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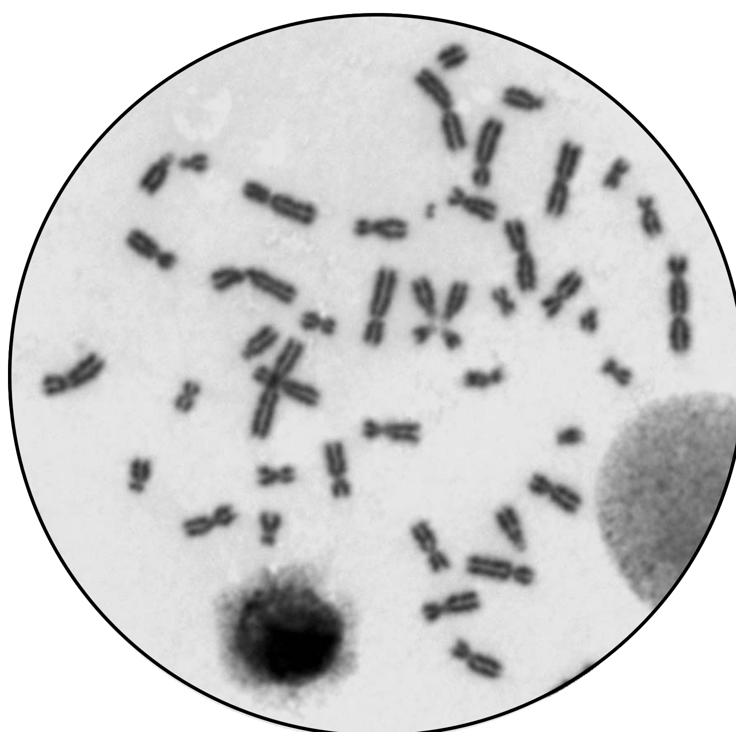
Scientific report

Daniela Stricklin, Eva Arvidsson, Thomas Ulvsand

Establishment of Biodosimetry at FOI: Dicentric Assay Protocol Development and ^{137}Cs Dose Response Curve



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Establishment of Biodosimetry at FOI:
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Abstract (not more than 200 words)

Biological assessment of radiation exposure, or biodosimetry, is critical in suspected radiation exposures when physical dosimetry is not available or uncertain and is still necessary to confirm physical dose estimates when available. Accurate dose estimates are crucial in making life saving medical decisions for exposed persons, for determining other health consequences, or for reassuring non-exposed persons. The dicentric assay is the most documented method for biological dose assessment and is now an accredited method for biodosimetry. We have established the dicentric assay at FOI by developing and optimizing protocols for our laboratory. A dose response curve for low LET γ radiation has been developed from *in vitro* ^{137}Cs irradiation of human lymphocytes. Our results are in good agreement with curves developed at other biodosimetry laboratories. Future efforts in the area of applied biodosimetry will be on developing methods such as fluorescence *in situ* hybridization, FISH, and premature chromosome condensation, PCC, that fill in the gaps of the dicentric assay.

Keywords
biodosimetry, dicentric assay, cytogenetics, radiation assessment

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Establishment of Biodosimetry at FOI:
Dicentric Assay Protocol Development and 137Cs Dose Response Curve

Sammanfattning (högst 200 ord)

Biodosimetri, en bestämning av stråldos utifrån analys av biologiskt material, är en viktig metod vid misstänkta strålningsexponeringar när fysikalisk dosimetri antingen inte är tillgänglig eller är osäker. Den är också nödvändig för att bekräfta doser erhållna med fysikalisk dosimetri. En korrekt uppskattning av stråldosen är en förutsättning för att viktiga, ibland livräddande, medicinska beslut för personer som blivit exponerade ska kunna tas. Den behövs också för att säkert kunna ge ett lugnande besked när en person inte blivit exponerad. Den dicentriska metoden är den mest dokumenterade för biodosimetriändamål och den är nu ackrediterad. Vi har etablerat den dicentriska metoden på FOI genom att utveckla och anpassa den för vårt laboratorium. En dosresponskurva för låg LET γ-strålning har tagits fram genom ^{137}Cs-bestrålning av humana lymfocyter in vitro. Våra resultat stämmer mycket bra överens med de kurvor som tagits fram vid andra biodosimetrilaboratorier. Framtida satsningar inom tillämpad biodosimetri kommer att handla om metodutveckling av fluorescens in situ hybridisering, s.k. FISH, och PCC (premature chromosome condensation). Båda dessa metoder kan komplettera den dicentriska metoden i de fall denna inte är tillräcklig.

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Table of Contents

Table of Contents 4
Introduction 5
  Background 5
  Dicentric Assay 5
  Chromosomal Damage and Evaluation 5
  Objectives 7
Materials and Methods 7
  Lymphocyte Collection 7
  In Vitro γ-irradiation 7
  Calibration of the Bilateral ^{137}Cs Chamber 8
  Culture 8
  Slide Preparation 9
  Aberration Scoring 9
  Statistical Methods 9
Results 9
  Culture Conditions 9
  Scoring Intra-Comparison 10
  Dose Response Curve 10
Discussion 11
  Culture Conditions 11
  Metaphase Evaluations 12
  Dose Response Curve 12
Limitations and Future Work 13
Acknowledgements 16
References 17
Appendix I: Protocols for Sample Processing 20
  Blood Collection 20
  Lymphocyte Separation 20
  Metaphase Culture Media Preparation 20
  Metaphase Cell Culture 20
  Cell Harvest 21
  Preparation of Giemsa Stain 21
  Dropping and Staining Slides 21
Appendix II: Distribution of Aberrations Observed in Analyses 23
Appendix III: Intra-Comparison 24
Appendix IV: Inter-Comparison of Dose Response Curves 26

Introduction

Background

Biodosimetry refers to the use of biological markers to estimate radiation exposure and dose. Biologically assessing dose is critical in suspected radiation exposures where physical dosimetry measurements are unavailable or uncertain. In addition, biodosimetry measurements may be used to confirm physical dosimetry estimates. In high exposure scenarios, a reliable and accurate assessment of dose is critical in order for medical personnel to make life-saving decisions, plan therapeutic treatment, and predict further health consequences. At lower doses, exposure assessment is useful in evaluating risk to late health effects such as cancer. Furthermore, a biological dose assessment may be used to reassure non-exposed persons that they have not received any significant exposure.

While a great many markers and methods exist for biological radiation dose assessment, the evaluation of dicentric chromosomes which dates back to the 1960's has been the most widely applied assay and is the most fully validated method available. Application of the dicentric assay over several decades and in nearly every radiation exposure accident has enabled assay optimization and has documented the merit and the limitations of the method. Today, the conventional dicentric assay has come under ISO standardization and has been incorporated into many radiological protection programs (IAEA 1986, 2001).

Dicentric Assay

In general, the dicentric assay is conducted by culturing blood lymphocytes, from an individual with a suspected exposure, for 48 hours to obtain metaphase chromosomes. The chromosomes of many metaphases, 500 - 1000 cells, are evaluated under a microscope for specific damage in the form of dicentric chromosomes, which are the result of chromosomal breakage from radiation interactions and subsequent abnormal rejoining. Dicentric chromosomes are the markers of choice for evaluation because they are easily identified, are quite specific to radiation, have low background frequency, and show a reproducible dose response relationship (Bauchinger 1984, Amundson 2001).

Chromosomal Damage and Evaluation

In a cytogenetic evaluation, all damages that are observed are recorded. In general, unstable aberrations are the type of damage usually observed, as these damages are very different in appearance as compared to normal chromosomes. These aberrations include dicentrics, acentrics, and ring chromosomes. Dicentric chromosomes contain two centromeres obtained from the joining of two broken chromosomes. An acentric chromosome is a fragment of a chromosome not containing a centromere, formed from a break in another chromosome. Rings are circular chromosomes formed from the joining of two breaks on separate arms of the same chromosome.

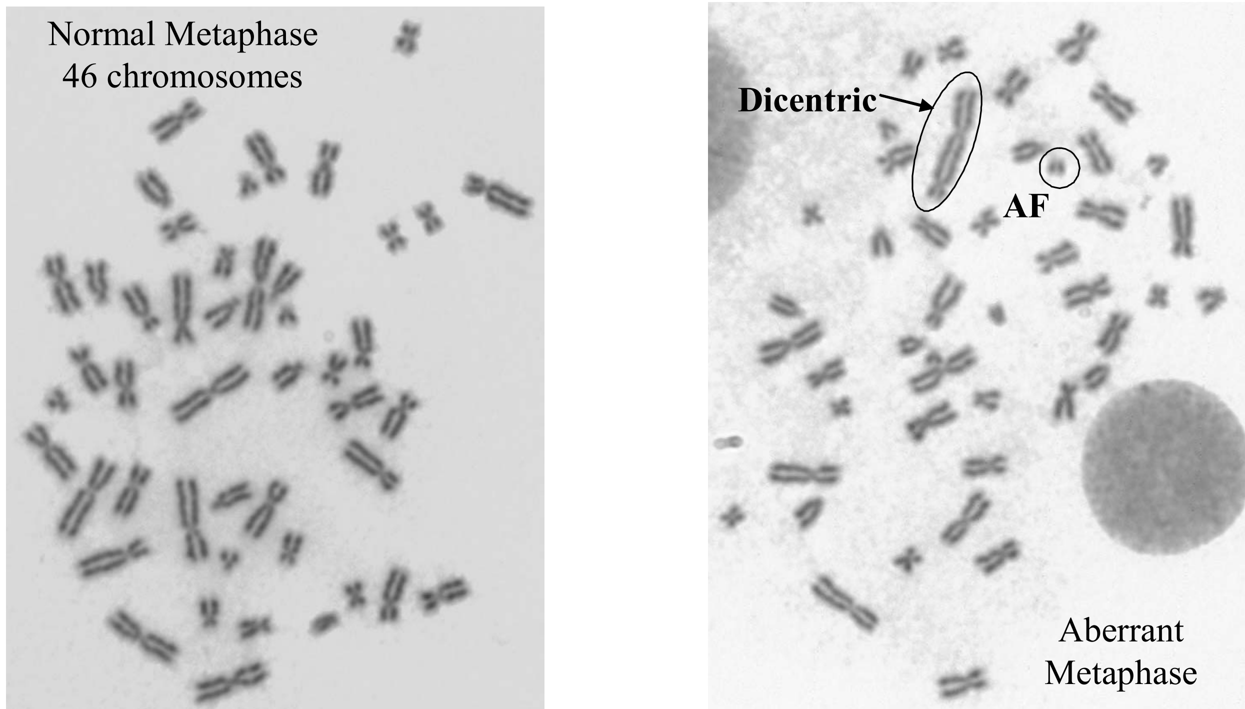

Figure 1 and 2: The picture above to the left represents a normal metaphase containing 46 normal chromosomes. The picture to the right is an abnormal metaphase containing radiation damage illustrating a dicentric chromosome and its accompanying acentric fragment (AF). These pictures are taken from FOI's *in vitro* dose response experiments, control and 4 Gy irradiated samples, respectively.

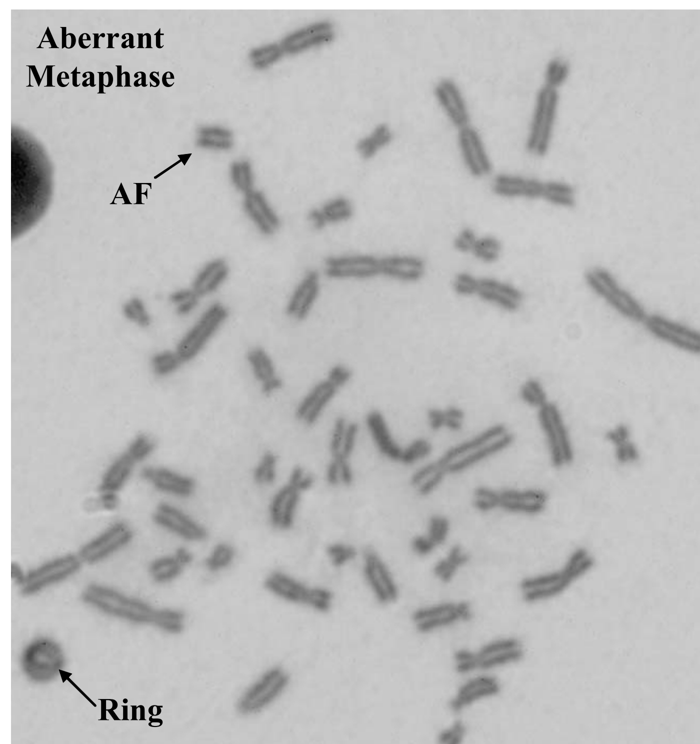

Figure 3: The picture above represents an abnormal metaphase illustrating a ring chromosome and its accompanying acentric fragment (AF). This picture is taken from FOI's *in vitro* dose response experiments, 4 Gy irradiated sample.

For an accurate biodosimetry evaluation, only cells in the first mitotic division should be evaluated, since subsequent division may result in loss of damaged chromosomes. For this reason, only dicentrics with accompanying acentric fragments are used in the analyses. While other aberrations may be observed and are recorded, the final analysis usually is based on the frequency of these dicentric chromosomes with accompanying acentric fragments, as these data have provided the most reliable and reproducible results due to distinct appearance and low background frequency. The

average background frequency of dicentrics is 0-2 per 1000 cells (Mettler 1995). The number of excess acentrics tends to be more variable but are also recorded. Centric rings are relatively rare and occur more often at the higher doses and with high LET radiations (Edwards 1997). Occasionally, the frequencies of centric rings have been used to give a better dose estimate of very high dose exposures (Hayata 2001).

Inter –laboratory variations in dose response curves, aberration yields, and dose estimates have been documented in collaborative exercises (Lloyd 1987). Due to the variable conditions in each lab (reagents, handling procedures, etc.) and to the subjective nature of the evaluation of the metaphase chromosomes, each biodosimetry lab is encouraged to create their own dose response curve for the dicentric assay to reduce uncertainty in assessments (IAEA 2001). Subtle variations to the process and handling of the samples can greatly affect the quality of metaphases produced. Therefore, each lab must establish their own protocols. Furthermore, consistent and reproducible scoring of metaphases requires some degree of technical expertise. Each person working in the lab evaluating cells should gain enough experience for reproducible data in agreement with each other person working in the lab. This should be documented in the form of intra-laboratory comparisons. Reliability may also be documented by participating in inter-laboratory comparisons; as such exercises are ongoing within many of the biodosimetry labs internationally. These steps are mandatory for ISO accreditation for application of the dicentric assay for biological dose assessment.

Objectives

The initial objective of the work presented was to establish and optimize protocols for the acquisition of blood samples, separation and culture of lymphocytes, preparation of metaphases, and slide preparation for the dicentric assay. The next aim was to develop standards for evaluating metaphases and to produce technically trained expertise within our laboratory. The final objective was to develop a dose response curve for the dicentric assay for γ-radiation at FOI.

Materials and Methods

Lymphocyte Collection

Blood samples were acquired from healthy volunteers via the local blood bank (Blodcentralen, Umeå) and from in-house volunteers (FOI, Umeå) in 5 - 10 ml Na Heparin vacutainer tubes (Becton Dickinson, NJ, USA) with ethical approval from the Research Ethics Committee, Umeå University (2003-03-12, §115/02, dnr 02-097). Tubes were inverted several times to dissolve and mix the blood and heparin. Lymphocytes were separated from whole blood using a density gradient (Histopaque-1077, Sigma Chemical Co., St. Louis, USA), washed and resuspended in culture media (RPMI-1640 with Glutamax, Gibco, NY, USA) in 15 mL polypropylene culture tubes.

In Vitro γ-irradiation

Samples were promptly irradiated using the bilateral ^{137}Cs Gammarad 900 chamber (Scanditronix, Uppsala, Sweden) at FOI, NBC Protection in Umeå with a dose rate of approximately 0.4 Gy/min (dose and dose rate were calculated for each experiment). The samples for the dose points were prepared in a total of 4 experiments, each with a control. Each sample, contained in a 15 ml centrifuge tube with 7 ml of media, was placed in the center of the cavity with its long axis joining a line between the two sources. The height of the blood in the tube is 5 cm, and it is symmetrically placed with the mid-point of the blood column in the center of the Gammarad cavity, see Figure 4.

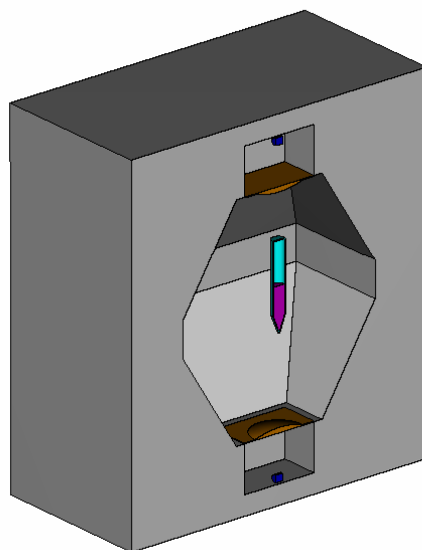

Figure 4. A schematic drawing of the cavity and placement of the sample. The blue dots are the ^{137}Cs sources. The distance between the sources is 0.42 meter. The schematic is compliments of Göran Ågren.

Calibration of the Bilateral ^{137}Cs Chamber

The dose was measured with an air filled ionization chamber with a measuring volume of 0.3 cc, calibrated against a reference chamber, Capintec PR06. The reference chamber was calibrated at the Swedish Secondary Standard Laboratory in Stockholm. Both chambers are made of tissue equivalent plastic. The calibration factor for the ionization chamber used was 79.5 mGy/nC. We used a calibrated dual-channel electrometer, Precitron Janus AC, to collect the liberated charge. To measure the dose, the ionization chamber was placed in the center of the Gammarad cavity and five measurements were taken (Table 1). The measurement time was 180 sec (3 minutes).

Table 1. Measurements in the Gammarad cavity.

Measurement No.	Reading (nC)
1	14.690
2	14.794
3	14.779
4	14.778
5	14.778
mean	14.76

The dose rate in air in the centre of the cavity was calculated to be 14.76 * 79.5/3 = **391 mGy/min**. The calibration measurements were compared to Monte-Carlo simulation results to support the calibration dose rate. The measurements were performed 15 - 20 December 2004. The dose rate was corrected for the radioactive decay of ^{137}Cs to the actual time of the irradiation of the blood samples.

Culture

Lymphocyte cultures were set up using isolated lymphocytes from 3-5 ml whole blood in 7 ml complete culture media (RPMI-1640 with Glutamax, Gibco, NY, USA) containing 10% fetal calf serum (Gibco), 2% phytohaemaglutinin (Sigma), 1% sodium heparin (Sigma), and 50 µg/ml

gentamycin (Sigma). The cultures were incubated at 37°C and 5% CO_2 for 48 hours. Colcemid (385 µl of 10 µg/ml stock solution, Sigma) was added for the last 1 hour and 45 minutes. For harvesting, cells were treated with hypotonic KCl solution (0.75 M) for 40 minutes at 37°C. The cells were fixed 2-3 times with Carnoy's solution (3:1 methanol / acetic acid) and stored at -20°C.

Slide Preparation

Samples were removed from -20°C and washed with fresh Carnoy's solution. All of the wash was removed except for 0.5 ml and the samples were re-suspended. 40 µl of each sample was dropped from at least 10 cm onto wet, pre-cleaned grease-free slides. Slides were allowed to dry and were then stained with a 4% Giemsa solution in fresh phosphate buffer (pH 6.8) for 7 minutes. Slides were then rinsed with distilled water and dried before evaluation.

Aberration Scoring

Slides were examined with a microscope at 40x to find metaphases. Metaphase images were captured at 100x using a Leica DMR microscope equipped with a Leica DC 200 camera and software which was imported into Photoshop 6.0. Images were saved using the metaphase coordinates, and notes taken for any unusual appearances. All metaphase spreads were analyzed at the computer by counting 46 centromeres or chromosomes and taking note of dicentrics, accompanying acentrics, excess acentrics, rings, and any other aberrations. Cells that were not distinct, not containing 46 chromosomes, or containing dicentrics without accompanying acentrics were considered non-scoreable and were not included in the data. The number of non-scoreable metaphases varied greatly between preparations and ranged from 2-20%. However, the number of cells containing dicentrics without acentrics, an indication of second division metaphases, was not more than 1 or 2 per slide (approximately 500 cells). Tricentrics were counted as two dicentrics. Triradials and one-armed fragments were considered chromatid damage and were ignored. Standards for metaphase evaluation were developed over time, approximately 6 months, via concurrent evaluations, comparisons, and discussions between scorers.

Statistical Methods

The aberration yields were evaluated to determine that they followed Poisson statistics. Then, the dose response curve fitting was conducted according to the Papworth (1975) method using Poisson weights and reiterative weighting of the least squares fit. The dose-response relationship for the yield of dicentrics was fitted according to the linear quadratic model $Y = C + \alpha D + \beta D^2$. Initial statistical evaluation and modeling was conducted with the aid of mathematical programs designed by National Radiological Protection Board, NRPB, in the UK. The programs were made available to FOI and were used for further updating the curve as more data became available. These programs will be used in the future as additional data is accumulated for the low dose points and for modeling additional curves.

Results

Culture Conditions

The initial phase of this work involving optimizing laboratory procedures and standards culminated in the production of working protocols for acquisition of blood samples, separation and culture of lymphocytes, preparation of metaphases, and procedures for slide preparation for the dicentric assay. In our work, critical points in the procedures were identified that greatly affected the quality of metaphases produced and eventual reliability in evaluation. These protocols which make note of the critical steps in sample processing are shown in Appendix I. All of the steps in the processing of the samples are important, but we have outlined the steps that may be variable and most often result in

problems. This includes layering of blood during lymphocyte separation, colcemid incubation time, cell bloating time, freshness of phosphate buffer in the Giemsa stain, and humidity during slide prearation. During lymphocyte separation, blood must be layered **very** gently on top of the histopaque. Our optimal incubation time with colcemid is the last 1 hour 45 minutes of the 48 hour culture time. Our optimal bloating time is 40 minutes. Fresh phosphate buffer at pH 6.8 is always used. If time allows, we drop slides on days with moderate humidity, see discussion.

Scoring Intra-Comparison

The initial phase also produced technical expertise for evaluating metaphases as demonstrated in intra-laboratory comparison between the two evaluators. 50 cells from the 4 Gy dose point were evaluated by each scorer. This dose point was chosen since the number of aberrations and technical difficulty is greatest at the high doses and therefore providing ample opportunity to compare judgment. In this exercise, the dicentric frequency obtained from each scorer was 1.12 and 1.10 / cell. Comparing each scorer on a cell by cell basis revealed that 46 out of 50 cells were in agreement, resulting in a 92% agreement between scorers, an 8% difference. In each case where there was a difference in agreement, a difference in judgement over one dicentric due to reasonable uncertainty was observed, see Appendix III for details. In such a comparison with a very high number of aberrations, a 10% difference is considered reasonable and acceptable.

Dose Response Curve

The data for the frequency of unstable aberrations in a total of 4501 cells for nine dose points with a critical number of cells evaluated for each dose point has been compiled. An initial dose response curve has been prepared based on the frequency of dicentrics with accompanying acentrics for this data. The frequencies of dicentrics are shown in Table 2 along with the standard error associated with each. The frequency distribution of dicentrics per cell refers to the number of dicentrics observed per cell. This information is useful, among other things, in calculating the ratio of the variance to the mean. This term is used as a test for the Poisson distribution, which the data should conform to. A value close to one confirms that the data follows the Poisson distribution and this is observed in our data. The highest dose point has the most deviant ratio; however, this is not uncommon at very high doses. The absolute numbers for the evaluations, as well as the information concerning other aberrations observed are listed in Appendix II.

Table 2. Dicentric frequencies after *in vitro* exposure of human lymphocytes to ^{137}Cs γ-irradiation.

Dose		Dicentrics / cell[1]	Frequency distribution of dicentrics / cell[2]					σ^2/Y[3]
Gy	Cells	± SE	0	1	2	3	4+	± SE
0	560	0.002 ± 0.0018	0.998	0.002				1.00 ± 0.00
0.2	519	0.013 ± 0.0051	0.988	0.012				0.99 ± 0.06
0.4	500	0.034 ± 0.0081	0.984	0.016				0.99 ± 0.06
0.8	781	0.052 ± 0.0082	0.949	0.050	0.001			1.00 ± 0.06
1.5	654	0.167 ± 0.0160	0.841	0.154	0.002	0.003		0.96 ± 0.06
2.3	599	0.392 ± 0.0256	0.679	0.257	0.057	0.005	0.002	1.03 ± 0.06
3.2	452	0.697 ± 0.0393	0.482	0.385	0.100	0.022	0.011	1.00 ± 0.07
4.0	321	1.05 ± 0.0572	0.312	0.411	0.209	0.059	0.009	0.85 ± 0.08
5	107	1.81 ± 0.1302	0.093	0.346	0.280	0.215	0.065	0.65 ± 0.13

[1]Only dicentrics with accompanying acentrics were scored.
[2]Refers to the frequency of cells observed with the corresponding number of dicentrics.
[3]σ^2/Y, variance/mean, used to test for Poisson distribution.

The dose response curve for dicentrics from this analysis was fitted by the method of reiteratively weighting of the least squares fit. The dose response relationship for dicentric yields for low LET radiation follows a linear quadratic curve, $y = C + \alpha D + \beta D^2$. The curve coefficients are shown in Table 3 and are compared to other published curve coefficients. In addition, an inter-comparison of

these curves in graphical form is illustrated in Figure 8 of Appendix IV. The FOI curve is plotted below with standard errors in Figure 5.

Table 3. Coefficients in the equation $y = C + \alpha D + \beta D^2$ after curve fitting of dicentric yields induced by low LET radiation.

Author	Source	C ± SE	$\alpha \pm$ SE Gy^{-1}	$\beta \pm$ SE Gy^{-2}
FOI (Stricklin 2005)	^{137}Cs	0.0025 ± 0.0016	0.013 ± 0.007	0.065 ± 0.003
STUK (Lindholm 1998)	^{60}Co	0.00055 ± 0.00024	0.0135 ± 0.0043	0.0544 ± 0.0034
NRPB (Lloyd 1986)	^{60}Co	-	0.014 ± 0.004	0.076 ± 0.003
Bauchinger 1983	^{60}Co	-	0.011 ± 0.004	0.056 ± 0.003
AFRRI (Prasanna 2002)	^{60}Co	-	0.098 ± 0.0209	0.044 ± 0.0093

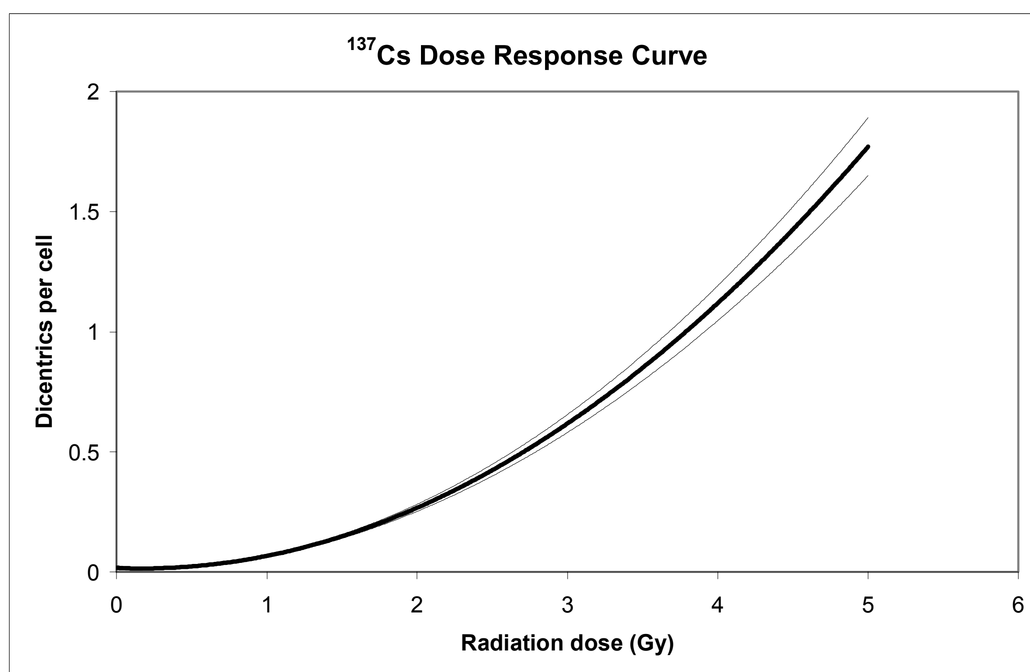

Figure 5. FOI dose-response calibration curve for dicentric yields in human lymphocytes with upper and lower 95% confidence limits for ^{137}Cs γ radiation.

^{60}Co curves should be approximately the same as ^{137}Cs curves and may be used for comparison (Bauchinger 1993). The values for the α and β coefficients observed from our curve fitting were in good agreement with three other published curves. Our laboratory has a high constant value in comparison due to the high background frequency observed in one of our control samples and partially due to the limited number of cells evaluated. However, the constant value will likely decrease as additional data for the control samples are added and is not in any way a hindrance to the application of the curve today.

Discussion

Culture Conditions

The conditions for handling and processing samples are outlined in a variety of literature sources (IAEA 1986, 2001, Prasanna 2002). However, many of the procedures are variable between laboratories and can greatly affect the results of the assay. Often, procedures for one lab may not be optimal for another lab, due to issues arising from the use of reagents from different vendors, different instruments, variations in techniques, etc. This is in part why it is suggested that each lab

create independent working protocols and dose response curves. The critical steps found in our work are explained further. In lymphocyte separation, if the resulting layer of blood is mixed in anyway with the histopaque, separation will be poor and it is likely to result in loss of cells. While some laboratories opt to forgoe lymphocyte separation rather than risk cell loss, we found that lymphocyte separation provided cleaner preparations with better overall yield of cells, given very careful separation procedures. The length of time used during colcemid arrest is variable among labs and may depend on the concentration used, manufacturer of the product used, etc. The colcemid incubation will greatly affect the quality of the metaphase chromosomes observed, i.e. whether chromosomes are too condensed to distinguish or too elongated, etc. Over bloating of the cells in hypotonic solution can cause cells to burst while too short incubation in hypotonic solution may result in cells that do not spread out and hence have many overlapping chromosomes. As phosphate buffer can have bacterial growth which affects the pH and hence the Giemsa stain, fresh buffer is necessary. Finally, the humidity during the dropping of cells onto slides had been reported to greatly affect the quality of metaphase spreads (STUK). Some labs use humidified chambers to drop slides and this is a consideration for the future.

Metaphase Evaluations

Metaphase evaluations are in part subjective and require some technical expertise as documented by inter-comparisons (Lloyd 1987, IAEA 2001, Lindholm 2002). Each laboratory has standards by which they evaluate the cells and these must be shared among all participants in the lab. Any new person that is to contribute to a laboratory's assessment must receive adequate training such that their judgment in scoring is comparable to the previous evaluations which can require up to 6 months or more of training. Furthermore, each person contributing to the evaluations must maintain competence by adding additional data, conducting new experiments, or participation in exercises, such as inter-comparisons. Training for 1-2 weeks each quarter (1-2 months per year) should be sufficient for each technical person to maintain competence.

The uncertainty in evaluating dicentrics is illustrated in Figure 7 of Appendix III. The chromosomes may take a variety of shapes and qualities, all of which are not perfectly clear and easy to discriminate. While some metaphases may be considered unscorable, it is not practical to exclude all cells that are not perfectly distinct. Instead, each lab develops standards for evaluations and exclusion of cells as mentioned previously. However, there are other methods that aid in evaluation and reduce uncertainty and hence increase reliability and sensitivity. The use of a pancentromeric DNA hybridization probe for centromere painting has been suggested for aiding in distinguishing centromeres and thus dicentrics (Kolanko 1993, Schmid 1995, Roy 1996). Conversely, this method requires incubation with a fluorescent probe, adding an additional day of laboratory work before analysis can begin and limits evaluation to a fluorescent microscope. Alternatively, some biodosimetry labs have incorporated a metaphase finder, software that recognizes and captures images of metaphases, to their microscope systems. This type of system includes software that estimates the identity of chromosomes based on their relative lengths and shapes. In this way, the system greatly facilitates analysis by speeding up the acquisition of metaphases, aids in identification of aberrant chromosomes, and subsequently decreases uncertainty. Therefore, the approach using metaphase software for addressing uncertainty is preferred for application of the dicentric assay in emergency preparedness.

Dose Response Curve

The dose response curve established by FOI is comparable to other published curves from eventual accredited biodosimetry laboratories (service biodosimetry laboratories are now preparing for accreditation based on ISO guidelines published in 2004). We have conducted the experiments for the dose response curve with ISO accreditation in mind, which includes but is not limited to documentation of procedures and competence. However, the dose response curve is considered an initial curve, for several reasons. For maintaining competence and expertise, additional dose points

may be added in the future. To increase statistical strength for lower dose estimates, more data will be added to the lower dose points. Laboratories may accumulate data over several years to have several thousands of cells at these points. Our lab will continue to add data for the low dose points in the future which is especially important for our statistics due to the high background frequency observed in our data. Furthermore, any additional personnel trained for evaluations must add comparable data to the curve which is a requirement under ISO guidelines (ISO, 2004). Hence, our dose response curve should be continually updated and maintained.

While the frequency of dicentric aberrations was comparable to other data, the frequency of other aberrations was more variable. The number of acentrics and rings observed vary much more among laboratories and is why assessments are not based on their frequencies. The variation is due to both greater variability in occurrence as well as variability in criteria for inclusion in the data. For example, some labs check for 46 pieces in a metaphase spread and do not evaluate the cell if there are more than 46 pieces and no obvious aberrations. This approach will result in a more conservative assessment of acentrics. However, our lab checks for 46 centromeres rather than pieces, includes the cell if there are 46 centromeres even if there are additional pieces, and counts the extra pieces as acentrics if appropriate. Our approach results in a higher acentric yield as reflected in our data. Another factor influencing acentric evaluation is cellular debris which can be partially removed by using RNase and this technique is currently being incorporated into protocols (Hayata 1993). The use of the RNase may facilitate scoring by removing substances which can be mistaken for acentric fragments or otherwise hinder the evaluation of cells.

Finally, the frequency of rings tends to be more variable due to the difficulty in evaluation. Only rings containing centromeres (centric rings) should be counted. However, the observation of a centromere is difficult in smaller rings. The evaluation of rings becomes important mainly with assessment at very high doses, an area which warrants further research. Since medical technology today enables the treatment of patients with quite high dose exposures, assessment at much higher doses has received more attention recently and will likely result in improved methods for these evaluations in the future, perhaps by evaluating rings in prematurely condensed chromosomes (Kanda 1999).

Limitations and Future Work

Because unstable aberrations are very different from normal chromosomes, they are eliminated with time and cell turnover. This is the reason the dicentric assay has limited utility over time, and may be applied for only a few months after an exposure. Stable aberrations, such as reciprocal translocations in which two broken chromosomes have exchanged pieces and rejoined with the other chromosome, may be evaluated for retrospective assessments occurring up to many years after an exposure (Lucas 1997, Lindholm 1998, Lindholm 2004). However, because these aberrations appear like normal chromosomes, special techniques such as fluorescence *in situ* hybridization, FISH, must be used to observe these damages. Since this method is more time consuming, expensive, and less sensitive, it is generally only applied at longer times after exposure. Experiments using this technique have been initiated at FOI and preparation of a dose response curve with this method will proceed in the future.

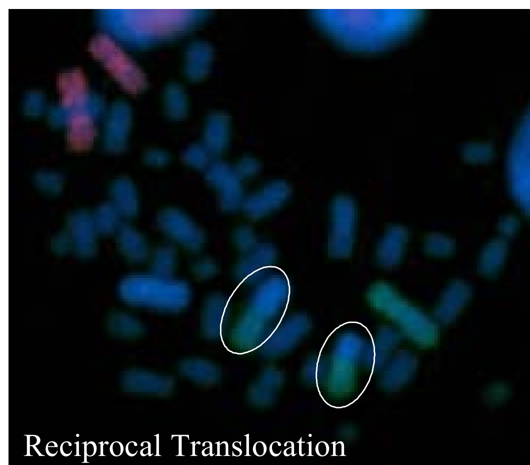

Figure 6: The picture above represents an abnormal metaphase illustrating stable aberrations, pair of reciprocal translocations, observed with FISH painting. This picture is taken from FOI's *in vitro* dose response experiment, 2.3 Gy irradiated sample.

A variety of other factors affect the reliability and application of the dicentric assay. For example, the energy and type of radiation will greatly impact the yield of aberrations formed as a result of exposure (Edwards 1997). X-rays produce more damage per dose than gamma irradiation, and high LET neutrons produce a much greater yield than either of those and include more complex rearrangements. While some idea of the type or ratio of radiations suspected can aid in dose assessment (Voisin 2004), IAEA suggests that each lab develop calibration curves for all three radiations mentioned if possible (IAEA 2001). Upon the acquisition of a microscope with a metaphase finder to provide more efficient data acquisition, our lab has planned to prepare a dose response curve for x-rays and has considered preparing a neutron curve through collaborative efforts.

Another consideration for biological dosimetry analysis is partial body exposures. Since the evaluation is conducted on the lymphocyte population, only the percentage of cells exposed will potentially show damage. Thus, if knowledge of the percentage and location of a partial body exposure is available, the partial exposure can be accounted for and a reliable dose estimate can be calculated. Otherwise, a dosimetric evaluation may still be performed and some estimate of dose obtained by observing and accounting for the over-dispersion of damage per cell (IAEA 2004). Since only a certain percentage of the body is damaged, the frequency of aberrations will be smaller than expected considering the number of damages observed per cell. Mathematical models are used with the data to ascertain dose estimates (Sasaki 2003). This method may be similarly applied in evaluation of exposure to unknown radiation to provide some additional information since higher LET radiations tend to produce more complex rearrangements than low LET radiations. However, for cases of unknown radiation type and partial body exposures, large uncertainty exists in the evaluations, and more research is needed to fully understand and accurately apply biodosimetry methods in these cases.

Finally, the dicentric assay has a range of application between 0.2 Gy and 5 Gy. The detection limit at the low end is restricted by the background frequency of aberrations in the population, and as a result, limits its application in very low dose radiation studies. On the other hand, the dicentric assay may not be applicable at the very highest doses received due to gross cell killing. In very high dose exposures, blood samples should be taken as soon after the exposure as possible, as the blood cells rapidly decline with time and may not yield enough viable cells for evaluation, and samples are not valid after blood transfusions begin. An evaluation also must be done as quickly as possible in this case, in order to provide information for medical treatment planning. In this area, research has been conducted on a variation of the dicentric assay by inducing prematurely condensed chromosomes, PCC's, either by fusion of human lymphocytes with mitotic hamster cells (Durante 1997) or chemical induction with phosphatase inhibitors (Coco-Martin 1997, Durante 1998,

Prasanna 2000). The potential of the method was demonstrated when applied during the Tokai-mura criticality accident as it provided useful and comparable results as compared to the dicentric assay (Hayata 2001). While several variations exist for both the culturing of the cells and for the analysis of the resulting PCCs, a standard protocol for the application of the method does not exist. Our lab has initiated PCC experiments and has proposed a joint project with the Finnish Radiation Protection Authority, STUK, and the Norwegian Radiation Protection Authority, NRPA, for determining the optimal parameters for application of the PCC method in emergency preparedness.

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Appendix I: Protocols for Sample Processing

Blood Collection

Collect blood in 5 or 10 ml Na or Li Heparin (green top) tubes. Invert the tube several times to dissolve and mix the blood and heparin.

Lymphocyte Separation

Using Histopaque®-1077 (Sigma)

1. To a 15-ml conical centrifuge tube, add 5 ml Histopaque®-1077 and bring to room temperature. Carefully layer 5 ml whole blood onto the Histopaque.
2. Centrifuge at 400xg (turn **brake off**!!) for 30 minutes at room temperature. Beckman centrifuge 1400rpm. Centrifugation at lower temperatures may result in cell clumping and poor recovery.
3. After centrifugation carefully aspirate, with a Pasteur pipette, the upper layer to within 0.5 cm of the opaque interface containing mononuclear cells. Discard upper layer.
4. Carefully transfer the opaque interface into a clean conical centrifuge tube. Add to this tube up to the 10 ml mark culture medium (RPMI) and mix gently by aspiration.*
5. Centrifuge at 250xg for 20 minutes. 1100rpm with Beckman centrifuge (**low brake**). Aspirate and discard. Leave 0.5 ml to re-suspend pellet.
6. Re-suspend cells by gentle agitation and bring up 7 ml in (complete) media*. With tops tightened, invert tubes to mix.

*For radiation experiments, at this point, cells should be in a media wash and **promptly** irradiated before incubation in complete media. Patient samples may be placed directly in complete media for incubation at this point.

Metaphase Culture Media Preparation

(30 ml):

RPMI 1640*	26 ml Glutamax
10% FBS/FCS	3 ml of stock
2% PHA-M (fridge)	600µl of 1mg PHA in 1 ml media
1% NaHeparin (RT)	300µl of 1mg NaHep in 1 ml media
50 µg/ml Gentamycin	150µl stock
2mM L-Glutamine*	na

* If using RPMI 1640 Glutamax media, addition of glutamine is not necessary.

1. **Thaw FBS** at room temperature.
2. Prepare **PHA** and **NaHeparin** solutions using aseptic techniques in sterile 1ml centrifuge tubes and weighing out 1 mg reagents with a spatula cleaned with alcohol. Mix reagents with 1ml media.
3. Using fresh stock media and aseptic techniques, first combine the media and FBS in sterile 50ml centrifuge tubes. Add other reagents and close centrifuge tubes tightly and mix.
4. Store media in refrigerator until ready to use.

Metaphase Cell Culture

Incubation

1. After lymphocyte separation and irradiation if applicable, **loosen tops** and place tubes in slanted sample rack in the incubator at 37ºC and 5% CO_2. Cells should be rotated once or twice during the next 46 hours.
2. At 46 hours and 15 min, add **colcemid** (385µl of 10µg/ml stock colcemid) to solution and invert tubes to mix and incubate for 1 hour 45 min.

Harvest cells at the end of incubation according to standard procedure.

Cell Harvest

Reagents:

Hypotonic KCl*	Dissolve 0.56g KCl in 100mL distilled water, warm to **37°C** (place in incubator)
Carnoy's Solution*	25mL acetic acid, 75mL methanol, **chill on ice**

*KCl lasts two weeks while Carnoy´s has to be prepared fresh every time.

1. Prepare reagents during colcemid incubation. At the end of the incubation period, centrifuge samples at 1000rpm for 5 min. Remove supernatant (leave about 0.5 ml) and vortex to resuspend pellet.
2. Slowly add 10mL (**dropwise**) KCL to each tube while gently **vortexing**. Incubate at 37°C for 30-45 min. Note that longer incubation time will bloat cells too much and may make them labile.
3. At the end of the incubation, add 400µL Carnoy's and mix well by inversion. Centrifuge at 1000rpm for 5 min. Remove supernatant (leave about 0.5 ml).
4. While **slowly** vortexing (so not to burst cells), add 2mL Carnoy's. Stop vortexing and add remaining 8ml Carnoy's (wash the walls to get cells down). Spin at 1000rpm for 5 min. Do at least 2 x 10 ml washing.
5. When cells are clean, freeze in full volume and with cells pelleted.

Preparation of Giemsa Stain

Giemsa Stain

96 ml Phosphate Buffer (pH 6.8)

4 ml Geimsa stock sol.

Phosphate buffer (pH 6.8)

50 ml	0.1M KH_2PO_4	(680.5 mg KH_2PO_4	50ml H_2O)
22.4ml	0.1M NaOH	(400 mg NaOH	100ml H_2O)

Buffer Preparation

1. Stock NaOH solution can be prepared, but phosphate solution should be prepared fresh.
2. The phosphate solution can be prepared in one bottle and the majority of the NaOH added.
3. Measure the pH while adding the remainder of the NaOH drop-wise. The pH is important, therefore the meter should be recently calibrated.

Stain Preparation*

1. Measure the amount of buffer you have prepared and calculate the amount of Giemsa stock solution needed.
2. Place the buffer in a well-labeled bottle indicating Giemsa stain, wrapped in Al foil. Add the amount of Giemsa stock solution required, place top securely on bottle, and mix.

*Always use freshly prepared stain. The stain should be no more than 1 week old.

Important: Giemsa is toxic and stains very easily. It is important to wear **gloves** and protective clothing when handling the stock solution and the stain solution.

Dropping and Staining Slides

1. Samples taken from freezer should be washed with fresh Carnoy's. Remove enough solution to resuspend cells in 0.5-1mL solution, depending on the number of cells present.
2. For Giemsa staining, regular slides with frosted edges may be used. For FISH or any fluorescence staining, high quality pre-cleaned slides should be used.

3. Label slides for samples and place slides in metal holder and put into glass cylinder with distilled water. Place slides one at a time on metal tray for dropping. It is very important that a water film remains on the slide while dropping the sample. Resuspend sample and pipette up 40μL. Drop the sample onto the slide from at least 1ft or about 30cm from pipette to slide. This will ensure metaphases adequately spread upon hitting surface of the slide. Shake excess water from slide and stand on its side to dry for at least 15min.
4. Place slides flat on a stand in a metal tray so that they are not touching. Add Giemsa drop-wise onto top of slide until surface is covered with Giemsa. Let stand for **7min**, then wash the entire slide gently and thoroughly with distilled water. Allow to dry for at least 15 min.

Appendix II: Distribution of Aberrations Observed in Analyses

Dose					Distribution of dicentrics[2]				
Gy	Cells	Dicentrics	Acentrics[1]	Rings	0	1	2	3	4+
0	560	1	15	1	559	1			
0.2	519	6	12	1	513	6			
0.4	500	8	47	0	492	8			
0.8	781	41	106	2	741	39	1		
1.5	654	109	134	6	550	101	1	2	
2.3	599	235	267	26	407	154	34	3	5
3.2	452	315	312	14	218	174	45	10	5
4.0	321	337	337	28	100	132	67	19	3
5	107	194	135	18	10	37	30	23	7

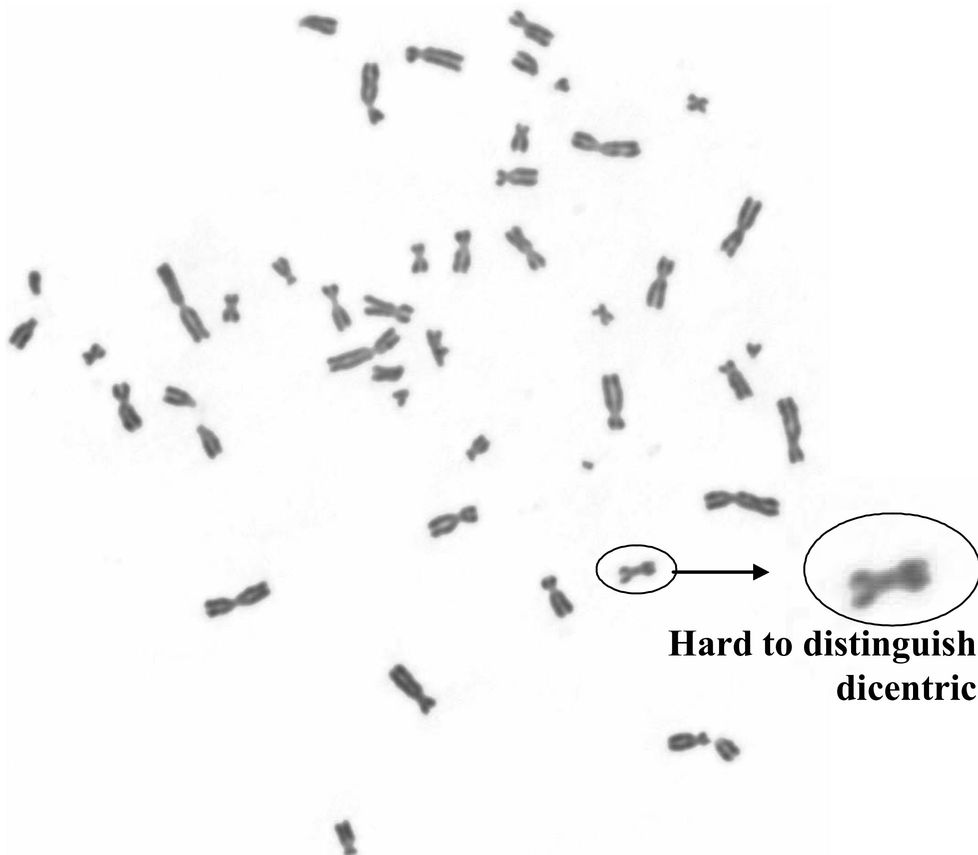
[1]Includes excess acentrics, not acentrics accompanying the dicentrics.
[2]Refers to the number of cells observed with the corresponding number of dicentrics.

Appendix III: Intra-Comparison

Sample: 4 Gy	Dicentrics*	
Coordinates	Scorer: DS	Scorer: EA
43.8 x 96.5		
44.4 x 95.1		
44.8 x 100.1	2	1
45.2 x 104.8	1	1
45.4 x 110.2	1	1
45.6 x 110.2		
46.1 x 109.3	1	1
46.1 x 96.2		
46.5 x 107.1	1	1
46.8 x 105.7	1	1
46.8 x 96.1	2	2
47.3 x 105.7	1	1
47.3 x 106.3	1	1
47.4 x 96.3	1	1
47.6 x 93.7		
48.3 x 106	1	1
48.3 x 111.1		
48.4 x 94.8	3	3
48.7 x 105.8	1	1
49.1 x 100.6	4	3
49.1 x 101.3	1	
49.5 x 108.9		
49.5 x 110.7	1	1
49.5 x 98.3		
49.5 x 106.1	1	1
49.7 x 99.2	2	2
49.8 x 102.2	1	1
49.8 x 103.8	2	2
50.1 x 102.6	1	1
51.3 x 93.2	2	2
51.4 x 105	2	2
51.4 x 110.4	1	1
51 x 106.3	2	2
52.2 x 99.6	2	2
52.5 x 94.4	1	1
52.8 x 97	2	2
52 x 107.4		
53.1 x 109.3	4	4
53.2 x 94.3	3	3
53.4 x 110.1	2	2
54.3 x 95.1	1	1
54.4 x 99.3	2	2
54.5 x 94.1		
54.7 x 93.5	2	2
54.7 x 98.4		
54.7 x 104.2		1
54.8 x 99.8	1	1
54.9 x 93		
54.9 x 94.6		
54.9 x 98.7	1	1
55.1 x 105.2		

*Dicentrics with accompanying acentric fragments.

Figure 7. Example of a differently scored metaphase due to reasonable uncertainty.



Appendix IV: Inter-Comparison of Dose Response Curves

Figure 8. Graphical representation of dose response curves from different laboratories based on published curve coefficients listed in Table 3.