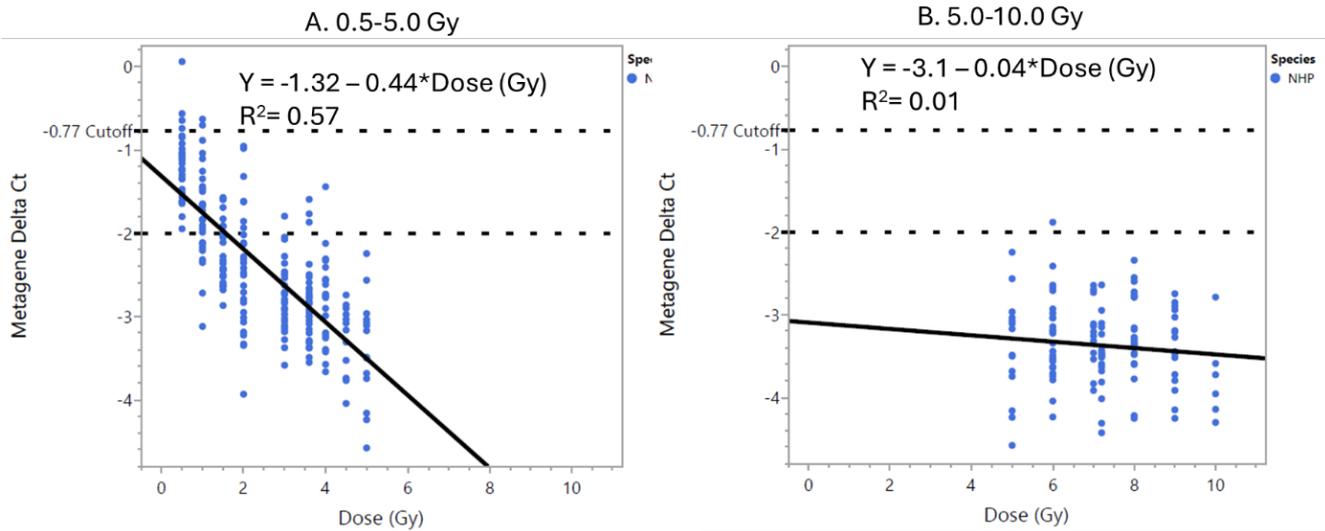
There is clear separation between irradiated and non-irradiated samples for NHP and human samples in all model systems. The limit of detection for the test is approximately 0.5 Gy. This is a very reasonable limit of

detection since there are no reported cases of acute radiation syndrome (ARS) from individuals irradiated with less than 0.5 Gy.

### Rapid Triage of Radiation Exposure Using a 4-Gene Real Time PCR Test

While the test was only designed to be qualitative for absorbed dose, there is a good correlation between delta Ct/fold change of the metagene and dose from 0.5 to 5.0 Gy ( $R^2=0.57$ ), and from 5.0 to 10.0 Gy there is

no correlation ( $R^2=0.01$ ) (Figure 2). Patients with delta Ct of less than -2 (greater than 4-fold increase) are more highly irradiated.



**Figure 2.** Plot of the Metagene Delta Ct versus dose for single dose in vivo NHP samples from A.) 0.5 to 5.0 Gy and B.) 5.0 to 10.0. The linear fit function of statistical software program jmp was used to calculate slope and line fit.

The fold change in the metagene peaks around 72 hours post-exposure and it is still upregulated at 168 hours post-exposure (7 Days). The 0.5 and 1.0 Gy dose

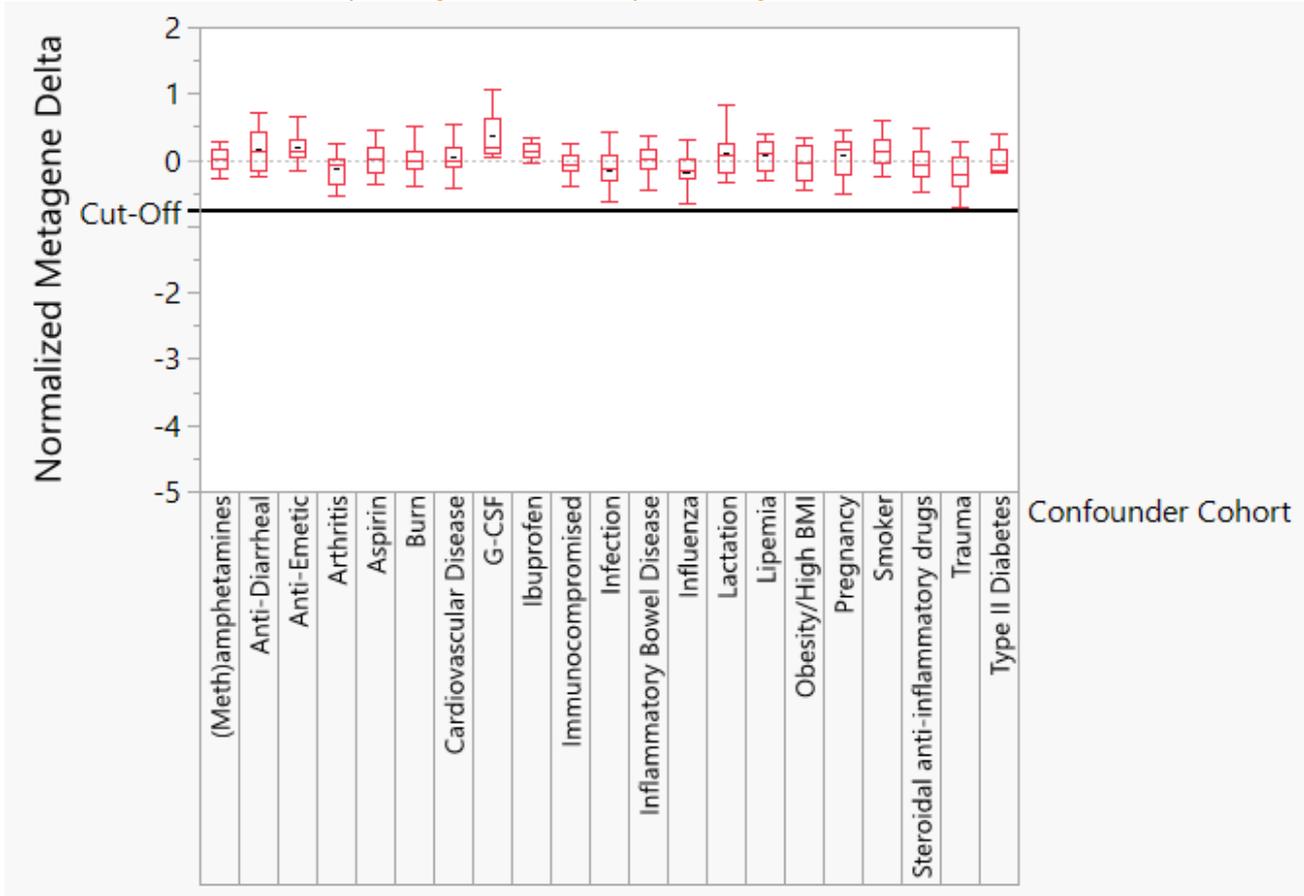
samples approach the 1.7-fold cut-off at day 7, while the higher dose samples remain significantly elevated (Table 7).

Time (Hours)	0.5 Gy	1 Gy	2 Gy	4 Gy	6 Gy	8 Gy	10 Gy
24	2.6	5.2	5.3	5.5	9.5	5.8	5.0
72	2.2	3.4	6.7	8.1	13.5	12.0	10.7
120	2.0	3.0	6.4	7.7	11.5	12.7	15.8
168	1.8	1.8	3.3	6.5	8.5	7.9	9.2

**Table 7.** Fold change calculations as a function of dose and time post irradiation for in vivo single dose NHP samples. Fold change equals  $2^{-\Delta Ct}$ .

The impact of potentially confounding chronic conditions and commonly taken therapeutics as well as trauma, burn, flu, infection and G-CSF treatment were investigated. Figure 3 shows the normalized mean metagene values for

the different cofounders investigated. The confounding condition samples showed minimal impact on the gene signature with an overall accuracy of 98.6%, (355 of 360 called correctly – Table 4).



**Figure 3.** Box and whisker plots of normalized Metagene Ct values for confounder cohort samples.

The test sensitivity is 99.0% and specificity is 96.5% (Table 8) based on 1486 samples consisting of 530 NHP TBI, 360 potentially confounding condition, 142 human

TBI, 150 human ex vivo, and 304 healthy human samples. The positive predictive value is 96.7%.

Threshold	Sensitivity	Specificity	Pos Predictive Value	Negative Predictive Value	Accuracy
0 Gy	99.0%	96.5%	95.3%	96.7%	97.1%
	[97.9-99.6]	[95.1-97.7]	[93.5-96.7]	[98.4-99.7]	[96.7-98.3]

**Table 8.** Performance summary for 4-mRNA RT-PCR Biodosimetry Test.

### Conclusions

A 4-mRNA biodosimetry test shows high sensitivity and specificity for identifying individuals that have absorbed more than 0.5 Gy of absorbed radiation. Samples must be collected into a blood RNA stabilizing device within 7 days post-exposure. The test is compatible with the large installed base of real time PCR testing equipment that

was used to respond to the COVID pandemic, and this test has the potential of being a valuable tool in responding to a mass scale nuclear incident. This test can be particularly useful for calming the public following a nuclear event and avoiding misallocation of scarce resources like hospital beds and Granulocyte Colony Stimulating Factor (G-CSF).

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