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**RENEB Inter-Laboratory Comparison 2021:
The FISH-based translocation assay**

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Abstract

Purpose: Translocation analysis using fluorescence *in situ* hybridization (FISH) is the method of choice for dose-assessment in case of chronic or past exposures to ionizing radiation. Although it is a widespread technique, unlike dicentrics, the number of FISH-based inter-laboratory comparisons is small. For this reason, although the current RENEb (Running the European Network of Biological and Physical retrospective Dosimetry) inter-laboratory comparison 2021 was designed as a fast response to a real emergency scenario, it was considered a good opportunity to perform an inter-laboratory comparison using the FISH technique to gain further experience.

Material and methods: The Bundeswehr Institute of Radiobiology provided peripheral blood samples from one healthy human volunteer. Three test samples were irradiated with blinded doses of 0, 1.2, and 3.5 Gy, respectively. Samples were then sent to the seven participating laboratories. The FISH technique was applied according to the standard procedure of each laboratory. Both, the frequency of translocations and the estimated dose for each sample were sent to the coordinator using a special scoring sheet for FISH.

Results: All participants sent their results in due time. However, although it was initially requested to send the results based on the full analysis, evaluating 500 equivalent cells, most laboratories only sent the results based on triage, with a smaller number of analyzed cells. In the triage analysis, there was great heterogeneity in the number of equivalent cells scored. On the contrary, for the full analysis, this number was more homogeneous.

For all three samples, one laboratory showed outlier yields compared to the other laboratories. Excluding these results, in the triage analysis, the frequency of translocations in sample #1 ranged from 0 to 0.013 translocations per cell, and for samples #2 and #3 the genomic mean frequency were 0.27 ± 0.03 and 1.47 ± 0.14 , with a coefficient of variation of 0.29 and 0.23 respectively.

Considering only results obtained in the triage analysis for sample #1, all laboratories, except one, classified this sample as the non-irradiated one. For sample #2, excluding the outlier value, the mean reported dose was 1.74 ± 0.16 Gy indicating a mean deviation of about 0.5 Gy to the delivered dose of 1.2 Gy. For sample #3 the mean dose estimated was 4.21 ± 0.21 Gy indicating a mean deviation of about 0.7 Gy to the delivered dose of 3.5 Gy.

Conclusions: In the frame of RENEB, this is the second FISH-based inter-laboratory comparison. The whole exercise was planned as a response to an emergency, therefore, a triage analysis was requested for all the biomarkers except for FISH. Although a full analysis was initially requested for FISH, most of the laboratories reported only a triage-based result. The main reason is that it was not clearly stated what was required before starting the exercise. Results show that most of the laboratories successfully discriminated unexposed and irradiated samples from each other without any overlap. A good agreement in the observed frequencies of translocations was observed but there was a tendency to overestimate the delivered doses. Efforts to improve the harmonization of this technique and subsequent exercises to elucidate the reason for this trend should be promoted.

Introduction

Among the different biomarkers of exposure to ionizing radiation (IR), the detection of translocations by means of fluorescence *in situ* hybridization (FISH) techniques is one of the widely accepted methods in biological dosimetry (1). After exposure to ionizing radiation, the number of induced dicentrics is assumed to be similar to the number of induced translocations (2-4). However, while dicentrics decrease with post-irradiation time, probably in a dose-dependent manner (5,6), translocation yield remains relatively constant, at least for doses below 3 Gy (7-10). For this reason, translocation-based dosimetry is proposed for chronic or past exposures to IR (1), and its usefulness has been proved in several studies (5,11,12). In addition, some authors pointed out the use of automated scoring for translocation-based dosimetry as automated analysis may have fewer problems to detect chromosome exchanges using fluorochromes than with uniform staining (13,14).

Contrary to the situation for the dicentric chromosome assay (DCA), where there are many inter-laboratory comparisons (ILCs), the number of ILCs is scarce for FISH-based translocations analysis. In the beginning, and as a part of a follow-up study of the Estonian accident (15), some comparisons among laboratories were done under the umbrella of a European Concerted Action (3). More recently, and under the RENEb network (Running the European Network of Biological and Physical retrospective Dosimetry), a first ILC for FISH was performed (16). The present RENEb ILC 2021 exercise was planned to simulate a real emergency scenario. Although compared to DCA, FISH-based translocations analysis should not be the first choice to be considered in an emergency for early categorization of potentially exposed individuals, it was considered a good opportunity to ensure the availability, quality, and efficiency of the FISH assay, and to identify needs for training and harmonization of the RENEb members.

Material and methods.

Blood sampling, radiation exposure, and distribution to participants

Irradiation conditions and blood shipment was done as described in the first (Interassay comparison) manuscript of this issue (17). In brief, the Bundeswehr Institute of Radiobiology provided peripheral blood samples from one healthy human donor. The doses corresponding to the blood samples were blinded and coded and are referred to as test sample #1 (0 Gy), #2 (1.2 Gy) and #3 (3.5 Gy). Samples were then shipped to participant laboratories, except for one laboratory that received unstained slides. This laboratory did the FISH staining technique and shared captured images with four further laboratories. As the same data and calibration curve was used for these five laboratories, they were grouped together as one laboratory (L2) for easier representation (Table1).

Sample preparation and analysis

Each laboratory followed its own standard protocol for blood culture. Lymphocytes were cultured for 48 or 50 h, and colcemid was added 24 to 2 h before culture harvesting. The FISH technique was applied according to the individual laboratory's procedure (Table 1). As different painting strategies cover different proportions of the genome, and to compare the frequencies of translocations between laboratories, the observed frequencies were converted to genomic frequencies (F_G), according to Lucas' and co-workers' proposed model (18,19).

By FISH painting techniques, chromosome aberrations can be described using different nomenclatures (i.e. one- or two-way, apparently simple, etc.). In the RENEBC 2021, all laboratories, except one, provided the type of translocations considered in the RENEBC scoring sheet. These translocations were in all cases one- and two-way translocations.

This categorization includes those translocations coming from complex aberrations (involving at least three breaks and affecting two or more chromosomes). Some laboratories also based their analysis on total apparently simple translocations, in this case, excluding those translocations arising from complex aberrations. Six participants provided results based on the observed frequencies of translocations. These frequencies have been scaled to full genome using the genomic conversion factor indicated in Table 1.

For dose estimation, almost all of the participants used their own calibration curve for translocations, and one used a dicentric curve. Some participants provided the calibration curve coefficients scaled to full genome, while others did not. For comparisons in this manuscript, all coefficients have been converted to full genome (Table 2 and Figure 1). Calibration curves were constructed using high-energy photons (X rays of 4 MV or gamma rays from Cobalt-60 with a mean energy of 1.25 MeV). For most of the laboratories, the doses used for calibration ranged from 0 to 4-5 Gy, with dose rates ranging from 0.29 to 0.75 Gy·min⁻¹. Of the seven calibration curves used by the participating laboratories, there were two calibration curves in which the doses used to calibrate, or the aberrations considered, differed clearly from the others; one where the highest evaluated dose was 1 Gy and where the dose rate was 0.026 Gy·min⁻¹ (L2) and another reported curve that was based on dicentric counts, not translocations (L6). Participants were requested to provide the dose based on both triage and full analysis (i.e. 50 or 500 equivalent cells respectively). Furthermore, for the five laboratories that shared images, only one compiled translocation frequency was reported for each sample (L2) instead of five separate scores, and dose estimation was performed with the same calibration curve.

Results

All laboratories sent their results within the established period (6 weeks). All laboratories reported results for the triage analysis, and in this case, there was a significant heterogeneity in the number of equivalent cells scored. For sample #1 this number ranged from 57 to 249 equivalent cells; for sample #2 from 32 to 161; and for sample #3 ranged from 2 to 41 equivalent cells. Three laboratories reported the full analysis (L1, L4, and L7), and in this case, the number of equivalent cells scored was more homogeneous. For sample #1 from 496 to 511; for sample #2 from 416 to 520; and for sample #3 from 69 to 185. In the full analysis, the number of translocations observed in samples #2 and #3 were higher than 100.

In the scoring sheet provided by the participating laboratories, most of the reported frequencies were the ones observed, and they were not scaled to full genome equivalent cells. For comparison purposes, observed frequencies have been converted to genomic frequencies (Figure 2). All converted results based on triage analysis are indicated in Figure 2A. For all three test samples, one laboratory (L6) showed outlier yields compared to the mean (outside the 95% confidence limit of the mean), for this reason in Figures 2B and 2C results from L6 were excluded. For the results based on triage analysis (Figure 2B), the mean values observed were 0.002 ± 0.005 , 0.27 ± 0.08 and 1.47 ± 0.33 for test samples #1, #2 and #3 respectively. The coefficient of variation (CV) for samples #2 and #3 was 0.29 and 0.23. The variability was higher for sample #3 with respect to sample #2, the inter-quantile range (IQR) was 0.30 and 0.10 translocations per cell for samples #3 and #2, respectively. Three laboratories reported full analysis based on about 500 equivalent cells (Figure 2C). For these three laboratories, the observed frequencies in the

full analysis did not differ significantly from those observed in the triage analysis. The major difference between both analyses was found for laboratory L7, where for sample #3 the observed yield of translocations per cell in the triage analysis was 1.61 while in the full analysis it was 1.81.

Clear information how the frequency of translocations was converted to an estimated dose was provided by all participants. However, the participants applied different ways to calculate the error of the estimated doses, and to unify, doses were re-calculated by Biodosetools (20) using the Merkle's method (21) and considering the 95% confidence limits of both, the observed frequencies of translocations and the reported dose effect-curves. The obtained results are shown in Table 3. All estimated doses based on triage analysis can be seen in Figure 3A. Like the results for the translocation yields, the estimated doses from laboratory L6 were also clearly outside the dose range estimated by the other laboratories. Considering only results obtained in the triage analysis for test sample #1, corresponding to the 0 Gy dose, except for L6, the other laboratories classified their samples as the non-irradiated one. For the irradiated samples (#2 and #3) all estimated doses were above the delivered ones. For sample #2, corresponding to the 1.2 Gy dose, in the triage analysis 4 laboratories reported a dose lower than 2 Gy; two laboratories reported doses between 2 and 2.5 Gy, and one laboratory reported a dose higher than 3 Gy (L6). For sample #2, considering all laboratories, the mean estimated dose in the triage analysis was 1.99 ± 0.28 Gy and the CV 0.37. If the value reported by L6 was excluded, the mean reported dose would be 1.74 ± 0.16 , and the CV 0.22 (Figure 3B). This indicates that for sample #2 there was a mean deviation of about 0.5 Gy to the delivered dose. The IQR was 0.55 Gy. When reported doses based on full analysis of the three participant laboratories are also considered L1, L4 and L7 (Figure 3C), only for L7,

the 95% confidence interval included the 1.2 Gy dose and the mean estimated dose is again 1.74 ± 0.14 Gy, which is comparable to the triage analysis value. For sample #3 estimated doses ranged from 3.6 to 4.7 Gy, with a clear outlier value of 7.7 Gy provided by L6. Excluding this value, the mean dose estimated was 4.21 ± 0.21 Gy and the mean deviation from the delivered dose was about 0.7 Gy. The CV for sample #3 was 0.12 and the IQR 0.94 Gy. When the full analysis was considered, the mean dose estimated by L1, L4, and L7 was 4.39 ± 0.24 Gy, and only for L7 the 95% confidence interval of the estimated doses included the 3.5 Gy dose.

Discussion

FISH-based translocation analysis is the method of choice in cases of chronic or past exposures to IR (1). It has been used for dose assessment in cases of occupational exposures (22), to estimate the dose from a blood sample obtained well after the accident (10), or for dose reconstruction in accidents that occurred in the past (23 ,24). The principle of the technique is based on translocations and dicentrics are induced at a similar frequency after exposure to IR, and they show a similar relationship with radiation dose. With appropriate dose-effect curves the yield of translocations observed in a blood sample can be converted to a dose. However, unlike dicentrics, translocations cannot be easily detected using conventional staining procedures. Most translocations go unnoticed with solid stained metaphases while the detection of radio-induced translocations is very laborious with banding techniques. For this reason and for biological dosimetry purposes, translocations are detected employing FISH techniques, using DNA-probes that cover whole chromosome pairs, the so-called chromosome *painting*. The selected chromosome pairs are those that cover an important part of the genome, such as chromosomes # 1 and # 2, and those that are morphologically different from each other, such as chromosomes

1 and # 12. The conversion factor to full genome equivalence as proposed by Lucas et al (18) ranged from 0.28 to 0.396 for the participating laboratories. This coverage agrees with the one suggested by the ISO standard 20046 (25).

All participating laboratories have sent triage analysis for this ILC while three laboratories have sent both triage and full analysis for dose assessment. There was great variability in the number of scored cells for the triage data. This can be partially attributed to the excel-based scoring sheet that has been provided to the laboratories. In that document, it was not clearly stated what was being asked. In addition, since the entire exercise was designed to respond to an emergency, it was very likely not clear enough that FISH required full analysis. Another factor that may have contributed to the fact that most laboratories only performed the triage analysis was the poor mitotic index obtained in the blood cultures by some participants.

In the previous ILC for FISH conducted by RENE B (16) two consecutive exercises were carried out after an initial exercise to harmonize the way to score translocations. In the first one, blood was irradiated at 2 Gy and the CV observed in the reported frequency of translocations by the 11 participating laboratories was 32%. In the second exercise, blood was irradiated at 0.85 and 2.7 Gy and the CV observed were 16 and 21%, respectively. In the present RENE B ILC 2021 from the 7 participants, three did not participate in the previous one, and the values reported for one of them were clearly too high. Excluding these outlier values, the CVs obtained were 29 and 23% for test samples #2 and #3 in which radiation dose delivered were 1.2 and 3.5 Gy. These results suggest that even though most laboratories have a similar way of scoring translocations, for future ILCs based on FISH analysis and avoiding atypical values, prior exercises or meetings would be necessary to indicate what is required clearly. FISH-based ILCs do not have the same

periodicity as dicentric analysis-based ILCs and the changes in the researchers involved can have a very significant effect on the study.

Biological dosimetry aims to evaluate the dose by interpolating the yield of a biomarker of radiation exposure to a pre-established calibration curve. As already reported for the DCA (16,26), there was significant variability in the reported calibration curves. This was also observed in the first FISH-based ILC and similar to what happened in the first ILC (16), in the present ILC one laboratory used a calibration curve based on dicentric chromosomes. It is assumed that the induction of dicentrics occurs with a similar frequency as translocations and that it was initially accepted that to evaluate chronic exposures the linear term for translocations could be inferred from the linear term of dicentric-based curves (27). It was also agreed that this should be used as an interim measure until FISH calibration data is available (28). This approach was not proposed for acute exposures, where the linear and quadratic terms are relevant. The dicentric curve for L6 is much lower than the curves from the other laboratories (Figure 1). Thus, for the same yield of translocations, the dose estimates based on this curve will be much higher than those estimated by the other curves based on translocation analysis. Another curve that needs to be discussed is the one where the maximum dose evaluated is 1 Gy (L2). In this case, the curve was constructed to estimate chronic low-dose exposures in occupationally exposed TEPCO clean-up workers. Moreover, the dose rate used during irradiation was one order of magnitude lower than the other reported curves. Despite the two curves being unsuitable for evaluating dose estimation reliability after acute exposures, estimated doses provided by extrapolation of observed frequencies to these curves have not been excluded to discuss all the results obtained.

As the exercise focused on triage, the samples were successfully categorized in most cases, except for L6, which reported very high outlier values. The scorer in L6 was new

to the FISH technique but decided to participate to gain practice. The high frequencies of translocations, and consequently, high reported doses, clearly demonstrate the requirement for scoring experience in FISH-based biological dosimetry. Scoring translocations in painted chromosomes is even more demanding than scoring dicentrics on Giemsa slides, so the deviating results of L6 are not surprising. For sample #1 all laboratories correctly classified this sample as the non-irradiated one. For sample #2 only two estimated doses were slightly higher than 2 Gy, and sample #3 was in all cases placed above 2 Gy. However, and similarly to what has been described for dicentrics and micronuclei in the present ILC, for samples #2 and #3, estimated doses were higher than the reference doses. The mean difference was about 0.5 and 0.7 Gy for samples #2 and #3 irradiated at 1.2 and 3.5 Gy, respectively. Similar deviations have been observed in the dicentric study of this ILC (0.5 and 0.9 Gy for samples #2 and #3). The deviation cannot be attributed to the low number of cells analyzed in the triage analysis. Because those laboratories that reported estimated doses based on the analysis of 500 equivalent cells or more than 100 translocations also estimated doses higher than the reference ones.

In the first FISH-based ILC (16) there was a general tendency to overestimate the delivered dose. In that ILC, blood samples were irradiated with a ^{137}Cs gamma-ray source at two different doses. For the low dose, 0.85 Gy, the mean estimated dose obtained by the eleven participant laboratories together was 1.17 Gy, 38% higher than the delivered one, and only one of the 10 reported values was equal to or lower than the delivered dose. For the high dose, 2.7 Gy, the mean estimated dose was 3.11 Gy, 15% higher than the delivered one, and in this case, only 2 of the 10 reported values were equal to or lower than 2.7 Gy. In the present study considering the triage results, the deviations are slightly higher 42% for sample #2 (1.2 Gy) and 20% for sample #3 (3.5 Gy). When the three values based on full analysis are considered, the deviations still increased by

55% for sample #2 and 25% for sample #3. Although several factors that may contribute to this difference, such as differences in radiation quality between irradiated samples and radiation used for calibration or the irradiation setup, have been extensively detailed in the dicentric paper of this special issue (29). Results of these two FISH-based ILCs indicate a tendency to overestimate using this technique. This trend still allows for the correct categorization of exposed individuals. Although FISH-based translocation analysis is not the technique of choice in an emergency, it should be applied for possible follow-up studies. For this reason, more studies are needed to correct this trend.

Conclusions

This is the second FISH-based ILC evaluating dose assessment using translocations as a biomarker. The whole exercise was planned as a response to an emergency and a triage analysis was requested for all the biomarkers except for FISH, where a full analysis was initially requested. However, most of the laboratories reported only one result based on triage. This can be attributed partly to the poor mitotic index indicated by some participants, but the main reason is that before starting the exercise it was not clearly stated what was required. In addition, some laboratories submitted their results based on genome equivalence while others did not. Although the scoring sheet allowed all values to be recalculated, future ILCs should clarify what is requested. Finally, although most of the laboratories successfully discriminated unexposed and irradiated samples, the analysis of translocations by FISH shows a tendency to overestimate the delivered doses, similarly to what has been seen with dicentrics and micronuclei. Although all aspects indicated in the dicentric manuscript can contribute to this overestimation, it should be noted that unlike dicentrics, where in most of the ILCs done, the mean dose estimate agrees with the delivered dose, for translocations the two ILCs performed so far have shown the same

tendency. Efforts to better harmonize this technique to avoid erroneous values and subsequent exercises to elucidate the reason for this trend should be promoted.

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Figure legends

Figure 1: Calibration data reported by participants. For comparisons and when needed calibration coefficients were converted to full genome. Although in some curves the maximum dose evaluated was 4 Gy. All curves have been drawn in the range of 0 to 5 Gy. Dotted curves represent the one constructed using dicentric data (*), and the one where the maximum dose evaluated was 1 Gy (**).

Figure 2: Genomic frequency of translocations reported by participant laboratories. **A)** boxplot including all reported frequencies based on triage analysis (black circles) and full analysis (white circles). **B)** boxplot excluding the L6 value and using only those frequencies based on triage. Boxplot **C** also includes the results based on full analysis.

Figure 3: Dose estimates reported by participant laboratories. Horizontal yellow lines indicate the delivered radiation dose. **A)** boxplot including all reported dose estimations based on triage analysis (black circles) and full analysis (white circles). **B)** boxplot excluding the L6 value and using only those point doses based on triage. Boxplot **C** also includes the results based on full analysis. Dotted lines indicated the delivered doses of 0, 1.2 and 3.5 Gy.

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**RENEB Inter-Laboratory Comparison 2021:
The FISH-based translocation assay**

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Abstract

Purpose: Translocation analysis using fluorescence *in situ* hybridization (FISH) is the method of choice for dose-assessment in case of chronic or past exposures to ionizing radiation. Although it is a widespread technique, unlike dicentrics, the number of FISH-based inter-laboratory comparisons is small. For this reason, although the current RENEb (Running the European Network of Biological and Physical retrospective Dosimetry) inter-laboratory comparison 2021 was designed as a fast response to a real emergency scenario, it was considered a good opportunity to perform an inter-laboratory comparison using the FISH technique to gain further experience.

Material and methods: The Bundeswehr Institute of Radiobiology provided peripheral blood samples from one healthy human volunteer. Three test samples were irradiated with blinded doses of 0, 1.2, and 3.5 Gy, respectively. Samples were then sent to the seven participating laboratories. The FISH technique was applied according to the standard procedure of each laboratory. Both, the frequency of translocations and the estimated dose for each sample were sent to the coordinator using a special scoring sheet for FISH.

Results: All participants sent their results in due time. However, although it was initially requested to send the results based on the full analysis, evaluating 500 equivalent cells, most laboratories only sent the results based on triage, with a smaller number of analyzed cells. In the triage analysis, there was great heterogeneity in the number of equivalent cells scored. On the contrary, for the full analysis, this number was more homogeneous.

For all three samples, one laboratory showed outlier yields compared to the other laboratories. Excluding these results, in the triage analysis, the frequency of translocations in sample #1 ranged from 0 to 0.013 translocations per cell, and for samples #2 and #3 the genomic mean frequency were 0.27 ± 0.03 and 1.47 ± 0.14 , with a coefficient of variation of 0.29 and 0.23 respectively.

Considering only results obtained in the triage analysis for sample #1, all laboratories, except one, classified this sample as the non-irradiated one. For sample #2, excluding the outlier value, the mean reported dose was 1.74 ± 0.16 Gy indicating a mean deviation of about 0.5 Gy to the delivered dose of 1.2 Gy. For sample #3 the mean dose estimated was 4.21 ± 0.21 Gy indicating a mean deviation of about 0.7 Gy to the delivered dose of 3.5 Gy.

Conclusions: In the frame of RENEB, this is the second FISH-based inter-laboratory comparison. The whole exercise was planned as a response to an emergency, therefore, a triage analysis was requested for all the biomarkers except for FISH. Although a full analysis was initially requested for FISH, most of the laboratories reported only a triage-based result. The main reason is that it was not clearly stated what was required before starting the exercise. Results show that most of the laboratories successfully discriminated unexposed and irradiated samples from each other without any overlap. A good agreement in the observed frequencies of translocations was observed but there was a tendency to overestimate the delivered doses. Efforts to improve the harmonization of this technique and subsequent exercises to elucidate the reason for this trend should be promoted.

Introduction

Among the different biomarkers of exposure to ionizing radiation (IR), the detection of translocations by means of fluorescence *in situ* hybridization (FISH) techniques is one of the widely accepted methods in biological dosimetry (1). After exposure to ionizing radiation, the number of induced dicentrics is assumed to be similar to the number of induced translocations (2-4). However, while dicentrics decrease with post-irradiation time, probably in a dose-dependent manner (5,6), translocation yield remains relatively constant, at least for doses below 3 Gy (7-10). For this reason, translocation-based dosimetry is proposed for chronic or past exposures to IR (1), and its usefulness has been proved in several studies (5,11,12). In addition, some authors pointed out the use of automated scoring for translocation-based dosimetry as automated analysis may have fewer problems to detect chromosome exchanges using fluorochromes than with uniform staining (13,14).

Contrary to the situation for the dicentric chromosome assay (DCA), where there are many inter-laboratory comparisons (ILCs), the number of ILCs is scarce for FISH-based translocations analysis. In the beginning, and as a part of a follow-up study of the Estonian accident (15), some comparisons among laboratories were done under the umbrella of a European Concerted Action (3). More recently, and under the RENEb network (Running the European Network of Biological and Physical retrospective Dosimetry), a first ILC for FISH was performed (16). The present RENEb ILC 2021 exercise was planned to simulate a real emergency scenario. Although compared to DCA, FISH-based translocations analysis should not be the first choice to be considered in an emergency for early categorization of potentially exposed individuals, it was considered a good opportunity to ensure the availability, quality, and efficiency of the FISH assay, and to identify needs for training and harmonization of the RENEb members.

Material and methods.

Blood sampling, radiation exposure, and distribution to participants

Irradiation conditions and blood shipment was done as described in the first (Interassay comparison) manuscript of this issue (17). In brief, the Bundeswehr Institute of Radiobiology provided peripheral blood samples from one healthy human donor. The doses corresponding to the blood samples were blinded and coded and are referred to as test sample #1 (0 Gy), #2 (1.2 Gy) and #3 (3.5 Gy). Samples were then shipped to participant laboratories, except for one laboratory that received unstained slides. This laboratory did the FISH staining technique and shared captured images with four further laboratories. As the same data and calibration curve was used for these five laboratories, they were grouped together as one laboratory (L2) for easier representation (Table1).

Sample preparation and analysis

Each laboratory followed its own standard protocol for blood culture. Lymphocytes were cultured for 48 or 50 h, and colcemid was added 24 to 2 h before culture harvesting. The FISH technique was applied according to the individual laboratory's procedure (Table 1). As different painting strategies cover different proportions of the genome, and to compare the frequencies of translocations between laboratories, the observed frequencies were converted to genomic frequencies (F_G), according to Lucas' and co-workers' proposed model (18,19).

By FISH painting techniques, chromosome aberrations can be described using different nomenclatures (i.e. one- or two-way, apparently simple, etc.). In the RENEBC 2021, all laboratories, except one, provided the type of translocations considered in the RENEBC scoring sheet. These translocations were in all cases one- and two-way translocations.

This categorization includes those translocations coming from complex aberrations (involving at least three breaks and affecting two or more chromosomes). Some laboratories also based their analysis on total apparently simple translocations, in this case, excluding those translocations arising from complex aberrations. Six participants provided results based on the observed frequencies of translocations. These frequencies have been scaled to full genome using the genomic conversion factor indicated in Table 1.

For dose estimation, almost all of the participants used their own calibration curve for translocations, and one used a dicentric curve. Some participants provided the calibration curve coefficients scaled to full genome, while others did not. For comparisons in this manuscript, all coefficients have been converted to full genome (Table 2 and Figure 1). Calibration curves were constructed using high-energy photons (X rays of 4 MV or gamma rays from Cobalt-60 with a mean energy of 1.25 MeV). For most of the laboratories, the doses used for calibration ranged from 0 to 4-5 Gy, with dose rates ranging from 0.29 to 0.75 Gy·min⁻¹. Of the seven calibration curves used by the participating laboratories, ~~there were two calibration curves clearly different from the others~~ there were two calibration curves in which the doses used to calibrate, or the aberrations considered, differed clearly from the others; one where the highest evaluated dose was 1 Gy and where the dose rate was 0.026 Gy·min⁻¹ (L2) and another reported curve that was based on dicentric counts, not translocations (L6). Participants were requested to provide the dose based on both triage and full analysis (i.e. 50 or 500 equivalent cells respectively). Furthermore, for the five laboratories that shared images, only one compiled translocation frequency was reported for each sample (L2) instead of five separate scores, and dose estimation was performed with the same calibration curve.

Results

All laboratories sent their results within the established period (6 weeks). All laboratories reported results for the triage analysis, and in this case, there was a significant heterogeneity in the number of equivalent cells scored. For sample #1 this number ranged from 57 to 249 equivalent cells; for sample #2 from 32 to 161; and for sample #3 ranged from 2 to 41 equivalent cells. Three laboratories reported the full analysis (L1, L4, and L7), and in this case, the number of equivalent cells scored was more homogeneous. For sample #1 from 496 to 511; for sample #2 from 416 to 520; and for sample #3 from 69 to 185. In the full analysis, the number of translocations observed in samples #2 and #3 were higher than 100.

In the scoring sheet provided by the participating laboratories, most of the reported frequencies were the ones observed, and they were not scaled to full genome equivalent cells. For comparison purposes, observed frequencies have been converted to genomic frequencies (Figure 2). All converted results based on triage analysis are indicated in Figure 2A. For all three test samples, one laboratory (L6) showed outlier yields compared to the mean (outside the 95% confidence limit of the mean), for this reason in Figures 2B and 2C results from L6 were excluded. For the results based on triage analysis (Figure 2B), the mean values observed were 0.002 ± 0.005 , 0.27 ± 0.08 and 1.47 ± 0.33 for test samples #1, #2 and #3 respectively. The coefficient of variation (CV) for samples #2 and #3 was 0.29 and 0.23. The variability was higher for sample #3 with respect to sample #2, the inter-quantile range (IQR) was 0.30 and 0.10 translocations per cell for samples #3 and #2, respectively. Three laboratories reported full analysis based on about 500 equivalent cells (Figure 2C). For these three laboratories, the observed frequencies in the

full analysis did not differ significantly from those observed in the triage analysis. The major difference between both analyses was found for laboratory L7, where for sample #3 the observed yield of translocations per cell in the triage analysis was 1.61 while in the full analysis it was 1.81.

Clear information how the frequency of translocations was converted to an estimated dose was provided by all participants. However, the participants applied different ways to calculate the error of the estimated doses, and to unify, doses were re-calculated by Biodosetools (20) using the Merkle's method (21) and considering the 95% confidence limits of both, the observed frequencies of translocations and the reported dose effect-curves. The obtained results are shown in Table 3. All estimated doses based on triage analysis can be seen in Figure 3A. Like the results for the translocation yields, the estimated doses from laboratory L6 were also clearly outside the dose range estimated by the other laboratories. Considering only results obtained in the triage analysis for test sample #1, corresponding to the 0 Gy dose, except for L6, the other laboratories classified their samples as the non-irradiated one. For the irradiated samples (#2 and #3) all estimated doses were above the delivered ones. For sample #2, corresponding to the 1.2 Gy dose, in the triage analysis 4 laboratories reported a dose lower than 2 Gy; two laboratories reported doses between 2 and 2.5 Gy, and one laboratory reported a dose higher than 3 Gy (L6). For sample #2, considering all laboratories, the mean estimated dose in the triage analysis was 1.99 ± 0.28 Gy and the CV 0.37. If the value reported by L6 was excluded, the mean reported dose would be 1.74 ± 0.16 , and the CV 0.22 (Figure 3B). This indicates that for sample #2 there was a mean deviation of about 0.5 Gy to the delivered dose. The IQR was 0.55 Gy. When reported doses based on full analysis of the three participant laboratories are also considered L1, L4 and L7 (Figure 3C), only for L7,

the 95% confidence interval included the 1.2 Gy dose and the mean estimated dose is again 1.74 ± 0.14 Gy, which is comparable to the triage analysis value. For sample #3 estimated doses ranged from 3.6 to 4.7 Gy, with a clear outlier value of 7.7 Gy provided by L6. Excluding this value, the mean dose estimated was 4.21 ± 0.21 Gy and the mean deviation from the delivered dose was about 0.7 Gy. The CV for sample #3 was 0.12 and the IQR 0.94 Gy. When the full analysis was considered, the mean dose estimated by L1, L4, and L7 was 4.39 ± 0.24 Gy, and only for L7 the 95% confidence interval of the estimated doses included the 3.5 Gy dose.

Discussion

FISH-based translocation analysis is the method of choice in cases of chronic or past exposures to IR (1). It has been used for dose assessment in cases of occupational exposures (22), to estimate the dose from a blood sample obtained well after the accident (10), or for dose reconstruction in accidents that occurred in the past (23 ,24). The principle of the technique is based on translocations and dicentric are induced at a similar frequency after exposure to IR, and they show a similar relationship with radiation dose. With appropriate dose-effect curves the yield of translocations observed in a blood sample can be converted to a dose. However, unlike dicentric, translocations cannot be easily detected using conventional staining procedures. Most translocations go unnoticed with solid stained metaphases while the detection of radio-induced translocations is very laborious with banding techniques. For this reason and for biological dosimetry purposes, translocations are detected employing FISH techniques, using DNA-probes that cover whole chromosome pairs, the so-called chromosome *painting*. The selected chromosome pairs are those that cover an important part of the genome, such as chromosomes # 1 and # 2, and those that are morphologically different from each other, such as chromosomes

1 and # 12. The conversion factor to full genome equivalence as proposed by Lucas et al (18) ranged from 0.28 to 0.396 for the participating laboratories. This coverage agrees with the one suggested by the ISO standard 20046 (25).

All participating laboratories have sent triage analysis for this ILC while three laboratories have sent both triage and full analysis for dose assessment. There was great variability in the number of scored cells for the triage data. This can be partially attributed to the excel-based scoring sheet that has been provided to the laboratories. In that document, it was not clearly stated what was being asked. In addition, since the entire exercise was designed to respond to an emergency, it was very likely not clear enough that FISH required full analysis. Another factor that may have contributed to the fact that most laboratories only performed the triage analysis was the poor mitotic index obtained in the blood cultures by some participants.

In the previous ILC for FISH conducted by RENE B (16) two consecutive exercises were carried out after an initial exercise to harmonize the way to score translocations. In the first one, blood was irradiated at 2 Gy and the CV observed in the reported frequency of translocations by the 11 participating laboratories was 32%. In the second exercise, blood was irradiated at 0.85 and 2.7 Gy and the CV observed were 16 and 21%, respectively. In the present RENE B ILC 2021 from the 7 participants, three did not participate in the previous one, and the values reported for one of them were clearly too high. Excluding these outlier values, the CVs obtained were 29 and 23% for test samples #2 and #3 in which radiation dose delivered were 1.2 and 3.5 Gy. These results suggest that even though most laboratories have a similar way of scoring translocations, for future ILCs based on FISH analysis and avoiding atypical values, prior exercises or meetings would be necessary to indicate what is required clearly. FISH-based ILCs do not have the same

periodicity as dicentric analysis-based ILCs and the changes in the researchers involved can have a very significant effect on the study.

Biological dosimetry aims to evaluate the dose by interpolating the yield of a biomarker of radiation exposure to a pre-established calibration curve. As already reported for the DCA (16,26), there was significant variability in the reported calibration curves. This was also observed in the first FISH-based ILC and similar to what happened in the first ILC (16), in the present ILC one laboratory used a calibration curve based on dicentric chromosomes. It is assumed that the induction of dicentrics occurs with a similar frequency as translocations and that it was initially accepted that to evaluate chronic exposures the linear term for translocations could be inferred from the linear term of dicentric-based curves (27). It was also agreed that this should be used as an interim measure until FISH calibration data is available (28). This approach was not proposed for acute exposures, where the linear and quadratic terms are relevant. The dicentric curve for L6 is much lower than the curves from the other laboratories (Figure 1). Thus, for the same yield of translocations, the dose estimates based on this curve will be much higher than those estimated by the other curves based on translocation analysis. Another curve that needs to be discussed is the one where the maximum dose evaluated is 1 Gy (L2). In this case, the curve was constructed to estimate chronic low-dose exposures in occupationally exposed TEPCO clean-up workers. Moreover, the dose rate used during irradiation was one order of magnitude lower than the other reported curves. Despite the two curves being unsuitable for evaluating dose estimation reliability after acute exposures, estimated doses provided by extrapolation of observed frequencies to these curves have not been excluded to discuss all the results obtained.

As the exercise focused on triage, the samples were successfully categorized in most cases, except for L6, which reported very high outlier values. The scorer in L6 was new

to the FISH technique but decided to participate to gain practice. The high frequencies of translocations, and consequently, high reported doses, clearly demonstrate the requirement for scoring experience in FISH-based biological dosimetry. Scoring translocations in painted chromosomes is even more demanding than scoring dicentrics on Giemsa slides, so the deviating results of L6 are not surprising. For sample #1 all laboratories correctly classified this sample as the non-irradiated one. For sample #2 only two estimated doses were slightly higher than 2 Gy, and sample #3 was in all cases placed above 2 Gy. However, and similarly to what has been described for dicentrics and micronuclei in the present ILC, for samples #2 and #3, estimated doses were higher than the reference doses. The mean difference was about 0.5 and 0.7 Gy for samples #2 and #3 irradiated at 1.2 and 3.5 Gy, respectively. Similar deviations have been observed in the dicentric study of this ILC (0.5 and 0.9 Gy for samples #2 and #3). The deviation cannot be attributed to the low number of cells analyzed in the triage analysis. Because those laboratories that reported estimated doses based on the analysis of 500 equivalent cells or more than 100 translocations also estimated doses higher than the reference ones.

In the first FISH-based ILC (16) there was a general tendency to overestimate the delivered dose. In that ILC, blood samples were irradiated with a ^{137}Cs gamma-ray source at two different doses. For the low dose, 0.85 Gy, the mean estimated dose obtained by the eleven participant laboratories together was 1.17 Gy, 38% higher than the delivered one, and only one of the 10 reported values was equal to or lower than the delivered dose. For the high dose, 2.7 Gy, the mean estimated dose was 3.11 Gy, 15% higher than the delivered one, and in this case, only 2 of the 10 reported values were equal to or lower than 2.7 Gy. In the present study considering the triage results, the deviations are slightly higher 42% for sample #2 (1.2 Gy) and 20% for sample #3 (3.5 Gy). When the three values based on full analysis are considered, the deviations still increased by

55% for sample #1 and 25% for sample #2. Although several factors that may contribute to this difference, such as differences in radiation quality between irradiated samples and radiation used for calibration or the irradiation setup, have been extensively detailed in the dicentric paper of this special issue (29). Results of these two FISH-based ILCs indicate a tendency to overestimate using this technique. This trend still allows for the correct categorization of exposed individuals. Although FISH-based translocation analysis is not the technique of choice in an emergency, it should be applied for possible follow-up studies. For this reason, more studies are needed to correct this trend.

Conclusions

This is the second FISH-based ILC evaluating dose assessment using translocations as a biomarker. The whole exercise was planned as a response to an emergency and a triage analysis was requested for all the biomarkers except for FISH, where a full analysis was initially requested. However, most of the laboratories reported only one result based on triage. This can be attributed partly to the poor mitotic index indicated by some participants, but the main reason is that before starting the exercise it was not clearly stated what was required. In addition, some laboratories submitted their results based on genome equivalence while others did not. Although the scoring sheet allowed all values to be recalculated, future ILCs should clarify what is requested. Finally, although most of the laboratories successfully discriminated unexposed and irradiated samples, the analysis of translocations by FISH shows a tendency to overestimate the delivered doses, similarly to what has been seen with dicentrics and micronuclei. Although all aspects indicated in the dicentric manuscript can contribute to this overestimation, it should be noted that unlike dicentrics, where in most of the ILCs done, the mean dose estimate agrees with the delivered dose, for translocations the two ILCs performed so far have shown the same

tendency. Efforts to better harmonize this technique to avoid erroneous values and subsequent exercises to elucidate the reason for this trend should be promoted.

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Figure legends

Figure 1: Calibration data reported by participants. For comparisons and when needed calibration coefficients were converted to full genome. Although in some curves the maximum dose evaluated was 4 Gy. All curves have been drawn in the range of 0 to 5 Gy. Dotted curves represent the one constructed using dicentric data (*), and the one where the maximum dose evaluated was 1 Gy (**).

Figure 2: Genomic frequency of translocations reported by participant laboratories. **A)** boxplot including all reported frequencies based on triage analysis (black circles) and full analysis (white circles). **B)** boxplot excluding the L6 value and using only those frequencies based on triage. Boxplot **C** also includes the results based on full analysis.

Figure 3: Dose estimates reported by participant laboratories. Horizontal yellow lines indicate the delivered radiation dose. **A)** boxplot including all reported dose estimations based on triage analysis (black circles) and full analysis (white circles). **B)** boxplot excluding the L6 value and using only those point doses based on triage. Boxplot **C** also includes the results based on full analysis. Dotted lines indicated the delivered doses of 0, 1.2 and 3.5 Gy.

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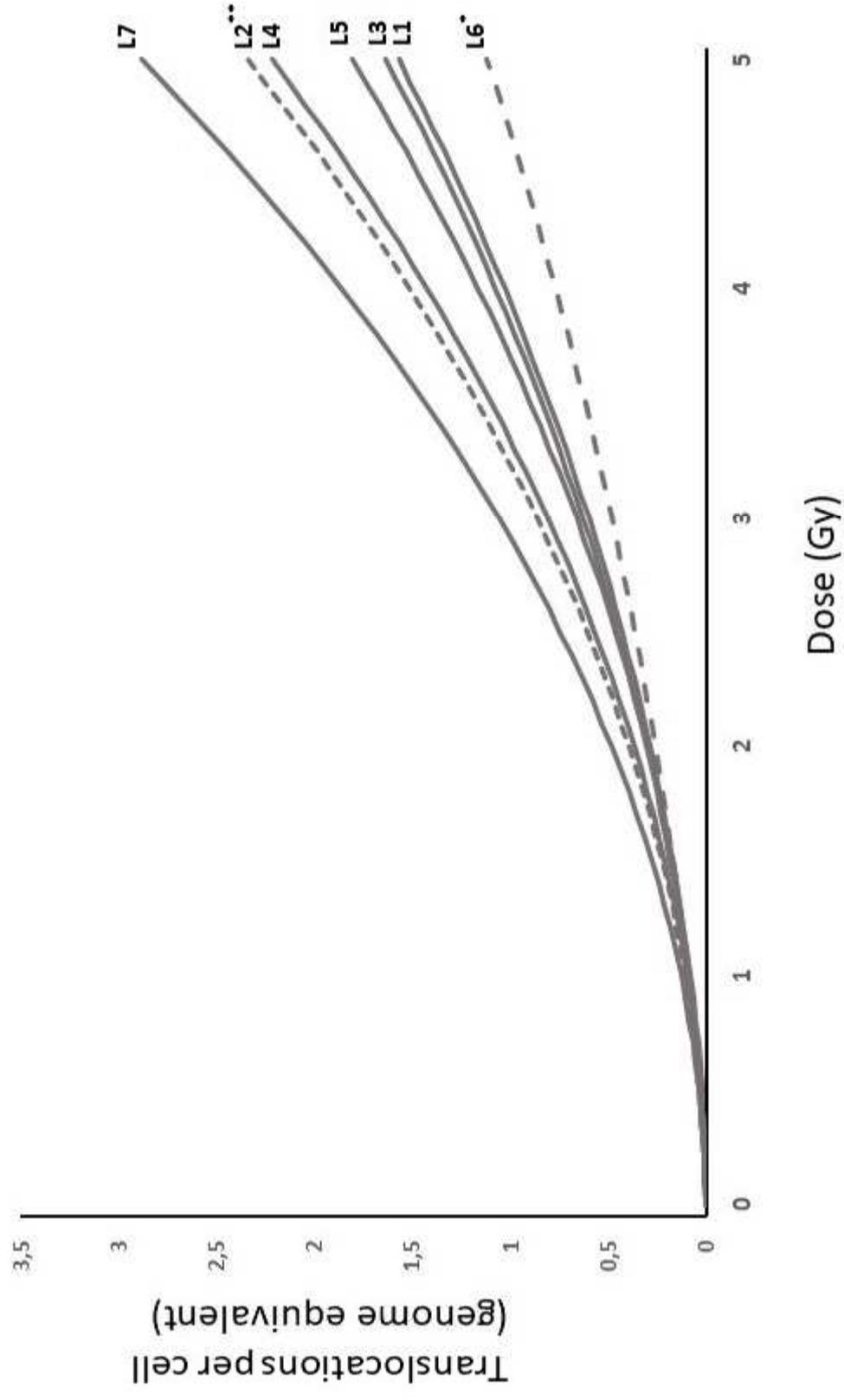
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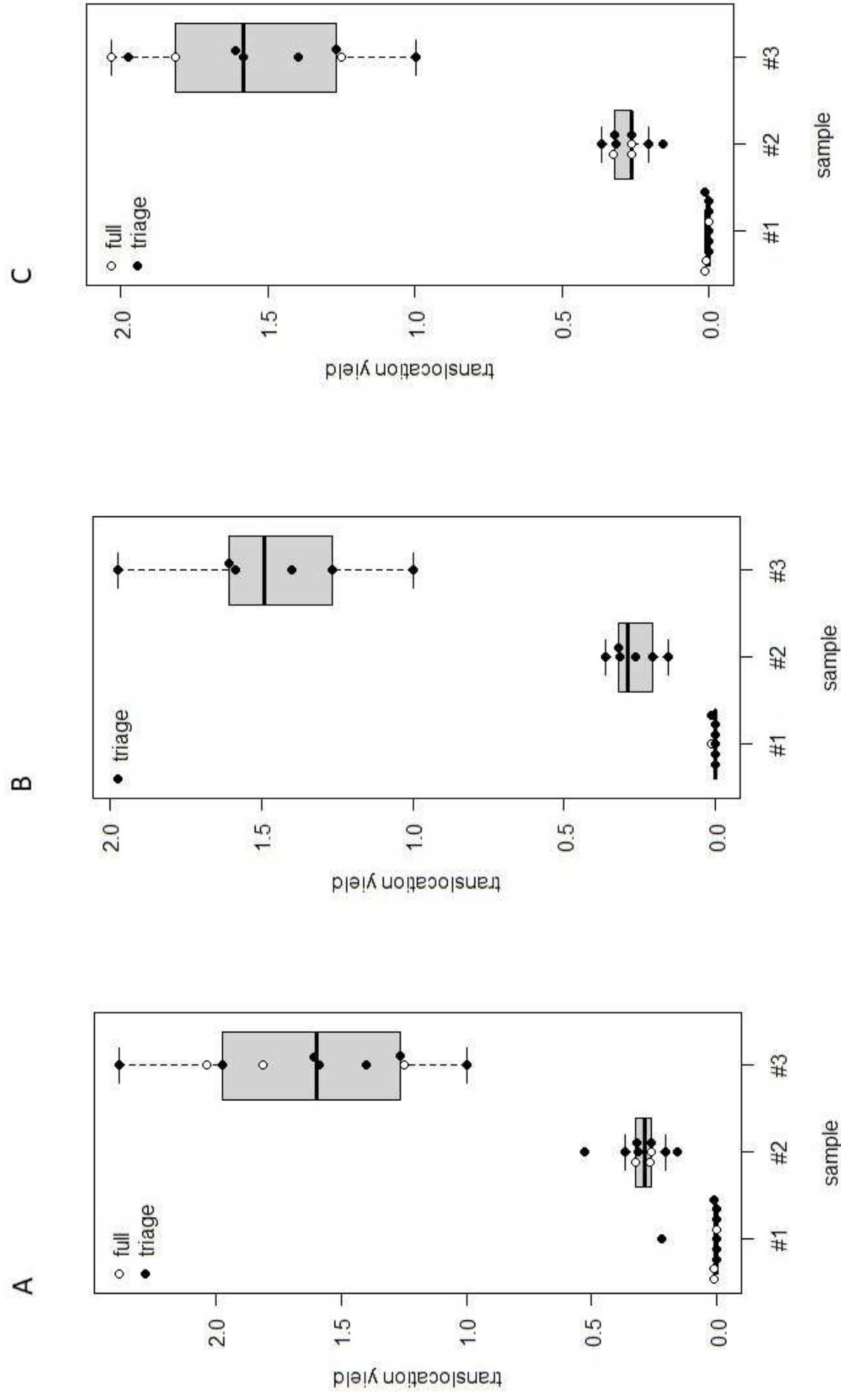
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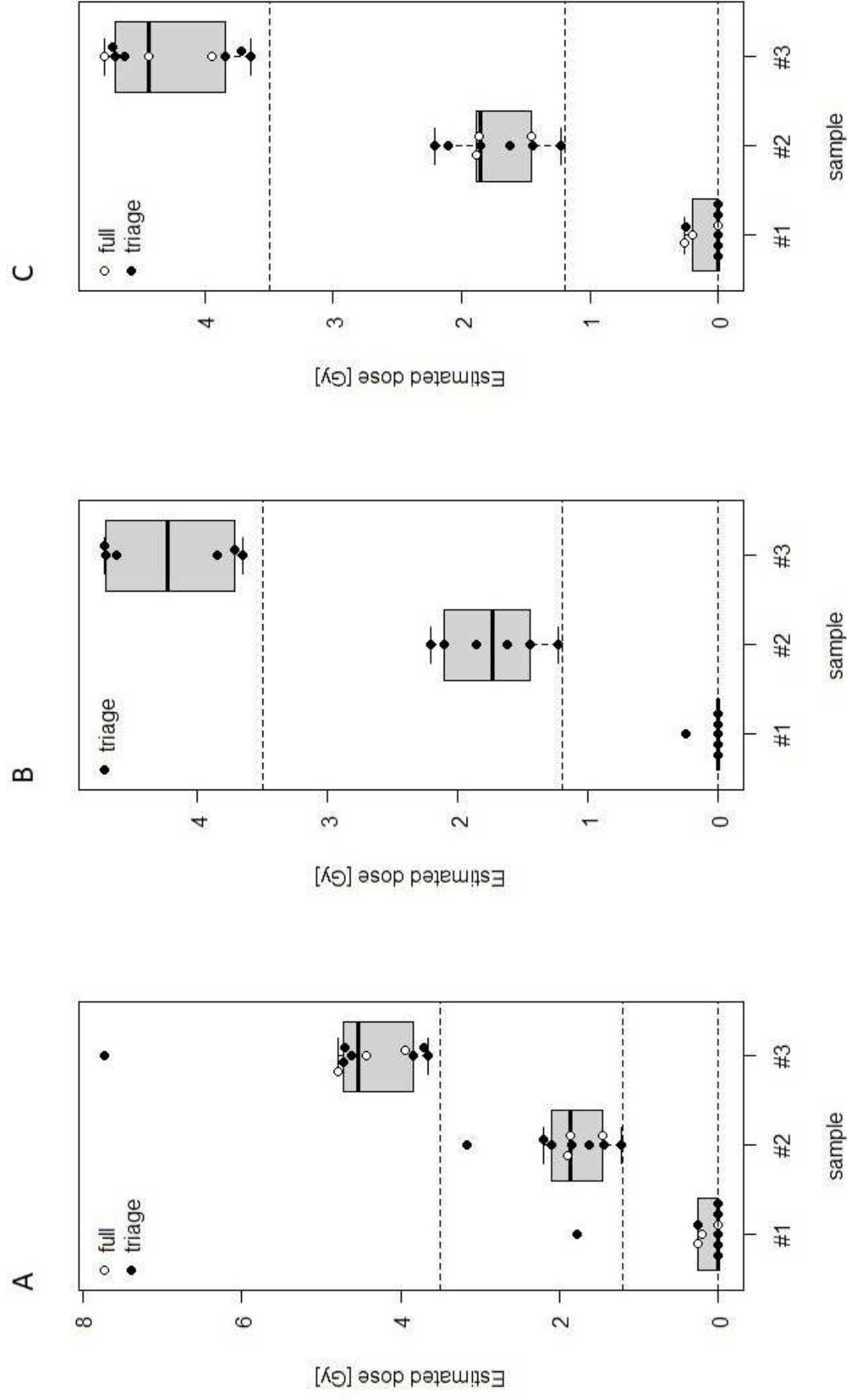


Table 1. Participant laboratories

Partners	Colcemid treatment (h)	Culture time (h)	Chromosomes painted	Painting strategy	C^(b)
L1	2	48	1, 4 & 11	One colour	0.319
L2	^(a)	^(a)	1, 2 & 4	Three colours	0.396
L3	24	48	1, 2 & 4	One colour	0.360
L4	3	48	1, 4 & 11	Two colours	0.337
L5	2	48	1 & 2	One colour	0.280
L6	24	48	1, 2 & 4	Three colours	0.396
L7	4	50	2, 4 & 12	Three colours	0.339

^(a) Analysis was done on captured images. ^(b) Full genome conversion factor according to the chromosome pairs painted, the painting strategy used, and the gender of the donor.

Table 2 reported calibration curves

Partners	Radiation quality	Dose-rate (Gy/min)	Dose-range (Gy)	C	±SE	α	±SE	β	±SE	
L1	Co 60	0.29-0.31	0-5	0.0021	±0.0006	0.0086	±0.0031	0.0182	±0.0015	
<i>FG values</i>				0.0065	±0.0020	0.0269	±0.0098	0.0573	±0.0046	
L2	Co 60	0.0263	0-1							
<i>FG values</i>				0.0005	±0.0001	0.0178	±0.0037	0.0901	±0.0054	
L3	Co-60	0.5	0-5	0.0021	±0.0002	0.0118	±0.0025	0.0211	±0.0013	
<i>FG values</i>				0.0058	±0.0004	0.0326	±0.0069	0.0587	±0.0353	
L4	Co-60	0.745	0-4	0.0015	±0.0010	0.0031	±0.0055	0.0293	±0.0032	
<i>FG values</i>				0.0044	±0.0029	0.0092	±0.0163	0.0867	±0.0096	
L5	Co 60	0.68	0-4	0.0005	±0.0004	0.0027	±0.0032	0.0202	±0.0025	
<i>FG values</i>				0.0019	±0.0143	0.0098	±0.0116	0.0703	±0.0088	
L6 ^(b)	Co-60									
<i>FG values</i>				0-3	0 0	0.069	±0.0561	0.031	±0.0197	
L7	X-rays (4MV)	0.5								
<i>FG values</i>				0-4	0.0009	±0.0002	0.0201	±0.0177	0.1112	±0.0072

^(a) Calibration curve based on dicentric analysis. Dose-range indicates the minimum and maximum doses used to construct calibration curves.

Table 3. Estimated doses with the 95% confidence interval

Partners	Sample					
	#1		#2		#3	
	D	(Dl, Du)	D	(Dl, Du)	D	(Dl, Du)
L1 (Tri)	0.00	(0.00, 0.34)	2.10	(1.66, 2.62)	4.70	(3.69, 6.03)
L1 (Fa)	0.00	(0.00, 0.33)	1.89	(1.58, 2.24)	4.43	(3.73, 5.35)
L2 (Tri)	0.00	(0.00, 0.00)	1.21	(0.58, 2.10)	3.64	(1.47, 6.90)
L3 (Tri)	0.00	(0.00, 0.68)	2.21	(1.80, 2.67)	3.84	(3.07, 4.81)
L4 (Tri)	0.00	(0.00, 0.00)	1.85	(1.50, 2.29)	4.72	(3.78, 6.10)
L4 (Fa)	0.20	(0.00, 0.61)	1.87	(1.55, 2.27)	4.79	(3.97, 5.99)
L5 (Tri)	0.00	(0.00, 0.71)	1.62	(1.17, 2.25)	4.62	(2.70, 7.77)
L6 (Tri)	1.79	(0.77, 4.90)	3.16	(1.77, 8.06)	7.73	(4.89, 20.89)
L7 (Tri)	0.25	(0.00, 0.80)	1.45	(1.11, 1.99)	3.71	(2.83, 4.84)
L7 (Fa)	0.26	(0.00, 0.50)	1.45	(1.12, 1.88)	3.95	(3.38, 4.66)

For each laboratory estimated doses (D) with the 95% confidence limits (Dose Lower, Dl, and Dose Upper, Du,) based on triage (Tri) or full analysis (Fa).