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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 analyse des micronoyaux par blocage de la cytokinèse (CBMN) dans les lymphocytes du sang périphérique

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Contents

Page

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 documents 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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT), see the following URL: Foreword — Supplementary information.

The committee responsible for this document is ISO/TC 85, *Nuclear energy, nuclear technologies, and radiological protection*, Subcommittee SC 2, *Radiological protection*.

Introduction

The purpose of this International Standard 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 full dose estimates or b) in a triage mode to populations to provide interim dose estimates for individuals.

The CBMN assay is an alternative cytogenetic technique, which is possibly simpler and faster to perform than the dicentric assay (ISO 19238:2014, ISO 21243:2008). It is also routinely used to demonstrate exposure to genotoxic agents, other than ionizing radiation, which is not covered in this International Standard. Although culture of the blood samples is slightly longer than for dicentrics, the scoring of micronuclei 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 radiation accidents. The blood volume required for sufficient number of scorable binucleated cells is similar than required for the dicentric assay. Again, the faster counting speed for micronuclei compensates for the extended culture time. In addition, the CBMN assay can be performed in an automated mode.

This International Standard 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 International Standard 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 event.

A part of the information in this International Standard 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 International Standard expands and standardizes the quality assurance and quality control, the criteria of accreditation and the evaluation of performance. This International Standard is generally compliant with ISO/IEC 17025 "*General requirements for the competence of testing and calibration laboratories*" with particular consideration given to the specific needs of biological dosimetry. The expression of uncertainties in dose estimations given in this International Standard complies with the "ISO-guide for the expression of uncertainty in measurement" (former GUM) and the ISO 5725-all parts.

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

1 Scope

This International Standard addresses the following:

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

b) laboratory safety requirements;

c) radiation sources, dose rates, and ranges used for establishing the calibration reference dose-effect curves allowing the dose estimation from CBMN assay yields and the minimum resolvable dose;

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

e) scoring criteria;

f) conversion of micronucleus frequency in binucleated cells into an estimate of absorbed dose;

g) reporting of results;

h) quality assurance and quality control;

i) informative annexes containing examples of a questionnaire, instructions for customers, a microscope scoring data sheet, a sample report and advice on strengths and limitations of current automated systems for automated micronucleus scoring.

2 Terms and definitions

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

2.1
acentric
chromosome fragment of varying size

Note 1 to entry: When it is formed independently of a dicentric or centric ring chromosome aberration, it is usually referred to as an excess acentric.

2.2
background level
spontaneous yield (or number) of micronuclei recorded in control samples or individuals

2.3
bias
statistical sampling or testing error caused by systematically favouring some outcomes over others

2.4
binucleated cells
cells that have completed one nuclear division after mitogen stimulation and cell type in which micronuclei are scored

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

2.5
CBMN laboratory
laboratory performing biological dosimetry measurements using the CBMN assay

2.6
centric ring
aberrant circular chromosome resulting from the joining of two breaks on separate arms of the same chromosome, generally accompanied by one acentric fragment

2.7
centromere
specialized constricted region of a chromosome that appears during mitosis joining together the two sister chromatids

2.8
chromosome
structure that carries genetic information

Note 1 to entry: Normally, 46 such structures are contained in the human cell nucleus. During nuclear division, they condense to form characteristically-shaped bodies.

2.9
chromatid
either of the two strands of a duplicated chromosome that are joined by a single centromere

Note 1 to entry: Chromatids separate during mitosis to become individual chromosomes.

2.10
confidence interval
statistical range about an estimated quantity within which the value of the quantity is expected to occur, with a specified probability

2.11
cytochalasin-B
Cyto-B
reagent used to block cytokinesis in dividing cells allowing once-divided cells to be identified as binucleated cells

Note 1 to entry: The binucleated cells are the cells in which micronuclei are specifically scored.

2.12
dicentric
aberrant chromosome bearing two centromeres derived from the joining of parts from two broken chromosomes, generally accompanied by an acentric fragment

2.13
fluorescence in situ hybridization
FISH
technique that uses specific sequences of DNA as probes to particular parts of the genome, allowing the chromosomal regions to be highlighted or “painted” in different colours by attachment of various fluorochromes

Note 1 to entry: This technique permits the detection of damage involving exchanges between differently painted pieces of DNA (usually whole chromosomes).

2.14
interphase
period of the cell cycle between the mitotic divisions

2.15
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

2.16
metaphase
second stage of mitosis when the nuclear membrane is dissolved, the chromatids are condensed to their minimum lengths and are aligned for division at the metaphase plate

2.17
micronucleus or micronuclei
MN
small nucleus that arises from lagging acentric chromosome fragments or whole chromosomes during nuclear division and chromosome 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.

2.18
minimum detection level
MDL
smallest measurable amount (e.g. yield or dose) that is detected with a probability β of non-detection (Type II error) while accepting probability α of erroneously deciding that a positive (non-zero) quantity is present in an appropriate background sample (Type I error)

2.19
minimum resolvable dose
lowest additional dose for which the lower 95 % poisson confidence limit is greater than 0, so that there is a 97,5 % chance that the dose received in excess of normal background is greater than 0

2.20
nuclear division index
index in the CBMN assay that is calculated from the relative frequencies of mononucleated, binucleated, and multinucleated cells

Note 1 to entry: This index provides a measure of inhibition of nuclear division.

2.21
precision
dispersion of measurements with respect to a measure of location or central tendency

2.22
quality assurance
planned and systematic actions necessary to provide adequate confidence that a process, measurement, or service has satisfied given requirements for quality

EXAMPLE Dose specified in a licence.

2.23
quality control
part of quality assurance intended to verify that systems and components correspond to pre-determined requirements

3 Micronucleus assay methodology used in this standard

3.1 General

In this International Standard, the frequency of micronuclei in cytokinesis block binucleated lymphocytes in cultured human peripheral blood lymphocytes 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 lymphocytes separated from the other blood components to be incubated in culture medium with a mitogen that would enable scoring of micronuclei in first-generation binucleated cells. A cytokinesis blocking agent, cytochalasin-B, is added at least 6 h before the first mitosis commences to arrest dividing lymphocytes at the binucleated cell 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.

Binucleated cells 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 binucleated cells and staining employed by a CBMN laboratory should be formally documented.

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

3.2 General requirement of the laboratory

The laboratory should be well-equipped with the required bio-hazard units, tissue culture, and standard laboratory equipment for lymphocyte tissue culture, cell separation, slide preparation, and microscopy scoring of cells and subcellular structures, such as micronuclei. The laboratory should maintain quality assurance documents including those describing periodic calibration of the equipment used for cell culture such as laminar flow hoods, pipettes, incubator, etc.

3.3 Requests for analysis and blood sampling

Depending on national regulations, the request for an analysis should normally be made by a doctor representing the patient, by the patient him/herself, or could be requested due to legal claims. In all cases where it is normally possible, the blood sampling for micronuclei analysis shall be made with the patient's informed consent. It is advisable that the laboratory head maintain the record of the patient's informed consent and the patient should also indicate who they will allow to receive the data. For minors, the informed consent should be obtained from the parent/guardian.

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. The purpose is to avoid having the blood sample sit for several hours from time of blood draw and before sample pickup for transportation.

The blood sample is collected using lithium or sodium heparin anticoagulant, maintained at room temperature (at approximately 20 °C) and cultured as soon as possible, but before 72 h. In some unavoidable circumstances involving a delay beyond 72 h, good sample preparation is still possible if the blood samples are stored with due precautions, such as using room temperature gel packs to maintain a temperature of 20 °C.

3.4 Cell culturing

The protocol for the CBMN assay shall be established and documented by each CBMN 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) Blood used to establish the calibration curves shall be incubated for 2 h at 37 °C immediately following irradiation and prior to culture of samples.

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 ± 0,5 °C either as whole blood, enriched lymphocyte suspension (buffy coat), or isolated lymphocytes.

d) Culture vessel shall be sterile and handled in a way to avoid microbial contamination.

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 $\mu g \cdot ml^{-1}$) is commonly used.

f) Mitogen [e.g. phytohaemagglutinin (PHA)] 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 concentration of at least 3,0 µg/ml and no more than 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 binucleated cells and minimize the number of mononucleated and multinucleated cells. It shall be adapted according to the standard culture conditions for each CBMN laboratory. The recommended culture time after mitogen stimulation for cell harvest is 72 h but under certain conditions (e.g. where mitotic delay is anticipated), longer time might be required. Typically, binucleated cells are harvested 24 h to 48 h after addition of cytochalasin-B.

i) Cells may be treated with a hypotonic solution such as 0,075 M KCl for 10 min to 15 min to swell the cells prior to fixation.

j) Cells may be fixed in suspension and then transferred to slides or alternatively, they may be transferred to slides by cyto-centrifugation and then fixed on the slide after air drying. In the former case, cells shall be fixed in freshly prepared fixative solution (i.e. 5:1 methanol:acetic acid) while agitating the cells to prevent clump formation and washed three times or four times with the same fixative until the cell suspension is clear. In the latter case, cells shall be fixed in absolute methanol.

k) If storage of fixed cells is required, then cell suspensions shall be kept in a −20 °C freezer.

l) Slides shall be prepared to ensure integrity of the cell membrane and allow an unambiguous identification of micronuclei in binucleated cells. Humidity and temperature conditions can be adjusted to increase the quality of the spreading.

m) Duplicate cultures should be performed from each blood sample per individual.

3.5 Staining

Cells shall be stained appropriately so that nuclei and micronuclei can be clearly visualized. Commonly used stains include, but are not limited to, Giemsa (for brightfield microscopy), DAPI, and acridine orange (for fluorescence microscopy). The stain used shall be specific for nuclei and micronuclei to avoid artefactual staining of other cellular structures that might resemble micronuclei (e.g. centrioles).

3.6 Microscopy

Use a fluorescence or brightfield microscope depending on the stain used. Observation of cells at a magnification of at least 400 × is required for scoring of cells and micronuclei. For optimal scoring, however, a higher magnification (e.g. 1 000 ×) is recommended.

3.7 Visual scoring of slides

3.7.1 General

Each sample shall be scored by two individuals, each scoring at least 500 binucleated cells (for a total of at least 1 000 binucleated cells) from different slides for the presence of micronuclei. Fewer binucleated cells can be scored for high dose samples or in triage mode (see Clause 10). The distribution of micronuclei amongst the binucleated cells should also be recorded. The slide scorers should be experienced in the scoring of micronuclei in lymphocytes (see Clause 9).

3.7.2 Criteria for scoring

3.7.2.1 Criteria for selecting binucleated cells which can be scored for micronucleus frequency

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

a) the cells shall be binucleated (BN);

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

c) the two nuclei in a BN cell shall 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 1/4 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 nuclei can be scored only if the nuclear boundaries of either nucleus are distinguishable;

f) the cytoplasmic boundary or membrane of a BN cell shall be intact and clearly distinguishable from the cytoplasmic boundaries of adjacent cells.

3.7.2.2 Criteria for scoring micronuclei

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 between 1/16 and 1/3 of the mean diameter of the main nuclei, which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively;

b) MN shall not be linked or connected to the main nuclei;

c) MN may touch but shall not 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;

f) micronuclei lying above or below the daughter nuclei shall not be scored.

3.7.2.3 Criteria for accepting scores

a) Inter-scorer variability is one of the key sources of variation in the micronucleus assay. It is therefore essential that the same scorers are maintained throughout a single assessment and ideally, two scorers are used, each providing a count from each of the duplicate cultures and their mean values calculated to take into account both experimental and scorer variation. However, it is also acceptable to use a single scorer if two experienced scorers are not available.

b) For duplicate samples in which more than 100 MN per 1 000 BN cells are induced, CVs should be less than 20 %.

3.7.3 Scoring data sheets

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

3.8 Automated analysis

Several systems for automated image analysis for the CBMN assay have been developed. Automation at present is beyond the scope of this International Standard. Efforts in this area are described in Annex B.

4 Confidentiality of personal information

4.1 Overview

Biological dosimetry investigations made by a CBMN laboratory shall be undertaken in accordance with national regulations regarding confidentiality. It would normally include the maintenance of confidentiality of the patient's identity, medical data, and social status. In addition, the commercial confidentiality of the patient's employer and any other organizations involved in a radiological accident/incident should be observed. This requirement extends to the following:

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

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

4.2 Applications of the principle of confidentiality

4.2.1 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.

4.2.2 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 (other requestors may be added here, especially for networking). In all cases where it is normally possible, the blood sampling for micronucleus analysis shall be made with the patient's informed consent and the patient should also indicate who they permit to receive the data. For minors, the informed consent shall be obtained from the parent/guardian. The laboratory head, depending on the national regulations, might be required to maintain a record of the patient's informed consent.

4.2.3 Transmission of confidential information

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

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

4.2.4 Anonymity of samples

The laboratory head needs to 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 CBMN laboratory. The coding is performed in an unambiguous way according to a standard procedure. The same code is to be used for all the stages of the analysis. The code is assigned by an authorized person, as defined in 8.2. Decoding, interpretation of results, and compiling the report are also to be performed by an authorized person.

4.2.5 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 another responsible person.

4.2.6 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 the data and results are stored. All laboratory documents relating to a case and which could permit the patient and/or employer to be identified shall be stored in a place only accessible to the authorized persons. Documents shall be retained in an appropriate place for at least 30 years for possible medical/legal re-evaluation of the case. Final disposal of any records shall be by secure means, such as shredding of paper records and complete deletion of electronic records.

5 Laboratory safety requirements

5.1 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.

5.2 Microbiological safety requirements

Handling human blood poses some risk of blood borne parasites and infections being transmitted to laboratory staff. All specimens should be regarded as being potentially infectious even if they are known to be derived from apparently healthy persons. Specimens shall be unpacked and manipulated in a class 2 microbiological safety cabinet. Setting up cultures in such a cabinet has the added benefit of minimizing culture failure due to microbial contamination. Use of hypodermic needles should be kept to a minimum to reduce the risk of injuries. Suitable disinfectants shall be available to deal with spills. All biological waste and used disposable plastic ware shall be sterilised (e.g. by autoclaving or incineration, before final disposal).

Staff should be offered available vaccination against blood borne diseases. The legal and ethical position regarding HIV 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 HIV negative.

5.3 Chemical safety requirements

Certain chemicals and pharmaceuticals are used routinely in the procedures covered in this International Standard. 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 hazard statements (H-Statements) according to the GHS Classification system are listed below.

Acridine Orange	H315, H319, H335, H340
Benzylpenicillin	H317
Bromodeoxyuridine	H351
Colcemid	H300, H310, H330, H340
Cytochalasin B	H300, H310, H330, H340, H361
DAPI	H302, H340, H350
Glacial Acetic Acid	H226, H314
Giemsa stain	H225, H301, H311, H331, H370
Heparin	H303
Hoechst stain (Bisbenzimide)	H302, H315, H319
Methanol	H225, H301, H311, H331, H370
Paraformaldehyde	H228, H302, H315, H317, H318
Phytohaemagglutinin	H303
Streptomycin sulfate	H302, H 332, H317, H334, H361

Keys

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
H303:	can be harmful if swallowed
H310:	fatal in contact with skin
H311:	toxic 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

H351: suspected of causing cancer

H361: suspected of damaging fertility or the unborn child

H370: causes damage to organs

5.4 Optical safety requirements

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

5.5 Safety plan

The laboratory head shall define written safety procedures for protection against microbiological, chemical, and optical hazards.

The laboratory head shall maintain a record of safety "near misses", accidents, and protocols or procedures to avoid repeating similar events.

6 Calibration source(s), calibration curve, and minimum resolvable dose

6.1 Calibration source(s)

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

a) description for all radiation source(s) used to generate *in vitro* calibration curves;

   EXAMPLE Philips 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).

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.

6.2 Calibration curve

A CBMN assay calibration curve is required for each laboratory performing biological dosimetry using the CBMN assay. In general, the same culturing conditions shall be used for establishing the calibration curve as used for analysis in a case of suspected overexposure. The curve should be produced from at

least six donors of varying age and gender with the same number of binucleated cells 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 range from 0 Gy to 0,5 Gy, 1Gy, 2 Gy, 3 Gy, and 4 Gy. Any substantial deviation from this dose range shall be justified. The inclusion of 0Gy data (i.e. data from blood samples unexposed to ionising 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 gender on base-line micronucleus frequencies.

For doses below 0,5 Gy, at least 2 000 binucleated cells should be scored for micronucleus frequency per dose for each donor and for doses above 0,5 Gy, at least 1 000 binucleated cells should be scored per dose for each donor. Observed frequencies of micronuclei in binucleated cells 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.

$$Y = C(\pm SEc) + \alpha D_T(\pm SE\alpha) + \beta D_T^2(\pm SE\beta) \quad (1)$$

where

Y is the micronuclei yield;

C is the population background;

α and β are equation coefficients;

D_T is the absorbed dose to tissue in Gy;

SE is the standard error of mean for each coefficient and constant.

For curve fitting, iteratively reweighted least squares should be used. For over-dispersed data, the weights shall take into account the over dispersion. 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. For calculating standard error of the curve, the standard errors coming from the donors should be combined, taking into account the possible over dispersion from the Poisson distribution.

The CBMN 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. CBMN laboratory radiobiologist or equivalent) that addresses the following issues:

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

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

6.3 Background micronucleus frequency

It has been well established that the background micronuclei frequency in individuals varies with age and gender and also due to various confounding factors (i.e. nutritional status, genotoxic exposures, lifestyle factors, malsegregation of sex chromosomes). For the purpose of radiation biodosimetry using MN frequency in lymphocytes, it is assumed that an individual's base-line MN frequency value prior to the ionising radiation exposure event is equivalent to the mean MN frequency value of unirradiated cells for their age and gender group. Ideally, the background micronucleus frequency would include results for at least three age groups separated into 1 year to 25 years, 26 years to 50 years, 51 years to 75 years and include at least three males and three females in each group to include base-line values for both genders across different age groups. The laboratory should generate its own database of base-line micronucleus frequencies. The mean MN value for the appropriate age and gender shall be subtracted from the observed MN frequency in lymphocytes after the radiation exposure event to deduce the likely induced MN frequency value. The latter value is then used to estimate the radiation exposure dose from the standard curve for radiation-induced MN.

6.4 Minimum resolvable dose measurement

The minimum resolvable dose is a function of the laboratory's measured control background levels of micronuclei, the calibration curve coefficients, and the number of cells scored in an analysis and is limited to the lowest dose used in the appropriate calibration curve. The minimum detection level can be as low as 0,18 Gy and as high as 0,26 Gy, depending on age and gender.

The CBMN laboratory shall provide a report reviewed and endorsed by a qualified expert (i.e. CBMN laboratory radiobiologist or equivalent) that describes the laboratory's micronuclei yield for the reference controls and measured radiation-induced levels for its proposed minimum resolvable dose measurement and the number of cells scored per sample.

7 Responsibility of the customer

This Clause includes items that are not controlled by the CBMN laboratory. Prior to blood sampling, coordination between the customer and CBMN laboratory shall occur. Essential requirements should be explained to the customer and this may be by a standardised instruction sheet as illustrated in Annex C. The essential features are the following:

a) blood sampling should use the collection system, containing lithium or sodium heparin as an anticoagulant, which has been sent or specified by the CBMN laboratory;

b) blood shall be collected (at least 5 ml), labelled accurately and unambiguously, maintained at approximately 20 °C (18 °C to 22 °C) and reach the CBMN laboratory as soon as possible within 72 h after collection;

c) precautions to ensure the integrity of the container and prevent leakage during shipment shall be observed. Packaging and labelling shall conform to national and international regulations and if air transportation is involved, a physical dosimeter should be included to monitor whether the sample has been exposed to ionising radiation in transit. Blood samples shall be kept cool during shipping with a recommended temperature range of 6 °C to 22 °C. A temperature recording device (recording both lowest temperature and highest temperature) should be included to document that the temperature during shipment was controlled properly;

d) the questionnaire provided by the CBMN laboratory shall be completed and returned promptly, preferably with the blood sample;

e) the CBMN laboratory shall be alerted of known infectious samples (HIV or hepatitis, especially).

8 Responsibility of the CBMN laboratory

8.1 Setup and sustainment of the QA program

The CBMN laboratory shall establish and maintain a QA program (see Clause 11), which covers all aspects of the service. The QA program shall include the following:

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

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

8.2 Responsibility during service

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

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

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

   2) a questionnaire that shall elicit patient consent and information on whole or partial body exposure, source and quality of the radiation, circumstances of the exposure, exposure location (country, city, company, etc.), date and time of exposure, previous occupational or medical exposures to radiation, intake of pharmaceuticals, infection, smoking habit, and significant exposures to any other DNA damaging agents, such as organic solvents or heavy metals (see Annex D);

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

b) in response to a request for service a blood collection system (e.g. 10 ml sterile tube) containing lithium or sodium heparin as the anticoagulant, shall be sent to the customer also including the appropriately labelled and addressed packaging material for the return of the sample to the CBMN laboratory. The packaging shall conform to national and/or international regulations for the transit of potentially infectious pathological specimens (see 11.3.3);

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

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

   2) code the blood sample;

   3) document the place and the duration and temperature of storage until the setting up of cultures;

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

   5) document with lot numbers as appropriate all reagents used for culturing;

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

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

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

   9) document the scoring and analysis results;

   10) store slides and case documents in an appropriate place for at least 30 years for possible medico-legal re-evaluation of the case;

d) the CBMN laboratory shall interpret results and prepare reports (see Annex E);

e) the CBMN laboratory should sustain a dialogue with the requestor, reprioritizing cases as required, and providing results to the requestor.

9 Accidental overexposure involving few individuals

9.1 Procedure for scoring micronuclei in binucleated cells

9.1.1 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.

9.1.2 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 complete analysis without scoring any cell more than once.

Criteria for scoring should be followed according to 3.7.

9.1.3 Laboratory scoring expertise

The laboratory head is responsible for ensuring that the scorers are appropriately trained to score slides according to the current standard. All scorers shall participate in intra-laboratory and inter-laboratory comparisons. A set of calibration slides with known micronucleus frequencies (e.g. slides used to generate a calibration curve) should be used routinely to verify that the scoring accuracy of scorers is well within the expected scoring range. Scorers shall be able to achieve acceptable coefficients of variation (no greater than 40 %) for repeat scores of standard control slides and absolute values that are within the Poisson 95 % confidence limits of the test reference yield expected from the laboratory's calibration curve.

9.2 Criteria for converting a micronucleus yield into an estimate of absorbed dose

9.2.1 Overview

The measured micronucleus 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 binucleated cells should be scored from the case specimen, unless the micronucleus yield is high (e.g. >1 micronucleus per cell), in which case, it is not necessary to proceed beyond scoring enough binucleated cells to observe 500 micronuclei. In the special case where there exists a high abundance of micronuclei but few binucleated cells, the dose can be reported after observing 250 micronuclei.

9.2.2 Comparison with controls

The CBMN laboratory shall provide in case reports the laboratory's background micronucleus frequency. If the measured micronucleus frequency is not significantly different from the background frequency, the best estimate of dose should be quoted as zero with its upper confidence limit. If the measured micronucleus frequency is significantly higher than the background level, then a dose estimate with its uncertainties shall be derived and reported (see 6.3 and 6.4).

An alternative approach is to subtract the mean background value of micronuclei for the appropriate age and gender category of the individual tested and use this corrected value to estimate the exposure dose from the calibration radiation dose-response curve of induced MN frequency in binucleated cells. If this approach is used to set up the calibration curve, it shall be specified and explained in the records/reports.

9.2.3 Determination of estimated dose and confidence limits

The CBMN assay laboratory shall provide in result reports the estimated whole body dose and confidence limits. Uncertainties would usually be expressed as 95 % confidence limits although other percentage values can be quoted, if judged appropriate to a particular case. If the lower confidence limit falls below zero dose, only the upper limit needs to be quoted.

The laboratory head shall define the methods used to determine confidence limits.

9.2.4 Acute and non-acute exposure cases

If an overexposure is known to have been received acutely (i.e. below 0,5 h), the dose estimate may be obtained by reference to an acute *in vitro* calibration curve. If an overexposure 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 overexposure 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 above 5 h. If below 5 h, an interaction factor should be estimated using a 2 h time constant.

The CBMN assay 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.

9.2.5 Testing the distribution of micronuclei per binucleated cell

The degree of over-dispersion for the distribution of micronuclei amongst binucleated cells should be tested. This should be done by the *u* test which is a normalized unit of the dispersion index $D = s^2/y$ (variance/mean), as shown in Formula (2). For a Poisson distribution D 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.

$$u = (D-1) \times \sqrt{\frac{N-1}{\left[2 \times \left(1 - \frac{1}{X}\right)\right]}} \qquad (2)$$

where

N is the number of binucleated cells analyzed;

X is the number of micronuclei detected.

9.3 Reporting of Results

9.3.1 General

Routinely, the report shall contain the micronuclei frequency per binucleated cell and its interpretation based on the current understanding of mechanisms for radiation-induced micronuclei 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.

9.3.2 Content of the report (see Annex E for a standard form)

The report should 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 binucleated cells scored, number of micronuclei found, distribution of micronuclei amongst binucleated cells;

j) interpretation of test results (see 9.3.3);

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

9.3.3 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 micronuclei, expressed in SI units of absorbed dose (Gy);

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

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

10 Population triage

10.1 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.

10.2 Use of a CBMN assay network for large scale exposures

To deal with mass casualty scenarios, cytogenetic biodosimetry networks can be established consisting of a reference laboratory supplemented by satellite laboratories, either nationally or internationally. ISO 21243 addresses the establishment of cytogenetic networks for the dicentric assay. This International Standard can equally be applied to the CBMN assay.

10.3 Procedure for scoring micronuclei in binucleated cells

The same procedure as that described in 9.1 should be used with modification as described below.

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 binucleated cells is sufficient, unless micronucleus yield is high in which case, it is adequate to score enough binucleated cells to observe 200 micronuclei. In the special case where there exists a high abundance of micronuclei but few binucleated cells, the dose can be reported after observing 100 micronuclei.

10.4 Criteria for converting a micronucleus yield into an estimate of absorbed dose

The same criteria as described in 9.2 should be used with the following exceptions:

a) only a maximum of 200 binucleated cells needs to be scored, unless 200 micronuclei are observed in a smaller number of binucleated cells;

b) a single average background level of micronuclei should be used as a control as described in the first paragraph of 9.2.2. Age matched background values are not required.

10.5 Reporting of results

The report should include as much content from 9.3.2 as possible. However, multiple samples can be tabulated in the same report if appropriate (see Annex F for multiple sample report).

11 Quality assurance and quality control

11.1 Overview

As a minimum, the quality assurance and quality control practices cited below apply to laboratories performing biological dosimetry by the CBMN assay.

11.2 Quality Assurance

11.2.1 Quality assurance plan

11.2.2 Responsible quality assurance person or organization

The quality assurance plan shall designate an organization or person, typically the laboratory head, with sufficient knowledge to identify quality assurance problems and with sufficient authority to initiate or recommend corrective actions and to provide verification of deficiency corrections.

11.3 Quality control

11.3.1 General

Performance checks shall be conducted to ensure the conformance of analytical processes, measurement equipment and procedures, and the facilities to predetermined operational requirements.

11.3.2 Quality control procedures

The laboratory shall have written quality control procedures to verify that the quality of estimation of absorbed dose measurements complies with the accuracy requirements specified in Clause 9. The quality control procedures should include the following:

a) use of traceable reference standards;

b) performance checks of measurement systems;

c) instrument calibration;

d) intra-laboratory analyses (e.g. known quantities, replicates, and blanks);

e) participation in available inter-laboratory intercomparison programmes;

f) computational checks;

g) review of procedures, specifications, and operating logs;

h) observation of operations and evaluation of quality control data;

i) evaluating conformance to the performance criteria of this International Standard;

j) evaluating quality control data to ensure the long-term consistency of analytical results;

k) verification of minimum detection level determinations.

11.3.3 Performance checks of sample transport integrity

In many cases, blood collection occurs at sites distant from the processing laboratory and transportation is necessary. If air transportation is used, one has to avoid the x-irradiation at the security checkpoints. 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 the customs office. All details concerning blood collection and storage should be recorded. Because of the risk for infectious diseases (hepatitis, HIV), appropriate precautions shall be followed when handling the blood samples.

11.3.4 Performance checks of sample integrity by CBMN laboratory

A system for recording the collection, transport, and storage of the blood samples should be established so that sample integrity is guaranteed. The use of coded samples is critical to avoid potential bias in the scoring. As internal quality assurance, a negative control randomly selected from a group of healthy unexposed subjects should be included to prove the reliability of the procedure. Blood from both exposed and unexposed individuals shall be handled in the same manner. The blood samples from both populations have to be taken concurrently and not successively.

11.3.5 Performance checks of instrumentation

Performance of the measurement equipment shall be checked and evaluated at regular intervals while the equipment is in use. For example, the stability of the temperature control of the incubators has to be controlled. These checks shall be sufficient to demonstrate that the measurement equipment is properly calibrated and that all components are functioning properly. Replicate *in vitro* measurements should also be made periodically. Techniques such as quality control or tolerance charts shall be used for the evaluation of instrument performance. A quality control measurement shall be performed prior to use of the instrument and the number of quality control measurements should represent at least 5 % of the total number of measurements.

11.3.6 Performance checks of sample protocol

The culture, fixation, and staining procedures shall be described in detail in the quality handbook. The composition of each reagent shall be described as accurately as possible in the quality handbook.

Samples containing known exposures of specific radiation dose and quality of interest shall be analysed to determine bias and precision of the analytical procedures. Replicate samples should also be processed periodically. Statistical techniques such as quality control charts shall be used to evaluate biological dosimetry cytogenetic procedure performance data. The number of quality control samples shall be equal to at least 5 % of the total samples analysed.

11.3.7 Performance checks of sample scoring

Before analysis, the microscopic slides or fixed cell suspensions should be stored in a manner that maintains their high quality. Uniform criteria for scoring shall be used. Scoring shall be performed by trained and experienced observers. If different scorers are involved, a balanced scoring design shall be used. Each scorer should analyse the same number of binucleated cells from the slides of all subjects rather than different scorers analysing all cells from different subjects. The identity of the scorer of the slides shall be recorded. A positive quality assurance standard should be included within the set of slides of the study. Independent of the service activity, internal quality assurance periodically involves the comparison of the scoring results of replicate samples between scorers. External quality assurance steps involve the sharing of replicate samples with other laboratories. Furthermore, external quality assurance requires the organization of multicentre intercomparisons at regular time intervals to guarantee the uniformity of the scoring results among the laboratories.

11.3.8 Performance checks of dose and confidence limits estimation

Non-parametric tests should be used for univariate statistical analysis because micronucleus data might not be normally distributed. The confidence limits of the exposure have to be calculated from the uncertainty on the micronucleus yields and the variation of the dose-response relationship among individuals, typically determined in a prior study. The dose-response relationship used for estimating confidence limits of exposure shall be selected appropriately depending on whether exposure was chronic or acute.

11.3.9 Performance checks of result report generation

Each study report to the customer shall be examined to ensure that it contains the necessary information as defined in this International Standard (see Clause 10), namely: subject and customer identifies, exposure information, exposure and sampling date, scoring results, interpretation of the results in terms of dose and its uncertainty and information of how this was derived.

Annex A
(informative)

Sample data sheet for recording micronuclei in binucleated cells

Sample No/Slide Code:

Microscope N°:

Scorer (Last Name, First Name):

Date (Day/Month/Year):

Slide No.	Micronucleus distribution in BN cells							Total No. of BN cells	Total No. of micronuclei
	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

BN: binucleated

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

Annex B
(informative)

Automation of micronuclei scoring

In the last 20 years, there have been several attempts to establish an automated MN scoring system, but none of the systems were adequately validated for routine diagnostic use. In the meantime, new developments in hardware and software have made advanced systems available, especially the fast progress in optical devices and larger data storage units allowed new approaches in automated image analysis.

The reason to promote the automation of the micronucleus assay has multiple motivations. An automated assay could reduce the uncertainties involved in human scoring and increase throughput of the samples. The ability to rapidly score a high number of samples would be of interest in all bio-monitoring projects (i.e. cohorts exposed to mutagenic agents such as DNA-reactive chemicals or radiation), in cancer research and radiation therapy (i.e. individual mutagen and/or radiation sensitivity, as predictive assay for individual cancer therapy), and as a screening bio-dosimetry tool in the case of a large scale radiation accident.

Although automation of MN assays is relevant to different cell types (i.e. buccal cells, erythrocytes), in this Annex, the focus is on peripheral human lymphocytes, the main cell type used for biological dosimetry. The end point of interest is restricted here to the CBMN assay, i.e. the frequency of micronuclei in binucleated cells (BN) in cytokinesis block cultures.

The manual quantification of the yield of micronuclei in BN cells can be very time consuming if a high number of samples need to be analysed after *in vivo* exposure. Furthermore, there is some bias/variability due to subjective interpretation of scoring criteria. The image analysis automation process can overcome this and can generate a high throughput system with great reproducibility once a set of classifiers to recognize binucleated cells and micronuclei within them are established and agreed for use across laboratories. A very important requirement for the use of automated scoring machines is knowledge about the uncertainties associated with the results and possible confounders.

An important feature to receive comparable data between different laboratories using the same automated system will be a standardized protocol (optimized for requirements of the system) for the culture of lymphocytes, slide preparation, slide staining, and image analysis classifiers.

There are three important systems with different background technologies and procedures in the field.

Flow cytometry has been used since the beginning of the 1990s as an approach to quantify micronuclei. The main limitation of this method results from the need to analyse lysed cells (resulting in destroyed plasma membranes) with MN in suspension. As a consequence, strategies to discriminate between cellular debris, apoptotic nuclear fragments, and MN were not always effective causing an increase in the number of false positive MN. Another restriction is the loss of information about the distribution of the MN in the cell population and, in addition, the measured samples cannot be stored for documentation purposes. Furthermore, it is not possible in a conventional flow cytometry system to restrict scoring to once-divided cells only which is essential for accurate micronucleus measurements without confounding by cell division kinetics in culture. However, a novel imaging flow cytometric system recently developed has the capacity to identify cytokinesis block binucleated cells and partially detect micronuclei within them.

Superior to this approach are the recently developed laser scanning cytometers (LSC) which provide fluorescence excitation with up to four different laser wavelengths. This method can analyse cells, fixed on microscope slides or in multi-well plates. A concurrent differential staining of DNA (e.g. PI) and proteins (e.g. FITC) offers a better MN identification than DNA staining alone. MN are characterized by their DNA content and the ratio of DNA/protein provides additional parameter to distinguish MN from artefacts. The software is able to identify the number of MN of each individual cell by the FISH spot counting protocol in the software and provides information about the distribution of the MN amongst mononucleated, binucleated, and multinucleated cells. Image analysis revealed that 93 % of the detected objects are MN. Binucleated cells are detected by their DNA content (DNA index of 2.2 to 3.8), confirmed

as >80 % true positive and MN scored specifically in such cells. There is a good correlation (r = 0,87) in MCF-7 cells between visual analysis and LSC.

Another strategy is using computerized microscopes as advanced image analysis systems. For instance, a system is equipped with a software module which scans CBMN assay samples full automatically for micronuclei using a 10 × microscope objective. Automated detection of mono-nucleated cells and binucleated cells provides information about cell proliferation. A motorised scanning stage and external slide feeder allows the automatic screening of 80 slides without further human interactions.

The binucleated cells and MN are detected by topographic parameters (e.g. shape and size of the nuclei, distance between nuclei, size of objects within region of interest) which are individually adapted to the preparation by classifiers defining the morphological cell parameters. Comparisons between visual counting and automated scoring reveals a lower MN frequency for the automated scoring, but the results are highly correlated, as determined by regression analysis (r = 0,96). The results obtained with the automated software can be used without further human evaluation. Applying a dose effect curve for gamma-rays, established with the automated system, a dose of 1 Gy can be detected with an accuracy of 0,2 Gy.

After a visual check of all binucleated cells identified, only 2 % of BN cells were misclassified with respect to their MN content.

NOTE 1 % of BN cells classified as BN cells with MN did not contain MN and about 1 % of BN cells classified as BN cells without MN contained MN. The percentage of false positive and false negative MN, with respect to all MN identified is, of course, higher.

The false positive rate of binucleated cells was 6,3 %, partly influenced by apoptotic cells. It is estimated that 60 blood samples (120 slides) can be processed in a 12 h shift. The detection rate of the automated system relative to visual scoring is about 87 %.

Another image-analysing system is comparable to the previous one but based on different detection algorithms. While the former detects binucleated cells by the proximity of two nuclei, in the latter system, the cytoplasm of cells is first detected and in a second step, the nuclei and micronuclei are searched for in the cell. This system shows a false positive value for MN detection higher than 1 % and therefore, an interactive visualization step is recommended as quality control and validation. The automated scoring resulted consistently in lower yields in dose effect curves than by manual scoring. The detection rate of the automated system is about 68,5 %.

Annex C
(informative)

Instructions for customer (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:

— before the blood sample is taken, the laboratory should be informed in order that it can prepare for its arrival and pick up. Schedule the time for blood draw with the sample pick-up for shipment to minimize this time;

— the phlebotomist shall be informed that all blood samples are to be collected into **lithium or sodium heparin tubes**, (at least 5 ml). They shall immediately gently rock the tubes for 2 min to ensure proper mixing and label the tubes with your name and date of birth;

— the subject shall complete the questionnaire, verify any prescription or over-the-counter medication being taken (drug, dosage- see bottom of this sheet), and document, if possible, any X-ray or nuclear medicine procedures undergone over the last 2 years (see bottom of sheet). The laboratory also needs to know if the subject smokes and, if so, for approximately how long (in years) and how many (per day);

— the blood sample shall be packaged carefully to prevent breakage of the tubes in transit. Also, the blood should be maintained around at 20 °C. **Blood samples shall not be frozen**. 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 further ensure that the samples do not freeze during transportation (e.g. Air-mail), the package shall be marked on the exterior with **URGENT DIAGNOSTIC SAMPLES - NOT TO BE FROZEN**. For air transport, packaging and labelling shall conform to the current regulations (XXX). The package and shipping documents shall be marked with "**NOT TO BE X-RAYED"** and a physical dosimeter shall be included in the package;

— immediately after blood collection, the sample shall be shipped by special transportation using overnight air express so that the laboratory can receive the blood early in the morning following sample collection. Contact the laboratory to confirm the shipment and inform of the Way Bill number. THIS IS IMPORTANT FOR TRACKING THE SAMPLE;

— for best results, blood shall be received within 24 h of collection.

(CBMN laboratory Head)

(CBMN laboratory address)

Phone: (XXX) XXX-XXX

Fax: (XXX) XXX-XXX

E-mail: name@company.com

Annex D
(informative)

Sample questionnaire

Exposure Information for Micronucleus Analysis

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

I, _____ (Name), born _____(dd/mm/yy) consent to giving a blood sample for the purpose of estimating the micronucleus yield induced by exposure to ionizing radiation.

_____

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 overexposure: _____ (dd/mm/yy - time)

2. Place: _____ Company: _____

3. Brief description of overexposure:

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. Previous exposure through medical practice:

Radiation therapy ○ Date, Part of Body _____

x-ray diagnoses ○ Date, Part of Body _____

Nuclear medicine ○ Date, Part of Body _____

2. Illness within the last 4 weeks before taking the blood sample: _____

3. Intake of medication: ○

Name of medication: _____ Dose: _____ Duration: _____

4. Smoker: no: ○ yes: ○ number/day: _____

5. Known diseases:

HIV ○ Hepatitis ○

_____

Results of micronucleus analysis to be sent to:

Name: _____

Address: _____

_____

Telephone #: _____

Annex E
(informative)

Sample of report for single assessment

(date)

(requestor name)

(requestor address)

Phones: (XXX) XXX-XXX

FAX: (XXX) XXX-XXX

Dose Estimates for Mr. John Doe:

Dear Mr. Y

This report is to inform you of cytogenetic assessment of the radiation dose received by Mr. John Doe, born *(date of birth)*. This cytogenetic assessment was requested *(date of order)* for the following reasons: *(hereafter all information on exposure circumstances relevant for biological dosimetry assessment).*

The cytokinesis block micronucleus assay is used to estimate the acute whole body radiation dose received by an individual. The assay was performed on blood samples received at the Service Laboratory on June 29, 1999 (blood collected from Mr. Doe June 28, 1999). The expected incidence of micronuclei in a **normal, unexposed individual** is 2 to 36 in 1 000 binucleated cells (first division).

The laboratory randomly assessed XXX binucleated cells and found XX micronuclei. Based on interpolation from a standard dose-response curve for Iridium-192 (appropriate radiation quality) and 95 % confidence levels, the laboratory estimates Mr. Doe received no more than XXX Gy (95 % upper confidence level) and no less than XXX Gy (95 % lower confidence level) with a mean dose of 0,XX Gy.

Should you have any further questions about this assessment please do not hesitate to contact the laboratory.

Yours sincerely,

(Service Laboratory Head)

(Service Laboratory address)

Phone: (XXX) XXX-XXX

Fax: (XXX) XXX-XXX

E-mail: name@company.com

Annex F
(informative)

Example group sample report

Dr. Y Medical information — Confidential

General Hospital

1 Main Street

Anytown, ON

A1A 1A1

Phone: **(555) 555-5555**

Fax: **(555) 555-4444**

REF: Dose estimates for all potentially irradiated

Dear Mr. Y

This report is to inform you of the cytogenetic assessment of the radiation dose received by 10 individuals. This cytogenetic assessment was requested on *(date of order)* for the following reasons: *(hereafter all information on exposure circumstances relevant for biological dosimetry assessment).*

The cytokinesis block micronucleus (CBMN) assay is used to estimate the acute whole body radiation dose received by an individual. The assay was performed on the blood samples and processed under the case code numbers provided by Dr. X using the routine culture method of the laboratory. The expected incidence of micronuclei in a **normal, unexposed individual** is XX micronuclei (range XX to XX) in 1 000 binucleated lymphocyte cells that completed nuclear division. The laboratory randomly assessed 200 binucleated cells (or 200 micronuclei) for each blood sample obtained from the 10 individuals. Listed below are the dose estimates including the upper and lower confidence levels for each individual. The laboratory used a 200 kVp X-ray standard curve established in the laboratory for estimating the doses. All doses below are X-ray equivalent dose estimates.

ID No.	No. of micronuclei	No. of binucleated cells assessed	Estimated dose Gy	Lower 95 % confidence limit	Upper 95 % confidence limit
123	5	200	0,1	0,0	0,6
124	14	200	0,7	0,3	1,1
125	35	200	1,5	1,1	1,9
126	22	200	1,1	0,7	1,5
127	7	200	0,3	0,0	0,7
128	12	200	0,6	0,2	1,0
129	18	200	0,9	0,5	1,3
130	26	200	1,2	0,8	1,6
131	46	200	1,8	1,5	2,3
132	8	200	0,4	0,0	0,8

Should you have any further questions about this assessment, please do not hesitate to contact the laboratory.

Yours sincerely,

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