RENEB

Realizing the European Network in Biodosimetry

THE

QUALITY

MANUAL

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Introduction

The purpose of this document is to define the use of the different biological and/or bio-physical assays as elements of the operational basis of the RENEB network. These assays are intended to be applied for accidental or malevolent exposures involving a) up to a few casualties to provide individual full dose estimates or b) in triage mode to populations to provide interim dose estimates for individuals.

A variety of methods are available which can be used as biodosimeters or markers of exposure. Currently, the best methods of biological dosimetry are based on the analysis of chromosomal damage (dicentric chromosomes and micronuclei) in peripheral blood lymphocytes and electron paramagnetic resonance spectroscopy (EPR) in bone and tooth enamel. These methods have been validated in international exercises and in a number of small-scale radiation accidents and have been shown to be reliable tools to detect an absorbed dose of radiation with sufficient precision. Indeed, the dicentric assay is regarded as the "gold standard" of biodosimetry. A number of new biodosimetric methods have recently been introduced, such as premature chromosome condensation (PCC), fluorescence in situ hybridisation (FISH) and γ-H2AX foci. As has been shown in a recent survey, these methods are established in many European laboratories, but what is lacking is networking to consolidate the standardisation and harmonisation of the assays. The network should include regular intercomparison studies and accident exercises that would guarantee the availability of a rapid response and the reliability of dose estimates.

The so-called "Realizing the European Network in Biodosimetry" (RENEB) is an EU FP7 funded project with the objective of setting up a network with an operational basis consisting of 7 established biodosimetric tools (the dicentric assay, the FISH assay, the micronucleus assay, the PCC assay, the γ-H2AX assay and EPR/optically stimulated luminescence - OSL). These assays will be organised and standardised in the participating laboratories, in the context of providing dosimetry in emergency situations. The network will be complementary to the MULTIBIODOSE project in that it will rely on MULTIBIODOSE guidelines on which biodosimetric tool(s) should be best applied for a specific accident scenario.

A Quality Assurance & Quality Management program is also included as an essential part of the Education & Training activities under RENEB. It is expected that each laboratory participating in the network should establish a QA & QM program relating to operation of each of the bioassays used in the lab. The resulting homogenetity of practises should guarantee that dose estimates produced by network members will be comparable irrespective of the labs' organisation and the specific emergency scenario. ISO standards 19238:2014 and 21243:2008 were written to provide a guideline to all laboratories to perform the dicentric assay, reproducibly and accurately, for overexposure assessment and mass radiological casualty assessment respectively, using documented and validated procedures. Similar procedures are supported by the new standard 13304-1:2013 on "Criteria for electron paramagnetic resonance (EPR) spectroscopy for retrospective dosimetry of ionizing radiation — Part 1: General principles" and by the ISO document 17099:2014"Performance criteria for laboratories using the cytokinesis-blocked micronucleus assay in blood lymphocytes for biological dosimetry". The different approaches described in these standards included pre-planning, active networking, reagent stockpiling, simplified sample processing, automation, medical management, radiation-protection management, record keeping and medical/legal requirements, qualification of staff and inter-comparison. Many parts of these standards can be easily adapted to the other assays proposed in RENEB.

This document is written in order to satisfy two aims developed in the course of RENEB:

1) To produce a common QA & QM manual, adapted from the ISO standards, describing performance criteria for each bio-assay implemented by the RENEB network laboratories;

2) To produce an integrated QA & QM manual, taking into account multiparametric approaches (alternative methods of triage) and relationships with first responders.

Part of the information in this document is contained in other international guidelines and scientific publications, primarily in the International Atomic Energy Agency's (IAEA) Technical Reports Series on Biological Dosimetry. However, this document expands and standardizes the quality assurance and quality control procedures, the criteria of recognition and the evaluation of performance, for the full range of assays. As such, this document will be of primary importance for sustaining the credibility of the network partners and readiness of the network to respond to requests from first responders, national and European authorities and R & D agencies.

1 Scope of the RENEB Quality Manual

This document addresses:

1) The position of the reference laboratory, the solicitation of dose estimates and the specific organisation of the network in case of emergency, including which assays should be applied;

2) the confidentiality of personal information, for the customer and the network's laboratories;

3) the laboratory safety requirements;

4) the radiation sources, dose rates and dose ranges used for establishing the reference calibration dose-effect curves for the different assays, and the minimum resolvable dose for each assay;

5) the performance of sample collection and sample preparation for each assay;

6) the observation criteria for each assay;

7) the conversion of observations into an estimate of absorbed dose for each assay;

8) the reporting and interpretation of results;

9) the organization of exercise and intercomparison

10) the criteria for evaluation and acceptance of new partner

11) the quality assurance and quality management.

2 Biological or Bio-physical assays used in the RENEB network

2.1 Overview

This manual is chiefly concerned with the assays currently used by the partners of RENEB network, however it is important to consider potential new assays which may be developed in future. QA & QM procedures will be designated and implemented for novel assays as soon as these have been validated in at least one RENEB partner laboratory. At the date of publication of the current edition of this document, the following assays are used by several partners' laboratories within the RENEB network:

- Dicentrics
- Micronuclei
- Translocations detected using the fluorescence *in situ* hybridisation (FISH)
- Prematurely condensed chromosomes (PCC)
- γ-H2AX foci
- Electron spin resonance spectroscopy (EPR)
- Optically stimulated luminescence (OSL)

A brief description of the respective pros and cons of the techniques above are given in the Annexes D to O, so that a clear material and methods of each technique such as practiced within the RENEB network by the reference laboratories.

2.2 General equipment of the laboratory

Each laboratory should be equipped with the required devices for physical dose assessment and with bio-hazard units, culture and standard laboratory equipment for biological dose assessment. Each laboratory should maintain quality assurance documents describing periodic maintenance and calibration of the necessary equipment (e.g. balance, laminar flux, etc, as defined in the technical Annexes for each assay).

2.3 Requests for analysis

Depending on national regulations, the request for an analysis should normally be made by a doctor representing the patient or by the patient him/herself, or the analysis could be requested as a result of a legal claim. In all cases where it is normally possible, sampling should be made with the patient's informed consent. For minors the informed consent should be obtained from the parent / guardian. It is advisable that the laboratory head maintain the record of the patient's informed consent and the patient and/or notifying medic should also be informed as to who will have access to the data.

It is the responsibility of the first responder/medical staff (doctor, nurse, etc…) to schedule material collection and shipping according to the guidelines and using equipment, if appropriate, provided by the laboratory, so as to assure sample is received by laboratory in the best possible conditions.

2.4 Sample processing

The protocol for all assays involved listed in section 2.1 should be established and documented by each laboratory practicing the assay in the framework of RENEB. The protocol used for the calibration curve and for dose estimates of patient samples should be identical across laboratories.

Where possible, each sample should be processed by a minimum of 2 operators, experienced in the assay.

Experimental and operator variability are the key sources of variation in results. It is therefore essential that the same operators are maintained throughout a single assessment and ideally 2 operators are used. Each operator should provide one result from one of at least 2 duplicate samples. Their mean value should then be calculated, to take into account both experimental and operator variation. For duplicate samples, coefficients of variation, CVs, should be less than 20 % (ISO 21238:2008).

It is acceptable to use a single operator if 2 experienced operators are not available or this approach is impractical, for instance in triage mode.

Several systems for automated assay analysis have been developed. Automation is authorized if the laboratory has validated the related methodology and created a suitable dose-effect calibration curve. The methodology and the related calibration curve should be documented.

3 Confidentiality of personal information

3.1 Overview

Biological dosimetry investigations made by a RENEB network member laboratory should be undertaken in accordance with national regulations regarding confidentiality. This would normally include the maintenance of confidentiality of the patient's identity, medical data and social status. In addition, commercial confidentiality of the patient's employer and any other organisations involved in the radiological accident/incident should be observed.

This requirement extends to 1) written, electronic or verbal communications between the laboratory and the person/organisation requesting the analysis and receiving the report and 2) the secure protection of confidential information held within the organisation where the laboratory is located.

3.2 Applications of the principle of confidentiality

The head of the laboratory should authorise a limited but sufficient number of laboratory staff to deal with documents related to the analysis. All the staff should have signed a commitment to confidentiality regarding their duties within the laboratory.

The laboratory head should maintain signed confidentiality agreements and ensure the security and safety of all confidential documents.

The laboratory head should have established protocols for maintaining the anonymity of samples. To avoid the identification of the patient while guaranteeing the traceability of the analysis, the samples should be coded upon arrival in the laboratory. The coding should be performed in an unambiguous way according to a standard procedure. The same code should use for all the stages of the analysis. Coding, decoding, interpretation of results and compiling the report should be performed by the smallest possible number of authorized persons.

Whatever the chosen means of communication, confidentiality should be ensured during the exchange of information and reports between the laboratory and the requestor of the analysis. The laboratory head should define all processes for information transmission and assurance of confidentiality.

The final report containing the results and their interpretation (when needed, specific to the individual assays*)* should be communicated to the requestor of the analysis. Depending on national regulations, further copies may, with appropriate approval, be passed to another responsible person.

3.2.1 Storage

The laboratory should store intact any remaining biological/processing samples to facilitate review/analysis by an external expert or another laboratory in the event of any dispute regarding the analysis.

Depending on national regulations, the laboratory head defines how data and results are stored. All laboratory documents relating to a case and which could permit the patient and/or employer to be identified should be stored in a place only accessible to the authorized persons. Documents should be retained in an appropriate, secure storage facility for at least 30 years for possible medical/legal re-evaluation of the case. Final disposal of documents will be by secure means such as shredding of paper records and permanent deletion of electronic records.

4 Laboratory safety requirements

4.1 Overview

Laboratory staff should conform to their national legislation and institutional regulations regarding safety in the laboratory. There are some particular features concerning safety in service laboratories that are worth highlighting. These include microbiological, chemical, and optical considerations.

All biological samples should be regarded as being potentially infectious even if they are known to be derived from apparently healthy persons. It should be noted that when blood samples are accepted from abroad, depending on the country of origin, airlines might require the sender to provide a certificate confirming that the samples have been tested and are HIV negative.

Certain dangerous chemicals and pharmaceuticals are used routinely in the procedures used within the network. The ISO documents (19238:2014 & 21243:2008 at least) and IAEA Techdocs (n°260 & 405 at least) describe precisely the risks associated with these products and their safe manipulation.

4.2 Safety plan

The laboratory head should document procedures for protection against microbiological, chemical, and optical hazards. The laboratory head should maintain protocols or procedures to use in case of accidents involving such hazards, with regard to institutional and national medical and safety requirements.

5 Reference source(s) & dose-effect calibration curve(s)

5.1 Reference source(s)

The head of the laboratory should be able to provide on request documentation which has been reviewed and endorsed by a qualified expert (i.e., radiation physicist or radiobiologist or laboratory head) that addresses the following issues:

1) characterization of the radiation reference source(s) used to generate each *in vitro* calibration curve or used to re-irradiate the samples (cf. OSL);

2) the calibration is performed in terms of absorbed dose in water for blood-based assays and in terms of air kerma for non-water equivalent tissue;

3) description of the procedure to certify the dosimetry method, to measure dose uniformity in the experimental area, and to verify dose and dose-rate determinations for individual experiments. The calibration in terms of dose in water should follow the IEAE protocol TRS-398. The calibration in terms of air kerma should follow the protocols described in ISO standard 4037-1.

4) The irradiation of samples is performed in the same configuration and condition than for the calibration of the beam

5) a summary dosimetry report for each calibration-source dose-response curve.

5.2 Dose-effect curve(s)

Except for OSL technique, a calibration curve is required for each laboratory performing dosimetry as part of the RENEB network. The same experimental conditions should be used to establish calibration curves and for analysis in a case of suspected overexposure. For biological assays, the curve should be produced from at least 6 donors of varying age and gender, where appropriate. For EPR, the calibration curve can be generated from a pool of samples from at least 5 samples from different origins or from at least 5 samples from different origin for each calibration dose. The selection of the calibration dose range depends on the assay, the radiation quality and the curve shape. These issues are addressed in the appropriate annexes; however, the typical doses for a low-LET calibration curve should range from 0.1 Gy to 4 Gy.

Specific methodological details and calculations for calibration curve fitting and to calculate statistical uncertainties associated with dose estimates can be extrapolated from the relevant IAEA manual and ISO standards for most of the RENEB assays.

The laboratory carrying out the assay should be able to provide documentation on the calibration source, the irradiation configuration, dose range and dose-response curve that has been reviewed and endorsed by a qualified expert (i.e., laboratory radiobiologist or equivalent), which addresses the following issues:

1) the experimental exposure set-up (sample holder, temperature control, etc.) and procedures to verify reproducibility of exposure set-up for individual experiments;

2) the *in vitro* calibration data and their fitting to a calibration curve.

5.3 Background frequency and signals

The background level of the observation of interest for each assay varies between individuals and/or with age, gender or other confounding factors (i.e nutritional status, genotoxic exposures, lifestyle factors) for biological samples or among manufacturers and production batches of for produced materials (cf. touch screen of mobile phone). For most assays, for the purpose of radiation biodosimetry, it can be assumed that an individual's base-line value prior to the ionising radiation exposure event is equivalent to the mean value of unirradiated samples for their age and gender group. Ideally the background level would include results for at least three age groups, separated into 1 to 25, 26 to 50, 51 to 75 years, and would include at least 3 males and 3 females in each group to provide information regarding base-line values for both genders across different age groups.

5.4 Minimum detection dose level

The minimum detection dose level is a function of each laboratory's measured control background levels, the calibration curve coefficients and the type of analysis and, furthermore, should be limited to the lowest dose used in the appropriate calibration curve.

The assay laboratory should be able to provide documentation, reviewed and endorsed by a qualified expert (i.e., laboratory radiobiologist or equivalent), that describes the laboratory's reference observation/control level and its measured radiation-induced observation levels which corresponds to the minimum detection dose.

6 Responsibility of the requestor

This section includes items that are not controlled by the laboratory. Prior to sampling, co-ordination between requestor and assay laboratory should occur. Essential requirements should be explained to the requestor such as through provision of a standardised instruction sheet. Precautions to ensure the integrity of the container and prevent leakage during shipment should be observed. Packaging and labelling should conform to national and international regulations and if air transportation is involved, the sample should not be exposed to ionising radiation in transit, and should be clearly labelled with instructions to prevent this. If temperature is a critical requirement, as with some assays, the temperature should be properly controlled and logged during shipment. A questionnaire provided by the assay laboratory should be completed by the requestor and returned to the laboratory promptly, preferably with the sample. The assay laboratory should be alerted if samples are known to be infectious.

7 Responsibility of the assay laboratory

7.1 Overview

The assay laboratory should establish and maintains a QA & QM program which covers all aspects of the assay. The QA & QM program should address the following issues:

1) the laboratory's QA & QM program should include periodic internal checks of equipment operations, reagent suitability and various performance checks (i.e., intra-comparison exercises, operator qualifications, sample protocol, integrity of results, dose estimations, report generation, etc.);

2) the laboratory's QA & QM program should include periodic external checks of the laboratory's operations in the form of external audits, including a review of the assay laboratory's documentation of equipment operations, reagent suitability, and various performance checks (i.e., inter-comparison exercises, operator qualifications, sample transport integrity, etc.).

7.2 Description of responsibility

The assay laboratory should retain documentation, reviewed and endorsed by a qualified expert (i.e. laboratory radiobiologist or equivalent) including the following:

1) where required, a collection system will be sent to the requestor which includes appropriately labelled and addressed packaging material for the return of the sample to the assay laboratory. The packaging will be conform to national and/or international regulations for the transit of potentially infectious pathological specimens;

2) after receipt of the sample, the following steps should be performed:

   i) document the receipt of the sample (date, time, operator);

   ii) code the sample;

   iii) document the place, duration and temperature of storage until the setting up of assay;

   iv) set up duplicate measurements in parallel and document date, time and operator;

   v) document with lot numbers, as appropriate, all reagents used;

   vi) document short- and long-term storage of sample(s);

   vii) document the results;

   viii) store sample and case documents in an appropriate place for at least 30 years for possible medico-legal re- evaluation of the case.

3) the assay laboratory interprets results and prepare reports;

4) the assay laboratory should sustain a dialogue with the requestor and providing results to the requestor.

8 Accidental overexposure involving few individuals

8.1 Procedure for assay

Few individuals is defined to be the number of individuals for whom dose estimation can be processed with the precision desired by the requestor (e.g. doctor) by the laboratory to which the request has been made without any external assistance (from other network members or otherwise; see Annex A). This number is variable and depends on the capacity of the assay laboratory.

When biological dosimetry is requested for a few individuals, the ISO standard 19238:2014 should be applied.

In brief, following national rules, the laboratory institution and the laboratory head are responsible for the decision regarding the choice of assay(s) used. The laboratory head should establish, implement and maintain procedures for each of the assays used for dosimetry purposes.

8.2 Responsibility of the assay laboratory

The laboratory head is responsible for ensuring that the individuals carrying out the assays (the operators) are appropriately trained. All individuals should participate in intra- and inter-laboratory comparisons. A set of calibration samples should be used routinely to verify that the accuracy of results is well within the expected range.

8.2.1 Overview

Except for OSL, the measured assay result should be converted to absorbed dose by reference to an appropriate *in vitro* calibration curve produced in the same laboratory with radiation of comparable quality in the same experimental conditions, according to the procedures laid out in section 5. In the simplest case, this will result in provision of an estimate of the mean whole-body dose for biological dosimetry or local dose for EPR and OSL on solid state materials.

The calibration is performed in terms of absorbed dose in water for blood-based assays and in terms of air kerma for non water equivalent tissue. The calibration in terms of dose in water should follow the IEAE protocol TRS-398. The calibration in terms of air kerma should follow the protocols described in ISO standard 4037-1.

For OSL, the signal is converted in dose by a factor determined by a re-irradiating of the samples at a known dose.

The assay laboratory should provide details of the laboratory's background level of the observation of interest in the written result report. If the assay result is not significantly different from the background, the best estimate of dose should be quoted as zero with its upper confidence limit. If the measured assay result is significantly higher than the background level, then a dose estimate with its uncertainties should be derived and reported. Uncertainties would usually be expressed as 95 % confidence limits although other percentage values may be quoted, if judged appropriate to a particular case.

An alternative approach is, when required, to subtract the mean background value of the individual tested and use this corrected value to estimate the exposure dose from the calibration radiation dose-response curve.

The laboratory should provide in the result report an estimated whole body dose and confidence limits. The laboratory head should define the methods used to determine confidence limits and whether reporting of other statistical quantities is desirable.

8.2.2 Partial body, localized and non-acute exposure cases

The degree of over-dispersion for biological assays can give an indication of inhomogeneity of exposure. If appropriate, and where possible, the degree of over-dispersion for the assay should be tested. When this approach is used, all conclusions should be carefully documented and justified. The comparison of solid state dose assessments with biological assays can give an indication on the level of heterogeneity of the dose distribution.

For biological dosimetry, further details of the procedure for non-acute found in the IAEA technical reports and the ISO 19238:2014 and 17099:2014 standards.

For physical dosimetry, further details of the procedure for non-acute found in the ISO 13304-1:2013 standard.

Where relevant, this approach may be adapted for partial body exposure dose estimates produced by the physical assays used in RENEB.

The assay laboratory should state in the report the method used to correct for non-acute exposure dose estimates and, when appropriate, also justify its assumptions.

8.3 Report of results

8.3.1 Overview

Routinely, the report should contain relevant information provided by the requestor since this may influence the interpretation of the findings in the laboratory carrying out the dose estimation.

8.3.2 Content of the report for each assay

The report should include information on the following points (ISO 19238:2014):

1) title of the report;

2) name and address of the laboratory performing the analysis;

3) identification of the report by a non-ambiguous reference;

4) name and address of the requestor, date of request;

5) identification of the method of analysis, i.e. providing the number and name of the method as described in the in-house quality system, and where relevant, any deviations from the test method;

6) unambiguous identification of the samples, i.e. names, internal code and date of birth of the exposed subjects;

7) description of the cases: all information provided by the requestor that is relevant to the interpretation of the results should be stated (possibly also in the section on interpretation of the results);

8) date and location of sampling, date of samples arrival in the laboratory, date of setting up assay (if different) and date of completion of analysis;

9) assay results: dose plus an assessment of the uncertainty in the dose (usually confidence intervals);

10) interpretation of assay results (see section 8.3.3);

11) name(s), title(s), position(s) and signature(s) authorizing the report and contact information.

8.3.3 Assay result and interpretation

This varies depending on the circumstances of each case but the report should include one or more of the following:

1) a dose estimate based expressed in SI units of absorbed dose (Gy) in water for biological assay and in terms of air kerma (Gy) for solid-state assessment ;

2) the assay background frequency of the laboratory and the coefficients of the calibration curve used for converting the dose from the assay result (except for OSL);

3) a quantification of the uncertainties on the dose estimate. This would normally be an upper, and where appropriate, a lower confidence limit, and the percent level of confidence;

4) a statement on whether the dose estimate was made assuming acute or protracted irradiation, whole or partial exposure and, if appropriate, how partiality/protraction has been accounted for;

5) if appropriate, the interpretation needs to consider the delay between the accident and sampling;

6) a summary of the essential key elements from the points above. If required, this would also include the best estimate of dose based on all the assay findings;

7) at the end of the report: an invitation for the requestor to contact the laboratory if he/she requires further clarification or explanation regarding the results and/or the methodology of dose estimation.

9 Population triage

9.1 General

The potential for nuclear and radiological emergencies involving mass casualties from accidental or malicious acts necessitates the requirement for procedures for emergency dose assessment as a component of the generic medical emergency response capabilities. A mass-casualty incident is defined here as an event that exceeds the local medical resources.

Each of the RENEB assays can be used in triage mode to evaluate radiation doses received by individuals in a rapid, approximate manner, in order to supplement the clinical categorization of casualties.

9.2 Use of the network for large scale exposures

In a mass casualty scenario, dosimetry under the RENEB network will be initiated by a reference laboratory, defined by the ISO 21243:2008 standard as the laboratory at which the request was first received, or the laboratory designated as the reference laboratory by the laboratory at which the request was first received. Dosimetry activities at the reference laboratory will be supplemented by associate laboratories, either nationally or internationally, upon request from the reference laboratory. ISO 21243:2008 addresses the establishment of bioassay networks for the dicentric assay, ISO 17099:2014 for the micronuclei assay and ISO 13304-1:2013 gives some indications for EPR dosimetry. These standards can equally be applied to the other assay involved in the RENEB network.

9.3 Emergency response of the reference laboratory

1) The head of the laboratory should prepare an emergency plan, defining roles for each member of staff;

2) The procedure to choose the assay should be defined (i.e. according to the MULTIBIODOSE decision tree, annex B); it should be possible for the decision to be made rapidly after receipt of the request for assistance, ideally before specific samples are requested, and certainly before samples processing begins;

3) Samples should ideally be sent directly to the laboratory where the processing for analysis will occur, as defined by the reference laboratory. The decision will depend on the location and the specific details of the emergency situation

4) The laboratory should processes the samples in the order of arrival;

5) Whatever the assay chosen, the analysis should be adapted to the emergency response according to predefined and documented procedures;

6) The dose estimate should obtained from an appropriate pre-existing calibration curve, except for OSL;

7) The first results of dosimetry should be made available as soon as possible.

9.4 Laboratories network definition and activation

1) In an emergency response scenario, the response of the RENEB network will consist of a single reference laboratory, with assistance from associate laboratories

2) When the reference laboratory receives a request for assistance which is outside the scope of its normal workload, the reference laboratory will activate the network by calling for assistance from one or more associate laboratories as defined in ISO 21243:2008.

3) The associate laboratories should be chosen by the reference laboratory to assist in the response on the basis of their specific expertise. Formal requests will be made and participation in the response as an associate laboratory will be on a voluntary basis.

4) The reference laboratory maintains responsibility for activating the network and for communication within the network.

5) The reference laboratory is primarily responsible for managing the response, including communication with emergency organisations and issuing dose estimation results and interpretation.

6) In cases where the event takes place in a country lacking a biodosimetry laboratory, the emergency officials may choose to contact any of the laboratories involved in the RENEB network to request assistance. Upon acceptance, the chosen laboratory will become the reference laboratory (ISO 21243:2008).

9.5 Preparedness of the laboratory/network

1) The network laboratories should make and maintain contact with one or more express delivery services, in order to ensure a system for sample transportation from the field to the reference laboratory and/or within RENEB is in place;

2) The standardization of techniques should be maintained in each network laboratory, in order that data can be pooled to provide a homogeneous combined response in the event of a mass casualty situation;

3) Frequent communication between RENEB laboratories should be maintained, for instance to address scientific and technical questions related to the assays (defined as part of the RENEB tasks);

4) The network should be maintained through consensus meetings which should be attended by at least one representative from each partner laboratory and which will take place at least every 2 years;

5) The laboratories of the network will prepare, maintain and make readily available to all partners the following documents:

    a. An information sheet containing full contact details (addresses, phones, fax, e-mail) of the laboratory staff involved in the network;

    b. A detailed information sheet, in the language of the country of origin, for medical staff or first responders that will carry out blood or physical sampling. This should include contact information, specification of sample size and container, requirements for packing/shipping and shipment tracking information.

    c. Detailed protocols for sample preparation, analysis and data interpretation for each of the assays used in the laboratory.

For the biological assays:

6) Each laboratory should maintain sufficient stocks of necessary consumables to be able to process at least 100 or more blood samples at short notice.

7) Stocks of specimen tubes together with information sheets should be stored in the laboratory, and/or deposited in appropriate locations in the country.

8) RENEB members will take part in periodic intercomparisons to test that protocols are correctly implemented and ensure consistency of analytical procedures and accuracy of dose estimates.

For the physical assays:

1) Each laboratory should maintain sufficient stocks of necessary consumables to be able to process at least 100 or more samples at short notice.

2) Stocks of specimen packs together with information sheets should be stored in the laboratory, and/or deposited in appropriate locations in the country.

3) RENEB members will take part in periodic intercomparisons to test that protocols are correctly implemented and ensure consistency of analytical procedures and accuracy of dose estimates.

9.6 Procedure for assay

In network mode, the following modifications from the individual mode (few individuals exposed) are applied:

1) Indicators of interest are chosen by the reference laboratory, with assistance from RENEB partners and according to the MULTIBIODOSE decision tree (annex B);

2) A single delivery company should be employed by the reference laboratory either to transport the samples from the field to the reference laboratory and/or to the other laboratories, as appropriate;

3) The reference laboratory should normally retain management responsibility and control of all the samples, even if associate laboratories have been specifically requested to directly receive samples;

4) The reference laboratory should attribute a unique code number to each sample (identical for physical and biological assays if required for the same individual) which will be distributed to associated laboratories for analysis;

5) The reference laboratory maintain the list of code numbers. The coding used for the dose reporting should be common and giving information on lab, technique and including sample code and ID code.

6) Each laboratory should use its own dose-effect curve (except for OSL);

7) Each laboratory should use its own treatment and analysis protocol, specifically designed for the observation of interest for the specific assay;

8) The dose assessment and associated interpretation for a specific sample should be carried out by the laboratory that processed that sample; however, final interpretation should be carried out by the reference laboratory;

9) The reference laboratory retains responsibility for communication with external agencies;

10) The reference laboratory is responsible for reporting the results to the individuals or agency which requested the emergency response assistance;

11) Automatic scoring systems may be used if appropriate QA and QC procedures have been defined;

12) If appropriate, images may be shared using the internet.

9.7 Reporting of results

The report should include as much information on the accident circumstances and on the individuals concerned by the expertise as possible. However multiple samples can be tabulated in the same report if appropriate.

10 Quality assurance of RENEB Laboratories

The minimum requirements are that the quality assurance and quality control practices detailed for each assay should be applied by the laboratories performing the assays as part of the RENEB network.

The laboratory institution should normally designate an individual or group of individuals, typically the laboratory head to lead a quality assurance programme. This individual should have sufficient knowledge to effectively lead the program and sufficient authority to initiate corrective actions when these are required.

The laboratory should maintain written quality control procedures to verify that the quality of estimation of absorbed dose measurements complies with the requirements specified in the ISO standards. The quality control procedures include the following:

1) Checks regarding sample transportation procedures;

2) Traceability of the samples and use of reference standards;

3) Performance checks for measurement systems;

4) Instrument calibration;

5) Intra-laboratory analyses and comparisons (including known quantities, replicates and blanks);

6) Automation checks;

7) Periodic review of procedures, specifications and maintenance of operating logs;

8) Observation of operations and evaluating quality control data to ensure the long-term consistency of analytical results;

9) Checks of minimum detection level determination methods.

11 Quality assurance program of the network

The minimum requirements are that the quality assurance and quality control practices cited in the ISO biodosimetry standards apply to the RENEB laboratories performing dose assessment for individuals exposures in mass casualties scenarios.

If the reference laboratory has solicited the services of associate laboratories, the reference laboratory will maintain responsibility regarding the quality assurance and quality control for all participant laboratories.

To ensure this is the case, the documents required in section 10 should be made available to all RENEB network participants. In addition, as required by the ISO standards, periodic inter-comparisons should take place between all laboratories actively using each of the RENEB assays (as defined in 11.3). In brief, samples containing known exposures of specific radiation dose and quality should be analysed by all partners to determine precision of the analytical procedures in each RENEB laboratory, and identify any further training needed.

11.1 Criteria on laboratory capabilities

To operate as a partner within the RENEB Network, a laboratory should fulfil the following criteria regarding their capabilities:

1) A level of expertise and experience using at least one assay for dosimetry purposes, based on the points listed below and a “Reporting Sheet for Potential New Members in RENEB” (Annex C) ;

2) The availability of suitable in-house calibration curves or calibration sources for OSL;

3) Documented QA & QC programs;

4) Documented protocols for the assays used as part of the network;

5) Successful participation in the last inter-comparison;

6) Evidence of sustained expertise, for instance training programs;

7) Compliance with appropriate national laws and regulations;

8) When possible, connection with national agencies responsible for emergency response/first responders;

For new member laboratories, sufficient expertise would usually be defined as completion of training in another RENEB partner laboratory and/or successful participation in at least one inter-comparison, as well as the other points listed above.

11.2 Criteria on laboratory capacity

To operate as a partner within the RENEB Network, a laboratory should have capacity criteria as follows:

1) Data on the capacity for an emergency response: X triage cases/week sustained for Y weeks, for each assay used as part of the network;

2) Documented evidence of the ability to operate at the capacity defined in 1), within the RENEB framework, for instance during an inter-comparison;

3) Readily available consumable resources (e.g. reagents, plastic-ware) to analyse at least 100 samples (ref ISO 21238:2008) or the ability to easily obtain such resources;

4) Following the initial triage of individuals, the ability to follow up with a more detailed analysis those cases who require further dose refinement to support clinical management.

11.3 RENEB exercise program

An exercise program between the laboratories within RENEB is essential for training and to maintain competence and readiness for emergency response. The RENEB exercise program consists of the following:

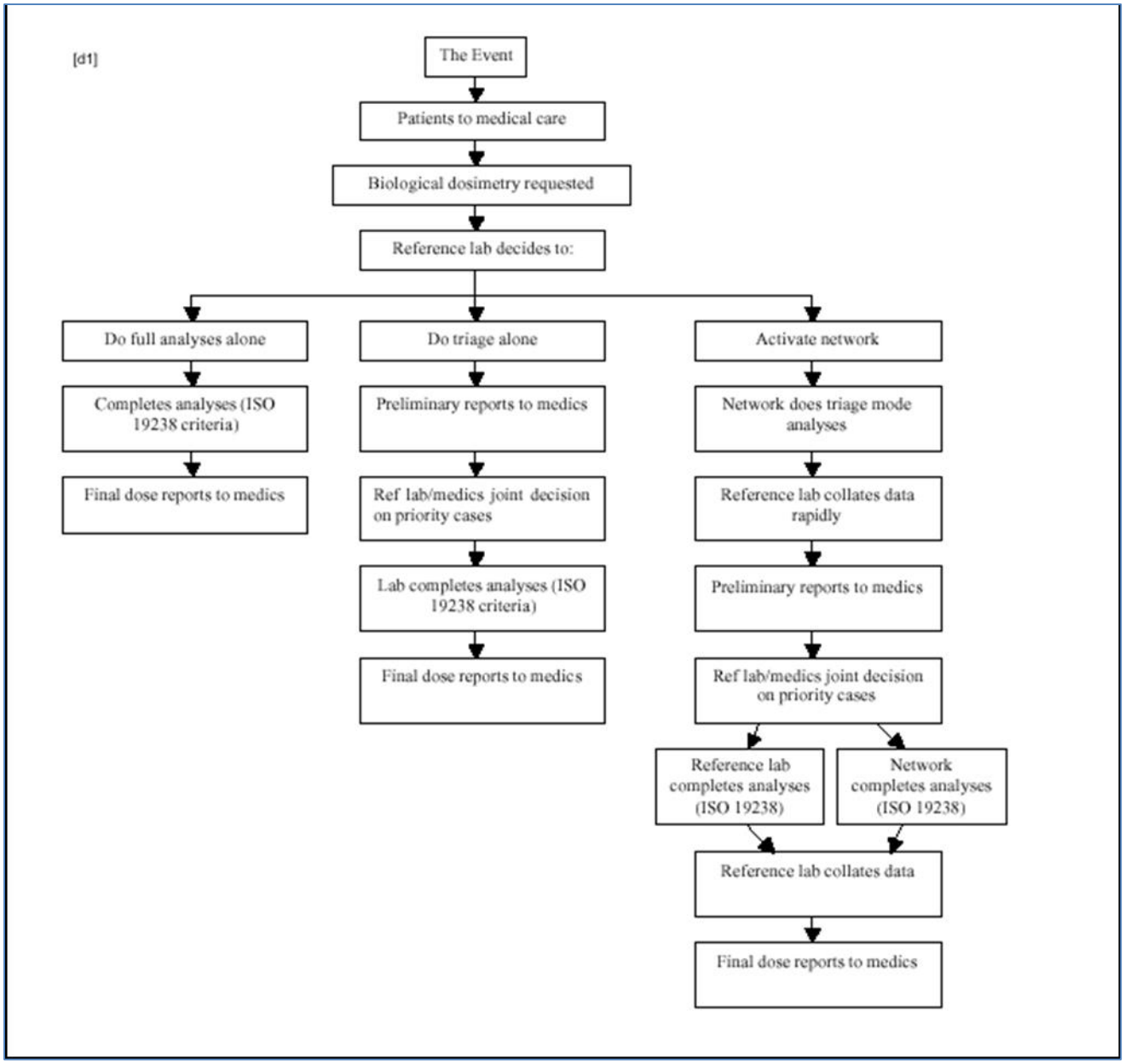
1) Intra-comparison exercises for each assay between RENEB laboratories to ensure compliance with the requirements of the network, the ISO standards and as a part of individual QA programs;

2) Inter-comparison exercises between different assays used within the RENEB network, and for different simulated emergency response scenarios;

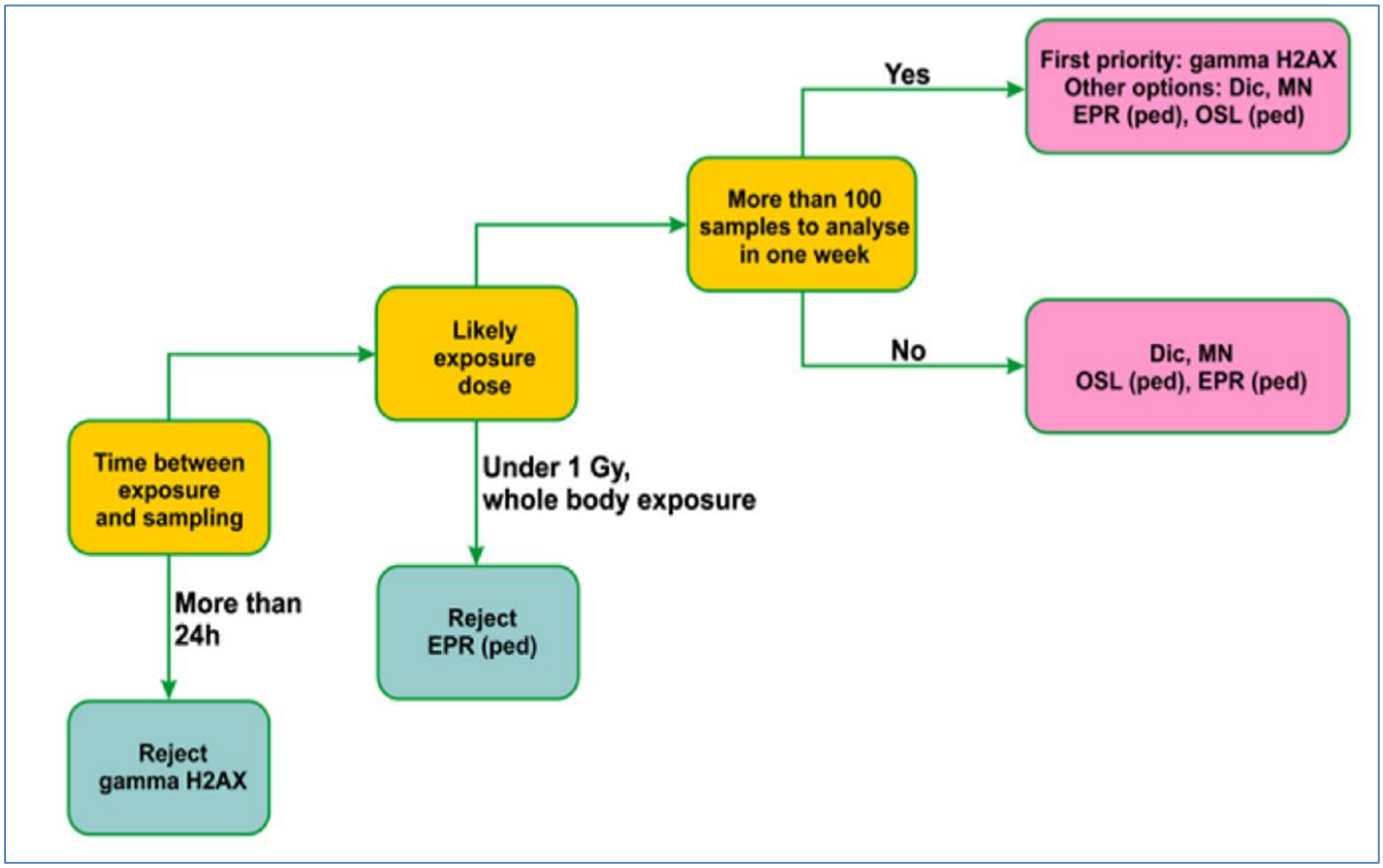
3) When appropriate, participation of RENEB laboratories in national and international emergency response exercises;

4) Table-top exercises on a laboratory and network level, to refine and maintain procedures and ensure maintenance of readiness for emergency response.

Annex A
Possible interactions between medical team, assay laboratory and RENEB network, according to the accidental situation (From ISO 21243:2008)



Annex B
Organisation of the dose assessment by assays, if the delay is known (from MUTIBIODOSE Guidance)



Annex C
Reporting Sheet for Potential New Members in RENEB

1. General information:

Institution/Department/Laboratory:
Head of institution:
Contact person:
Address:
Country:
Email:
Telephone:

2. Biodosimetric methods currently used in your lab

dicentric assay FISH assay micronucleus assay PCC assay
Gamma-H2AX EPR/OSL assay
For what radiation qualities is your lab calibrated?
Do you use automated systems (e.g. foci counting, metaphase finder)?
Statistical methods used in dose estimation?
Which quality assurance and quality control procedures do you have?
In which methods do you have published experience?

3. Lab infrastructure

Number of staff members:
Processing capacity of samples/week:
Availability of assay capacity (days to result):
Has your laboratory already dealt with cases of (suspected) exposure?

4. Networking

Do you have collaboration with current RENEB members?
Has your laboratory the capacity to host trainees in biodosimetry?
Are you already involved in a national/international network for biodosimetry?

5. others

Are you developing or using other methods for biodosimetry?
Funding basis: are you university, NGO, SME, research center, other?
Do you have further comments?

Annex D
Dicentric Assay

1.- Background

The dicentric chromosome assay is regarded as the “gold standard” of biological dosimetry (Blakely et al 2005) because of the high specifity for radiation and the low spontaneous frequency of the dicentric chromosomes. The technical performance of this assay has been described in detail in international guidelines and scientific publications, primarily in the International Atomic Energy Agency (IAEA), Safety Standards, EPR-Biodosimetry, 2011. ISO standards provide criteria for cytogenetic service laboratories performing the dicentric chromosome assay in its routine involving at most a few casualties (ISO 19238: 2014) or for rapid cytogenetic triage involving mass-casualty incidents (ISO 21243: 2008).

The technique is capable of estimating whole body doses of low LET radiation down to about 100 mGy based on the analysis of 1000 cells. (Romm et al., 2009) Mathematical procedures exist to perform dose estimations in case of dose protraction or after partial body exposure (IAEA, 2011).

General aspects of applying the dicentric assay as an element of the operational basis of the RENEB network are described in the Quality Manual.

2. - Dicentric assay laboratory procedure

The dicentric assay is an already highly standardized and harmonized technique but there is not a “universal” protocol available which is commonly used by all biodosimetry laboratories. Each laboratory has to establish its own protocol to achieve optimal results, but it is important to use the same culturing conditions for establishing the calibration curve as for analyzing dicentric chromosomes in case of suspected overexposure. Cell cycle controlled scoring method is recommended as this permits the analysis of first division metaphases. Short term (2-3 hours) colcemid treatment using Fluorescence plus Giemsa (FpG) staining and long term (24 hours) colcemid treatment and simple Giemsa staining are the techniques suggested (Romm et al., 2013).

A protocol (Annex 1) is attached and can assist laboratories to start or to optimize the procedure in their laboratory. Also several critical aspects are addressed. For more detailed technical information use the literature mentioned above.

3. - Calibration

For biological dosimetry purposes an appropriate calibration curve must be used for dose estimation. Dose–response curves for the yield of dicentric chromosomes are generated by irradiation of unstimulated lymphocytes in vitro under conditions as close as possible to the in vivo situation. The dose response relationship for dicentric chromosomes is very reproducible and stable over time for a given radiation quality and dose rate. Laboratories performing biological dosimetry should establish their own calibration curves for gamma radiation sources (^{60}Co or ^{137} Cs) and/or X rays. (e.g. 200 – 250 kVp). The dose range should cover 10 or more doses between 0.25 – 5.0 Gy. For high LET radiation a maximum of 2.0 Gy is suggested. The IAEA manual gives clear examples on how to construct dose effect curves for low LET gamma radiation and high LET alpha radiation.

4. - Blood sampling

For the analysis of dicentric chromosomes, blood should be sampled as soon as possible post exposure since after a delay of more than a few weeks the frequency of dicentrics begins to decrease particularly following large doses (IAEA, 2011) . If the radiation exposure occurred with lower doses the sampling time can be extended to about six months. In the case of partial-body or non-uniform exposure the sample should not be drawn before about 24 h after the exposure in order to assure a homogeneous mixture of irradiated and unirradiated lymphocytes.

5.- Transport

It is recommended that blood samples are shipped using an express service and declared as UN 3373 Biological Substance Category B. Packaging and labeling must conform to national and international regulations. A temperature logger and a dosemeter to monitor the temperature and any dose received by the samples during transport are

advisable. A standardized sample instruction sheets, (ISO 19238, 2014), can be used to inform customers of the correct procedure.

6. – Scoring

Dicentric chromosomes can be scored manually under the microscope or semi-automatically with the help of an image analysis system coupled to a microscope equipped with a motorized stage.

Scoring strategies have recently been adapted to enhance the throughput by reducing the number of cells to be analysed from the normal 500 or 1,000 cells down to 50 cells (Triage Mode) (Lloyd et al., 2000; Romm et al., 2011) or by scoring cells in a less restrictive manner (QuickScan Mode),(Flegal et al. 2010, 2012). Also evaluation of metaphase images distributed via the internet can be accelerated by taking advantage of world-wide expertise (Sugarman et al., 2014; Romm et al., 2014). The semi automated dicentric scoring with special software tools have the potential to improve the dicentric assay as a helpful tool to screen large numbers of blood samples in case of a large scale radiation emergency.(Romm et al., 2013). The different scoring strategies have been tested successfully in the frame of the MULTIBIODOSE project (Annex 2, MULTIBIODOSE, Deliverable 1.2, dicentric assay)

7. - Data analysis

The analysis of metaphases has to be performed by trained and experienced scorers recording all chromosomal abnormalties in a standardized scoring sheet. The scoring criterias and qualification of the staff has to be documented. Details for scoring expertise by performance checks through laboratory intercomparison studies and periodic checks of individual scorers are described in ISO 19238: 2014.

For biological dosimetry special software tools exist, for example CABAS (Deperas et al., 2007) or Dose Estimate (Ainsbury & Lloyd, 2010) for fitting calibration data and include additional tools that assist dose estimation (IAEA).

8. - References:

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Annex E
Dicentric Assay Protocol

MATERIAL

- Lithium-heparin whole blood samples
  - ➢ *Caution! EDTA coated tubes used for blood sampling often result in poor cell growth.*

TRANSPORT

- Use express shipment service
- Shipments of blood samples according to UN 3373 Biological Substance Category B and PI 650 regulations.
  - ➢ *During transportation temperature should be ideally between 18 and 24°C*

Temperature logger and dosimeter to monitor the temperature and any dose received by the samples during transport are advisable.

REAGENTS FOR CULTURING

- Culture medium with stable glutamine, for example RPMI - 1640
  - ➢ *Choice of culture medium has an influence on the cell growth and the number of second in vitro metaphases (M2)*

- Antibiotics: Penicillin/Streptomycin
- Fetal calf serum (FCS) commercially available and stored frozen (- 20°C)
- Phytohaemagglutinin (PHA-L)
  - ➢ *Use of PHA-P is not necessary*

- 5-Bromo-2-deoxyuridine (BrdU)
  - ➢ *For cell cycle control using FPG staining*

- Colcemid
  - ➢ *Add Colcemid after 45 h for 3 h or after 24 h for cell cycle control (permanent Colcemid) using a lower Colcemid concentration*

STEP BY STEP DESCRIPTION OF THE METHOD

BLOOD CULTURES

To set up 100 ml culture medium, remove 10 ml from a 100 ml RPMI bottle and add:

- 10 ml Fetal calf serum (FCS), stored at - 20°C
- 0.4 ml Penicillin/streptomycin, stored at - 20°C
- 2.5 ml PHA-L, prepare it just before use

Mix the medium

I. Per blood sample set up several parallel **culture tubes** each containing:

- 5 ml prepared culture medium (see above)
- 0.5 ml blood (collected in a heparin tube)
- 0.1 ml BrdU, stored in the fridge (only for FpG staining)

- *: final concentration not > 50 µM (15.4 µg/ml -IAEA recommendation)*

II. Place the culture tubes in the incubator at 37 °C.

a.) In the case of Fluorescence plus Giemsa staining (FpG) add 100 µl Colcemid **45 h** after culture set up. Return the culture tubes to the incubator for further 3 h. Harvest the cultures after 48 h culture time in total.

b.) In the case of permanent Colcemid add 40 µl **24 h** after culture set up. Return the culture tubes to the incubator for further 24 h. Harvest the cultures after 48 h culture time in total.

- *final concentration: 0.05 – 0.1 µg/ml (IAEA recommendation)*

Contents of culture tube	
RPMI1640	4.5 ml
Foetal calf serum	10%
PHA	2%
Penicillin / streptomycin	10000 U or µg/ml, resp.
BrdU	10 mM
Colcemid	a) 0.1 µg/ml b) 0.08 µg/ml
Whole blood	0.5 ml
IN TOTAL	**5,5 ml**

CULTURE STOP AND CELL FIXATION

1. After a culture time of 48 h the tubes are taken out of the incubator and put in the centrifuge to spin for 10 min at 200 g.
2. Remove carefully the supernatant just above the red pellet by suction; do not remove the red pellet!!!
3. Add 4 – 6 ml of 0.075M KCl solution (prewarmed to 37ºC) and mix it on the vortex for some seconds.
4. Place the culture tubes for 15 min in a water bath or incubator (37ºC) for incubation.
5. Centrifuge the tubes for 10 min at 200 g.
6. Remove the supernatant carefully down to the white layer.
7. Add 1 ml of fixative (Methanol:Acetic acid 3:1) in drops very slowly until you get a change of colour from red to black, then add a further 6 ml of fixative. Mix it on the vortex thoroughly.
8. Centrifuge the tubes for 10 min at 200g.
9. Remove the supernatant above the white pellet.
10. Add 6 – 8 ml of fixative.
11. Repeat fixation steps (8-10) 2-3 times until the fixative is clear. Overnight storage in the refrigerator is possible. Concentrate the cells according to the cell yield.
12. Drop the cells on clean slides. (Slide preparation and handling of the cell suspension see below).

PREPARATION OF THE SLIDES

Wash the slides in aqua dest., add 5ml of methanol (> 99,9 %) and store them in the fridge

HANDLING OF THE CELL SUSPENSION, SLIDE PREPARATION

- After the last washing, remove the supernatant carefully and resuspend the pellet to make a slightly milky suspension.
- Allow 2 drops of the suspension to fall in a line on the slide and let it dry
- Check the quality and quantity of mitoses under the microscope. The metaphases should be well spread with few cross-overs, possibly with no cytoplasm.
- Several slides can be made from one culture.

FpG (FLUORESCENCE PLUS GIEMSA) STAINING

- The slides are placed for 12 min at room temperature in Hoechst 33258 (0.0125g in 250 ml aqua dest.; avoid contact with Hoechst, toxic)
- Rinse the slides in aqua dest.
- Mount 2 – 3 drops of PBS-buffer on the slides and cover them with coverslips
- Place the slides for 20 min on a heating plate (60°C) under black light
- Rinse the slides in aqua dest.
- Stain the slides for 3 min in Giemsa-solution (20 ml Giemsa & 230ml PBS-buffer)
- Rinse the slides 3 times in aqua dest.
- Let the slides dry overnight at room temperature and cover them with mounting-medium and coverslips the next day

Annex F
Gamma-H2AX Assay

1.- BACKGROUND

A limitation of the dicentric analysis for biodosimetry is the delay caused by the need to culture cells for two days before metaphases can be obtained. Also, the assay cannot easily be adapted for rapid high throughput screening of hundreds or thousands of people in a large scale radiation emergency scenario. For these reasons, a rapid triage tool such as the gamma-H2AX assay could potentially complement conventional chromosome dosimetry by enabling a multi-tiered approach in which people are initially screened using the rapid (but less precise) gamma-H2AX assay, followed by prioritised chromosome dosimetry for cases with high foci counts (Ainsbury et al., 2011; Rothkamm & Lloyd 2014).

2.- GAMMA-H2AX LABORATORY PROCEDURE

There is no a single procedure of gamma-H2AX for biological dosimetry purposes. The technique of the gamma-H2AX immunoassay is variable in reagents and methods (Rothkamm & Horn, 2009). First, the antibodies and reagents can be purchased from different companies. Second, the protocols supplied by the companies are different and not always work efficiently, so the laboratories must modify the protocols until obtaining good results. For these reasons it is not realistic to propose a unique protocol for the gamma-H2AX assay. However, a common protocol (Annex 1) can be used as a starting point to optimize the technique in each laboratory. Local adaptations of this protocol or its earlier versions were used successfully by the participants in the first RENEB-gamma-H2AX intercomparison exercise (Barnard et al., 2014), the Multibiodose exercises (Rothkamm et al., 2013a; Ainsbury et al., 2014) and a NATO biodosimetry exercise (Rothkamm et al., 2013b,c). Further information is available in the Multibiodose Deliverable 3.1 (Annex 2) which also discusses an optimised 96-well high throughput version of the assay for finger-prick blood samples (Moquet et al., 2014) and issues around blood sampling and transport.

Dual-colour immunostaining for two separate foci biomarkers such as gamma-H2AX and 53BP1 should be considered to reduce the risk of assay failure (a second marker is available for scoring in case one marker fails) and also to enhance the sensitivity in the case of low dose exposure by scoring coinciding foci and thus reducing the impact of staining artefacts (Rothkamm et al, 2007; Chua et al., 2011; Horn et al., 2011; Barnard et al., 2013).

3.- CALIBRATION

For biological dosimetry it is important to use appropriate calibration curves to convert the biomarker signal into a dose estimate. Whilst the dose response relationship for cytogenetic endpoints such as dicentrics or micronuclei has been found to be very reproducible and stable over time for a given radiation quality, gamma-H2AX foci induction seems to be much more variable both between laboratories and even within one laboratory over time. For this reason, calibration experiments should be performed frequently and individually in each laboratory and for each scorer using volunteer blood samples. Further, any biodosimetric tests should ideally include at least two reference samples (zero dose and one known dose) to confirm or, if necessary, adjust the dose response relationship (Rothkamm et al 2013a).

It is important to note that the time between radiation exposure and blood sampling has a huge influence on the yield of gamma-H2AX foci observed, due to the fast kinetics of foci loss which follow the time course of DNA double-strand break repair (Horn et al., 2011; Barnard et al., 2013). The implications of this effect for blood sampling and shipment are discussed in Annex 2. However, another implication is the need to obtain calibration curves for different time points, simulating any additional shipment-related effects, to enable an accurate exposure assessment.

4.- SCORING METHODOLOGY

Whilst several intensity-based gamma-H2AX biodosimetry approaches have been developed and explored (e.g. Pope et al., 2011; Turner et al., 2011; Horn & Rothkamm, 2011; Horn et al., 2011; Rothkamm et al., 2012), they have been found to be far less sensitive than fluorescence microscopic scoring of gamma-H2AX foci, especially beyond the first few hours post exposure, and are therefore not regarded as suitable for biodosimetry in unplanned exposure scenarios. Annex 3 contains the Multibiodose Deliverable 3.2 which discusses the different approaches available for gamma-H2AX quantification.

Foci scoring can be performed manually through the eye pieces of a microscope, manually using digital images obtained with a microscope camera or automatically using image analysis software packages. See e.g. Barber et al. (2007); Rothkamm & Horn (2009); Rothkamm et al. (2012) for a more detailed discussion.

Whatever scoring method is used, its optimisation should focus on the establishment of consistent scoring criteria and frequent practical testing using coded samples, in order to achieve good repeatability and reproducibility. As mentioned above, any unknown samples to be scored should ideally be accompanied by reference samples, the scoring of which could be used to adjust the dose response calibration. In practice, however, it will be difficult to exactly reproduce the shipment conditions and timings; therefore consistency of scoring is of utmost importance.

5.- DATA ANALYSIS AND INTERPRETATION

Software tools such as Dose Estimate (Ainsbury & Lloyd, 2010) or CABAS (Deperas et al., 2007) which were developed to support chromosome dosimetry, can also be used to fit calibration curves, estimate doses and calculate confidence intervals for gamma-H2AX foci data. The Multibiodose deliverable 6.4, included here as Annex 4, provides a detailed discussion of dose and uncertainty estimation for different biodosimetry methods. The gamma-H2AX assay is specifically addressed in Appendix A.3.

Time-, dose-, and radiation quality-dependent changes in gamma-H2AX foci counts need to be considered when converting foci yields into dose estimates. Partial body exposures are associated with overdispersed foci distributions which can therefore be used as an indicator of non-uniform exposures. The same mathematical methodologies that has been established for estimating the irradiated fraction and its associated dose based on the distribution of chromosome aberrations among lymphocytes (IAEA 2011) can also potentially be used for foci distributions (Rothkamm et al., 2007; Horn et al., 2011; Blakely et al., 2011). However, over- and underdispersed data sets have been observed also for uniform exposures, especially when using automated foci scoring methods (Rothkamm et al., 2013a).

The rapid time course of foci loss following irradiation implies that gamma-H2AX-based dose estimation is associated with very large uncertainties in the case of protracted or intermittent radiation exposures. In such a case as well as in situations where the exact timing between exposure and blood sampling is unknown, the method may only be able to highlight a recent exposure qualitatively (Rothkamm et al., 2013a). Multiplexing this biomarker with a marker of a different biological response such as apoptosis induction which has much slower kinetics, may improve the accuracy of foci-based dose estimations for unknown post-exposure times (Horn et al., 2013).

Importantly, one should regard the gamma-H2AX assay as less reliable than conventional cytogenetic methods. Its main purpose in radiation emergency management is to provide a rapid screening tool in order to i) quickly identify those patients who may be at risk of developing acute radiation syndrome and ii) help prioritise chromosome dosimetry based on observed foci counts. In this respect, its main function is that of a qualitative indicator of exposure rather than a precise dosimetry tool. The fast signal decay limits its use to the first few days post exposure, a significant limitation to its usefulness which must be carefully considered before it is deployed.

6.- REFERENCES

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Annex G
Gamma-H2AX Assay Protocol

1. Aim of the method:	Define standard procedures and conditions for obtaining gamma-H2AX-immunostained lymphocyte samples suitable for biological dosimetry based on microscopic foci scoring.
2. Background:	Immunofluorescence staining and microscopic scoring of gamma-H2AX foci in blood lymphocytes enables rapid detection of recent radiation exposure in unstimulated blood lymphocytes.
3. Sample requirements:	
3.1 Nature of sample	Human peripheral blood lymphocytes, gamma-irradiated and kept in RPMI1640 medium + 10% heat-inactivated FCS[1]; incubated for 4 h and 24 h at 37°C post irradiation.
3.2 Container:	1.5 ml screw-cap tubes
3.3 volume	~0.5 – 1 million cells per sample; Four doses (0, 1, 2, 4 Gy) each at 4h and 24h for calibration, if required. Four unknown doses each at 4h and 24h.
3.4 Transport conditions:	Ship tubes overnight in bubble wrap on wet ice (0-4°C) or with a frozen cold pack and temperature logger; follow UN Regulation 650 for packaging
3.5 Actions if requirements are not met:	Request new sample; test old sample, discard if no good. Note the potential for continued signal loss in warm samples.
4. Materials:	
4.1. Equipment and supportive materials:	• Cytospin with filter paper and slide holder, e.g. Shandon (or use alternative step 1A) • Microcentrifuge • Microscopy slides with surface coating to enhance cell adhesion, e.g. SuperFrostPlus; • Fluorescence/phase contrast microscope with at least x40 lens; • Moist chamber (e.g. lidded plastic box with wet paper); • Coplin jars; micro-pipettors and tips; tissues, cover slips, e.g. 24x60 mm^2; Parafilm stripes.
4.2. Reagents:	• Phosphate buffered saline (PBS), without Mg & Ca; • Deionized H_2O; • 0.25% Triton-X100, 0.1% glycine in PBS; • 2% formaldehyde (FA), acid free, in PBS; • Blocking solution (BS; 1xPBS, 1% BSA, 0.1% Tween20); • Anti γ-H2AX antibody (e.g. Millipore 05-636, 1:500 in BS); • Fluorophore-conjugated 2^{nd} antibody (e.g. AlexaFluor488 goat anti-mouse, Invitrogen A-11029, 1:500 in BS); • Mounting medium with antifade and fluorescent DNA counterstain: e.g. Vectashield with DAPI;

5. Sample processing: Cytospin	***All incubations are at ambient temperature unless stated otherwise*** (1) Resuspend pellet in 2 ml PBS. Adjust cell concentration to cytospin 60-100 000 cells onto a slide for 1 min at 500 RPM.[1]
[Alternative: manual spotting] Fixation and permeabilisation Antibody staining Mounting	***1A) [Alternative: Resuspend pellet in 0.1 ml PBS and spread 25 µl onto a slide. Allow cells to adhere for ~15 min.][2]*** ***(2) Inspect slides using a phase contrast microscope for suitable density, even distribution and integrity of cells.[3]*** ***(3) Fix cells with 2% FA for 5 min.*** ***[Optional: Store slides overnight in 0.5% FA/PBS at 4°C]*** ***(4) Extract cells in 0.25%Triton-X100, 0.1% glycine/PBS for 5 min[4]*** ***(5) Drain and incubate slides in BS for 10min.*** ***(6) Remove excess liquid, apply 90µl γ-H2AX Ab solution, cover with Parafilm and incubate in moist chamber for 45 min.[5]*** ***(7) Remove Parafilm with forceps and wash 3 x 2 min in BS.*** ***(8) Remove excess liquid, apply 90µl 2nd Ab solution, cover with Parafilm and incubate in moist chamber in the dark for 30 min.*** ***(9) Remove Parafilm and wash 3 x 2 min in PBS in the dark.*** ***(10) Drain excess liquid, add 18µl mounting medium with DNA counterstain[6] and apply a cover slip.*** ***(11) Store slides at 4°C until anaylsis.[7]*** ***(12) Score foci per cell in random fields of view, using at least an x40 lens. Record results for 10, 20, 30, 40 and 50 scored cells if scoring manually. For automated scoring, record results for 20, 50, 100, 150 and 200 cells, always using the provided scoring/reporting sheet.***
Notes	***1) Exact cell numbers, volumes and centrifugation time/RPM depend on the specifications of the cytospin equipment. Keep remaining cells on ice or at 4°C as backup, in case something goes wrong.*** ***2) 25 µl suffice to cover a ~100mm2 area on the slide. Smaller volumes should be used in combination with smaller slide areas, e.g. on multi-well slides. Avoid complete drying of the sample. Keep remaining cells on ice or at 4°C as a backup, in case something goes wrong.*** ***3) Especially make sure cells form a monolayer. At high densities cells can form multiple layers on top of each other which complicates foci scoring, especially using unsupervised automated approaches.*** ***4) Glycine captures unbound FA, thus eliminating the need for additional washing steps.*** ***5) Avoid drying of the cell area, as that would result in non-specific staining.*** ***6) DNA counterstain can alternatively be added to the 2nd Ab solution to reduce non-specific background fluorescence.*** ***7) For long-term storage, slides should be sealed with nail varnish and stored at 4ºC for weeks/months or at -20ºC for longer periods. Repeated freezing and thawing should be avoided.***

Annex H
FISH Assay

1.- INTRODUCTION

A limitation of the dicentric analysis for biodosimetry is that cells carrying dicentrics are mitotically unstable and disappear from peripheral blood due to cell renewal, but also to deadth by apoptosis during interphase. For this reason, in cases of chronic or long term exposures the analysis of more persistent chromosome aberrations, like translocations, is a reliable method for biodosimetry. An easy way to score translocations for biodosimetric purposes is by fluorescence *in situ* hybridisation techniques (FISH). Using fluorescent whole chromosome probes that hybridise with entire chromosomes (chromosome painting), inter-chromosome aberrations, such as translocations between "painted" and "unpainted" chromosomes, can be detected.

2.- FISH TECHNIQUE

There is no a single procedure of FISH for biological dosimetry purposes. The technique of FISH is variable in reagents and methods. First, the probes and reagents can be purchased from different companies. Second, the protocols supplied by the companies are different and do not always work efficiently, so each laboratory must modify the protocols until good results are obtained. For these reasons it is not realistic to propose a unique protocol for FISH. However, a common protocol (Annex 1) can be used as a starting point to optimize the technique in each laboratory. This protocol was used by the participants in the RENEB-FISH training meeting in BfS (Munich, May 2014) with optimal results.

3.- CHROMOSOMES TO BE PAINTED

As indicated in the IAEA manual (EPR-Biodosimetry, 2011), *painting three of the larger chromosomes (i.e. #1 to #12), representing about 20% of the genome leads to about 33% efficiency in detecting translocations when a single colour is used*. The percentage of each chromosome probes cocktail relative to the whole genome is estimated from the physical length of chromosomes [Morton, *PNAS 88:7474, 1991*].

For biological dosimetry by translocationsscoring, it is recommended that single colour painting is used with a triple cocktail of target chromosomes. Alternatively two to three-colour painting can be used to also detect translocations between painted chromosomes, giving a slightly increased detection efficiency.

4.- SCORING CRITERIA AND NOMENCLATURE

When chromosome painting is applied, a metaphase should be scored if it contains the 46 centromeres and the entire painted portion. However, in order to speed up the analysis, and according to the IAEA *well-spread metaphase cells are considered suitable for scoring if the cells appear to be intact, the centromeres are morphologically detectable and present in all painted chromosomes, and the fluorochrome labelling is sufficiently bright to detect exchanges between chromosomes labelled in different colours*. *Those cells that are obviously deficient in a large portion of painted material or labelled centromeres should be excluded from the scoring*.

In the IAEA manual different nomenclatures are proposed because with partial genome analysis the conventional terminology proved inadequate. Nowadays, the most common method describes each abnormal metaphase as a unit [Knehr et al. *Int J Radiat Biol 73:135, 1998*] using a modification of the PAINT nomenclature [Tucker et al Mutat Res 347:21, 1995]. Abnormal chromosomes are described using abbreviations, indicating if the chromosome contains painted (B, b) or counterstained (A, a) material. Capital letters indicate the presence of a centromere.

Chromosome aberrations are classified as simple or complex, the latter is observed when three or more breaks in two or more chromosomes have produced the observed chromosome aberration. Aberrations are considered as complete when all the painted broken pieces are rejoined and as incomplete when one or more painted pieces appeare to be unrejoined. However, painting a set of chromosomes does not allow the origin of all the rejoined portions to be identified, therefore simple aberrations are considered as "apparently" simple.

As an example, a reciprocal translocation between a painted chromosome and a counterstained one is described as t(Ab) plus a t(Ba), and scored as one "apparently simple complete translocation", this can be also called simple complete or a two-way translocation. A dicentric formed by a painted and a counterstained

chromosomes is described as dic(BA) ace(ab) and scored as one “apparently simple complete dicentric”, this can be also called simple complete or a two-way dicentric.

5.- DATA HANDLING AND DOSE ESTIMATION

When the frequency of translocations must be compared between laboratories using a different combination of chromosome probes, the total genomic translocation frequency can be estimated according to Lucas et al. [*Int J Radiat Biol 62:53, 1992*]. For a detailed description, the IAEA manual can also been consulted.

5.1. Calibration curve

Using FISH painting it is possible to score different types of translocations and it is also possible to produce different dose-effect curves of translocations for biodosimetry. As examples, some laboratories have producedcurves for “total translocations in all cells”, “apparently simple translocations in stable cells”, “two-way translocations in all cells”, “two-way translocations in stable cells” and more.

The establishment of the dose-effect curve coefficients is the same as described for dicentric dose effect curves.

5.2. Dose estimation

For estimating dose each laboratory must score the translocations in the same way as was used for the curve elaboration and with the same probe cocktail. When a laboratory has more than one dose-effect curve, it will be able to estimate the dose as many times as dose-effect curves it has. Dose estimations shall follow the same procedure described for dicentrics.

Theoretically translocations and dicentrics are induced by ionising radiation at the same frequency. If a laboratory hasn’t a dose-effect curve for translocations, it can transform the frequency of total translocations in all cells to genomic frequency. Then, to estimate the dose it is possible to use the dose-effect curve for dicentrics.

Annex I
FISH Assay Protocol

1 PROCEDURE TO PREPARE BLOOD SAMPLES FOR CHROMOSOMAL ANALYSES WITH FISH (BfS)

Cell culturing and preparation were performed according to IAEA recommendations (IAEA 2011) and ISO standards (ISO 19238)

1.1. MATERIAL

- Lithium-heparin whole blood samples

1.2. REAGENTS

- RPMI-1640 medium with stable glutamine (Biochrom, Berlin)
- Antibiotic solution, Penicillin/streptomycin (10000 U*/ml* bzw. IJg/ml) (Biochrom, Berlin)
- Fetal calf serum (FCS) (Biochrom, Berlin)
- Phytohaemagglutinin (PHA-L) (Biochrom, Berlin)
- 5-Bromo-2-deoxyuridine (BrdU) (Sigma, Steinheim)
- Colcemid, 10 µg/ml (Roche, Mannheim)
- Hypotonic solution KCI (75mM, 4°C) (Merck, Darmstadt)
- Methanol/Acetic acid *(3/1* 4°C) (Merck, Darmstadt)
- Giemsa (Merck, Darmstadt)

1.3. BLOOD CULTURES

To set up 100 ml culture medium, remove 10 ml of RPMI-medium from a 100 ml RPMI bottle and add:

- 10 ml Fetal calf serum (FCS), stored at - 20°C
- 0.4 ml Penicillin/streptomycin, stored at - 20°C
- 2.5 ml PHA-L, prepare it just before use

Mix the medium

I. Per blood sample set up six **culture tubes** containing:

- 5 ml prepared RPMI medium
- 0.5 ml blood (collected in a heparin tube)
- 0.1 ml BrdU, stored in the fridge (only for FpG staining)

II. Place the culture tubes in the incubator at 37ºC.

a.) In the case of Fluorescence plus Giemsa staining (FpG) add 0.1 ml Colcemid **45 h** after culture set up. Return the culture tubes to the incubator for further 3 h. Harvest the cultures after 48 h culture time in total.

b.) In the case of permanent Colcemid add 40 µl **24 h** after culture set up. Return the culture tubes to the incubator for further 24 h. Harvest the cultures after 48 h culture time in total.

Contents of culture tube		
RPMI1640	Biochrom	4.5 ml
Foetal calf serum	Biochrom	10%
PHA	Biochrom	2%
Penicillin / streptomycin	Biochrom	10000 U bzw. µg/ml
BrdU	Sigma	10 mM
Colcemid	Roche	a) 0.1 µg/ml b) 0.04 µg/ml
Whole blood		0.5 ml
	IN TOTAL	**5,5 ml**

1.4. CULTURE STOP AND CELL FIXATION

13. After a culture time of 48 h the tubes are taken out of the incubator and put in the centrifuge for 10 min / 1000 rpm.
14. Remove carefully the supernatant just above the red pellet by suction; do not remove the red pellet!!!
15. Add 2 pipettes of prewarmed (*37ºC)* 0.075M KCl solution and mix it on the vortex.
16. Place the culture tubes for 15 min in the water bath or incubator *(37ºC)* for incubation.
17. Centrifuge the tubes for 10 min / 1000 rpm.
18. Remove the supernatant down to the white layer; be very careful; do not remove the layer, because the lymphocytes are here.
19. Add 1 pipette of fixative (Methanol:Acetic acid 3:1) in drops very slowly until you get a change of colour from red to black, then add 2 further pipettes of fixative. Mix it on the vortex thoroughly.
20. Centrifuge the tubes 10 min / 1000rpm.
21. Remove the supernatant over the white pellet at the bottom.
22. Add 2 pipettes of fixative.
23. Repeat fixation steps (8-10) 2-3 times until the fixative is clear. Overnight storage in refrigerator is possible. Concentrate the cells according to the cell yield.
24. Drop the cells on clean slides. (Slide preparation and handling of the cell suspension see below).

1.5. PREPARATION OF THE SLIDES

Wash the slides in aqua dest., add 5ml of methanol and store them in the fridge

1.6. HANDLING OF THE CELL SUSPENSION

- After the last washing, remove the supernatant carefully and resuspend the pellet to make a slightly milky suspension.

- Allow 2 drops of the suspension to fall in a line on the slide and let it dry

- Check the quality and quantity in the microscope. The metaphases should be well spread with few cross-overs, intact with no cytoplasm.

- Several slides can be made from one culture.

2. FISH (Fluorescence in situ hybridisation) staining

➢ Cell Cycle control for FISH Painting using FpG -method

Solution required:

- Bisbenzimid stock solution: 5 mq Bisbenzimid (Bisbenzimide H 33258, Serva) in 100 ml Aqua

bidest.

Painting treatment:

- Overlay slides in Quadriperm with1 ml Bisbenzimid stock solution + 49 ml PBS.
- Leave in dark at room temperature for 15 min.
- Put the slides under "blacklight" for 20 min.
- Wash 1 x in PBS.
- Then continue with normal FISH painting.

➢ **Painting procedure (recommended by MetaSystems and modified for use in BfS lab)**

Chromosome paint mix: XCPmix for # 2, # 4, # 8 whole chromosome paints marked with Texas Red.

2.1. First Possibility

Equipment: Waterbath

Solution required: 70% formamid in 2 x SSC, pH 7.0 - 7.3; Denaturation solution.

- 70% , 90% , 100% ethanol, -20 ºC

Procedure:

➢ **Probe denaturation:**

- Denature the probe (25 µl / slide) by incubating at 72 ºC for 5 min (waterbath).
- Incubate at 37ºC for 30 min.

➢ **Dehydrate slides in cold 70%, 90%, 100% ethanol for 2 min at room temperature (RT)**

➢ **Denaturation of chromosome slides**

- Prewarm the denaturation solution to 68ºC.
- Immerse the slides into the denaturation solution, incubate for 2 min.
- Transfer to cold 70%, 90% and 100% ethanol, incubate for 2 min each.
- Dry the slides using nitrogen.

➢ **Probe denaturation and hybridisation**

- Pipette the denatured and prehybridised probe mix onto the denatured chromosome slides.
- Overlay with a coverslip.
- Seal with rubber cement.
- Incubate in a humidified chamber at 37ºC overnight in a waterbath.

➢ **Posthybridization treatment (2nd day)**

- Remove rubber cement carefully.
- Place the slides in 4 x SSC + 0.05% Tween 20 (pH: 7.0 - 7.3), room temperature (RT).
- Incubate for about 2 min then remove coverslip carefully.
- Transfer slides to prewarmed (68ºC) 2 x SSC (pH: 7.0 - 7.3) for 5 min.
- Transfer slides to 4 x SSC (pH: 7.0 - 7.3) (RT) for 5 min.
- Apply about 50 µl of DAPI Vectashield (vector laboratories H-1200) (antifade mounting solution) to each slide.
- Overlay with a coverslip.

2.2. Second Possibility

Equipment: ThermoBrite (Slide denaturation */* hybridization system)

See the ThermoBrite User Guide for more information about how to use the instrument.

Protocol used in BfS:

- **Denaturation / Hybridization treatment (1st Day)**
  - Dehydrate slides in cold 70%, 90%, 100% ethanol for 2 min at room temperature (RT).
  - Apply 25 µl of probe mixture to the target area of your slides and overlay with a coverslip.
  - Seal each coverslip with rubber cement.
  - Place the slides in the ThermoBrite.
  - Run the denaturation / hybridization programme (2 min denaturation at 68ºC / 24 h at 37ºC hybridization).
- **Posthybridization treatment (2nd day)**
  - Remove rubber cement carefully.
  - Place the slides in 4 x SSC + 0.05% Tween 20 (pH: 7.0 - 7.3), room temperature (RT) for about 2 min.
  - Remove coverslip carefully.
  - Transfer slides to prewarmed (68ºC) 2 x SSC (pH: 7.0 - 7.3) for 5 min.
  - Transfer slides to 4 x SSC (pH: 7.0 - 7.3) (RT) for 5 min.
  - Apply about 50µl of DAPI Vectashield (vector laboratories H-1200) (antifade mounting solution) to each slide.
  - Overlay with a coverslip.

Annex J
Cytokinesis-block-Micronucleus (CBMN) Assay

1. BACKGROUND

The CBMN assay is an alternative cytogenetic technique, which is simpler and faster to perform than the dicentric assay (Vral et al. 2011, Fenech 2010). It has been recommended by the IAEA as a reliable and robust biodosimeter for measuring radiation exposure. The CBMN assay is capable of estimating whole body doses of low LET ionizing radiation between about 0.2 Gy and 4 Gy and allows investigations up to weeks or months after an in vivo exposure (IAEA: EPR-Biodosimetry, 2011; ISO 17099, 2014). The CBMN assay is also routinely used to demonstrate exposure to genotoxic agents, other than ionizing radiation (Fenech 2007).

In case of large-scale radiation accidents, the CBMN assay is also a valuable biodosimetric tool for emergency triage, thanks to the easy technology and the availability of automated scoring by computerized imaging (Willems et al. 2010). Automated scoring procedures have been under investigation in EU projects (MultiBiodose, RENEB) and the results achieved so far are very promising.

General aspects of applying the CBMN assay as a tool of the operational basis of the RENEB network are described in the common part of the Quality Manual.

2. LABORATORY PROCEDURE OF THE CBMN ASSAY

Although the CBMN assay, like the dicentric assay, is an already highly standardized technique, there is not a 'universal' protocol available that is commonly used by all biodosimetry laboratories performing the CBMN assay. Differences present in the used MN protocols can be reduced to the mode of fixation (fixation of lymphocytes in suspension or on cytospin slides), staining (Giemsa, acridine orange, DAPI, …) and the use of whole blood versus isolated lymphocyte cultures. Each laboratory however has to use its own protocol to achieve optimal results. Important is that the same protocol is used for establishing the calibration curve as for estimating the dose in case of a suspected overexposure. A standard protocol that is used by most of the participants in the RENEB network, and that is also described extensively in the recently published ISO document for the MN assay (ISO 17099, 2014) and in the IAEA manual (EPR-Biodosimetry, 2011), is attached (Annex 1). This protocol can be used by other labs as a starting point to optimize the technique. The differential and additional steps that are needed to perform the MN assay in an automated mode for emergency triage, using the Metasystems software, are also indicated in the protocol in Annex 1. The slides made for automated scoring can be further processed to highlight centromeres using FISH and a pan-centromeric FISH probe. This procedure is recommended when dealing with low-dose exposures. For a detailed protocol of the combined MN-centromere FISH staining we refer to the IAEA manual (EPR-Biodosimetry, 2011). Also the analysis of telomeres in MN by FISH technology can be helpful.

3. SCORING CRITERIA AND NOMENCLATURE

Micronuclei can be scored manually under the microscope after conventional staining with Giemsa for light microscopy or after e.g. acridine orange staining for fluorescence microscopy. For triage mode, MN can be stained for instance by DAPI and scored automatically or semi-automatically with the help of an image analysis system coupled to a fluorescent microscope equipped with a motorized stage. In the MultiBiodose and RENEB programmes, the Metafer4 of Metasystems has been used successfully for automated MN analysis.

Manual scoring of slides

In case of an accidental overexposure involving few individuals, per sample, duplicate cultures are set-up and per culture at least 500 BN cells are scored (this means a total of at least 1000 binucleated (BN) cells per sample from 2 different slides). The distribution of micronuclei amongst the BN cells should also be recorded. In addition, it can also be valuable to score a nuclear divison index (NDI). Details on how to calculate the NDI are given in EPR-Biodosimetry (2011).

In triage mode, involving a large number of exposed individuals, fewer BN cells may be scored (ISO 17099, 2014).

Automated/semi-automated scoring of slides

Several systems for automated image analysis for the CBMN assay have been developed and can be used for measuring accidental overexposures, esp. for triage purposes to assess approximately and rapidly radiation doses higher then 1 Gy, received by individuals in order to supplement the clinical categorization of casualties. For automated or semi-automated analysis, which implies the visual inspection of the gallery of MN positive objects, 1 slide per duplicate culture is scanned and analyzed (1 scanned slide can contain approximately 1000 BN cells). In total, 2 slides per sample are analysed.

Automated and semi-automated MN scoring techniques (based on DAPI stained slides) have been tested successfully in the frame of the MultiBioDose and RENEB programmes. Within the Multibiodose project it has been shown that semi-automated scoring represents important added value to the automated MN assay in view of accuracy of dose estimates in the low dose range (0-1 Gy) and the use of the σ^2/μ overdispersion parameter for dose assessment reliability in case of partial body exposures (Thierens et al. 2014).

For manual and semi-automated scoring ideally 2 scorers should be involved, however one scorer is also acceptable. The scorers should be experienced in the CBMN assay and their expertise should be checked periodically (see ISO 17099, 2014). Online training exercises for manual and (semi)automated scoring, consisting of candidate micronucleus-positive BN objects which have to be evaluated, have been developed and are available on the RENEB website and in Annex 2 and 3.

The MN data have to be recorded in a standardized scoring sheet (examples are given in ISO and IAEA) and the scoring criteria should be documented. Detailed scoring criteria have been published (Fenech 2007; ISO 17099, 2014; EPR-Biodosimetry, 2011) and should be followed.

5. DATA HANDLING AND DOSE-ESTIMATION

For dose-estimations in case of an accidental exposure, a micronucleus calibration curve is required for each lab performing biodosimetry using the MN assay. The same culture protocol and staining method should be used for establishing the calibration curve as for dose-assessment in a case of suspected overexposure. The procedures for producing *in vitro* dose-response calibration curves are comparable with those used for dicentrics. However, for MN, the curves should be based on data obtained from different donors (n>5), with a same number of BN cells scored for each donor (ISO 17099, 2014).

For the fitting of calibration curves and for dose-estimation in a case of an overexposure, special software tools exist, like Dose Estimate (Ainsbury & Lloyd, 2010) and CABAS (Deperas et al. 2007), which can be used. For a more general description on 'basic statistics for cytogenetic analysis' the IAEA manual can be consulted (EPR-Biodosimetry, 2011).

Due to the high inter-individual variability of the spontaneous MN frequencies, it is important that each lab performing the CBMN assay has its own reference data set of MN background frequencies. This reference group should ideally contain a substantial number of individuals of both sexes and different age groups.

In general, the MN assay only detects *in vivo* exposures in excess of 0,2 – 0,3 Gy (Vral et al. 2011; EPR-Biodosimetry, 2011; ISO 17099 2014). A better low-dose assessment can however be achieved by analysing the MN for the presence of centromeres and telomeres (Baeyens et al. 2013, EPR-Biodosimetry, 2011).

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Annex K
Protocol cytokinesis-block micronucleus assay

ASSAY Reagents

- RPMI---1640 medium (Gibco)
- L---glutamine (200mM) (Gibco)
- Penicillin/streptomycin (50U/ml + 50µg/ml) (Gibco)
- Complete RPMI---1640 medium (cRPMI) : 500 ml RPMI---1640 + 5 ml L---glutamine + 2.5 ml penicillin/streptomycin
- Fetal calf serum (FCS) (Gibco)
- PHA---M , 10 ml solution (Ref: 10576---015, Gibco)
- Cytochalasin B (Sigma)
- Hypotonic solution KCl (75mM, 4°C) (Sigma)
- Ringer (9 g NaCl + 0.42 g KCl + 0.24 g CaCl2 for 1 liter)
- Methanol/Acetic acid/Ringer solution (10:1:11 (**manual** scoring) or 4/1/5 (**automated** scoring), 4°C)
- Methanol/Acetic acid (10/1 (**manual**) 4/1 (**automated**), 4°C)
- Giemsa stain solution (giemsa's azur eosin methylene blue solution, Merck) (**manual** scoring)
- DAPI Vectashield vector laboratories H---1200 store at 4°C (antifade mounting solution) (**automated** scoring)

Blood cultures

1. Venous blood samples are collected in heparine tubes
2. In case you want to perform *in vitro* irradiations to set up a calibration curve: put 1 ml of blood into a plastic falcon tube and irradiate *in vitro* at 37°C. Controls are sham irradiated. (for setting up a calibration curve doses between 0,2 and 4 Gy should be used)
3. Per sample set up two 25 cm^2 falcons containing:
    - 4 ml cRPMI
    - 0.5 ml FCS
    - 0.5 ml blood + 100 µl PHA---M
4. Place the falcons upright in the 5% CO2 incubator.
5. After **24 h** culture time, add 20 µl cytochalasin B (final concentration 6 **µ**g/ml), gently mix.
6. Harvest the cultures after **70 h** culture time.

Culture stop and cell fixation and staining

1. Decant the blood cultures in centrifuge tubes and centrifuge during 8 min at 1000 rpm.
2. Discard supernatans.
3. Add slowly and while vortexing 7 ml of cold KCl.
4. Centrifuge the cells directly during 8 min at 1000 rpm.
5. Discard supernatans.
6. Add slowly and while vortexing **7 ml** methanol/acetic acid/ringer solution and store overnight at 4°C.
7. Centrifuge during 10 min at 1000 rpm.
8. Discard supernatans.
9. Add slowly and while vortexing **5 ml** cold methanol/acetic acid.
10. Centrifuge during 8 min at 1000 rpm.
11. Discard supernatans.
12. Repeat fixation steps (9---11) three times more until fixative is clear. Overnight storage in refrigerator is possible. Concentrate the cells according to the cell yield.
13. Drop the cells (40µl) on dry and clean slides.

Manual MN analysis

When the slides are dry, stain them for 20 min with Giemsa (6% solution in HEPES buffer (0.03M; pH 6.5).
If the cells are not enough swollen (resulting in to dark staining of the cytoplasm) the cell suspension can be fixed once more in fixative containing more acetic acid e.g. 8:1 or 6:1.
• Per sample MN are scored in 1000 binucleate cells (500 binucleate cells per slide per culture).

Criteria for selecting binucleated (BN) cells which can be scored for micronucleus frequency
The cytokinesis-block cells that are scored for MN frequency should have the following characteristics:
a) The cells should be binucleated.
b) The two nuclei in a BN cell should have intact nuclear membranes and be situated within the same cytoplasm.
c) The two nuclei in a BN cell should have a regular shape and be approximately equal in size, staining pattern and staining intensity.
d) The two nuclei within a BN cell may be unconnected or may be attached by one or more fine nucleoplasmic bridges, which are no wider than 1/4th of the nuclear diameter.
e) The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nucl ei can be scored only if the nuclear boundaries of either nucleus are distinguishable.
f) The cytoplasm of a BN cell should be somewhat visible and distinguishable from the cytoplasm of adjacent cells.

Criteria for scoring micronuclei (MN)
a) MN are morphologically identical to but smaller than the main nuclei.
b) The diameter of MN in human lymphocytes is variable. The largest MN should be smaller than 1/3rd of the diameter of the main nuclei; on the other hand MN can also be very small.
c) MN are non-refractile and can therefore be readily distinguished from artefacts such as staining particles.
d) MN are not linked or connected to the main nuclei.
e) MN may touch or slightly overlap the main nuclei but the micronuclear boundary should be distinguishable from the nuclear boundary.
f) MN usually have the same staining intensity as the main nuclei but occasionally staining may be more or less intense.

Automated MN analysis

Apply 40 µl DAPI Vectashield to coverslip and apply to slides. Per culture 1 or 2 slides are scanned.

Criteria for binucleated (BN) cells:
The automatically selected BN cells shown in the gallery should have the following characteristics:
(a) The cells should be binucleated.
(b) The two nuclei in a BN cell should have intact nuclear membranes and be situated within a circular area defined around the two nuclei of the BN cell (size of this circular area is defined in the classifier). No other nuclei or artefacts should be in too close proximity of the BN cell.
(c) The two nuclei in a BN cell should have a regular shape and be approximately equal in size, staining pattern and staining intensity.
(d) The two nuclei within a BN cell may be unconnected or may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of either nucleus are distinguishable.

Criteria for micronuclei (MN):
The automatically selected MN in the gallery pictures should have the following characteristics:
(a) MN are morphologically identical to but smaller than the main nuclei: the diameter of MN in human lymphocytes is usually smaller than 1/3rd of the diameter of the main nuclei; they can also be very small.
(b) MN are non-refractile and can therefore be readily distinguished from artefacts such as staining particles.
(c) MN are not linked or connected to the main nuclei.
(d) MN may touch the main nuclei but the micronuclear boundary should be distinguishable from the nuclear boundary.
(e) MN usually have the same staining pattern as the main nuclei but occasionally staining may be more or less intense.

METAFER 4 (METASYSTEMS) FOR AUTOMATED MN ANALYSIS

Press the icon on the desktop, the program Metafer 4 will initiate

Load the slides

- Stage → move to… → slide center 8 →
- Place the slides in the upper left side of the slot

To scan the slides :

MODE

- MSearch
- Fluorescence

SETUP

- Activate the slides – click once (make sure the information will be saved in the **correct data path**)
- Type the name of the slide
- Classifier: DAPI UGENT Biodosimetry
- Sensitivity : 9
- Search window: Center N%
- Size : 90
- Max. count: depends on the nbr you want to score (1000 – 2500 BN)
- Search speed : medium
- Search report: none
- On search end : continue/shut down

M SEARCH

Prepare the microscope : Make sure the 10 x objective is in place, DAPI filters activated and the shutter open (on the touch screen of the microscope)
To focus: center on an object with the mouse and focus Press
OK to initiate
First the system will focus on various (24) spots on the field, then the slides will be analyzed according to the classifier

To set up the classifier:

- Go to Msearch
- Micronuclei Setup
- Adapt parameters as described below:

Classifier Name : biodosimetry ...
Classifier Description : fluorescence1

–Nudei----------

Image Processing Operations :
Sharpen(3,4):
IP operation groups = Sharpening
IP operation in groups = Sharpen
Range = 3
Power = 4

Object Threshold (in %) :15
Minimum Area (µm^2) : 80
Maximum Area (µm^2) : 1000
Maximum Relative Concavity Depth : 0.160
Maximum Aspect Ratio : 1.370
Maximum Distance (µm) : 25
Maximum Area Asymmetry (in %) : 80
Region of Interest Radius (µm) : 30
Maximum Object Area in ROI (µm^2) : 35

--Autosperate----------

Use Automatic Separation of Binucleate Cells ? : 0
AutoSep Concavity Regression Radius (in 1/10 µm): 10
AutoSep Minimum Concavity Contour Angle (in °): 45
AutoSep Minimum Relative Concavity Contour Distance (in %): 40

--Micronuclei----------
Micronuclei : Image Processing Operations :

MedianV(3)
IP operation groups = Smoothing
IP operation in groups = Median V
Range = 3
MedianH(3)
IP operation groups = Smoothing
IP operation in groups = Median H
Range = 3
Average(3,1)
IP operation groups = Smoothing
IP operation in groups = Average
Range = 3
Power = 1
Sharpen(5,5)
IP operation groups = Sharpening
IP operation in groups = Sharpen
Range = 5
Power = 5

Object Threshold (in %): 7
Minimum Area (µm^2): 1
Maximum Area (µm^2): 40
Maximum Relative Concavity Depth: 0.700
Maximum Aspect Ratio: 1.700 Maximum Distance (µm): 25

Annex L
Biodosimetry using the cell fusion mediated Premature Chromosome Condensation (PCC) Assay

1. Background

Premature chromosome condensation (PCC) in non-stimulated peripheral blood lymphocytes, mediated by their fusion with Chinese Hamster Ovary (CHO) mitotic cells in the presence of polyethylene glycol (PEG), is an alternative assay for biological dosimetry in radiation emergency medicine (Terzoudi and Pantelias, 2006) where rapid and accurate estimation of absorbed dose is considered to be a high priority (IAEA Safety Standards, EPR-Biodosimetry, 2011). Specifically, following exposure to ionizing radiation the PCC-fusion assay enables one to measure the chromosomal aberrations directly in G0 lymphocytes shortly after blood sampling, without the need for their mitogen stimulation or culturing (Pantelias and Maillie, 1983). This assay was first reported as an alternative assay for biodosimetry in 1984 (Pantelias and Maillie, 1984). Indeed, Giemsa staining of lymphocyte prematurely condensed chromosomes (PCCs) allows a rapid quantification of an exposure by means of PCC fragments and rings, using appropriate calibration curves (Pantelias and Maillie, 1985; Lamadrid Boada at al., 2013). Particularly, when PCC is combined with C-banding (Pantelias et al., 1993) or more recently with fluorescence in situ hybridization (FISH) techniques using specific DNA libraries or telomere-centromere (TC) staining with peptide nucleic acid (PNA) probes, the assay permits detection of PCC fragments and translocations as well as of dicentrics and centric ring chromosomes, enabling thus their rapid and accurate analysis (M'kacher et al., 2014).

The assay can be used to determine exposure to low doses as well as to life threatening acute doses of low and high LET radiation. Moreover, it can discriminate accurately between total and partial body exposures since the numbered of normal cells, as determined directly in G0 lymphocytes without the need for their mitogen stimulation or culturing, reflects more accurately the proportion of unirradiated lymphocytes in the peripheral blood. Even a small spared fraction as low as 5% can be efficiently detected by means of this assay. Most importantly, in the case of large-scale radiation accidents, the PCC-fusion assay could be also proved to be a valuable tool for emergency triage particularly when combined with TC-staining and the availability of automated scoring by computerized imaging and specialized software such as the recently developed PCC-TCScore software (M'kacher et al., 2014).

For the purpose of the present RENEB QUALITY MANUAL, the basic laboratory procedure for cell-fusion mediated PCC induction in peripheral blood lymphocytes is attached in Annex1. In addition, scoring criteria, data analysis and dose estimation based on Giemsa-stained excess lymphocyte PCC fragments (i.e. PCCs in excess of 46) and PCC-ring chromosomes as well as PCC-dicentric and centric ring chromosomes obtained using TC staining with PNA probes and appropriate calibration curves, are presented.

2. Cell-fusion mediated PCC Laboratory Procedure

Sendai virus-mediated fusion between mitotic and interphase mammalian cells causes the rapid condensation of the interphase chromosomes into distinct structures, a phenomenon termed premature chromosome condensation (PCC) (Johnson and Rao, 1970). This virus-mediated PCC-fusion protocol has been widely used in cytogenetic fields and particularly in radiation biology because induction of PCC enables visualization and analysis of chromosomal damage directly in interphase nuclei, without the need of irradiated cells to proceed to metaphase (Hittelman and Rao, 1974; Waldren and Johnson, 1974; Cornforth and Bedford, 1983). However, cell fusion by means of Sendai virus requires cells with membranes especially receptive to the virus particles, and it is known that G0 lymphocytes cannot be satisfactorily fused using Sendai virus. This difficulty was overcome for the purpose of biological dosimetry with the development of a PCC assay using polyethylene glycol (PEG) as a fusing agent to induce PCC in G0 lymphocytes by fusing them to mitotic Chinese hamster ovary (CHO) cells (Pantelias and Maillie, 1983).

A detailed cell-fusion mediated PCC protocol is attached (Annex 1) and it can be used to optimize the PCC-assay in every biological dosimetry laboratory. A similar PCC protocol is also described in the recently published IAEA manual (EPR-Biodosimetry, 2011). A brief description of this protocol is presented in a recent report (Sebastia et al., 2014). Also, the protocol for the very promising new approach for biological dosimetry in radiation emergency medicine where the rapid and accurate detection of dicentrics is a high priority using automated scoring, has been recently described (M'kacher et al., 2014). In fact, the introduction to the PCC-fusion assay of telomere-centromere (TC) staining with peptide nucleic acid (PNA) probes and the development of PCC-TCScore software, has made possible the rapid scoring of unstable chromosome aberrations, including dicentrics, with a level of accuracy and ease not previously possible.

3. Blood sampling and lymphocyte isolation

Following exposure to ionising radiation, peripheral blood should be sampled in heparinised vials as soon as possible since the initial induced chromosomal PCC breaks decrease with time and after a delay of a few weeks the frequency of dicentrics also decreases. If necessary the sampling time can be extended to about six months. For non-uniform or partial body exposures a post irradiation time period of about 24 h is allowed so that irradiated and unirradiated lymphocytes are homogeneously mixed. Packaging, labelling and shipment of blood samples shall be conformed to national and international regulations. Temperature logger, dosimeter to record the temperature and any dose received by the samples during transport and the use of standardized sample instruction sheets (ISO 19283, 2014) are advisable. For the isolation of lymphocytes to be used for the PCC assay, the whole blood can be simply layered on top of equal amount of Ficoll-Paque gradient in a test tube before centrifugation. The procedure is described in the detailed protocol for the PCC assay, presented in Annex 1.

4. Analysis and scoring criteria

PCC spreads can be located manually and their analysis can be greatly facilitated using semi-automated image analysis systems like those provided by MetaSystems. The analysis involves counting the number of chromosome elements which appear as single chromatids lighter stained and can be discriminated easily from the CHO mitotic chromosomes which are darkly stained following Giemsa staining. In unirradiated lymphocyte PCCs, 46 elements can be scored. The number of chromosome elements in the exposed samples is recorded, and the induced frequency is estimated by simply subtracting the value obtained in unirradiated samples. Generally, a number of 30 to 50 PCC-spreads is considered to be adequate for dose estimation following a single exposure. In case of high doses, rings can be also easily scored and quantified in PCC spreads directly by microscopy or by means of image analysis systems. A circular shaped chromatid with a central opening is scored as a PCC ring. Small circular chromatids with or without central openings are also considered as rings (Lamadrid Boada at al., 2013). When the blood sampling is taking place at least 8 hours post-irradiation, C-banding of lymphocyte PCC spreads has made possible the visualization and quantification of dicentrics, acentric fragments and centric ring chromosomes (Pantelias et al., 1993). However, the recent introduction to the PCC-fusion assay of telomere-centromere (TC) staining with peptide nucleic acid (PNA) probes enables the scoring of unstable chromosome aberrations, including dicentrics, with a level of accuracy and ease not previously possible (M'kacher et al., 2014).

5. Calibration curve and dose estimation

Appropriate calibration curves must be constructed for dose estimation for each of the three different endpoints of chromosomal aberrations analysed; excess PCC fragments and PCC-rings using Giemsa staining of PCC-speads, as well as PCC-dicentric and centric ring chromosomes using TC staining with PNA probes. The dose-response curves are generated by in vitro irradiation of unstimulated lymphocytes under conditions as close as possible to in vivo situation. Such dose-response calibration curves are reproducible and stable over time for a given radiation quality and dose-rate. Laboratories specialized in biological dosimetry performing the PCC assay should construct their own calibration curves mainly for gamma radiation sources such as Co-60 or Cs-137 and X-rays. The dose range should cover at least 4 dose points between 0.25 to 1.0 Gy and at least 4 dose points above 1.0 Gy and up to 5 Gy for whole body exposures. For partial body exposures the dose range should be extended up to 20 Gy. For excess PCC fragments and PCC-rings using Giemsa staining linear dose-response curves are obtained. However, using TC staining in lymphocyte PCCs, the dose-response curves for PCC-dicentric and centric ring chromosomes are described by linear-quadratic relationships. In this case, specialized softwares such as "CABAS" or "Dose Estimate" used for the conventional dicentric assay can be also employed for dose estimation (IAEA Safety Standards, EPR-Biodosimetry, 2011).

6. References

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Annex M
PCC (Premature Chromosome Condensation) Assay Protocol

The following detailed PCC protocol is based on the publication "A Simple Method for Premature Chromosome Condensation Induction in Primary Human and Rodent Cells Using Polyethylene Glycol" by Pantelias and Maillie (Somatic Cell Genetics, Vol. 9 No. 5, 1983, pp 533-547), and first used for Biological Dosimetry purposes in 1984 (Pantelias G.E. and H.D. Maillie, "The use of peripheral blood mononuclear cell prematurely condensed chromosomes for biological Dosimetry", Radiat Res., 99, 1984, pp 140-150).

MATERIALS

- Culture medium (McCoy's 5A, RPMI 1640 [+] HEPES or any other medium) supplemented with 10 % fetal bovine serum and antibiotics.
- Phosphate buffered saline (PBS) or Hank's buffered salt solution (HBSS) calcium and magnesium free.
- Polyethylene glycol (PEG) 1500, Boehringer Mannheim, No. 783 641 (readymade solution: PEG 50%w/v, 75 mM Hepes) or PEG 1450 (powder or flakes) Sigma-Aldrich, P5402 (40-50% w/v in RPMI 1640 [+] HEPES but w/o serum).
- Colcemid stock solution (10^{-5}, or 10 μg/ml).
- Hypotonic solution (0.075 M, KCl)
- Fixative: Three parts methanol and one part acetic acid glacial.
- 14 ml round bottomed tubes.
- Heparinized tubes (BD Vacutainer) are used for blood collection.
- Ficoll-Paque gradient for lymphocyte isolation (Biocoll Separating Solution L6113, Density 1.077 g/ml, BIOCHROM).
- Pasteur pipettes, microscope slides, glass cover slips no. 1 (24x50 mm), and mounting medium (Entellan, MERCK, 107961).
- Test tube rack lined with a folded paper towel.

PROCEDURES

A. Preparation of PEG solution

1. In a test tube mix 1gr PEG (MW 1450, Sigma-Aldrich) with 1.5ml RPMI[+] HEPES w/o serum (PEG 40% w/v). PBS can be also used instead of RPMI.

2. The mixture is warmed up in a water bath (>37^{o}C) and well mixed until the solution is clear. A microwave oven (20sec) can be also used instead of the water bath.

3. The solution is cooled down and ready to be used (150μl per fusion). Allternatively it can be kept in the refrigerator until the time to be used.

B. Preparation of mitotic cells

Mitotic cells obtained by standard selective detachment (shake off) procedure are kept always with colcemid in ice or frozen properly until needed for fusion with interphase cells and PCC induction.

A large number of mitotic cells can be routinely obtained by subculturing plateau phase CHO cells and blocking them at metaphase in their first or second cell division: Colcemid (50μl from stock solution

for each 25cm^2 flask containing 5ml culture medium) is added to the cells approximately 14h (for 1st division) or 26h (for 2nd division) after having been subcultured. Mitotics are harvested by the shake off procedure 4h later on or at even later times until many CHO cells enter mitosis and get round and detached from the bottom of the flask, as can be observed using an inverted microscope. After collecting the first wave of mitotics you can add culture medium plus colcemid in the same flasks so that 2-3 hours later you can harvest by shake off the second wave of mitotics and so on until practically you collect at metaphase all the CHO cells. Routinely if the first harvest is satisfactory, the metaphase cells from one 25cm^2 flask can be used for 2 fusions (2 different experimental points). Of course, for preparing a large number of mitotic cells the 75cm^2 flasks are more appropriate.

C. Isolation of lymphocytes

Lymphocytes are isolated from peripheral blood by means of Ficoll-Paque gradient following the procedures suggested by the manufacturer. Routinely, 10ml of heparinized peripheral blood is collected. For this purpose in a 14ml tubes we add 5ml of Biocoll Separating Solution and we layer carefully and gently 5ml of whole blood without any dilution using a 5ml pipette, so that we do not disturb the interphase between the separating solution and the whole blood (i.e. avoid any mixing). After centrifugation at 1800 rpm for 20min, the lymphocytes are concentrated at interfase of the gradient, collected with a 5ml pipette and diluted in 10ml RPMI[+] HEPES culture medium with serum. Lymphocytes are then centrifuged at 1500 rpm for 10min, the cell pellet is resuspended in RPMI full medium and lymphocytes are then ready to be used for the different exoerimental points. The isolated lymphocytes from 10 ml of whole blood are preferentially kept in RPMI[+] HEPES culture medium and can be used for 10 or maximum 20 experimental points. For this purpose appropriate number of CHO mitotic cells must be prepared for cell fusion.

D. Fusion of CHO mitotics with isolated peripheral blood lymphocytes

1. In a 14 ml round bottomed culture tube, mix lymphocytes, resuspended in medium without serum, with mitotic CHO cells also resuspended in medium without serum containing colcemid and centrifuge for 6 min at medium rpm (eg. 1000rpm) so that the mixed cells in the pellet are not too packed.

2. If air bubbles are formed on top of the medium in the tube after centrifucation in the previous step, they should be removed by suction or by means of a Pasteur pipette, so that the air bubbles do not stick to the pellet when supernatant is discarded.

3. Pour off supernatant, keeping always the tube inverted. Blot on a paper towel and place the tube upside down in the lined test tube rack (this prevents the residual fluid from the walls of the tube to cover the cells in the pellet). Reblot tube until cells to be fused are cover with minimum fluid.

4. After a few minutes and while holding inverted the tube, use a 200 µl pipette to get near to the cell pellet with the tip, inject 150 µl PEG straight to the cells to be fused and immediately turn the tube upright and place it in the test tube rack without shaking the tube. The main goal in this step is to inject PEG solution directly to cell membanes by avoiding to have any fluid between cells and PEG (that is why we keep tubes inverted until the addition of PEG). At this point, the cell pellet should appear detached from the tube bottom forming clumps in the PEG solution (for this purpose we prefere round bottom and not conical tubes, and we use up to 1000 rpm centrifugation). If the pellet remains attached to the bottom of the tube, the fusion may not be suscessful.

5. If the experimental design has several experimental points, the procedures 1-4 described above should be repeated for all samples, so that all tubes are brought to the point described in 4.

6. Subsequently, 1.5 ml of PBS or RPMI without serum containing colcemid is added slowly to the tubes and mixed with the PEG solution by gently shaking so that the solution is homogenious with small agrigations of CHO cells with the lymphocytes.

7. Centrifuge for 6 min at 1000 rpm.

8. Pour off supernatant, blot and add 0.7 ml of pre-warmed RPMI growth medium, containing colcemid (10µl from stock solution) to hold the CHO cells at metaphase. The medium is added very slowly by tapping tube gently so that CHO cells wih lymphocytes remain suspended in small agrigations. If you break pellet to single cell suspension, cell fusion may not be successful. To optimize cell fusion particularly when a low number of lymphocytes is available, 0.7 ml of complete lymphocyte growth

medium (RPMI) containing PHA(2%) and colcemid can be used, as initially proposed by Pantelias and Maillie (Somatic Cell Genetics, Vol. 9 No. 5, 1983, pp 533-547). In the detailed protocol described in their 1983 publication, readymade Chromosome Medium 1A containing PHA (Gibco, No. 120-1672) was used.

9. Tubes are finally placed in test tube rack or in a glass beaker and incubated at 37^{o}C for 75 min. By that time cell fusion and premature chromosome condensation in lymphocytes is completed so that we can visualize 46 distinged single chromatid chromosomes in control lymphocytes. If you incubate for less time the chromosomes are more extended. More incubation time will allow maximum packing of the lymphocyte chromosomes

10. Subsequently, chromosome preparations are obtained using standard procedures: Hypotonic KCl (0.075 M), Carnoy's fixative (1:3 v/v Acetic acid / Methanol), and air dryed chromosome spreads on slides are stained with 3% Giemsa.

Annex N
Electron Paramagnetic Resonance (EPR) spectroscopy

1. Recommendation to elaborate laboratory procedures

In a general manner the procedures established for each laboratory should follow the ISO standard 13304-1 and RENEB quality manual recommendations. In addition of these recommendations, specific recommendations for the glass analysis are made below.

a. Storage

It is recommended to keep all the samples including those uses for calibration purposes in the dark. Additional signals could be induced by natural or artificial light sources.

b. Sample preparation

The sample preparation aims to remove all unwanted materials that could cause parasitic signals and to have the sample in a form that could be measured by EPR.

The part of the touch glass which is analyzed is the external slice of glass that is usually made in alkali-alumino-silicate glass. The external touch screen slice has to be separate from the other slices of the touch screen.

It is important to remove all paintings, glues, or other materials that could be applied on the surface of the glass sheet to eliminate all possible un-wanted signals. The cleaning can be performed with ethanol or acetone. The use of ultrasound bath is not recommended unless if it is established that the frequency, power and duration of the bath has no influence on the signal of interest.

The glass can be cut or crush in order to obtain grains or fragments that would fit in the EPR tubes. The cut of glass with nippers does not induce mechanical induced signal. Whatever the method used, it should be checked that it does not induced an additional component.

c. Sample measurements

The measurements should be performed in X-band in continuous wave mode. For the recording of the spectra, it is recommended to use a high microwave-power as the signal of interest saturates only at very high micro-wave power. For example, with a high Q resonator, a microwave above 20 mW is recommended. The recording parameters that have an influence on the signal to noise ratio, on the reproducibility of the measurements and the measurements time have to be optimized. It concerns at minimum the micro-wave power, the modulation amplitude, the conversion time, time constant, number of accumulation, the sweep width. The number of repeated measurements is depending on the best compromise between accuracy and rapidness following the exigencies of the requestor(s).

d. Signal analysis and dose estimation

Whatever the method used, the method selected has to be validated through an intercomparison. For a large number of samples that has to be evaluated in short delay, it is recommended to use a calibration curve(s) to estimate the dose. The calibration curve should take into account the variability among samples that have to be analyzed. Pre-established curves for the main types of touch screens could be also an option. For expertise with low number of samples, a calibration curve established with the glass of the same origin than the samples that have to evaluated can be preferred.

Although the EPR signal induced by irradiation is considered as stable over weeks, the delay between the irradiation and the EPR measurements should not be too different for calibration sample and sample under analysis. It is on the responsibility of the laboratory to possibly select the more appropriated calibration curve.

2. Quality control

The minimum quality controls requested are described in the ISO standard 13304-1 relative to the minimum criteria of EPR dosimetry. The quality assurance manual of the laboratory should describe the quality control practice.

3. Documentation

The minimum documents required for QA and QC are listed in the ISO standard 13304-1 as well as the information on samples, measurements, dose estimation that have to be recorded.

Annex O
Optically Stimulated Light (OSL)

Protocol using resistors removed from the circuit board of mobile phones

1. Background

In the framework of the EU-FP7 MULTIBIODOSE project, two protocols using optically stimulated luminescence (OSL) of resistors removed from the circuit board of mobile phones were developed with the aim to use the resistors as fortuitous dosimeters in case of radiological accident. With the first protocol ("fast-mode" protocol), no preheat process is performed on the sample, so that measurements are much faster. This protocol could be suitable for a first triage in a mass casualty radiological emergency. In the second protocol ("full-mode" protocol), a preheat of the sample aims to make the signal more stable. The procedure is slower but higher precision is expected. This protocol could be more appropriate for an accurate dose-assessment process.

2. Sample requirements

SIM cards and batteries can be removed because not needed. All samples have to be coded or identified to insure connection and traceability between estimated doses and mobile phone owner identity. Collected mobile phones should be stored and transported in dark (opaque boxes or bag).

3. Equipment and supportive materials

- Screwdrivers, scalpels, or utility knives to open the mobile phones and extract the resistors
- Sample holders ("cups")
- Silicone oil
- OSL reader equipped with a blue light source
- External or internal radiation source

4. Sample preparation *under red light*

The mobile phone is disassembled and the resistors are removed from the circuit boards.
Resistors are put on sample holders previously sprayed with silicone oil.

5. Sample measurements

For both protocols, a first measurement is performed to obtain the signal related to the unknown dose (the so-called "accident" signal). Then a calibration dose (5 Gy) is delivered to the sample and the resulting OSL signal is recorded (the "calibration" signal).

OSL measurement parameters:

- Using the "fast-mode" protocol: OSL acquisition at room temperature for 30 s
- Using the "full-mode" protocol: preheat: 10 s at 120°C, OSL acquisition at 100 °C for 30 s.

6. Signal analysis and dose estimation

OSL signal from resistors is unstable with time. It decreases with time after irradiation (fading). Fading correction factors were derived from two fading curves (one for each protocol) calculated by the three MULTIBIODOSE project partners (HMGU, IRSN and ISS). An analysis template allow to calculate the signal intensities (integration windows of 0-6 s for signal and 6-12 s for background, respectively) and the dose corrected for fading.

Uncertainties are calculated from the combination of the error estimation due to counting statistics for the instrument signal and background and the uncertainty resulting from fitting of the fading curves to the experimental data.

7. Quality control

The quality assurance manual of the laboratory should describe the quality control practices (source calibration, control of the photomultiplier performances, control of background level…).