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Calibration curve for radiation dose estimation using FDXR gene expression biodosimetry – premises and pitfalls

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Abstract

Purpose

Radiation-induced alterations in gene expression show great promise for dose reconstruction and for severity prediction of acute health effects. Among several genes explored as potential biomarkers, FDXR is widely used due to high upregulation in white blood cells following radiation exposure. Nonetheless, the absence of a standardized protocols for gene expression-based biodosimetry is a notable gap that warrants attention to enhance the accuracy, reproducibility and reliability of such methods. The primary objective of this study was to evaluate the sensitivity of transcriptional biodosimetry to differences in protocols used by different laboratories and establish guidelines for the calculation of calibration curve using FDXR expression data. Additionally, we aimed to investigate the feasibility of extrapolating a calibration curve derived from samples irradiated with doses exceeding 0.1 Gy to doses below this threshold.

Material and Methods

Two sets of irradiated blood samples generated during RENE B exercise were used. The first included samples irradiated with known doses including: 0, 0.25, 0.5, 1, 2, 3 and 4 Gy. The second set consisted of three 'blind' samples irradiated with 1.8 Gy, 0.4 Gy and a sham-irradiated sample. After irradiation, samples were incubated at 37 °C over 24 h. Subsequently, aliquots were filled into PAXgene tubes and sent to participating laboratories where RNA isolation and FDXR expression analysis by qPCR were performed.

Results

Calibration curves were generated using non-linear and linear regression. Dose estimates for nonirradiated sample and sample irradiated with 0.4 Gy showed remarkable consistency across all laboratories, closely approximating the true doses regardless methodological variations. For sample irradiated with 1.8 Gy the differences were more pronounced but remained within an acceptable margin for triage within the context of high dose range.

Conclusion

Methodological differences in reference genes and primers/probes used for FDXR expression measurement do not have a significant impact on the dose estimates generated, provided that all reference genes performed as expected and the primers/probes target a similar set of transcript variants. The preferred method for constructing a calibration curve based on FDXR expression data involves employing linear regression to establish a function that describes the relationship between the logarithm of absorbed dose and FDXR ΔC_t values. However, one should be careful with using non-irradiated sample data as these cannot be accurately represented on a logarithmic scale. A standard curve generated using this approach can give reliable dose estimations in a dose range from 50 mGy to 4 Gy at least.

Keywords: FDXR, gene expression, biological dosimetry, qPCR

Introduction

Biological dosimetry is an important component of radiation protection and emergency response during radiation accidents. It can be used to assess the radiation dose received by individuals when information from physical dosimetry is not available or not reliable. It supports the medical countermeasures and estimation of the long-term health effects of radiation exposure (Sproull et

al. 2017). Apart from gold-standard cytogenetic methods used by biodosimetry laboratories, complementary approaches are emerging with gene-expression assays being one of the most promising (Kultova et al. 2020; Abend et al. 2023; Port et al. 2023;).

Radiation-induced changes of gene expression show great promise for dose reconstruction and for severity prediction of acute health effects (Ostheim et al. 2021). Gene expression-based biodosimetry is a field of intensive research around the world. Different methodologies of gene expression analysis are routinely compared during exercises organized under the umbrella of RENEb or NATO (Badie et al. 2013; Manning et al. 2016; Abend et al. 2021; Abend et al. 2023). Among several genes analyzed and used as potential biomarkers, FDXR is always the highest upregulated in white blood cells following radiation exposure and is widely used by many laboratories as recently shown during an interlaboratory comparison organized by RENEb (Abend et al. 2023). Such exercises reveal that different laboratories use not only different sets of radiation responsive and reference genes, but also different approaches to construct standard curves used for dose estimation.

In 2017, during an interlaboratory comparison organized by RENEb, FDXR gene expression data were generated by three participating laboratories. As it stands, there isn't a standardized procedure/protocol for gene expression based biodosimetry. These unpublished data provided the opportunity to assess to what extent transcriptional biodosimetry is sensitive to differences in procedures and protocols used by different laboratories for the generation of qPCR data and calculation of the calibration curves. Moreover, we used available literature data to extend our analysis with an attempt to answer the question whether gene expression-based biodosimetry is applicable in the low-dose range (below 0.1 Gy) and examined whether calibration curve based on samples irradiated with doses > 0.1 Gy can be extrapolated below 0.1 Gy.

Materials and methods

Sample preparation and irradiation

Two sets of irradiated blood samples were generated and delivered. The first one served to generate a calibration curve. It included samples irradiated with known (communicated) doses including: 0, 0.25, 0.5, 1, 2, 3 and 4 Gy. The second set consisted of three ‘blind’ samples (radiation dose was unknown to the participants) irradiated with 1.8 Gy, 0.4 Gy and a sham-irradiated sample. Within this exercise these blind samples were coded as samples A, C, and B, respectively. The blood irradiation was performed as described previously (Gregoire et al. 2021). Briefly, a blood samples from a female donor (Etablissement Français du Sang (EFS), France; Agreement CPSL UNT N°13/EFS/123) was irradiated at 37 °C in a water bath with 4-MV X-rays delivered by a Linear medical accelerator (Elekta Synergy, IRSN, Fontenay aux Roses, France) at 0.5 Gy min⁻¹, dose in water. The irradiation field was 30 x 30 cm and the distance between the source and the sample was of 1.07 m. Radiation field mapping and dosimetry was confirmed using cylindrical ionization chamber (0.125 cc n° 4920) calibrated in dose to water. After irradiation, samples were incubated at 37 °C over 24 h. Subsequently, 2.5 ml aliquots were filled into PAXgene tubes (preserves RNA), kept 24 h at -20 °C and sent to participating laboratories in dry ice.

RNA isolation and qPCR

RNA isolation and qPCR were performed by three participating laboratories using established protocols which differed among them: Bundeswehr Institute of Radiobiology (BIR, Germany), Institute of Nuclear Chemistry and Technology (INCT, Poland), United Kingdom Health Security Agency (UKHSA, United Kingdom). Description of RNA isolation and qPCR methods used by

each laboratory is given in Table 1. The main methodological difference between laboratories was due to the fact that different reference genes were used for normalization and different primers and probes were used for FDXR gene expression measurements.

Statistical methods

Statistical analysis was performed using R Statistical Software (version 4.3.1; R Foundation for Statistical Computing, Vienna, Austria) (R Core Team 2023). Standard error of regression, also known as residual standard error, was calculated as a goodness-of-fit measure, because commonly used R-square values are not valid for nonlinear regression (Spiess and Neumeier 2010).

Results

Derivation of a calibration curve delineating the relationship between the absorbed dose of radiation and FDXR expression.

The normalized FDXR gene expression values (ΔC_t values) of the calibration samples, were plotted against radiation dose on a linear scale and separately for each laboratory (Figure 1). The relationship was not linear. Instead, it was accurately described by either an exponential (Figure 1, left side) or logarithmic function (Figure 1, right side), depending on which variable was regarded as the dependent one (plotted on the y-axis). In the context of biological dosimetry, the objective is to infer the radiation dose based on modifications in gene expression values. Consequently, in the models employed for dose estimation within this segment of the study, the dose was regarded as a dependent variable and was plotted on the y-axis.

Data from laboratory 1 and laboratory 2 were fitted using an exponential function resulting in standard error of regression (S) values of 0.048 and 0.032, respectively. For

laboratory 3 data, S value was about 10-times higher (0.363). This was driven by the 2 Gy irradiated sample, which was therefore regarded as an outlier and excluded from subsequent analysis. Without the 2 Gy sample, S value for laboratory 3 halved (0.157) and became closer to the ones obtained for the other two laboratories (Figure 1, lower graphs). The calculated exponential functions (Figure 1, left side) were used to reconstruct the absorbed doses of the blind samples. Resulting dose estimates for each laboratory are presented in Table 2.

Dose estimates for blind samples “B” and “C” showed remarkable consistency across all laboratories, closely approximating the true doses regardless methodological variations. Notably, for sample “A”, the differences were more pronounced, particularly for laboratory 3. Nevertheless, even in this instance, the 0.6 Gy difference between the estimated and true dose remains within an acceptable margin for triage within the context of high dose range (Lloyd et al. 2000).

It is interesting to note that for all three laboratories, the dose estimates for non-irradiated samples were around 20 mGy which is very close to the real true dose. To be noted, as an exponential function (1) was used for dose estimation, y (Dose) decreases with increasing ΔCt values but never reaches zero. Therefore, dose estimates for non-irradiated samples will consistently yield small values greater than zero.

$$Dose = 2^{a+b \times FDXR \Delta Ct} \quad (1)$$

In practice, it is easier to work with linear than exponential functions. Exponential function (1) can be converted to a linear function by taking a logarithm of both sides of the equation. Resulting function (2) describes the linear relationship between logarithm of dose and FDXR expression measured in terms of ΔCt values.

$$\log_2 Dose = a + b \times FDXR\Delta Ct \quad (2)$$

Data for non-irradiated sample (0 Gy) cannot be directly used during fitting function (2) to FDXR expression data, as the logarithm of zero is undefined. To avoid using non-linear regression for calculation of parameters “a” and “b”, two possible solutions can be considered: (i) performing linear regression without data for nonirradiated sample, (ii) substituting 0 Gy with another value. In the latter case, the obvious question is: ‘what value should be used?’

Figure 2 and table 3 shows the results of the analysis based on data from laboratory 1. Three linear calibration curves were generated. The first one (in blue) did not include the non-irradiated sample, whereas the second and third ones used respectively 0.01 Gy (in green) or 0.001 Gy (in orange) values as representations of non-irradiated sample. The corresponding dose estimates based on each of the calibration curves are given in Table 3.

The standard curve generated without data for non-irradiated sample provided the best fit of the data (highest R^2 value, lowest S value) and resulted in the most precise dose estimates, which were similar to the dose estimates obtained using exponential calibration curve (Tables 2 and 3). Therefore, representing the non-irradiated sample as a 0.01 Gy or 0.001 Gy resulted in a significant shift of the slope of the curve and an associated bias in the dose estimation.

Dose – FDXR expression relationship at doses below 0.1 Gy.

Functions of the standard curves described above give a very precise description of the relationship between absorbed radiation dose and FDXR expression in the dose range 0.25 - 4 Gy covered by the data generated during the RENEB exercise. Equation (3) is a function related to a linear standard curve calculated from laboratory 1 data without 0 Gy sample (Figure 2).

$$\log_2 Dose = -2.204 - 1.419 \times FDXR\Delta Ct \quad (3)$$

If we transform equation (3) to a form in which FDXR ΔCt is on the left side of the equation, we obtain a function (4) in which for every doubling or halving of the absorbed dose, the FDXR ΔCt changes by a value of 0.705, equal to a 1.63 fold change as shown in (5). In other words, when absorbed dose increases by 100%, the FDXR expression increases by 63%.

$$FDXR\Delta Ct = -1.553 - 0.705 \times \log_2 Dose \quad (4)$$

$$2^{0.705} = 1.63 \quad (5)$$

This relationship is valid for the data covering the dose range from 0.25 to 4 Gy. The question arises, whether it is also valid in the lower dose range, 0-0.25 Gy. It is unlikely, since in the equation (4), when the dose approaches zero, FDXR ΔCt becomes infinitely large, i.e FDXR expression becomes infinitely low. Obviously, this doesn't correspond to the experimental data in which FDXR ΔCt for non-irradiated sample (0 Gy) is not infinitely large but is well defined (1.788 in the case of laboratory 1 data). As shown in table 2 and 3, both exponential and linear approaches to standard curve calculation and dose estimation, resulted in a dose estimates around 20 mGy for non-irradiated sample. Equation (4) predicted that following a 10 mGy dose exposure, FDXR ΔCt will be equal to 3.129 which is higher than the value observed for the non-irradiated sample and therefore is unlikely to be the correct prediction of FDXR expression level after low dose of radiation exposure. We concluded that equations (3) and (4) correctly describe the relationship between absorbed dose and FDXR transcriptional gene expression in a dose range 0.25-4 Gy but not for doses below 20 mGy.

To further explore this issue, we used the data from the paper by Manning et al. (2013), in which FDXR expression was analyzed in human blood from healthy donors irradiated *ex vivo* by high (0.1-4 Gy) and low (5-100 mGy) X-ray doses. Experiments in a low and high dose ranges

were performed separately on a different set of donors. In the present analysis, we treated donors no. 18 and 19 as outliers based on the interquartile rule and excluded from the original dataset, as the mean FDXR Δ Ct values for these donors were more than 1.5 interquartile range below the first quartile (Figure 3).

We analyzed the FDXR Δ Ct values from Manning et al. (2013) using the procedure described in the previous sub-section. First, the mean FDXR Δ Ct values from both low-dose and high-dose experiments were plotted against log₂ of dose (Figure 4A). For the high-dose experiment, a linear relationship between log₂ of dose and FDXR Δ Ct was observed similarly to data described in the previous sub-sections. However, in the case of low-dose experiment, only three points corresponding to the highest doses in the range (50, 75, 100 mGy) form a straight line with a slope similar to the one observed for high-dose experiment, thus suggesting that the relationship between FDXR expression and dose in the low-dose range is not as straightforward as in the high-dose range. Moreover, data points from high and low dose experiments were significantly shifted from each other. Both experiments included samples irradiated with 0.1 Gy and the mean Δ Ct values differed for more than one PCR cycle (Figure 4A). A different set of donors in each experiment and/or technical differences during performing the experiments may have caused this difference. To get some insight into the relationship between FDXR expression and absorbed dose in a range from 5 mGy to 4 Gy, we decided to adjust data from the low-dose experiment to the data from the high-dose experiment. To this end, the difference between Δ Ct values for samples irradiated with 0.1 Gy in both experiments was calculated, and the resulting value was subtracted from Δ Ct's of low-dose range experiment and presented in Figure 4B. For data points down to 50 mGy, a linear relationship between FDXR Δ Ct and log₂ of dose was observed. In this case, with every doubling of the dose, FDXR Δ Ct decreases by 0.559 which

corresponds to 47% increase in mRNA level (1.47 fold change). This relationship is not valid below 50 mGy, as below this dose FDXR expression is stable, not dose dependent, and therefore not statistically different from the control (0 Gy) (Figure 5A).

Discussion

Gene expression analysis in blood cells holds great promise as a rapid, high-throughput biological dosimetry technique. Moreover, it has high potential for automatization and development of deployable, point of care device. At present, no standardized protocols have been agreed on and no analytical frameworks for gene expression analysis in the context of biological dosimetry have been established yet. Although the number of laboratories working in this field is growing, the methodologies used vary significantly in terms of the set of genes used as a radiation biomarker, genes used for normalization, as well as methods for calibration curve calculation. This diversity is clearly visible in RENEB exercises, where it is often difficult to attribute observed dose estimate differences to specific differences in methodologies used by participating laboratories (Abend et al. 2016; Manning et al. 2017; Abend et al. 2021; Abend et al. 2023).

Among genes suitable for gene expression-based biodosimetry studies, the most used is FDXR, validated by qPCR experiments (O'Brien et al. 2018) and confirmed in the radiation signature generated by long read sequencing (Cruz-Garcia et al. 2019). It is important to note that laboratories have so far used their favourite primer designs for amplification by qPCR without checking for the specific amplified FDXR transcript (splice variants). This can be a source of discrepancies as radiation-responsive alternative FDXR transcripts are up-regulated at different levels (Cruz-Garcia et al. 2020).

FDXR encodes mitochondrial flavoprotein ferredoxin reductase. The reason for this popularity of FDXR among biodosimetry researchers is due to large shifts in its mRNA level in peripheral blood lymphocytes following irradiation in a dose dependent manner. FDXR is regulated by p53 family of proteins and its upregulation sensitizes cells to apoptosis. It seems therefore, that FDXR upregulation after irradiation is a step in the molecular cascade pushing lymphocytes toward apoptotic death (Liu et al. 2002).

In the present work, we used FDXR expression data to study the role of data analysis methods in calibration curve calculation; we demonstrated that very close dose estimates can be obtained regardless of differences in qPCR methodology and reference genes used. We showed that a reliable calibration curve may be generated by means of a non-linear regression based on absorbed dose and corresponding ΔC_t values. However, we demonstrated that the more convenient approach consists of a linear regression based on the logarithm of dose and ΔC_t values. While following this approach, one should be careful with using non-irradiated sample data as these cannot be represented on a logarithm scale. Arbitrary applying low positive values (such as 0.01 Gy or 0.001Gy) as a representation of non-irradiated samples may seem a reasonable approach from a biological point of view since e.g. 0.001 Gy represent a negligible dose at which levels, no detectable biological effects are expected. However, such representation of non-irradiated samples may result in a biased standard curve shape, hence resulting in a suboptimal dose estimation as shown in Figure 2 and Table 3. A proposed solution was to exclude data for non-irradiated sample from calibration curve calculation. Another option is to use an ‘artificial’ dose value for the nonirradiated sample for which R^2 of the resulting curve is the highest (i.e. the curve is best fitted to the data).

Interestingly and importantly, despite the differences in protocols between laboratories, the robustness of FDXR gene expression-based biodosimetry was evident with dose estimates based on data from participating laboratories being practically identical for non-irradiated sample and 0.4 Gy sample (Table 2). Slightly bigger differences were observed for a high dose sample (1.8 Gy), especially for laboratory 3 and may be attributable to an observed unusual clotting of blood in PaxGene tubes, which was probably caused by insufficient tube shaking to solve the blood in the solution stored in the PaxGene tubes. The main methodological differences between participating laboratories were due to different reference genes used and different sequences of primers and probes used for FDXR expression measurement. Analyses described in the present work demonstrate that such methodological differences did not have a significant impact on the dose estimates generated, provided that all reference genes under study performed as expected. Similarly, different sets of primers and probes for FDXR recognize different exon regions and, therefore, transcripts. However, all these exons revealed comparable radiation-induced gene expression changes, indicating that for this gene, differently used gene expression assays are of less importance (Schüle et al. 2022). To be noted, two radiation-responsive FDXR transcripts were only detected by nanopore long read sequencing and do not have detectable background expression (Cruz-Garcia et al. 2020b)

Linear models (3) and (4) described the relationship between absorbed dose of radiation and FDXR expression in terms of the amount of change of ΔCt value with every doubling of the dose. According to laboratory 1 data, there was a 63% increase in FDXR expression when the absorbed dose was doubled. To the best of our knowledge, this is the first time when FDXR expression changes after irradiation were described in such terms. We propose to use this percentage change as a parameter, termed the “FDXR Radiation Responsiveness Index (FRRI)”.

Several questions can be asked concerning the characteristics of FRRI. For instance: How consistent is it for a specific donor? What influence does the donor's health or inflammatory status exert on its value? How does it vary across multiple donors? What values does it exhibit in diverse cell types, including cell lines? Is there any correlation between FRRI and radiosensitivity? Hopefully, future research will answer these questions.

Clearly, models such as (3) and (4) cannot reasonably describe expression change between non-irradiated sample and any irradiated sample, as zero cannot indeed be doubled or halved. However, such a technical issue was not the only reason why models (3) and (4) were not applicable to low range of radiation doses. The second reason was of biological nature and was due to the fact that after *ex vivo* irradiation with doses below 50 mGy, the relationship described by models (3) and (4) was not valid as shown by reanalyzing the data from Manning et al. (2013) (Figure 5). Our results are in line with the original outcome of this paper where no significant changes in FDXR expression were found below 50 mGy, 24 hours following in vitro exposure. To be noted, recently, Cruz-Garcia et al. have studied for the first time the transcriptional dynamics of FDXR *in vivo*, in patients' blood during radiotherapy. This was performed after the first fraction of treatment; FDXR transcriptional up-regulation was dose dependent from the lowest irradiated percentage of the body/dose per fraction for the neck (0.038-0.045 Gy) to the highest for the pelvis area (0.155-0.169 Gy) pelvic zone) reaching a peak at 6–8 h. Interestingly, the radiation response of other genes classically used in gene expression-based biodosimetry (i.e. CDKN1A, GADD45, CCNG1, DDB2, MDM2, PHPT1, SESN1, and PUMA) was not strong enough to provide meaningful information after such low doses (Cruz-Garcia et al. 2022). Hence, one can argue, that the reason why we were unable to detect changes in FDXR expression after low doses in vitro is due to technical limitations of qPCR and not to biological regulation. Such technical limitations may be of the following nature: (1) gene expression changes below 50 mGy

were too low to be accurately measured by means of qPCR, (2) mRNA level (copy number) in samples irradiated with doses lower than 50 mGy (including nonirradiated sample) was too low to allow reliable measurements by qPCR, (3) time point for analysis was not optimal as the peak of FDXR gene expression is expected 6-8 hours post exposure and not at 24 hours, (4) limitations of in vitro exposure and behavior of cells. Points 1 and 2 described above are unlikely to fully explain the results described in the present paper. Firstly, standard curve presented in figure 4, predicted that in a dose range 0.05Gy – 4 Gy, FDXR expression increases by 47% with every doubling of the dose. Such change can be easily measured by qPCR. If the relationship was also valid below 50 mGy, then we should observe 1,47 fold change between doses 0.005 Gy and 0.01Gy, or between 0.01 and 0.02 Gy, or more than 1.47 fold change between doses 0.02 and 0.05 Gy. None of these was true as shown in Figure 5. In contrast, fold change between doses 0.05 and 0.1 Gy is 1.66 which is reasonably close to the prediction. Secondly, FDXR Ct values for samples <50 mGy from Manning et al. (2013) are in the range of 26-28 with exception of two donors for which Ct's were in the range 28-30, but these donors were regarded as outliers and excluded from the present analysis. Ct values in the range 26-28 represent mRNA copy numbers which can be reliably measured by means of qPCR as it gives highly reliable and reproducible results for Ct values up to, at least, 30. Therefore, the reason why we didn't see significant changes of FDXR expression below 50 mGy cannot be explained by low expression level in the conditions to which the samples were kept. While the peak of FDXR expression is expected 6-8 hours post-exposure, it has been demonstrated that following this peak, FDXR expression declines rather slowly over next several hours (Ostheim et al. 2021; Nosel et al 2013), hence the suboptimal time point cannot fully explain observed differences in FDXR expression dynamics at doses below and above 50 mGy. It seems therefore, that the observed results are due to

differences in the biological regulation of FDXR expression in lymphocytes in a low (<50 mGy) and high (between 50 mGy and 4 Gy) dose ranges, at least when exposed *in vitro*.

Available studies on FDXR expression after doses ≤ 50 mGy vary in their conclusions (Table 4). It seems that the results are highly influenced by the factors such as research model used (*in vivo*, *ex vivo*) and time after irradiation. It was demonstrated that during *ex vivo* experiments a minimum of 4 hours is necessary for a biological response, leading to a significant upregulation of FDXR (Abend et al. 2021). Moreover, significant uncertainty may be associated with dose estimation, especially *in vivo*.

Among available studies on gene expression analysis in biodosimetry, two approaches can be identified. One is based on individual genes (most often on FDXR as it shows the highest induction after irradiation), and the other one uses a set of genes. From the one hand it seems justified to use FDXR alone as it can give a reliable dose estimation as in the present study and other papers (Badie et al. 2013; Abend et al. 2016; Manning et al. 2017; O'Brien et al. 2018; Abend et al. 2021; Abend et al. 2023). Moreover, even if we base our dose estimation on mean ΔCt value from several genes, the FDXR will probably (most certainly in the lower range of the doses, below 0.5 Gy) have the greatest contribution to the final results because the fold changes after irradiation are the highest for FDXR and consequently, its expression is the most informative.

On the other hand, biodosimetry based on expression of just one gene may be prone to errors and biases. For example, if a patient has a mutation/polymorphism in the promoter region of FDXR (or other gene used a single biomarker) that can affect its regulation after irradiation, it could lead to bias in dose estimate. If we use more genes than just FDXR, the dose estimate will still be affected by FDXR inter-individual polymorphisms or promoter methylation levels

(epigenetic differences) but would be counter balanced by the other genes expression values. Furthermore, it is essential to consider inter-individual differences in both the basal FDXR expression and its response to radiation, even though the *in vivo* data collected from radiotherapy patients thus far has shown relatively consistent results. Of course, other factors must also be taken into account while developing new biodosimetry methods. For example, costs will be lower when using one gene and the use of a single calibration curve simplify dose estimations. In conclusion, the identity and number of genes used for biological dosimetry purposes will ultimately be the result of a trade-off between reliability, feasibility, simplicity, and cost. It may finally turn out that using FDXR alone is indeed the best option but at the current stage of research in the field of gene expression-based biodosimetry it is too early to conclude such discussion, and further analyses are required to conclude on the optimal combination of genes for dose estimation.

It is important to keep in mind that the relationships described here are valid for different fractions of circulating leukocytes (Nasser et al. 2021). For other cells, regulation of FDXR expression after irradiation may look slightly different and therefore conclusions of this study should not be transferred to other cellular systems too hastily. Also, regulation of other genes regarded as a potential biodosimetry markers may be (and probably is) different from that of FDXR.

Lack of standardization should be addressed to enhance the accuracy, reproducibility and reliability of gene expression-based biodosimetry. We hope that the present report is a valuable step in this process. However, continued research and collaborative efforts are crucial to harness the full potential of gene expression analysis in leukocytes to provide a robust and widely applicable biological dosimetry method.

To conclude, the present study showed that the best approach to calculation of calibration curve based on FDXR expression data is to use a linear regression to obtain a function that best describes the relationship between the logarithm of absorbed dose and FDXR ΔC_t . This study also showed that standard curve generated in such a way can give reliable dose estimations in a dose range from 50 mGy to 4 Gy and that one should be careful with including data for nonirradiated sample in such calculations.

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Disclosure statement.

The authors report there are no competing interests to declare.

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Figure Legends

Figure 1. Calibration curves based on the FDXR expression data from each participating laboratory with dose plotted on y- or x-axis. For laboratory 3, two curves were calculated: As indicated in the graph titles, the first included the 2 Gy exposed sample, while the second one was generated without 2 Gy sample. Equations of the calibration curves are shown together with standard error of regression (S) values.

Figure 2. Linear standard curves generated using laboratory 1 data with non-irradiated sample excluded from analysis (solid blue line) or represented as 0.01 Gy (dashed green line) or 0.001 Gy (dotted red line).

Figure 3. Box plots of mean FDXR Δ Ct values for donors used in high-dose and low-dose experiments from Manning et al. (2013). Median, first quartile, third quartile, minimum and maximum values are shown. Donors 18 and 19 were regarded as outliers as the mean FDXR Δ Ct values for these donors fall more than 1.5 interquartile range below the first quartile.

Figure 4. (A) Mean FDXR Δ Ct values \pm SD from 8 donors (high dose range experiment, red circles) or 6 donors (low dose range experiment, blue triangles) were plotted against log₂ of dose. Regression function was calculated using data from high-dose experiment only. (B) Data from low-dose experiment were adjusted to high-dose experiment based on gene expression values at 0.1 Gy. Blue dotted line represents expression level in nonirradiated sample from low-dose experiment, while red dashed line represents expression level in nonirradiated sample from high-dose experiment. Regression function is the same as in (A). Data from Manning et al. (2013).

Figure 5. Mean fold changes of FDXR expression in blood after irradiation in a low-dose range (A) and high-dose range (B). Bars represent 95% confidence interval. * <0.05 versus control (t-test). n=6 (low-dose range, panel A) or n=8 (high-dose range, panel B). Data from Manning et al. (2013).