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Development of a Point-of-Care Microfluidic RNA Extraction Slide for Gene Expression Diagnosis after Irradiation

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In times of war, radiological/nuclear emergency scenarios have become a reemphasized threat. However, there are challenges in transferring whole-blood samples to laboratories for specialized diagnostics using RNA. This project aims to miniaturize the process of unwieldy conventional RNA extraction with its stationed technical equipment using a microfluidic-based slide (MBS) for point-of-care diagnostics. The MBS is thought to be a preliminary step toward the development of a so-called lab-on-a-chip microfluidic device. A MBS would enable early and fast field care combined with gene expression (GE) analysis for the prediction of hematologic acute radiation syndrome (HARS) severity or identification of RNA microbes. Whole blood samples from ten healthy donors were irradiated with 0, 0.5 and 4 Gy, simulating different ARS severity degrees. RNA quality and quantity of a preliminary MBS was compared with a conventional column-based (CB) RNA extraction method. GE of four HARS severity-predicting radiation-induced genes (*FDXR*, *DDB2*, *POU2AF1* and *WNT3*) was examined employing qRT-PCR. Compared to the CB method, twice as much total RNA from whole blood could be extracted using the MBS (6.6 ± 3.2 µg vs. 12.0 ± 5.8 µg) in half of the extraction time, and all MBS RNA extracts appeared DNA-free in contrast to the CB method (30% were contaminated with DNA). Using MBS, RNA quality [RNA integrity number equivalent (RIN^e)] values decreased about threefold (3.3 ± 0.8 vs. 9.0 ± 0.4), indicating severe RNA degradation, while expected high-quality RIN^e $\geq$ 8 were found using column-based method. However, normalized cycle threshold (Ct) values, as well as radiation-induced GE fold-changes appeared comparable for all genes utilizing both methods, indicating that no RNA degradation took place. In summary, the preliminary MBS showed promising features such as: 1. halving the RNA extraction time without the burden of heavy technical equipment (e.g., a centrifuge); 2. absence of DNA contamination in contrast to CB RNA extraction; 3. reduction in blood required, because of twice the biological output of RNA; and 4. equal GE performance compared to CB, thus, increasing its appeal for later semi-automatic parallel field applications. © 2024 by Radiation Research Society

INTRODUCTION

Political conflicts of the 21st century have reemphasized the threat posed by radiological/nuclear (R/N) scenarios. Some of the most prominent categories of R/N incidents in the recent past, such as Hiroshima and Nagasaki atomic bomb (*1*), environmental disasters in civil power reactors (*2*, *3*), and occupational or public accidents (*4*, *5*) led to severe and large-scale emergencies, that affected numerous individuals (*6*). Early diagnosis of affected radiation-exposed individuals is required since early treatments improve the prognosis. Consequently, medical response systems are imperative to provide life-saving patient triage treatment in minimum time. An early and high throughput diagnostic is required to differentiate and group potential victims into clinically relevant hematologic acute radiation syndrome (HARS) severity degree categories according to MEdical TREatment ProtocOLs (METREPOL): No HARS (H0), low (H1), medium (H2), severe (H3) and fatal (H4) HARS. Only H2–4 HARS require immediate hospitalization and intensive therapy. Long-term surveillance after an H1 HARS diagnosis classification is recommended because of an increased risk for chronic diseases such as cancer or non-cancer health outcomes (e.g., cardiovascular disease) (*7*). Unexposed individuals should be identified as well so that limited clinical resources can be devoted to those that might truly benefit from them.

During the last decades, multiple methods have developed, whereby gene expression (GE) analysis, mainly provided by specific quantitative real-time PCR (qRT-PCR) as the gold standard method (*8*), offers early and high-throughput opportunities for biodosimetry purposes as well as clinical outcome prediction (*9*). A radiation-responsive GE signature (*FDXR*/*DDB2* and *POU2AF1*/*WNT3*) was first identified using a baboon model (*10*) in combination with ex vivo experiments (*8*) as well as measurements on healthy donors (*11*), and validated with radiotherapy patients (*12*), confirming its functionality in triaging radiation-exposed individuals. Regarding its applicability in

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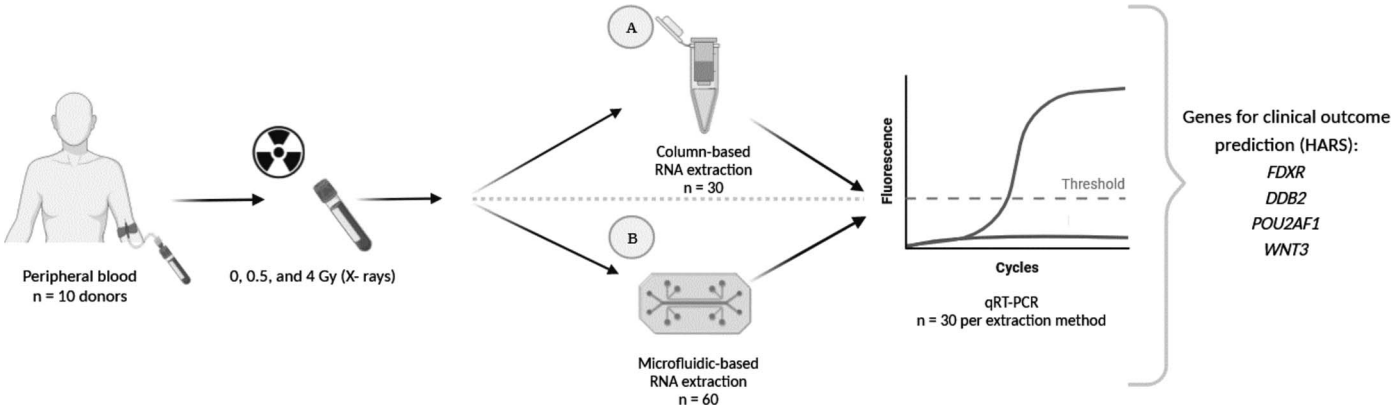

FIG. 1. Study design (created with BioRender.com), starting from the ex vivo X-ray irradiation of withdrawn blood (left) (0, 0.5 and 4 Gy representing doses to induce various severities of HARS) to the gene expression analysis of a radio-responsive gene set (*FDXR*, *DDB2*, *POU2AF1* and *WNT3*) using the gold-standard qRT-PCR method (right). The intermediate step comparing the two RNA extraction methods [conventional column-based (panel A) vs. preliminary microfluidic-based module (panel B) are visualized in the middle portion of the figure.

early and high throughput exercises, a blinded study showed that 1000 patients could be grouped accordingly (*13–15*) in less than 30 h (*16*), demonstrating its usefulness in triage and early classification within the diagnostic window of 4–72 h after exposure (*8*). Nevertheless, an impaired infrastructure will impede the transit of patient samples to professionally equipped labs, making radiobiological triage very difficult. Due to the lack of triage information, hospitals would be overwhelmed with patients. This issue could be countered by developing a point-of-care diagnostic device and revising and minimizing the well-established benchtop operating procedures.

Microfluidics is a technology that can be used to miniaturize laboratory steps bioengineered onto a chip. In its full version, it can integrate an entire GE analysis workflow, including RNA extraction and qRT-PCR, onto a microfluidic card (lab-on-a-chip). A microfluidic slide for RNA extraction is thought to be a preliminary step towards a microfluidic card. Generations of microfluidic devices cover various fabrication technologies, such as glass, silicon, metal, paper, and polymer materials (*17*). Nevertheless, polymer-based compounds have the advantages of fast production and low cost. Known applications can be found for pregnancy testing or (biomarker-wise) for SARS-CoV-2 quick-testing (*18*). Further applications are known as "organ-on-a-chip," replacing animal models with cell culture (*19*) controlled microenvironments (*20*) for testing drugs (*21*). Using microfluidics as a field-deployable tool by fusing biology with technology could, therefore, also enable acceleration in determining time-dependent GE changes for radiobiological triage.

Recently, the Bundeswehr Institute of Radiobiology, in collaboration with ChipShop, developed a microfluidic-based slide (MBS) to extract RNA from whole-blood samples. For this study, whole blood was provided from healthy donors and irradiated to simulate various HARS severity degrees. Subsequently, the RNA was extracted using an MBS and compared with RNA isolated using a conventional silica membrane-based method. In addition to the qualitative and quantitative RNA analysis, the HARS severity predicting genes *FDXR*, *DDB2*, *POU2AF1* and*WNT3* were examined for further methodological comparison purposes.

This project aimed to develop a stand-alone system for a (semi-)automated, user-friendly, and reproducible RNA extraction platform with transportable tools to bypass current bottlenecks of RNA extraction and enable field care. Such an RNA extraction slide could enable on-site diagnostics even without an intact infrastructure in the background. In later developmental stages, this new diagnostic setting could also benefit third-world countries with poor health care and a lack of specialized laboratories for specific transcriptional screenings, such as a bedside test for human genetics or diagnosis of RNA microbes.

MATERIALS AND METHODS

Sample Collection and Irradiation

Peripheral whole blood from ten healthy donors (six male and four female volunteers) was collected using six S-Monovette® 4.9 ml EDTA tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) per donor. Subsequently, two EDTA blood tubes (one for each extraction method investigated) were ex vivo irradiated with either 0 (sham), 0.5, or 4 Gy single X-ray exposures. A mean photon energy of 100 keV (Maxishot SPE cabin, Yxlon, Hamburg, Germany) was used with 3 mm beryllium and 3 mm aluminum filters. The absorbed dose was measured using a UNIDOS webline 10021 dosimeter (PTW, Freiburg, Germany). The dose rate was 1.0 Gy/min at 13 mA with an accelerating potential of 240 kV (maximum photon energy of 240 keV). Simulating an in vivo scenario, whole blood was incubated in EDTA tubes for 7 h at 37°C (biological response time) to allow for radiation-induced gene expression (GE) changes to occur (*8*). The samples were divided into two extraction groups: A conventional CB RNA extraction via the Invitrogen mirVana kit and a preliminary MBS RNA extraction (Fig. 1). For the CB extraction, 2.5 ml of whole blood was transferred into a PAXgene® Blood RNA tube (BD Diagnostics, PreAnalytiX GmbH, Hombrechtikon, Switzerland). Two PAXgene tubes per dose were collected with one of them serving as a backup sample. The tubes were kept at room temperature for at least 2 h to avoid RNA degradation and stored at –20°C until further processing ($n = 30$ used, $n = 30$ backup). For the MBS, the RNA was extracted from irradiated and incubated EDTA tubes without any prior transfer into PAXgene tubes. A backup extraction of each sample was made due to the MBS RNA extraction method's lower input volume (250 µl) and the impossible storage for later

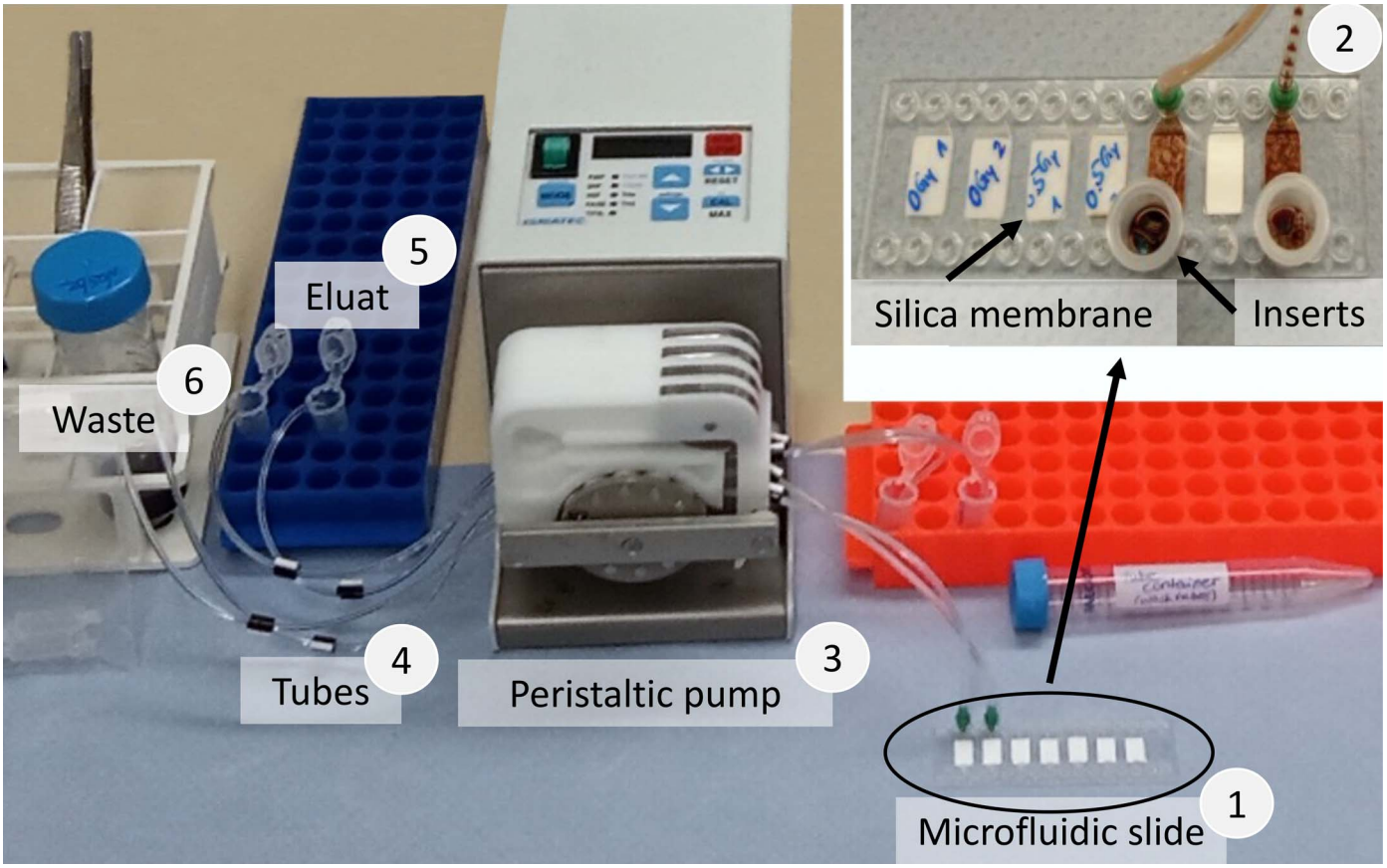

FIG. 2. Preliminary microfluidic slide module for RNA isolation purposes. The system consists of a microfluidic slide (*1*) comprised of inserts attached to input channels and silica membranes (*2*), operated by a peristaltic pump (*3*) that pulls the supernatant and eluate (*5*) through tubes (*4*) into the corresponding reservoir/waste (*6*).

extraction later extraction as it would be for PAXgene samples (n = 60). Due to the minimally-invasive collection and the fully anonymized processing of the samples, the local ethical commission (Ethics committee, Bayerische Landesärztekammer, Munich, Germany) decided that the experiments meet ethical standards and do not require additional approval. Donors signed written informed consent.

MBS RNA Extraction

RNA was isolated from EDTA blood tubes using a preliminary MBS comprised of a slide (designed in cooperation with microfluidic ChipShop, Jena, Germany) in its developmental stage combined with a peristaltic pump (Peristaltic Pump Relgo Digital, Ismatec Fisher Scientific, Schwerte, Germany) (Fig. 2). The polymer-based microfluidic slide was set up with loading tanks and tubes fitted inside a circular pump casing comprised of 12 spring rollers pumping the fluids through the tube by rotation and compression (Fig. 2). In brief, 250 µl EDTA blood was mixed with Proteinase K and lysis buffer, followed by a 10-min incubation prior to loading onto the MBS built-in horizontal silica membrane. After several washing steps using a flow rate of 500 µl/min prior and post-DNA digestion using 75 µl/min (with the supernatant pumped into the waste bin), the RNA was eluted in 80 µl of elution buffer, leading into a collection vessel (Eppendorf). Afterwards, the samples were stored at –20°C until further processing.

CB RNA Extraction

Prior to the RNA extraction, the PAXgene tubes had to be prepared by thawing, pelleting nucleic acids, washing, as well as digesting proteins using proteinase K (BD Diagnostics). The RNA, including microRNAs, was extracted (miRNA Isolation Kit, Invitrogen, ThermoFisher Scientific, Braunschweig, Germany). Briefly, cells were lysed, and DNA, protein, and RNA were phase-separated via Acid-Phenol-Chloroform. The RNA was precipitated via ethanol and transferred onto a vertical column-based silica membrane. After the RNA had been washed and centrifuged (10,000 g) prior to and post-DNA digestion for 15 min, the RNA was eluted with 100 µl hot water.

RNA Quantity and Quality Analysis

RNA (CB n = 30; MBS n = 60) was quantified spectrophotometrically (NanoDrop™, PeqLab Biotechnology, Erlangen, Germany), and RNA integrity was assessed via the RNA integrity number (RIN^e) using the 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RIN^e was measured by using the 28S to 18S rRNA ratio (*22*). Possible sample contamination by genomic DNA was inspected by conventional PCR using β-actin primers.

Gene Expression Analysis

Total RNA (0.5 µg) (CB n = 10; MBS n = 10) was reverse transcribed for each sample via the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Life Technologies, Darmstadt, Germany). The qRT-PCR itself was performed using commercially available TaqMan assays (*FDXR* (*Hs01031617_m1*), *DDB2* (*Hs00172068_m1*), *POU2AF1* (*Hs01573371_m1*), and *WNT3* (*Hs00902257_m1*). For *FDXR*, *DDB2*, and *POU2AF1*, 2.5 ng, and *WNT3*, *25* ng cDNA per reaction was mixed with the TaqMan® Universal PCR Master Mix. Subsequently, the prepared samples were run in a 96-well format as duplicates using a QuantStudio™ 12K OA Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA). Each gene's cycle threshold (Ct) value was normalized relative to the housekeeping gene (HKG) 18S rRNA (ThermoFisher Ref: 4310893E) (*9*). The HKG was diluted down to 0.0025 ng per reaction. After the qRT-PCR had run, the threshold was set to 0.05, and the fold change (FC) was

TABLE 1
The Generalities and the Framework of the Microfluidic-Based (MBS) Compared to the Column-Based (CB) RNA Extraction Method

Feature	Microfluidic-based slide (MBS)	Column-based (CB)
General framework		
Market	Costumary, in development	Standard
Workspace	Onsite/Point-of-care	Laboratory-bound
System	Closed	Open
Technical equipment	Microfluidic slide, tanks, pump, and final collection tube	Falcon, PAXgene, and collection tubes, ice, filter-column membrane, 2x centrifuge, heat block, and working bench with safety cabinet
Procedure	Semi-automated	Manual
Equipment mobility	Portable	Stationed
Reagent storage	Room temperature (only DNase at -20°C)	4°C (only DNase at -20°C)
Harmful toxins among reagents	None	Phenol-Chloroform
RNA extraction/results		
Biological samples	EDTA blood (till now)	EDTA and PAX gene blood
Sample input volume	250 µL	2.5 mL
Time	50 min	1h 45 min
Extraction steps until Elution	~12 steps	> 39 steps (without repetition steps)
RNA quantity (extrapolated for MB)	12.0 ± 5.8 µg	6.6 ± 3.2 µg
RNA quality (RIN)	3.3 ± 0.8	9.0 ± 0.4
DNA contamination	None	Occured in almost 30%
Gene expression	As expected	As expected

Note. Provided is general information (top) and RNA extraction and results (bottom) containing procedure, sample input, setup, as well as output.

calculated relative to the unexposed sample (sham = 0 Gy) using the –ΔΔCt-approach (FC = $2^{-\Delta\Delta Ct}$) (*23*, *24*). While a FC = 1 implies no change in GE as in unexposed samples, a FC ≥ 2 and a FC ≤ 0.5 was considered to represent an up- or downregulation. FCs ≤ 2 were considered to represent methodological variance, which could not be discriminated from control values.

Statistical Analysis

Mean values ± standard deviations (SD) were calculated using Excel (Microsoft, Redmond, WA). Both parametrical (Welch's t-test) and non-parametrical tests (Mann-Whitney Rank Sum Test) were performed where applicable via SigmaPlot (Version 14.5, Jandel Scientific, Erkrath, Germany). A P value ≤0.05 indicated significant differences among the compared groups.

RESULTS

General Framework of RNA Extraction

In its customary developmental stage, the MBS required fewer reagents (one kit) compared to the CB method, which required two kits and consisted of potentially harmful toxins (Table 1). The MBS RNA extraction workflow took 50 min and involved 12 extraction steps, while the CB method took 1.75 h, and included more than 39 steps. A comparative overview, including reagents, input volume, and technical equipment required in the MBS and CB workflow, is provided in Table 1.

RNA Quantity

When extrapolating the insert volumes accordingly, from 250 µl (MBS) to 2.5 ml (CB), the total RNA (per 2.5 ml whole blood) doubled significantly when using the MBS method (12.0 ± 5.8 µg), compared to the CB method (6.6 ± 3.2 µg; Fig. 3A and Table 2). This significant method-related difference in isolated RNA quantities was found at 0.5 Gy (P = 0.003) and 4 Gy (P = 0.014), respectively. The RNA quantity was not significantly associated with the radiation dose for both RNA isolation methods (Table 2).

RNA Quality

For the CB method, average RIN^e values of 9.0 ± 0.4 could be calculated. These values were significantly higher (P < 0.001) compared to about three-fold decreased mean RIN^e values of 3.3 ± 0.8 when using the MBS method (Fig. 3B and Table 2). The RIN^e values were not significantly associated with the radiation dose in both RNA isolation methods (Table 2). Corresponding gel electrophoresis images revealed multiple RNA bands for the MBS method and two discrete bands (referring to the 28S rRNA and 18S rRNA species) using the CB method (Fig. 4).

DNA Contamination

After the primary DNA digestion round, none of the MBS samples (n = 60, including the backup samples) presented genomic DNA, but about one-third of CB samples (9 out of 30) required a second DNase digestion round (Supplementary Table S1,[2] https://doi.org/10.1667/RADE-23-00169.1.S1).

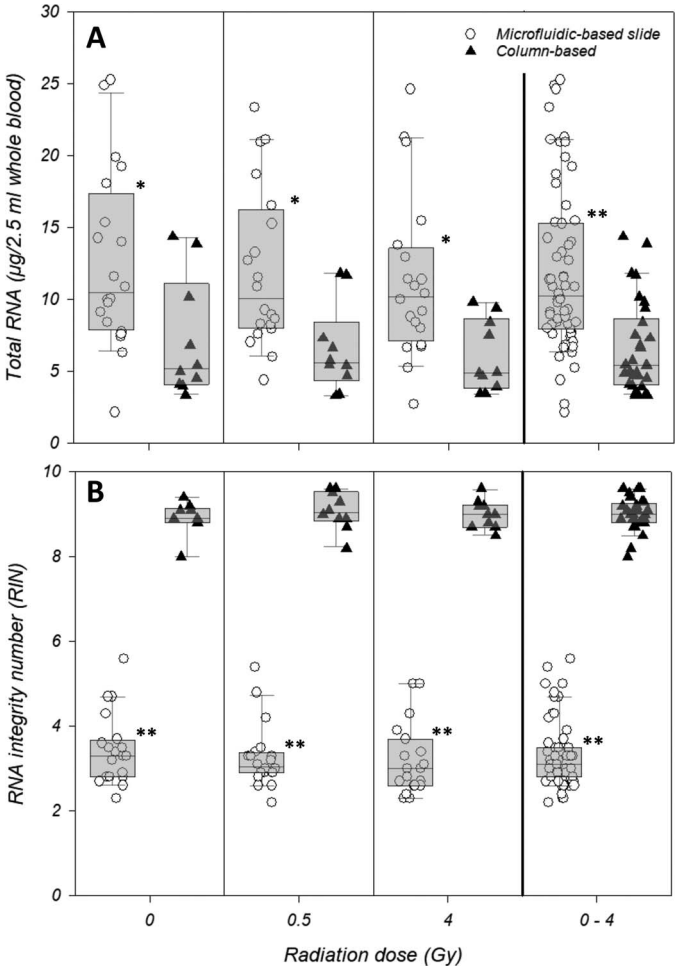

FIG. 3. RNA quantity (panel A) and quality (panel B) measurements (provided as RIN^e values) using two RNA extraction methods: Microfluidic-based and conventional column-based RNA extraction. The symbols of the jitter plot represent measurements for each examined sample of the preliminary microfluidic-based (gray circles) and the conventional column-based RNA extraction (black triangles) method. A corresponding box plot reflects the distribution of data. Results from both methods are presented for different radiation doses (Y scale). P values refer to different significance levels of *$P \leq 0.05$ and **$P \leq 0.001$.

Gene Expression Analysis

Using MBS, significant changes in *FDXR* GE were found after irradiation, and a median fold change of 1, 15, and 31 was measured at 0, 0.5 and 4 Gy, respectively (Fig. 5, Table 3). These GE changes were comparable and not statistically different when employing the CB RNA isolation method. A similar pattern was found for *DDB2*, but the dose-dependent significantly upregulated fold changes were smaller compared to *FDXR* (e.g., for MBS, the median fold change was 1, 6, and 12 at 0, 0.5 and 4 Gy, respectively) but similar using both methods (Fig. 5, Table 3). Using MBS, a significant downregulation of *POU2AF1* was observed, and median fold change of 1 at 0 Gy decreased to 0.9 and 0.6 at 0.5 and 4 Gy, respectively (Fig. 5, Table 3). These GE changes were comparable and not statistically different when employing the CB RNA isolation method. A similar pattern was found for *WNT3*, but a decreased fold change at 0.5 (FC = 0.8) and 4 Gy (FC = 0.8) was comparable and not statistically different. Corresponding fold changes using both methods were comparable as well, and not statistically different (Fig. 5). Detailed descriptive statistics of GE measurements are presented in Table 3.

[2] Editor's note. The online version of this article (DOI: https://doi.org/10.1667/RADE-23-00169.1) contains supplementary information that is available to all authorized users.

DISCUSSION

Radiological or nuclear scenarios will cause an impaired infrastructure that demands point-of-care (POC) tools so that specialized diagnostic tests can be applied in the field, thus avoiding the transport of irradiated biological material to specialized laboratories. Large-scale biodosimetry interlaboratory exercises repeatedly reported undesirable delays and difficulties in transporting biological material, even in the presence of a functional infrastructure (*25*). Radiation-induced GE changes can be used for biodosimetry (*26*) as well as clinical outcome prediction of acute health effects such as the life-threatening acute radiation syndrome (*27*, *28*). This technique has certain advantages, including early and high-throughput diagnostic capability as well as a high degree of automation. One of the first steps for GE analysis is the isolation of RNA. This can be routinely accomplished in laboratories using heavy equipment such as centrifuges or working benches with safety cabinets. For POC GE-based diagnostics, performing this important RNA isolation step with the same quality but less infrastructure in the field would be advantageous. In collaboration with ChipShop, the Bundeswehr Institute of Radiobiology developed a microfluidic-based slide (MBS) device. It represents a preliminary version for semiautomatic RNA isolation (Fig. 2) that would lead to eventual development of a microfluidic card permitting automatic RNA isolation. In this study, we systematically examined the RNA quantity and quality and performed qRT-PCR on ex vivo irradiated blood samples from healthy donors using the newly developed MBS in comparison to an established conventional laboratory workflow, including Phenol-chloroform separation of RNA and RNA extraction via column-based silica membranes (Fig. 1, Table 1).

A four-gene set (*FDXR*, *DDB2*, *POU2AF1* and *WNT3*) which predicts the development of the potentially life-threatening HARS was used to prove the applicability of the RNA isolates for downstream qRT-PCR. Using the MBS compared to the established CB method, half of the RNA extraction time without the burden of heavy technical equipment (e.g., a centrifuge) was required, and twice as much RNA without detectable DNA contamination could be isolated in contrast to CB RNA extraction. Also, with MBS, the input of blood needed (250 µl) was tenfold smaller, making more invasive intravenous blood draws dispensable, and GE performance was comparable to the CB method.

TABLE 2
Isolated Total RNA (µg) and RNA Quality (RNA Integrity Number, RIN[e]) for the Conventional Column-Based (CB) and the Microfluidic-Based Slide (MBS) Methods After Radiation Exposure

A	RNA extraction method		Total RNA (µg)			RNA integrity (RIN)		
		Dose (Gy)	n	Mean	SD	n	Mean	SD
	Microfluidic-based slide	0	20	12.6	6.2	20	3.4	0.8
		0.5	20	12.0	5.5	20	3.3	0.8
		4	20	11.3	5.6	20	3.2	0.8
	Column-based	0	10	7.2	4.1	8	8.9	0.4
		0.5	10	6.6	3.0	10	9.1	0.4
		4	10	6.1	2.5	8	9.0	0.3

B	Comparison (p-values)		Total RNA (µg)			RNA integrity (RIN)		
		Dose (Gy)	MBS	CB	MBS vs. CB	MBS	CB	MBS vs. CB
	Dose comparison	0 vs 0.5	ns	ns		ns	ns	
		0 vs 4	ns	ns		ns	ns	
		0.5 vs 4	ns	ns		ns	ns	
	Method comparison	0			0.014			<0.001
		0.5			0.003			<0.001
		4			0.005			<0.001
		0-4 combined			<0.001			<0.001

Notes. Section A shows mean fold changes (FC), ± standard deviation (SD), and number of examined samples (n) of four genes employing two RNA extraction methods. Section B shows the statistical analysis and their corresponding P values.

The halved RNA extraction time was due to a threefold reduction in the number of extraction steps (39 steps using CB vs. 12 steps using MBS) (Table 1). Only small and light devices such as microfluidic slides, waste tanks, a small pump, and collection tubes were required for MBS RNA isolation, which contrasts with the established CB workflow comprising heavy centrifuges, workbenches, and consumables. These results are in line with the cited work, indicating that MBS not only utilizes a portable and miniaturized format but also avoids the multitude of steps involving manual handling of samples as found during the use of pregnancy and SARS-CoV-2 diagnostics (*18*). Furthermore, automating lab processes and minimizing the risk of procedural error offers the potential to reduce the need for specialized lab technicians (*17*, *18*).

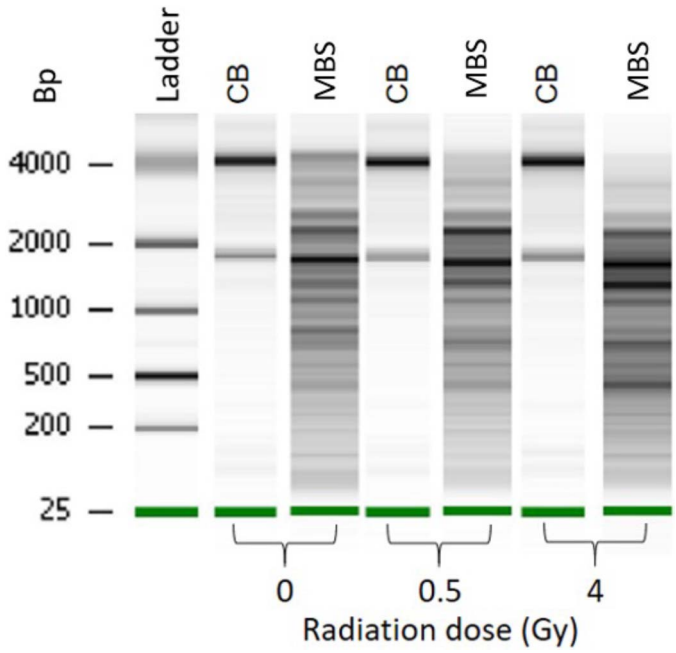

FIG. 4. The virtual image of an electrophoresis run employing Agilent Bioanalyzer for different radiation exposures (X scale) and two RNA extraction methods: The conventional column-based (CB) and the microfluidic-based slide method (MBS). An RNA size standard (ladder) and corresponding band sizes in base pairs (Bp) are shown to the left.

The doubled RNA amount without detectable DNA contamination using MBS provides another interesting feature. The reason for this could be that the closed MBS system contained the fluids and their reactions in a small lateral dimension, presenting a laminar flow (low Reynolds number) that controlled the environment exceptionally well (*17*). Also, when inspecting possible sample contamination by genomic DNA via PCR using β-actin primers, MBS samples were DNA-free throughout all MBS samples; this contrasted with CB samples, where DNA contamination occurred, and DNase re-digestion was indispensable.

Tenfold lower sample input volumes of only 250 µl whole blood for the MBS method allows the avoidance of venipuncture in the future by further downscaling the volume input using capillary blood. Furthermore, loading the patient's blood onto the MBS spares the personnel from working with a potentially harmful mixture of phenol and chloroform, which is used for phase separation of DNA, RNA, and proteins during the RNA extraction phase (*29*).

The total RNA output seemed strongly degraded in MBS samples compared to the conventional method (Fig. 3B). To obtain the hypothesized RNA degradation by MBS, we followed the recommendation of an unknown reviewer and carried out an additional experiment. Three RNA samples previously extracted

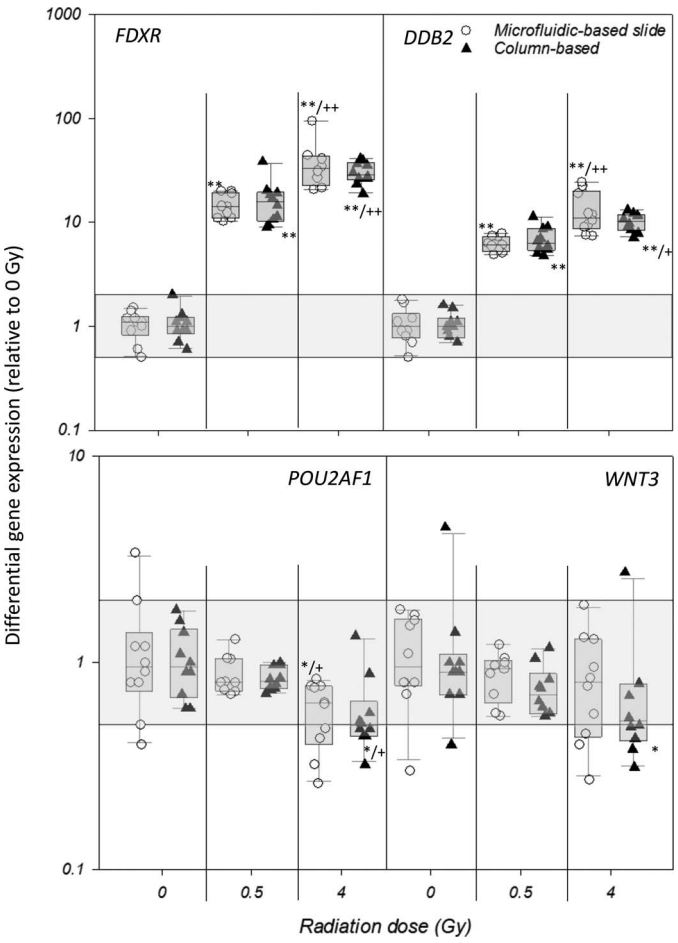

FIG. 5. Differential gene expression changes (abscissa) after irradiation (ordinate) for four genes relative to control values used as the reference. Jitter plots representing single measurements and superimposed box plots representing the corresponding data distribution are provided for two RNA isolation methods: The conventional column-based (black triangles) and the microfluidic-based slide (white-filled circles) methods. P values refer to different significance levels of *P $\leq$ 0.05 and **P $\leq$ 0.001. Stars represent significant comparisons relative to the unexposed corresponding RNA-isolation technology. + represents comparison relative to 0.5 Gy of the corresponding RNA-isolation technology.

via the CB method and presenting satisfying RIN values (RIN $>$ 8) were re-processed using the MBS. The results showed almost similar RIN values as after CB-extraction and no signs of RNA degradation (data not shown). This also excludes the possible incompatibility between the MBS reagents and the Agilent Bioanalyzer chemistry. We assume an interaction in the system during the lysis of whole blood leads to large rRNA species getting stuck as they cross the silica membrane during extraction. Since the conventional extraction has a sample purification step using phenol-chloroform, we anticipate that impurities and cell components from the original blood sample are the cause of factitious degradation. This was confirmed by the re-extraction of the pure RNA on the silica membrane, which does not cause any RNA degradation. Without the sample purification, possible changes in the three-dimensional configuration of rRNA species might have additionally occurred, thus mimicking lower-sized "fragments" visible as multiple bands on the gel and falsely indicating RNA degradation (Fig. 4). Furthermore, similar fold change in GE using both methods and showing expected gene up- (*FDXR* and *DDB2*) and downregulation (*POU2AF1* and *WNT3*) after the respective radiation dose (Fig. 3), as previously seen (*30*) argue against RNA degradation (Fig. 4). Also, *18S rRNA* copy numbers quantified via qRT-PCR in MBS samples corresponded to the expected number of *18S rRNA* copy numbers in undegraded RNA samples, indicating that RNA from MBS samples was not degraded.

When investigating the GE of possible patients in the field, this MBS could be combined with automated qRT-PCR pipetting and cycling machines called MYRA (a liquid handling robot) and MIC (magnetic induction thermal cycler) (made by Biomolecular Systems), which have shown their operational capabilities as part of a mobile laboratory during SARS-CoV-2 field testings (*31*). Another possibility of further processing would be via Nanopore technology (Oxford Nanopore Technology). Another group used the GridION to classify SARS-CoV-2 strains by whole genome sequencing (*32*). Cruz-Garcia et al., on the other hand, investigated radiation exposure signatures via the third generation long-read sequencing method (*33*). Either way, as mentioned previously, this MBS could be combined with various technological applications, including but not limited to the gold standard qRT-PCR using the QuantStudio™ 12K OA Real-Time PCR System.

The current MBS comprises limitations concerning the sensitive tube setup connected with a peristaltic pump due to its preliminary module phase stage and the simulated RNA degradation. Furthermore, extending and widening the MBS slides by fusing them with MBS qRT-PCR slides as a one-step PCR will be the next step to creating a stand-alone device in terms of an integrated microfluidic card. Until then, a couple of issues must be addressed before the development of such a transportable MBS card: Sample uptake, reagent storage, sample preparation, amplification of nucleic acids, separation, detection of the analyte of interest, and waste handling. (*34*). Microfluidics is still a complex technology needing continuous and steady machining before the simple benchtop method can be replaced with a commercialized methodology (*35*). Nevertheless, afterwards, it should be more efficient economically and scientifically. The storage of scarce reagents in blister-like channels of the MBS reduces reagents and assay costs due to fixed implementation (*36*).

In conclusion, this proof-of-principle study showed that microfluidic-based slides for RNA extraction are less time-consuming, don't require heavy equipment, provide twice as much output of high-quality mRNA free of DNA, and show GE results comparable to those using conventional RNA extraction. These results are an indicator that a corresponding integrated, semi-automatic microfluidic card should be developed for future point-of-care-diagnostics, not only for biodosimetry purposes and clinical outcome prediction in R/N

TABLE 3
Gene Expression Analysis for Both the Conventional Column-Based (CB) and the Microfluidic-Based Slide (MBS) Method after Radiation Exposure

A	RNA extraction method	Dose (Gy)	Gene of interest	n	mean FC	SD
	Microfluidic-based slide	0	*FDXR*			
			DDB2			
			POU2AF1			
			WNT3			
		0.5	*FDXR*	10	14.7	3.7
			DDB2	7	6.1	1.1
			POU2AF1	10	0.9	0.2
			WNT3	10	0.8	0.2
		4	*FDXR*	10	31.4	9.1
			DDB2	10	12.4	5.4
			POU2AF1	10	0.6	0.2
			WNT3	9	0.8	0.4
	Column-based	0	*FDXR*			
			DDB2			
			POU2AF1			
			WNT3			
		0.5	*FDXR*	10	16.9	8.7
			DDB2	10	7.0	2.1
			POU2AF1	10	0.8	0.1
			WNT3	10	0.7	0.2
		4	*FDXR*	10	30.6	7.3
			DDB2	10	10.2	2.0
			POU2AF1	10	0.6	0.3
			WNT3	10	0.8	0.7

B			P-value comparisons		
	Genes of interest	Dose (Gy)	MBS	CB	MBS vs. CB
	FDXR	0 vs 0			ns
		0 vs 0.5	<0.001	<0.001	
		0 vs 4	<0.001	<0.001	
		0.5 vs 4	<0.001	0.001	
		0.5 vs 0.5			ns
		4 vs 4			ns
	DDB2	0 vs 0			ns
		0 vs 0.5	<0.001	<0.001	
		0 vs 4	<0.001	<0.001	
		0.5 vs 4	<0.001	0.003	
		0.5 vs 0.5			ns
		4 vs 4			ns
	POU2AF1	0 vs 0			ns
		0 vs 0.5	ns	ns	
		0 vs 4	0.01	0.002	
		0.5 vs 4	0.01	0.01	
		0.5 vs 0.5			ns
		4 vs 4			ns
	WNT3	0 vs 0			ns
		0 vs 0.5	ns	ns	
		0 vs 4	ns	0.02	
		0.5 vs 4	ns	ns	
		0.5 vs 0.5			ns
		4 vs 4			ns

Notes. Section A shows mean fold changes (FC), ± standard deviation (SD), and number of examined samples (n) of four genes employing the RNA extraction methods. Section B shows the statistical analysis and their corresponding P values.

scenarios, but also as a bedside test in human genetic and clinical applications, e.g., for microbiological matters.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. Raw data of 10 donors investigated when comparing the conventional column-based (CB, gray background) with the microfluidic-based slide (MBS) RNA extraction from whole blood samples after irradiation (0, 0.5 and 4 Gy). The quantitative data was measured via the NanoDrop. The qualitative data was acquired by measuring the RNA integrity number (RIN) via the Agilent Bioanalyzer and running a β-actin PCR and gel electrophoresis to determine possible genomic contamination (black background).

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