CytoRADx: A High-Throughput, Standardized Biodosimetry Diagnostic System Based on the Cytokinesis-Block Micronucleus Assay

Authors: Capaccio, Chris, Perrier, Jay R., Cunha, Lídia, Mahnke, Ryan C., Lörch, Thomas, et al.

Source: Radiation Research, 196(5) : 523-534

Published By: Radiation Research Society

URL: https://doi.org/10.1667/RADE-20-00030.1

RADIATION RESEARCH **196**, 523–534 (2021)
0033-7587/21 $15.00

DOI: 10.1667/RADE-20-00030.1

CytoRADx: A High-Throughput, Standardized Biodosimetry Diagnostic System Based on the Cytokinesis-Block Micronucleus Assay

Chris Capaccio,[a] Jay R. Perrier,[a,b] Lídia Cunha,[b] Ryan C. Mahnke,[a] Thomas Lörch,[c] Michael Porter,[a] Chris L. Smith,[a] Ken Damer,[a] J. Daniel Bourland,[d] Bart Frizzell,[d] Jennifer Torelli,[e] Marie Vasquez,[e] Jeremy B. Brower,[f] Melanie Doyle-Eisele,[f] Maria Taveras,[b], Helen Turner,[b] David J. Brenner[b] and Richard Kowalski[a,1]

[a] *ASELL, LLC, Owings Mills, Maryland;* [b] *Columbia University, Center for Radiological Research, New York, New York;* [c] *MetaSystems, GmbH, 68804 Altlussheim, Germany;* [d] *Wake Forest School of Medicine, Departments of Radiation Oncology, Physics, and Biomedical Engineering, Winston-Salem, North Carolina;* [e] *Helix3, Inc., Morrisville, North Carolina; and* [f] *Lovelace Biomedical Research Institute, Albuquerque, New Mexico*

Capaccio, C., Perrier, J.R., Cunha, L., Mahnke, R.C., Lörch, T., Porter, M., Smith, C.L., Damer, K., Bourland, J.D., Frizzell, B., Torelli, J., Vasquez, M., Brower, J.B., Doyle-Eisele, M., Taveras, M., Turner, H., Brenner, D.J. and Kowalski, R. CytoRADx: A High-Throughput, Standardized Biodosimetry Diagnostic System Based on the Cytokinesis-Block Micronucleus Assay. *Radiat. Res*. 196, 523–534 (2021).

In a large-scale catastrophe, such as a nuclear detonation in a major city, it will be crucial to accurately diagnose large numbers of people to direct scarce medical resources to those in greatest need. Currently no FDA-cleared tests are available to diagnose radiation exposures, which can lead to complex, life-threatening injuries. To address this gap, we have achieved substantial advancements in radiation biodosimetry through refinement and adaptation of the cytokinesis-block micronucleus (CBMN) assay as a high throughput, quantitative diagnostic test. The classical CBMN approach, which quantifies micronuclei (MN) resulting from DNA damage, suffers from considerable time and expert labor requirements, in addition to a lack of universal methodology across laboratories. We have developed the CytoRADx™ System to address these drawbacks by implementing a standardized reagent kit, optimized assay protocol, fully automated microscopy and image analysis, and integrated dose prediction. These enhancements allow the CytoRADx System to obtain high-throughput, standardized results without specialized labor or laboratory-specific calibration curves. The CytoRADx System has been optimized for use with both humans and non-human primates (NHP) to quantify radiation dose-dependent formation of micronuclei in lymphocytes, observed using whole blood samples. Cell nuclei and resulting MN are fluorescently stained and preserved on durable microscope slides using materials provided in the kit. Up to 1,000 slides per day are subsequently scanned using the commercially based RADxScan™ Imager with customized software, which automatically quantifies the cellular features and calculates the radiation dose. Using less than 1 mL of blood, irradiated *ex vivo*, our system has demonstrated accurate and precise measurement of exposures from 0 to 8 Gy (90% of results within 1 Gy of delivered dose). These results were obtained from 636 human samples (24 distinct donors) and 445 NHP samples (30 distinct subjects). The system demonstrated comparable results during *in vivo* studies, including an investigation of 43 NHPs receiving single-dose total-body irradiation. System performance is repeatable across laboratories, operators, and instruments. Results are also statistically similar across diverse populations, considering various demographics, common medications, medical conditions, and acute injuries associated with radiological disasters. Dose calculations are stable over time as well, providing reproducible results for at least 28 days postirradiation, and for blood specimens collected and stored at room temperature for at least 72 h. The CytoRADx System provides significant advancements in the field of biodosimetry that will enable accurate diagnoses across diverse populations in large-scale emergency scenarios. In addition, our technological enhancements to the well-established CBMN assay provide a pathway for future diagnostic applications, such as toxicology and oncology.

INTRODUCTION

Injuries resulting from radiation exposures are complex, severe, and difficult to diagnose at critical early stages. The effects of acute radiation syndrome (ARS) are also challenging to treat and life-threatening in the most serious cases (1). In a large-scale catastrophe, such as a detonation of a nuclear device in a major city, hundreds of thousands of people could require medical intervention for radiation absorption, while many more might be healthy but concerned about potential exposure (2). Thus, it will be crucial to accurately screen large numbers of people to direct scarce medical resources to those in greatest need, and to determine how to best treat a diverse population of victims exposed to varied doses of radiation (3).

[1] Address for correspondence: ASELL, LLC., Product Development, 11515 Cronridge Drive Suite Q, Owings Mills, MD 21117; email: rkowalski@asell.com.

Currently no FDA-cleared test is available to quantify radiation absorption, i.e., to conduct biodosimetry. Existing techniques involve observations of the severity and time course of certain ARS symptoms, such as nausea and vomiting (3). Other well-characterized indicators include a decline in absolute lymphocyte counts over time. However, these hematological change-based approaches typically require testing of two or more sequential blood samples, drawn hours or days apart (4). These types of lower precision, lower throughput biodosimetry methods would prove impractical in the event of a large-scale radiation exposure event, with the potential need to screen hundreds of thousands of people at diverse locations in short order. Recent efforts have been dedicated to developing more effective biodosimetry tools, some of which have focused on the appearance of chromosomal aberrations. One such approach, the dicentric chromosome assay (DCA), currently accepted as the gold-standard for biodosimetry (5), requires precise chromosome preparation, time consuming imaging at high magnification, and complex image analyses (6), necessitating skilled laboratorians, controlled environmental conditions, and multi-pass imaging time. While companies, including MetaSystems, have automated image capture and analyses, the remaining complexities of the DCA technique hamper its practicality for rapid, large-scale biodosimetry. Alternatively, the cytokinesis-block micronucleus (CBMN) assay has shown diagnostic potential for various medical indications involving genomic damage, including radiation absorption (7), while demonstrating feasibility for reproducible, high-throughput use (8).

The CBMN assay identifies chromosomal abnormalities resulting from exposure to genotoxic agents, such as ionizing radiation, mediated through the creation of double-stranded DNA breaks (9, 10). In the CBMN assay, target cells are induced to proliferate *in vitro* followed by inhibition of daughter-cell separation (cytokinesis block) to enrich the sample with mitotically active cells and to control for those cells that have undergone mitosis. This process enables visual identification of dividing, bi-nucleated (BN) cells and aberrations, such as micronuclei (MN), from a population of cells that are normally quiescent. MN form when chromosome fragments from damaged DNA are encased in a miniature nuclear envelope during cytokinesis (7). Although MN are only present temporarily, halting cytokinesis allows fixation of these features. Quantities of MN in peripheral blood lymphocytes have been correlated to the extent of radiation absorption (7, 11). However, the classical CBMN approach suffers from the use of lab-specific calibration curves and sample preparation methodologies that vary from lab to lab. In addition, considerable time and expert labor are required to manually identify BN and MN on microscope slides, some of which have been reduced by semi-automated platforms like Metafer (12–14). Furthermore, the historic quantitative range of CBMN-based biodosimetry has been limited to approximately 0 to 5 Gy (15). Another approach, imaging flow cytometry (IFC), is under development for triage biodosimetry, but the dose range may be similarly restricted to under 5 Gy, there are a limited number of deployed instruments, and the final readout still requires 15 min of image acquisition per sample, despite recent advancements (16). We developed the CytoRADx System to address numerous drawbacks of the CBMN assay and related methods, by implementing a standardized reagent kit and assay protocol, fully automated microscopy and image analysis, and integrated dose estimation. The system quantifies a wider range of absorption, from 0 to 8 Gy, which includes important medical decision points (i.e., 2 Gy and 6 Gy) that are critical to determining the optimal therapeutic regimen for radiation accident victims (17). In addition, the system uses existing commercial equipment, including the imaging instrument, that is already deployed in clinical laboratories across the country, which could enable rapid mobilization in case of emergencies.

For the U.S. Food and Drug Administration (FDA) to allow marketing of an *in vitro* diagnostic device, assessing the safety and effectiveness of the device typically employs the intended use population. For biodosimetry applications, this target population consists of the general public receiving a large single partial- or whole-body dose of radiation due to detonation of a nuclear device or other large-scale exposure event. Since this human population cannot be safely or ethically tested in a clinical trial, a two-phase method of clinical samples from humans and non-clinical samples from non-human primates (NHPs) was established. NHPs (specifically *Macaca mulatta)* were the chosen animal model to support validation of the CytoRADx System for several reasons: chromosomal damage caused by ionizing radiation within both human and NHP lymphocytes is apparent as micronuclei within binucleated cells; human and NHP lymphocytes can be cultured *in vitro* from heparinized whole blood samples; and human and NHP lymphocytes are morphologically similar and easily visualized with DAPI stained cells by fluorescence microscopy. The ability to analyze *ex vivo* human, as well as *ex vivo* and *in vivo* NHP models, could enable the use of CytoRADx in additional studies of medical countermeasures (18), toxicology (19*)* and oncology (20, 21).

METHODS

The CytoRADx System

The CytoRADx System consists of the CytoRADx Assay and the RADxScan™ Imager (Fig. 1A and C). In addition, the CytoRADx System also utilizes other common laboratory equipment, including a humidified CO_2 incubator, a centrifuge for deep-well plates, and a biological safety cabinet. The CytoRADx Assay is an optimized version of the CBMN assay, based on the variant described by Fenech (7). The CytoRADx Assay Kit contains proprietary media formulations, cytokinesis blocking agent, fluorescent stain, hypotonic solution, microtiter plates, and microscope slides and coverslips to process 192 samples. Briefly, 600 μL of whole blood shipped and stored at room temperature (never frozen) was combined with culture medium containing a proprietary mixture of mitogens and growth

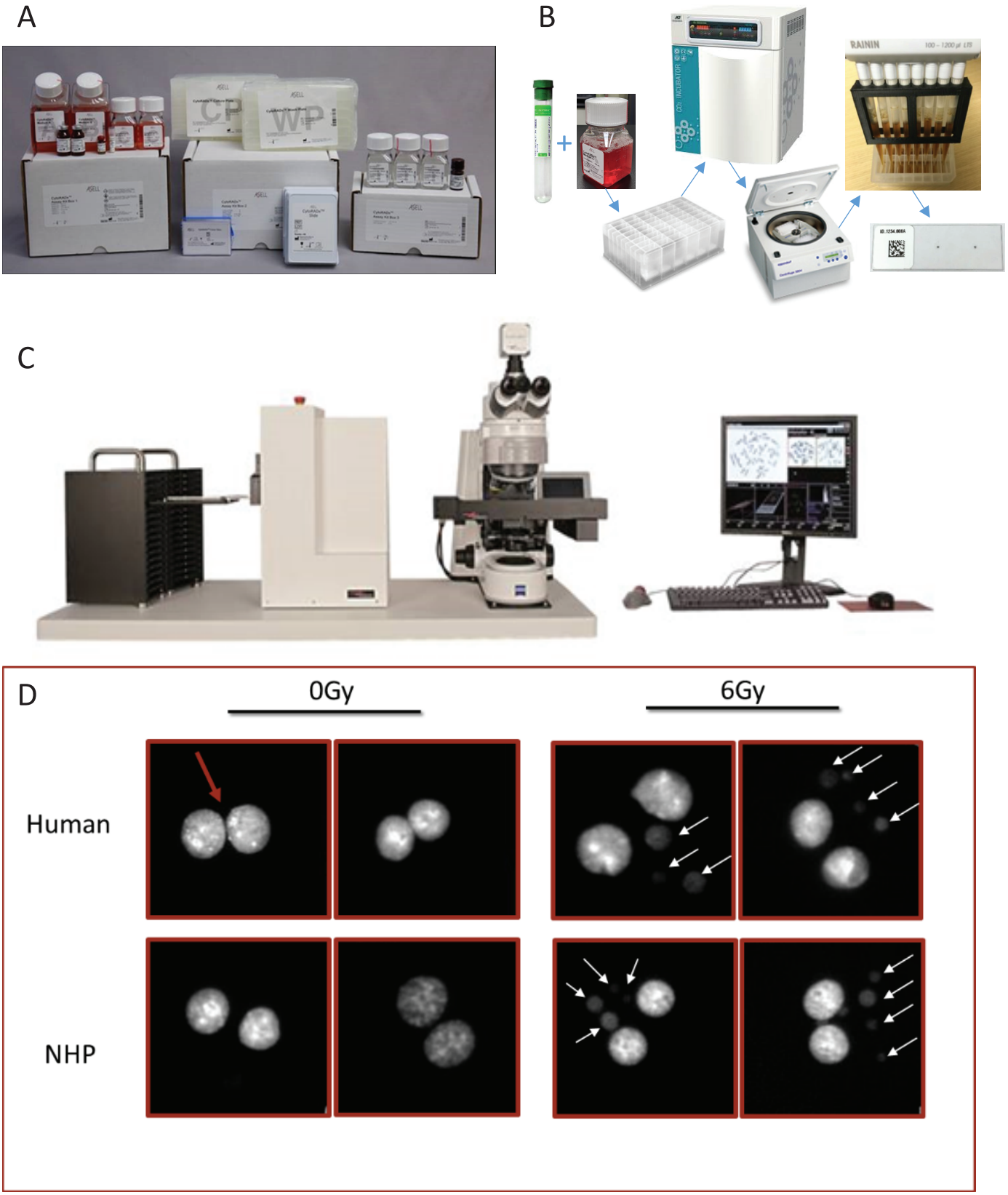

FIG. 1. CytoRADx System. Panel A: CytoRADx™ Assay Kit ; Panel B: CytoRADx major processing steps; Panel C: RADxScan™ imager; and Panel D: Gallery of images showing BN with MN, which were automatically scanned, detected, and quantified (human and NHP).

TABLE 1

Population	No. of subjects tested	Sample source	Characteristics
Healthy adults	261	Commercial and CUIMC	Age range 19–59 years, males and females
Healthy pediatric	50	Columbus Regional Research Institute, Commercial	Age range 2–7 years, males and females
Healthy adolescent	50	Commercial	Age range 8–18 years, males and females
Healthy geriatric	50	Commercial	Age range > 59 years, males and females
Burn	16	Maricopa, MedStar, WFSM	Any partial-thickness burn larger than 10% of total-body surface area (TBSA) or third-degree burns covering more than 3% of TBSA
Infection–sepsis	44	Albert Einstein, MedStar, MultiCare, WFSM,	Medically diagnosed; with WBC <4,000 or >12,000 cells/mm^3
Trauma	34	Albert Einstein, Maricopa, MedStar, MultiCare, Univ. of Kentucky	Any requiring ER admittance, such as car accidents, explosion injuries, brain injuries (concussions) with a severity score greater than 10
HIV	52	Commercial, Albert Einstein	HIV positive with CD4 count <350 cells/μL
IBD	50	Commercial	Medically diagnosed with inflammatory bowel disease including ulcerative colitis and Crohn's disease
Rheumatoid arthritis	28	Commercial	Medically diagnosed with rheumatoid arthritis with ACR score >6
Type 2 diabetes	50	Commercial	Medically diagnosed with type 2 diabetes
Pregnancy	50	Commercial	Positive pregnancy test

factors in a 48-well plate (5 mL volume per well) and incubated humidified at 37°C with 5% CO_2. The CytoRADx Assay uses the same reagents, consumables, and detection of biomarkers for both human and NHP samples. After culturing (44 h for human or 68 h for NHP), medium was exchanged with fresh medium containing a cytokinesis blocking agent and cells were cultured for an additional 26 h. Previous work showed that NHP lymphocytes required additional time compared to human lymphocytes to reach adequate proliferation prior to cytokinesis blocking (data not shown). Cultures were then treated with potassium chloride, washed and fixed with methanol/glacial acetic acid fixative. Fixed cells were dropped on microscope slides, dried and nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI; Fig. 1B). The overall assay procedure was approximately 72 h from receipt of blood samples, which is comparable to other cytogenetic assays. Slides generated from the CytoRADx Assay were analyzed using the RADxScan Imager, a modified version of the MetaSystems Metafer platform, which has been previously used to image and quantify BN and MN. The imager scans each microscope slide using a 10× objective with epifluorescence detection of DAPI stained objects on a slide. Fluorescent objects that met criteria for size, shape, intensity, and clustering were analyzed using a customized, proprietary version of MetaSystems' software to score the number of BN and mononucleated cells (MO), the total number of BN with MN, and the number of MN within each BN. These scores were used in a proprietary, multivariate algorithm by the RADxScan Imager to estimate the amount of total body radiation absorbed, without use of background subtraction or a test- or laboratory-specific calibration curve. Figure 1D illustrates the high degree of morphological similarity between human and NHP binucleated cells produced by the CytoRADx System. Also shown are multiple micronuclei within binucleated cells. Up to 1,000 slides can be scanned per day per instrument.

Human and Non-Human Primate Blood Specimens

All whole blood samples were collected from human subjects under IRB approved protocols. The various human subject populations described herein are listed in Table 1. Blood specimens were obtained either commercially through Bioserve Technologies (Beltsville, MD), HemaCare (Van Nuys, CA), BioIVT (Westbury, NY), designated as ''Commercial'', or through site-specific protocols at participating centers. Centers providing human specimens for these studies included Einstein Medical Center, Philadelphia, PA; Columbus Regional Research Institute, Columbus, GA; Columbia University Irving Medical Center, New York, NY (CUIMC); Valleywise Health (formerly Maricopa Medical Center), Phoenix, AZ; MedStar Washington Hospital Center, Washington, DC; MultiCare Health System, Tacoma, Washington; Wake Forest School of Medicine, Winston-Salem, NC (WFSM); and University of Kentucky Medical Center, Lexington, KY.

NHP whole blood specimens were obtained commercially from Worldwide Primates (Miami, FL) or Alpha Genesis (Yemassee, SC) under institutional animal care and use committee (IACUC) approved protocols. NHPs consisted of an equal mix of males and females with ages between 3 and 6 years inclusive with body weight ≥4 kg. All whole blood specimens were collected into standard commercially available evacuated blood collection tubes containing sodium heparin.

Blood Irradiations Ex Vivo

Whole blood specimens were shipped overnight at room temperature in insulated shippers (human blood: Sonoco ThermoSafe, PN: 609UPS; NHP blood: Pelican BioThermal, PN: Series 22-248) to CytoRADx System testing labs. Blood specimens were aliquoted into samples of appropriate volume to conduct irradiations at either Columbia University Irving Medical Center (CUIMC) or Wake Forest School of Medicine (WFSM). Blood irradiations at CUIMC were conducted using a commercial radioisotope research irradiator (Cs^{137}, 0.662 MeV γ rays, 15 mL sample tubes oriented horizontally, nominal dose rate of 0.73 Gy/min) (22). This device was calibrated based on dosimetry measurements with a thermoluminescent dosimeter device. Blood irradiations at WFSM were conducted using a commercial radioisotope clinical irradiator typically used for blood sterilization [Cs^{137}, 0.662 MeV γ rays, sample tubes oriented vertically in continuously rotating ABS tube holder (Fig. 2), nominal dose rate of 4 Gy/min; Gammacell® 3000 Elan Irradiator, Best Theratronics, Ottawa, Ontario, Canada]. Quality assurance procedures established dose rates and dose homogeneity at both sites. With both irradiators,

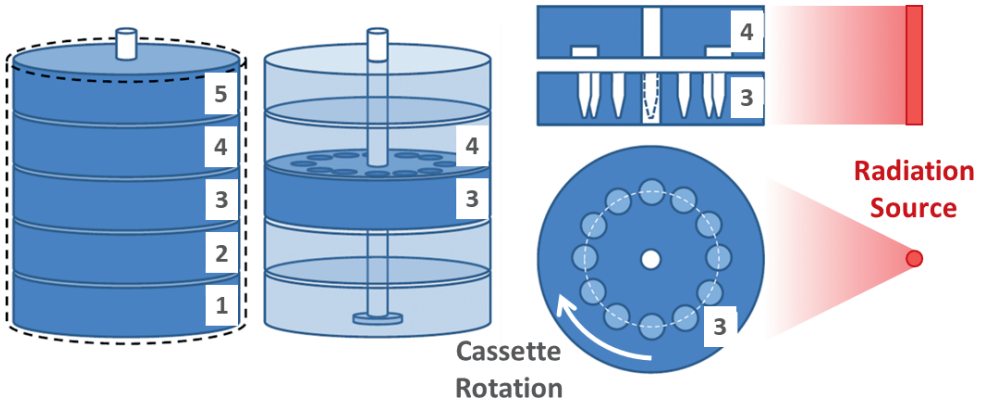

FIG. 2. *Ex Vivo* irradiation fixture. Custom fabricated ABS tube holder for blood irradiations *ex vivo*.

γ-ray dose levels ranged from 0 (non-irradiated sham controls) to 11 Gy. Volumes of blood per 1.7 mL conical tube ranged from 0.5 to 1.4 mL and 2 to 15 mL for 15 mL conical tube.

Irradiation of NHPs In Vivo

Rhesus macaques were obtained commercially from Worldwide Primates and shipped under IACUC approved conditions to Lovelace Biomedical and Environmental Research Institute (LBERI, Albuquerque, NM). All animal welfare procedures followed Public Health Service Policy on Humane Care and Use of Laboratory Animals, according to the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. Animals were deemed healthy by veterinary staff at LBERI upon successful completion of quarantine procedures prior to irradiations.

Irradiations were conducted at LBERI using a research-dedicated 6 MV linear accelerator (LINAC; Clinac 600C, Varian Medical Systems, Palo Alto, CA), a device designed to treat human cancer patients. The LINAC was calibrated in accordance with the American Association of Physics in Medicine (AAPM) Task Group Reports 51 (23) and 106 (24). The LINAC was calibrated to ± 2% absolute using a NIST-traceable PTW ionization chamber. Each NHP was positioned entirely inside the light field of the instrument. Prior to each day of animal irradiation, the instrument output was verified using a tissue-equivalent phantom and calibrated ionization chamber.

Animals fasted overnight, were sedated with 10 mg/kg (±0.5 mg/kg) ketamine (intramuscular), prepped for irradiation, and moved to the irradiation room within the LINAC facility. Animals were then anesthetized with isoflurane (1–3% for maintenance, via face mask inhalation), wrapped with cellophane and tissue-equivalent bolus for build-up of surface dose, and placed into a sling. Animals received a single irradiation (day 0) except for the 0 Gy (sham) group. Animals in the sham group were transferred to the LINAC facility and anesthetized but not irradiated. Irradiations consisted of the mid-plane-target dose delivered through a pair of right and left lateral opposed fields, each delivering one-half of the dose (Fig. 3). Diode detectors for *in vivo* dosimetry were placed on each side of the animal to measure the summed entrance and exit doses. After irradiation, isoflurane was removed, and animals were returned to holding cages and monitored until recovered. After recovery, animals were returned to the housing room.

In addition to standard care of NHPs, irradiated NHPs were administered oral antibiotics (Baytril, 5 mg/kg), Flintstones™ vitamins, and nutritional support (e.g., bananas, apples, oranges) daily from days 1–30 postirradiation. Animals received fluid support (Prang), special diet (moistened biscuits), and anti-diarrheals as necessary. Additional support consisted of Tylenol® for fever and possible discomfort. At scheduled necropsy or in cases of morbidity, animals were euthanized under strict criteria, in compliance with Public Health Service Policy on Humane Care and Use of Laboratory Animals established by the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health.

Preirradiation blood specimens obtained prior to irradiations were shipped to WFSM for irradiation *ex vivo* at 0, 1, 2, 3, 4, 6 and 8 Gy, and then shipped to NeoGenomics located in Fort Myers, FL for CytoRADx System testing. On day 0, animals received total-body irradiation as described above at a dose of 0, 1, 2, 3, 4, 5, 6 or 8 Gy. After irradiation, blood specimens were collected from animals at set timepoints over the span of 28 days and sent to NeoGenomics laboratories located in Fort Myers, FL and Aliso Viejo, CA.

Human Subjects Receiving Partial-Body Irradiation

Male subjects (N = 4) aged 64 to 77 years receiving medically prescribed radiation therapy using a clinical linear accelerator to treat prostate cancer were enrolled. Independent of the stage of cancer, inclusion criteria specified a minimum of 3% of a subject's total body volume received a cumulative dose of 30 Gy or greater over the course of their treatment schedule. The centers in this sponsor-specific protocol were Wake Forest School of Medicine and the Medical Center of the University of South Carolina (Charleston, SC). Radiation treatment regimens consisted of a single radiation treatment administered daily, 5 days a week over the course of 7 to 8 weeks. Blood specimens were collected into blood collection tubes containing sodium heparin prior to the start of radiation therapy, at weekly intervals during treatment, and for an additional 4 weeks after treatment was completed. Samples were shipped overnight to NeoGenomics (Fort Myers, FL) for CytoRADx System testing.

RESULTS

Accuracy and Precision with Human and NHP Samples Irradiated Ex Vivo

Since the CytoRADx Assay is based on formation of micronuclei within proliferating lymphocytes cultured *in vitro*, whole blood samples irradiated *ex vivo* provide a useful model for assessing radiation-induced chromosomal damage (7, 22). This is demonstrated in Fig. 4, which shows the CytoRADx results using blood specimens from 24 apparently healthy adult human subjects and 34 NHPs irradiated *ex vivo* at doses of 0, 1, 2, 3, 4, 5, 6 and 8 Gy using the custom designed irradiator system at WFSM. The estimated dose to delivered dose linear regression had a R^2 value of 0.95 with a slope of 1.04 for human samples and a

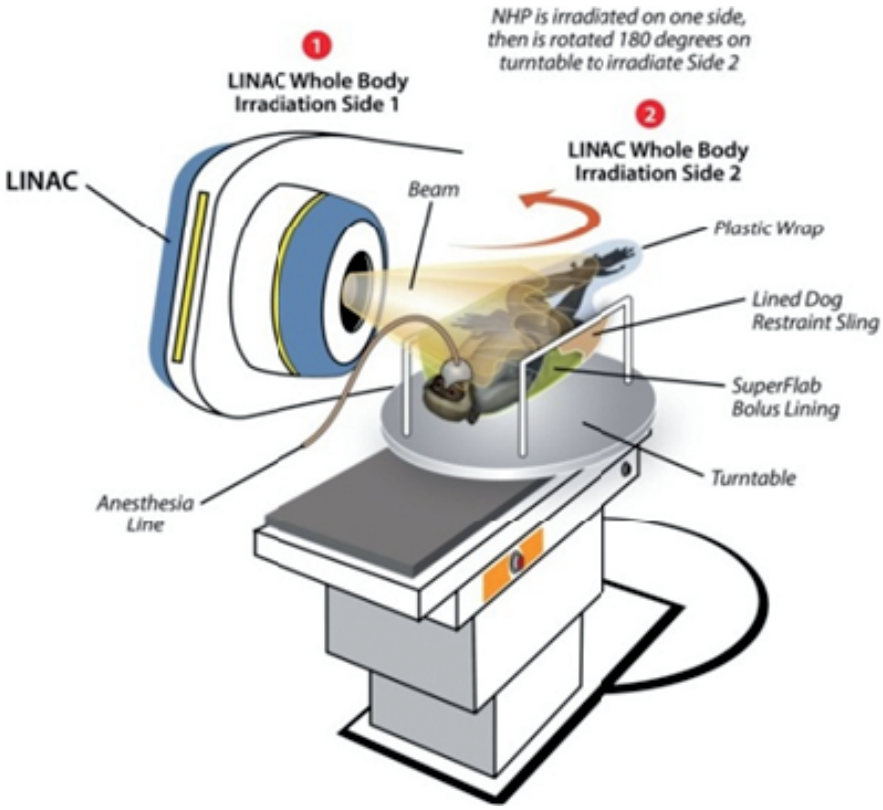

FIG. 3. NHP *in vivo* irradiation. *In vivo* irradiation methodology for NHPs, performed using LINAC at LBERI. (1) and (2) indicate schematically the location of whole-body irradiations from left and right sides, respectively.

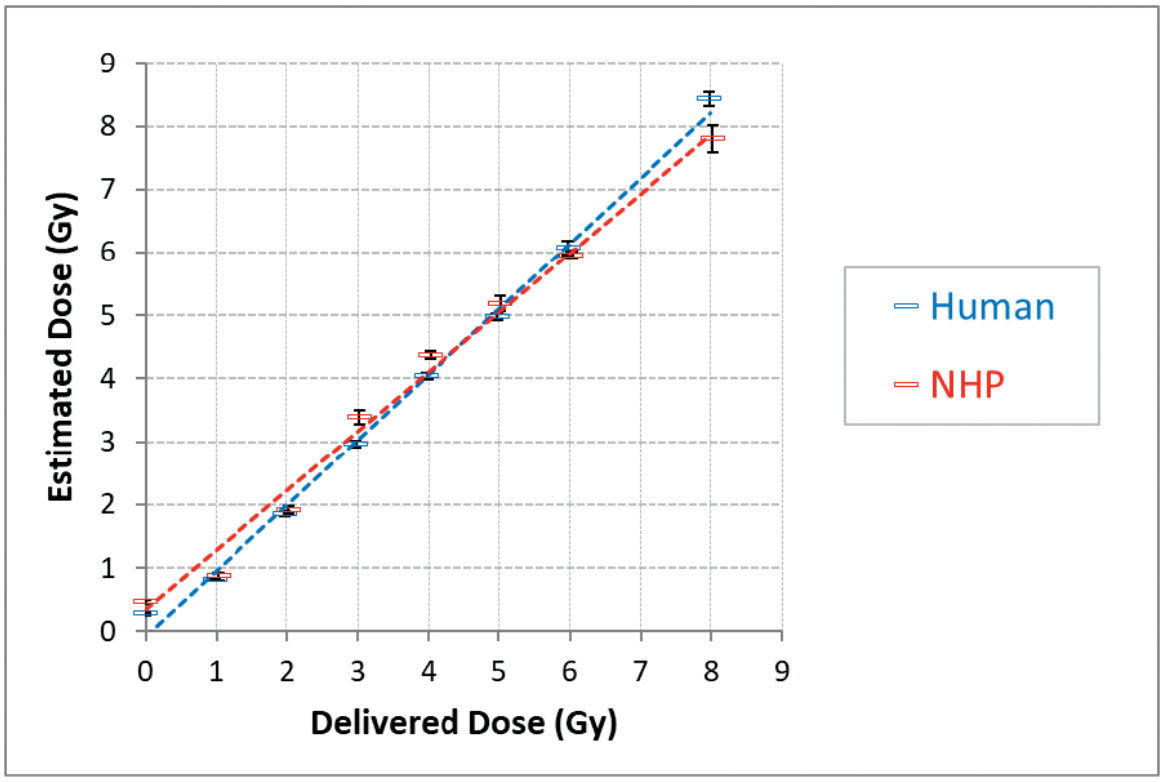

FIG. 4. Analysis of *ex vivo* irradiated samples. Estimated dose for human and NHP samples irradiated *ex vivo* one day after blood draw and processed with CytoRADx one day after irradiation. Data shown as Mean ± Standard Error (N = 24 human donors and 34 NHP animals).

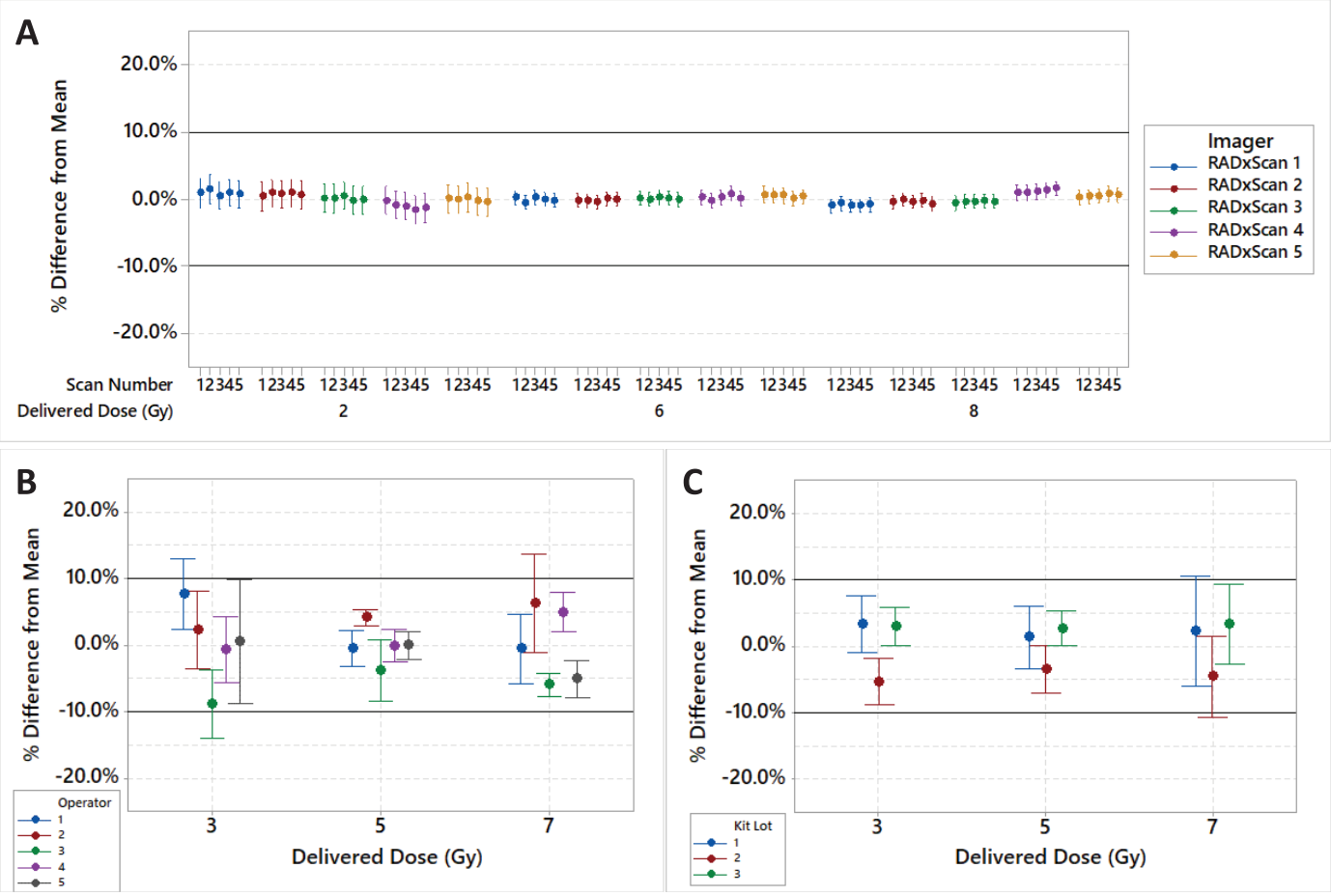

FIG. 5. Repeatability and reproducibility studies. Panel A: Comparison of 48 slides per dose scanned 5 times each on each of 5 imagers (144 total slides). Panel B: Comparison across 5 newly trained operators in the same lab. Panel C: Comparison across 3 different lots of the reagent kit after storage of 16 months (lot 1), 14 months (lot 2) or 12 months (lot 3).

R^2 value of 0.91 with a slope of 0.94 for NHP samples. The accuracy for human samples and NHPs samples resulted in more than 90% of dose estimates within 1.2 Gy of delivered dose. For the data presented, all whole blood samples were irradiated *ex vivo* one day after blood draw, and subsequently processed using the CytoRADx Assay one day after irradiation.

Repeatability and Reproducibility

The performance of the CytoRADx System was examined across multiple variables that are required by FDA for clearance of a diagnostic test, e.g., varied equipment, technical personnel, and manufactured product lots. To assess the repeatability of results on the RADxScan Imager, 144 slides were prepared using the CytoRADx Assay from human blood samples irradiated at CUIMC at 2, 6, and 8 Gy. Each slide was scanned 5 times under the same conditions, on each of five RADxScan Imagers at four different geographical locations. As shown in Fig. 5A, the repeatability as measured by the percentage change from mean demonstrated highly consistent results. Figure 5B shows the reproducible inter-operator performance of the assay at the same laboratory site. Five operators each processed the same set of human blood samples irradiated *ex vivo* at 3, 5 and 7 Gy. Data are plotted as the percentage change from the mean value of all operators per dose level. The mean for each operator fell within 10% of the global mean across all operators, thus demonstrating consistent results across this critical variable. The performance across multiple CytoRADx Assay kit lots was tested using blood samples irradiated at 3, 5, and 7 Gy (Fig. 5C). For each comparison, one-way ANOVA was completed at each dose to investigate the significance of differences in the means of the variables using a type I error of 5%. Tests resulted in no statistical significance between slide scan iterations, RADxScan Imager units, operators, or assay kit lots.

System Performance Among Potential Confounding Populations

To verify that comorbid conditions likely to result from detonation of a nuclear device, various pre-existing medical conditions, and an individual's age do not affect CytoRADx System results, human subjects in the cohorts listed in Table 1 were tested on the CytoRADx System. Figure 6 shows the

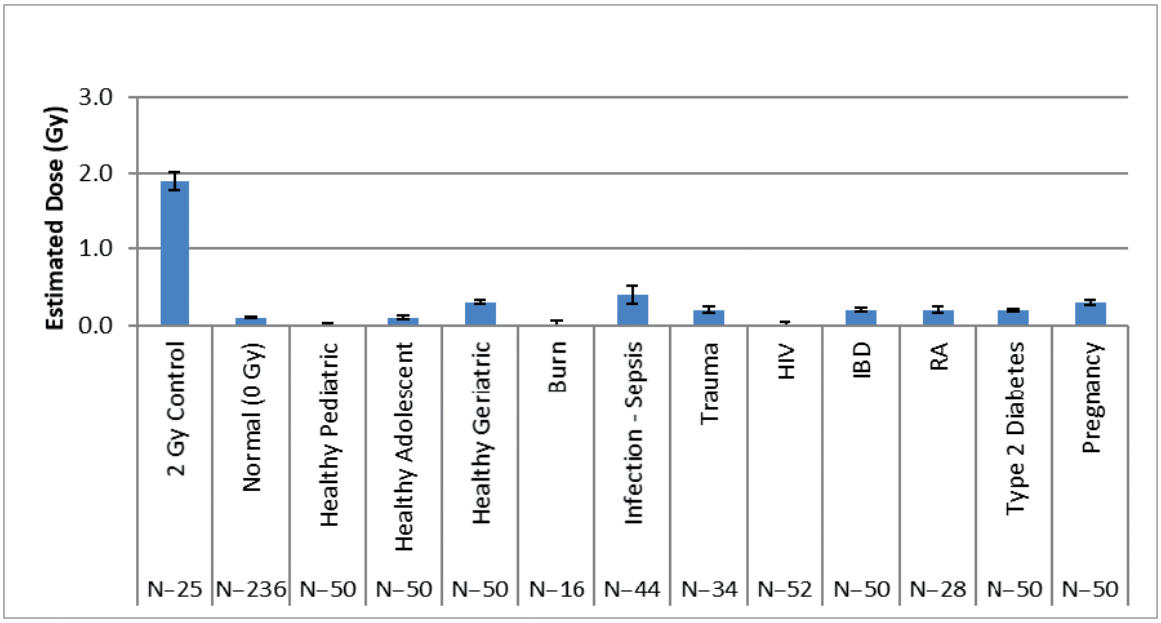

FIG. 6. Special populations, injuries, and medical conditions. Special populations (N = number of donors per group). Y-axis represents the estimated dose based on calculations by the CytoRADx System. Bars represent mean values of replicate samples from each subject tested. Error bars represent the standard error of mean. Note, the 2 Gy control consisted of results from blood samples irradiated *ex vivo* at 2 Gy from 25 healthy human adult subjects.

dose estimation of non-irradiated blood specimens obtained from these populations. For comparison purposes, human blood samples from apparently healthy adults were irradiated *ex vivo* at 2 Gy (N = 25) or non-irradiated (N = 236). None of the potential confounding populations showed average estimated dose values greater than 0.5 Gy, demonstrating minimal impact on the dose estimations.

In Vivo NHP Studies

To demonstrate that the CytoRADx System is useful for estimating the amount of ionizing radiation received by individuals potentially exposed to clinically significant levels of radiation, an *in vivo* NHP study was conducted using 32 rhesus macaques. Accurate estimated doses were obtained over 0–8 Gy for samples collected from days 1–28 postirradiation (Fig. 7, $R^2 = 0.77$, slope = 1.12, samples collected on days 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 14, 21, and 28). These results confirm the accuracy of the system for measuring *in vivo* radiation exposure up to 28 days postirradiation.

Stability of Results Over Time

After detonation of a nuclear bomb or other wide-spread radiation exposure, it may take many days or weeks before a blood sample can be collected and tested on a CytoRADx System. To demonstrate the stability of the *in vivo* biomarker signal, tests were performed using samples from four human radiation therapy patients being treated for prostate cancer. As shown in Fig. 8, the CytoRADx System's estimated dose generally increased with each weekly sample drawn during radiation treatment, and then remained stable for 4 weeks after the final treatment (the last sample collected during treatment is noted using a vertical line on each patient's plot).

The stability of human whole blood specimens stored at room temperature was also examined and demonstrated stable results for at least 72 h of specimen storage (data not shown). These results demonstrate the utility of the CytoRADx System in response to a nuclear event, where subsequent response activities and blood sample collection is likely to take many days or weeks due to the chaotic and logistically challenging environment.

DISCUSSION

When mobilizing limited resources in response to a large-scale radiation exposure event, it will be critical to have medical screening/diagnostic and interventional equipment that suits the circumstances – accurate and precise, standardized and distributable across dispersed laboratory sites, and able to produce reproducible results across a diverse population of victims for extended periods of time. While experimental methods like the CBMN assay have demonstrated feasibility for biodosimetry applications, these approaches face logistical hurdles for large-scale emergency use. The CytoRADx System fills these gaps by employing a standardized user-friendly assay kit that can be distributed to geographically disparate laboratory sites. The CytoRADx Assay Kit is optimized for use with the RADxScan Imager, a modified version of the commercial Metafer platform, which is currently in use at numerous laboratories

FIG. 7. Results from NHPs irradiated *in vivo*. Estimated dose for NHPs exposed to total-body irradiation *in vivo* (N = 32 total animals). X-axis represents the dose delivered to the animals based on physical dosimetry. Y-axis represents the estimated dose based on calculations by the CytoRADx System. Symbols represent mean values of replicate samples for animals receiving the prescribed doses. Bars represent the standard error of mean. Dotted line is a best-fit linear regression through the mean values.

worldwide and is capable of imaging micronuclei. Furthermore, the combination of the standardized assay kit with automated scanning of up to 1,000 slides per day, image analysis, and radiation dose estimation provide a robust, reliable, high-throughput system needed to determine the amount of ionizing radiation absorbed by victims of mass-casualty disaster scenarios.

Performance of the CytoRADx System

Blood samples irradiated *ex vivo* provide a model for assessing the performance of the CytoRADx System. As shown Fig. 4, the actual exposure of human blood samples to gamma rays is linearly correlated to CytoRADx System estimated doses. This same correlation is observed with NHP samples irradiated *ex vivo* (Fig. 4). Using our optimized assay protocol and customized analysis software, the analytical range of the CytoRADx System (0–8 Gy) is greater than that achieved ($\leq$5 Gy) by previous methods. To identify the best medical intervention during a large-scale disaster, it is critical to assess individuals for absorption at the decision points of 2 and 6 Gy. This allows limited resources to be dedicated to those in greatest need, wherein $<$2 Gy requires no professional medical assistance, 2–6 Gy requires treatment with acute radiation syndrome medical countermeasures (MCM), and $>$6 Gy requires more invasive intervention, e.g., a bone marrow transplant (2, 17). The use of *ex vivo* irradiation models are also critical to validating the system performance since the intended use population, single dose radiation exposure victims, cannot be ethically examined.

In the event of large-scale radiation exposure event, numerous laboratories are required to screen large numbers of victims as quickly as possible. The *ex vivo* human irradiation model further demonstrates the repeatability and reproducibility of results across different RADxScan Imagers, operators, and CytoRADx Assay Kit lots (Fig. 5). While not statistically significant, the greatest variability was observed between operators compared to RADxScan Imagers and CytoRADx Assay Kit lots. In each case, the observed variability falls within tolerances for hematology tests such as lymphocyte counts. The low variability observed in these studies demonstrates that the CytoRADx System produces the robust results necessary for deployment in response to a disaster scenario.

The population of victims after a radiation exposure disaster in a major city will include numerous demographics and a variety of medical conditions that could confound results. Human subjects with medical conditions likely to be prevalent after detonation of a nuclear device and the subsequent ‘‘shelter-in-place’’ period consisted of subjects with burns, trauma, or sepsis (Table 1). These conditions are expected to represent a significant number of comorbidities in addition to radiation absorption. The CytoRADx System relies on the ability of lymphocytes contained in a whole blood sample to proliferate *in vitro*. Therefore, individuals with pre-existing medical conditions consisting of HIV

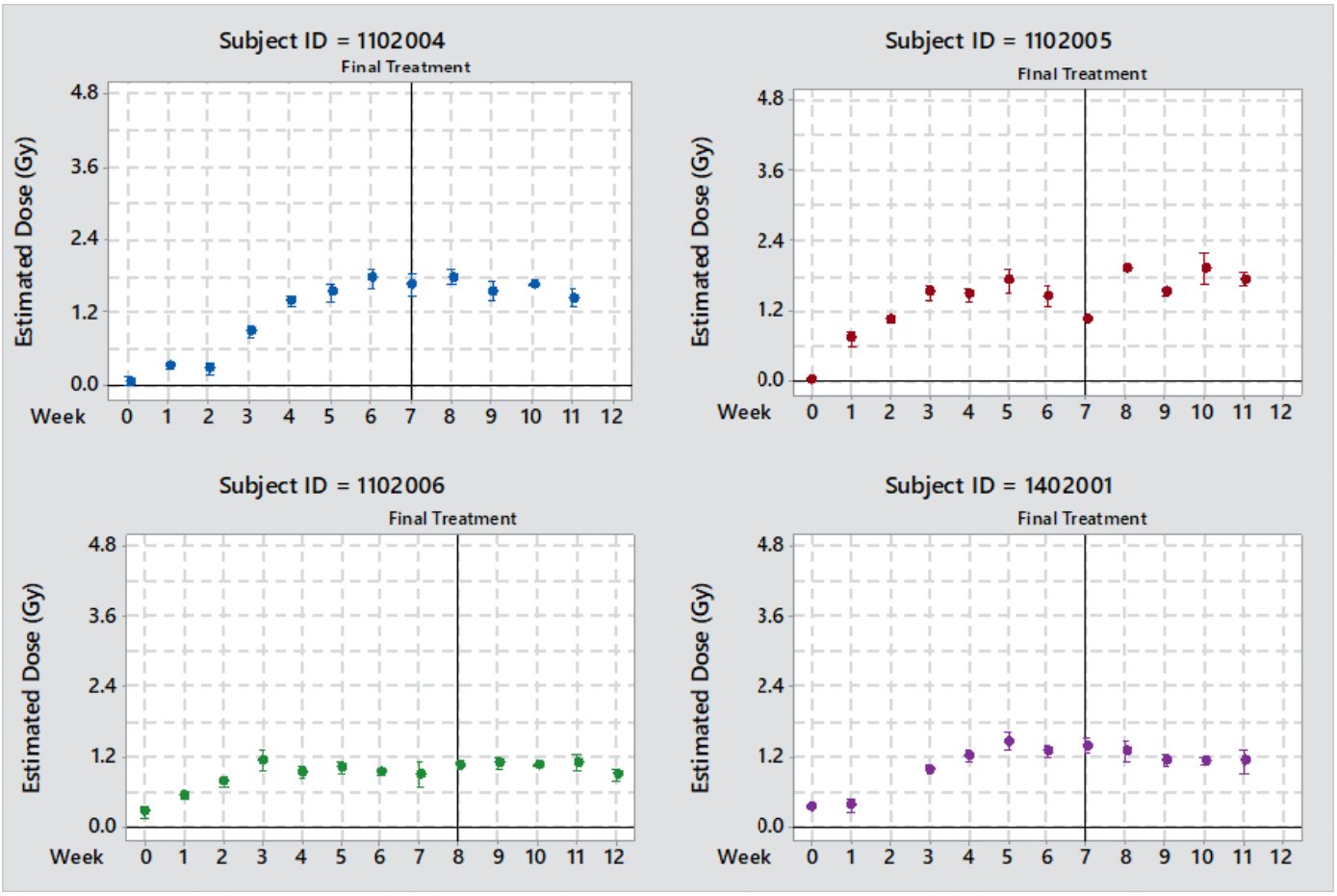

FIG. 8. Estimated dose over time for 4 subjects undergoing radiation treatment for prostate cancer. Data points represent the mean of three replicates. Error bars represent the standard error of the mean. Patient 1402001′s sample from week two was not tested. Vertical lines represent the last sample taken during radiation treatment.

infection, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), type 2 diabetes, and pregnancy were tested along with different age groups (25) to assess for potential interference with the CytoRADx System. The effectiveness of a biodosimeter would be greatly reduced if individuals with these types of conditions produced erroneous results. The data shown in Fig. 6 indicate that the distribution of CytoRADx System estimated doses from all populations tested, were like the healthy adult control group and did not display increased background levels. This indicates that these populations would be unlikely to generate a false positive result on the CytoRADx System which could cause an individual to receive treatment for radiation absorption unnecessarily. These findings show that the CytoRADx System produces reliable results across a diverse population of individuals likely to be encountered during a large-scale radiation exposure event.

To further investigate system performance using additional models of the intended use population, animal studies were employed. When the CytoRADx System estimates absorbed dose of blood samples from NHPs receiving single dose total-body irradiation (*in vivo*), using algorithm parameters established from NHP blood samples exposed *ex vivo*, the dose estimations are linear from 0 to 8 Gy, and time independent up to 28 days postirradiation. This time independence shows that the CytoRADx System estimates an accurate dose even during the profound lymphocyte depletion that occurs after total-body irradiation (3). This finding is crucial, since the complex emergency conditions after a radiological disaster would present difficulty in testing all potential victims immediately after exposure.

Human *in vivo* models of radiation exposure using patients undergoing radiation therapy for prostate cancer, independent of stage, were also tested. This population presented limitations as a model, due to the fractioned regimen being delivered only to a small portion of the subject's body, which results in a comparatively low cumulative delivered dose to the total body. This effect precluded examination of accuracy, precision, or the full quantitative range of the CytoRADx assay. However, these data further demonstrated the notable stability of results over at least 28 days postirradiation.

Future Directions

These efforts were focused on the performance and application of the CytoRADx System for biodosimetry in large-scale radiation exposure scenarios. However, the underlying basis of the technology, a high-throughput adaptation of the CBMN assay with automated analysis, would prove useful in several other applications. Related studies of newly emerging radiation MCMs rely heavily upon methods to accurately quantify the severity of radiation injury (18). Standardized biodosimetry methods are useful in this context to assess the efficacy of new countermeasures. In addition, the CBMN assay has been utilized in toxicology studies to study genotoxic effects of novel drug compounds and chemicals (19). This classic technique has also shown promise as a diagnostic tool for various types of cancer (20), including lung cancer, wherein the method shows great potential when combined with standard Computed Tomography imaging results (21, 26). These approaches suffer from the lack of a standardized, reproducible, automated tool, and the CytoRADx System can satisfy this need.

CONCLUSION

The CytoRADx System has advanced the CBMN assay beyond a labor-intensive method that requires highly skilled cytologists, is throughput-limited, and requires species-dependent media formulations. Earlier versions of the CytoRADx Assay transitioned from 15 mL conical tubes into a deep-well microplate format to permit parallel processing and increase throughput possibilities (22). The results of continued improvements to the CytoRADx System have been described. Improvements include a standardized assay procedure with proprietary and optimized kit components; able to test both human and NHP blood samples with similar performance. The CytoRADx System produces repeatable and reproducible results that are not affected by many factors tested in these studies, including pre-existing medical conditions, an individual's age, and injuries likely to be caused by detonation of a nuclear bomb. Multiple laboratories across the country already have the commercial equipment needed to run the assay and can be quickly mobilized if the need arises. This optimized assay increases the measurement range to 8 Gy and up to 28 days postirradiation to more fully span key medical decision points and greatly increase the available window for performing testing during the response to a large-scale radiation exposure event. In addition, the ability of the CytoRADx System to provide similar performance with animal samples irradiated *ex vivo* and *in vivo* facilitates a more comprehensive validation of the technology and subsequent pursuit of appropriate regulatory approvals to permit large scale use.

ACKNOWLEDGMENTS

In addition to the authors, the following Principal Investigators provided human blood samples under site-specific or centrally approved IRB protocols: Joseph Herres, DO., Jeffery Jenkins, MD, Kevin Foster, MD, Jeffrey Shupp, MD, Simon Mahler, MD and Cynthia Talley, MD. This project has been funded in whole or in part with Federal funds from the Biomedical Advanced Research and Development Authority, Office of the Assistant Secretary for Preparedness and Response, Office of the Secretary, United States Department of Health and Human Services, under Contract No. HHSO100201700022C.

Received: February 2, 2020; accepted: August 26, 2021; published online: September 13, 2021

REFERENCES

1. Singh VK, Newman VL, Seed TM. Colony-stimulating factors for the treatment of the hematopoietic component of the acute radiation syndrome (H-ARS): a review. Cytokine. 2015; 71(1):22-37.
2. Knebel AR, Coleman CN, Cliffer KD, Murrain-Hill P, McNally R, Oancea V, et al. Allocation of scarce resources after a nuclear detonation: setting the context. Disaster Med Public Health Prep. 2011; 5 Suppl 1:S20-31
3. J Waselenko JK, MacVittie TJ, Blakely WF, Pesik N, Wiley AL, Dickerson WE, et al. Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. Ann Intern Med. 2004; 140(12):1037-51.
4. D Bolduc DL, Bünger R, Moroni M, Blakely WF. Modeling H-ARS using hematological parameters: a comparison between the non-human primate and minipig. Radiat Prot Dosimetry. 2016; 172(1-3):161-173.
5. Singh VK, Newman VL, Romaine PL, Hauer-Jensen M, Pollard HB. Use of biomarkers for assessing radiation injury and efficacy of countermeasures. Expert Rev Mol Diagn. 2016; 16(1):65-81.
6. Royba E, Repin M, Pampou S, Karan C, Brenner DJ, Garty G. RABiT-II-DCA: A fully-automated dicentric chromosome assay in multiwell plates. Radiat Res. 2019; 192(3):311-323.
7. Fenech M. Cytokinesis-block micronucleus cytome assay evolution into a more comprehensive method to measure chromosomal instability. Genes (Basel). 2020; 11(10):1203.
8. Rodrigues MA, Beaton-Green LA, Wilkins RC, Fenech MF. The potential for complete automated scoring of the cytokinesis block micronucleus cytome assay using imaging flow cytometry. Mutat Res Genet Toxicol Environ Mutagen. 2018; 836(Pt A):53-64.
9. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, et al. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis. 2011; 26(1):125-32.
10. Nagel ZD, Engelward BP, Brenner DJ, Begley TJ, Sobol RW, Bielas JH, et al. Towards precision prevention: Technologies for identifying healthy individuals with high risk of disease. Mutat Res. 2017; 800-802:14-28.
11. Vral A, Fenech M, Thierens H. The micronucleus assay as a biological dosimeter of in vivo ionising radiation exposure. Mutagenesis. 2011; 26(1):11-7.
12. Shuryak I, Turner HC, Perrier JR, Cunha L, Canadell MP, Durrani MH, et al. A high throughput approach to reconstruct partial-body and neutron radiation exposures on an individual basis. Sci Rep. 2020; 10(1):2899.
13. Sommer S, Buraczewska I, Kruszewski M. Micronucleus assay: the state of art, and future directions. Int J Mol Sci. 2020; 21(4):1534.
14. Publications (Keyword: Scoring Micronuclei), MetaSystems, January 2021. Online. Available: https://metasystems-international.com/us/publications/micronuclei. Accessed 1 March 2021.
15. Romm H, Barnard S, Boulay-Greene H, De Amicis A, De Sanctis S, et al. Laboratory intercomparison of the cytokinesis-block micronucleus assay. Radiat Res. 2013; 180(2):120-8.

16. Wang Q, Rodrigues MA, Repin M, Pampou S, Beaton-Green LA, Perrier J, et al. automated triage radiation biodosimetry: integrating imaging flow cytometry with high-throughput robotics to perform the cytokinesis-block micronucleus assay. Radiat Res. 2019; 191(4):342-351.

17. Coleman CN, Knebel AR, Hick JL, Weinstock DM, Casagrande R, Caro JJ, et al. Scarce resources for nuclear detonation: project overview and challenges. Disaster Med Public Health Prep. 2011; 5 Suppl 1:S13-9.

18. Singh VK, Olabisi AO. Nonhuman primates as models for the discovery and development of radiation countermeasures. Expert Opin Drug Discov. 2017; 12(7):695-709. Erratum in: Expert Opin Drug Discov. 2017; 12 (8):875.

19. S. Knasmüller and M. Fenech, The Micronucleus Assay in Toxicology. Cambridge, UK: The Royal Society of Chemistry, 2019.

20. Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, et al. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis. 2007; 28(3):625-31.

21. El-Zein R, Vral A, Etzel CJ. Cytokinesis-blocked micronucleus assay and cancer risk assessment. Mutagenesis. 2011; 26(1):101-6.

22. Lue SW, Repin M, Mahnke R, Brenner DJ. Development of a high-throughput and miniaturized cytokinesis-block micronucleus assay for use as a biological dosimetry population triage tool. Radiat Res. 2015; 184(2):134-142.

23. Almond PR, Biggs PJ, Coursey BM, et al. AAPM's TG-51 protocol for clinical reference dosimetry of high-energy photon and electron beams. Med Phys. 1999; 26(9):1847-1870.

24. Das IJ, Cheng CW, Watts RJ, et al. Accelerator beam data commissioning equipment and procedures: report of the TG-106 of the Therapy Physics Committee of the AAPM. Med Phys. 2008; 35(9):4186-4215.

25. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. Proc Biol Sci. 2015; 282(1821):20143085.

26. El-Zein RA, Etzel CJ, Munden RF. The cytokinesis-blocked micronucleus assay as a novel biomarker for selection of lung cancer screening participants. Transl Lung Cancer Res. 2018; 7(3):336-346.