A new biodosimetric method: branched DNA-based quantitative detection of B1 DNA in mouse plasma

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ABSTRACT. A simple and accurate method for measuring the biological effects of radiation is of increasing importance, especially in mass casualty scenarios. We have therefore developed a new biodosimetric technique targeting circulating B1 DNA in mouse plasma by branched DNA signal amplification for rapid quantification of plasma DNA. This technology targets repetitive elements of the B1 retrotransposon in the mouse genome, followed by signal amplification using Panomics Quantigene 2.0 reagents. Evaluation was conducted concerning precision, accuracy and linearity. Plasma samples were collected from mice 0–24 h after 0–10 Gy total body irradiation (TBI). The average inter- and intra-assay coefficients of variance were 8.7% and 12.3%, respectively. The average recovery rate of spiked DNA into plasma was 89.5%. This assay revealed that when BALB/c and NIH Swiss mice were exposed to 6 Gy TBI, plasma B1 DNA levels increased significantly at 3 h post-TBI, peaked at 9 h and gradually returned toward baseline levels in 24 h. A dose-dependent change in plasma DNA was observed at 9 h post-TBI; the dose–response relation was monotonic, exhibiting linearity for BALB/c mice from 3 to 6 Gy ($r=0.993$) and NIH Swiss mice from 3 to 7 Gy ($r=0.98$). This branched DNA-based assay is reliable, accurate and sensitive in detecting plasma B1 DNA quantitatively. A radiation dose-correlated increase in plasma B1 DNA was demonstrated in BALB/c and NIH Swiss mice in the dose range from 3 to 6 Gy, suggesting that plasma B1 DNA has potential as a biomarker for radiation biological effect.

Received 28 September 2010

Revised 23 April 2010

Accepted 28 April 2010

DOI: 10.1259/bjr/49886569

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Our awareness of the potential risk of accidental and intentional radiation exposure in the form of a terrorist attack has grown in recent years, as unrest, warfare and terrorism have spared few countries or continents. Environmental and ever-growing medical radiation exposures as common components of modern life are also a continued public health concern. Furthermore, plans for space exploration demand better understanding and evaluation of cosmic radiation exposure risks.

Physical dosimeters measure radiation exposure, but, unlike biodosimeters, they cannot measure biological effects or individual differences in radiation sensitivity. There is an acknowledged dearth of rapid and effective methods for biodosimetry, especially in the mass casualty scenario. Thus, identifying biomarkers for accurate biodosimetry and assessing their implications are topics of increasing importance [1].

The chromosomal aberration assay remains the “gold standard” for biodosimetry and an important part of clinical evaluation of radiation exposure [1, 2] despite newly emerging techniques, such as gene expression profiles [3], proteomics [4], metabolomics [5] and

mutation frequency [6]. Unfortunately, there are several aspects of this test as it is currently performed that make it less useful in mass casualty screening and in the acute medical management setting. These include time- and labour-intensive procedures, and the requirement for professional facilities and personnel [1, 2, 7]. The limitations of existing methods prompted us to seek correlated biomarkers and accurate methods to provide a better biological estimation of absorbed dose.

The observation that circulating DNA is detected after irradiation was first made by Russian scientists in 1987, when sera from 8–100 Gy irradiated rats were assessed with 0.8% agarose gel indicating an increase in nucleosome DNA [8]. Since then, radiation-released low molecular weight genomic DNA in plasma has been actively studied by another Russian group [9–12]. In 2006, studies of radiation-induced release of mitochondrial DNA into plasma were also reported by Russian scientists [13]. In these experiments, the total circulating DNA in plasma was measured by electrophoresis or by targeting particular housekeeping genes via polymerase chain reaction (PCR) techniques, which are not suitable for a mass casualty situation.

Here, we introduce a new approach for biodosimetry through the targeting of the B1 component of plasma DNA, a potential cell or gene stress indicator in the mouse. Through the establishment of the branched DNA

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(bDNA)-based quantitative assay, we have demonstrated a correlation between plasma B1 DNA and absorbed dose over a specific dose range. In addition, bDNA technology permits direct plasma measurement, circumventing complicated nucleic acid processing procedures. B1 is also called “7SL RNA gene-related non-autonomous retrotransposon” and has been recently identified as being actively involved in the early post-stress genetic response [14]. Since B1 evolutionarily exists in all rodents and mammals, this assay can be extended easily to humans and other species.

Methods and materials

Animals and treatments

Male BALB/c or NIH Swiss mice, 6–8 weeks old, were divided into groups of five. Control mice were not irradiated, and other groups received a single dose of total body irradiation (TBI). Animals were immobilised with the aid of a plastic restrainer during exposure to a ^{137}Cs γ -ray source at a dose rate of $\sim 2.23 \text{ Gy min}^{-1}$ (homogeneity $\pm 6.5\%$). Mice were housed in a clean environment using microisolator technology. All protocols were approved by the University of Rochester Institutional Animal Care and Use Committee (IACUC), and all experiments were carried out in accordance with United States Public Health Service guidelines.

Preparation of samples

Approximately 50–100 μl of blood was collected in 0.5-ml ethylene diamine tetra-acetic acid (EDTA)-treated

polypropylene tubes from the mouse lateral saphenous vein at various times after irradiation, as described previously [12]. The blood was centrifuged (500 g for 10 min at 4°C), followed by separation of 25–50 μl of plasma into fresh polypropylene tubes, with care taken to leave the cellular portion undisturbed. The samples were stored at -20°C .

Quantigene 2.0 bDNA-based B1 assay

The bDNA-based signal amplification is a multiple “sandwich hybridisation” assay employing bDNA molecules to achieve signal amplification. It includes two fundamental parts: target capture and signal amplification (Figure 1).

Target capture

A 20- to 40-fold dilution by nuclease-free water of mouse plasma was fully denatured at 95°C for 10 min and then immediately placed on ice until sample loading. A working probe solution was prepared in diluted Lysis Buffer (Panomics, Fremont, CA; cat. no. QG0504) with proteinase K at a final concentration of $16.7 \mu\text{g ml}^{-1}$, together with 0.1% blocking reagent (Roche, Nutley, NJ; cat. no. 1096176), capture extender at 250 pM and label extender at 1000 pM. 5 μl of prepared mouse plasma was added into the capture plate well containing 95 μl of working probe solution and the plate was sealed with a foil seal (Panomics; cat. no. QG0524). After an overnight hybridisation at 55°C , the plate was washed three times with washing buffer ($0.1 \times$ standard citrate saline containing 0.3 g l^{-1} lithium lauryl sulphate) and all traces of wash buffer were removed by tapping the inverted plate on a clean paper towel.

Figure 1. Schematic representation of the QuantiGene branched DNA assay for plasma B1 DNA quantitation. CE, capture extender; LE, label extender; black solid arrow represents hybridisation reaction.

Signal amplification and detection

The standard QuantiGene protocol (Panomics, QuantiGene 2.0 reagent system, user manual) was followed. Briefly, sequential hybridisations were conducted at 55°C for 1 h in 100 µl of 1:1000 diluted bDNA pre-amplifier (Panomics; P/N15528), then at 55°C for 1 h in 100 µl of a 1:1000 diluted bDNA amplifier (Panomics; P/N15527), and finally at 50°C for 1 h in 100 µl of 1:1000 dilution of alkaline phosphatase-labelled probe (Panomics; P/N10202). All the bDNA probes were diluted in probe diluent (Panomics; cat. no. QS0503). Wells were washed with wash buffer after each hybridisation. After the final wash, 100 µl of the alkaline phosphatase substrate (Panomics; P/N14557) was added to each well and the plate was left at room temperature for 10 min. The developed luminescence signal was measured in relative light units (RLUs) on a Perkin Elmer Victor 3V (PerkinElmer, Watham, MA) instrument, using a 0.2 s integration time.

Probe set design

The B1 consensus sequence was obtained from Giri (<http://www.girinst.org/replibase>). Four capture extenders (CEs) and two label extenders (LEs) were constructed to cover almost the entire length of the B1 consensus sequence (Figure 2). Roughly half of each CE's sequence is complementary to the targeted regions on B1, and the other half is complementary to the capture oligos immobilised in the well. Likewise, roughly half of each LE's sequence is complementary to targeted sections on B1, whereas the other half is complementary to the bDNA pre-amplifier.

Linearity and sensitivity

Serial two-fold dilutions of mouse genomic DNA (Promega, Madison, WI; cat. no. G3091) were prepared in Tris EDTA buffer and used to construct a standard curve expressed as RLU *vs* the input

concentration of mouse genomic DNA. The optimal linear range was determined, and the assay sensitivity was defined as two standard deviations (SDS) above the background means. In addition, a serial two-fold dilution of the plasma with a high level of plasma DNA selected from that of the irradiated mice was used to test for any effect of the plasma matrix on the assay.

Reproducibility

Two internal high and low DNA content quality control samples were prepared from mouse plasma, subdivided and stored at -20°C. The "high" DNA concentration test sample was set around the high limit of the linear range and the "low" test sample near the low limit of the linear range. The high and low test samples were subjected to four runs with four repeats per run. Both the intra- and inter-assay reproducibility were calculated. Statistically, the interassay variance is obtained by the subtraction of intra-assay variance from total variance, according to the variance sum law.

Recovery of spiked DNA in plasma

A 20-fold dilution of the plasma pool prepared from normal mice was used as the matrix, and a calibrated amount of Promega mouse genomic DNA was added to create spiked DNA standards. The recovery test was conducted with both high and low levels of spiked DNA and the recovery rate was calculated. These experiments calibrate the system and identify any masking effect on DNA detection in plasma.

Data analysis

Analysis of variance (ANOVA) was used to ascertain whether there was a significant difference between mice

Figure 2. Schematic representation of probe sets design. 135 bp B1 consensus sequence (direct strand) is shown and the target regions of the probe sets are marked on it. The nucleotide sequence of four CEs and two LEs are shown. CE, capture extender; LE, label extender.

groups at different data points. The significance level was set at $p < .05$.

Results

Assay development and evaluation

Plasma DNA has recently been explored as a biomarker of cell death and damage in numerous physiological and pathological conditions [15]. Utilising B1 DNA as an index of cell death or damage, as well as a responder of cell or gene stress, allows for the further potential of interpreting the biological effect of radiation.

The QuantiGene Reagent System is based upon bDNA technology from Bayer. bDNA amplifies the readout signal rather than the target DNA without requiring DNA purification, thus simplifying the workflow and increasing the accuracy of the assay [16, 17]. An early version of this technology has been cleared by the US Food and Drug Administration for use in detecting human immunodeficiency virus (HIV) and hepatitis C virus in plasma. Relying on the mechanism of bDNA, a probe set for mediating capture and signalling of B1 DNA was designed (see Methods).

Linearity

In the range of DNA concentrations between 1.6 and 100 ng ml⁻¹, excellent linearity was shown ($r^2=0.998$). At 150 ng ml⁻¹, the signal was saturated. In addition, plasma with a high level of B1 DNA from an irradiated mouse was serially diluted to detect any disruption of the B1 DNA measurement by the plasma matrix. Good linearity ($r^2=0.992$) demonstrates that there is little or no matrix interference, consistent with the relatively small amount of sample that was loaded into the reaction system (5- μ l sample into the 95- μ l reaction solution) (Figure 3). The lowest limit of detection, as calculated

from background measurements (see Methods), was 0.67 ng ml⁻¹.

Precision and reproducibility

To test the reproducibility, we included many different reagents and various time intervals between assays. At a higher concentration level, the intra-assay and interassay coefficients of variances were 10% and 4%, respectively. At a lower concentration level, they were both 14% (Table 1). This is consistent with increased imprecision corresponding to decreased concentration.

Recovery

The accuracy was tested by measuring the recovery of spiked DNA in normal mouse plasma. The recovery rates at low and high levels of spiked DNA were 85.3% and 90.4%, respectively (Table 2). The high recovery rate at both low and high levels also illustrates the fact that a plasma matrix effect does not interfere significantly with the assay.

Application in quantifying plasma DNA

First, the dynamic change in plasma DNA over time after 6 Gy TBI in BALB/c mice was investigated. Plasma B1 DNA levels were examined at different time-points after irradiation; the results were compared among the irradiated groups and with a non-irradiated control group. Following 6 Gy TBI, plasma B1 DNA concentration increased with time, peaked at 9 h and then declined rapidly towards baseline levels at 24 h (Figure 4). We noticed that the mean level of plasma B1 DNA in BALB/c mice was low (91.2 ± 39.6) in the non-irradiated control group, but in irradiated groups it increased nearly twofold (178.9 ± 61.3) at 3 h, fivefold (427.9 ± 147.3) at 5 h, 15-fold (1422.6 ± 225.8) at 7 h and 38-fold (3500.1 ± 715.0) at 9 h post irradiation. The levels of plasma B1

Figure 3. Linearity of the branched DNA-based assay of plasma B1 DNA. (a) Standard curve with mouse genomic DNA. Solid line, a regression line of the middle seven points. Broken line, connection line of all the nine points. (b) Dilution curve from mouse plasma. Dilution factor, 1/dilution folds. 2E+06, scientific notation of 2×10^6 . RLU, relative light unit. This experiment was conducted with two in-run replicates and repeated three times. The error bar amount is equal to the standard error of mean. Analysis of variance was conducted for the linear regression and $p < .05$.

Table 1. Reproducibility of the mouse plasma B1 assay

a. LQC ($34.37 \pm 6.67 \text{ ng ml}^{-1}$)				
Runs				
1	31.8	41.9	34.2	43.3
2	32.8	38.9	39.9	44.3
3	25.4	33.0	24.0	22.9
4	31.3	30.5	37.7	38.1
Source of variation	df	Variance	SD	CV
Interassays	3	21.54	4.64	0.14
Intra-assays	12	23.00	4.80	0.14
Total	15	44.54	6.67	0.19

b. HQC ($118.25 \pm 13.30 \text{ ng ml}^{-1}$)				
Runs				
1	107.1	91.4	115.3	119.6
2	110.9	127.9	114.4	114.3
3	129.6	114.4	127.2	^a
4	106.2	139.2	142.9	113.3
Source of variation	df	Variance	SD	CV
Interassays	3	22.98	4.79	0.04
Intra-assays	11	153.95	12.41	0.10
Total	14	176.93	13.30	0.11

HQC, high-quality control; LQC, low-quality control; df, degree of freedom; SD, standard deviation; CV, coefficient of variation.

^aA repeat was discarded because of incorrect sample loading.

DNA at different time-points are significantly different from those of the previous point ($p < .05$). Similar results were observed in NIH Swiss mice.

Second, the dynamic change in plasma B1 DNA concentration with dose of TBI was determined in BALB/c and NIH Swiss mice. For this experiment, mice received TBI at a dose of 0–10 Gy and samples were collected 9 h after irradiation. For BALB/c mice, the concentration of plasma B1 DNA increased with dose up to at least 6 Gy. The average concentration of plasma B1 DNA scaled up monotonically from 68 ± 23 to $3754 \pm 637 \text{ ng ml}^{-1}$, as dose increased from 0 to 6 Gy. The concentrations at 0, 3, 4, 5 and 6 Gy were significantly different from each other ($p < .05$). A linear regression analysis, performed between doses and plasma B1 DNA concentration in the range 3–6 Gy, demonstrated an excellent linearity with $r^2 = 0.993$ ($p < .05$). For NIH Swiss mice, a similar dynamic change in plasma B1 DNA was seen, except in the linear range from 3 to 7 Gy, with $r^2 = 0.98$ ($p < .05$) and approximately twice as much

DNA detected per unit dose as that for BALB/c mice (Figure 5).

Discussion

Plasma DNA, also called circulating cell-free DNA, was discovered nearly 60 years ago, but its potential utility in cancer and other clinical disorders was not realised until relatively recently [15]. Since the attraction of blood-based, non-invasive tests is obvious, a new upsurge is in the making in characterisation of the nature of plasma DNA in various settings. Although relatively limited studies of plasma DNA as a molecular biomarker of radiation response have been reported by Russian scientists, their methodology targeted low molecular weight DNA and was mainly confined to gel electrophoresis and spectrometry. This necessarily rendered their assays less sensitive and resulted in poor correlations and a requirement for high radiation dose exposure levels [8–12].

Targeting the B1 component of plasma DNA as a biodosimetry marker provides our assay with inherently high sensitivity and potentially a better correlation. B1 repeats, originating from the 7SL RNA gene, are a family of short interspersed nuclear elements (SINEs) specific to the genomes of rodents. There are an estimated 150,000 B1 copies in the *Mus* genome, accounting for 2.7% of the whole genome mass [14, 18]. The abundance of B1 repeats in the mouse genome amplifies the sensitivity and allows us to use as little as 5 μl of 10-fold diluted plasma without requiring DNA purification. Aside from serving as merely a structural genetic marker of cell death and damage in plasma, the retrotransposon is also expected to act functionally as an early responder to cell or gene stresses since the increased retrotransposon activity of SINEs and LINEs (long interspersed nucleic elements) is one of the early manifestations of exposure to stress [19–21]. The post-stress activation of retrotransposon in cells may also play some part in the repair of double-stranded DNA breakage through retrotransposon reverse transcriptase-mediated repair [22]. Although the goal was to use the circulating B1 repeats as a measure of total circulating DNA, the existence of high concentrations of B1 DNA in the circulation, which normally features low levels of cell-free DNA, provides evidence for these reported functions of B1 DNA. It is reasonable to predict that different stresses might raise the circulating levels of retrotransposons. Likewise, it is known that circulating DNA can increase as a result of many illnesses [14, 19, 22]. The levels of DNA, however, that are observed in the irradiated animals are many times greater than what we have seen with other stresses, including major surgery (*i.e.* splenectomy), corticosteroids, lipopolysaccharide (LPS) and others.

bDNA is a hybridisation-based method that uses multiple oligonucleotide probes to capture target DNA and multiple bDNA multimers to amplify the readout signal. Compared with real-time PCR, which amplifies the target using enzymatic reaction, bDNA's "sandwich hybridisation"-based reaction not only avoids errors resulting from enzyme susceptibility to environmental factors, but also bypasses the preparation of nucleic acid samples and particular probe labelling, while achieving

Table 2. Recovery test

Conc. (ng ml^{-1})	Baseline (plasma pool)	Low	High
Repeats			
1	10.0	26.0	105.5
2	7.4	27.4	109.3
3	10.9	30.7	92.6
4	12.4	24.8	94.8
Mean	10.2	27.2	100.6
SD	2.1	2.6	8.1
Addition conc.		20.0	100.0
Recovery conc.		17.1	90.4
Recovery rate (%)		85.3	90.4

Conc., concentration; sb, standard deviation.

Figure 4. Plasma B1 DNA time-dependent change after 6 Gy total body irradiation (TBI). (a) Box-plot representation in BALB/c mice. The central “box” extends from the 1st to the 3rd quartile (encompassing data points within the 25th and 75th percentiles). The line in the middle of the box indicates the mean (50th percentile of the data set), while the “whiskers” extend to the most extreme data values. (b) Box-plot representation in NIH Swiss mice. Plasma was collected from mice at the indicated time points after 6 Gy TBI and then subjected to plasma B1 assay (five mice in each experimental and control group). Denoted are the mean, median, 25th and 75th percentile and 95% confidence limits. *Different from the previous time-points ($p < .05$) in one-way analysis of variance. IR, irradiation.

sufficient sensitivity and accuracy [16, 17]. In our experiments, 5 μ l of 10-fold diluted plasma (equal to 0.5 μ l of original plasma) was directly input into wells containing hybridisation buffer immediately after denaturation. Skipping the complex and error-promoting procedures of nucleic acid extraction provides our assay with the advantage of increased stability and allows for complete automation. It is believed that the B1 DNA target exists in plasma in a protein-coupled state: this would make the target template less accessible for hybridisation. In our assay, the uncoupling of DNA and protein was achieved using a special hybridisation buffer that works on the plate in parallel with hybridisation.

Following a radiation exposure incident such as detonation of an improvised nuclear device, it will be important to have access to a population-based rapid screening biodosimetry test within the acute phase (less than 72 h). This allows the stratification of patients according to total exposure and permits optimal treatment planning. A critical need exists for methods that (1) can be used within the first 24 h of exposure, (2) allow for high-throughput automation and (3) can measure damage over the entire clinically relevant radiation exposure range (0–10 Gy). The current 96-well plate-based assay described in this manuscript is simple to perform and has the potential to be developed into a

Figure 5. Plasma B1 DNA dose-dependent change 9 h after irradiation. (a) Box-plot representation in BALB/c mice. (b) Box-plot representation in NIH Swiss mice. The central “box” extends from the 1st to the 3rd quartile (encompassing data points within the 25th and 75th percentiles). The line between the 1st and 3rd indicates the median (50th percentile of the data set), while the “whiskers” extend to the most extreme data values. Plasma was collected from mice at 9 h post-total body irradiation of the indicated doses and subjected to the plasma B1 assay (five mice in each experimental and control group). *Different from the previous dosage ($p < 0.05$) in one-way analysis of variance.

high-throughput automated device. Our method also has the potential to be improved further to produce final results within 2 h. Recently, the QuantiGene reagent system has also been adapted to a Luminex multiplex bead array platform, which will provide the basis for a future, radiation-related, multimarker evaluation system [16].

The origin of plasma DNA remains controversial and has yet to be elucidated fully. Although many reports have implicated apoptosis as the source of free circulating DNA in serum and plasma, it may not be the dominating force or first source of the DNA. Specifically, there is incomplete evidence to support the correlation between apoptosis and circulating DNA, other than the inconsistent demonstration of a nucleosome ladder profile from plasma of patients with normal or various diseased conditions. It is therefore commonly believed that more than one mechanism is involved in producing the circulating DNA, including active release by living cells [15, 23–26]. Here, our data provide fresh insight. It is well known that the fragmentation of nuclear DNA in apoptotic cells usually takes place 4–72 h after radiation [27, 28]. However, the plasma B1 DNA was already greatly increased just 3 h post 6 Gy of TBI in our experiments, reaching levels of $179 \pm 61 \text{ ng ml}^{-1}$ (BALB/c)/ $288 \pm 102 \text{ ng ml}^{-1}$ (NIH Swiss) and $428 \pm 127 \text{ ng ml}^{-1}$ (BALB/c)/ $1675 \pm 683 \text{ ng ml}^{-1}$ (NIH Swiss) at time points of 3 h and 5 h, respectively; notably, both of these levels are significantly higher than pre-irradiation levels. These data suggest that a large release of DNA into the circulation precedes apoptosis, and its likely source is either mitotic cell death processes from cells already in G2/M (probably gut and bone marrow), or from active DNA secretion from viable cells. The latter hypothesis might suggest a physiological response to the circulating retrotransposon as an early adaptive response potentially helpful in the repair of radiation-induced DNA double-strand breaks in the remaining cells, all of which have DNA damage in need of repair. (The physics of radiation dictate that there will be several thousand single-strand breaks and several dozen double-strand breaks in every cell after a 1 Gy of radiation exposure.) Several recently proposed retrotransposon-activating functions could help the cell cope with the physiological stresses following radiation [22, 29, 30].

In the present studies, the increase of plasma B1 DNA concentration slowed for radiation doses over 6 Gy. In other experiments, we have seen steady increases in double-stranded total DNA over 6 Gy. The level of circulating DNA is a complex function of various sources of release into the circulation and various mechanisms of clearance (e.g. DNase, cellular uptake, reticuloendothelial cell mediated filtration) [15, 31]. There may also be a differential rate of degradation of B1 DNA compared with double-stranded DNA, or non-B1 DNA. In future studies, we will test the impact of a number of factors on the kinetics of B1 and total circulating DNA including animal age, gender and strain.

In humans, Alu is the evolutionary counterpart of B1. It constitutes the most abundant repetitive element in the human genome, with 1.1×10^6 copies representing 10.6% of the whole genome mass [18, 21]. Translational detection of Alu should outperform that of B1 given the lower number of repeats of the latter in the mouse genome (150 000 copies).

Conclusion

This study demonstrates the feasibility of a potential biomarker for acute life-threatening radiation exposure. The B1 component of plasma DNA was measured by a bDNA-based quantitative assay, not only as a marker of cell death or damage, but also as a potential indicator of the radiation-related stress response of adaptation. A dose- and mouse strain-dependent increase in plasma B1 DNA load was demonstrated up to 6 Gy. In addition, since this mouse-based assay is extremely promising for biodosimetry, translation to other species, including human, will be the next stage of our efforts.

Statement of Interest: The authors acknowledge that a conflict of interest exists; Drs Paul Okunieff, Lurong Zhang and Auigo Zhang are co-owners of the technology development company DiaCarta LLC, which owns intellectual property related to the measurement of radiation exposure using circulating DNA. Dr Auigo Zhang is also affiliated to the technology company Panomics/Affymetrix, which manufactures many of the reagents needed for this assay. Dr Yunqing Ma is an employee, with financial interest, of both DiaCarta and Panomics/Affymetrix.

Acknowledgments

This research was supported by the Centers for Medical Countermeasures against Radiation program, U19-AI067733, National Institute of Allergy and Infectious Diseases (NIAID). The authors wish to thank Dr Bruce Fenton, Dr Rob Howell and Amy K. Huser for thoughtful editing contributions.

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