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Anna Francès-Abellán
Universitat Autònoma de Barcelona
Pablo Carrasco
Hospital de la Santa Creu i Sant Pau
Jaime Pérez-Alija
Hospital de la Santa Creu i Sant Pau
Gemma Armengol
Universitat Autònoma de Barcelona
Pere Puig
Universitat Autònoma de Barcelona
Joan Francesc Barquinero
Francesc.Barquinero@uab.cat
Universitat Autònoma de Barcelona

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Statistical methods for dose-response calibration curves with multiple donors for Dicentric Chromosome Assay.

Anna Francès-Abellán[a], Pablo Carrasco[b], Jaime Pérez-Alija[b], Gemma Armengol[a], Pere Puig[c,d], Joan Francesc Barquinero[a*].

[a]Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, Bellaterra E-08193, Catalonia, Spain.

[b]Servei de Radiofísica i Radioprotecció, Hospital de la Santa Creu i Sant Pau, Barcelona, Catalonia, Spain.

[c]Department of Mathematics, Universitat Autònoma de Barcelona, Bellaterra E-08193, Catalonia, Spain.

[d]Centre de Recerca Matemàtica, Universitat Autònoma de Barcelona, Bellaterra E-08193, Catalonia, Spain.

*Corresponding author: Joan Francesc Barquinero, Francesc.Barquinero@uab.cat

ABSTRACT

Biological dosimetry is an essential tool for estimating the absorbed radiation dose in individuals suspected of exposure to ionizing radiation. In this field, the dicentric chromosome assay (DCA) is considered the “gold” standard, but its labour-intensive and time-consuming nature limits its immediate application during large-scale emergencies. Consequently, innovative strategies have arisen to increase scoring throughput in radiation mass casualty incidents. This work follows these operational changes by successfully implementing and validating a three-donor semi-automatic scoring curve, paired with a quick scan methodology, within the UAB biodosimetry laboratory. In addition, a manual curve using the same three donors was completely rebuilt and validated. Furthermore, this research integrates statistical methodologies for curve comparison to find significant differences between donors or laboratories. It also introduces robust alternatives for curve fitting complying with international ISO guidelines, which specify that DCA calibration curves should be constructed from at least two donors. These statistical methodologies will be openly available in following versions of Biodose Tools at https://www.reneb.net/software/.

KEYWORDS: Biological dosimetry, Dicentric Chromosome Assay, Multiple donors, Curve comparison, Curve fitting.

1. INTRODUCTION

Biological dosimetry is recognized as a valuable methodology for estimating the absorbed radiation dose in individuals suspected of exposure to ionizing radiation (IR). It is particularly useful in cases where there are reasons to believe there has been exposure without wearing a dosimeter, in cases where there is no unequivocal dosimetric evidence, or if there is the legal need to assess the exposure over an individual's working life [1]. In addition, it is a tool which can aid during emergency situations where clinical triage of dose exposure is needed for medical management [2,3,4] or provide dose estimates when a nuclear or radiological emergency is investigated [5].

Among biological dosimetry techniques, the Dicentric Chromosome Assay (DCA) is still considered the 'gold standard' [6,7] and has become a routine component of the radiation protection programmes of many countries, due to its specificity, strong dose-response correlation, low background frequency, and stability even months post-exposure [1,8]. In order to use the DCA for dose estimation, a calibration curve with the frequency of dicentrics per cell across different radiation doses needs to be generated. To determine the best fit coefficients of this curve, the suggested statistical technique in the IAEA manual [1] is the maximum likelihood estimation [9,10] using the method of iteratively reweighted least squares (IRLS). In practice, this can be easily achieved using the *glm()* function in R to fit a Generalized Linear Model (GLM). Furthermore, ISO guidelines [11] specify that DCA calibration curves should be constructed from at least two adult, healthy donors and data should be pooled from them. This procedure relies on the assumption that there are no significant differences in radiation-induced dicentrics between donors [12], and hence, individual curves do not differ significantly from each other or from the curve obtained after pooling. However, in practice, this condition may not always be satisfied; thus, a statistical approach to systematically compare and validate the existence of differences is needed. In addition, comparing curves may also be necessary when evaluating results from different laboratories, or when a single laboratory aims to compare curves obtained using different radiation qualities or generated over time.

The DCA is a time-consuming technique, which includes 48 h of cell culture, along with the scoring of typically 500 metaphases to achieve accurate dose estimates. This is feasible for individual dose estimates but can become an issue for large-scale events where hundreds or thousands of individuals may require biodosimetry analysis. In the past years, several strategies have been developed to increase the technique capability,

like the creation of international mutual assistance networks [13] or the implementation of semi-automatic (SA) scoring methods through assisted computer image analysis (e.g. [14,15,16]).

The objectives of this work are to (a) present an updated manual DCA dose-response curve elaborated with three donors; (b) apply the SA scoring methodology to obtain a dose-response curve using three donors to increase the analysis capacity of our laboratory; (c) propose statistical methodologies to detect differences between donors and to fit a dose-response curve combining data from multiple donors; (d) validate these curves using blood samples irradiated at unknown doses; and finally (e) incorporate these statistical methodologies in the biological dosimetry specialized software Biodose Tools [17,18] available at https://www.reneb.net/software/.

2. MATERIAL AND METHODS

2.1 Blood collection and irradiation of the samples

Peripheral blood samples from three different donors, two males and one female (aged 59, 43, and 37, respectively) were obtained to construct the calibration curves. For the validation test, samples were obtained from two additional donors, one male and one female, aged 56 and 48 respectively. In all cases blood samples were obtained under informed consent by venipuncture and collected into heparinized tubes. The study was carried out in accordance with relevant guidelines and regulations, including the Declaration of Helsinki and Spanish legislation on biomedical research (Law 14/2007). This procedure was approved by the Universitat Autònoma de Barcelona (UAB) Ethics Committee on Animal and Human Experimentation (reference number: CEEAH 6587).

To obtain the calibration data for the dose-response curves, blood samples from each of the three donors were irradiated *ex vivo* at doses of 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 Gy. For the validation test, the corresponding samples were irradiated at 0.5 and 3 Gy. Irradiation was performed using 6 MV photon beams from a TrueBeam linear accelerator (Varian Medical Systems, CA, USA) at *Hospital de la Santa Creu i Sant Pau* (Barcelona, Spain). To ensure dose homogeneity, an isocentric setup with two opposed fields (0° and 180°) was used. Samples were placed into two cavities drilled in a solid 20 × 20 cm² polymethyl methacrylate phantom with a thickness of 20 cm in the beam direction, providing full electron equilibrium at the sample position. The required monitor units (MU) were calculated to deliver the prescribed dose to the samples. All irradiations were

delivered at a dose rate of 1 $Gy \cdot min^{-1}$ (100 $MU \cdot min^{-1}$). The attenuation effect of the treatment couch was included in the dose calculations. The linear accelerator output was verified daily using two independent quality assurance systems: Daily QA3 (Sun Nuclear, WI, USA) and Machine Performance Check (Varian Medical Systems). Following irradiation, blood samples were incubated at 37°C for 2 h, in accordance with IAEA recommendations to allow cellular repair mechanisms to take place. Right after, blinded and coded samples were transported at ambient temperature to the UAB for culture and analysis.

2.2 Culture conditions

Cultures were made in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (GIBCO, Thermo-Fisher, Eugene, OR, USA, ref.: 52400-017) supplemented with 20% foetal calf serum (GIBCO, ref.: 10270-106), 1.5% Penicillin/Streptomycin 10,000 U/mL (Biowest, Nuaillé, France, ref.: l0022-100), 2% L-Glutamine 200mM (Biowest, ref.: X0550-100), 5% phytohemagglutinin (GIBCO, ref.: 10576-015) and 1% Heparin 1,000 U/mL (Laboratorio Reig Jofré, Barcelona, Spain, ref.: 608737.4). Each culture of 5 mL was incubated at 37°C at 5% humidity in a CO_2 incubator for 50 hours with 0.7 mL of blood. After 24 hours, 0.05 mL of KaryoMAX Colcemid solution (GIBCO, ref.: 15212-046) was added. Lymphocyte metaphase spreads were prepared and stained using Giemsa (Sigma-Aldrich, Darmstadt, Germany, ref.: 1.09204.0500) for subsequent microscopic analysis.

2.3 Dicentric analysis

The microscopy setup included a Zeiss Axio Imager Z2 microscope coupled to a Metafer4 Slide Scanning System version 3.12.11 (MetaSystems Hard & Software GmbH, Altlussheim, Germany). For the manual curve, chromosomes were counted by hand for each donor: up to 2,500 metaphases for 0 Gy; up to 1,000 for doses from 0.1 to 0.5 Gy, and for doses from 0.75 to 5 Gy dicentrics were counted up to 100 or at least 100 metaphases were counted per dose. Chromosome analysis was conducted exclusively on metaphases containing 45-46 centromeres. For the SA calibration curve, the frequency data was generated by scanning the same slides using the DCScore module integrated into Metafer4 (MetaSystems Hard & Software GmbH). Two DCScore classifiers were evaluated: one trained using metaphases generated in our laboratory, and a classifier kindly provided by Dr. Martin Bucher from the German Federal Office of Radiation

Protection (BfS). As the BfS classifier demonstrated a slightly superior performance, it was selected for the present study.

Obtaining enough suitable metaphases for SA analysis was challenging. Ambient humidity and temperature levels likely affected chromosome spread quality, which led to the adoption of a modified protocol. Rather than validating only automatically detected candidates, a quick scan analysis (QS-DCA) was applied across all metaphases [19,20]. This approach applied to SA analysis recovered analysable images discarded by the DCScore classifier as well as metaphases containing undetected dicentric chromosomes (false negatives). By applying this procedure, although chromosomes were not specifically counted, only metaphases that seemed complete were accepted. The QS-DCA analysis was applied in both the SA dose-response curves and the SA dose estimates. Full QS-DCA protocol used for SA analysis is provided in Supplementary material, section 1.

2.4 Curve fitting

The corresponding individual linear-quadratic curve for each of the three donors was fitted using the dicentric Fitting module from Biodose Tools version 3.7.2. A total of six curves were generated, three using the manual data and three using the SA data.

For the comparison of curves, two statistical approaches are proposed: (a) the Likelihood Ratio Test (LRT) and (b) the Wald Test. Both tests can be adjusted for multiple comparisons using either the Bonferroni or the Benjamini–Hochberg correction. The LRT can only be applied when a true likelihood function is estimated for all curves (e.g. when all curves are fitted using the Poisson model). If one curve is fitted using the quasi-Poisson model to accommodate overdispersion (model dispersion > 1) the Wald test should be applied instead. A full detailed description of these methods is provided in Supplementary material, section 2.

To derive a final dose-response curve combining multiple donors two methods are proposed: the multivariate random-effects model (MRM) and the multivariate inverse-variance weighted model (MWM).

2.4.1 Multivariate random-effects model

This model is indicated for when there are significant differences among the curves we want to pool. It combines evidence from multiple studies (e.g. donors) to make inference about one or more parameters of interest (e.g. regression coefficients). In particular, the

187 *rma.mv()* function available in R [21] can be used to fit suitable multivariate models and
188 account for subject variation.

189 To begin with, for each donor i, we have the estimated coefficients for its curve
190 $(\widehat{C}_i, \widehat{\alpha}_i, \widehat{\beta}_i)$, as well as the corresponding estimated variance-covariance matrix S_i. It is
191 assumed that, for each donor i, the mean number of dicentrics, λ_i, depends on the dose D
192 according to a quadratic function:

193 $$\lambda_i = \widehat{C}_i + \widehat{\alpha}_i D + \widehat{\beta}_i D^2 \qquad (1)$$

194 where the estimated coefficients vector $\hat{\theta}_i = (\widehat{C}_i, \widehat{\alpha}_i, \widehat{\beta}_i)$ can be written as:

195 $$\hat{\theta}_i = \mu + u_i + e_i \qquad (2)$$

196 where $\mu = (\mu_C, \mu_\alpha, \mu_\beta)$ is the fixed-effects vector representing the population-average
197 coefficients; $u_i = (u_{Ci}, u_{\alpha i}, u_{\beta i})$ is the vector of random-effects, describing how donor i
198 deviates from the population mean, and e_i represents the uncertainty or estimation error,
199 which follows a multivariate normal distribution:

200 $$e_i \sim N(0, S_i) \quad (3)$$

201 where S_i is the within-donor covariance matrix of the estimated coefficients of the
202 corresponding curve.

203 The random-effects are assumed to follow a multivariate normal distribution:

204 $$u_i \sim N(0, \Psi) \quad (4)$$

205 The model allows selected coefficients $\{C, \alpha \; or \; \beta\}$ to exhibit between-donor
206 heterogeneity through the variance components τ_C^2, τ_α^2, and τ_β^2. In Biodose Tools, the user
207 can easily specify in which coefficients there are differences after analysing the curves
208 with the LRT or Wald test.

209 $$\Psi = \begin{bmatrix} \tau_C^2 & 0 & 0 \\ 0 & \tau_\alpha^2 & 0 \\ 0 & 0 & \tau_\beta^2 \end{bmatrix} \qquad (5)$$

210 The diagonal structure assumes that any between-donor variation in C, α, and β is
211 independent. However, the estimated coefficients within each donor may be correlated
212 through the covariance matrices S_i:

213 $$\hat{\theta}_i \sim N(\mu, \Psi + S_i) \qquad (6)$$

To estimate the unknown parameters $\mu = (\mu_C, \mu_\alpha, \mu_\beta)$ and $\tau = (\tau_C^2, \tau_\alpha^2, \tau_\beta^2)$ the model is fitted via restricted maximum-likelihood (REML). The resulting estimated curve is:

$$\hat{\lambda} = \widehat{\mu_C} + \widehat{\mu_\alpha}\, D + \widehat{\mu_\beta} D^2 \quad (7)$$

The uncertainty around the estimated dose-response curve is quantified using numeric simulation. A total of 10,000 sets of coefficients are randomly created from a multivariate normal distribution defined by the estimated coefficients vector $\mu = (\mu_C, \mu_\alpha, \mu_\beta)$ and the variance-covariance matrix $\Sigma = \Psi + S_i$. For each simulated set of coefficients, a predicted yield is calculated across the range of doses 1-5 Gy. Then, the 2.5th and 97.5th percentiles of the simulated predictions are calculated at each dose to obtain the corresponding 95% confidence interval.

2.4.2 Multivariate inverse-variance weighted model

This model is for when there are no significant differences between curves, but distribution data is not available (i.e. only the coefficients of the curves and the covariance matrix are available), making it impossible to use the usual GLM approach. In this model, the coefficient vector of each curve, $\hat{\theta}_i = (\widehat{C_i}, \widehat{\alpha_i}, \widehat{\beta_i})$, is weighted according to the inverse of its estimated covariance matrix, so that more precisely estimated curves receive greater weight. The variability of each estimated coefficient vector is represented by its covariance matrix S_i. The covariance matrix of the combined estimate is $\Sigma = (\sum_i S_i^{-1})^{-1}$, from which confidence intervals are calculated as described in the previous method. The coefficient vector of the resulting estimated curve, $\hat{\theta} = (\hat{C}, \hat{\alpha}, \hat{\beta})$, is given by:

$$\hat{\theta} = \left(\sum_i S_i^{-1}\right)^{-1} \left(\sum_i S_i^{-1}\, \hat{\theta}_i\right) \quad (8)$$

2.5 Curve validation

The resulting pooled curves were validated by irradiating the test samples from the two additional donors, labelled as A and B, and assessing their ability to detect total body irradiation at low (0.5 Gy) and high doses (3 Gy) after scoring 500 cells or 100 dicentrics. Dose estimates were calculated using the newly elaborated curves for manual and for SA scoring. In addition, doses were estimated using a previously established curve from our laboratory [22] to evaluate potential differences. All dose estimations were obtained using

the dicentric Dose estimation module from Biodose Tools v3.7.2 and confidence intervals were calculated using the Merkle's 83% option.

3. RESULTS

3.1 Dose-response curves per donor

The results of the manual scoring analysis for the three donors are presented in Fig. 1. For donor 1 and donor 3, the curve was estimated assuming a quasi-Poisson model due to overdispersion (dispersion index (σ^2/y) > 1), while for donor 2 the curve fitting was done assuming a Poisson model ($\sigma^2/y \leq 1$). The detailed distribution data is provided in Supplementary material, Tables S1–S3.

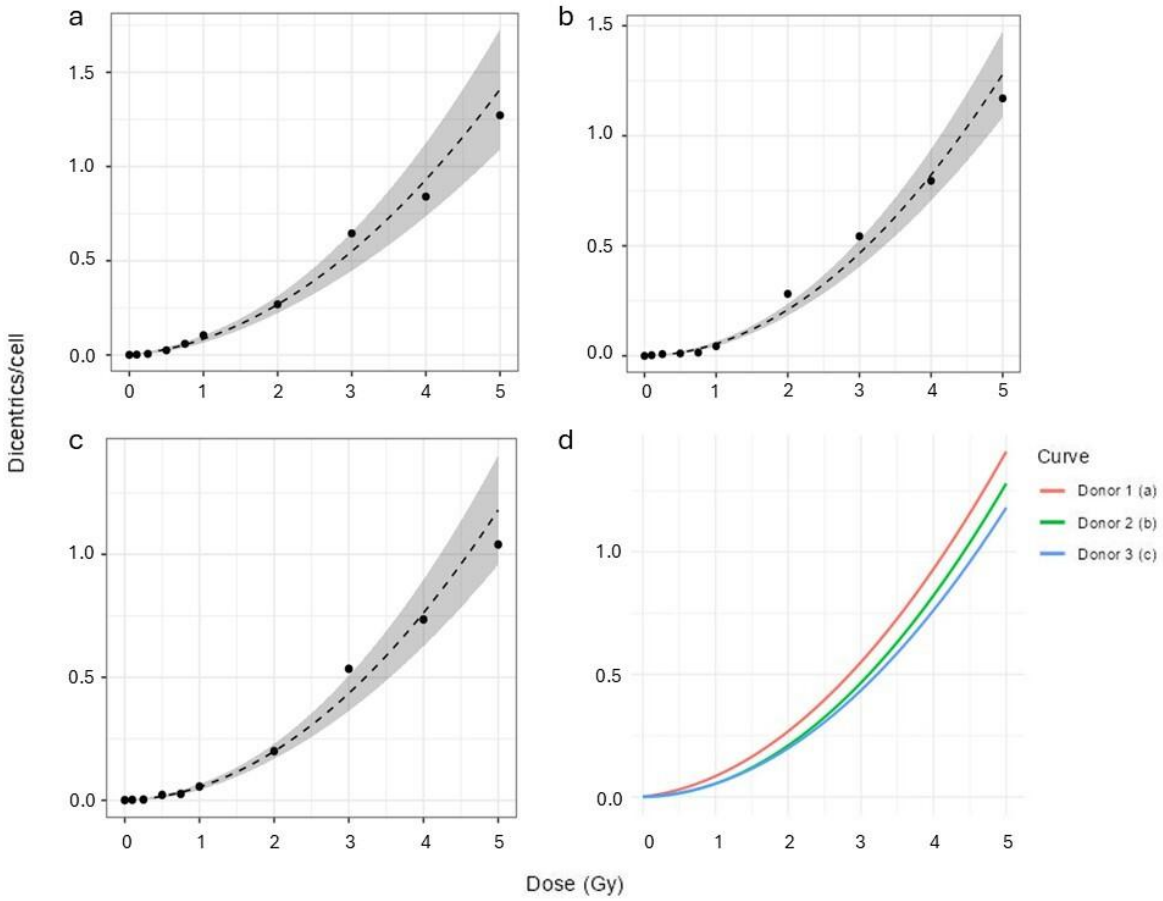


Figure 1. Manual dose-response curve with 95% confidence interval (CI) for *a.* donor 1. *b.* donor 2. *c.* donor 3. *d.* the three curves plotted together.

For donor 1 the obtained coefficients correspond to:

$$\lambda = 0.001\ (\pm 0.0007) + 0.035\ (\pm 0.0101)D + 0.049\ (\pm 0.0058)D^2 \quad (9)$$

For donor 2:

$$\lambda = 8.74 \cdot 10^{-07} (\pm 0.0009) + 4.34 \cdot 10^{-03} (\pm 0.007) D + 5.03 \cdot 10^{-02} (\pm 0.0037) D^2 \quad (10)$$

For donor 3:

$$\lambda = 7.3 \cdot 10^{-04} (\pm 0.0006) + 8.09 \cdot 10^{-03} (\pm 0.006) D + 4.56 \cdot 10^{-02} (\pm 0.0039) D^2 \quad (11)$$

The values in parentheses indicate their respective standard uncertainty. The time required for the manual analysis of 24,882 cells from the three donors was approximately 622 hours.

The results for the SA analysis for the three donors are presented in Fig. 2. For donor 1 and donor 2, the curve was estimated assuming a quasi-Poisson model due to overdispersion ($\sigma^2/y > 1$), while for donor 3 the curve fitting was done assuming a Poisson model ($\sigma^2/y \leq 1$). The detailed distribution data is provided in Supplementary material, Tables S4–S6.

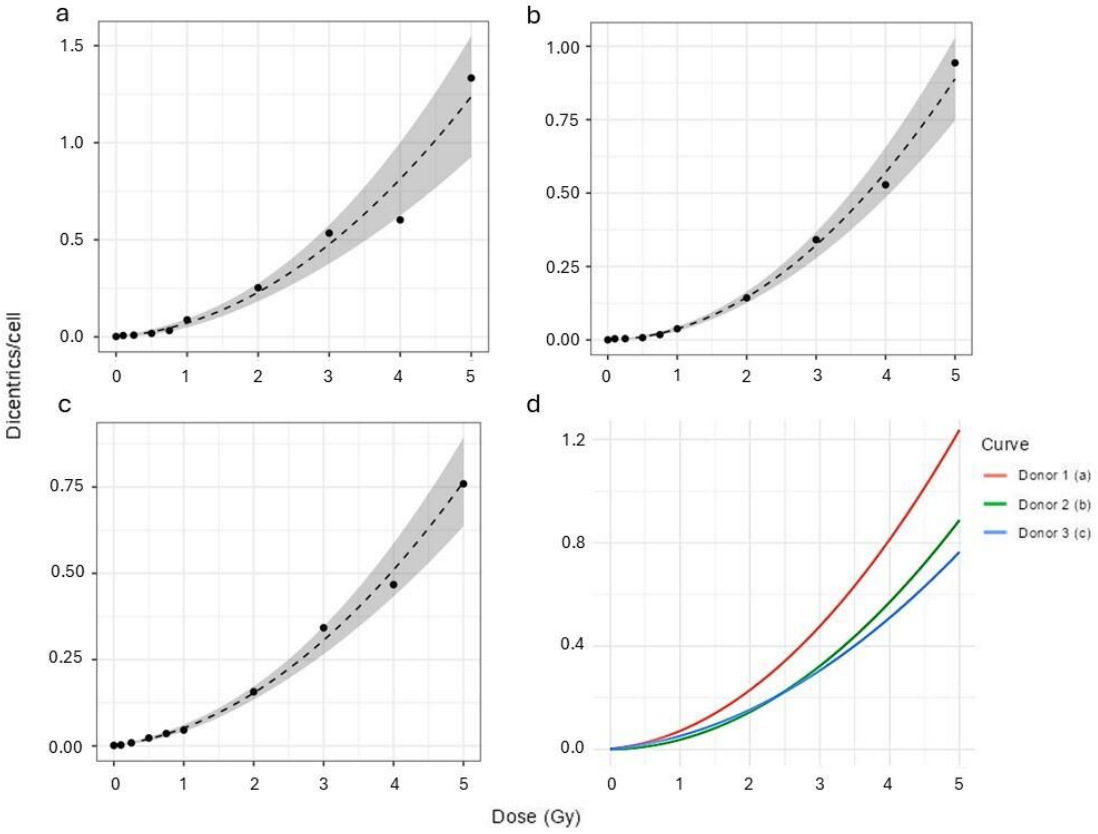


Figure 2. SA dose-response curve with 95% confidence interval (CI) for *a.* donor 1. *b.* donor 2. *c.* donor 3. *d.* the three curves plotted together.

For donor 1 the obtained coefficients correspond to:

$$\lambda = 0.002 (\pm 0.0014) + 0.025 (\pm 0.0119) D + 0.044 (\pm 0.0059) D^2 \quad (12)$$

For donor 2:

$$\lambda = 0.0008\ (\pm 0.0005) + 0.002\ (\pm 0.0047)D + 0.035\ (\pm 0.0026)D^2 \quad (13)$$

And for donor 3:

$$\lambda = 0.0013\ (\pm 0.0006) + 0.025\ (\pm 0.0053)D + 0.025\ (\pm 0.0025)D^2 \quad (14)$$

The time required for the SA analysis of 26,883 cells from the three donors was approximately 42 hours, representing a near 15-fold decrease compared to the manual scoring analysis.

3.2 Curve comparison

Because a Poisson distribution could not always be assumed, and therefore the true likelihood function was not obtained for all the curves, their comparison was performed using the Wald test. To account for multiple tests, a Bonferroni correction was selected. The results of the Wald test for manual scoring (Table 1) indicated significant differences between the curves of donors 1 and 2, and between donors 1 and 3, whereas no significant differences were detected between donors 2 and 3. Table 2 shows how between donors 1 and 2, the difference was attributable to the linear coefficient α. Between donors 1 and 3, although the overall Wald test was significant, none of the individual model coefficients differed significantly, suggesting that the observed difference arose from their joint effect rather than from a single parameter.

For the SA methodology, the Wald test indicated significant differences among the curves of all three donors pairwise (Table 1). Between donors 1 and 3, the differences were associated with the quadratic coefficient (β), whereas between donors 2 and 3, significant differences were observed in both α and β. Between donors 1 and 2, no individual model coefficient showed sufficient statistical evidence to account for the observed difference, as seen in donors 1 and 3 of the manual curves (Table 2).

3.3 Final curve fitting

For the manual scoring methodology, the Wald test indicated significant differences between donors (Table 1) specifically in the linear component (Table 2), therefore the MRM was applied using the coefficient α as the random effect. For the SA scoring methodology, the Wald test also indicated significant differences between donors, and the

MRM was applied including α and β as random effects (Tables 1 and 2). A MWM model was also fitted to show an example of this methodology, however, note that while all three donors were used for the MRM, the MWM could only be fitted for manual scoring using donors 2 and 3, as no significant differences were detected between them (Table 1). Since all three SA curves presented significant differences, the MWM could not be used to fit the SA final curve. Fitted curves are presented in Fig. 3 together with the corresponding GLMs fitted by the IRLS method, which represent the standard approach currently used in most laboratories, and does not consider differences between donors. Full dose-response curves coefficients are provided in Supplementary material, section 3, Tables S7-S12.

Table 1. Wald test statistic for the comparison of manual and semi-automatic scored curves between donors with the associated p-value. A p-value < 0.05 indicates significant differences between curves.

Scoring method	Donor	Statistic	P-value
manual	1-2	17.3185	0.0018*
manual	2-3	2.0155	1
manual	1-3	15.4942	0.0043*
semi-automatic	1-2	24.4101	0.0001*
semi-automatic	2-3	13.4483	0.0113*
semi-automatic	1-3	20.5683	0.0004*

Table 2. Significant Wald test statistic (p-value < 0.05) for the comparison of curve coefficients between donors for manual and semi-automatic scoring methods.

Scoring method	Donor	Coefficient	Statistic	P-value
manual	1-2	α	6.1985	0.0384*
semi-automatic	1-3	β	8.4908	0.0107*
semi-automatic	2-3	α	10.357	0.0039*
semi-automatic	2-3	β	6.7861	0.0276*

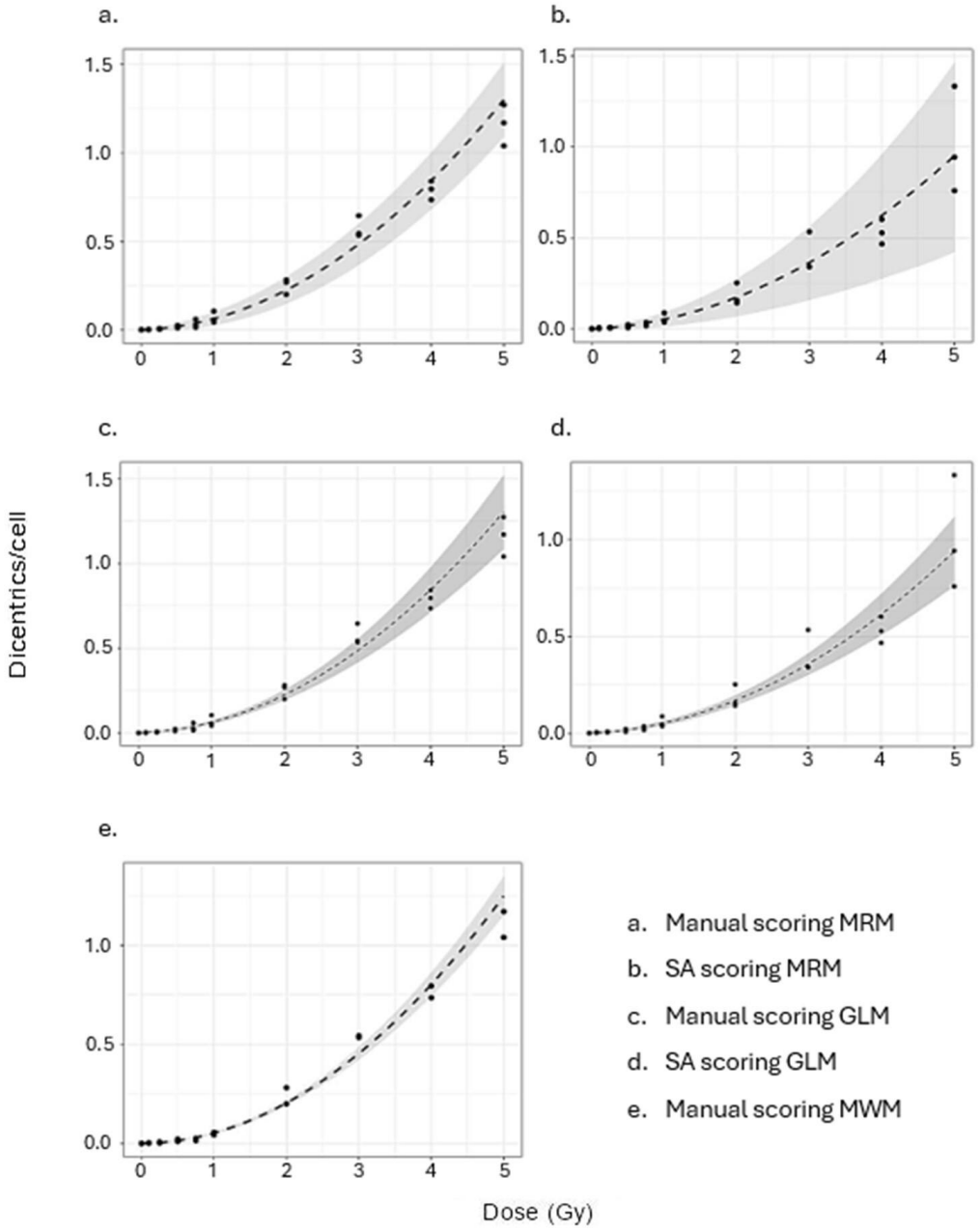


Figure 3. Dose-response curves and confidence intervals with three donors: *a*. manual scoring fitted using a Multivariate Random-effects Model (MRM). *b*. semi-automatic (SA) scoring fitted using a MRM. *c*. manual scoring fitted using a Generalized Linear Model (GLM). *d*. SA scoring fitted using GLM. *e*. manual scoring fitted using a Multivariate inverse-variance Weighted Model (MWM).

3.4 Curve validation

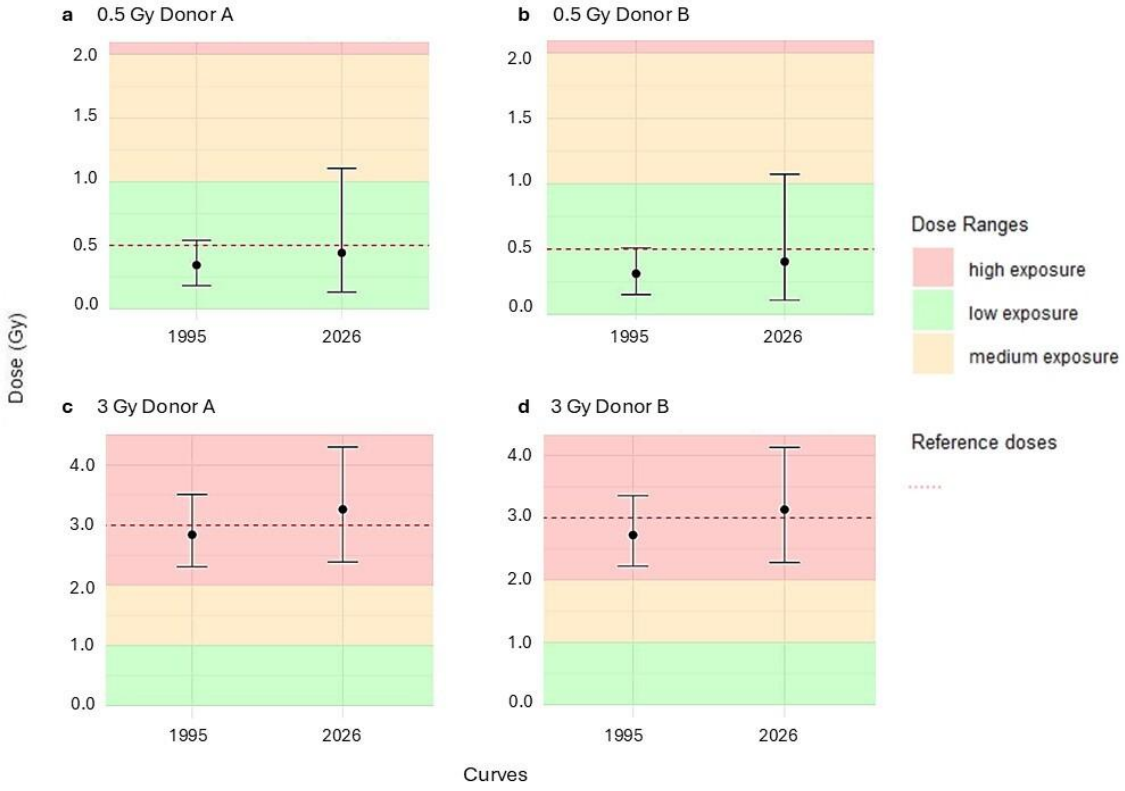
For both the manual and SA scoring of 500 cells (or 100 dicentrics), the true dose was contained within the 95% confidence interval for all estimated doses (Table 3). The MRM produced dose estimates closest to those derived from the GLM with wider confidence

intervals. For the four samples analysed, the manual method required approximately 50 hours, whereas the SA method reduced the required analysis time to only 3 hours. Full dicentric distribution and cell count is provided in Supplementary material, section 3, Table S13.

Table 3. Dose estimates with confidence interval (Merkle's 83%) for doses 0.5 and 3 Gy, for donors A and B using different fitting methodologies (GLM = Generalized Linear Model; MRM = Multivariate Random-effects Model; MWM = Multivariate inverse-variance Weighted Model) for manual and semi-automatic scoring.

Dose	Donor	Scoring method	GLM	MRM	MWM
0.5	A	manual	0.44 (0.24-0.68)	0.44 (0.13-1.1)	0.53 (0.33-0.76)
0.5	B	manual	0.40 (0.20-0.65)	0.40 (0.11-1.07)	0.49 (0.29-0.72)
3	A	manual	3.27 (2.65-4.04)	3.27 (2.39-4.3)	3.31 (2.73-4.03)
3	B	manual	3.13 (2.55-3.86)	3.13 (2.28-4.13)	3.17 (2.62-3.85)
0.5	A	semi-automatic	0.5 (0.27-0.78)	0.49 (0.15-1.5)	-
0.5	B	semi-automatic	0.54 (0.30-0.82)	0.52 (0.17-1.55)	-
3	A	semi-automatic	3.42 (2.78-4.23)	3.39 (2.17-6.65)	-
3	B	semi-automatic	3.4 (2.78-4.18)	3.37 (2.18-6.57)	-

The comparison of the new manual calibration curve fitted using a MRM, with the one established in the same laboratory in 1995, revealed a statistically significant difference in the β coefficient (*p-value* = 0.003). The results indicated that the new curve provided a better fit for low-dose estimations than the curve from 1995 (Fig. 4), while at higher doses the previous curve showed a tendency to underestimate and the newly generated curve tended to overestimate.



342

343 **Figure 4.** Dose estimates with confidence interval (Merkle's 83%) for: *a*. Donor A 0.5 Gy. *b*.
344 Donor B 0.5 Gy. *c*. Donor A 3 Gy and *d*. Donor B 3 Gy, obtained with the curve from Barquinero
345 et al. [22] (1995, left) and the manual Multivariate Random-effects Model (2026, right).

346

347 **4. DISCUSSION**

348 Several laboratories have already included the SA analysis as part of their standard
349 protocol, with both manual and SA scoring being compared during interlaboratory
350 comparisons (e.g. [23,24,25]). The SA analysis procedure includes automatic metaphase
351 finding, image capture at high resolution, and detection of dicentric candidates through a
352 previously trained classifier. These automatically detected dicentric candidates are then
353 evaluated by a trained human scorer. However, in the present study the quality of the
354 obtained metaphases difficulted the correct operation of the classifier. Even when the
355 classifier was trained with comparable quality metaphases, the results were misleading.
356 In this situation, where most of the dicentrics were not even detected and a limited number
357 of metaphases were available to analyse, a quick scan (QS-DCA) was applied across all
358 images. This step recovered false negatives and analysable images that had been

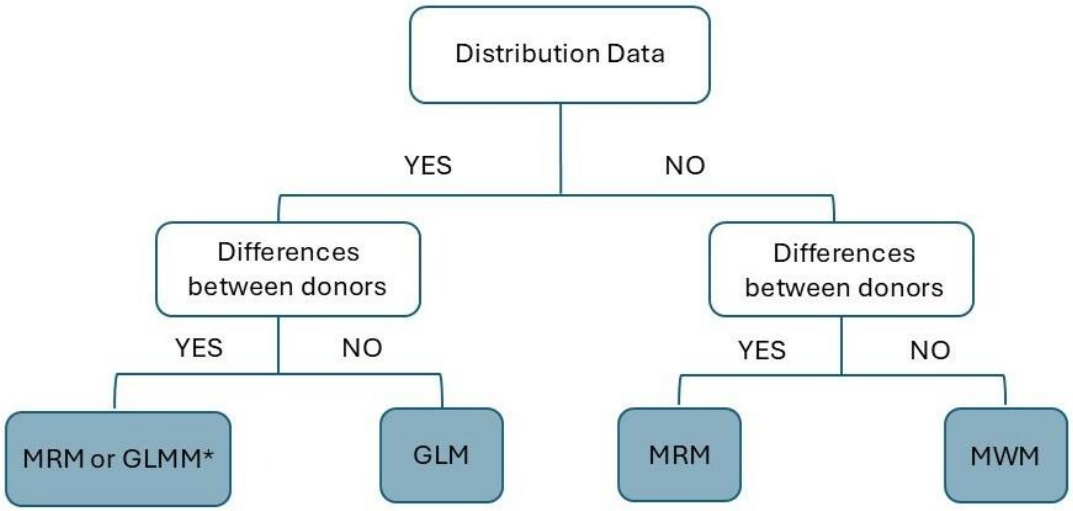
discarded by the algorithm, maximizing the limited data in both the SA curves and SA dose estimates. The QS-DCA technique which has been previously validated (e.g. [20,26]) has also proven effective for SA analysis. However, a closer examination of Fig. 1 and 2 reveals greater inter-donor variability in the semi-automatically scored calibration curves compared with the manually scored curves. It can also be observed how, despite the implementation of the modified SA scoring protocol, the semi-automatically scored calibration curve exhibited a lower quadratic coefficient than the manually scored curves, consistent with the pattern reported for other conventional SA curves (e.g. [14,27]). This behaviour was not observed in Romm et al. [28], where the calibration curves obtained using a manual and a QS-DCA scoring method were very similar, with no significant differences in the linear-quadratic coefficients. Our increased variability may be attributable to the rapid nature of the quick-scan itself on an already machine-analysed sample, which can influence the scorer outcome. Therefore, it seems that when using this modified SA scoring procedure is even more important to apply methods that account for donor variability when using multiple donors. In any case, the dose estimates reported with the QS-DCA applied to the SA analysis were satisfactory (i.e. inside a ± 0.5 Gy limit).

This SA scoring methodology is expected to substantially increase our laboratory's analytical capacity compared with the previous manual approach. Under our current laboratory configuration, which includes two analysis stations, only one or two samples per day can be analysed manually scoring 500 metaphases, whereas up to 22 samples could be processed per day using the SA analysis. Even when applying a manual triage protocol based on scoring 50 metaphases per sample [29,30,31] the throughput remained limited to approximately 12 samples per day. In contrast, implementing the SA approach would increase the daily capacity to approximately 192 samples, representing a major improvement for emergency biodosimetry response. Furthermore, if we take the validation data from the SA scoring (available in Table S11) and re-estimate the doses 0.5 Gy and 3 Gy using only 50 metaphases, the results obtained for donor A (0.83 Gy and 2.89 Gy, respectively) and donor B (0.52 Gy and 2.98 Gy, respectively) still fall within their correct clinical triage categories (low exposure: 0–1 Gy, medium exposure: 1–2 Gy, and high exposure: >2 Gy) supporting that the increase in analytical capacity is achieved without compromising clinical triage effectiveness for rapid dose categorization.

Following the IAEA guidelines [1], the best-fitting value of each coefficient of a dose-response curve is obtained by maximizing the likelihood function using the IRLS method, under the assumption that the observations follow a Poisson distribution. The *glm()* function from the *stats* package in R approximates a GLM using the IRLS method from non-normal response variables (i.e. Poisson). Since ISO guidelines [11] specify the use of at least two healthy donors when constructing the dose-response curve using DCA, the intuitive default procedure can be to pool the data collected for each donor to fit a joint calibration curve. However, using GLMs would be only valid when there are no statistical differences between donors, as in Romm et al. [14], or if all data originates from a single donor. This is because a GLM approach treats inter-donor variability as global residual noise, thereby, if such biological heterogeneity exists, it is entirely ignored [32, 33]. The results from the comparison among donors in Tables 1 and 2 show how, although inter-individual variation of dicentric counts between adults is commonly assumed to be almost negligible for conventional manual scoring [12], these differences can be significant and must be checked before pooling data together. For SA scoring there is not a stablished consensus [34] but due to the observed greater inter-donor variability compared with the manually scored curves, it seems even more important to check for differences between donors when using this type of scoring method. In the present study, we propose two methods to evaluate variations between donors, the LRT and the Wald test. Since some of our curves presented overdispersion, a quasi-Poisson model was used and only the Wald test could be applied. However, since this will not be always the case, both tests will be included in Biodose Tools.

To fit a dose-response curve (Fig. 5) in the presence of significant inter-donor differences, a General Linear Mixed Model was explored first. However, fitting this model using our dicentric data introduced significant computational difficulties, and frequently led to non-convergence limiting their viability for a general use. Therefore, an alternative option was taken into consideration, and a two stage meta-analytic structure was explored, first fitting independently for each donor, and then treating each donor's fitted curve as an independent study. This resulted in a MRM, where curves are first fitted independently, and their resulting parameters are combined accounting for both the correlation within each donor curve, and the heterogeneity between donors through the inclusion of a random-effects structure. This methodology has also been used to generate pooled dose-response curves using curves from different laboratories to analyse the effect of distinct

radiation types (J. Einbeck, personal communication). Moreover, this meta-analytic approach can also be applied when there are no differences between curves, but the usual GLM is not applicable because dicentric distribution data is unavailable. In this case, the MWM offers a meta-analytic alternative by aggregating separate curves into a unified model, where each curve represents an individual donor from which only the curve coefficients and the covariance matrix are available (e.g. old curves or curves from different laboratories). If no statistically significant differences are detected, the MWM can be applied to generate a pooled curve retaining all donors (i.e. curves) and assigning greater influence on curves with a better fit and lower uncertainty, reducing the disproportionate influence of highly variable curves. This allows to combine data from more than two donors satisfying the ISO standard [11] requirement. Nevertheless, because it lacks the capacity of random-effects models to incorporate inter-donor variability, its application is restricted to cases where the curves show no statistically significant differences. For cases where there are significant differences, the MRM is the best option.



445 **Figure 5.** Decision making diagram for choosing the best fitting model under different scenarios.
446 Distribution data refers to raw aberration distribution and cell counts. When distribution data is
447 not provided it means that only the curve coefficients (and the covariance matrix) are supplied.
448 *The available models in Biodose Tools are: GLM = Generalized Linear Model; MRM =
449 Multivariate Random-effects Model; and MWM = Multivariate inverse-variance Weighted
450 Model.

451 In this context, a statistical module in Biodose Tools was developed to facilitate the whole
452 process involving the comparison of dose–response curves and the fitting using multiple
453 donors. The Multiple donors module provides two complementary approaches for curve
454 comparison: the Wald test, which identifies coefficient(s) exhibiting significant
455 variability, and can be applied to curves fitted using a quasi-Poisson model; and the LRT,
456 a widely used method for comparative model evaluation. Following the identification of
457 significant differences between curves, an informed decision regarding the fitting method
458 can be made. Both the MRM and the MWM are available in the Multiple donors module,
459 and the GLM in the Fitting module.

460 The comparison of the newly generated manual calibration curve fitted using a MRM,
461 with the one established in the same laboratory in 1995 indicated that the new curve
462 provided a better fit for low-dose estimations (Fig. 4), while at higher doses the previous
463 curve from 1995 showed a tendency to underestimate and the newly generated curve
464 tended to overestimate. This difference is attributable to methodological changes
465 introduced over the years, particularly improvements in microscopy technologies and the
466 refinement of scoring criteria, which have resulted in a more restrictive criteria for the

identification and acceptance of dicentric chromosomes. These changes have influenced the observed dicentric yield and, consequently, the shape of the calibration curve and dose estimation.

The overall results obtained from donors A and B support the validity and performance of the newly generated calibration curves, with an increase in the confidence intervals for dose estimates (Table 3, column MRM) to accommodate the observed donor variability. Moreover, the incorporation of the SA scoring methodology in our laboratory provides a valuable tool with the potential to assist during large-scale radiation events where the timely processing of numerous samples is essential. However, the transition towards a fully automated procedure capable of both rapidly and reliably analyse multiple samples will require the development and validation of additional methodologies. In particular, the integration of artificial intelligence-based tools (e.g. [35,36,37,38]) may offer significant opportunities to improve both the capacity and accuracy of automated DCA in a near future.

5. CONCLUSIONS

This work has successfully implemented the SA methodology to the UAB biodosimetry laboratory and has updated the manual curve in accordance with the actual scoring protocol. Furthermore, this work offers the application of statistical methodologies for curve comparison in the online free platform Biodose Tools, along with alternatives for curve fitting in accordance with ISO guidelines.

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AUTHOR CONTRIBUTIONS

AFA: Investigation, Methodology, Software, Writing-Original Draft; PC: Resources, Writing-Review and Editing; JPA: Resources, Writing-Review and Editing; PP: Methodology, Writing-Review and Editing; GA: Writing-Review and Editing; JFB: Writing-Review and Editing, Supervision, Project administration.

DATA AVAILABILITY

All relevant data are available from within the manuscript and the supplemental information files, or the Catalan Open Research Area (CORA) repository https://dataverse.csuc.cat/

ADDITIONAL INFORMATION

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE LEGENDS

Figure 1. Manual dose-response curve with 95% confidence interval (CI) for *a.* donor 1. *b.* donor 2. *c.* donor 3. *d.* the three curves plotted together.

Figure 2. SA dose-response curve with 95% confidence interval (CI) for *a.* donor 1. *b.* donor 2. *c.* donor 3. *d.* the three curves plotted together.

Figure 3. Dose-response curves and confidence intervals with three donors: *a*. manual scoring fitted using a Multivariate Random-effects Model (MRM). *b*. semi-automatic (SA) scoring fitted using a MRM. *c*. manual scoring fitted using a Generalized Linear Model (GLM). *d*. SA scoring fitted using GLM. *e*. manual scoring fitted using a Multivariate inverse-variance Weighted Model (MWM).

Figure 4. Dose estimates with confidence interval (Merkle's 83%) for: *a*. Donor A 0.5 Gy. *b*. Donor B 0.5 Gy. *c*. Donor A 3 Gy and *d*. Donor B 3 Gy, obtained with the curve from Barquinero et al. [22] (1995, left) and the manual Multivariate Random-effects Model (2026, right).

Figure 5. Decision making diagram for choosing the best fitting model under different scenarios. Distribution data refers to raw aberration distribution and cell counts. When distribution data is not provided it means that only the curve coefficients (and the covariance matrix) are supplied. *The available models in Biodose Tools are: GLM = Generalized Linear Model; MRM = Multivariate Random-effects Model; and MWM = Multivariate inverse-variance Weighted Model.

TABLES

Table 1. Wald test statistic for the comparison of manual and semi-automatic scored curves between donors with the associated p-value. A p-value < 0.05 indicates significant differences between curves.

Scoring method	Donor	Statistic	P-value
manual	1-2	17.3185	0.0018*
manual	2-3	2.0155	1
manual	1-3	15.4942	0.0043*
semi-automatic	1-2	24.4101	0.0001*
semi-automatic	2-3	13.4483	0.0113*
semi-automatic	1-3	20.5683	0.0004*

Table 2. Significant Wald test statistic (p-value < 0.05) for the comparison of curve coefficients between donors for manual and semi-automatic scoring methods.

Scoring method	Donor	Coefficient	Statistic	P-value
manual	1-2	α	6.1985	0.0384*
semi-automatic	1-3	β	8.4908	0.0107*
semi-automatic	2-3	α	10.357	0.0039*
semi-automatic	2-3	β	6.7861	0.0276*

Table 3. Dose estimates with confidence interval (Merkle's 83%) for doses 0.5 and 3 Gy, for donors A and B using different fitting methodologies (GLM = Generalized Linear Model; MRM = Multivariate Random-effects Model; MWM = Multivariate inverse-variance Weighted Model) for manual and semi-automatic scoring.

Dose	Donor	Scoring method	GLM	MRM	MWM
0.5	A	manual	0.44 (0.24-0.68)	0.44 (0.13-1.1)	0.53 (0.33-0.76)
0.5	B	manual	0.40 (0.20-0.65)	0.40 (0.11-1.07)	0.49 (0.29-0.72)

3	A	manual	3.27 (2.65-4.04)	3.27 (2.39-4.3)	3.31 (2.73-4.03)
3	B	manual	3.13 (2.55-3.86)	3.13 (2.28-4.13)	3.17 (2.62-3.85)
0.5	A	semi-automatic	0.5 (0.27-0.78)	0.49 (0.15-1.5)	-
0.5	B	semi-automatic	0.54 (0.30-0.82)	0.52 (0.17-1.55)	-
3	A	semi-automatic	3.42 (2.78-4.23)	3.39 (2.17-6.65)	-
3	B	semi-automatic	3.4 (2.78-4.18)	3.37 (2.18-6.57)	-

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Supplementary Files

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