RADIATION RESEARCH **184**, 134–142 (2015)
0033-7587/15 $15.00

DOI: 10.1667/RR13991.1

Development of a High-Throughput and Miniaturized Cytokinesis-Block Micronucleus Assay for Use as a Biological Dosimetry Population Triage Tool

Stanley W. Lue,[a] Mikhail Repin,[a] Ryan Mahnke[b] and David J. Brenner[a,1]

[a] *Center for Radiological Research, Department of Radiation Oncology, Columbia University Medical Center, New York, New York 10032; and* [b] *Northrop Grumman, Elkridge, Maryland 21075*

Lue, S. W., Repin, M., Mahnke, R. and Brenner, D. J. Development of a High-Throughput and Miniaturized Cytokinesis-Block Micronucleus Assay for Use as a Biological Dosimetry Population Triage Tool. *Radiat. Res.* 184, 134–142 (2015).

Biodosimetry is an essential tool for providing timely assessments of radiation exposure. For a large mass-casualty event involving exposure to ionizing radiation, it is of utmost importance to rapidly provide dose information for medical treatment. The well-established cytokinesis-block micronucleus (CBMN) assay is a validated method for biodosimetry. However, the need for an accelerated sample processing is required for the CBMN assay to be a suitable population triage tool. We report here on the development of a high-throughput and miniaturized version of the CMBN assay for accelerated sample processing.

INTRODUCTION

The threat of radiation exposure due to a large-scale radiological accident or detonation of small radiological dispersal or improvised nuclear devices (IND) in a mass-casualty setting is a serious public health concern (*1, 2*). In addition to the aforementioned, natural disasters causing the meltdown of nuclear reactors such as in the Fukushima Daiichi Nuclear Power Plant catastrophe are also matters of concern. In such cases, it may be imperative to screen tens or hundreds of thousands of individuals for radiation exposure, both to identify and prioritize individuals that would benefit from medical treatment and to alleviate the concerns of the ''worried well'' (*3–5*). The threat of large-scale radiological incidents has led to a number of recent developments in the fields of biological and physical dosimetry (*6*). However, to triage potentially large numbers of people exposed to ionizing radiation, the development of high-throughput approaches for radiation biodosimetry has been identified as a high priority.

Ionizing radiation induces chromosomal damage, which can be tested through the utilization of cytogenetic assays. The ''gold standard'' in radiation biodosimetry for individuals accidentally exposed to ionizing radiation is the analysis of dicentric chromosomes (*7*). Although a very powerful technique in radiation biological dosimetry, a major drawback in dicentric chromosome analysis is that it is very time consuming. Skilled cytogeneticists or technicians can analyze 200–500 metaphase cells per day (*8–10*) or for triage purposes, 50 metaphases can be analyzed in 15–20 min using simplified scoring rules (*11, 12*) or a semi-automated approach (*13*). For large-scale radiation accidents it is necessary to develop biodosimetry methods with higher throughput and, potentially, full automation including image analysis, to eliminate the subjectivity associated with manual or semi-automated analysis of cytogenetic samples.

In the past 20 years, a variety of new and faster biodosimetric assays have been developed (*14–16*), including the well-established cytokinesis-block micronucleus (CBMN) assay (*17*). The CBMN assay enables the scoring of micronuclei in cells that have undergone a single nuclear division. Micronuclei are induced by ionizing radiation and are acentric chromosome fragments and whole chromosomes that are unable to interact with the spindle fibers in the nucleus. These micronuclei lag behind at anaphase, and as a result are not included in the main daughter nuclei, remaining in the cytoplasm as small circular entities. Compared to the labor-intensive dicentric assay, the easy and rapid scoring of micronuclei in the CBMN assay makes this method very attractive for population triage in the case of large-scale radiation accidents, as well as for large-scale assessment of genetic damage in radiation workers receiving a high dose of radiation (*18*). Obtaining immediate results will be critical after a large-scale accident because of the need to identify, at an early stage, those individuals who will benefit from medical involvement and those who will not. However, there still remains an unmet need to speed up sample processing in the CBMN assay for

[1] Address for correspondence: Center for Radiological Research, Department of Radiation Oncology, Columbia University Medical Center, New York, NY 10032; e-mail: djb3@cumc.columbia.edu.

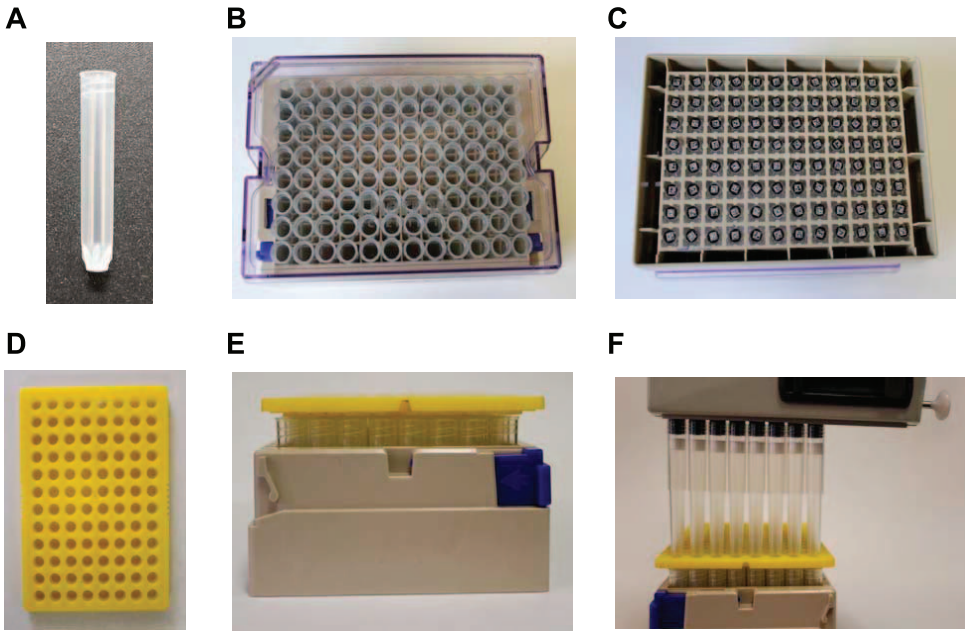

FIG. 1. Panel A: 1.4 mL 2D-barcoded microtube. Panel B: 96-tube rack (8 × 12) containing 1.4 ml 2D-barcoded microtubes with lid. Panel C: Bottom of 96-tube rack showing barcodes. Panel D: ''Tip regulator'' for aspirating solutions from microtubes, generated by removing protrusions from underneath the Eppendorf tip holder. Panel E: Tip-regulator placed on top of 96-tube rack. Panel F: 1,250 µl filter tips on electronic multichannel pipet with tip-regulator (8 or 12 channel pipet can be used).

triaging tens or hundreds of thousands of individuals who are accidentally exposed to radiation.

We report here on the development of a high-throughput and miniaturized version of the CMBN assay. Our approach to the problem of accelerated sample processing was to reduce the volume of human whole blood used to 50 µl, which would then be cultured, treated with hypotonic solution and fixed into individual 1.4 ml barcoded microtubes. These microtubes are conveniently organized into an industry standard 8 × 12 microtiter plate format allowing for simultaneous processing of 96 samples using common laboratory equipment (Fig. 1). We then fully automated the analysis of binucleates and micronuclei on slides using the Metafer software. With this new format, processing of hundreds or even thousands of samples can be accomplished by one individual within a day after culturing. This study aimed to optimize, assess and validate the new assay as a tool for population triage purposes after a mass-casualty radiation event.

MATERIALS AND METHODS

Human Whole Blood (50 µl) CBMN Assay Protocol

Heparinized blood samples (3 ml) were collected from ten healthy volunteers (6 females and 4 males) in the age range of 24–50 years after informed consent was obtained. For each donor, 50 µl blood samples were pipetted into 1.4 ml 2D-barcoded tubes and placed into a 96-tube rack (8 × 12) and the tubes were covered with the supplied top (Thermo Fisher Scientific Inc., Waltham, MA). For each donor, duplicate samples were prepared and analyzed. The covered tubes in the racks were transported to a Gammacell 40 ^{137}Cesium irradiator (Atomic Energy of Canada, Ltd., Mississauga, Canada) and exposed to 1, 2, 3, 4 and 5 Gy gamma rays. After irradiation, 500 µl of PB-MAX™ karyotyping media (Life Technologies, Grand Island, NY) was added to the 50 µl human whole blood samples and mixed three times [or self-prepared in house media was used: RPMI 1640, 10% fetal bovine serum, 2% phytohemagglutinin-M (PHA-M) and 2% penicillin/streptomycin (all from Life Technologies)]. Two different media types were utilized in this protocol to compare and determine the efficacy of each in a large-scale radiological event.

Culturing tubes were placed into a 37°C/5% CO_2 incubator for 44 h. After 44 h of incubation, cytochalasin-B (Sigma-Aldrich® LLC, St. Louis, MO) was added to cultures to block cytokinesis of proliferating lymphocytes at a final concentration of 6 µg/ml and a final concentration of 0.5% DMSO, and cells were gently mixed five times to break up pellets. Samples were placed back into a 37°C/5% CO_2 incubator for an additional 26 h. After 70 h, plate(s) containing whole blood samples in 1.4 ml 2D-barcoded tubes were removed from the incubator. The media above the settled cell pellet was aspirated without disturbing the pellet using a multichannel electronic pipet (Eppendorf or Thermo Scientific) and a ''tip regulator'' leaving approximately 150 µl behind (Fig. 1). The tip-regulator was used for all aspiration steps. A timer was set for 10 min. Room temperature 0.075 *M* KCl (850 µl) (Life Technologies) was added and the timer was started after the first addition of KCl. While the timer was running, a multichannel electronic pipet was set to 500 µl for gentle mixing, samples were then gently mixed six times. After 10 min, 200 µl of ice-cold 4:1 fixative (methanol:acetic acid) was added and then immediately gently mixed three times with the electronic pipet set at 500 µl. Using a centrifuge adaptable for spinning plates, samples were centrifuged at 1,200 rpm for 3 min. Supernatant (850 µl) was removed without disturbing the pellet using the multichannel electronic pipet. A manual 300 µl multichannel pipet set at 200 µl was then used to mix the hard dark pellet eight times. Ice-cold 4:1 fixative (850 µl) was added and gently mixed three times using the multichannel electronic pipet. Samples were then centrifuged at 1,200 rpm for 3 min. The steps consisting of the removal of supernatant, addition of ice-cold fixative and centrifugation were repeated two additional times or until

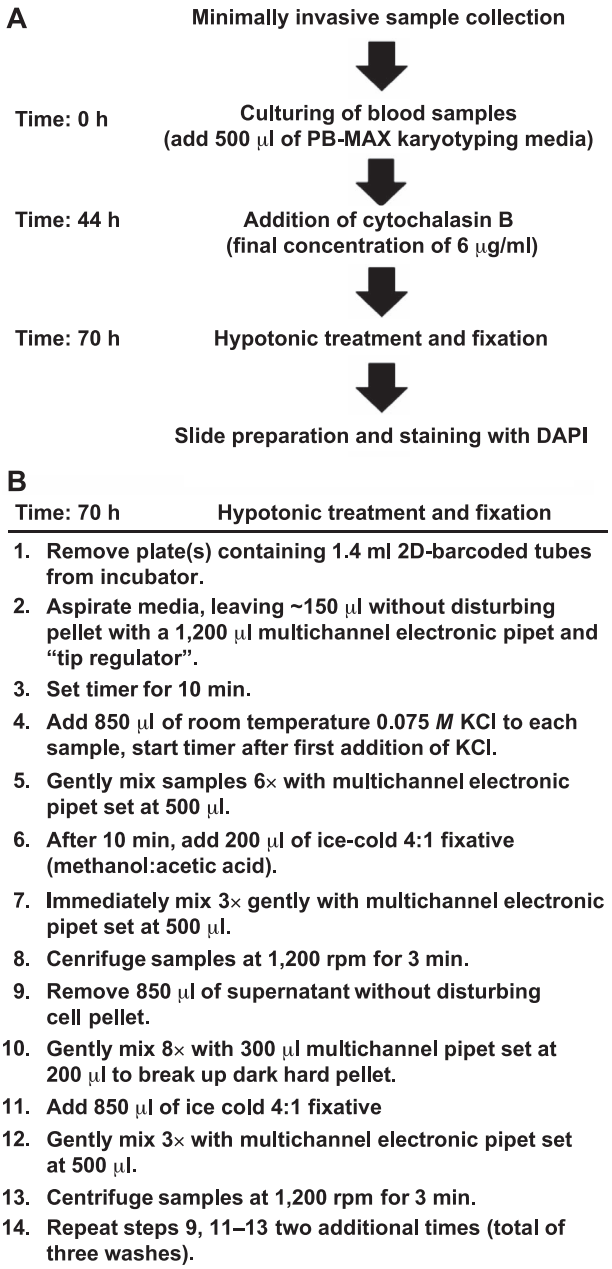

FIG. 2. Panel A: General scheme for 50 μl human whole blood micronucleus assay protocol. Panel B: Specific steps for hypotonic treatment and fixation for 50 μl human whole blood micronucleus assay protocol.

samples were clear (Fig. 2B). After the last fixative wash, supernatant was removed leaving 50 μl of sample without disturbing the pellet. The temperature of the room where slides were to be made was confirmed to be between 65–70°F. A single channel manual pipet was used to resuspend the pellet 10 times and to aliquot 25 μl of sample onto the bottom third of the slide where it was allowed to evaporate for 15–30 s. The remaining 25 μl of the sample was added to the top third of the same slide. Complete evaporation of all slides was allowed for at least 10 min before staining. Finally, 25 μl of Vectashield® media containing DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA) was added onto the slides and a coverslip was placed on top of each slide. Slides were placed in the dark for 10 min to allow the stain to spread evenly throughout the sample, then stored at 4°C until analysis.

Human Lymphocyte Isolation Protocol

Heparinized blood samples (3 ml) were collected from five healthy volunteers (3 females and 2 males) in the age range of 24–50 years after informed consent was obtained. For each donor, 500 μl blood samples were pipetted into 15 ml conical tubes. For each donor, duplicate samples were prepared and analyzed. The tubes were irradiated with gamma rays (2, 4, 5 and 6 Gy) using a Gammacell 40 ^{137}Cesium irradiator (Atomic Energy of Canada Ltd.). After irradiation, 1.5 ml of prewarmed RPMI 1640 media (Life Technologies) was added to the 500 μl human whole blood samples and mixed thoroughly. The blood sample was carefully layered on top of 2 ml of pre-aliquoted Histopaque®-1077 (Sigma-Aldrich). Once layered, samples were centrifuged at 1,220 rpm for 45 min at ambient temperature. Complete media (5 ml, RPMI 1640), 10% fetal bovine serum, 2% PHA-M and 2% penicillin/streptomycin (all of which were acquired from Life Technologies) was added into 6-well plates (Corning Inc., Corning, NY). Once centrifugation was completed, the white ring of lymphocytes was aspirated and transferred into culture media in the 6-well plates and the plates were swirled for good mixing. The 6-well plates that contained isolated lymphocytes were placed into a 37°C/5% CO_2 incubator for 44 h. After 44 h of incubation, cytochalasin-B (Sigma-Aldrich) was added at a final concentration of 6 μg/ml and a final concentration of 0.5% DMSO to cultures, and the plates were swirled to mix. At 70 h, samples were removed from the incubator and the contents of each well were transferred into properly labeled 15 ml conical tubes. The wells were washed with 3 ml of phosphate buffered saline (PBS; Life Technologies) and then added to the correct 15 ml conical tubes. The conical tubes were then filled to 10 ml with PBS. The samples were then centrifuged for 10 min at 1,000 rpm. Once centrifugation was completed, the supernatant was aspirated leaving ~1 ml. Plastic transfer pipets were used to gently push air into the solution to break the pellet; this ''air bubbling'' technique was performed 40–50 times. Hypotonic solution (1 ml of 0.075 *M* KCl; Life Technologies) was added to all samples, then a timer was started which was set for 10 min. The cell suspension was air bubbled in KCl three to four times with a transfer pipet and then the volume was brought up in each sample to 8 ml using KCl. After 10 min, 2 ml of ice-cold 3:1 (methanol:acetic acid) fixative was added, then the tubes were capped and inverted two times to mix. The samples were then centrifuged for 10 min at 1,000 rpm. After centrifugation, the supernatant was aspirated with a transfer pipet leaving 2 ml of sample. Transfer pipets were used to air bubble the samples 30 times. Then 2 ml of ice-cold fixative was added and air bubbled twice to mix. The final volume in the conical tube was brought up to 12 ml using ice-cold fixative, and the tubes were then capped and inverted twice to mix. The samples were allowed to incubate at room temperature for 10 min, centrifuged at 1,000 rpm for 10 min at ambient temperature. After centrifugation, the supernatant was aspirated leaving 1 ml of sample, then 5 ml of ice-cold fixative was added. The samples were kept in the refrigerator overnight to ensure the cells were completely fixed. On the next day, the samples were centrifuged at 1,000 rpm for 10 min at room temperature. The supernatant was removed by uncapping and inverting the tube for 3 s and the tube was placed back upright. The caps were placed back onto each tube, which was then allowed to sit undisturbed for 10 min, enabling the liquid to settle down. The pellet was resuspended by air bubbling 10 times with a glass Pasteur pipet equipped with a 1 ml dropper bulb. A micropipet was used to aspirate 25 μl of cell suspension and dropped onto the center of the glass slide and swirled. All slides prepared were allowed to air dry completely, which took about 10 min in a room at 65–70°F. Once dried, 25 μl of Vectashield media containing DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories) was added onto each slide and a coverslip was placed on top of the sample. The slides were placed in the dark for 10 min to allow the stain to spread throughout the sample, then stored at 4°C until analysis.

TABLE 1
Classifier Settings Used in Automated Image Analysis of Micronuclei in Human Lymphocytes

Object	Parameter of classifier	Current study
Nuclei	Object threshold (%)	15
	Minimum area (μm²)	80
	Maximum area (μm²)	1,000
	Maximum relative concavity depth	0.16
	Maximum aspect ratio	1.37
	Maximum distance (μm)	25
	Maximum area asymmetry (%)	90
	Region of interest radius	40
	Maximum object area in region of interest (μm²)	45
	Image processing operations	Sharpen (3,4), median V(3), median H(3), average (3,2)
Micronuclei	Object threshold (%)	6
	Minimum area (μm²)	1
	Maximum area (μm²)	50
	Maximum relative concavity depth	0.7
	Maximum aspect ratio	1.72
	Maximum distance	60
	Image processing operations	Median V(3), median H(3), average (3,1), sharpen (5,5) SBHistoMax

Automated Scoring by Metafer

Entire slides were scanned at 10× magnification with Metafer 4 software (MetaSystems, Altlussheim, Germany) consisting of a microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and motorized stage capable of scanning eight slides unattended. The Metafer classifier (Table 1) was run on the Metafer MSearch platform version 3.5 software. Images were captured using a high-resolution, monochrome megapixel charge coupled device (CCD) camera. Details concerning slide-scanning principles, stage movements, focusing and image acquisition can be found in earlier publications (*19–22*).

RESULTS

Comparison between Media Types

The CBMN assay, as well as other cytogenetic assays, requires culturing cells to divide. In some of the recently published manuals by the International Atomic Energy Agency (IAEA), emphasis was placed on the choice of culturing media, its components (serum, mitogens and antibiotics) and its testing and optimization before setting up the complete culturing media (*7, 23*). Traditionally, cytogenetic studies use a combination of RPMI solution, a mitogen, antibiotic and serum as part of its growth media. However, this self-prepared in house media is not quality-controlled tested and there is a question of reproducibility among new batches of media made. Recently, complete cGMP manufacturer-compliant, quality-control-tested cytogenetic media have been made commercially available. In particular, PB-MAX karyotyping media is a commercially made pre-prepared growth media for *in vitro* diagnostic use that contains proprietary concentrations of growth factors and antibiotics in an RPMI base.

Experiments were conducted to compare the self-prepared in house media preparation (RPMI 1640, 10% fetal bovine serum, 2% phytohemagglutinin-M and 2% penicillin/streptomycin) against the PB-MAX karyotyping media. Comparisons of media types followed the general protocol shown in Fig. 2A and B. Automated dose-response curves were generated for each media type examined. Metafer was used to analyze entire slides and scored a variable number of binucleated cells for each dose, which is related to the fact that the number of dividing cells decreases with increasing radiation dose. A dose-related increase of binucleate cells with micronuclei ratio (Mn/BN) was observed for both media types investigated (Fig. 3). Both curves were fitted with a linear quadratic equation $Y = ax^2 + bx + c$. The data obtained showed a good correlation between PB-MAX karyotyping and traditional self-prepared in house culturing media at every dose tested.

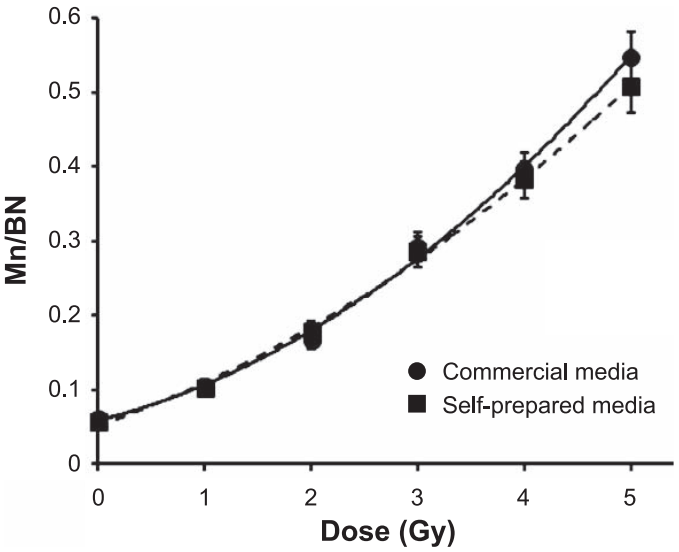

FIG. 3. Automated dose-response curve comparing commercial media (PB-MAX karyotyping media) and self-prepared in house media formulation (RPMI 1640, 15% FBS, 2% PHA and 2% penicillin/streptomycin) for the 50 μl human whole blood CBMN assay. Data shown for both curves represent average values obtained from 10 healthy donors. Error bars are ± standard error of mean (SEM).

TABLE 2A
Average Number of Detected Binucleated Cells (BN), Micronuclei (Mn) and the Calculated Number of Micronuclei per Binucleated Cell (Mn/BN) when Comparing PB-MAX Karyotyping and Self-Prepared In House Media in the 50 µl Human Whole Blood CBMN Assay in 1.4 ml 2D-Barcoded Tubes

	PB-MAX karyotyping media			Self-prepared media formulation		
Dose (Gy)	BN	Mn	Mn/BN	BN	Mn	Mn/BN
0	1,468	95	0.065 ± 0.008	1,743	104	0.061 ± 0.004
1	1,182	129	0.11 ± 0.01	1,645	170	0.103 ± 0.006
2	1,102	179	0.16 ± 0.01	1,258	210	0.16 ± 0.01
3	761	211	0.28 ± 0.02	982	254	0.26 ± 0.02
4	641	260	0.41 ± 0.02	703	260	0.37 ± 0.02
5	515	273	0.53 ± 0.03	550	278	0.50 ± 0.03

Currently, the IAEA considers a minimum of 200 binucleated cells as appropriate to be scored in the CBMN assay for triage purposes (*7, 24*). Although 50 µl of human whole blood was used, the number of analyzed cells per dose point meets the standard set by the IAEA. The average number of binucleates analyzed per slide ranged between 500 to 1,500 and was dose dependent. Unirradiated samples (0 Gy) for both media types exhibited over 1,000 binucleated cells scored and most interestingly greater than 500 binucleated cells scored at 5 Gy (Table 2A). The results indicate that the commercially available formulation is comparable to self-prepared in house media and is therefore very attractive for use in a high-throughput CBMN assay in the case of large-scale radiological event for population triage, since it minimizes human error in supplement additions and there is minimal lot variability.

The main variability in the assay may be due to individual radiosensitivity and to the variation in cell cycle duration among individuals. This variability in radiosensitivity, expressed as micronuclei frequencies in peripheral lymphocytes, is widely described in the literature. This effect, reflecting a variation in chromosomal instability, is associated with the interindividual variation in cellular defense systems, mainly the DNA repair capacity, deputed to protect the genome integrity (*25*). These remarkable interindividual differences can lead to variations in baseline levels, as well as radiation-induced micronuclei frequency. One possible drawback with automated analysis concerns the background level of Mn/BN for unirradiated samples (0 Gy). While our fully automated analysis for background level of Mn/BN is similar to some laboratories (*26*), it is higher compared to others (*19*). Several reasons could factor into a higher background level, one of which is a different protocol used to process the samples through the hypotonic and fixative steps, which has been reported in other studies (*27*). In addition, laboratories routinely culture a minimum of 0.5 ml of whole blood but only a portion of the sample is spotted on a slide. However, in our protocol an entire sample is placed on a slide without swirling, which may lead to more false-positives due to a larger density of binucleated cells in one area. Another reason is the use of different classifier settings on Metafer to analyze binucleates and micronuclei. If the 50 µl human whole blood assay protocol conducted in 1.4 ml microtubes is to be adopted for use as a population triage tool in the case of a large-scale radiological event, all laboratories involved must agree on how the samples will be processed, what automated analysis system will be used and what the algorithm and classifier settings will be to achieve consistent background results. Furthermore, micronuclei induction is not radiation specific, and due to various confounders may lead to a variable background frequency of micronuclei in unirradiated blood samples (*26, 28–30*). Although higher background levels of Mn/BN have been observed for automatic scoring compared to visual scoring,

TABLE 2B
Average Number of Detected Binucleated Cells (BN), Micronuclei (Mn) and the Calculated Number of Micronuclei per Binucleated Cell (Mn/BN) when Comparing the 50 µl Human Whole Blood CBMN Protocol in 1.4 ml 2D Barcoded Tubes Versus the 500 µl Isolated Human Lymphocyte CBMN Protocol

	High-throughput human whole blood CBMN protocol in 1.4 ml tubes (50 µl)			Conventional isolated human lymphocyte CBMN protocol (500 µl)		
Dose (Gy)	BN	Mn	Mn/BN	BN	Mn	Mn/BN
0	1,130	98	0.08 ± 0.01	1,516	104	0.07 ± 0.01
2	849	176	0.21 ± 0.01	1,085	170	0.18 ± 0.02
4	524	222	0.42 ± 0.02	701	210	0.43 ± 0.03
5	418	229	0.55 ± 0.03	526	254	0.50 ± 0.03
6	329	207	0.63 ± 0.04	375	260	0.67 ± 0.03

Note. The same donors were used in both methods.

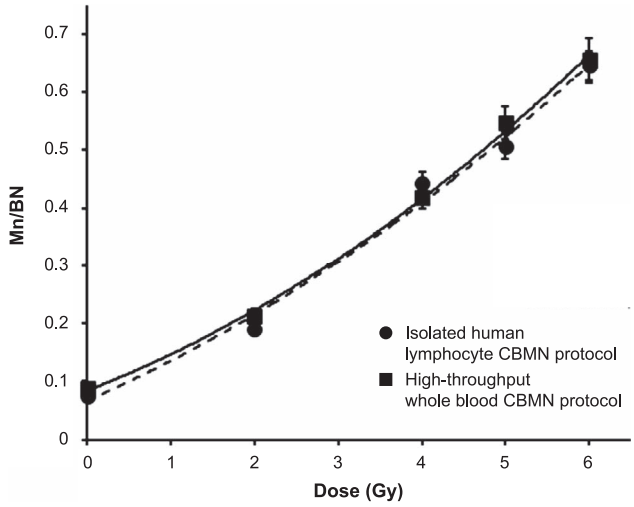

FIG. 4. Automated dose-response curve comparing a 500 μl isolated human lymphocyte CBMN protocol with the 50 μl human whole blood CBMN assay. Data shown for both curves represent average values obtained from 5 healthy donors. Error bars are ± standard error of mean (SEM).

it has been reported statistically that both are tightly correlated between the two scoring techniques (*22*).

Human Whole Blood (50 μl) Assay vs. Lymphocyte Isolation Protocol

To demonstrate the robustness of the 50 μl human whole blood assay, *ex vivo* dose-response curves were generated and a side-by-side comparison between the manually isolated lymphocyte and 50 μl whole blood protocol was performed. Although the entire 50 μl whole blood sample and a portion of the 500 μl manually isolated lymphocyte samples were dropped onto individual slides, both methods investigated resulted in similar dose-response curves being generated (Fig. 4). The average range of binucleates analyzed for the 50 μl whole blood assay was 300–1,100 and for the isolated lymphocyte method was 300–1,500, respectively. The number of binucleates observed was dose dependent. As expected, the percentage level of micronuclei increased with dose and the percentage level of binucleates decreased with dose, due to irradiation and cell death, respectively. Table 2B shows the frequency of micronuclei and binucleated cells in peripheral human lymphocytes at increasing doses of gamma rays, from 0, 2, 4, 5 and 6 Gy. Interestingly, both the 50 μl human whole blood and isolated lymphocyte protocols yielded similar Mn/BN ratios and number of micronuclei and binucleated cells detected. In Table 2B, the data reported represent the average number of binucleates and micronuclei observed for each whole slide scanned and analyzed for each individual donor at 0, 2, 4, 5 and 6 Gy. All doses examined met the standard, set by the IAEA, of a minimum of 200 binucleates to be scored for triage purposes. Micronuclei frequencies were obtained with our classifier settings on Metafer, and image analyses are strictly dose dependent with very little variation between the two methods. From a data standpoint, the 50 μl human whole blood protocol is comparable to the traditional lymphocyte isolation approach for the CBMN assay.

DISCUSSION AND CONCLUSION

Micronuclei stem from chromosome breaks or when entire chromosomes fail to engage with the mitotic spindle fibers and lag behind when cells divide. In 1985, Fenech established the CBMN assay in human peripheral blood lymphocytes (*17*). The assay has several advantages, such as speed and ease of analysis, no requirement for metaphase cells and reliable identification of cells that have completed only one nuclear division (*31, 32*). The CBMN assay is now a well-established cytogenetic dosimetry method. In an emergency situation, radiation dose estimates should be provided as soon as possible with sufficient accuracy to support clinical decision-making. In the current study we developed a high-throughput and miniaturized version of the CMBN assay, which allows for a single individual to process thousands of blood samples from the end of culturing to the point of image analysis. This new methodology is coupled with an automated image analysis approach using MetaSystems software to allow for rapid and objective analysis of slides.

We established a protocol for the CBMN assay using 1.4 ml 2D-barcoded tubes in a 96 microtiter format, which allows for sufficient throughput needed in population triage. The actual throughput of this assay is made possible by culturing, harvesting and fixing lymphocytes in microtubes. In the case of population triage due to a radiological accident, blood collection would be performed by finger-stick using a standard lancet and samples transferred into individually barcoded microtubes using heparinized capillaries having a set volume (50 μl) (*33*). Use of barcoded tubes allows for identification of individuals.

Throughput was our primary reason to use a small volume of human whole blood and to convert to the use of microtubes in this new miniaturized version of the CBMN assay. However, to develop a method for minimizing human error, we compared commercially available tested cytogenetic media to in house ''self-prepared'' media where a technician/scientist would individually add supplements to make a complete media formulation. The results demonstrate that a ''ready-to-use'' complete media, i.e., PB-MAX karyotyping media, produces results that are satisfactory, reproducible and comparable to our in house self-prepared media counterpart. Another advantage of using the commercially available pre-made media is that it has undergone strict quality control testing by the vendor, which ensures reliable and reproducible results. This is of significant importance when performing biological dosimetry on a large population. One vital strategy to accelerate sample processing in the CBMN method and increase throughput was the use of 50 μl human whole blood. During the inception of the CBMN assay, larger volumes of whole

blood were originally used to isolate lymphocytes before culturing. Also, there is the need for additional measures, such as the use of lymphocyte separation media and the transfer to culturing apparatus. In addition, many laboratories today culture using larger volumes of human whole blood (0.5–1 ml), which is not conducive for high-throughput sample processing for population triage in a large-scale radiological event. Setting up cultures is simplified when small volumes of whole blood are used, requiring only the addition of ''ready-to-use'' complete media to microtubes containing the blood and placement of the tube racks into an incubator. The use of small volumes also allows for accelerated processing of a significantly larger number of samples.

Higher throughput of the CBMN assay was achieved with the modifications to the hypotonic/fixative steps. According to standard CBMN assay procedures, lymphocytes must be treated with hypotonic solution and then fixed. These steps involve separate washes with potassium chloride and fixative by centrifugation. Using traditional methods, 5–10 ml blood cultures are transferred into 15 ml conical tubes and 15–30 min centrifugation times are used (*33*). With the use of microtubes in a 96 microtiter format, centrifugation times we narrowed down to a few minutes, thus saving a significant amount of time from the end of culturing to when slides are prepared. An individual can process 96 samples during the hypotonic treatment and fixation step in 40–45 min. We estimate that a single individual can process at least 1,500–1,700 samples in a 12 h shift, which amounts to sixteen to eighteen 96 microtiter formatted racks containing microtubes. Whereas, the traditional method of using 5–10 ml blood cultures would take an individual 12 h to process 96 samples. One can envision if the assay is adopted by cytogenetic biodosimetry laboratories, such as the World Health Organization's BioDoseNet network (*34*), the ability to process samples would be drastically improved compared to our current standards if a mass-casualty radiological event occurred.

Automated scoring of binucleates using sophisticated image analysis systems is another promising strategy to increase throughput of the CBMN assay for achieving a rapid and reliable dose assessment for triage purposes in large-scale nuclear or radiological incidents. Several automated systems are available on the market and have been tested (*35–38*). In our study, we coupled our high-throughput miniaturized assay with the MetaSystems scoring system. A MetaSystems 10× objective scanner can analyze an entire slide in 13 min using our classifier settings. This is significantly faster than the manual scoring of 200 binucleated cells, which takes around 15 min (*35*). Although, automated scoring is the bottleneck in the process for population triage, it generates reasonable, reliable and objective dose curves in addition to being more expeditious than human scorers. Although still relatively new, the findings from automated analysis clearly show potential advantages in large-scale studies (*27, 28, 39*). Future advancements in the development of faster image analysis software will benefit the scope of higher throughput sample processing in the case of large population analysis.

To truly make the assay high throughput, full automation of the cytogenetic dose assessment method would be needed. Currently, to track the demanding throughput required for screening in drug discovery, pharmaceutical and biotechnology companies are applying automation and robotic technologies such as liquid handling systems. They are being used for multiple sample preparations to increase the number of molecules that can be biologically assayed (*40–42*). The RABiT is an example of an advanced throughput system developed at Columbia University Medical Center for the purpose of biological dosimetry applications (*2, 5, 43, 44*). To make our method described here even more higher in throughput, the next generation of our assay format would be to fully automate the method using robotics.

Acquiring immediate results will be crucial after a large-scale radiological incident because of the need to detect, at an early stage, those individuals who will benefit from medical involvement and those who will not. The studies reported here indicate the development of a high-throughput and miniaturized version of the CMBN assay, which has the capability of rapid sample processing for critical data retrieval. The new method allows for the culturing, hypotonic and fixation treatments of blood samples in a singular tube, which is an advancement in throughput of sample processing when using the CBMN assay. The results from this 50 μl human whole blood assay are comparable to traditional methods that typically use larger volumes of blood. Furthermore, this new formatted CBMN assay will fast track the identification and prioritization of individuals who would benefit from immediate medical treatment. Once highly exposed individuals have been identified, they may receive treatment for acute radiation syndrome, which causes damage to vital organs such as bone marrow and the gastrointestinal tract (*45*). Exposed individuals may benefit from the use of new pharmacological countermeasures such as PHD inhibitors as a treatment for accidental exposure to ionizing radiation (*46*).

In this work, the high-throughput method preparation of CBMN assay samples from 50 μl of whole blood volume was developed by miniaturizing and using an ANSI/SLAS microplate format. The developed method allows for a single individual to process thousands of blood samples from the end of culturing to the point of image analysis. The developed protocol can be used in a traditional laboratory setting for multiple sample investigation in radiological biodosimetry where either self-prepared in house or commercially available media can be used. Additionally, the method is versatile enough to use in biodosimetry laboratories for other cytogenetic assays with a few modifications. In conclusion, our new high-throughput and miniaturized CBMN method will expedite sample

processing to treatment regimen in the case of a large-scale radiation event.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Allergy and Infectious Disease, National Institutes of Health, grant no. U19 A1067773. Federal funds in whole or in part were also provided by the Biomedical Advanced Research and Development Authority (BARDA), Office of the Assistant Secretary for Preparedness and Response, Office of the Secretary, Department of Health and Human Services, under contract no. HHSO100201000002C. We would like to thank Helen Turner, Jay Perrier and Maria Taveras from the Center for Radiological Research, Department of Radiation Oncology, Columbia University Medical Center for their excellent technical assistance.

Received: December 11, 2014; accepted: May 3, 2015; published online: July 31, 2015

REFERENCES

1. Singh VK, Ducey EJ, Brown D, Whitnall MH. A review of radiation countermeasure work ongoing at the Armed Froces Radiobiology Research Institute. Int J Radiat Biol 2012; 88:296–310.
2. Garty G, Chen Y, Salerno A, Turner H, Zhang J, Lyulko O, et al. The RABIT: a rapid automated biodosimetry tool for radiological triage. Health Phys 2010; 98:209–17.
3. Grace MB, Moyer BR, Prasher J, Cliffer KD, Ramakrishnan N, Kaminski J, et al. Rapid radiation dose assessment for radiological public health emergencies: Role of NIAID and BARDA. Health Phys 2010; 98:172–8.
4. Rea ME, Gougelet RM, Nicolalde RJ, Geiling JA, Swartz HM. Proposed triage categories for large-scale radiation incidents using high-accuracy biodosimetry methods. Health Phys 2010; 98:136–44.
5. Garty G, Chen Y, Turner HC, Zhang J, Lyulko OV, Bertucci A, et al. The RABiT: a rapid automated biodosimetry tool for radiological triage. II. Technological developments. Int J Radiat Biol 2011; 87:776–90.
6. Ainsbury EA, Al-Hafidh J, Bajinskis A, Barnard S, Barquinero JF, Beinke C, et al. Inter- and intra-laboratory comparison of a multibiodosimetric approach to triage in a simulated, large scale radiation emergency. Int J Radiat Biol 2014; 90:193–202.
7. Cytogenetic dosimetry: applications in preparedness for and response to radiation emergencies. IAEA emergency preparedness and response series. Vienna: International Atomic Energy Agency; 2011. p. 1–229.
8. Baeyens A, Swanson R, Herd O, Ainsbury E, Mabhengu T, Willem P, et al. A semi-automated micronucleus-centromere assay to assess low-dose radiation exposure in human lymphocytes. Int J Radiat Biol 2011; 87:923–31.
9. Romm H, Oestreicher U, Kulka U. Cytogenetic damage analyzed by the dicentric assay. Ann Ist Super Sanita 2009; 45:251–9.
10. Vaurijoux A, Gruel G, Pouzoulet F, Gregoire E, Martin C, Roch-Lefevre S, et al. Strategy for population triage based on dicentric analysis. Radiat Res 2009; 171:541–8.
11. Repin MV, Repina LA. Rapid dicentric assay of human blood lymphocytes after exposure to low doses of ionizing radiation. (article in Russian) Radiats Biol Radioecol 2011; 51:411–8.
12. Flegal FN, Devantier Y, McNamee JP, Wilkins R. Quickscan dicentric chromosome analysis of radiation biodosimetry. Health Phys 2010; 98:276–81.
13. Romm H, Ainsbury E, Barnard S, Barrios L, Barquinero JF, Beinke C, et al. Automatic scoring of dicentric chromosomes as a tool in large scale radiation accidents. Mutat Res 2013; 756:174–83.
14. Ossetrova NI, Sandergren DJ, Blakely WF. Protein biomarkers for enhancement of radiation does and injury assessment in nonhuman primate total-body irradiation model. Radiat Prot Dosimetry 2014; 159:61–76.
15. Rothkamm K, Beinke C, Romm H, Badie C, Balagurunathan Y, Barnard S, et al. Comparison of established and emerging biodosimetry assays. Radiat Res 2013; 180:111–9.
16. Brengues M, Paap B, Bittner M, Amundson SA, Seligmann B, Korn R, et al. Biodosimetry on small blood volume using gene expression assay. Health Phys 2010; 98:179–85.
17. Fenech M, Morley AA. Measurement of micronuclei in lymphocytes. Mutat Res 1985; 147:29–36.
18. Vral A, Fenech M, Thierens H. The micronucleus assay as a biological dosimeter of in vivo ionising radiation exposure. Mutagenesis 2011; 26:11–7.
19. Willems P, August L, Slabbert J, Romm H, Oestreicher U, Thierens H, et al. Automated micronucleus (MN) scoring for population triage in case of large scale radiation events. Int J Radiat Biol 2010; 86:2–11.
20. Schunck C, Johannes T, Varga D, Lorch T, Plesch A. New developments in automated cytogenetic imaging: unattended scoring of dicentric chromosomes, micronuclei, single cell gel electrophoresis, and fluorescence signals. Cytogenet Genome Res 2004; 104:383–9.
21. Varga D, Johannes T, Jainta S, Schuster S, Schwarz-Boeger U, Kiechle M, et al. An automated scoring procedure for the micronucleus test by image analysis. Mutagenesis 2004; 19:391–7.
22. Seager AL, Shah UK, Brüsehafer K, Wills J, Manshian B, Chapman KE, et al. Recommendations, evaluations and validation of a semi-automated, fluorescent-based scoring protocol for micronucleus testing in human cells. Mutagenesis 2014; 29:155–64.
23. Cytogenetic analysis for radiation dose assessment. IAEA technical reports series no. 405. Vienna: International Atomic Energy Agency; 2001. p. 1–127.
24. McNamee JP, Flegal FN, Greene HB, Marro L, Wilkins R. Validation of the cytokinesis-block micronucleus (CBMN) assay for use as a triage biological dosimetry tool. Radiat Prot Dosimetry 2009; 135:232–42.
25. Wu X, Soitz MR, Amos CI, Lin J, Shao L, Gu J, et al. Mutagen sensitivity has high heritability: evidence from a twin study. Cancer Res 2006; 66:5993–6.
26. Beinke C, Port M, Abend M. Automatic versus manual lymphocyte fixation: impact on dose estimation using the cytokinesis-block micronucleus assay. Radiat Environ Biophys 2015; 54:81–90.
27. Rossnerova A, Spatova M, Schunck C, Sram RJ. Automated scoring of lymphocyte micronuclei by the MetaSystems Metafer image cytometry system and its application in studies of human mutagen sensitivity and biodosimetry of genotoxin exposure. Mutagenesis 2011; 26:169–75.
28. Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The Human MicroNucleus Project - an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat Res 1999; 428:271–83.
29. Tucker JD, Valdapalli M, Joiner MC, Ceppi M, Fenech M, Bonassi S. Estimating the lowest detectable dose of ionizing radiation by the cytokinesis-block micronucleus assay. Radiat Res 2013; 180:284–91.
30. Fenech M, Morley AA. Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low-dose X-irradiation. Mutat Res 1986; 161:193–8.

31. Fenech M. The advantages and disadvantages of the cytokinesis-block micronucleus method. Mutat Res Genet Toxicol Environ Mutagenesis 1997; 392:11–8.

32. Yang H, Feng R, Liu J, Wang H, Wang Y. Increased frequency of micronuclei in binucleated lymphocytes among occupationally pesticide-exposed populations: a meta-analysis. Asian Pac J Cancer Prev 2014; 15:6955–60.

33. Repin M, Turner HC, Garty G, Brenner DJ. Next generation platforms for high-throughput biodosimetry. Radiat Prot Dosimetry 2014; 159:105–10.

34. Maznyk NA, Wilkins R, Carr Z, Lloyd D. The capacity, capabilities and needs of the WHO BioDoseNet member laboratories. Radiat Prot Dosimetry 2012; 151:611–20.

35. Romm H, Barnard S, Boulay-Greene H, De Amicis A, De Sanctis S, Franco M, et al. Laboratory intercomparison of the cytokinesis-block micronucleus assay. Radiat Res 2013; 180:120–8.

36. De Sanctis S, De Amicis A, Di Cristofaro S, Franchini V, Regalbuto E, Mammana G, et al. Cytokinesis-block micronucleus assay by manual and automated scoring: calibration curves and dose prediction. Health Phys 2014; 106:745–9.

37. Frieauff W, Martus HJ, Suter W, Elhajouji A. Automatic analysis of the micronucleus test in primary human lymphocytes using image analysis. Mutagenesis 2013; 28:15–23.

38. Fenech M, Kirsch-Volders M, Rossnerova A, Sram R, Romm H, Bolognesi C, et al. HUMN project initiative and review of validation, quality control and prospects for further development of automated micronucleus assays using image cytometry systems. Int J Hyg Environ Health 2013; 216:541–52.

39. Bonassi S, Neri M, Lando C, Ceppi M, Lin YP, Chang WP, et al. Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project. Mutat Res 2003; 543:155–66.

40. Fonsi M, Orsale MV, Monteagudo E. High-throughput microsomal stability assay for screening new chemical entities in drug discovery. J Biomol Screen 2008; 13:862–7.

41. Shi S, Condon RG, Deng L, Saunders J, Hung F, Tsao YS, et al. A high-throughput automated platform for the development of manufacturing cell lines for protein therapeutics. J Vis Exp 2011; 55:1–5.

42. Youdim KA, Saunders KC. A review of LC-MS techniques and high-throughput approaches used to investigate drug metabolism by cytochrome P450s. J Chromatogr B Analyt Technol Biomed Life Sci 2010; 878:1326–36.

43. Turner HC, Brenner DJ, Chen Y, Bertucci A, Zhang J, Wang H, et al. Adapting the gamma-H2AX assay for automated processing in human lymphocytes. 1. Technological aspects. Radiat Res 2011; 175:282–90.

44. Turner HC, Sharma P, Perrier JR, Bertucci A, Smilenov L, Johnson G, et al. The RABiT: high-throughput technology for assessing global DSB repair. Radiat Environ Biophys 2014; 53:265–72.

45. Lee L, Moding E, Kirsch DG. Reining in radiation injury: HIF2α in the gut. Sci Transl Med 2014; 6:1–3.

46. Taniguchi CM, Miao YR, Diep AN, Wu C, Rankin EB, Atwood TF, et al. PHD inhibition mitigates and protects against radiation-induced gastrointestinal toxicity via HIF2. Sci Transl Med 2014; 6:1–9.