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Radiological protection — Performance criteria for laboratories using the cytokinesis-block micronucleus (CBMN) assay in peripheral blood lymphocytes for biological dosimetry

Radioprotection — Critères de performance pour les laboratoires pratiquant la dosimétrie biologique par l'analyse des micronoyaux par blocage de la cytodiérèse (CBMN) dans les lymphocytes du sang périphérique

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 85, *Nuclear energy, nuclear technologies, and radiological protection*, Subcommittee SC 2, *Radiological protection,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 430, *Nuclear energy, nuclear technologies and radiological protection,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 17099:2014), which has been technically revised.

The main changes are as follows:

— minor edits to text throughout;

— reorganization of document to better harmonize with other biological dosimetry standards;

— addition of 7.2.7 on data security plan;

— additional requirements added for the report on the conditions of the exposure for the calibration curve in 10.2;

— relaxation of the number of individuals required for each age group for establishing background micronucleus frequency, leaving the determination up to the head of the laboratory (10.3);

— addition of details on determining the minimal resolvable dose (10.4), the absorbed dose (11.2.4) and the uncertainty (11.2.5);

— removal of reference to coefficient of variance when determining scoring expertise, focussing on the use of 95 % confidence intervals to determine expertise (11.1.3);

— addition of reference to other exposure scenarios (11.2.8);

— removal of Annex on automated micronuclei scoring as it was deemed outside of the scope of the standard;

— addition of a sample group report (see Annex E);

— addition of a detailed annex (see Annex F) for calculating the decision threshold and detection limit along with a sample calculation and R script for performing these calculations.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The purpose of this document is to define the use of the cytokinesis-block micronucleus (CBMN) assay with human peripheral blood lymphocytes for biological dosimetry of exposure to ionizing radiation. This assay is intended to be applied for accidental or malevolent exposures involving

a) up to a few casualties to provide individual whole-body dose estimates, or

b) in a triage mode to populations to provide rapid, lower accuracy dose estimates for individuals that can be improved with more accurate analysis at a later time.

The CBMN assay is an alternative cytogenetic technique, which is possibly simpler and faster to perform than the dicentric assay[1][2]. It is also routinely used to demonstrate exposure to genotoxic agents, other than ionizing radiation, which is not covered in this document. Although culture of the blood samples is slightly longer than for dicentrics, the scoring of micronuclei (MN) in binucleated lymphocytes is easier.

As was done with the dicentric assay, the CBMN assay has been adapted for the emergency triage of large-scale multi-casualty nuclear or radiological incident. The blood volume required for a sufficient number of scorable binucleated cells (BNCs) is similar to that required for the dicentric assay. Again, the faster counting speed for MN compensates for the extended culture time. However, it has to be considered that factors such as age, sex, diet and environmental mutagens can have an influence on the results particularly after low dose exposures[3][4][5]. In addition, the CBMN assay can be performed in an automated mode using various cytometric technologies but these are outside the scope of this document.

This document provides a guideline on how to perform the CBMN assay for dose assessment using documented and validated procedures. Dose assessment using the CBMN assay has relevance in medical management, radiation-protection management, record keeping, and medical/legal requirements. This document is divided into two parts, according to the use of CBMN assay: radiation exposure of a few individuals or population triage in a large radiological or nuclear event.

A 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, the criteria of accreditation and the evaluation of performance. This document is generally in conformity with ISO/IEC 17025[6] with particular consideration given to the specific needs of biological dosimetry. The expression of uncertainties in dose estimations given in this document complies with ISO/IEC Guide 98-3[15] (former GUM) and the ISO 5725 (all parts)[7].

Radiological protection — Performance criteria for laboratories using the cytokinesis-block micronucleus (CBMN) assay in peripheral blood lymphocytes for biological dosimetry

1 Scope

This document gives guidance on

a) confidentiality of personal information for the customer and the laboratory,

b) laboratory safety requirements,

c) calibration sources and calibration dose ranges useful for establishing the reference dose-response curves that contribute to the dose estimation from CBMN assay yields and the detection limit,

d) performance of blood collection, culturing, harvesting, and sample preparation for CBMN assay scoring,

e) scoring criteria,

f) conversion of micronucleus frequency in BNCs into an estimate of absorbed dose,

g) reporting of results,

h) quality assurance and quality control, and

i) informative annexes containing sample instructions for customers, sample questionnaire, a microscope scoring data sheet, and a sample report.

This document excludes methods for automated scoring of CBMN.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
background frequency
background level
spontaneous yield (or number) of MN in BNCs recorded in control samples or individuals who are not abnormally exposed to genotoxins including ionizing radiation

3.2
binucleated cells
BNCs
cells that have completed one nuclear division after mitogen stimulation but have been blocked from performing *cytokinesis* (3.6) and are the cell type in which *micronuclei* (3.9) are scored in the CBMN assay

Note 1 to entry: These cells are accumulated in culture using cytochalasin-B which is an inhibitor of cytokinesis.

3.3
chromosome
structure that comprises discrete packages of DNA and proteins that carries genetic information which condense to form characteristically shaped bodies during nuclear division

3.4
confidence interval
statistical range about an estimated quantity within which the value of the quantity is expected to occur, with a specified probability

3.5
cytochalasin-B
Cyto-B
reagent used to block *cytokinesis* (3.6) in dividing cells allowing once-divided cells to be identified as binucleated cells

3.6
cytokinesis
physical process of cell division, which divides the cytoplasm of a parental cells into two daughter cells

3.7
dicentric
aberrant *chromosome* (3.3) bearing two centromeres derived from the joining of parts from two broken *chromosomes* (3.3)

Note 1 to entry: It is generally accompanied by an acentric fragment.

3.8
linear energy transfer
LET
quotient of dE/dl, as defined by the International Commission on Radiation Units and Measurements (ICRU), where dE is the average energy locally imparted to the medium by a charged particle of specific energy in traversing a distance of dl

3.9
micronucleus
MN
small nucleus that arises from lagging acentric *chromosome* (3.3) fragments or whole chromosomes during nuclear division and *chromosome* (3.3) segregation at mitosis during anaphase/telophase

Note 1 to entry: More that 90 % of the micronuclei induced by ionizing radiation arise from lagging acentric chromosome fragments.

3.10
non-refractile
process by which materials do not have the ability to refract or scatter light

3.11
precision
dispersion of measurements with respect to a measure of location or central tendency

3.12
quality assurance
planned and systematic actions necessary to provide adequate confidence that a process, measurement, or service has satisfied given requirements for quality

3.13
quality control
part of *quality assurance* (3.12) intended to verify that systems and components correspond to pre-determined requirements

3.14
service laboratory
laboratory performing biological dosimetry measurements

4 CMBN assay methodology used in this document

4.1 General

In this document, the frequency of MN in cultured human lymphocytes blocked in cytokinesis and scored by microscopy is used for dose estimation after suspected exposure to ionizing radiation.

Lymphocytes are cultured by a method that permits once-divided cytokinesis-block cells to be recognized by their binucleated appearance for analysis. This requires whole blood or isolated lymphocytes to be incubated in culture medium with a mitogen that would enable scoring of MN in first-generation BNCs. A cytokinesis blocking agent, cytochalasin-B, is added at least 6 h, i.e. approximately 24 h after the start of the culture, before the first mitosis commences in order to arrest dividing lymphocytes at the BNC stage after nuclear division is completed. The duration of the cell culture and the timing of addition of the arresting agent are optimised to ensure an adequate frequency of binucleated cells.

BNCs are recovered from the cultures by centrifugation, placing in a hypotonic salt solution and fixing in a mixture of methanol and acetic acid. Fixed cells are placed on microscope slides and stained. In the case of isolated lymphocytes, it is also acceptable to prepare slides by cytocentrifugation of cells onto slides, followed by air-drying, fixation with methanol, and staining. The exact protocol for cell culture, harvesting BNCs and staining employed by a CBMN assay laboratory should be formally documented.

Microscope slides containing stained cells are scanned to identify suitable BNCs. The frequency of MN observed in an appropriate number of scored BNCs is converted to an estimate of radiation dose by reference to calibration data.

4.2 Requests for analysis and blood sampling

Depending on national regulations, the request for an analysis should normally be made by a physician representing the patient, or the analysis could be requested by another authority due to legal claims. In all cases where it is normally possible, the blood sampling for MN analysis shall be made with the patient's informed consent. The laboratory head, depending on the national regulations, may be required to maintain the record of the patient's informed consent.

It is the responsibility of the medical staff (e.g. doctor, nurse, etc.) to schedule blood draw and shipping so as to ensure that the blood sample is received by the laboratory in the best possible conditions (see 13.2.4). The purpose is to avoid having the blood sample sit for several hours from time of blood draw and before sample pickup for transportation (see Clause 5 for details).

5 Responsibility of the requestor

This clause includes items that are not controlled by the service laboratory. Prior to blood sampling, an initial conversation between the requestor and the service laboratory should occur to co-ordinate the sample collection and shipment. Specific requirements regarding sample collection and shipment should be explained to the requestor including the approximate delivery time for the assay result(s). A standard

instruction sheet (illustrated in Annex B) explaining the requirements should be sent to the requestor. The requirements include:

a) Blood sampling shall use Vacutainer®[1)] tubes containing lithium or sodium heparin as the anticoagulant and the Vacutainer® tubes should either be sent or specified by the service laboratory.

b) Blood shall be collected (ideally about 5 ml), labelled accurately and unambiguously, maintained at room temperature (around 20 °C), and sent to the service laboratory as soon as possible.

c) Precautions shall be taken to ensure the integrity of the container to prevent leakage during shipment. Blood samples shall be kept at ambient temperature during shipment, i.e. 11° C to 30 °C. A temperature recording device shall be included to ensure that the temperature during shipment is controlled. Packaging and labelling shall conform to national and international regulations. If air transportation is involved, a physical dosimeter shall be included to monitor whether the sample was irradiated in transit.

d) A questionnaire (see Annex C) provided by the service laboratory shall be completed and returned prior to the start of blood culturing.

e) The laboratory shall be alerted of biologically contaminated and/or infectious samples so that extra precautions can be taken when handling the sample.

6 Responsibility of the service laboratory

6.1 Setup and sustainment of the quality assurance program

The service laboratory shall establish and maintain a quality assurance (QA) program (see Clause 13), which covers all aspects of the service. The laboratory's QA program shall address the following issues:

a) It shall include periodic internal checks of equipment operations, reagent suitability, and various performance checks, i.e. intra-laboratory comparison exercises, operator qualifications, sample protocol, scoring, dose estimations, report generation, etc.

b) It shall include periodic external checks of the laboratory's operations. The external audits shall include a review of the service laboratory's documentation of equipment operations, reagent suitability, and various performance checks, i.e. inter-comparison exercises, operator qualifications, sample transport integrity, etc.

6.2 Responsibility during service

The service laboratory shall provide necessary guidance, procedures, and timely reporting to provide dose assessment by the CBMN assay with a request for service. The service activities shall address the following issues:

a) The service laboratory shall have documentation, reviewed and endorsed by a qualified expert, i.e. service laboratory radiobiologist or equivalent, including the following:

   1) an instruction sheet to be sent to the customer describing shipping procedures (see Annex B);

   2) a questionnaire that shall elicit patient consent and all available information regarding the patient and the exposure scenario (see Annex C);

   3) step by step procedures for processing the blood sample from receipt of the sample to reporting of the dose;

b) The service laboratory is not responsible for sample transport; however, they should provide advice regarding sample transfer. If required, a kit for the collection of at least 5 ml whole blood in tubes containing lithium or sodium heparin as the anticoagulant shall be sent to the requestor with the

1) Vacutainer is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

appropriately labelled and addressed packaging material for the return of the sample to the service laboratory. The packaging shall conform to national and/or international regulations for the transit of potentially infectious pathological specimens (see 13.2.4).

c) After receipt of the blood sample, the following steps shall be performed:

   1) document the receipt of the blood sample (date, time, recipient);

   2) check for conformity of the sample (blood volume, integrity of the tubes);

   3) mark the blood sample with a unique code;

   4) store samples at room temperature and document the place of storage until the setting up of cultures;

   5) set up cultures in parallel as soon as possible and document date, time, and operator;

   6) document all the reagents used for culturing with appropriate lot numbers and expiry dates;

   7) document the addition of reagents and the end of the culture (date, time, operator);

   8) document the short- and long-term storage of the sample until slide making;

   9) document the slide codes, number of slides, and location of storage;

   10) document the results from scoring;

   11) store the slides and case documents in an appropriate place for possible medico-legal re-evaluation of the case;

d) The service laboratory shall interpret the results and prepare reports (see Annex D).

e) The service laboratory shall sustain a dialogue with and provide results to the requestor.

7 Confidentiality of personal information

7.1 Overview

Biological dosimetry investigations made by a service laboratory shall be undertaken in accordance with national regulations regarding confidentiality. This would normally include the maintenance of confidentiality of all of the patient's information including identity, medical data, etc. In addition, the commercial confidentiality of the patient's employer and any other organizations involved in a radiological or nuclear accident/incident should be observed.

This requirement extends to

a) written, electronic, or verbal communications between the laboratory and the person/organization requesting the analysis and receiving the report, and

b) the secure protection of confidential information held within the organization where the service laboratory is located.

7.2 Applications of the principle of confidentiality

7.2.1 Delegation of responsibilities within the laboratory

The head of the laboratory may authorize a limited number of laboratory staff to deal with documents related to the analysis. Persons with this authority shall have signed a commitment to confidentiality regarding their duties within the laboratory.

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

7.2.2 Requests for analysis

Depending on national regulations, the request for an analysis should normally be made by a physician representing the patient, or the analysis could be requested by another authority due to legal claims. In all cases, the blood sampling for CBMN analysis shall be made with the patient's informed consent. The laboratory head, depending on the national regulations, may be required to maintain the record of the patient's informed consent.

7.2.3 Transmission of confidential information

Whatever the chosen means of communication, confidentiality shall be ensured during the exchange of information and reports between the service laboratory and the requestor of the analysis.

The laboratory head shall define all processes for information transmission and assurance of confidentiality.

7.2.4 Anonymity of samples

The laboratory head shall have established protocols for maintaining the anonymity of samples. To avoid the identification of the patient while guaranteeing the traceability of the analysis, the blood samples should be coded upon arrival in the service laboratory. The coding should be performed in an unambiguous way according to a standard procedure. The same code is to be used for all the stages of analysis. The code is assigned by an authorized person, as defined in 7.2. Decoding, interpretation of results, and compiling the report are also to be performed by an authorized person.

7.2.5 Reporting of results

The final report containing the results and their interpretation (when needed) is communicated to the requestor of the analysis. Depending on national regulations, further copy may, with appropriate approval, be passed to other responsible persons.

7.2.6 Storage

The laboratory shall store the cultured cell pellet and slides to facilitate review/analysis by an external expert or another laboratory in the event of any dispute regarding the analysis.

The laboratory head shall define how fixed cells, slides, data and results are stored. Retention periods are defined by the laboratory head according to national regulations/policies for possible medico-legal re-evaluation of the case. All laboratory records relating to a case, which could permit the patient and/or employer to be identified, shall be stored in a place only accessible to the authorized persons. Final disposal of records shall be conducted by secure means such as shredding.

7.2.7 Data security plan

A data security plan should be established with written procedures for safeguarding data that contains personal identifiable information. This should include provisions for the storage of written and electronic data, results and reports in a secure location accessible only to authorized persons. A plan for secure disposal of data should also be included.

8 Laboratory safety requirements

8.1 Overview

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

8.2 Microbiological safety requirements

Handling human blood poses some risk of blood borne parasites and infections being transmitted to laboratory staff. Specimens shall be unpacked and manipulated in a class 2 microbiological safety cabinet to minimizing culture failure due to microbial contamination. Suitable disinfectants shall be available to deal with spills. All biological waste and used disposable plastic ware shall be sterilised, for example by autoclaving or incineration, before final disposal.

The legal and ethical position regarding pathogen testing of blood samples upon receipt differs between countries, and researchers should follow their national requirements. 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 pathogen negative.

8.3 Chemical safety requirements

Certain chemicals and pharmaceuticals are routinely used in the procedures covered in this document. When present in cultures or used in staining procedures they are mostly used in small volumes and in dilutions that generally present no health hazard. They are however prepared and stored in concentrated stock solutions. The main reagents of concern and their internationally agreed upon hazard statements (H-Statements) according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) are listed in Table 1. Note that commercially available products can vary depending on the physical form/quantity/composition. Always check the hazard and precautionary statements on safety data sheets available from suppliers for your own reagents.

Table 1 — List of reagents and corresponding hazard statement

Reagent	Hazard statement
Acridine orange	H302, H312, H319, H335, H340
Benzylpenicillin	H317 H334
Cytochalasin B	H300, H310, H330, H361
Glacial acetic acid	H226, H314
Giemsa stain	H225, H301, H311, H331, H370
Heparin	H315, H319, H334
Methanol	H225, H301, H311, H331, H370
Paraformaldehyde	H228, H302, H315, H317, H318, H332, H334

Table 1 *(continued)*

Reagent	Hazard statement
Phytohaemagglutinin	H302, H317, H332
Streptomycin sulfate	H302, H317, H332, H334, H361

Key
H225: highly flammable liquid and vapour
H226: flammable liquid and vapour
H228: flammable solid
H300: fatal if swallowed
H301: toxic if swallowed
H302: harmful if swallowed
H310: fatal in contact with skin
H311: toxic in contact with skin
H312: harmful in contact with skin
H314: causes severe skin burns and eye damage
H315: causes skin irritation
H317: can cause an allergic skin reaction
H318: causes serious eye damage
H319: causes serious eye irritation
H330: fatal if inhaled
H331: toxic if inhaled
H332: harmful if inhaled
H334: can cause allergy or asthma symptoms or breathing difficulties if inhaled
H335: can cause respiratory irritation
H340: can cause genetic defects
H361: suspected of damaging fertility or the unborn child
H370: causes damage to organs

8.4 Optical safety requirements

When ultraviolet lamps are used in sterilising the interior of microbiological safety cabinets, shielding and working procedures shall be in place to avoid direct irradiation of the skin or eyes of laboratory staff. When using microscope with fluorescent lamps, shielding shall be in place to avoid direct irradiation of the eyes.

8.5 Safety plan

The laboratory head or responsible person as defined by national regulations shall define written safety procedures for protection against microbiological, chemical, and optical hazards.

The laboratory head shall maintain a record of accidents and lessons learned and modify protocols or procedures to avoid repeating similar accidents.

Radiation protection considerations can also apply when the sample contains radioactive substances in significant quantities as defined by national radiation protection regulations.

9 Sample processing

9.1 Culturing

The protocol for the CBMN assay shall be established and documented by each service laboratory. The protocol used for the calibration curve and for dose estimates of patient samples shall be identical. There are several critical aspects that shall be adhered to:

a) for calibration curves, blood shall be incubated for approximately 2 h at 37 °C ± 1 °C immediately following irradiation and prior to culture;

b) cultures should be set up in duplicate to allow the determination of the intra-experimental coefficient of variation;

c) cells shall be cultured at 37 °C ± 1 °C either as whole blood or enriched lymphocyte suspension (buffy coat layer which includes a mixture of lymphocytes and monocytes) obtained by density gradient centrifugation (e.g. Ficoll-Paque, Lymfoprep);

d) culture vessels shall be sterile and handled in a way to avoid microbial contamination. Mostly, cells are cultured in round bottom cell culture tubes or T25 cell culture flasks. For lymphocyte microcultures multiwell plates can also be used[8];

e) specific culture media that allow peripheral blood lymphocytes to proliferate shall be used;

EXAMPLE RPMI-1640, Ham's F10, MEM, or McCoy supplemented with Foetal Bovine Serum (FBS) between 10 % and 20 %, 200 mM L-glutamine, and penicillin/streptomycin (100 IU·ml^{-1}/100 µg·ml^{-1}) is commonly used.

f) mitogen [e.g. phytohaemagglutinin (PHA)], at a concentration determined by the laboratory (e.g. 20 µg/ml), shall be added to the media to stimulate lymphocytes into mitosis;

g) cytochalasin-B (Cyto-B) shall be added, 24 h to 44 h after mitogen stimulation at a final concentration between 3,0 and 6,0 µg/ml to the cell culture to block cytokinesis in cells during their first nuclear division after mitogen stimulation;

h) the timing of harvest is crucial to maximize the number of BNCs and minimize the number of mononucleated and multinucleated cells. It shall be adapted according to the standard culture conditions for each service laboratory. The recommended culture time after mitogen stimulation for cell harvest is between 68 h to72 h but under certain conditions (e.g. where mitotic delay is anticipated due to old age and/or high radiation dose >6 Gy), longer culture time, use of mitotic delay inhibitors and additional CBMN assay biomarkers might be required to further optimise biological dosimetry. A modification of the CBMN assay to overcome mitotic delay is to use inhibitors of the G2/M and spindle checkpoints, such as caffeine and/or ZM447439 (an Aurora kinase inhibitor) respectively and a slightly delayed harvest time of 74 h to 78 h. This technique allows cells with high levels of DNA damage to complete nuclear division and be scored as BNCs in the CBMN assay and was shown to improve biological dosimetric accuracy up to 10 Gy from photons and 4 Gy of mixed neutrons-photons[9]. For high radiation dose cases additional biomarkers in the standard CBMN assay such as the average micronucleus frequency in micronucleus-positive BNCs has been shown to be a better biological dosimeter for low-LET radiation in the range of 5 Gy to 15 Gy compared to using MN frequency in BNCs[10];

i) after culture, cells are centrifuged in order to separate the cells from the medium. Thereafter, cells shall be treated with a hypotonic solution such as cold (4 °C) 0,075 M KCl to lyse red blood cells and/or swell the lymphocytes prior to fixation;

j) after the addition of hypotonic solution and centrifugation, the supernatant shall be removed and cells shall be fixed in freshly prepared cold fixative I, consisting of methanol:acetic acid:0,9 %NaCl (Ringer's solution) (ratio should be determined by the laboratory) while agitating the cells to prevent clump formation. Next, the cells are fixed with another three rounds of cold fresh fixative II (methanol:acetic acid) until the cell suspension is clear[11]. Additionally, formaldehyde (1 %) can be added to the first fixative; for isolated lymphocyte cultures, it is advisable to add the formaldehyde immediately after hypotonic treatment[12];

k) Optimally, slides should be prepared and stained within a few days after fixation. However, if storage of fixed cells is required, then cell suspensions shall be kept in the fridge or in a −20 °C freezer;

l) slides shall be prepared to ensure integrity of the cell membrane and allow an unambiguous identification of MN in BNCs. Humidity and temperature conditions can be adjusted to increase the quality of the spreading;

m) alternatively, when working with lymphocyte cultures, cells may also be transferred to slides by cyto-centrifugation and then fixed on the slide with absolute methanol after air drying [13].

9.2 Staining

Cells shall be stained appropriately so that nuclei and MN can be clearly visualized. Commonly used stains include, but are not limited to, Giemsa (for brightfield microscopy), and acridine orange (for fluorescence microscopy). DNA-specific stains such as DAPI (4′,6-diamidino-2-phenylindole) may also be used. The stain used shall be specific for nuclei and MN to avoid artefactual staining of other cellular structures that can resemble MN (e.g. centrioles). After staining, slides should be covered with a coverslip using an appropriate mounting medium.

9.3 Microscopy

Use a fluorescence or brightfield microscope depending on the stain used. Observation of cells at a magnification between ×400 and ×1 000 is required for reliable visual scoring of cells and MN. For computer-aided scoring an objective of ×10 magnification or greater can be used.

9.4 Scoring of slides

9.4.1 General

Each sample should be analysed by 2 scorers, each scoring 500 BNCs per slide per culture (for a total of at least 1 000 BNCs scored on 2 slides) for the presence of MN. Fewer BNCs can be scored for high dose samples or in triage mode (see Clause 12). The distribution of MN amongst the BNCs should also be recorded. The slide scorers should be experienced in the scoring of MN in lymphocytes (see 11.1.3).

9.4.2 Criteria for scoring

9.4.2.1 Criteria for selecting BNCs which can be scored for MN frequency

The cytokinesis-block cells that shall be scored for MN frequency should have the following characteristics:

a) the cells shall be binucleated (BNC);

b) the two nuclei in a BNC shall have intact nuclear membranes and be situated within the same cytoplasmic boundary;

c) the two nuclei in a BNC shall be approximately equal in size, staining pattern, and staining intensity;

d) the two main nuclei in a BNC 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;

e) the cytoplasmic boundary or membrane of a BNC shall be intact and clearly distinguishable from the cytoplasmic boundaries of adjacent cells.

9.4.2.2 Criteria for scoring MN

MN are morphologically identical to, but smaller than, the main nuclei. They also shall have the following characteristics:

a) the diameter of MN in human lymphocytes shall be smaller than 1/3 of the mean diameter of the main nuclei;

b) MN shall lie freely in the cytoplasm of the BNC and shall not be linked to the main nuclei by means of a stalk of nuclear material;

c) occasionally, MN may touch but shall not fully overlap the main nuclei and the micronuclear boundary shall be distinguishable from the nuclear boundary;

d) MN usually have the same staining intensity as the main nuclei but occasionally, staining may be less intense;

e) MN are non-refractile and can therefore be readily distinguished from artefacts such as staining particles.

9.4.3 Scoring data sheets

An example of a scoring data sheet is provided in Annex A.

9.5 Automated analysis

Several systems for automated image analysis for the CBMN assay have been developed. Automation at present is beyond the scope of this document.

10 Calibration source(s), calibration curve, and minimum detectable dose

10.1 Calibration source(s)

The service laboratory shall provide a report, reviewed and endorsed by a qualified expert, i.e. radiation physicist or the service laboratory head, that addresses the following issues:

a) description of radiation quality [e.g. X-ray machine with a 2,1 mm Cu half value layer (HVL), 250 kVp, filament current 12,5 mA, and a source-to-surface distance (SSD) of 50 cm] and irradiation set up for each radiation calibration source used to generate in vitro calibration curves;

b) characterization of the radiation calibration source(s) used to generate each in vitro calibration curve and traceability to a national/international radiation standard;

c) description of the dosimetry protocol, the procedure to certify that the dosimetry method is calibrated to a standard, the method used to measure dose uniformity in the experimental array, and the written procedures and documentation to verify dose and dose-rate determinations for individual experiments;

d) provision of a summary dosimetry report for each calibration-source dose-response curve.

For more details on irradiation parameters and conditions please refer to the IAEA manual [11].

10.2 Calibration curve

It is important to ensure that the interpretation of the results of a dose assessment using the CBMN assay takes into account the quality of the radiation in the case and the calibration source used to generate the dose-response calibration curve. The selection of the radiation sources for the calibration curve should reflect the most likely cases that are analysed. Typically, acute dose-rates of above 0,3 Gy/min should be chosen.

A CBMN assay calibration curve is required for each laboratory performing biological dosimetry using the CBMN assay. The same culturing conditions shall be used for establishing the calibration curve as used for analysis in a case of suspected unplanned exposure. The curve should be produced for at least three age groups separated into 1 to 25, 26 to 50, 51 to 80 years and include at least 1 individual (preferably more) of each sex in each group with the same number of BNCs being enumerated from each donor. The selection of the calibration dose range depends on the radiation quality. In the case of low-LET radiation, more than seven doses shall be selected, distributed equally among the linear and quadratic component of the dose response curve. The typical doses for a low-LET calibration curve should include at least 0 Gy, 0,1 Gy, 0,2 Gy, 0,5 Gy, 1 Gy, 2 Gy, 3 Gy, and 4 Gy. Any substantial deviation from this dose range shall be justified. The inclusion of 0 Gy data, i.e. data from blood samples unexposed to ionizing radiation, in the calibration dose-response curve is important because it allows the intercept to be determined and takes account of the effect of age and sex on base-line MN frequencies[4].

For doses less than or equal to 0,5 Gy, at least 2 000 BNCs should be scored for MN frequency per dose for each donor and for doses higher than 0,5 Gy, at least 1 000 BNCs should be scored per dose for each donor.

The following definitions are used for further calculations:

— y: observed MN: The absolute number of MN observed in n scored BNCs;

— μ: observed yield of MN: The observed number of MN per BNC.

Observed frequencies of MN in BNCs should be fitted to the linear or linear-quadratic models as shown in Formula (1). For most high-LET radiation types, a linear model should be more appropriate.

$$\mu = C\left(\pm S_{\mathrm{E},C}\right) + \alpha\left(\pm S_{\mathrm{E},\alpha}\right)D + \beta\left(\pm S_{\mathrm{E},\beta}\right)D^2 \quad (1)$$

where

C is the population background;

α and β are the linear and quadratic equation coefficients;

D is the absorbed dose to tissue in Gy;

S_{E} is the standard error of the mean for each coefficient and constant.

For curve fitting, iteratively reweighted least squares should be used. For overdispersed data, the weights shall consider the overdispersion. When the obtained value of chi-squared is higher than the degrees of freedom, standard errors should be increased by (chi-squared/degree of freedom)$^{1/2}$ or by using generalized linear models with distributions accounting for overdispersion (e.g. negative binomial).

The effect of age, sex and donor shall be assessed and appropriately accounted for the estimation of standard errors on the curve, if required (e.g. by including age or sex as parameters in Formula (1) or by estimating calibration curves stratified by age and/or sex).

The service laboratory shall provide a report on the calibration source, dose range, and dose-response curve and it shall be reviewed and endorsed by a qualified expert, i.e. service laboratory radiobiologist or equivalent, that addresses the following issues:

a) full description of radiation quality [e.g. X-ray machine with a 2,1 mm Cu half value layer (HVL), 250 kVp, filament current 12,5 mA, and a source-to-surface distance (SSD) of 50 cm] and irradiation set up for each radiation calibration source used to generate in vitro calibration curves;

b) characterization of the radiation calibration source(s) used to generate each in vitro calibration curve and traceability to a national/international radiation standard;

c) description of the dosimetry protocol including details of the quantity that has been measured, the procedure to certify that the dosimetry method is calibrated to a standard, the method used to measure dose uniformity in the experimental array, and the written procedures and documentation to verify dose and dose-rate determinations for individual experiments;

d) provision of a summary dosimetry report for each calibration-source dose-response curve.

For more details on irradiation parameters and conditions please refer to the IAEA manual[11].

10.3 Background MN frequency

It has been well established that the background MN frequency in individuals varies with age and sex and also due to various confounding factors, i.e. nutritional status, genotoxic exposures, lifestyle factors, mis-segregation of sex chromosomes[11]. For the purpose of radiation biological dosimetry using MN frequency in lymphocytes, it is assumed that an individual's base-line MN frequency value prior to the ionizing radiation exposure event is equivalent to the mean MN base-line value obtained across different age groups. Ideally each laboratory collects its own set of background MN frequency data, and this data should include a sufficient number of individuals from each sex in each of several age groups (e.g. 1 to 25, 26 to 50, 51 to 80 years) to be representative for this population. This depends on the characteristics of the specific population

(age, sex and exposure to mutagens) and the exposure scenario. As such, this should be determined by the head of the laboratory with input from a statistician.

10.4 Comparison with the background level: Characterisation of the minimum detectable dose

In ISO 11929-1[14], two characteristic limits are defined:

— the decision threshold, y_d, or minimum resolvable number of MN, which is the threshold above which the physical effect is assumed to be quantifiably present. In other words, the decision threshold can be understood as the minimum number of MN that is significantly different from the expected background number of MN for a given significance level α (e.g. $\alpha = 0{,}05$);

— the detection limit, y_z, is the smallest true value of the measurand (number of MN), which can be detected. It gives information on the practical operating range of the assay and is thus used to assess whether the measurement procedure is appropriate in a particular set of circumstances.

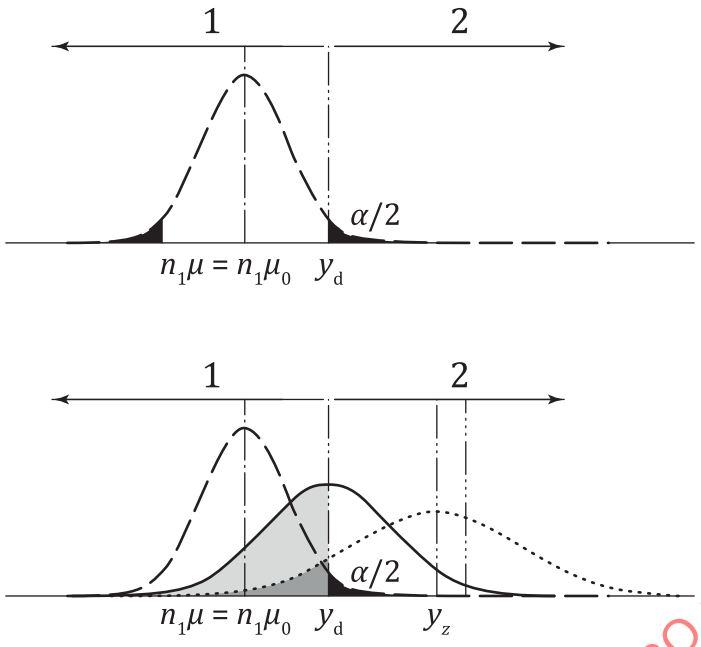
The decision threshold and the detection limit should be calculated before the measurement is performed.

Table 2 — Error rates for the inference of positive and negative conclusions

		Inferred conclusion	
		Test person exposed H_0 rejected	Test person not exposed H_0 accepted
True state	Test person exposed H_0 false	True positive	Type 2 error (β) False negative
	Test person not exposed H_0 true	Type 1 error $\frac{\alpha}{2}$ False positive	True negative

If a test person shows a number of MN greater than the decision threshold, y_d, it can be concluded that the patient was exposed and a dose estimate should be made. In other words, the null hypothesis, H_0, that the test person was not exposed or that the observed MN yield is equal to the background MN yield, can be rejected with a type 1 error rate $\frac{\alpha}{2}$. However, an important parameter to consider is the fact that the dose value of zero should not be present within the 95 % confidence interval formed from the dose-response curve used. If this were to be the case, one could not conclude that the person has been exposed. If the observed number of MN, y, is below the decision threshold, y_d, it is decided that it cannot be concluded that the person was exposed. Nevertheless, depending on the unknown true state (see Table 2) the probability for a false negative result can be relatively high and, therefore, it cannot be concluded that the person was not exposed (see Figure 1).

In cases where the observed number of MN is below the decision threshold, the detection limit shall be reported. The detection limit provides valuable information about the true dose (or number of MN) above which fewer than 100 β % false negative results are expected. The detection limit can be interpreted in the following way: Given the true state that a test person was exposed (see Table 2), if the true dose or number of MN of the test person was higher than the detection limit, the probability of observing a number of MN lower than the decision threshold and thus inferring a false negative conclusion would be less than β.



Key

1	H_0 accepted	———	sampling distribution under H_1 (mean at y_d)
2	H_0 rejected	·····	sampling distribution under H_1 (mean at y_z)
— —	sampling distribution under H_0	—·····—	detection limit y_z
—··—	decision threshold y_d		

Figure 1 — Illustration of the decision threshold and the detection limit (μ_0 = *constant*)

Top panel: Given the null hypothesis H_0 (test person not exposed) is true, i.e. MN yield, μ, of the test person is equal to the background MN yield, μ_0, the probability for wrongly rejecting H_0 or for observing a MN number that exceeds the decision threshold, y_d, is less than $\frac{\alpha}{2}$.

Bottom panel: Given that the alternative hypothesis H_1 (test person exposed) is true, if the true dose or the true number of MN, respectively, is equal to the decision threshold, y_d, the probability for wrongly accepting H_0 is approximately 0,5 (light grey area under the curve). If the true dose or the true number of MN, respectively, is greater than the detection limit, the probability for wrongly accepting H_0 is lower than a predefined type 2 error rate β (e.g. β = 0,1).

To define the decision threshold, it is assumed that the test person has not been exposed, i.e. the MN yield of the test person μ is equal to the background MN yield $\mu_0(j)$ for a person of j years old. The null hypothesis is given by $\mu = \mu_0(j)$ [or $n\mu = n\mu_0(j)$], respectively. Assuming that the background MN yield is constant, i.e. has no uncertainty, and that the number of MN follows a Negative Binomial distribution of type I with dispersion parameter τ, the decision threshold, y_d, can be defined by the following probability:

$$P(y > y_d | n_1\mu = n_1\mu_0(j), \tau) = \sum_{k=y_d+1}^{\infty} \frac{\Gamma\left(k + \frac{n_1\mu_0(j)}{\tau}\right)\tau^k}{\Gamma\left(\frac{n_1\mu_0(j)}{\tau}\right)\Gamma(k+1)(1+\tau)^{k+\frac{n_1\mu_0(j)}{\tau}}} = \tag{2}$$

$$1 - \sum_{k=0}^{y_d} \frac{\Gamma(k + n_1\mu_0(j)/\tau)\tau^k}{\Gamma(n_1\mu_0(j)/\tau)\Gamma(k+1)(1+\tau)^{k+n_1\mu_0(j)/\tau}} \leq \frac{\alpha}{2}$$

where

y is the observed number of MN;

y_d is the decision threshold;

μ_0 is the expected background yield of MN;

j is the age of the individual in years

Γ is the gamma function, with $\Gamma(n)=(n-1)!$ For positive integer numbers n or $\Gamma(z)=\int_0^\infty t^{z-1}e^{-t}dt$ for positive real numbers z.

n_1 is the number of cells scored for the test person;

τ Is the dispersion parameter of the Negative Binomial distribution of type I;

α is the probability for a type 1 (false positive) error, which is generally taken to be 5 %.

Again, assuming that the number of MN of the test person follows a Negative Binomial distribution of type I, the detection limit for the number of MN, y_z, is defined by the Formula (3):

$$P(y \le y_d | n_1 \mu = y_z, \tau) = \sum_{k=0}^{y_d} \frac{\Gamma(k + y_z/\tau)\tau^k}{\Gamma(y_z/\tau)\Gamma(k+1)(1+\tau)^{k+y_z/\tau}} = \beta \quad (3)$$

The latter (Formula 3) can be solved iteratively (see example in Annex F)

where β is the type 2 (false negative) error, which would generally be between 5 % and 20 %;

A detailed example for the calculation of the decision threshold and detection limit can be found in Annex F.

For the CBMN assay, the decision threshold and detection limit are a function of number of factors including the laboratory's appropriate measured or chosen control background levels of MN, and the number of cells chosen for analysis. Therefore, the detection limit and decision threshold shall be assessed on a case-by-case basis. The detection limit can be converted to a 'minimum detectable dose' by application of the calibration curve, as shown in Annex F. However, in all cases, the minimum detectable dose cannot be lower than the lowest dose used in the appropriate calibration curve.

If the observed number of MN exceeds the decision threshold, this means that there is evidence to refute the null hypothesis of no significant difference between the background and observed numbers of MN. The calculation of observed absorbed dose should then be carried out and reported according to 11.2.4. If not, then the detection limit should be reported.

If the true number of MN is higher than the detection limit (or the true dose is higher than the minimum detectable dose), the probability of observing a lower number of MN than the decision threshold (or the probability to infer false negative conclusions) is lower than β.

11 Accidental exposure involving few individuals

11.1 Procedure for scoring MN in BNCs

11.1.1 Coding of samples and slides

All samples, slides, and intra-laboratory or inter-laboratory validation standards shall be coded. Complete records of coding shall be maintained.

11.1.2 Scoring techniques

The laboratory head shall establish and implement procedures for the scoring techniques used. When scoring is at least partially performed with computer assisted image analysis, the system used should have been previously subjected to quality assurance trials with results documented.

Methodical scanning of slides is crucial to ensure the scoring of any given cell only once without duplication.

Criteria for scoring should be followed according to 9.4.2.

11.1.3 Laboratory scoring expertise

Analyses shall be conducted by trained and experienced scorers fully familiar with the scoring of MN in BNCs and the handling of software tools used in biological dosimetry. Documentation validating their expertise shall be maintained.

The laboratory head is responsible for maintaining the scoring criteria and the qualifications of the individual scorers. All scorers shall participate in intra-laboratory and inter-laboratory comparisons regularly.

A scorer is regarded as qualified, if the 95 % confidence intervals of the doses estimated based on the MN yield of the test sample and on the calibration curve of the lab, include the reference doses.

See below additional details for the assessment of scoring expertise by performance checks through laboratory inter-comparison studies (see 13.2.2) and periodical checks of individual scorers (see 13.2.3).

11.2 Criteria for converting a MN yield into an estimate of absorbed dose

11.2.1 Overview

The measured MN frequency is converted to absorbed dose by reference to an appropriate in vitro calibration curve produced in the same laboratory with radiation of comparable quality with regards to dose rate and linear energy transfer. This provides an estimate of the mean whole-body dose. In conventional cases, at least 1 000 BNCs should be scored from the case specimen, unless the MN yield is high (e.g. >1 MN per cell), in which case, it is not necessary to proceed beyond scoring enough BNCs to observe 500 MN. In the special case where there exists a high yield of MN but insufficient BNCs to observe 500 MN, the dose can be reported after observing 250 MN.

11.2.2 Comparison with controls

The service laboratory shall provide in case reports the laboratory's background MN frequency for the relevant age and sex group for the sample being tested. If the measured MN frequency is not statistically significantly different from the background frequency, the best estimate of dose should be quoted as zero with its upper confidence limit. If the measured MN frequency is statistically significantly higher than the background level, i.e. higher than the decision threshold, then a dose estimate with its uncertainties shall be derived and reported (see 10.3 and 10.4).

11.2.3 Confidence limits on the number of MN

For the derivation of confidence intervals for the yield of MN, overdispersion with respect to Poisson shall be taken into account, by increasing the Poisson-derived uncertainty by the square root of the ratio of variance to mean. Alternatively, more appropriate models to describe the distribution of MN can be used, such as the Negative Binomial, in order to consider both the mean yield of MN and the dispersion coefficient. The laboratory shall provide justification for and validation of the chosen model(s), with input from a qualified statistician as required.

11.2.4 Calculation of absorbed dose for whole-body exposures

If the observed excess number of MN satisfactorily exceeds the decision threshold as defined above, then the absorbed dose should be calculated by comparison with an appropriate calibration curve, created as

described in 10.2 as defined by Formula (4). It shall be ensured that the data used for the estimation of the calibration curve is representative for the test person and that confounding factors (e.g. age and sex) have been accounted for appropriately. Ideally, the service laboratory has curves for different age and sex groups and should use a curve matching the age and sex of the test person as closely as possible. Alternatively, if age or sex were included as a parameter in the model for curve fitting, the formula for dose estimation shall be adjusted accordingly. The absorbed dose, D, is calculated by solving the linear or quadratic equations, which is possible using freely or commercially available software tools. The information on how the calculations are performed within the software should be documented and updates performed periodically.

The absorbed dose can be obtained by solving Formula (4):

$$D = \frac{-\alpha + \sqrt{\alpha^2 + 4\beta(\mu - C)}}{2\beta} \tag{4}$$

where

C, α, β are the coefficients of the calibration curve;

μ is the observed MN yield of the test person.

11.2.5 Calculation of uncertainty on absorbed dose

The resulting estimated absorbed dose represents the best estimate possible given the associated variability, which arises from the experimental and intrinsic uncertainties. Therefore, the measurement uncertainty shall be estimated using appropriate methods.

The general procedure for assessing uncertainty relies on formal combination of all the sources of experimental uncertainty according to standard methods of error propagation (ISO/IEC Guide 98-3)[15]. In brief, the relationship between the absorbed dose and the input quantities should first be clearly defined, the sources of uncertainty relevant to the particular case should be identified and quantified and then the combined uncertainty should be calculated (ISO 5725-1)[16].

In practice, the recommended methodology to calculate uncertainty on absorbed dose in the context of the CBMN assay is to combine the confidence limits on the MN frequency with the uncertainties on the calibration curve coefficients. In many cases, these represent the dominant sources of uncertainty. This can be achieved by using Merkle's method[17]. If the MN data shows signs of overdispersion, this shall be included for the estimation of uncertainties of the curve as well as for the MN frequencies of the test person by using appropriate distributional assumptions. Additional parameters, such as donor effects, scorer effects or experimental conditions can also increase the uncertainty and should be accounted for if necessary.

In all cases, the laboratory head shall define the methods used to determine the confidence limits on dose. The service laboratory shall report the method used to determine the standard uncertainty (the standard deviation) and the expanded uncertainty (which gives the 95 % confidence interval). The laboratory should also retain records of the uncertainty budget (a list of the uncertainty components and how they were evaluated) together with details of any systematic uncertainties accounted for and all other corrections and constants employed[15].

11.2.6 Acute and non-acute exposure cases

If an exposure is known to have been received acutely, i.e. within 0,5 h, the dose estimate may be obtained by reference to an acute in vitro calibration curve. If an exposure is known to have been protracted beyond 24 h, the dose estimate may be obtained by reference to just the background level and linear coefficients of the acute calibration curve. For exposures of 0,5 h to 24 h, if available, the measured yield may be interpreted from an appropriate non-acute calibration curve. Alternatively, the full acute curve may be used but with a reduction of the dose-squared coefficient. This may be calculated by the G-function method.

NOTE Further explanations of the G-function can be found in the IAEA technical reports.

If an exposure is known to have been intermittent, its individual fractions may be assumed to be independent i.e. their effects are additive, if the interaction interval is greater than 5 h. If less than 5 h, an interaction factor should be estimated using a 2 h time constant.

The service laboratory shall state in the result reports the method used to correct for non-acute exposure dose estimates and, when appropriate, also justify its assumptions.

11.2.7 Testing the distribution of MN per BNC

The degree of overdispersion for the distribution of MN amongst BNCs should be tested. This should be done by the *u* test which is a normalized unit of the dispersion index $\delta = s^2/\mu$ (variance/mean), as shown in Formula (5). For a Poisson distribution δ should be unity, u values higher than 1,96 indicate an overdispersion (significance level, α = 0,025). The degree of overdispersion can give an indication of inhomogeneity of exposure, however, the use of this approach for partial body exposure estimation using the CBMN assay needs further investigation. Nevertheless, if the laboratory decides to use this approach, all conclusions shall be carefully documented and justified.

$$u = (\delta - 1) \times \sqrt{\frac{N-1}{\left[2 \times \left(1 - \frac{1}{y}\right)\right]}} \tag{5}$$

where

N is the number of BNCs analysed;

y is the number of MN detected.

11.2.8 Other exposure scenarios

When there is a high degree of uncertainty in the conversion of the measured yield to dose, calculation of the dose may not be prudent. In this case, it is possible to use the comparison of radiation induced frequency of MN to the background to give an indication of whether or not an exposure has occurred (e.g. in the case of internal exposures).

Examples of several different exposure scenarios in which the CBMN assay has been applied can be found in the literature[18][20].

11.3 Reporting of results

11.3.1 General

Routinely, the report shall contain the MN frequency per BNC and its interpretation based on the current understanding of mechanisms for radiation-induced MN formation. Relevant information provided by the customer (e.g. circumstances of suspected exposure, independent measure of dose, etc.) shall be reported since this can influence the interpretation of the findings in the service laboratory.

The report should be subdivided into the following sections.

11.3.2 Content of the report (see Annex D for a standard form)

The report shall include information on the following points:

a) title of the report, i.e. “test report”;

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

c) identification of the report by a unique number, i.e. a specific document number provided by the institutional registry;

d) name and address of the customer, date of order;

e) 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;

f) unambiguous identification of the sample, i.e. name, internal code, and date of birth of the exposed subject;

g) description of the case: all information provided by the customer that is relevant to the interpretation of the result shall be stated (can also be dealt with in the interpretation of the results);

h) date and location of blood sampling, date of sample arrival in the laboratory, date of setting up cultures (if different), and date of completed analysis;

i) assay results: number of BNCs scored, number of MN found, distribution of MN amongst BNCs;

j) interpretation of test results (see 11.3.3);

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

11.3.3 Interpretation of the results

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

a) dose estimate based on the frequency of MN, expressed in SI units of absorbed dose (Gy);

b) statement on the likelihood that any MN used in dose estimation relate to this particular radiological incident;

c) the MN background frequency of the laboratory and the coefficients of the calibration curve used for converting the dose from the MN frequency;

d) 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;

e) statement on whether the dose estimate was made assuming acute or protracted irradiation and, if the latter, how protraction had been accounted for;

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

g) summary of the essential key elements from the points above. This would normally include the best estimate of dose based on the cytogenetic findings;

h) at the end of the report: an invitation for the customer to contact the laboratory if he/she requires further clarification or explanation about the results and/or the assay.

12 Population triage

12.1 General

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

The CBMN assay in triage mode evaluates and assesses approximately and rapidly radiation doses received by individuals in order to supplement the clinical categorization of casualties.

12.2 Use of a CBMN assay network for large scale exposures

To deal with mass casualty scenarios, cytogenetic biological dosimetry networks can be established consisting of a reference laboratory supplemented by satellite laboratories, either nationally or

internationally. ISO 21243[2] addresses the establishment of cytogenetic networks for the dicentric assay. This document can equally be applied to the CBMN assay.

12.3 Procedure for scoring MN in BNCs

The same procedure as that described in 11.1 should be used with modifications as follows.

In triage mode, if only individuals exposed to at least 1 Gy of radiation need to be identified rapidly and there is an overwhelming number of subjects to be tested, scoring of a minimum of 200 BNCs is sufficient, unless MN yield is high in which case, it is adequate to score enough BNCs to observe 200 MN. In the special case where there exists a high abundance of MN but few BNCs, the dose can be reported after observing 100 MN.

12.4 Criteria for converting a MN yield into an estimate of absorbed dose

The same criteria as described in 11.2 should be used with the following exceptions:

a) only a maximum of 200 BNCs shall be scored, unless 200 MN are observed in a smaller number of BNCs;

b) a single average background level of MN should be used as a control as described in the first paragraph of 11.2.2. Age matched background values are not required but can be used if available.

12.5 Reporting of results

The report should include as much content from 11.3.2 as possible. However, multiple samples can be tabulated in the same report if appropriate (see Annex E for group sample report).

13 Quality assurance and quality control

13.1 Overview

Quality management shall ensure a continued improvement of operations. This document requires that the quality assurance (QA) and quality control (QC) procedures required by ISO/IEC 17025 shall be followed in each laboratory[6]. This document defines quality assurance and quality control procedures specific for laboratories performing biological dosimetry by cytogenetics.

13.2 Specific requirement

13.2.1 General

QA and QC programs comparing the proficiency and competency with other certified or suitably qualified cytogenetic biological dosimetry laboratories shall be established and these evaluations have to be performed at regular intervals. In most instances, either annual or bi-annual assessments are performed and the results recorded.

ISO 5725 (all parts)[7] is dedicated to statistical analysis to test the reliability and the precision of a technique. Each test proposed requires a minimum number of samples.

13.2.2 Performance checks by laboratory inter-comparisons

Proficiency tests are essential tools for the quality assurance of the laboratories as they constitute an objective evaluation of its performance, from both human and technical viewpoints.

Variations in assessment values of individual laboratories that appear to be inconsistent with all other laboratories may change the outcome of assay results (dose estimates). To discard or correct inconsistent values, two approaches can be used (ISO 5725-2[19]/ISO 5725-5[21]):

a) numerical outlier tests (e.g. Cochran and Grubbs tests): To discard data that after a statistic test exceeds the critical value of the test at the 1 % significance level;

b) robust methods for data analysis: To yield robust values of the average and standard deviation of the data.

The procedure is as follows:

c) the outlier test for laboratory inter-laboratory comparison performance requires a minimum of five laboratories for statistical robustness (ISO 5725-1[16]);

d) estimation of the mean value and the standard deviation once the inter-laboratory outliers are discarded or corrected. The preferred method is the calculation of the robust parameters;

e) determination of the laboratory's performance by calculating z-score parameter from the laboratory results, the reference value, and the estimated standard deviation. To determine u-score parameter (this evaluation includes both participant measurements and reference value uncertainties).

13.2.3 Periodical performance check of scorer qualification

A list of qualified scorers is established at least every second year by intra-comparison of laboratory personnel.

To be qualified, each scorer shall score a sample of lymphocytes exposed to a radiation dose above 1 Gy (acute photons) and a sample of lymphocytes exposed to a radiation dose below 1 Gy (acute photons) according to the standard practice of the laboratory. Scoring of 500 cells is preferable depending on the practice of each service laboratory.

A laboratory scorer is regarded as qualified if the measured MN yield in BNCs is within the Poisson 95 % confidence limits of the test reference yield expected from the laboratory's calibration curve. For example, a scorer finds that his/her measured yield in a test sample is 48 MN in 1 000 cells (95 % LCL: 0,035, UCL: 0,063) for a reference level test and agrees with the laboratory's calibration curve that predicts a yield of 0,05 MN per BNC.

13.2.4 Performance checks of sample transport integrity

In many cases, blood collection occurs at sites distant from the service laboratories and therefore transportation is necessary. The requestor is responsible for assuring the blood samples are transported in optimal temperature conditions (from 18 °C up to 30 °C). Temperature control should also be maintained using appropriate packaging material and/or tracking system. When air transportation is used, the X-irradiation at the security checkpoints should be avoided. A physical dosimeter may be included in the shipping package to verify whether or not the sample is exposed to X-rays. For international transport, the appropriate permits shall be obtained in advance and included in the shipment to avoid delays at customs including declaration about the biosafety of samples. For international shipments a Pro Forma or commercial invoice is also required. All details concerning blood collection and storage should be recorded.

13.2.5 Performance checks of sample integrity by service laboratory

A system for recording the collection, transport, and storage of the blood samples shall be established so that sample integrity is guaranteed. The use of barcoded samples (a barcode can be assigned for the sample(s) either by the requestor or by the designated service laboratory) is critical to maintain impartiality while scoring. If possible, chain of custody can be established for sample tracking.

13.2.6 Performance checks for instrumentation

Performance of all the equipment used for the assay shall be checked and evaluated at regular intervals while the equipment is in use.

Examples of critical equipment include incubators, water baths, weighing machines, thermometers, pipettes, centrifuges and freezers.

For example, the stability of the temperature control and humidity of the incubators has to be monitored and registered in a logbook on a daily basis. If used, the weighing machine has to be checked periodically

for accuracy. These regular checks shall be sufficient to demonstrate that the assay equipment is properly calibrated and that all the components are functioning properly.

13.2.7 Performance checks of sample protocol

As an internal quality assurance, negative controls from unexposed individuals and, where possible, internal positive controls should be included in the study to prove the reliability of the procedure. Blood from both exposed and unexposed individuals shall be handled in the same manner. The samples of both groups (exposed and unexposed) have to be taken concurrently and not successively.

For the interpretation of results, it can be useful to prepare a slide for differential count from each blood sample before starting the cultures to determine whether lymphocyte counts are in the normal range. The culture, fixation, and staining procedures shall be described in detail. It is recommended that the same lot of media and reagents be used throughout the study. The expiry dates for growth media and reagents need to be checked prior to their use. The composition of all reagents shall be described as accurately as possible. A quality check for lymphocyte proliferation may be required if different batches of serum are used.

13.2.8 Performance checks of sample scoring

Uniform criteria for scoring shall be used. Scoring shall be performed by trained and experienced scorers. If different scorers are involved, a balanced scoring design shall be used. Each scorer should analyse the same number of BNCs from the same slides of all the subjects rather than different scorers analysing all the cells from different subjects. Cross-validation of scoring results is required. The identity of the scorer of the slides shall be recorded.

13.2.9 Performance checks of dose and confidence limits estimation

Non-parametric tests should be used for univariate statistical analysis. The confidence interval of the exposure has to be calculated from the uncertainty on the MN yields and the variation of the dose-response relationship among individuals, typically determined in a prior study. The dose-response relationship used for chronic and acute exposures has to be appropriate. The results of the negative and positive internal quality assurance controls are used to demonstrate the reliability of the methodology and scoring.

13.2.10 Performance checks for result report generation

The test reports to requestors (preferably physicians who order the cytogenetic biological dosimetry testing) shall be prepared in a manner to ensure that they contain the necessary information defined in this document (see 11.3), namely: subject and requestor identifiers, exposure information, exposure and sampling dates, scoring results, interpretation of the results in terms of dose and its uncertainty, and information on how these were derived.

Annex A
(informative)

Sample data sheet for recording MN in BNCs

Sample No./Slide Code:

Microscope No.:

Scorer (Last Name, First Name):

Date (Day/Month/Year):

Slide No.	Micronucleus distribution in BNCs							Total No. of BNCs	Total No. of MN
	0 MN	1 MN	2 MN	3 MN	4 MN	5 MN	>5 MN		
1	468	27	4	1	0	0	0	500	38
2	472	26	2	0	0	0	0	500	30
1 + 2	940	53	6	1	0	0	0	1 000	68
Remarks:									

MN: micronucleus

BNCs: binucleated cells

NOTE The numbers in the table are only given as an example.

Annex B
(informative)

Instructions for requestor (sample)

PROCEDURES FOR COLLECTING BLOOD FOR MICRONUCLEUS ANALYSIS

Analysis of micronucleus yield in human peripheral lymphocytes blood is an alternative to dicentric analysis for the biological assessment of radiation exposure. It is similarly used when a person's physical dosimeter is absent or inoperative or when the reading of the physical dosimeter is missing or disputed. To optimize the recovery of lymphocytes from the blood, it is very important that the blood be collected and shipped according to the following protocol:

— Notify laboratory before the blood sample so that we can prepare for its arrival and pick up.

— Collect about 5 ml of blood into **lithium or sodium heparin tubes**. Gently rock the tubes for 2 min to ensure proper mixing. Label the tubes unambiguously and complete the questionnaire.

— Package the blood sample carefully to prevent breakage of the tubes in transit.

  — Blood samples should be packaged to avoid extreme heating or cooling (18 °C up to 30 °C). One method of maintaining blood at room temperature is to place the tubes on a gel pack that has been allowed to stay at room temperature for several hours to ensure that the samples do not freeze during transportation. If temperature extremes are likely to be encountered, a minimum-maximum thermometer should be included in the package. **Blood samples shall not be frozen**.

— Mark on the external packaging and the shipping documents **Biological substances- Category B- DO NOT FREEZE**

— Avoid X-irradiation at the security checkpoints when air transportation is used. A physical dosimeter should be included in the shipping package to verify this. For international transport, the appropriate permits shall be obtained in advance and included in the shipment to avoid delays at customs. For air transport, packaging and labelling should conform to the current International Air Transport Association (IATA) regulations. These require that blood samples should be packed to conform to UN 3373 infectious materials. The package itself and the 'Nature and Quantity of Goods' box of the air waybill should show the following wording: "Biological Substances – Category B packed in compliance with IATA packing instruction 650".

— Mark the package and shipping documents DO NOT X-RAY.

— Ship the sample immediately after blood collection, by **special transportation** using **overnight air express so that we can receive the blood early in the morning following sample collection.**

— Contact the laboratory to confirm the shipment and inform us of the **waybill** number. THIS IS IMPORTANT FOR TRACKING THE SAMPLE.

— For best results blood should be received within 24 h of sampling. All details concerning blood collection and storage should be recorded.

(Service laboratory Head)

(Service laboratory address)

Phone: (XXX) XXX-XXX

Fax: (XXX) XXX-XXX

E-mail:

Annex C
(informative)

Sample questionnaire

Exposure Information for Micronucleus Analysis

[TO BE FILLED OUT BY THE SUBJECT or representative (e.g. the medical doctor in charge)]

I, _____ (Name), born _____(dd/mm/yy) consent to giving a blood sample for the purpose of estimating the radiation exposure dose by measuring the micronucleus frequency in lymphocytes.

_____
Signature

_____

Blood sample taken by: _____ Laboratory name: _____

Laboratory address: _____

Telephone #: _____ Fax: _____ E-mail: _____

Date and time blood sample taken: _____ (dd/mm/yy)

Exposure Data: _____ Radiation worker_____ or Non-Radiation Worker

1. Date and time of exposure: _____ (dd/mm/yy - time)

2. Place: _____ Company: _____

3. Brief description of exposure:

4. Whole-body exposure	☐	Partial body exposure	☐	Internal contamination	☐
Dose value: _____		Part of body: _____		Nuclide	
		Dose value: _____		Dose value:	
Acute	☐	Fractionated	☐	Protracted	☐

How is this dose value obtained?

5. Type of radiation:

X-ray	☐	kV
γ	☐	nuclide?
α	☐	nuclide?
Neutrons	☐	source

Patient Data:

1. Patient sex: ☐ Male ☐ Female
2. Previous exposure through medical practice:

Radiation therapy ☐ Date, part of body _____

X-ray diagnoses ☐ Date, part of body _____

Nuclear medicine ☐ Date, part of body _____

3. Illness within the last 4 weeks before taking the blood sample: _____
4. Intake of medication: ☐

Name of medication: _____ Dose: _____ Date and duration: _____

5. Smoker: no: ☐ yes: ☐ number/day: _____
6. Known diseases:

HIV ☐ Hepatitis ☐

_____

Results of micronucleus analysis to be sent to:

Name: _____

Address: _____

_____

Telephone #: _____

Annex D
(informative)

Sample of report for single assessment

Laboratory name, address

Contact data

Test report of biological dose assessment/CBMN assay (report form should be in accordance with the requirements of ISO 17025[6])

ID No of report (unambiguous) and date of issue of report:

Requestor name, contact data:

Date of request:

Sample received

Sample	Sampling date/location Arrival date	Condition of a sample
Code, name and date of birth of exposed subject		

Description of case

Method(s) of analysis: CBMN assay according to Standard Procedure no. issued (date) in compliance with ISO 17099. The dose is estimated using calibration curve for (type of radiation).

Date of beginning of cell culture and micronucleus frequency analysis

All tests followed a standard method and there were no environmental conditions which may bear upon the results (any deviations shall be described in the report).

Results

Sample code	No. of binucleated cells analysed	No. of micronuclei

Dose assessment with units and uncertainties (The dose is estimated using a calibration curve for type of radiation)

Results reported by
Position, name
Date, Signature

Dose assessment by
position, name
date, signature

Approved by
position, name
date, signature