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# Validation of the Cytokinesis-Block Micronucleus (CBMN) assay for use as a triage biological dosimetry tool.

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# Acknowledgements

**Introduction**

This study was conducted as part of the ongoing agenda of CRTI project #06-0146RD, entitled "Rapid Identification of Radiologically-Exposed Individuals for Medical Casualty Management". The overall objective of this CRTI project is to develop rapid tools to identify individuals exposed to clinically significant doses of ionizing radiation following a mass casualty radiological/nuclear event. Traditionally, the dicentric chromosome assay (DCA) has been used to derive biological-dose estimates for potential unknown exposures. However, this assay is labour intensive and requires a great degree of technical expertise to be conducted appropriately. Efforts to increase the throughput of the DCA assay and establish a network of competent laboratories, the National Biological Dosimetry Response Plan (NBDRP), was initiated in CRTI project #0027RD and maintained in the current CRTI project #0146RD. However, alternative high-throughput assays are being evaluated as possible initial screening tools to identify potentially exposed individuals. These could conceivably increase the rate of sample turnover and reduce the number of samples required to be scored by the high-accuracy, low-throughput DCA technique.

One proposed high-throughput alternative to the DCA assay is the cytokinesis-block micronucleus (CBMN) assay. The CBMN assay essentially measures the same genetic endpoints as the DCA assay (e.g. clastogenic damage), however a slightly different culturing procedure and scoring technique is applied. Unlike the DCA assay, the CBMN assay is subject to variation in the background level of clastogenic aberrations among un-exposed subjects due to a variety of factors including: age, gender, smoking and diet. However, it has been proposed that at clinically relevant doses of ionizing radiation (e.g. > 1 Gy), the assay is sufficiently sensitive to identify potentially exposed individuals from those with elevated background clastogenic damage and this assay may serve as a valid screening tool.

The primary goal of the current study was to establish dose-response curves for the CBMN assay in each of three NBDRP laboratories [Health Canada (HC), Atomic Energy of Canada Ltd (AECL), and Defence Research Establishment Ottawa (DRDC)]. Blood from each of 6 donors was aliquoted into 8 separate tubes, with each tube receiving a different dose (0, 0.1, 0.25, 0.5, 1, 2, 3 and 4Gy) of γ-radiation. The same donors were used for the HC and DRDC samples, while AECL used a separate set of donors. The participating laboratories processed the samples according to a common CBMN protocol (See Appendix A). Once the assay was completed, the slides were blinded and scored by 6 individuals at HC, 4 individuals at AECL and 2 individuals at DRDC, resulting in a total of 12 individual scorers each assessing 48 blinded samples for use in establishing dose-response curves.

From this data, the variability within and between labs was evaluated to determine whether a single cross-laboratory curve could be applied to the data or whether each laboratory needed a lab-specific curve. Furthermore, an evaluation of the individual scorers in each lab was evaluated to determine whether all individuals within a given laboratory score samples similarly. Finally, a sensitivity analysis was conducted on the CBMN data to determine the lowest dose significantly different from background when the total number of cells scored varied between 50 and 500. Table 1 presents a brief outline of the experiment.

**Methodology**

*Collection of Blood*
All procedures related to the collection and handling of blood samples from human volunteers were conducted according to a protocols approved by the appropriate institutional human ethics committees. Consent forms and questionnaires were obtained for each donor to capture information about age, gender and recent therapeutic history. Blood was collected by venipuncture of the cubital or cephalic vein into 7 ml lithium-heparinized (100 U/tube) Vacutainer tubes (Becton Dickinson, NJ). Three male and three female donors were recruited, with one donor per gender at approximately 20, 40 or 60 years of age. The blood samples from each donor were aliquoted into 8 separate tubes and each tube was assigned to one of 8 γ-irradiation exposures (0, 0.1, 0.25, 0.5, 1, 2, 3 and 4Gy) using a GammaCell40 (Atomic Energy of Canada Ltd; Cs[137], dose rate ~82 cGy/min).

*Cytokinesis-block Micronucleus Assay*
Briefly, whole blood samples were diluted 1:9 with RPMI 1640 culture medium (Invitrogen, Burlington, ON) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, Oakville, ON) to achieve 10 mL cultures in 25 cm$^2$ vented flasks (Nalge-Nunc, Rochester, NY) with a leukocyte cell concentration of approximately 0.5-1.0 x 10$^6$ cells/ml. The cultures were mitogen-stimulated by addition of 100 µL phytohemagluttinen (PHA) (Invitrogen). After stimulation with 1% PHA, blood cultures were incubated (37$^o$C, 5% CO$_2$) for 44 h before addition of cytochalasin B (final concentration: 4 µg/ml) (Sigma). After an additional 28 h incubation period, cell suspensions were transferred to 15 ml centrifuge tubes and the cells were centrifuged for 8 min at 200 x *g*. The supernatant was removed and the cell pellets were then re-suspended in 10 ml of 75 mM KCl and incubated for 5 min at room temperature before addition of 2 ml of fixative (5:1 methanol:glacial acetic acid). The samples were allowed to stand for a further 10 min and were then centrifuged for 8 min at 200 x *g*. The supernatant was again removed and the cell pellets were re-suspended in 10 mL of fixative and allowed to stand for 10 min. This fixation step was repeated twice more to remove cellular debris. Finally, the fixed cell suspension was re-suspended in a small volume (~200 µl) of fixative to achieve the desired cell concentration for slide preparation. The cell suspensions were dropped onto pre-cleaned Fisher*finest* glass slides (Fisher Scientific, Ottawa, ON) maintained in pre-warmed/humidified Hanabi Metaphase Spreader (Adstec Inc., Japan), then dried overnight on a 37$^o$C slide-warmer. Slides were stained with 10 µg/ml acridine orange (Sigma) on the day of analysis, visualized at 200X magnification under fluorescence and micronuclei (MN) were scored according to the criteria outlined in Appendix A. MN were quantitated in 50 to 500 binucleated (BN) cells for each slide by each donor. A total of 48 slides were scored in a blinded fashion by 12 independent scorers across three laboratories. The results were not decoded until all samples were analyzed.

*Statistical Analysis*
The first objective of this study was to establish dose-response curves for each of the three participating NBDRP laboratories. To accomplish this objective, the following analyses were conducted: (i) establish the appropriate statistical model to fit the CBMN

dose-response data, (ii) evaluate the intra- and inter-laboratory variability, (iii) compare scorers within each laboratory, and (iv) compare laboratories.


*(i) Dose Response Modeling*

The Poisson distribution was used to characterize the ratio of MN cells to BN cells scored. Both Generalized Linear Models (GLM) and Generalized Linear Mixed Models (GLMM) were used to fit the dose response relationship of rate MN cells using the SAS macro GLIMMIX (SAS web link:  http://support.sas.com/kb/25/030.html).  The advantage of using GLMM over GLM is the ability to explain the random variability within and between labs due to scorers and donors. Three models were considered in order to characterize the dose response relationship of rate MN per 500 BN cells scored. Traditionally in Poisson Regression, the natural log of the rates are modelled, therefore the first two models considered were the linear Poisson regression model (LPRM) and the quadratic Poisson regression model with log link (QPRM-L). These two models are defined as follows: LPRM: $y = \exp(a_1 + b_1 x)$, and QPRM-L: $y = \exp(a_2 + b_2 x + c_2 x^2)$, where *y* is the rate of MN cells per 500 TC scored, *x* is the dose of radiation (*x*=0, 0.1, 0.25, 0.5, 1, 2, 3, 4 Gy), and *exp* is the exponential function. The regression coefficients $a_1$ and $a_2$ describe the background rate of MN cells scored in their respective models. The regression coefficients $b_1$ and $b_2$ indicate the rate of increase of MN cells with respect to dose, in their respective models. Finally the regression coefficient $c_2$  in the QPRM-L characterizes the curvature of the data. The third model that was considered was a quadratic Poisson Regression model with identity link (QPRM-I). This last model is traditionally used to characterize DCA data (Merkle, 1983). The QPRM-I model is defined as: $y = a_3 + b_3 x + c_3 x^2$, where *y* and *x* are defined above, and the regression coefficients are interpreted similar to those for QPRM-L. Without loss of generality the error terms are omitted from the models for convenience of notation, here and throughout, and are assumed to follow the Poisson distribution.

In order to assess the goodness of fit from these three models the following was considered: the scaled deviance for each model along with a graph of the model fit to the data and biological consideration. Models with smaller deviance were considered to be a better fit.  Models exhibiting over-/under- dispersion were corrected using the Pearson scaled deviance. Based upon the dose response modeling analysis (see Results Section), the QPRM-I model was used as the model of choice for the remainder of the analysis.


*(ii) Intra- and Inter-laboratory Variability*

Each of the three labs involved in the study had different scorers.  As well two of the labs used one set of donors, whereas the third lab used a different set of donors. Both scorers and donors introduce variability in the study that could potentially explain variability within and between labs. In order to investigate this, generalized linear mixed models (GLMMs) with two random effects scorers ($s_i$, *i*=1, 2, ..., *I*) and donors ($d_j$, *j*=1, 2, ..., *J*) were fit the data.

Each lab was initially analyzed separately to characterize the within lab variability. The QPRM-I model was used to relate the rate of MN cells per 500 BN cells scored to dose (*x*), with scorer ($s_i$) and donor ($d_j$) as random effects.

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$y = a + bx + cx^2 + s_i + d_j$ (1). The intercept *a* corresponds to the background rate of MN cells, *b* is the slope of the dose response curve and *c* is the curvature of the dose response curve. A value of *b=0* corresponds to a no dose effect whereas *b>0* reflects an increasing trend in the rate of MN cells per 500 TC scored with increasing dose. A value of *c=0* corresponds to a linear relationship, whereas *c>0 (c<0)* reflects a concave (convex) quadratic relationship. The parameters *a*, *b* and *c* are unknown fixed effects. The random effects $s_i$ and $d_j$ are assumed to be mutually independent and normally distributed variates with mean zero and variances $\sigma_s^2$ and $\sigma_d^2$, respectively (Drum and McCullagh,1993). The variance component $\sigma_s^2$ measured the degree of heterogeneity between scorers and $\sigma_d^2$ measured the degree of heterogeneity between donors. The Restricted Maximum Likelihood method (REML) was used to estimate the variance components for the random effects and Maximum Likelihood method (ML) was used to estimate fixed effect parameters *a*, *b* and *c* (Wolfinger and O'Connell, 1993).

The global null hypothesis $H_0 : \sigma_d^2 = \sigma_s^2 = 0$ was tested using the Likelihood Ratio Test (LRT). Failure to reject the global null hypothesis would imply that the data followed the conventional QPRM-I. The LRT is (-2) times the difference of the Restricted Log Likelihood (RELL) of the full model (1) and the reduced QPRM-I (2), where we assume no extra variance due to scorers and donors: $y = a + bx + cx^2$ (2). The LRT follows the $\chi^2$ distribution with degrees of freedom equal to the number of positive parameter estimates being tested. If the global null hypothesis is rejected ($p(\chi^2)<0.05$) in favour of the alternative (*H_a*: *at least one of the covariance components is not equal to zero*) then individual variance components test statistics were conducted ($H_o : \sigma_k^2 = 0$, $H_a : \sigma_k^2 \neq 0$, *where k = s or d*). For example, suppose one is interested in testing for no donor level over-/under- dispersion after accounting for scorer level over-/under- dispersion. The null hypothesis is $H_o : \sigma_d^2 = 0$ with $\sigma_s^2$ unspecified. This individual variance component test statistics required fitting the quadratic Poisson regression mixed model under the null hypothesis without the donor random effect $d_j$:
$y = a + bx + cx^2 + s_i$ (3). Again the LRT would be used to compare the restricted log likelihood of the full model (1) to the reduced model (3). Similarly, if the interest is to test for no scorer level over-/under- dispersion ($H_o : \sigma_s^2 = 0$ with $\sigma_d^2$ unspecified) then the LRT would be used to compare the restricted log likelihood of the full model (1) with the reduced model: $y = a + bx + cx^2 + d_j$ (4). A similar methodology was applied when characterizing the between lab variability.

*Comparison of scorers*

To compare scorers within a lab, simultaneous QPRM-I were fit to the data within a lab. In this setting scorers were treated as a fixed effect since the objective was to allow a different dose response curve for each scorer and then compare them. The random effect of donor ($d_j$) was kept in the model in order to account for the variability due to donors. The model was $y = a + bx + cx^2 + a_i + b_i x + c_i x^2 + d_j$ (5), where *y=a+bx+cx²* is the QPRM-I for the lab under consideration. The parameters $a_i$, $b_i$, $c_i$ *(i=1,2, … , I)* are the background rate, slope and curvature, respectively, of the QPRM-I for each scorer *i*. If the scorers within a lab have the same background rate then the F-

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test for the null hypothesis ($H_0$: $a_i$=0, $i$=1,2,..$I$) would be insignificant, i.e. p>0.05. Conversely, if the null hypothesis is rejected in favour of the alternative ($H_a$: *at least one of scorers has a different background rate from the rest*), then multiple comparison tests were carried out to compare all possible pairs of scorers. Bonferroni corrections were used to ensure the overall Type 1 error rate was less than 0.05.  A similar approach was used when assessing the slope and curvature parameters of the model.

*Comparison of Labs*

A similar methodology was applied when comparing labs. Again simultaneous QPRM-I were fit to the data, where each lab was allowed its own dose response curve. The random effects of scorers and donors were included in the model in order to account for the variability due to scorers and donors in each lab.   The following simultaneous QPRM-I was fit to the data, for all three labs simultaneously, $y = a + bx + cx^2 + a_l + b_l x + c_l x^2 + s_i + d_j$  (6). Again,  $y=a+bx+cx^2$ is the usual QPRM-I across all three labs and the parameter  $a_l, b_l$ and $c_l$ ($l$=1, 2, 3) are the background rate, slope and curvature respectively of the QPRM-I  for each lab $l$.  If labs have similar background rates then the F-test for the null hypothesis $H_0$: $a_l$=0, $l$=1, 2, 3 would be insignificant i.e., p>0.05.  Conversely, if the null hypothesis is rejected in favour of the alternative ($H_a$: *at least one of labs has a different background rate from the others*), then multiple comparison tests were carried out to compare all possible pairs of labs. Bonferroni corrections were used to ensure the overall Type 1 error rate was less than 0.05. A similar approach was used when assessing the slope and curvature parameters of the model.

*Sensitivity analysis*

The second objective of this study was to determine the sensitivity of the CBMN assay to detect a 1Gy difference over background levels when the total number of cells scored varied between 50 and 500.  Two approaches were considered for this analysis. The first approach considered the sensitivity of the CBMN assay at the lab level (v) and the second approach was at the scorer level (vi). The sensitivity analysis at the lab level takes into account the data from each scorer. Therefore sample size is increased, and overall variability is decreased. The sensitivity of the assay at the lab level is expected to be greater (i.e., detect differences between lower dose groups and background rates) at the lab level than at the scorer level.

*(v) Lab level*

Traditionally Poisson regression models are used for the sensitivity analysis (similar to analysis of variance for continuous data). The average rate of MN per $n$ cells scored ($n$ = 50, 100, 200, 300, 400, 500) was compared among the 8 dose groups ($\tau_k$, $k$ = 0, 0.1, 0.25, 0.5, 1, 2, 3 and 4Gy). The rate of MN per $n$ cells scored was analyzed separately for each level of $n$.   The objective was to determine the smallest dose significantly different from background when $n$ changed. Each donor's blood was exposed to all levels of radiation and each scorer scored each donor's blood. Therefore, donors and scorers were kept in the model as random blocking effects. The Poisson
$$\log(MN_{ijk}) = \log(n_{ijk}) + \mu + \tau_k + s_i + d_j + a_{ijk}$$ (7), where log($MN_{ijk}/n_{ijk}$) is the natural log rate of *MN* cells per  $n$ cells scored in the $k^{th}$ dose level from the $i^{th}$ scorer ($s_i$, $i$=1, 2, ..., $I$) of the $j^{th}$ donor ($d_j$, $j$=1, 2, ..., $J$), in each lab. The overall average rate of MN in the

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lab is indicated by the parameter $\mu$ and $\varepsilon_{ijk}$ is assumed to follow the Poisson distribution. The models are corrected for over-/under- dispersion using Pearson deviance scale. If the overall effect of dose was significant (i.e., p<0.05) then multiple comparison tests were carried out to compare each dose group to the control group. Bonferroni corrections were used to ensure the overall Type 1 error rate was less than 0.05.

*(vi) Scorer level*

A similar sensitivity analysis was conducted for each scorer $i$ ($i$ = 1, 2, ..., $I$) in each lab. Model (7) is modified to include only the random blocking effect of donors $\log(MN_{jk}) = \log(n_{jk}) + \mu + \tau_k + d_j + \varepsilon_{jk}$ (8), where the parameter $\mu$ is the overall average rate of MN for the $i^{th}$ scorer. Again, if the overall effect of dose was significant (i.e., p<0.05) then multiple comparison tests were carried out to compare each dose group to the control group. Bonferroni corrections were used to ensure the overall Type 1 error rate was less than 0.05.

## Results

*Dose Response Modelling*
Figures 1-3 present the fit of the three models (LPRM, QPRM-I, QPRM-L) to the three labs respectively. In all three labs, QPRM-L and QPRM-I appeared to fit the data well (i.e., the model predicted the rate of MN cells per 500 BN cells scored in each dose group close to the observed average rates). The average observed rates in each dose group were marked with X in the graphs. Table 2 presents the goodness of fit test results from the three models fit to the data, in each lab. The scaled deviance from the QPRM-L and QPRM-I models were similar, with the deviance for QPRM-I being slightly smaller (HC: $p(\chi^2_{285} \geq 275.79)$=0.64; DRDC: $p(\chi^2_{93} \geq 95.17)$=0.42; AECL: $p(\chi^2_{189} \geq 195.25)$=0.36 ). Finally, the model QPRM-I was preferred as it characterized the dose-response relationship of the CBMN assay more accurately. The QPRM-L model reaches a plateau at high dose levels which is not biologically correct for this data. Therefore, the base dose-response model that was used in the remainder of the analysis was the QPRM-I.

*Intra- and Inter-laboratory Variability*
The QPRM-I with two random effects donors and scorers (1) was fit to all three labs separately, in order to characterize the within lab variability. Based on the LRT, for the global hypothesis ($H_0: \sigma^2_s = \sigma^2_d = 0$), there was a significant amount of variability due to at least one of these random effects in both the HC ($\chi^2_2$ = 29.51, p<0.0001) and DRDC lab ($\chi^2_2$ = 8.22, p=0.0164). However, in the AECL lab, the variation contributed by two random effects together was not significant ($\chi^2_1$ = 2.72, p=0.0985). Note, for lab AECL there was only one degree of freedom for the global test since the parameter estimate of the random effect for donor variability was estimated to be zero.

Individual variance component tests were carried out in the HC and DRDC lab in order to further examine the source of variability within the labs. In the HC lab, a significant amount of variability was explained by both scorers ($\chi^2_1$ = 5.35 , p=0.0207),

and donors ($\chi_1^2 = 21.39$ , p<0.0001).  Whereas, in the DRDC lab, the variability due to scorers was insignificant ($\chi_1^2 = 1.13$, p=0.2888), there was a significant amount of variability due to donors ($\chi_1^2 = 7.20$, p=0.0073).  Both HC and DRDC lab used the same donors to prepare their slides for the MN assay.

In order to investigate the variability between labs the QPRM-I with two random effects donors and scorers (1) was fit to all three labs simultaneously.  Based on the LRT, for the global hypothesis ($H_0: \sigma_s^2 = \sigma_d^2 = 0$), there was a significant amount of variability due to at least one of these random effects ($\chi_2^2 = 47.30$, p<0.0001).  Individual variance component tests verified that a significant amount of variability was due to both scorers ($\chi_1^2 = 22.41$, p<0.0001) and donors ($\chi_1^2 = 24.83$, p<0.0001) across all three labs.

Table 3 presents the parameter estimates for the fixed effect (dose) and random effects (scorers and donors) in each of the three labs, as well as in all labs combined. Note in Table 3, the estimate of the variance component due to donors in the AECL lab was zero, indicating no variability between donors in this lab.  It is important to point out that the AECL lab used a different set of donors than the other two labs (HC and DRDC).

*Comparison of Scorers*

The QPRM-I presented in Equation (5) was fit to the data from each lab in order to compare the scorers within a lab.  The objective here was to compare scorers, not to characterize the within lab variability due to scorers.  In order to compare scorers, each scorer was allowed its own dose response model, and then the models were compared (simultaneous Poisson regression models).  Table 4 present the results from the overall F-test to compare the background rate, slope and curvature from each scorer within a lab.

In the HC and DRDC labs, the background rate, slope and curvature were statistically similar for all scorers within their respective labs. The overall F-tests for the background rate, slope and curvature parameter estimates were not significant (p>0.05 in all cases) within these two labs.  Figures 4a, and 4b present the dose response models for each scorer in the HC and DRDC lab, respectively.

In lab AECL, the models for each scorer were considered similar with respect to background rate and slope (p>0.05 for both these parameter estimates, Table 4).  The curvature parameter was marginally significantly different for at least one of the scorers in this lab (p=0.0463).  Upon closer examination of the graph for the dose response models for each scorer in this lab (Figure 4c) scorer AECL2-F appeared to have a slightly different curve from the rest.  Data from this one scorer was removed and model (5) was refit to the remainder 3 scorers in the lab.  The remainder three scorers had similar dose-response models (Table 4, lab AECL with AECL2-F removed, overall F-test for background rate, slope and curvature were no longer statistically significant, i.e., p>0.05).  Again, it is important to note that the difference from the one scorer was marginal, and in only the one parameter (curvature of the dose response model).

*Comparison of Labs*

Simultaneous Poisson regression model (6) was fit to the data from all three labs in order to determine if the dose response model from all three labs were similar.  Table 4 presents the results from the overall F test to compare the background rate, slope and curvature parameters from the three labs, and Figure 5 presents the dose response

curve from each lab. The background rate from all three labs was statistically similar ($F_{(2,548)} = 1.26$, p=0.2844), the slope was marginally different for at least one of the labs ($F_{(2,548)} = 3.03$, p=0.0490), and the curvature parameter was significantly different for at least one of the labs ($F_{(2,548)} = 25.33$, p<0.0001). Multiple comparison tests for the slope indicated that there was a marginal statistical difference between the slopes of the dose response model for AECL and HC ($F_{(1,456)} = 6.12$, p=0.0411). With respect to the curvature of the model, all labs were significantly different from one another (AECL vs. DRDC: $F_{(1,268)} = 53.75$, p<0.0001; AECL vs. HC: $F_{(1,456)} = 23.14$, p<0.0001; and DRDC vs. HC: $F_{(1,367)} = 12.45$, p=0.0015).

*Sensitivity Analysis*

As previously mentioned the objective in the sensitivity analysis was to determine the smallest dose significantly different from background, when the total number of cells scored varied between 50 and 500. For multiple comparisons, p-values between 0.01 and 0.05 were considered to be marginal, and possibly due to a low power of the test, for this reason the next highest dose group with p<0.01 was also reported.

*Sensitivity analysis: Lab level*

Poisson regression model (7) was fit to the rate of MN for each total number of *n* cells scored (*n*=50, 100, 200, 300, 400, 500). The overall effect of dose was highly significant for each lab at each level of *n* (p<0.0001, results not presented). Table 6 reports the results from the sensitivity analysis for each lab. In the AECL lab, the smallest dose significantly different from background was 0.5Gy regardless of the number of cells scored (*n*=50 p=0.0006; *n*=100, …, 500 p<0.0001). In a few cases, the 0.25Gy dose was marginally significantly higher over background rates. In the DRDC lab, the smallest dose significantly different from background was 0.5Gy, regardless of the number of cells scored (p<0.0041). Finally, in the HC lab, as the number of cells scored increased, the dose level significantly different from background decreased. For example, when only 50 BN cells were scored, a dose of 1Gy was significantly higher than background rates (p<0.0001). As the number of BN cells scored increased to 500, then 0.5Gy was significantly different over background rates, and 0.25Gy was marginally significantly different over background rates.

These results are not surprising since at the lab level the model is taking into account the observations from all the scorers. Therefore, the repeated measures reduce the overall variability of the model thereby making the tests much more sensitive to detect differences between the lower dose groups and background rates.

*Sensitivity analysis: Scorer level*

Poisson regression model (8) was fit to the rate of MN for each total number of *n* cells scored (*n*=50, 100, 200, 300, 400, 500) for each scorer in each lab. The overall effect of dose was highly significant for each scorer at each level of *n* (p<0.0001, results not presented). Table 7 reports the results from the sensitivity analysis for each scorer. In general, as the total number of cells scored increased from 50 to 500, the sensitivity of the assay increased. When the total number of cells scored was 200, all scorers were able to detect a 1 Gy dose (or lower) over background rates.

**Discussion**

The primary objective of this study was to establish CBMN curves in each of the three participating laboratories using a common CBMN protocol. This involved 12 individual scorers across three labs. To accomplish this goal, the variability within and between labs was evaluated in an attempt to establish the appropriate statistical model to fit the data. It was found that in the HC lab there was a significant amount of variability due to scorers as well as donors. In the DRDC lab, there was variability due to donors, while for the AECL lab there were no significant differences attributable to scorers or donors. It is important to note that HC and DRDC used the same donors, however HC had 6 scorers while DRDC had only two. AECL used a separate set of donors and had 4 scorers in their lab.

Comparison of dose response curves for each scorer indicated that all scorers within labs were statistically similar to all other scorers within their lab. However, when the dose response curves for each lab (HC, AECL and DRDC) were compared, they were observed to be significantly different from one another. Based on the analysis, we were able to conclude that each lab should have its own lab-specific dose response curve as there was no single model that would appropriately summarize the dose response data across all three labs (Figure 5). There are several possible explanations for these lab-specific discrepancies (including differences in training levels, equipment, culturing conditions etc…), however these differences are not a reason for particular concern as this phenomena has been previously observed in international inter-comparison studies using the DCA (Wilkins et al., 2008). For this reason the International Organization for Standardization (ISO) subcommittee on biodosimetry (ISO 19238 TC85/SC2/WG18: 2004) recommends that each laboratory using the DCA for biological dose-estimates should generate their own lab-specific calibration curve. Our data suggests that a similar practice should be implemented for the CBMN assay.

The second objective of the study was to determine the lowest dose significantly detectable from background (unexposed samples) when the total number of BN cells scored varied between 50 and 500. This objective was undertaken in an attempt to identify the minimum number of BN cells that were required to be scored to be able to statistically predict doses ≥1 Gy (clinically relevant doses). Reducing this number as low as possible without reducing clinically-relevant sensitivity would increase the through-put of this assay and thereby increase the turnaround time in a mass casualty scenario. It was observed that most of the scorers across all three labs were able to detect a difference of 1Gy (or less) when a total of 200 BN cells were scored.

In conclusion, statistically-based lab-specific CBMN dose-response (calibration) curves have been generated in 3 participating laboratories (HC, AECL, DRDC) in support of CRTI project #0146RD. All scorers in all labs have been statistically demonstrated to be capable of detecting radiological doses ≥1 Gy (in relation to un-irradiated samples), when assessing only 200 BN cells using a common CBMN protocol. Initial estimates indicate that this approach is approximately 10 times faster (on a sample-by-sample basis) than the DCA assay and may provide a useful screening tool in the event of a radiological emergency.

**Future Direction:** Now that the CBMN assay has been established at each participating laboratory, this technique will be evaluated alongside the DCA in CRTI#06-0146RD Exercise #30701).

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**Table 1: Brief outline of project.**

| Lab | Scorers | Donors | Response Variate |
|---|---|---|---|
| HC | HC1<br>HC2<br>HC3<br>HC4<br>HC5<br>HC6 | A,B,C,D,E,F | rate=MN/BN cells, where BN cells=50,100,200,300,400, 500 |
| DRDC | DR1<br>DR2 | A, B, C, D, E, F | |
| AECL | AE1<br>AE2<br>AE3<br>AE4 | G, H, I, J, K, L | |

**Table 2: Goodness of Fit test results for model comparison.**

| Lab | n | Model | d.f | Scaled deviance | p-value | conclusion |
|---|---|---|---|---|---|---|
| AECL | 192 | LPRM | 190 | 298.0978 | 8.8e-07 | |
| | | QPRM | 189 | 196.7236 | 0.335 | |
| | | QPRM | 189 | 195.2518 | 0.362 | Best model for AECL |
| DRDC | 96 | LPRM | 94 | 97.6825 | 0.377 | |
| | | QPRM | 93 | 96.2275 | 0.389 | |
| | | QPRM | 93 | 95.1741 | 0.418 | Best model for DRDC |
| HC | 288 | LPRM | 286 | 298.0978 | 0.299 | |
| | | QPRM | 285 | 276.5331 | 0.629 | |
| | | QPRM | 285 | 275.7911 | 0.641 | Best model for HC |

**Table 3: Parameter Estimates of fixed effects (dose), random effects (scorers and donors) and LRT for covariance components**

| LAB | $a$(s.e.) | $b$(s.e.) | $c$(s.e.) | $\sigma_s^2$ (s.e.) | $\chi_1^2$ (p-value) | $\sigma_d^2$(s.e.) | $\chi_1^2$ (p-value) |
|---|---|---|---|---|---|---|---|
| HC | 0.0181 (0.0037) | 0.0453 (0.0060) | 0.0382 (0.0020) | 1.6e-05 (1.6e-05) | 5.35 (0.0207) | 4.7e-05 (3.8e-04) | 21.38 (<0.0001) |
| DRDC | 0.0271 (0.0082) | 0.0505 (0.0120) | 0.0540 (0.0039) | 6.3e-05 (1.1e-04) | 1.13 (0.2888) | 1.43-04 (1.3e-04) | 7.2 (0.0073) |
| AECL | 0.0226 (0.0032) | 0.0675 (0.0063) | 0.0243 (0.0020) | 2.5-e05 (2.9e-05) | 2.73 (0.0985) | 0 | |
| All Labs | 0.0214 (0.0032) | 0.0528 (0.0050) | 0.0364 (0.0016) | 5.9e-05 (3.4e-05) | 22.41 (<0.0001) | 3.1e-05 (2.0e-05) | 24.83 (<0.0001) |

**Type 4: Overall F-test based upon simultaneous Poisson regression models to compare scorers within a lab, as well as to compare labs.**

| Lab | $a_i$ F(p-value) | $b_i$ F(p-value) | $c_i$ F(p-value) |
|---|---|---|---|
| HC $F_{(5,265)}$ | 0.32 ( 0.9018) | 1.43 (0.2151) | 0.42 (0.8374) |
| DRDC $F_{(1,85)}$ | 0.63 (0.4310) | 1.07 (0.3029) | 0.02 (0.8876) |
| AECL $F_{(3,175)}$ | 0.49 (0.6920) | 2.14 (0.0965) | 2.72 (0.0463) |
| AECL* $F_{(2,130)}$ | 0.26 (0.7698) | 2.19(0.1159) | 0.18(0.8320) |
| All Labs $F_{(2,548)}$ | 1.26(0.2844) | 3.03(0.0490) | 25.33(<.0001) |

* Note: AE2 removed from AECL model.

**Table 5: Final parameter estimates for the dose response models of each lab.**

| Lab | a (s.e.) | b (s.e.) | c (s.e.) | $\sigma_s^2$ (s.e.) | $\sigma_d^2$ (s.e.) | Φ |
|---|---|---|---|---|---|---|
| HC | 0.018 (0.0037) | 0.045 (0.0060) | 0.038 (0.0020) | 1.6e-05 (1.6e-05) | 4.7e-05 (3.8e-04) | 0.0118 |
| DRDC | 0.027 (0.0060) | 0.051 (0.0122) | 0.054 (0.0040) | | 1.4-04 (1.2e-05) | 0.0120 |
| AECL | 0.022 (0.0020) | 0.068 (0.0064) | 0.024 (0.0020) | | | 0.0078 |

**Table 6: Sensitivity analysis: Lowest Dose (Gy) significantly different from background for each lab.**

| Lab | Number of BN cells scored | | | | | |
|---|---|---|---|---|---|---|
| | n=50 Dose (p-value) | n=100 Dose (p-value) | n=200 Dose (p-value) | n=300 Dose (p-value) | n=400 Dose (p-value) | n=500 Dose (p-value) |
| AECL | 0.5 (0.0006) | 0.25 (0.0401) 0.5 (<.0001) | 0.25 (0.0418) 0.5 (<.0001) | 0.5 (<.0001) | 0.5 (<.0001) | 0.25 (0.0378) 0.5 (<.0001) |
| DRDC | 0.5 (0.0041) | 0.5 (0.0004) | 0.5 (0.0002) | 0.5 (0.0002) | 0.5 (<.0001) | 0.5 (<.0001) |
| HC | 0.25 (0.0127) 0.5 (0.0111) 1 (<0.0001) | 0.5 (0.0074) | 0.5 (<.0001) | 0.25 (0.0381) 0.5 (<.0001) | 0.25 (0.0415) 0.5 (<.0001) | 0.25 (0.0179) 0.5 (<.0001) |

**Table 7 Sensitivity analysis: Lowest Dose (Gy) significantly different from background for each scorer.**

| Lab | Scorer | Number of BN cells scored | | | | | |
|---|---|---|---|---|---|---|---|
| | | n=50 Dose (p-value) | n=100 Dose (p-value) | n=200 Dose (p-value) | n=300 Dose (p-value) | n=400 Dose (p-value) | n=500 Dose (p-value) |
| AECL | AE1 | 1 (0.0076) | 0.5 (0.0081) | 0.5 (0.0493) 1 (0.0006) | 0.5 (0.0487) 1 (0.0003) | 0.5 (0.0088) | 0.5 (0.0010) |
| | AE2 | 0.5 (0.0110) 1 (0.0127) 2 (<.0001) | 0.5 (0.0037) | 0.5 (0.0010) | 0.5 (0.0038) | 0.5 (0.0048) | 0.5 (0.0030) |
| | AE3 | 0.5 (0.0323) 1 (0.0553) 2 (0.0002) | 0.5 (0.0119) 1 (0.0005) | 0.5 (0.0127) 1 (<.0001) | 0.5 (0.0346) 1 (<.0001) | 0.5 (0.0123) 1 (<.0001) | 0.5 (0.0033) |
| | AE4 | 1 (0.0013) | 1 (<.0001) | 0.5 (0.0169) 1 (<.0001) | 0.5 (0.0226) 1 (<.0001) | 0.25 (0.0498) 0.5 (0.0093) | 0.25 (0.0448) 0.5 (0.0006) |
| DRDC | DR1 | 0.25 (0.0237) 0.5 (0.0007) | 0.25 (0.0265) 0.5 (0.0008) | 0.25 (0.0177) 0.5 (0.0009) | 0.5 (0.0058) | 0.5 (0.0050) | 0.5 (0.0034) |
| | DR2 | 1 (0.0261) 2 (<.0001) | 1 (0.0087) | 0.5 (0.0482) 1 (0.0010) | 0.5 (0.0206) 1 (0.0006) | 0.5 (0.0053) | 0.5 (0.0076) |
| HC | HC1 | 2 (<.0001) | 1 (0.0064) | 1 (<.0001) | 0.5 (0.0246) 1 (0.0001) | 0.5 (0.0059) | 0.5 (0.0060) |
| | HC2 | 1 (0.0012) | 1 (<.0001) | 1 (<.0001) | 1 (<.0001) | 1 (<.0001) | 1 (<.0001) |
| | HC3 | 0.5 (0.0213) 1 (0.0015) | 0.5 (0.0197) 1 (0.0003) | 0.5 (0.0016) | 0.5 (0.0015) | 0.5 (0.0024) | 0.5 (0.0024) |
| | HC4 | 2 (0.0001) | 1 (0.0016) | 0.5 (0.0072) | 0.5 (0.0045) | 0.5 (0.0056) | 0.5 (0.0015) |
| | HC5 | 2 (<.0001) | 1 (0.0022) | 0.5 (0.0258) 1 (0.0004) | 0.25 (0.0364) 0.5 (0.0080) | 0.25 (0.0414) 0.5 (0.0079) | 0.25 (0.0427) 0.5 (0.0063) |
| | HC6 | 1 (0.0097) | 1 (0.0152) 2 (<.0001) | 1 (0.0038) | 1 (0.0020) | 1 (0.0009) | 1 (0.0007) |

Figure 1: Comparison of dose response models for HC lab
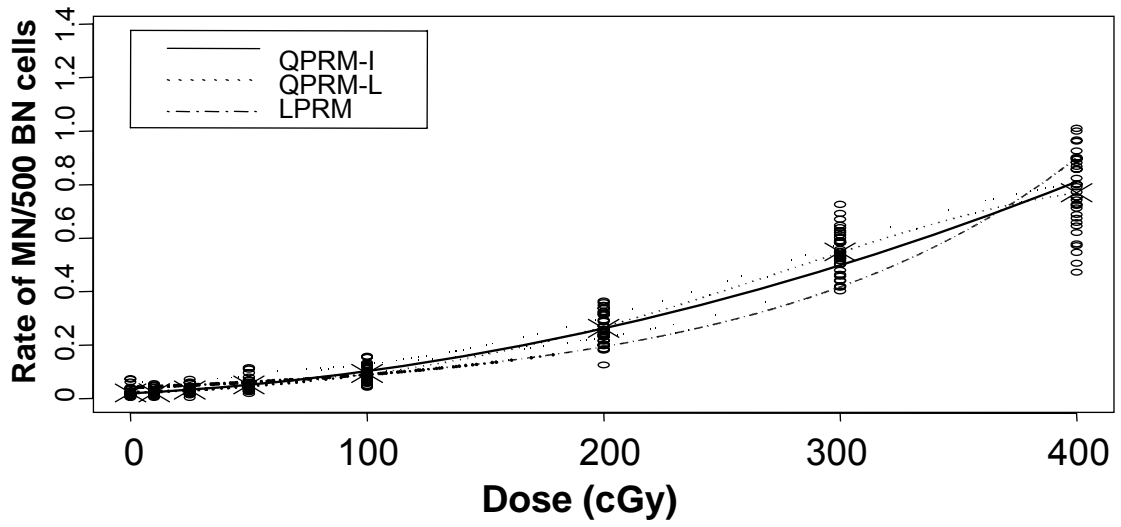


Figure 2: Comparison of dose response models for DRDC lab
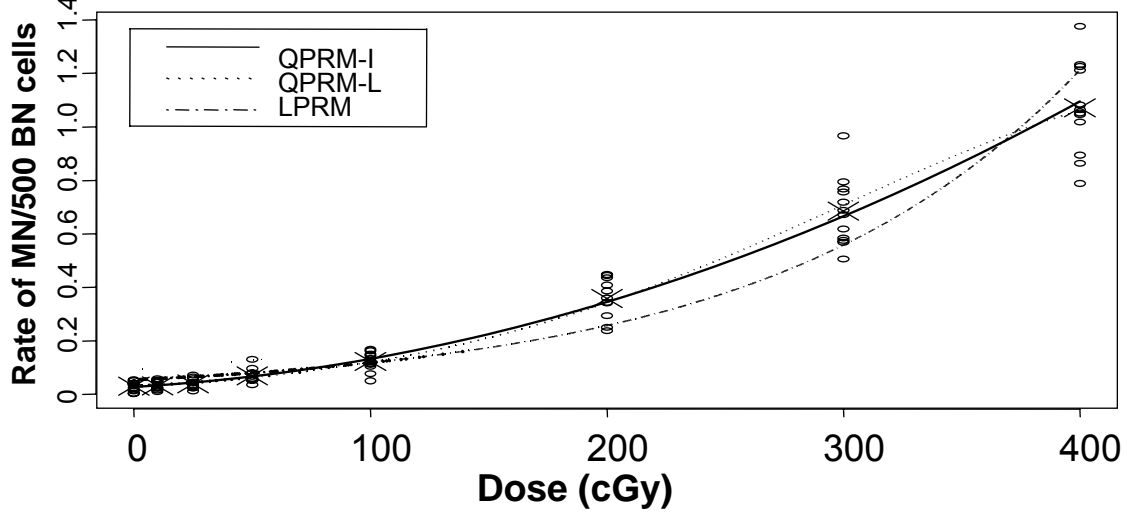


Figure 3: Comparison of dose response models for AECL lab
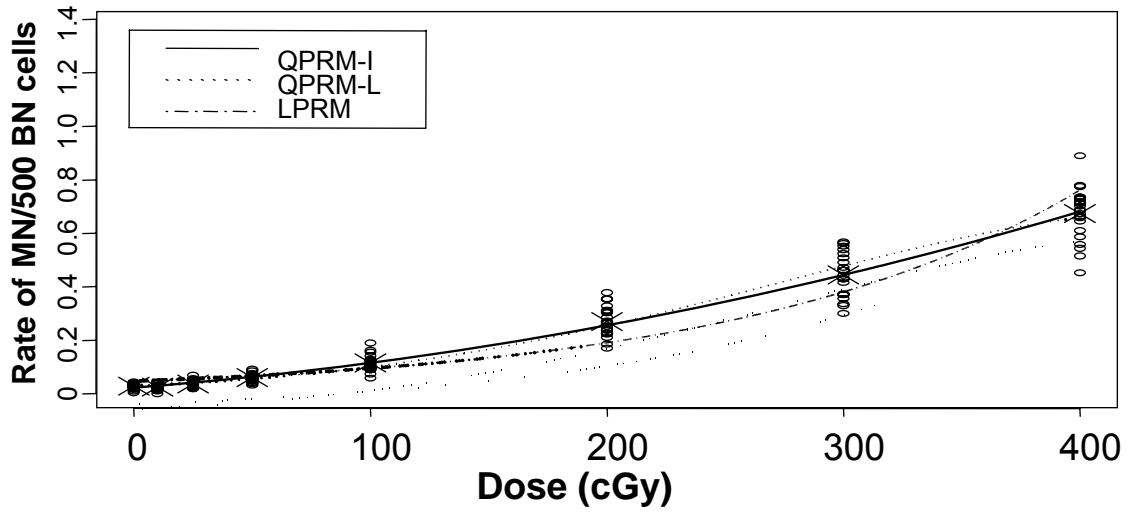
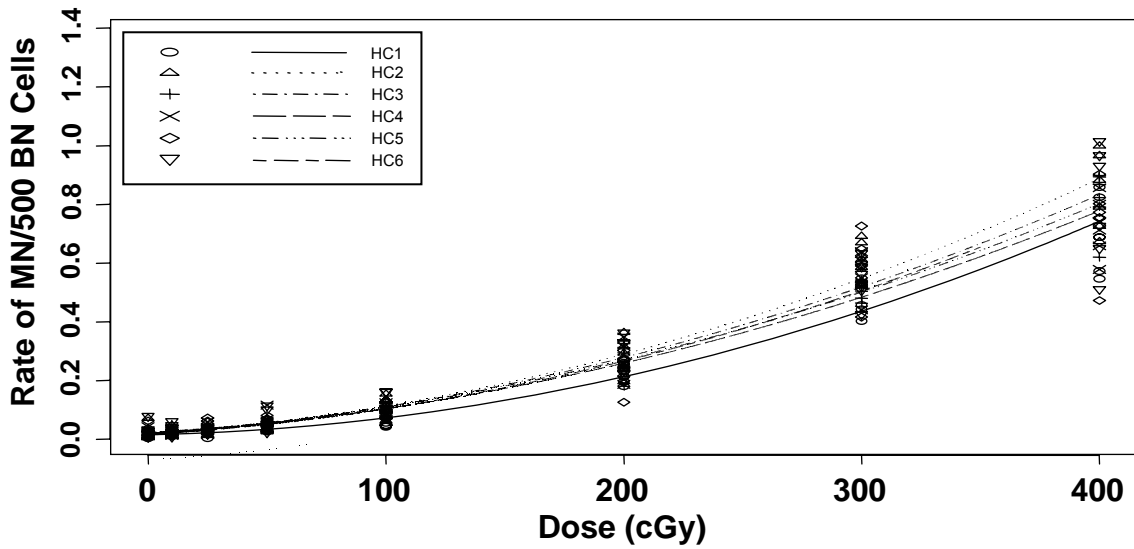Figure 4a: Comparison of dose response curves for each scorer in HC lab



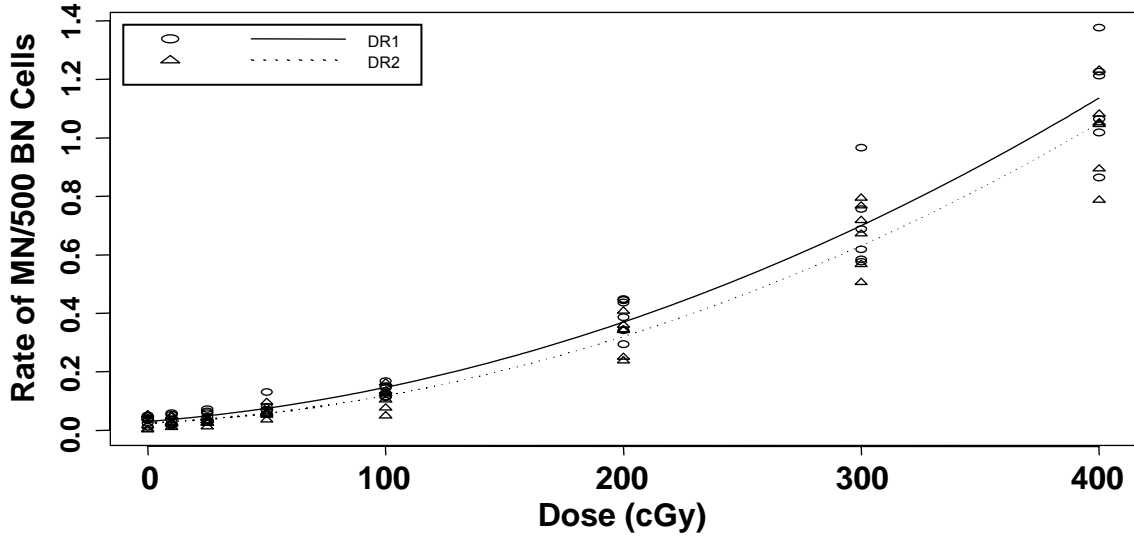Figure 4b: Comparison of dose response curves for each scorer in DRDC lab



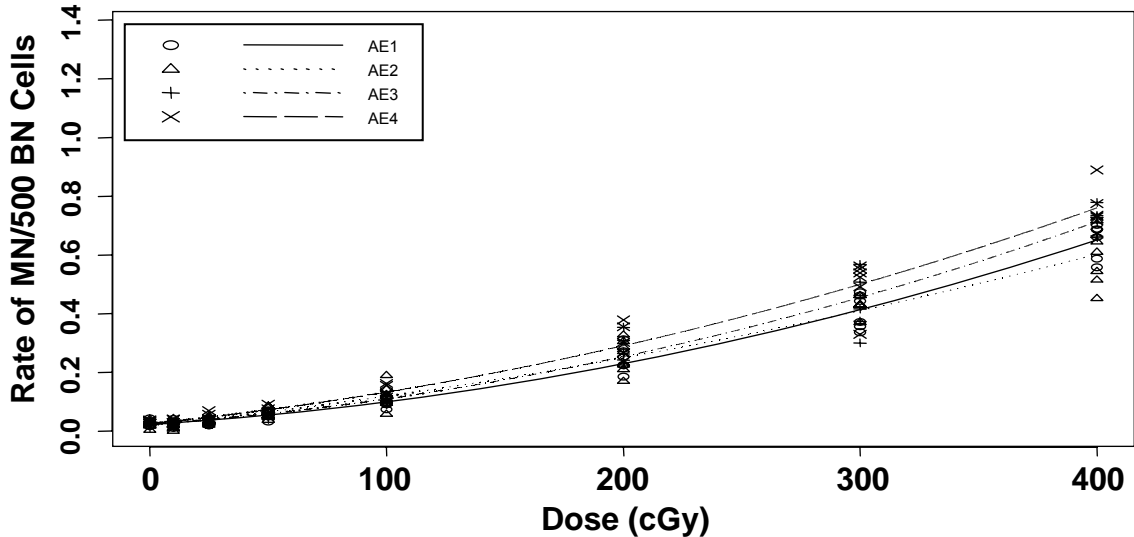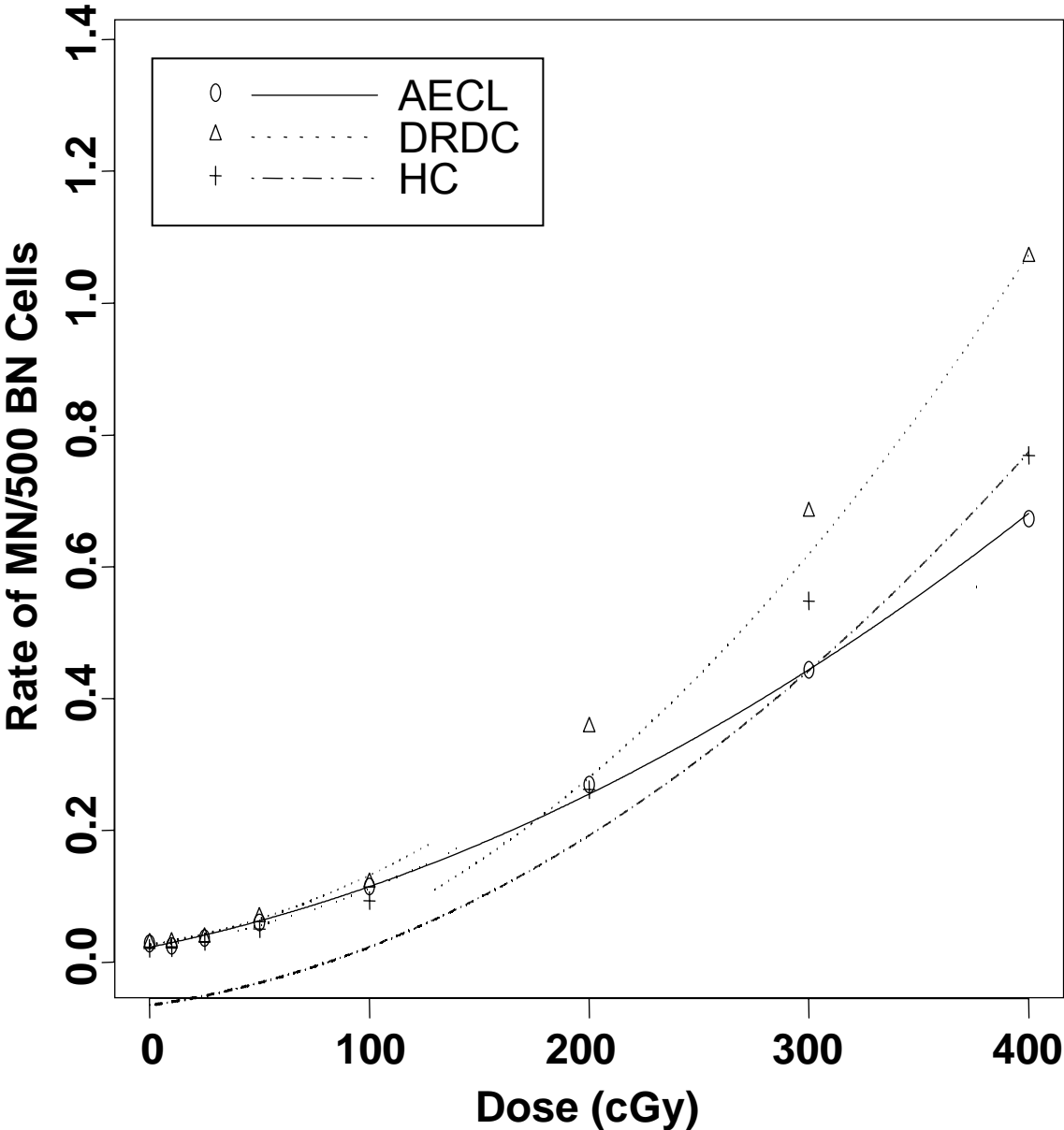Figure 4c: Comparison of dose response curves for each scorer in AECL lab

**Figure 5: Dose response curves for each lab**

## 1.1: Blood Culturing

### Reagents and Materials:

**Complete RPMI Medium: (at 37°C) containing:**
> 400 mL RPMI Medium
> 45 mL Fetal Bovine Serum (final concentration =10%)
> 4.5 mL Pen-Strep/Glutamine Solution (100X solution, Sigma Cat G-1146, [stock is 10000 IU/mL penicillin, 10 mg/mL streptomycin sulfate, 200 mM L-Glutamine]

**Phytohaemagglutinin-M** (PHA) (i.e.: Invitrogen product #10576-015 – solutions of PHA are considered '100%' as supplied. Small aliquots are stored at –20 °C. Typically, a final concentration of 1% v/v in the blood cultures is used).

**Cytochalasin B** (from *Helminthosporium dematioideum*, Sigma #C-6762, stock made up at 1.5 mg/mL in 100% DMSO, protected from light, store in small aliquots at –20 °C)

1 –2 mL peripheral blood in a sodium heparin vacutainer tube.

25 cm$^2$ Vented Culture Flasks (Nunc "T-25 Flasks").

### Procedure

1. Aliquot **9 mL** of complete RPMI media to each T-25 Flask. Distribute **1 mL** of well-mixed blood to each flask containing media (1:9 blood to media dilution).

2. Add **100 μL** PHA (final concentration 1% v/v in blood culture), mix gently.

3. Culture blood for 44 h in 37°C/5%$CO_2$ incubator.

4. Add Cytochalasin B to achieve a final concentration of **4 μg/mL** (using the 1.5 mg/mL stock solution in DMSO). For a 10 mL culture, add **26.7 μL**.

5. Culture for another 28 h in 37°C/5% $CO_2$ incubator.

6. Proceed to cell harvest (see below).

## 1.2: Cell Harvest

### Reagents and Materials:

– Hypotonic Solution (0.075 M KCl (at Room Temperature (RT) – remove from cold room 1 –2 h before needed))
– **5:1** methanol:acetic acid at RT (i.e.: 250 mL MeOH and 50 mL glacial acetic acid – make this solution fresh at the beginning of the procedure)
– 15 mL Polypropylene centrifuge tubes

1. Begin cell harvest 28 h after Cytochalasin B addition (and 72 hours after PHA addition).

2. Transfer diluted whole blood suspensions to 15 mL polypropylene tubes.

3. Rinse each culture dish with **2 mL** Complete RPMI media and add to the appropriately labeled polypropylene tube.

4. Centrifuge tubes at 200xg for 8 min.

5. Remove supernatant and re-suspend the pellet by flicking pellet. It is critical to re-suspend the pellet fully at this point.

6. Add **10 mL** Hypotonic solution to the re-suspended pellet.

7. Incubate for 5 minutes at RT.

8. After the 5-minute incubation, gently re-suspend the cells in the hypotonic solution and then add **2 mL** 5:1 fixative. Invert to completely mix the fixative.

9. Incubate for 10 minutes at RT.

10. Centrifuge at 200xg for 8 min.

11. Aspirate supernatant to a **container separate from bleach** and re-suspend pellet by flicking. Again it is critical to fully re-suspend the pellet at this point or clumping of the cells will be observed in the final product.

12. Add **10 mL** 5:1 fixative to the re-suspended pellet, invert to mix.

13. Incubate for 10 minutes RT.

14. Centrifuge at 200xg for 8 min.

15. Aspirate supernatant to a **container separate from bleach** and re-suspend pellet by flicking.

16. Add **10 mL** 5:1 fixative, invert to mix.

17. Let stand 10 minutes RT.

18. Centrifuge at 200xg for 8 min.

19. Aspirate supernatant to a **container separate from bleach** and re-suspend pellet by flicking.

20. Repeat Steps 16 through 19 until solution is clear (typically 2 more times).

21. At this point samples are either ready to make slides or ready for storage (top up with **10 mL** of 5:1 fixative). Samples may be stored at 4$^{o}$C for up to 2 weeks or at –20 $^{o}$C indefinitely.

## 1.3: Slide Making

### Slide Preparation

*Note: MN slides of good quality (without acid washing) have been prepared using Fisherfinest Premium Microscope Slides (Fisher catalog #125443).*

1. Soak slides overnight in 70% EtOH with 2.5 % HCl in CorningWare dish in fume hood (cover with aluminum foil).

2. On the next day, remove slides individually and rub with a KimWipe until all residue is removed.

3. Store slides in 100% EtOH in the freezer (5 slides/50 mL centrifuge tube)

### Preserving Cytoplasm Prior to Making Slides

1. When ready to make slides, remove 'fixed' samples from refrigerator or freezer.

2. Add **250 µL** of 37% formaldehyde solution (Fisher BP531-500) to each 10 mL 'fixed' sample. Do not add formaldehyde to more samples than you will be able to complete making slides for within 2 h.

3. Mix by inversion. Centrifuge at 200xg for 8 min.

4. Aspirate supernatant, re-suspend pellet by flicking.

5. Add ~ **50 µL** 5:1 fixative (cell suspension should be cloudy) to each sample. Return all samples to 4 $^{o}$C except for the sample from which slides will be currently made. The volume of 5:1 fixative may have to be increased once the first slide is examined (dependant upon the number of binucleated cells in a 400x view (typically we aim for between 8 to 15 BN in a single field of vision)).

## HANABI Slide-Making Technique

*Note:*  *It is possible to use a traditional drop technique (See Appendix 1) similar to hand-dropped slides used for metaphase spread slides, however, using the Hanabi Slide Maker generally results in slides of higher quality.*

1. Turn the HANABI on. Adjust settings for optimal MN slide preparation (~BASE temp: 10; WALL temp: 12; BATH temp: 10; Dry index: 2 lines from the left).
2. Fill the humidifying tank (removable stainless steel bath at rear of machine) with distilled water.
3. Rinse the pre-washed slides (stored in 100% ethanol in the freezer) in ice-cold water to remove all the ethanol, then stand them on their side to dry.
4. Place dry/cleaned slides on the cassette with the etched side up and towards the top, close the lid.
5. Push the READY button (humidifies the chamber) – bright green light turns on.
6. When the green spreading LED comes on, the READY button will flash – "Ready for samples".
7. Slide window open, place pipettor with **10 μL** of well-mixed sample in the guide position and dispense sample onto slide.
8. Close the window between samples to maintain appropriate conditions.
9. Once all samples have been dispensed, close window and press the dry button (takes approximately 90 seconds).
10. Check the slides by phase contrast microscopy to assess the cell density. Adjust concentration if required.
11. Always make sure that the water level is sufficient when making large numbers of slides.
12. Once dried, place 'dropped' slides on a $37^{o}$C slide warmer to complete drying process.
13. After 5-10 min, slides can be stained with Acridine Orange (see below) or maintained at RT indefinitely.
14. Make at least 5 slides per sample.

## Staining Samples with Acridine Orange

*Note:*  *A 10 mg/mL Acridine Orange Stock Solution is available from Sigma (Cat # A8097-10ml), which can be stored at $4\,^{o}C$, and its shelf life has been proven for at least 1 year now.*

1. Dilute the Acridine Orange Stock Solution (10 mg/mL) to a final concentration of 50 μg/mL with Milli-Q water, and dispense Working Acridine Orange Solution into a foil covered plastic coplin jar (protect solution from light whenever possible and store at $4\,^{o}$C).
2. Set up three 50 mL tubes with **fresh** Milli-Q $H_2O$ for rinsing.
3. Dip slides in stain for 10 – 30 seconds, followed by three 5 s rinses in Milli-Q $H_2O$.
4. Mount cover slip (Fisher*finest* cat #12-545-88) with $H_2O$ and pat dry.
5. Visualize and score at 400x power using fluorescence excitation (~530nm) (if possible use a filter set that will allow simultaneous red/green detection).
6. Refer to Section 1.4 for microscopy scoring criteria.

## 1.4: CBMN Scoring Criteria

The cytokinesis-blocked micronucleus (CBMN) assay is a measure of chromosome breakage and loss. It is important to specify the acceptance criteria for binucleate cells (BNC), and micronuclei (MN) within BNC, so that consistent scoring can be achieved between different scorers and laboratories. This will enable better precision of the assay and increase the reliability of the method for comparing DNA damage between samples.

This section describes the criteria that we would like our four core labs to follow for emergency biodosimetry purposes. The criteria are very similar, but not exactly the same as, the criteria described by Fenech *et al.*, (Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., and Zeiger, E. HUMN project: detailed description of the scoring criteria for cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mut. Res.* 534 65-75 (2003)). This paper provides some useful pictures of Giemsa-stained CBMN cells that can be used for reference when learning the technique. However, we find Acridine Orange-stained cells far easier to score in comparison to Giemsa-stained cells as Acridine Orange-stained cells will stain the nucleus green/yellow and the cytoplasm red.

CBMN scoring can be divided into three steps:

1. Determine if a cell may be considered for scoring (e.g. Is the cell an intact binucleate cell (BNC)?).

2. Determine if the BNC contains a MN (or more than one MN). MN are morphologically identical, but smaller than, the main nuclei.

3. After the desired number of BNC have been scored, determine the frequency of mono-, bi-, tri- and quadra-nucleated cells to allow the Proliferative Index and Binucleate Frequency to be calculated.

**1) Determining Eligible Binucleated Cells**

Cells that have undergone one nuclear (but not cytoplasmic) division, after whole blood culture and subsequent cytochalasin B block, will be binucleated. These BNC are the cells of interest for MN scoring. A BNC is considered eligible for scoring if it has a relatively intact cytoplasmic boundary with two clearly defined nuclei. A small degree of tearing in the cytoplasm is tolerated (an artifact of slide preparation), provided that the scorer is confident that any MN that might have been present within the torn area would still be associated with the cell in question. Any cells that have the look of having committed toward apoptosis should not be scored as an eligible BN cell for the CBMN assay.

Specifically, the following criteria must be met for a BNC to be enumerated:

1. The cytoplasmic boundary of the cell should be intact and clearly distinguishable from adjacent cells.

2. The cell is binucleated.

3. The nuclei are round or oval-shaped, however one 'dent' in each nuclei (kidney-shaped) is tolerated.

4. The two nuclei must have intact nuclear membranes.

5. Both nuclei must be situated within the same cytoplasmic boundary.

6. The two nuclei should be of approximately the same size, staining pattern and staining intensity.

7. The two nuclei may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter (see below).

8. The two nuclei may touch, but ideally they should not overlap. If the nuclei do overlap, they may still be scored as a BNC as a long as at least one nuclear membrane is discernable in the overlap area.

9. The binucleated cell in question should not appear to have committed toward apoptosis.

**2) Micronucleus (MN) Scoring Criteria**

MN are scored only within eligible BNC. Typically, 1000 BNC are scored per sample for dose-response curves or experimental studies. However, a lower number of BNC may be suitable for triage situations and experiments planned over the next 3 months will evaluate this possibility.
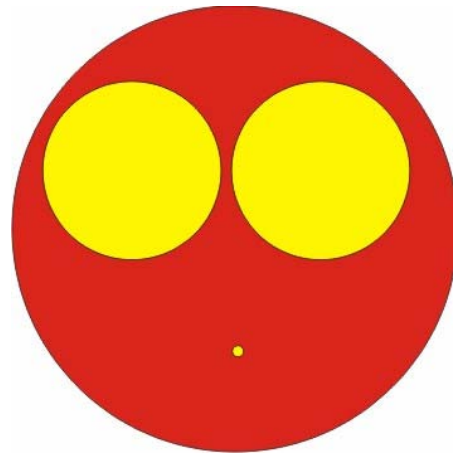
Specifically, the following criteria must be met for a MN to be enumerated:

1. The MN diameter should range from 1/2.5 to 1/16th of the diameter of the main nuclei of the BNC.

2. MN are round or oval in shape.

3. MN are non-refractile and therefore can be distinguished from artefacts such as staining debris.

4. The MN membrane is not linked or connected to the main nuclei (these are classified as nuclear buds).

5. MN may touch the main nuclei, but the MN boundary must be distinguishable from the nuclear boundary.

6. MN should have approximately the same staining intensity as the main nuclei, however, in our experience we have noted that micronuclei can sometimes stain with less intensity than the main nuclei. These MN are currently scored both at HC and AECL.

7. More than one MN may be present within a BNC. BNC bearing one, two and three MN should be enumerated separately (on separate keys of the tally) as this allows greater flexibility in presenting the scoring data. BNC bearing more than 3 MN are not included in either the MN tally or the BNC tally, as there is a risk that these cells may actually be undergoing apoptosis.

The pictures below may be useful for visualizing the approximate size of a typical BNC containing the maximum and minimum size of scorable MN.
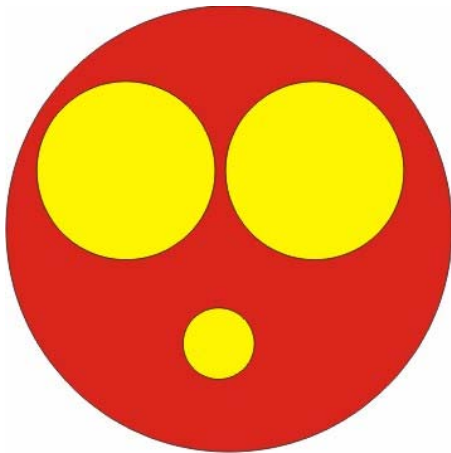


| **1/2.5 diameter MN to nucleus** | **1/16 diameter MN to nucleus** |

Occasionally, nucleoplasmic bridges (NPB) may be observed in binucleated cells. They are thought to originate from rearranged chromosomes with more than one centromere (e.g.: dicentric chromosomes). A separate tally of BNC containing NPB may be kept for reporting purposes.

BNC bearing NPB may still be considered for scoring provided the following criteria are met:

1. The NPB is a continuous link between the two nuclei

2. The width of the bridge does not exceed one-fourth of the diameter of the nuclei

3. The bridge has the same staining characteristics as the main nuclei

4. More than one bridge may be observed within the BNC, however the one-fourth rule will apply for the sum of the bridges.

5.  A BNC with an NPB may contain a MN, and can be scored as such, as long as the MN meets the criteria described above.

## 3) Proliferative Index (PI) and Binucleate Frequency (BNF)

It is useful to score slides not only for the presence of BNC, but also for the ratio of BNC cells to mono-, tri- and quadra-nucleated cells. This can give an indication of the health of the culture, the appropriateness of the culture conditions and whether cytochalasin B was added at the optimum time. While BNC frequency is quoted most often, but we have found that calculating the Proliferative Index (PI) is a more descriptive indicator. At Health Canada, typically a PI of 1.8 to 2.2 is achieved.

BNF and PI can be determined during a separate set of scoring (after CBMN scoring) of approximately 500 cells. Choose a position near the middle of a slide to scan. Every lymphocyte is scored, whether mono-, bi-, tri-, or quadranucleated (with the exception of apoptotic cells), provided that the nuclei are clearly contained within a cytoplasmic boundary. All lymphocytes (whether they would be appropriate for CBMN scoring or not) are tallied (i.e.: torn cells are still counted). Neutrophils and cell debris are ignored.

BNC Frequency (or %BNC) is calculated by the following formula:

$$\%BNC = (\# \ BNC / Total \ Lymphocytes) * 100\%$$

Proliferative Index (or PI) is calculated by the following formula:

$$PI = \frac{((1 * \# mononucleated) + (2 * \# binucleated) + (3 * \# trinucleated) + (4 * \# quadranucleated))}{Total \ \# \ of \ Lymphocytes \ evaluated}$$

## Appendix 1:

### Traditional Slide Making using the Drop Technique

Prepare by pre-warming a water-bath to 60°C and cover with foil except for small (10 cm$^2$) area. Prepare 500 mL of ice-cold water by mixing an equal volume of ice with cold water.

1.  Re-suspend cells in small volume of 5:1 fixative (should be cloudy).
2.  Draw up **10 μL** of cell sample in pipette.
3.  Dip the cold slide in ice cold water until sheet of water forms.
4.  Drop cell sample onto wet slide from 3-5 cm with slide held at about 30°.
5.  Immediately flush the slide 3 times with 5:1 fix using a Pasteur pipette.
6.  Blot the end of the slide and wipe the back of the slide with KimWipe.
7.  Hold the slide over steam (about 3 cm above 60°C water bath) for 20 sec.
8.  Wave the slide through the air and wipe the back of the slide.
9.  Hold the slide over steam for a further 20 sec.
10. Check slide under phase contrast to check the density of cells.
11. Place slide on slide warmer at 37°C for 5-10 min or maintain at RT indefinitely.
12. Make at least 5 slides per sample.

| | | |
|---|---|---|
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