

Cytokinesis-block micronucleus cytochrome assay

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The cytokinesis-block micronucleus cytochrome assay is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated (BN) cells and include (a) micronuclei (MNI), a biomarker of chromosome breakage and/or whole chromosome loss, (b) nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions, and (c) nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios. Further information regarding mechanisms leading to MNI, NPBs and NBUDs formation is obtained using centromere and/or telomere probes. The assay is being applied successfully for biomonitoring of *in vivo* genotoxin exposure, *in vitro* genotoxicity testing and in diverse research fields such as nutrigenomics and pharmacogenomics as well as a predictor of normal tissue and tumor radiation sensitivity and cancer risk. The procedure can take up to 5 days to complete.

INTRODUCTION

MNI originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division (Fig. 1)^{1–6}. The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MNI in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided BN cells, which are the cells that can express MNI^{3–6}. In the CBMN assay, once-divided cells are recognized by their BN appearance after blocking cytokinesis with cytochalasin-B (Cyt-B), an inhibitor of microfilament ring assembly required for the completion of cytokinesis^{3–7}. Restricting scoring of MNI in BN cells prevents confounding effects caused by suboptimal or altered cell division kinetics, which is a major variable in micronucleus (MN) assay protocols that do not distinguish between non-dividing cells that cannot express MNI and dividing cells that can^{6,8,9}. Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells.

Over the past 17 years, the CBMN assay has evolved into a comprehensive method for measuring chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis (Figs. 1–5)^{3–6,8–22}. This method is now also used to measure NPBs, a biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair, and to measure NBUDs, a biomarker of gene amplification^{20–39}. The significance of these developments and the concept of the CBMN assay as a “cytome” assay of chromosomal instability are explained in the following sections. The “cytome” concept implies that every cell in the system studied is scored cytologically for its viability status (necrosis, apoptosis), its mitotic status (mononucleated, BN, multinucleated) and its chromosomal damage or instability status (presence of MNI, NPBs, NBUDs and number of centromere probe signals among nuclei or MNI of BN cell if such molecular tools are used in combination with the assay) (Figs. 1–5). For these reasons, it is now appropriate to refer to this technique as the cytokinesis-block MN cytochrome (CBMN Cyt) assay.

We have proposed that NPBs between nuclei in BN cells should be scored in the CBMN assay because they provide a measure of chromosome rearrangement, which is otherwise not achievable in

this assay if only MNI are scored^{3,22,23,35–39}. NPBs occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. Rarely is it possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis, which ultimately results in breakage of the NPB when the daughter cells separate²⁶. However, in the CBMN assay, BN cells with NPBs are allowed to accumulate because cytokinesis is inhibited and the nuclear membrane is eventually formed around the chromosomes allowing an anaphase bridge to be observed as an NPB.

Various mechanisms could lead to NPB formation following DNA misrepair of strand breaks in DNA³⁹. Typically, a dicentric chromosome and an acentric chromosome fragment are formed that result in the formation of an NPB and an MN, respectively (Figs. 1–3 and 5). Misrepair of DNA strand breaks could also lead to the formation of dicentric ring chromosomes and concatenated ring chromosomes which could also result in the formation of NPBs³⁹. An alternative mechanism for dicentric chromosome and NPB formation is telomere end fusion caused by telomere

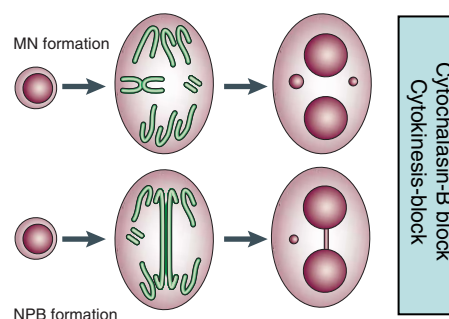


Figure 1 | MN and NPB formation in cells undergoing nuclear division. MN originate from either lagging whole chromosomes or acentric chromosome fragments. NPBs originate from dicentric chromosomes that may be caused by misrepair of double strand DNA breaks or telomere end fusions. These events can only be observed in cells completing nuclear division, which are recognized by their BN appearance after cytokinesis blocking with Cyt-B.

shortening, loss of telomere capping proteins or defects in telomere cohesion⁴⁰, but in this case the NPB is not necessarily accompanied by an acentric chromosome fragment or an MN. The formation of anaphase bridges and NPBs has been observed in models of rodent and human intestinal cancer *in vivo* and shown to correlate with telomere length, indicating that NPB formation may also be used as a surrogate measure of critically short telomeres²⁵. Real-time *in vitro* studies showed that anaphase bridges may be broken in more than one region during late anaphase, leading to the formation of acentric chromosome fragments and eventually MNi^{26,27}. Therefore, some MNi may also originate from broken anaphase bridges although whether this actually happens in cytokinesis-blocked cells remains unclear. The importance of scoring NPBs should not be underestimated because it provides direct evidence of genome damage resulting from misrepaired DNA breaks or telomere end fusions, which is otherwise not possible to deduce by scoring MNi only, which could originate from either acentric chromosome fragments or chromosome loss.

Over the past decade, another unique mechanism of MNi formation, known as nuclear budding, has emerged. This process has been observed in cultures grown under strong selective conditions that induce gene amplification as well as under moderate folic acid deficiency^{27–37}. Shimizu *et al.*^{31,32} showed, using *in vitro* experiments with mammalian cells, that amplified DNA is selectively localized to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MNi during S phase of mitosis. Amplified DNA may be eliminated through recombination between homologous regions within amplified sequences forming mini-circles of acentric and atelomeric DNA (double minutes), which localize to distinct regions within the nucleus, or through the excision of amplified sequences after segregation to distinct regions of the nucleus. The process of nuclear budding occurs during

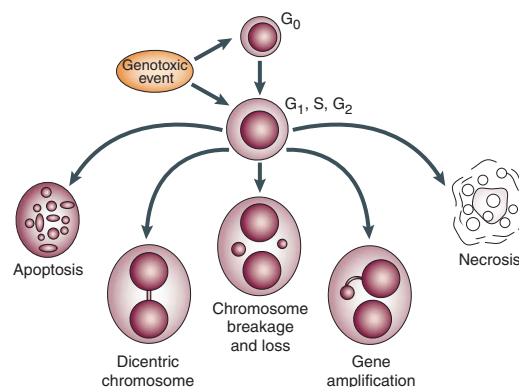


Figure 2 | The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents. Using these biomarkers within the CBMN assay, it is possible to measure the frequency of chromosome breakage (MN), chromosome loss (MN), chromosome rearrangement, for example, dicentric chromosomes (NPB), gene amplification (NBUDs), necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of mono-, bi- and multinucleated cells.

S phase and the NBUDs are characterized by having the same morphology as an MN with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Figs. 2 and 3h). The duration of the nuclear budding process, and the extrusion of the resulting MN from the cell remain largely unknown. MNi may also be formed by a budding process following exposure to γ -irradiation³³. In this process, Rad 51 recombination protein complexes are detectable throughout the entire nucleus 3 h after irradiation and then concentrate into distinct foci before being extruded from the nucleus as NBUDs.

In a series of studies on folic acid deficiency in long-term primary human lymphocyte cultures, we carefully quantified the interrelationship between MNi, NPBs and NBUDs in an attempt to validate the use of these biomarkers and to determine more comprehensively the impact of folic acid deficiency, within the physiological range, on various aspects of genomic stability^{35–38}. Folic acid concentration correlated significantly ($P < 0.0001$) and negatively ($r = -0.63$ to -0.74) with all these markers of

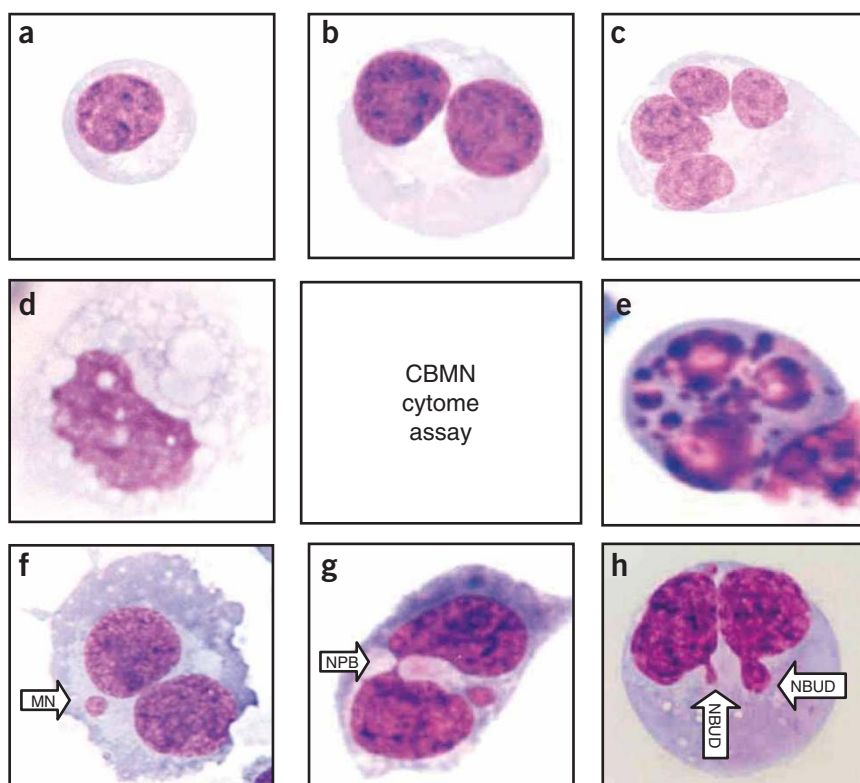
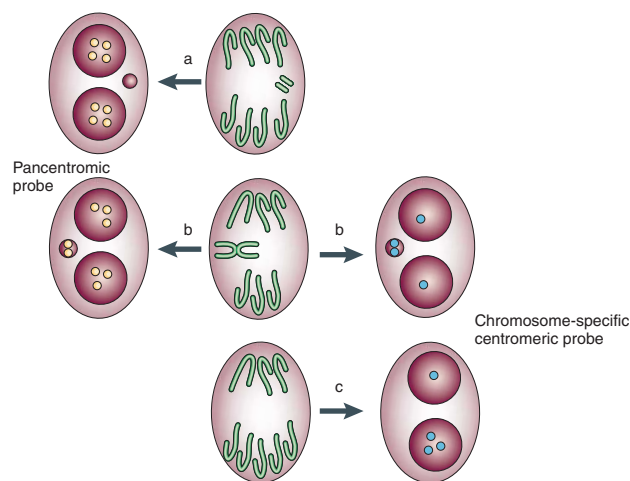


Figure 3 | Photomicrographs of the cells scored in the CBMN "cytome" assay. (a) Mononucleated cell; (b) BN cell; (c) multinucleated cell; (d) early necrotic cell; (e) late apoptotic cell; (f) BN cell containing one or more MNi; (g) BN containing an NPB (and a MNi); (h) BN cell containing NBUDs. The ratios of mononucleated, BN, multinucleated, necrotic and apoptotic cells are used to determine mitotic division rate or NDI (a measure of cytostasis) and cell death (cytotoxicity). The frequency of BN cells with MNi, NPBs or NBUDs provides a measure of genome damage and/or chromosomal instability. For a wider selection of photomicrographs of different types of cells and biomarkers scored in the CBMN Cyt assay, refer to Fenech *et al.*⁷⁵.

Figure 4 | Centromeric probes to determine mechanism of MN formation and aneuploidy. The use of molecular techniques for identifying (a) an MN originating from a lagging acentric chromosome fragment, (b) a MN originating from a lagging whole chromosome and (c) non-disjunction of a specific chromosome leading to aneuploid daughter nuclei. The yellow spots in the nuclei and MNi of the BN cells on the left of each panel show the centromeric or kinetochore pattern of staining when pancentromeric probes or kinetochore antibodies are used. The light blue spots in the nuclei and MNi of the BN cells on the right of each panel show the pattern of centromeric staining when a centromeric probe specific to the chromosomes involved in MN formation or non-disjunction events is used. The example shown is for a hypothetical cell with only two pairs of chromosomes. Pancentromeric probes should be used only for distinguishing between MNi originating from chromosome breaks (centromere negative) and chromosome loss (centromere positive). Chromosome-specific centromere probes should be used only to measure malsegregation (owing to non-disjunction or chromosome loss) involving unique chromosomes. It is important to note that pancentromeric probes cannot be used to determine non-disjunction because of the difficulty in reliably counting all centromeres within nuclei.



chromosome damage, which were minimized at 60–120 nM folic acid. The strong cross-correlation between MN, NPB and NBUD frequency ($r = 0.65\text{--}0.77$; $P < 0.001$) suggests a common mechanism initiated by folic acid deficiency-induced DNA breaks, the most plausible being chromosomal instability generated by breakage–fusion–bridge cycles⁴¹.

Expression of MNi could also be used as a surrogate marker of DNA hypomethylation. For example, the DNA methylation inhibitor 5-azacytidine induces undercondensation of the heterochromatin regions of chromosomes 1, 9 and 16, and the specific loss of these chromosomes as MNi in human lymphocytes *in vitro*^{42,43}. The immunodeficiency, centromeric region instability, facial anomalies syndrome, which is caused by mutation in the DNA methyl transferase DNMT3B gene, is characterized by despiralization of heterochromatin of chromosomes 1, 9 and 16 and loss of this chromatin into NBUDs and MNi⁴⁴. These events are likely to be relevant to the aging process because Suzuki *et al.*⁴⁵ demonstrated that *in vitro* aging of normal human fibroblasts results in concomitant demethylation of satellite 2 and satellite 3 DNA (which is abundant in the juxta-centromeric DNA of chromosomes 1, 9 and 16) and the increasing frequency of MNi that specifically

contained these sequences. It has been conclusively shown that *in vivo* aging leads to an increased MN frequency in lymphocytes and that loss of the X chromosome in females and males and loss of the Y chromosome in males are among the primary mechanisms explaining this increase^{6,45,46}.

Although it is evident that MN expression could be increased as a result of hypomethylation of satellite DNA, it is also possible that increased genome damage may be caused by hypermethylation of CpG islands within or adjacent to the promoter regions of house-keeping genes involved in cell-cycle check points and DNA repair. For example, CpG island hypermethylation of mitotic spindle check point genes such as APC, BUBR1 and hCDC4 could reduce their expression and therefore increase the possibility of chromosome malsegregation leading to MN formation^{47,48}. Silencing or loss of function of ATM, FANC and BRCA1 or BRCA2 genes involved in DNA repair could also result in higher baseline MN frequencies in human cells^{49–51}.

To take full advantage of the CBMN Cyt assay and obtain deeper understanding of the genotoxic mechanism, it is essential to distinguish between MNi originating from whole chromosomes and those originating from acentric fragments as well as to determine whether malsegregation of chromosomes is occurring between nuclei in a BN cell that may or may not contain MNi. This is best achieved by using methods that can detect centromeric regions of chromosomes. The use of MN size as a discriminant is not recommended for human cells or other cell types in which the size of chromosomes is heterogenous because a small MN may contain either a fragment of a large chromosome or a whole small chromosome. Centromeric regions of chromosomes can be detected indirectly using antibodies to the kinetochore proteins that are assembled on centromeres^{52,53} but this approach does not distinguish between unique chromosomes and may not detect

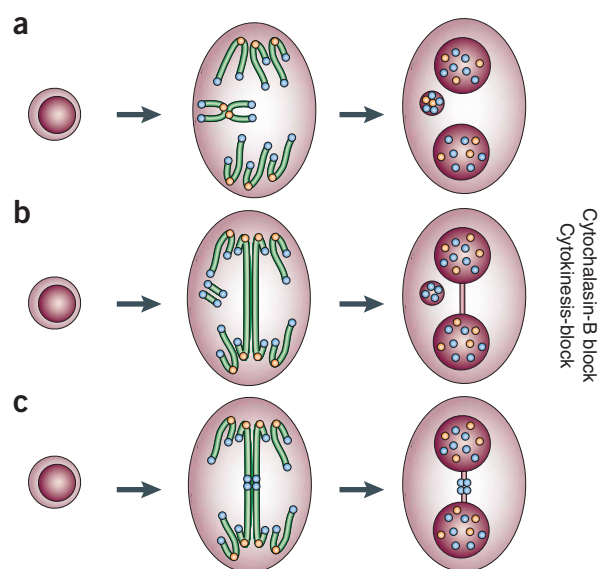


Figure 5 | Centromeric and telomeric probes to determine mechanism of MN and NPB formation. Pancentromeric and telomeric probes can be used to distinguish (I) between MNi originating from whole chromosome loss (a) and MNi originating from acentric chromosome fragments (b) and (II) NPBs from dicentric chromosomes resulting from misrepair of DNA strand breaks (b) and dicentric chromosomes caused by telomere end fusions (c). The yellow dots represent probes that hybridize to the centromeric region of chromosomes. The light blue dots represent probes that hybridize with the telomeric sequences in chromosomes.

chromosome loss occurring due to absence of kinetochores on defective centromeres⁵⁴. The preferred method is to use *in situ* hybridization (ISH) to identify centromeric regions using DNA or peptide nucleic acid pancentromeric probes, which allows MNi containing whole chromosomes to be reliably identified. Furthermore, use of centromeric probes that are specific for a unique chromosome can be used to detect non-disjunctional events (i.e., unequal distribution of unique homologous chromosome pairs in daughter nuclei) in BN cells^{12–19,55}. The types of results that can be expected with the various techniques are illustrated schematically in **Figure 4**. It is also possible to use a combination of pantelomeric and pancentromeric probes to determine the mechanism of MN and NPB formation as explained in **Figure 5**. For example, an NPB originating from a telomere end fusion would be expected to have a telomere signal within the bridge, but an NPB originating from a DNA misrepair event is less likely to exhibit a telomere signal within the bridge because double strand breaks are expected to occur more frequently in the non-telomeric sequences because they are much more abundant.

Most chemical agents and different types of radiation have multiple effects at the molecular, cellular and chromosomal level, which may occur simultaneously and to varying extents depending on the dose. Interpretation of genotoxic events in the absence of data on effects on nuclear division rate and necrosis or apoptosis can be confounding because observed increases in genome damage may be due to indirect factors such as inhibition of apoptosis or defective/permissive cell-cycle checkpoints leading to shorter cell-cycle times and higher rates of chromosome malsegregation. Furthermore, determining nuclear division index (NDI) and proportion of cells undergoing necrosis and apoptosis provides important information on cytostatic and cytotoxic properties of the agent being examined that is relevant to the toxicity assessment. In human lymphocytes, the NDI also provides a measure of mitogen response, which is a useful biomarker of immune response in nutrition studies and may also be related to genotoxic exposure^{3,23,36–38,56}. The cytochrome approach in the CBMN Cyt assay is important because it allows genotoxic (MNi, NPBs and NBUDs in BN cells), cytotoxic (proportion of necrotic and apoptotic cells) and cytostatic (proportion and ratios of mono-, bi- and multinucleated cells, NDI) events to be captured within one assay.

In conclusion, the CBMN method has evolved into an efficient “cytochrome” assay of DNA damage and misrepair, chromosomal instability, mitotic abnormalities, cell death and cytostasis, enabling direct and/or indirect measurement of various aspects of cellular and nuclear dysfunction such as

- unrepaired chromosome breaks, DNA misrepair, acentric chromosome fragments and asymmetrical chromosome rearrangement (MN or NPB accompanied by MN originating from acentric chromosomal fragments),
- telomere end fusions (NPB with telomere signals in the middle of the bridge and possibly without accompanying MN),
- malsegregation of chromosomes due to spindle or kinetochore defects or cell-cycle checkpoint malfunction (MN containing whole chromosomes or asymmetrical distribution of chromosome-specific centromere signals in the nuclei of BN cells),
- nuclear elimination of amplified DNA and/or DNA repair complexes (NBUDs),
- chromosomal instability phenotype and breakage–fusion–bridge cycles (simultaneous expression of MNi, NPBs and NBUDs)

- DNA hypomethylation (specific elevation in the frequency of MN containing whole chromosomes 1, 9 and 16),
- altered mitotic activity and/or cytostasis (NDI) and
- cell death by necrosis or apoptosis (ratios of necrotic and apoptotic cells)

The Micronucleus Assay Working Group of the International Workshop on Genotoxicity test procedures concluded that demonstration of cell proliferation in both control and treated cells is compulsory for the acceptance of the test because MN expression is compromised when the proportion of dividing cells is reduced by cytostatic or poor tissue culture conditions^{9,57,58}. It is evident that the best way to eliminate any possible confounding effects of altered cell division kinetics is to use the CBMN Cyt assay regardless of the cell type used by scoring MNi in BN cells, which in addition allows measurement of NPBs as well as NBUDs, cytotoxicity and cytostatic effects. The choice not to use Cyt-B is justifiable only in those rare situations in which cytokinesis defects are being specifically investigated.

The CBMN assay has already been proven to be an effective tool for the study of cellular and nuclear dysfunction caused by *in vitro* or *in vivo* aging, micronutrient deficiency or excess, genotoxin exposure and genetic defects in genome maintenance. More recent studies indicate that this method is likely to also prove fruitful in the emerging fields of nutrigenomics and toxicogenomics and their combinations, as it becomes increasingly clear that nutrient status also impacts on sensitivity to exogenous genotoxins⁵⁹. The ultimate test of the CBMN Cyt assay is its ability to predict disease outcomes prospectively. The Human Micronucleus (HUMN) project (www.humn.org), an international collaborative project aimed at determining the variables affecting MN frequency in humans and the pathological significance of this biomarker⁶⁰, has recently completed a cohort study involving a total of 6,718 subjects from ten countries, screened for MN frequency between 1980 and 2002; a significant increase of all cancer incidence was found for subjects in the groups with medium (RR = 1.84; 95% CI: 1.28–2.66) and high MN frequency (RR = 1.53; 95% CI: 1.04–2.25) relative to those with low MN frequency⁶¹. Results from a case–control study of lung cancer in smokers showed that both spontaneous and nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced MNi are associated with increased lung cancer risk (OR 2.06 and 2.32, respectively) and the association with NPB (OR 29.05 and 45.52, respectively) and NBUDs (OR 6.53 and 10.10, respectively) was even greater⁶². These initial results indicate the potential predictive value of the CBMN Cyt assay with respect to cancer risk and validate its use as a test for detecting nutritional, environmental and genetic factors that are potentially carcinogenic.

Use of the CBMN Cyt assay in peripheral blood lymphocytes provides the opportunity for a harmonized approach for studying genotoxicity and cytotoxicity both *in vitro* and *ex vivo*, which is important for modeling and predicting *in vivo* effects in humans. This provides the opportunity to test, using the same system, the extent to which the *in vitro* test is predictive of *in vivo* genotoxic events. The adoption of the CBMN Cyt assay by the pharmaceutical industry^{57,58,63}, its application in human biomonitoring of genotoxic exposures^{24,60,61} and its increasing application in preventive medicine and nutrition^{62,64,65} and the increased investment in the automation of the CBMN Cyt assay (see

<http://www.imstar.fr/applications/genotoxicity/micronucleiassay/>; http://www.metasystems.de/products/metafer/metafer_msearch.htm; <http://www.biocompare.com/techart.asp?id=1247> are indicative of the increasing importance of this test^{66–71}.

MATERIALS

REAGENTS

- Ficoll–Paque, sterile (Amersham Pharmacia Biotech; #17-0840-03, 500 ml, #17-0840-02, 100 ml) **! CAUTION** This product is very stable if bottle remains unopened; however, it can deteriorate if exposed to air for long periods of time. For best results, always date the bottles, use 100 ml bottles and remove from the bottle slowly using a needle and syringe without breaking the seal. Store at 4 °C. Use at room temperature (20–22 °C).
- Hank's balanced salt solution (HBSS), sterile, with calcium and magnesium, without phenol red (Trace, #11-101-0500V). Store at 4 °C **▲ CRITICAL** Must be used at room temperature (20–22 °C).
- RPMI 1640 medium, without L-glutamine, sterile liquid (Trace, #11-066-0500V, 500 ml or #11-066-100V, 100 ml). Store at 4 °C. Use at 37 °C when preparing cultures.
- Fetal bovine serum (FBS), heat-inactivated, sterile (Trace, #15-010-0100V, 100 ml). Store frozen at –20 °C. Thaw in a 37 °C water bath before adding to the culture medium. Once thawed FBS will remain stable at 4 °C for 3–4 weeks **! CAUTION** Avoid repeated refreezing of the thawed FBS.
- L-Glutamine solution, 200 mM sterile solution, cell-culture tested (Sigma, #G7513, 20 ml). Store frozen at –20 °C in 1.1 ml aliquots. Thaw at room temperature before adding to the culture medium
- Sodium pyruvate solution, sodium pyruvate 100 mM, sterile solution (Trace, #21-154-0100V, 100 ml). Store frozen at –20 °C in 1.1 ml aliquots. Thaw at room temperature before adding to the culture medium
- Cyt-B (Sigma, #C6762) **! CAUTION** This material is toxic and a possible teratogen. It must always be purchased in sealed vials. The preparation of this reagent must be carried out in a cytoguard cabinet and the following personal protection must be used: Tyvek gown, P2 dust mask, double nitrile gloves and safety glasses.
- DMSO (Sigma, hybrimax, sterile-filtered, #D2650)
- Phytohemagglutinin (PHA; Abbott Murex, #HA15 8E27)
- Isoton II (Coulter Electronics, #8546719)
- Zapoglobin (Coulter Electronics, #9366013)
- DePex (or DPX) mounting medium (Ajax, 3197)
- 1% (w/v) hypochlorite solution for disinfecting cytocentrifuge cups. Change weekly
- Diff-Quik staining set (Lab-Aids LP64851, supplied by Surgical and Medical). Consists of fixative, solution 1 (orange) and solution 2 (blue). Stains can be reused and can last several months depending on usage. Diff-Quik is a modified Wright-Giemsa stain
- Methanol (Ajax Univar, #318) for fixing cells. This fixative can be used in place of the Diff-Quik fixative once it runs out
- 0.85% (w/v) isotonic saline, sterile, for the preparation of PHA solution: add 4.25 g sodium chloride to 500 ml Milli-Q water, autoclave at 121 °C for 30 min and then store at 4 °C

EQUIPMENT

- Sterile vacutainer blood tubes with lithium heparin as anticoagulant (e.g., Greiner vacuette tubes 9 ml, #455084 No gel)
- 70 ml polystyrene gamma sterile containers (e.g., Sarstedt 75.9922.333)
- 10 ml graduated sterile pipettes (Falcon #7551)
- Preferred tube for Ficoll separation—dependent on the blood volume: 50 ml polystyrene, sterile, conical (Falcon #2073), 15 ml polypropylene, sterile, conical (Falcon #2096), 10 ml polystyrene, yellow capped, sterile, V-bottom (Disposable Products #21829)
- Sterile plugged Pasteur pipettes 9" (22–23 cm) (Chase #93P)
- 200–1,000 µl Finn timer + sterile 1 ml tips (Lab systems, Edward Instruments Co. #1040-800 Blue)
- 40–200 µl Finn timer + sterile 200 µl tips (Lab systems, Edward Instrument Co. #1030-800 Yellow)
- Biological safety cabinet (e.g., Gelman Sciences Class II BH Series)

The standard method for the CBMN Cyt assay as applied to isolated lymphocyte cultures and its various modifications as used with other culture systems and cell types are described in detail in the next sections. Timing information can be found in **Table 1**.

- Bench top centrifuge, biosafe with sealed buckets, capable of spinning at 1,000g (e.g. MSE Mistral 2000R)
- Cell counter (e.g., Coulter Electronics model ZB1). A hemocytometer can be used instead if an electronic cell counter is not available
- Counting vials 15 ml (Johns diluent vials #DSV002)
- Preferred tube for culturing cells is dependant on the final culture volume. Use 6 ml polystyrene, vented cap, gamma-sterilized, round bottom culture tube (Falcon #2058), for 750 µl isolated lymphocyte culture
- Single-use 0.22 µm filter units, syringe-driven (Millipore-GS, #SLGS0250S)
- 0.2 µm hydrophobic filter (Millipore, Dualex #SSLOVS25XS)
- Cytocentrifuge (e.g., Shandon Cytocentrifuge from Thermo Electron Corporation, <http://www.thermo.com>)
- Microscope slides, frosted end, 76 × 26 mm (3 × 1"), 1 mm thick (HD Scientific MZO2 110)—wiped with alcohol and allowed to dry before use
- Filter cards—Shandon 3 × 1", thick, white, boxes of 200 (from Thermo Electron Corporation)
- Cytocentrifuge cups—supplied with instrument from Thermo Electron Corporation. Must be clean and completely dry before assembly
- Metal slide holders—12 supplied with instrument from Thermo Electron Corporation
- Slide holder opener—supplied with instrument from Thermo Electron Corporation
- Staining racks and containers
- Coverslips, no. 1, 22 × 50 mm (HD Scientific MZ LD2250)
- Tissues or Whatman no. 1 filter paper, no. 371001240, 24 cm diameter (from Lab Supply)
- Microscope with excellent optics for bright-field and fluorescence examination of stained slides at ×1,000 magnification (e.g., Leica DMLB, Nikon Eclipse 600)
- Slide storage boxes (Kartell, no. 278)

REAGENT SETUP

Culture medium Prepare by adding 90 ml of RPMI 1640 medium with 10 ml of 100% FBS, 1 ml of 200 mM L-glutamine and 1 ml of 100 mM sodium pyruvate solution.

Prepare the culture medium in sterile tissue-culture-grade glass or plastic bottles. Smaller volumes of culture medium may be prepared depending on the number of cultures required. Culture medium can be stored for 1 week at 4 °C before use. It is not usually necessary to sterilize the prepared culture medium if rigorous aseptic technique is followed; however, to be absolutely certain, medium may be sterile-filtered using a 0.22 µm filter.

Cyt-B Dissolve 5 mg solid in 8.33 ml DMSO to give a Cyt-B solution concentration of 600 µg ml^{–1} as follows:

- (1) Remove the Cyt-B vial from –20 °C and allow to reach room temperature. Do not remove the seal.
- (2) Sterilize the top of the rubber seal with ethanol and allow the ethanol to evaporate.
- (3) Pipette 8.33 ml of DMSO into a 50 ml sterile Falcon tube.
- (4) Vent the vial seal with a 25 G needle and a 0.2 µm hydrophobic filter to break the vacuum.
- (5) Using a 5 ml sterile syringe and another needle, inject 4 ml of the 8.3 ml DMSO through the seal.
- (6) Mix contents gently. Cyt-B should dissolve readily in DMSO. Leave syringe and needle in place.
- (7) Remove the 4 ml from the vial into the syringe and eject into a labeled sterile TV10 tube.
- (8) Aspirate the remaining 4.3 ml of DMSO into the syringe and inject into the vial as before.
- (9) Remove this final volume from the vial and eject into the TV10 tube.
- (10) Mix and dispense 100 µl aliquots into sterile, 5 ml polystyrene yellow capped tubes labeled Cyt B with the date.
- (11) Store at –20 °C for up to 12 months. The vials of powder are guaranteed by Sigma for 2 years if stored at –20 °C.

PHA The preparation of this reagent has been modified slightly from the instructions in the product insert to suit this protocol. 45 mg solid is dissolved in 20 ml sterile isotonic saline to make a solution of 2.25 mg ml^{-1} as follows:

- (1) Remove the vial from the refrigerator and allow to reach room temperature. Do not remove the seal.
- (2) Sterilize the top of the rubber seal with ethanol and allow the ethanol to evaporate.
- (3) Pipette 20 ml of sterile isotonic saline into a 50 ml sterile Falcon tube.
- (4) Vent the seal with a 25G needle and a $0.22 \mu\text{m}$ hydrophobic filter to break the vacuum.
- (5) Using a 10 ml sterile syringe and another needle, inject 5 ml of the 20 ml isotonic saline into the vial and dissolve the solid.

- (6) Remove the 5 ml solution from the vial into the syringe and eject into another labeled sterile 50 ml Falcon tube.
- (7) Add another 5 ml of isotonic saline to the vial, using the syringe and mix gently.
- (8) Remove this volume from the vial and eject into the labeled Falcon tube.
- (9) Add the remaining 10 ml of isotonic saline to the labeled Falcon tube.
- (10) Dispense 500 μl aliquots into sterile, labeled 1.2 ml vials (cryovial type).
- (11) Store at 4°C for up to 6 months.

Note: The 500 μl aliquots are single-use only. Discard any remaining solution in vial. Discard any vial with signs of bacterial contamination. Slight turbidity may occur but this does not affect the activity of PHA.

PROCEDURE

Isolation and counting of leukocytes ● **TIMING** approximately 3 h

1| Collect fresh blood by venipuncture into vacutainer blood tubes with lithium heparin anticoagulant. Keep blood tubes at room temperature (i.e., $20\text{--}22^\circ\text{C}$). The volume taken is dependent on the number of cells needed. (Usually one can expect to collect up to 1×10^6 leukocytes per 1 ml blood.)

! CAUTION All procedures until slide preparation must be carried out aseptically in a class II Biological Safety Cabinet.

2| Dilute whole blood 1:1 with HBSS ($20\text{--}22^\circ\text{C}$) and gently invert to mix.

3| Gently overlay diluted blood onto Ficoll–Paque using a ratio of 1:3 (e.g., 2 ml Ficoll–Paque:6 ml diluted blood) being very careful not to disturb the interface.

4| Weigh and balance the centrifuge buckets before spinning the tubes at 400g for 30 min at $18\text{--}20^\circ\text{C}$.

5| Remove the leukocyte layer located at the interface of Ficoll–Paque and diluted plasma into a fresh tube, using a sterile, plugged, Pasteur pipette, taking care not to remove too much Ficoll–Paque.

6| Dilute the leukocyte suspension (which includes a mixture of lymphocytes and monocytes) with $3\times$ its volume of HBSS at room temperature ($20\text{--}22^\circ\text{C}$), mix gently and then centrifuge at 180g for 10 min.

7| Discard the supernatant and resuspend the cell pellet (slightly beige color) in $2\times$ the volume removed of HBSS, using a Pasteur pipette, then centrifuge at 100g for 10 min at $18\text{--}20^\circ\text{C}$.

8| Discard the supernatant and gently resuspend the cells in 1 ml culture medium at room temperature, using a Pasteur pipette.

9| Count the cells using the Coulter Counter.

10| Set instrument settings for counting human lymphocytes (e.g., Coulter Counter Model ZB1; threshold: 8, attenuation: 1, aperture: $1/4$, manometer: 0.5 ml).

11| Dilute 15 μl cell suspension into 15 ml isoton + five drops of zapoglobin to lyse any residual red blood cells.

12| Perform the count in triplicate to determine the mean. If the cell count is $>15,000$, count 7.5 μl cells instead, then multiply by 2. Multiply the value by 2,000 (dilution factor of 1,000 and counting volume of 0.5 ml) to give the number of cells per ml.

13| To set the cells up at 1×10^6 per ml in 750 μl culture medium, divide 750 (μl) by the number of cells per ml and multiply by 1. This gives the volume of cells to use in “ μl ”. Make this volume up to 750 μl with medium.

Example: mean cell count = 12,586

$12,586 \times 2,000 = 25.2 \times 10^6$ cells per ml

$750 \mu\text{l} / 25.2 (\times 10^6) \times 1 (\times 10^6) = 29.8 \mu\text{l}$

$29.8 \mu\text{l}$ cell suspension + $720.2 \mu\text{l}$ medium = cell suspension at 1×10^6 per ml in 750 μl .

■ **PAUSE POINT** Cells may be left in medium at this point at room temperature in the dark for 1–2 h with the tubes sealed.

Culture of lymphocytes, addition of Cyt-B and harvesting of cells ● **TIMING** 72 h

14| Using the above calculation (Step 13), resuspend the cells at 1×10^6 per ml into the culture medium. Add the calculated volume of medium into labeled 6 ml round-bottomed culture tubes, then add the cells into the medium to make up a final volume of 750 μl . Set up duplicate cultures per subject and/or treatment studied (**Fig. 6**).

PROTOCOL

15| Stimulate mitotic division of lymphocytes by adding PHA. Add 10 μl of the PHA solution to 750 μl culture to give a final concentration of 30 $\mu\text{g ml}^{-1}$. Discard the remaining PHA after use.

16| Incubate the cells at 37 $^{\circ}\text{C}$ with lids loose in a humidified atmosphere containing 5% CO_2 for exactly 44 h.

▲ **CRITICAL STEP** Cyt-B must be added exactly 44 h after PHA stimulation.

Addition of Cyt-B to culture

17| Thaw out stock vial containing 100 μl solution of Cyt-B in DMSO at 600 $\mu\text{g ml}^{-1}$.

! **CAUTION** Carry out all manipulations of the Cyt-B solutions in a fume hood with the base glass window down to chest level and use the following personal protection: Tyvek gown, double nitrile gloves and safety glasses. These precautions are necessary for protection against biohazardous and cytotoxic aerosols.

18| Aseptically add 900 μl of culture medium equilibrated to room temperature to the vial to obtain a 1,000 μl solution of 60 $\mu\text{g ml}^{-1}$ Cyt-B.

19| Remove 56 μl of medium from the top of the 750 μl culture and replace with 56 μl of the 60 $\mu\text{g ml}^{-1}$ Cyt-B solution to give a final Cyt-B concentration of 4.5 $\mu\text{g ml}^{-1}$.

20| Return cultures to the incubator and incubate the cultures for a further 28 h. Discard the remaining Cyt-B solution.

21| At 24–28 h after addition of Cyt-B, harvest cells for slide preparation and scoring according to the procedure given below. The chosen harvest time should maximize the proportion of BN cells and minimize the frequency of mononucleated and multinucleated (three or more nuclei) cells.

Harvesting of cells using cytocentrifugation ● **TIMING** Approximately 30 min

22| Follow the manufacturer's instructions for assembly of the slides, filter-cards and cytocentrifuge cups within the cytocentrifuge rotor. Cytocentrifugation is performed at 18–20 $^{\circ}\text{C}$.

23| Prepare a concentration of cells that is sufficient to produce a monolayer of cells on each spot. It may be necessary to spin down a culture gently and take off a proportion of the supernatant to obtain an optimal concentration of cells on the slide following cytocentrifugation. In the case of lymphocyte cultures in round-bottomed tubes, it is only necessary to take off the supernatant (approximately 300 μl from a 1 ml culture), as the cells tend to settle in the incubator.

! **CAUTION** Loading and cytocentrifugation of cell culture sample (Steps 23–28) must be carried out in a fume hood or preferably in an approved cytoguard cabinet. Appropriate safety protection including gloves must be worn.

24| Resuspend the cells well using a Pasteur or Gilson pipette to disaggregate the cells. It is best to suspend all the cultures to be harvested first, then return to each one to resuspend just before loading into the sample cup.

25| Working quickly, add 100–200 μl of cell suspension to the well of each sample cup. The typical volume for cytokinesis-blocked lymphocyte cultures is 120 μl . The required volume may need slight adjustment depending on the concentration of cells in the culture and the optimal cell density for slide scoring, which is determined by trial and error.

26| Replace the lid on the rotor and press down on the button in the center to lock. Place the rotor in the cytocentrifuge and click the main lid shut. Set the time and speed parameters recommended by the manufacturer.

27| Press START. The machine will run the program, stop and slow automatically.

28| Returning the rotor to the fume hood, follow the manufacturer's procedure for opening each slide holder.

Drying, fixing and staining of cells and slide preparation ● **TIMING** Approximately 30 min

29| Dismantle each slide holder, taking care not to smear the spots. Discard the filter cards into the biohazard bin. Place the sample cups in the 1% hypochlorite solution to soak for at least 10 min before rinsing carefully six times in pure water and drying. Do not use direct heat to dry the cups.

! **CAUTION** Appropriate protection including nitrile gloves must be worn while staining samples.

30| Place the slides horizontally on a slide tray and allow the cells to air-dry for exactly 10 min at room temperature.

▲ **CRITICAL** Do not air-dry slides for longer than 10 min, otherwise cellular and nuclear morphology is altered making it difficult to score the slides.

31| Place slides vertically in a dry staining rack and place in methanol or Diff-Quik fixative for 10–15 min. The methanol can be reused.

32| Transfer the rack without delay to Diff-Quik solution 1 (orange) and stain for 6 s while moving the rack back and forth. Increase staining time if required.

33| Transfer the rack without delay to Diff-Quik solution 2 (blue) and stain for another 6 s while moving the rack back and forth. Again, staining time can be increased if required.

▲ CRITICAL STEP Staining time is determined by trial and error depending on how fresh the stains are. The aim is to obtain optimal contrast between nuclear and cytoplasmic staining so that various biomarkers in the CBMN Cyt assay are easily and unequivocally scored.

34| Keeping the slides in the rack, wash the slides very briefly and gently with tap water and rinse with pure water, making sure that the slides are not destained by residual water droplets on cells.

35| Immediately place the slides face-down on paper tissues (or better, Whatman no. 1 filter paper) to blot away any residual moisture. Do not place any pressure or rub on the cell spots.

36| Place the slides on a slide tray and allow to dry for about 10–15 min.

37| Examine the cells at $\times 100$ and $\times 400$ magnification to assess efficiency of staining and the density of the cells, remembering that for the CBMN Cyt assay, it is necessary to have at least 1,000 BN cells to score. Restaining can be carried out at this stage if the staining is too light by repeating Steps 30–37. If the cell density is too heavy or light, concentrate or dilute the cells as necessary and repeat the spinning and staining steps.

38| If the slides are satisfactory, seal the tubes or flasks containing the cultures and discard in the biohazard bin.

39| Leave the slides to dry completely for at least 30 min before putting coverslips on.

■ PAUSE POINT Slides can be left overnight at room temperature to dry.

Coverslipping and storage

40| Place the slides to be coverslipped on tissue or paper and set out one coverslip alongside each.

! CAUTION Carry out coverslipping in a fume hood to avoid inhalation of organic solvent in DePex and leave slides there until completely dry.

41| Put two large drops of DePex (use a plastic dropper) on each of the coverslips in the approximate area where the spots correspond.

! CAUTION Wear nitrile gloves when applying DePex medium.

42| Invert the slide over the coverslip and allow the DePex to spread by capillary action. Slide the coverslip gently to and fro to expel any excess DePex and air bubbles. Ensure that the spots do not have air bubbles over them.

43| Wipe excess DePex from the edges of the slide and ensure that the medium or glass does not cover any of the frosted label area, as a coding label will not stick on these.

44| Place the slides on a tray and leave overnight in the fume hood to dry.

45| Store slides in slide boxes at room temperature and code with a sticky label over the frosted area before scoring.

46| Score the biomarkers in the CBMN Cyt assay using the criteria described in **Box 1**. See **Box 2** for details regarding the data that should be obtained from each slide. **Box 3** explains how the NDI is calculated, as well as the purpose of doing the measurement. The basic elements of a typical score sheet are listed in **Table 2** and an example of a score sheet is given in **Table 3**. For a comprehensive photographic gallery of the various cell types scored in the CBMN Cyt assay, refer to Fenech *et al.*⁷². **Figures 7–10** provide diagrammatic explanations of the criteria for selecting BN cells suitable for scoring presence of MNi, NPBs and NBUDs (**Fig. 7**), the other various cell types that are observed and scored in the CBMN Cyt assay (**Fig. 8**), typical size and appearance of MNi (**Fig. 9**) and other structures in BN cells that resemble MNi but in fact represent other abnormal cellular events (**Fig. 10**).

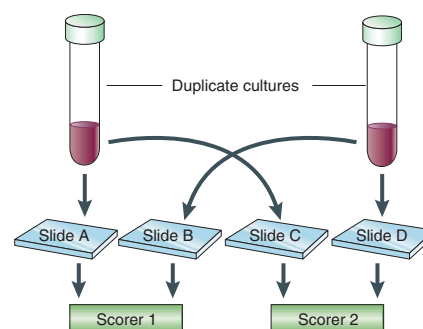


Figure 6 | An optimal sampling and scoring plan for the CBMN Cyt assay that enables an estimation of experimental variation (results for A + C versus B + D) as well as the effect of scorer bias (results for A + B versus C + D).

BOX 1 | SCORING CRITERIA

Criteria for scoring viable mono-, bi- and multinucleated cells

Frequency of viable mono-, bi- and multinucleated cells is measured to determine cytostatic effects and the rate of mitotic division, which can be calculated using the nuclear division index (see **Box 3**). These cell types have the following characteristics:

- Mono-, bi and multinucleated cells are viable cells with an intact cytoplasm and normal nucleus morphology containing one, two and three or more nuclei, respectively.
- They may or may not contain one or more MNi or NBUDs and in the case of bi- and multinucleated cells they may or may not contain one or more NPBs.

Necrotic and apoptotic cells should not be included among the viable cells scored.

On rare occasions, multinucleated cells with more than four nuclei are observed if cell-cycle time is much shorter than normal or the cytokinesis-blocking time is too long.

Figures 2, 3 and 7–10 illustrate some typical examples.

Criteria for scoring apoptotic cells

Apoptotic lymphocytes are cells undergoing programmed cell death. They have the following characteristics:

- Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear membranes.
 - Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane.
 - Staining intensity of the nucleus, nuclear fragments and cytoplasm in both kinds of apoptotic cell is usually greater than that of viable cells.
- Figures 1, 2, 3e and 8** illustrate typical examples of apoptotic cells.

Criteria for scoring necrotic cells

Necrosis is an alternative form of cell death that is thought to be caused by damage to cellular membranes, organelles and/or critical metabolic pathways required for cell survival such as energy metabolism. Necrotic lymphocytes have the following characteristics:

- Early necrotic cells can be identified by their pale cytoplasm, the presence of numerous vacuoles (mainly in the cytoplasm and sometimes in the nucleus), damaged cytoplasmic membrane and a fairly intact nucleus.
- Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary.
- Staining intensity of the nucleus and cytoplasm in both types of necrotic cell is usually less than that observed in viable cells.

Figures 1, 2, 3d and 8 illustrate typical examples of necrotic cells.

Criteria for selecting BN cells suitable for scoring MNi, NPBs and NBUDs

The cytokinesis-blocked BN cells that may be scored for MN, NPB and NBUD frequency should have the following characteristics:

- The cells should be binucleated.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
- The two nuclei within a BN cell may be attached by a nucleoplasmic bridge, which is no wider than 1/4th of the nuclear diameter.
- 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 each nucleus are distinguishable.
- The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

Examples of the types of binucleated cells that may be scored are illustrated diagrammatically in **Figures 1–5 and 7**. The cell types that should not be scored for the frequency of MNi, NPBs and NBUDs frequency include mono- and multinucleated (with three or more nuclei) cells and cells that are necrotic or apoptotic (illustrated in **Fig. 8**).

Criteria for scoring micronuclei

MNi are morphologically identical to but smaller than nuclei. They also have the following characteristics:

- The diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.
- MNi are non-refractile and they can therefore be readily distinguished from artifact such as staining particles.
- MNi are not linked or connected to the main nuclei.
- MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

Examples of typical MNi that meet the criteria set above are shown in **Figures 1–5 and 9**. Examples of cellular structures that resemble MNi but should not be classified as MNi originating from chromosome breakage or loss are illustrated in **Figure 10**.

Criteria for scoring nucleoplasmic bridges

An NPB is a continuous DNA-containing structure linking the nuclei in a binucleated cell. NPBs originate from dicentric chromosomes (resulting from misrepaired DNA breaks or telomere end fusions) in which the centromeres are pulled to opposite poles during anaphase. They have the following characteristics:

- The width of an NPB may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell.
- NPBs should also have the same staining characteristics as the main nuclei.

BOX 1 | SCORING CRITERIA (CONTINUED)

- On rare occasions, more than one NPB may be observed within one binucleated cell.
- A binucleated cell with an NPB may contain one or more MNi.
- BN cells with one or more NPBs and no MNi may also be observed.

Examples of BN cells with NPBs are illustrated in **Figures 1, 2, 3g, 5, 7c, 7d and 9c**.

It may be more difficult to score NPBs in BN cells with touching nuclei, and it is therefore reasonable to specify whether NPBs were scored in all BN cells regardless of proximity of nuclei within a BN cell or whether they were scored separately in those BN cells in which nuclei were clearly separated and those BN cells with touching nuclei. There is not enough evidence yet to recommend scoring NPB only in BN cells in which nuclei do not touch.

Criteria for scoring nuclear buds

An NBUD represents the mechanism by which a nucleus eliminates amplified DNA and DNA repair complexes. NBUDs have the following characteristics:

- NBUDs are similar to MNi in appearance with the exception that they are connected with the nucleus via a bridge that can be slightly narrower than the diameter of the bud or by a much thinner bridge depending on the stage of the extrusion process.
- NBUDs usually have the same staining intensity as MNi.
- Occasionally, NBUDs may appear to be located within a vacuole adjacent to the nucleus.

If it is difficult to determine whether the observed nuclear anomaly is an MN touching the nucleus or a nuclear bud, it is acceptable to classify it as the latter.

A small protrusion of nuclear material from the nucleus without an obvious constriction between the nucleus and the protruding nuclear material should not be classified as a nuclear bud. This type of event is called a nuclear bleb (see **Fig. 10d**). The significance of this type of event is unknown. Typical examples of NBUDs are shown in **Figures 2, 3h and 10c**.

● TIMING

Timing information can be found in **Table 1**.

? TROUBLESHOOTING

Blood and cell storage conditions

The published evidence available suggests that storage of blood between 5 and 22 °C for up to 24 h has no significant impact on baseline or radiation-induced MN frequency⁷³; however, these observations need to be further verified and replicated.

It is possible to perform the CBMN Cyt assay using cryopreserved lymphocytes, but there are conflicting reports on whether cryopreservation alters the frequency rate of MN in BN cells^{74,75}, which means that it is essential to optimize and verify that the freezing and thawing protocol used does not induce DNA damage.

Mitogen stimulation

The optimal PHA concentration for maximizing proportion of BN cells should be verified for each batch of PHA using the BN frequency ratio and NDI to assess mitogenesis. The success of mitogen stimulation can be determined visually 24 h following PHA stimulation. If lymphocytes have been successfully stimulated, cultures will appear clumpy and grainy. Unstimulated cultures will appear cloudy and silty.

Time of addition of Cyt-B and Cyt-B concentration

The critical aspect regarding the time of addition of Cyt-B is to ensure that it is added before the first mitotic cells start to appear so that all the observed BN cells that are captured are in fact once-divided cells only. This is important because MN tend to get lost in subsequent divisions and the MN frequency in a second division cell is likely to be less than that in a first division cell after a genotoxic insult^{4,5}. Cyt-B may take up to 6 h before it starts to exert its cytokinesis-blocking action (unpublished observations), which means it should be added at least 6 h before cells start to enter M phase of the cell cycle. The optimal

TABLE 1 | Timing.

Timeline (days)	Timeline (h)	Activity	Duration of activity based on 12 lymphocyte cultures
Day 1 (e.g., Monday)	—4	Isolate lymphocytes and prepare cultures	240 min
	0	Add PHA	5 min
Day 3 (e.g., Wednesday)	44	Add Cyt-B	10 min
Day 4 (e.g., Thursday)	72 ^a –73	Harvest cells and prepare slides using cytocentrifugation	30 min
		Air-drying of slides	10 min
		Fixation and staining of cells	20 min
Anytime		Slide scoring	480 min (40 min per slide)

^aAn earlier harvest time (e.g., 68 h) is acceptable if this is required to maximize the proportion of BN cells and minimize the proportion of multinucleated cells.

BOX 2 | EXAMINATION OF SLIDES AND SLIDE SCORING FOR ASSESSMENT OF CBMN CYT ASSAY BIOMARKERS

Slides should be coded before scoring by a person not involved in the experiment so that the person who scores the slides is not aware of the treatment conditions, individual or groups to which the cells on the slides belong. Slides are best examined at $\times 1,000$ magnification using a good-quality bright-field or fluorescence microscope (e.g., Leica DMLB, Nikon Eclipse E 600). A score should be obtained for slides from each duplicate culture ideally from two different scorers using identical microscopes (**Fig. 6**). The number of cells scored should be determined depending on the level of change (effect size) in the MN index that the experiment is intended to detect and the expected standard deviation of the estimate. The optimal way to score slides in the CBMN cytome assay is to first determine the frequency of mononucleated, binucleated and multinucleated viable cells as well as the necrotic and apoptotic cells in a minimum of 500 cells; then the frequency of DNA damage biomarkers (MNI, NPBs and NBUDs) are scored in a minimum of 1,000 binucleated cells. For each slide, the following information should therefore be obtained:

1. the number of viable mononucleated, binucleated, multinucleated (with three or more nuclei) cells per 500 cells scored (from this information the Nuclear Division Index can be derived; see **Box 3**);
2. the number of apoptotic cells per 500 cells;
3. the number of necrotic cells per 500 cells;
4. the number of MNI in at least 1,000 binucleate (BN) cells;
5. the frequency of BN cells containing MNI in at least 1,000 BN cells;
6. the frequency of BN cells containing NPBs in at least 1,000 BN cells;
7. the frequency BN cells containing NBUDs in at least 1,000 BN cells.

The distribution of BN cells with zero, one or more MNI may be useful to record if abnormal distribution of MNI among cells may be expected owing to partial body radiation in the case of ionizing radiation exposure or if it is expected that the agent being tested might induce multiple MNI in affected cells (e.g., spindle poisons such as colcemid). Because most BN cells with more than one NPB or more than one NBUD are rare, it is usually not necessary to record distribution of NPBs and NBUDs among BN cells.

The frequency of MNI and NBUDs in non-divided mononuclear cells can also be scored if the level of pre-existing DNA damage needs to be measured; however, this approach cannot be used instead of the CBMN Cyt assay, because it does not account for DNA damage accumulated in the bulk of lymphocytes *in vivo* while circulating in the quiescent phase, it cannot be used to measure NPBs and does not give the same results as scoring MN and NBUDs in BN cells^{3,90,91,97}.

time to add Cyt-B with lymphocytes is usually 44 h after PHA stimulation; however, earlier addition of Cyt-B is acceptable if the culture conditions used cause an earlier than expected mitotic wave to occur.

To capture all once-divided cells as BN cells, it is also essential to verify that the Cyt-B concentration is optimal to maximize the ratio of cytokinesis-blocked cells by doing a dose–response across the concentration range of 2–10 $\mu\text{g ml}^{-1}$ of Cyt-B using a 24 h cytokinesis-blocking time. The dose–response should yield a plateau in response within the optimal concentration range for cytokinesis-blocking. Choose a concentration that is at least a dose-point past the inflection point of the dose–response and on the plateau. Experience has shown that concentrations of 4.5 and 6 $\mu\text{g ml}^{-1}$ are optimal for isolated lymphocyte and whole blood lymphocyte cultures, respectively. These concentrations are usually also optimal for mammalian cell lines but this should be checked for each cell line.

Cytokinesis-blocking time

In maximizing the number of cytokinesis-blocked BN cells by increasing exposure time to Cyt-B, there is the risk of also increasing the proportion of cytokinesis-blocked multinucleated cells, which arise from BN cells that attempt another nuclear division while cytokinesis-blocked. Ideally, the proportion of BN cells among cytokinesis-blocked cells should be in excess of

BOX 3 | CALCULATION OF NUCLEAR DIVISION INDEX (NDI)

The NDI provides a measure of the proliferative status of the viable cell fraction. It is therefore an indicator of cytostatic effects and, in the case of lymphocytes, it is also a measure of mitogenic response, which is useful as a biomarker of immune function²².

NDI is calculated according to the method of Eastmond and Tucker¹⁰⁷. Score 500 viable cells to determine the frequency of cells with 1, 2, 3 or 4 nuclei, and calculate the NDI using the formula

$$\text{NDI} = (M_1 + 2M_2 + 3M_3 + 4M_4)/N,$$

where M_1 – M_4 represent the number of cells with 1–4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells). The NDI is a useful parameter for comparing the mitogenic response of lymphocytes and cytostatic effects of agents examined in the assay.

The lowest NDI value possible is 1.0, which occurs if all of the viable cells have failed to divide during the cytokinesis-block period and are therefore all mononucleated. If all viable cells completed one nuclear division and are therefore all binucleated, the NDI value is 2.0. An NDI value can only be greater than 2.0 if a substantial proportion of viable cells have completed more than one nuclear division during the cytokinesis-block phase and therefore contain more than two nuclei. For example, if 50% of viable cells are binucleated, 10% trinucleated and 10% quadrinucleated, the NDI value is 2.2.

TABLE 2 | Information that should be included on a score sheet for the CBMN Cyt assay.

1. Name of the person scoring the slides
2. Code number of each slide
3. Number of BN cells scored
4. Frequency of MNi in 1,000 BN cells
5. Frequency of BN cells containing MNi in 1,000 BN cells
6. Frequency of BN cells with NPBs in 1,000 BN cells
7. Frequency of BN cells with NBUDs in 1,000 BN cells
8. Frequency of viable mono-, bi-, tri- and tetranucleated cells and necrotic and apoptotic cells in a total of 500 cells
9. Nuclear division index
Optional additions to the scoresheet include (a) distribution of BN cells with 0, 1, 2, 3 or more MNi in 1,000 BN cells and (b) coefficient of variation for duplicate estimates of the above parameters

80%. The proportion of BN and multinucleated cells among all cells will depend on the proportion of dividing cells in the culture and the cell-cycle time, which in turn depends on the cell line and the culture conditions. For human cells, a cytokinesis-blocking period of 24 h is usually optimal for most conditions. In situations when mitotic delay may be expected owing to high levels of DNA damage, a longer cytokinesis-block period may be considered to capture late dividing cells as BN cells.

Controls and important variables

It is important to include a concurrent control sample from a single individual or cell line with each assay run throughout a study period to verify that there are no major variations in experimental conditions that may have impacted on the observed results. Furthermore, a positive control in which cells are challenged with an MN-inducing agent (e.g., ionizing radiation, mitomycin-C) is also recommended to verify that the system responds predictably from one assay batch to the next.

Historical control data are particularly useful in human biomonitoring studies aimed at establishing normal range values for the CBMN assay and to identify key variables affecting the observed frequency of CBMN assay biomarkers, which typically include age, gender, B vitamin status (particularly folate and vitamin B12) and smoking status. It is important in case-control studies that the groups are matched properly for these variables. For more information on the use of controls in biomonitoring studies refer to Albertini *et al.*⁷⁶ and for important variables affecting MN frequency in the CBMN assay in human lymphocytes refer to Bonassi *et al.*⁷⁷. The appropriate use of controls in *in vitro* studies is discussed in detail in the report of the *In Vitro* Micronucleus Assay Working Group⁵⁷.

Hypotonic treatment of cells

Hypotonic treatment for slide preparation is not recommendable because it may destroy necrotic cells and apoptotic cells, making them unavailable for assay. Inclusion of necrosis and apoptosis is important for the accurate description of mechanism of action and measurement of cellular sensitivity to a chemical or radiation. Isolated lymphocyte culture assay or culture of cell lines does not require hypotonic treatment of cells for slide preparation, thus making it possible to preserve the morphology of both necrotic and apoptotic cells.

Slide preparation and staining

In our experience, most of the problems in the CBMN Cyt assay arise during slide preparation and staining. This is because the quality of the score depends on the quality of the slide. Main points to note: (i) avoid cell clumps by gently resuspending cells before harvest and transfer to slides; (ii) maintain a moderate cell density so that it is relatively easy to identify cytoplasmic boundaries; (iii) stain only one slide initially to ensure that staining is optimal before staining the whole batch.

Number of BN cells that should be scored

One of the most common questions is the number of BN cells to be scored in the CBMN assay. The accepted protocol is to score a minimum of 1,000 BN cells per treatment or time point although reports vary between 500 and 2,000 BN cells. An alternative

TABLE 3 | Example of CBMN Cyt assay score sheet.

Study:											
Date:											
Scorer:											
Page no.											
Cytostatic/cytotoxicity score							DNA damage indeces in BN cells				
Slide no. or code	Total no. of cells scored	No. of mononucleated cells	No. of BN cells	No. of multinucleated cells	No. of apoptotic cells	No. of necrotic cells	Total no. of BN cells scored	No. of BN cells with MN	Total no. of MN in BN cells	No. of BN cells with NPB	No. of BN cells with NBud

approach is to keep on scoring BN cells until a fixed number of MNi are observed (e.g., 45 MNi). The latter has the advantage that more BN cells are scored when fewer MNi are induced, thus maintaining similar statistical power across different treatments. The main disadvantage is that more than 2,000 cells may have to be scored in cultures with low MN frequency. In our experience, scoring 1,000 BN cells from each of the duplicate cultures (total 2,000 BN cells) always yields robust results.

Slide scoring

With respect to scoring slides, it is best to first score the frequency of mononucleated, BN, multinucleated, apoptotic and necrotic cells to determine the NDI and frequency of apoptotic and necrotic cells and then focus on scoring BN cells for the presence of MNi, NPBs and NBUDs to determine the genome damage rate. Note that it is best to skip scoring a cell if one is uncertain on how to classify it.

Attention should be given to ensure that MNi, NPBs and NBUDs are scored only in BN cells and not in multinucleated cells because multinucleated cells are not once-divided cells and tend to have greatly elevated MNi frequencies relative to BN cells⁷⁸, which would result in inaccurate genome damage estimates. This issue is particularly important in dense cell preparations, because it may be difficult to distinguish between the two cell types. The elevated MNi frequency in multinucleated cells is artifactual and caused by inability of the spindle to properly segregate the large number of chromosomes in multinucleated cells. For these reasons, it is important to optimize the CBMN Cyt assay protocol to maximize the frequency of BN cells and at the same time minimize the frequency of multinucleated cells.

Reproducibility

The use of duplicate cultures is critical for producing robust results because it also allows the measurement of the intraexperimental coefficient of variation (CV). Cytogenetic assays should be subject to the same rigor as analytical assays, which typically reject duplicate results with a CV greater than 10%. Owing to the visual scoring, greater latitude in the acceptable CV is understandable. However, in our experience and the results of international interlaboratory scoring comparison⁷⁹, CVs greater than 40% are not acceptable for baseline data. With radiation-exposed cultures in which more than 100 MN per 1,000 BN cells are induced, CVs less than 20% are expected. Acceptable CVs for automated systems have yet to be established, but CVs of between 5.4% and 9.5% have been reported recently (see <http://www.imstar.fr/applications/genotoxicity/micronucleiassay/>). Scores from inexperienced personnel (e.g., students, new staff) should not be relied upon until

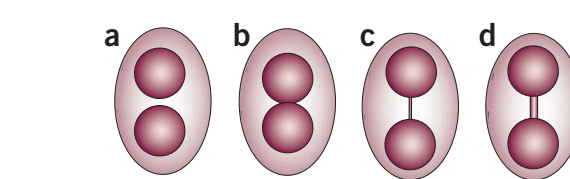


Figure 7 | Criteria for choosing binucleate cells in the cytokinesis-block MN assay. (a) Ideal binucleate cell; (b) binucleate cell with touching nuclei; (c) binucleate cell with narrow NPB between nuclei; (d) binucleate cell with relatively wide NPB. Cells with two overlapping nuclei may be considered suitable to score as BN cells if the nuclear boundaries are distinguishable. Occasionally, BN cells with more than one NPB are observed.

they are able to achieve acceptable CVs (no greater than 40%) for repeat scores of standard control slides and absolute values that are within close range to those obtained by experienced personnel. Interscorer variability is one of the key sources of variation in the MN assay⁷⁹. It is therefore essential that the same scorers are maintained throughout a single study 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. An alternative approach is to calibrate scorers by using a common set of standard slides with “low”, “medium” and “high” MN frequencies. The

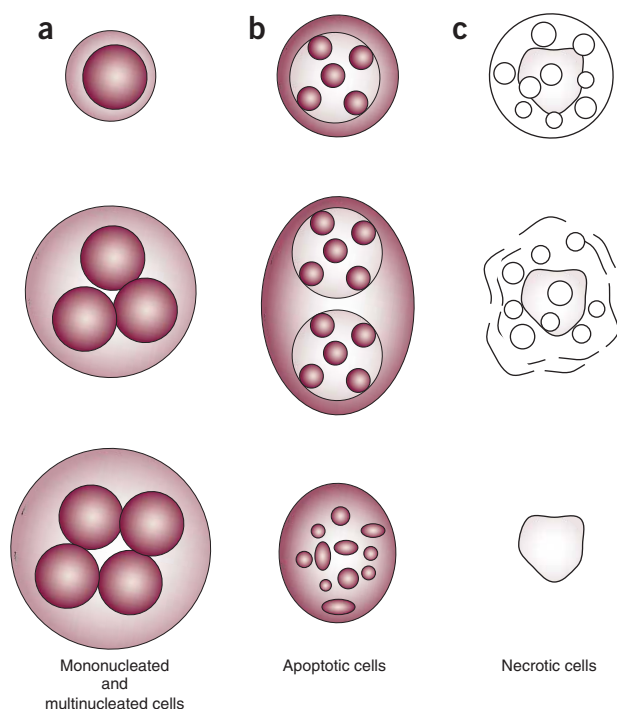


Figure 8 | The various types of cells that may be observed in the *in vitro* cytokinesis-block MN assay excluding BN cells. These cell types shown should not be scored for MN frequency: (a) viable mono-, tri- and quadrinuclear cells; (b) mononucleated and BN cells at early stage of apoptosis when chromatin condensation has occurred but nuclear membrane has not disintegrated and late-stage apoptotic cells with intact cytoplasm, no nucleus and apoptotic chromatin bodies within the cytoplasm; (c) cells at the various stages of necrosis including early stages showing vacuolization, disintegration of cytoplasmic membrane and loss of cytoplasm with an intact nucleus and late stages in which cytoplasm is partially or completely lost and nuclear membrane is visibly damaged and nuclear material is commencing to leak from the remnant nucleus.

scores of each scorer on the standard slides can then be used to calculate a corrected value. The latter approach is still in development but worth noting as an option because it can take account of differences in the visual capacity of scorers within the same laboratory and between laboratories. Another important source of variability between scorers and between laboratories is the quality of the microscopes and their optics. In our experience, scoring of NPBs is influenced by the quality of the microscope because fine bridges can be missed with low-quality optics. The main issue here is for scorers to avoid switching microscopes during experiments and for the laboratory manager to upgrade the optics of the microscopes to a uniform and high level whenever possible. An optimal sampling and scoring schedule for the CBMN Cyt assay is shown in Figure 6.

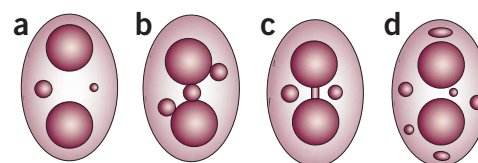


Figure 9 | Typical appearance and relative size of MNi in BN cells. (a) Cell with two MNi one with 1/3 and the other 1/9 the diameter of one of the main nuclei within the cell. (b) MNi touching but not overlapping the main nuclei. (c) A BN cell with NPB between main nuclei and two MNi. (d) A BN cell with six MNi of various sizes; this type of cell is rarely seen in cells that are not exposed to high doses of genotoxins.

Is it necessary to heat-inactivate FBS in the CBMN Cyt assay?

Heat-inactivation (56 °C for exactly 30 min) is only required if it is considered necessary to destroy heat-labile complement proteins that may cause cell lysis. Triglia and Linscott⁸⁰ determined that levels of complement in FBS are a fraction of adult levels and in ten samples of commercial FBS no hemolysis was detected even in undiluted serum. Furthermore prewarming of FBS to 37 °C is enough to inactivate heat-labile complement. In addition, other heat-labile components such as certain vitamins (e.g., folic acid), growth factors, amino acids, etc. may be diminished by the heat treatment. Therefore, the choice to use heat-inactivated FBS or nontreated FBS is optional and depends on whether the heat inactivation of FBS causes any significant effects on growth and genome stability of cells tested in the CBMN Cyt assay. The CBMN Cyt assay for lymphocytes can be performed using untreated FBS.

ANTICIPATED RESULTS

The anticipated results with the CBMN Cyt assay depend on the culture conditions, level of exposure to genotoxic or cytotoxic agents and their potency, the nutrient composition of the medium and the genetic background, age and gender of the donor of the cells being tested.

For normal peripheral blood lymphocytes cultured under optimal conditions using the protocol described above, one can usually expect the following ranges of values

- frequency of BN cells: 30–60%,
- NDI: 1.3–2.2,
- necrotic cells: 0–9%,
- apoptotic cells: 0–7%,
- MNi per 1,000 BN cells: 0–30,
- NPBs per 1,000 BN cells: 0–10,
- NBUDs per 1,000 BN cells: 0–5.

MNi frequency in human peripheral blood lymphocytes measured using the CBMN Cyt assay increases with age and tends to be higher in females relative to males by a factor of approximately 1.4 owing to the random loss of the inactive X chromosome, which may lag at anaphase as a result of defective centromere or kinetochore function^{6,13,65,46}.

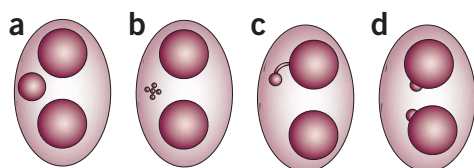


Figure 10 | Occasionally, BN cells (or cells that resemble BN cells) may contain structures that resemble MNi but should not be scored as MNi originating from chromosome loss or chromosome breakage. These situations include (a) a trinucleated cell in which one of the nuclei is relatively small but has a diameter greater than 1/3 the diameter of the other nuclei; (b) dense stippling in a specific region of the cytoplasm; (c) NBUD that appears like an MN with a narrow nucleoplasmic connection to the main nucleus and (d) nuclear blebs consisting of nuclear material protruding from the nucleus but without an obvious constriction or bridge between the protruding nuclear material and nucleus.

The frequency of the biomarkers in the CBMN Cyt assay may vary in their extent and relative to each other depending on the genotoxic and cytotoxic mechanism of specific chemicals, radiation type and nutrient deficiency at different doses. For example, X-rays are efficient in inducing genome damage biomarkers with relatively small effects on NDI and cell death, whereas on the other hand, exposure to hydrogen peroxide tends to induce high levels of cell death with relatively low levels of DNA damage biomarkers in human lymphocytes^{6,20,23,39}. Cells with genetic defects in DNA repair, antioxidant response or apoptosis may exhibit abnormally high levels of MNi, NPBs, NBUDs, necrosis and apoptosis as well as reduced NDI.

Risk of false negative results if the assay is performed without cytokinesis-block

There has been an interest in exploring the possibility of performing the *in vitro* MN assay without Cyt-B to minimize concern of a possible confounding effect of Cyt-B while running the potential risk of obtaining a false negative result because of inadequate control of cell division kinetics because, for example, inhibition of nuclear division by the agent being tested is likely to lead to an underestimate of MN expression given that both once-divided and nondivided cells are included in the denominator of the MN frequency ratio^{9,81}. One of the reasons for considering performing the MN assay without Cyt-B is the concern that Cyt-B, used to accumulate BN cells, may interfere with the expression of MN⁶³. However, studies with normal cells do not show an induction of MNi by Cyt-B or a dose-response effect of Cyt-B with MN frequency in BN cells at doses that are usually used to block cells in cytokinesis^{5,78,82–84}. One study suggested that MN expression induced by spindle poisons may be less than expected in cytokinesis-blocked BN cells because of pole-to-pole distance shortening, which may increase the probability of reinclusion of lagging chromosome fragments or whole chromosomes back into a nucleus, but this did not diminish the effectiveness of the CBMN assay⁸⁵.

Although the evidence of obtaining a false positive result with the CBMN assay in normal cells is lacking, there is already adequate evidence that performing the MN assay in a manner that does not account for inhibition of nuclear division can lead to false negative results or an underestimate of MN induction in human lymphocyte cultures^{5,6,8,81,84}. An example of false negative result of MN assay without Cyt-B is shown in **Figure 11**, which would also have been predicted from the mathematical model that was developed to demonstrate the effect of altered cell division kinetics on MN assay outcomes⁹. Nevertheless, recent studies comparing the MN assay with or without Cyt-B suggest that if cell lines with good growth characteristics are used and culture and nuclear division conditions are optimal, it is possible to obtain comparable results between the CBMN assay and the MN assay without Cyt-B when strong clastogens are tested⁸⁶. However, genotoxicity tended to be detected at lower concentrations with the CBMN assay because of the greater statistical power afforded by the fact that BN cells contain twice the number of MNi per cell than would be the case for their mononucleated daughter cells^{81,84,87,88}. Furthermore, without a cytokinesis-block it becomes impossible to score NPBs, which can only be observed in BN cells and this would result in a critical genotoxic event being missed. In addition, non-disjunction events studied using chromosome-specific centromeric probes are best observed in BN cells when the actual distribution of chromosomes between daughter nuclei within one cell after anaphase can be observed directly^{15–19,55}. In addition, the use of Cyt-B makes it easier to score apoptotic cells because it inhibits the disintegration of apoptotic cells into smaller apoptotic bodies because the latter process requires microfilament assembly⁸⁹, which is readily inhibited by Cyt-B⁷.

Finally, a mathematical model of MN expression predicts (i) that scoring MN in BN cells is the most reliable way of determining MN frequency and (ii) scoring MNi in mononucleated cells in cultures without cytokinesis-block is likely to generate false negative results when nuclear division is significantly inhibited by the chemical tested or the culture conditions do not allow an optimal number of dividing cells⁹. Consequently, results for MN frequency obtained by scoring MNi in mononucleated cells in cultures without Cyt-B cannot be considered conclusive and a negative result with this system should be confirmed using the CBMN Cyt assay. The mathematical model paper⁹ is an essential reading to have a thorough appreciation of the effect of nuclear and cell division kinetics on MN frequency.

When is it justifiable to also score MNi in mononucleated cells in the CBMN Cyt assay?

It is justifiable to also score MNi in mononucleated cells in the CBMN Cyt assay under the following circumstances:

- (1) Information on the frequency of MNi already present within lymphocytes *in vivo* may be required in chronic exposure situations because this reflects the expression of MNi in lymphocyte precursor cells (in the thymus, bone marrow, spleen and lymph nodes) exposed to the genotoxic agent. This information may also be useful if it is considered necessary to correct the results of the CBMN Cyt assay for MNi already present within lymphocytes; however, this may not be justified because it remains unclear whether lymphocytes with pre-existing MNi can divide *ex vivo*. The best time point to measure MNi already present in lymphocytes is approximately 24 h after PHA stimulation when activated lymphocytes have a larger cytoplasmic area, which facilitates MNi scoring^{90,91}.
- (2) The agent being examined in an *in vitro* assay is suspected to cause mitotic slippage and thus prevent formation of BN cells following DNA replication⁹¹.

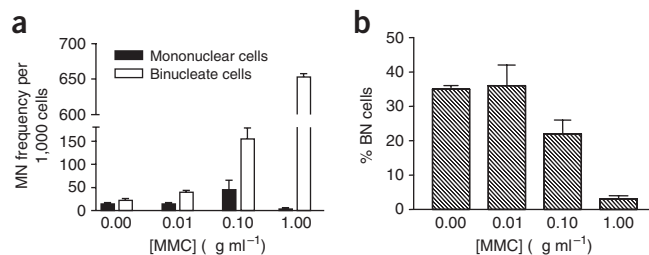


Figure 11 | False negative result if Cyt-B is not used. **(a)** Comparison of the MN dose-response in human lymphocytes exposed *in vitro* in G1/S/G2 to mitomycin-C (MMC) measured either in mononucleated cells in cultures without Cyt-B (solid black bars) or in BN cells in cultures with Cyt-B (white bars). **(b)** The level of dividing cells assessed by measuring the percentage of BN cell in the cytokinesis-blocked cultures. It is evident that the assay without Cyt-B underestimates the extent of genetic damage induced by MMC, particularly at doses that inhibit nuclear division. The data represent the mean \pm 1 s.e. of three replicate cultures.

Other important aspects of the CBMN Cyt assay

In this manuscript, only the CBMN Cyt assay protocol for isolated lymphocyte cultures is described in detail; however, similar methods can be applied to whole blood lymphocyte cultures, other cell culture systems and other cell types, which are of similar importance as the isolated lymphocyte system in terms of usage. Furthermore, the CBMN Cyt assay includes other important aspects such as the use of molecular cytogenetic techniques to determine the mechanism of formation and DNA content of MNI and NPBs and abnormal distribution of chromosomes among nuclei in a BN cell. Therefore, it is the purpose of the following sections to provide further information and details on these other important aspects of the CBMN Cyt assay.

CBMN Cyt assay in other cell culture systems

Whole blood cultures for human lymphocytes

The CBMN Cyt assay in human lymphocytes can also be performed using whole blood cultures. Typically, 0.5 ml of whole blood is added to 4.5 ml of culture medium (e.g., RPMI 1640) supplemented with fetal calf serum containing L-glutamine, antibiotics (optional) and PHA. Cyt-B is added at 44 h after PHA stimulation. The recommended optimal concentration of Cyt-B for accumulating BN cells in whole blood cultures is $6 \mu\text{g ml}^{-1}$. The BN lymphocytes are harvested 24 h after adding Cyt-B as follows.

- (1) the cells are centrifuged gently (300g) for 5 min and the supernatant culture medium is removed;
- (2) the cells are hypotonically treated with 7 ml of cold (4°C) 0.075 M KCl to lyse red blood cells and centrifuged immediately (300g) for 8 min;
- (3) the supernatant is removed and replaced with 5 ml fixative consisting of methanol:acetic acid (3:1) (the fixative should be added while gently agitating the cells to prevent clumps forming);
- (4) the cells are then centrifuged again at 300g for 8 min and washed with two further changes of fixative;
- (5) the cells are resuspended gently and the suspension is dropped onto clean glass slides and allowed to dry.

As an alternative, it is also possible to isolate the BN lymphocytes directly from the whole blood culture using Ficoll gradient separation, followed by one wash in culture medium, and then transfer cells to slides by cyto centrifugation before fixation and staining. This approach precludes the requirement for hypotonic treatment and enables optimal preservation of the cytoplasm. The Ficoll separation method is preferable to the hypotonic treatment protocol for slide preparation because hypotonic treatment could result in loss of morphology and lysis of necrotic and apoptotic cells. Staining of cells can be carried out using either 10% Giemsa in potassium phosphate buffer (pH 7.3) or Diff-Quik for light microscopy or acridine orange ($10 \mu\text{g ml}^{-1}$ in phosphate-buffered saline, pH 6.9) for fluorescence microscopy.

Murine lymphocyte cultures

Lymphocytes are isolated either from the spleen or peripheral blood and cultured according to the procedures described by Fenech *et al.*⁹². Because murine lymphocytes have shorter cell division cycles than human lymphocytes, it is essential to add Cyt-B no later than 18 h after stimulation by mitogen and to harvest the cells 20–30 h later. Depending on the culture conditions, it is possible to obtain good binucleate ratios at 42 h after mitogen stimulation.

Other primary cell cultures

The CBMN Cyt assay can be readily adapted to other primary cell types to assess DNA damage induced *in vitro*, *in vivo* or *ex vivo*. The most important points to remember are (a) to ensure that MNI, NPBs and NBUDs are scored in the first nuclear division following the genotoxic insult and (b) to perform preliminary experiments to determine the concentration of Cyt-B and incubation time at which the maximum number of dividing cells will be blocked at the binucleate stage. It is also important to remember that Cyt-B may take up to 6 h before it starts to exert its cytokinesis-blocking action (unpublished observation).

When using established or primary cell lines from dividing cell populations, it is usual to add Cyt-B shortly after exposure to genotoxin to capture all cells undergoing their first nuclear division as BN cells—this usually requires an incubation period of about 24–48 h, depending on the cell-cycle time, before harvesting the cells.

Attached cells can be trypsinized and then prepared by cyto centrifugation as described for human lymphocytes. Specific methods have been described for use with nucleated bone marrow cells⁹³, lung fibroblasts⁹⁴, skin keratinocytes⁹⁵ and primary tumor cell cultures⁹⁶.

It is generally more practical to assess *in vivo* induction of MNI by blocking cytokinesis in dividing cells after the cells have been isolated from the animal and placed in culture medium in the presence of Cyt-B; this approach has proven to be successful with a variety of cell types including fibroblasts, keratinocytes and nucleated bone marrow cells^{93–96}.

Treatment schedules for *in vitro* genotoxicity testing

The cytokinesis-block MN assay has become one of the standard methods for determining the safety of chemicals and pharmaceuticals^{57,58,63,81,84,86}. Ideally, each chemical under investigation should be tested for its genotoxic potential at the various stages of the cell cycle. Because human peripheral blood lymphocytes are in the G_0 phase when collected, they can be used for assessing damage induction at this stage. However, cells are expected to be more sensitive to genotoxic effects during S, G_2 and M phase, and for this purpose, it is essential to expose cell cultures when most cells are dividing. Because MN expression requires one nuclear division to be completed, the period between treatment and harvest time has to allow for this.



PROTOCOL

With human peripheral blood lymphocytes treated in G_0 , it is necessary to accumulate BN cells as early as possible and for as long as possible to ensure that even cells experiencing mitotic delay are examined. Typically, the standard protocol of adding Cyt-B at 44 h and harvesting cells at 72 h should suffice for this purpose; however, a second harvest of cells at 96 h may maximize the number of late-dividing BN cells available for analysis.

If treatment of cells in G_1 , S, G_2 and M phases is required, as would be the case with cell lines, then exposure to the chemical should occur during the logarithmic growth phase of the culture, followed shortly afterwards by Cyt-B to accumulate dividing cells, and cells are then harvested 6 and 48 h later depending on the stage of the cell cycle that is being examined and whether the treatment causes substantial mitotic delay. At the very early harvest times, mainly cells exposed in G_2 or late S phase are accumulated as BN cells, whereas at the later harvest time, cells exposed in all stages of the cell cycle are blocked in the binucleate stage. Thus, the harvest time relative to Cyt-B addition would affect the type of cell examined.

The most efficient way to do the MN assay in a manner that minimizes false negative results is to use the Cyt-B method and a treatment phase of 24 h duration to cover all phases of the cell cycle and harvesting cells 24 and 48 h after Cyt-B addition in the recovery phase to allow for any cell-cycle delay in damaged cells as outlined in **Figure 12**. In the case of lymphocytes, it would be preferable to delay the start of the treatment phase to 48 h after mitogen stimulation at which point a large proportion of the mitogen-activated cells would have progressed from G_1 to S, G_2 and M phases of the cell cycle. By scoring MN in both mononucleated and BN cells, it should be possible to account for MN expressed before Cyt-B block as well as cells with MN that fail to divide and cells that undergo mitotic slippage^{91,97}. The suggested protocol also allows for harvesting of cells at the end of the treatment phase before Cyt-B addition if there is a need to test whether the chemical inhibits cytokinesis. Examples of other typical schedules for use of the CBMN Cyt assay for *in vitro* genotoxicity testing are summarized in **Table 4**. The use of a metabolic activation system such as S9 mix should be included as

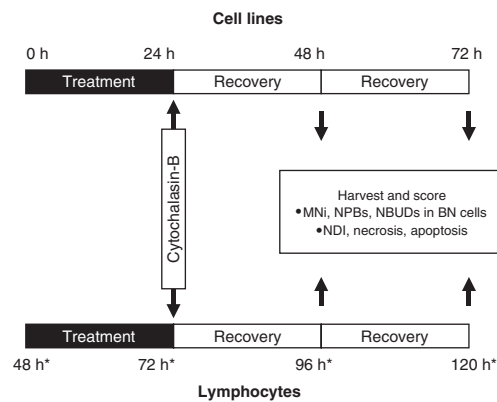


Figure 12 | A scheme for the *in vitro* MN assay that is intended to minimize the risk of false negative results in both cell lines and lymphocytes by allowing for an adequately long treatment period (24 h) during all stages of the cell cycle as well as a sufficiently long recovery period with two harvest time points (24 and 48 h after completion of the treatment) to allow adequate time for cells to complete nuclear division post exposure and make sure that cells with a long cell-cycle delay are not missed in the assay. The protocol also allows the harvesting of cells immediately before addition of Cyt-B if it was necessary to test for induction of polyploidy via multinucleation or cytokinesis-blocking action of the agent tested or if it was considered useful to score MN in cells without the Cyt-B block. It is assumed that the cell lines are already in log phase growth when tested. *The time points for lymphocytes refer to number of hours after mitogen stimulation. MNi, micronuclei; NPBs, nucleoplasmic bridges; NBUDs, nuclear buds; BN, binucleated; NDI, nuclear division index.

TABLE 4 | Typical protocols used for testing the genotoxic effects of a chemical or radiation using the CBMN Cyt assay.

	Peripheral blood human lymphocytes				Cell lines in log phase
Culture time (h)	CBMN assay, G_0 exposure	CBMN assay, G_1 /S exposure	CBMN assay, G_1 /S/ G_2 /M exposure*	CBMN/ARA assay, G_0 exposure	CBMN assay, G_1 /S/ G_2 /M exposure*
-24	Start exposure to test agent			Start exposure to test agent	Start exposure to test agent
0	(i) Wash out test agent (ii) Add PHA	Add PHA	Add PHA	(i) Wash out test agent (ii) Add PHA (iii) Add ARA	Add Cyt-B
16				(i) Wash out ARA (ii) Fresh medium with IL-2 and DC	
20		Start exposure to test agent			
24					Harvest cells
44	Add Cyt-B	(i) Wash out test reagent (ii) Add Cyt-B	Start exposure to test agent Add Cyt-B	Add Cyt-B	
48					Harvest cells
72	Harvest cells	Harvest cells	Harvest cells	Harvest cells	
96	Harvest cells	Harvest cells	Harvest cells	Harvest cells	

*In these protocols the test agent is retained in the culture medium even after Cyt-B is added. However, it may also be desirable to remove test chemical by replacing culture medium (a) after exposure period to test chemical or (b) just before addition of Cyt-B. IL-2 may be added to fresh medium for lymphocyte cultures. ARA = cytosine arabinoside; Cyt-B = cytochalasin-B; DC = deoxycytidine; IL-2 = interleukin-2; PHA = phytohemagglutinin.

an option when testing new chemicals^{57,58,63} but this could limit the exposure period owing to the possible cytotoxicity of S9 to the target cells. A better option may be the use of metabolically competent cells such as genetically modified MCL-5 cells⁹⁸.

Molecular techniques for studying mechanisms for MN and NPB formation and non-disjunction in BN cells

To take full advantage of the CBMN assay, it is essential to distinguish between MNi originating from whole chromosomes or acentric fragments. This can be achieved by using probes that are specific for the centromeric DNA or antibodies that bind to the kinetochore proteins that are assembled at the centromeric regions of active chromosomes. The use of MN size as a discriminant is not recommended for human cells or other cell types in which the size of chromosomes is heterogenous because a small MN may contain either a fragment of a large chromosome or a whole small chromosome. The anti-kinetochore antibody method^{52,53} has some limitations because this approach does not distinguish between unique chromosomes and may not detect chromosome loss occurring due to absence of kinetochores on inactive centromeres as may occur in the case of the inactive X chromosome⁵⁴. The use of ISH to identify centromeric regions is preferable because it does not suffer from these limitations. In addition, using centromeric probes that are specific for one or more unique chromosomes allows detection of non-disjunctional events (i.e., unequal distribution of homologous chromosomes in daughter nuclei) in BN cells, which provides further information on events causing aneuploidy and altered gene dosage^{12–19}. For details on the use of the kinetochore detection methods, refer to the paper of Fenech and Morley⁵³, and for methods relating to centromere detection by ISH, refer to the papers by Farooqi *et al.*¹², Hando *et al.*¹³, Ehajouji *et al.*^{15,17}, Schuler *et al.*¹⁸ and Wang *et al.*⁹⁹. The types of results that can be expected with the various techniques are illustrated in **Figure 4**.

A combination of pancentromeric and pantelomeric probes can also be used to distinguish between (a) MN containing whole chromosomes or acentric chromosome fragments and (b) NPBs originating from misrepair of double strand breaks in non-telomeric DNA and NPB caused by telomere end fusion of chromosomes (**Fig. 5**). The use of molecular probes to determine the content of MNi has been extensively reviewed recently⁴⁶.

Measurement of excision-repaired DNA lesions in G₀/G₁ human lymphocytes using the cytosine arabinoside MN assay in human lymphocytes

After assessing the MN response in human G₀ lymphocytes following exposure to a variety of genotoxins, it became evident that the extent of MN formation in relation to cytotoxicity was low for chemicals and ultraviolet radiation which mainly induce base lesions and adducts on DNA rather than strand breakage or spindle damage¹⁰⁰. We hypothesized that this was due to either efficient repair of the lesions or that such sites, if left unrepaired, do not convert to a double-stranded break in DNA following one round of DNA synthesis. Furthermore, we reasoned that inhibition of the gap-filling step in excision repair by cytosine arabinoside (ARA) would result in the conversion of such base lesions to a single-stranded break that would become a double-stranded break following DNA synthesis leading to the production of an acentric fragment, which would then be expressed as an MN within one division cycle^{100,101}.

Using this concept (illustrated in **Fig. 13**), we showed that addition of ARA during the first 16 h of lymphocyte culture (i.e., before DNA synthesis) did result in a dramatic increase (tenfold or greater) in the MN dose-response following ultraviolet or *N*-nitroso-*N*-methyl urea treatment. However, the ARA-induced increase following X-ray exposure was only 1.8-fold as would be expected from the proportion of DNA adducts or base lesions relative to the induction of DNA strand breaks. This method has since been used to identify pesticides that induce excision repair and to distinguish between genotoxic agents that do or do not induce excision repair¹⁰².

The ARA protocol is an important adjunct to the basic CBMN assay in lymphocytes and should be attempted particularly if strong cytotoxic effects are observed in conjunction with weak MN induction. Precise measurement of excision-repaired DNA lesions using the ARA method is only possible using the CBMN assay because (a) the conversion of excision-repaired DNA lesions to MN occurs only in cells that have completed nuclear division and (b) the addition of ARA may also result in significantly altered cell division kinetics, which could confound results in MN assays without Cyt-B.

ARA inhibition of DNA polymerase may cause DNA strand breaks in cells undergoing replicative DNA synthesis. Therefore, it is only possible to use this method in PHA-stimulated G₀ lymphocytes with ARA exposure occurring during the G₁ phase and before S phase, because excision repair is activated during G₁. In practice, this means that lymphocytes are cultured in the presence of ARA during the first 16–20 h after PHA stimulation, following which the cells are washed to remove ARA and incubated in culture medium containing deoxycytidine to reverse ARA inhibition of DNA polymerase. After these steps,

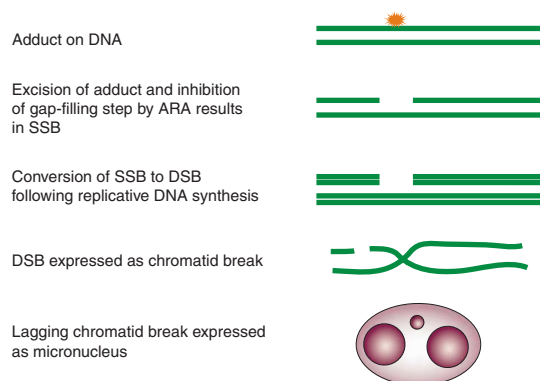


Figure 13 | A schematic diagram explaining the mechanism for the conversion by ARA of an excision-repairable DNA lesion to an MN within one division cycle. DSB, double strand break; SSB, single strand break.

the standard CBMN protocol (described above) is followed with the exception that interleukin-2 is added to replenish this mitogenic cytokine, which is generated by activated lymphocytes but otherwise lost during the washing process. For more details on the procedure and typical results, refer to Fenech and Neville¹⁰⁰ and Surrales *et al.*¹⁰².

Future developments

It is evident that the *ex vivo/in vitro* MN assay has evolved into a robust assay for genetic damage with applications in ecotoxicology¹⁰³, nutrition¹⁰⁴, radiation sensitivity testing both for cancer risk assessment¹⁰⁵ and optimization of radiotherapy^{64,106}, biomonitoring of human populations⁶⁰ and importantly testing of new pharmaceuticals and other chemicals^{63,86}. There is little doubt that there is a need for an automated scoring system for quicker and more reliable non-subjective data acquisition, which would ideally be based on the scoring of slides also prepared for visual scoring—this should enable consistent results to be obtained that are not influenced by the inter-individual and temporal variability of human scorers. For this goal to be achieved, it is essential that scoring criteria are well developed and that a robust slide preparation protocol be put in place and that slide preparations be permanent so that they can be re-examined visually if necessary. Automated image analysis systems have recently been developed for scoring of MNi in mammalian cells^{66–71} (see <http://www.imstar.fr/applications/genotoxicity/micronucleiassay/>; http://www.metasystems.de/products/metafer/metafer_msearch.htm#Micronucleus; <http://www.biocompare.com/techart.asp?id=1247>) but these systems do not take into account other important events such as NPBs, NBUDs, necrosis, apoptosis and cytostasis, which are essential for the correct interpretation of the result obtained. In the future, we should expect to have an automated system that can score reliably the various end points possible with the CBMN Cyt assay outlined in this paper.

Finally, it is essential to keep abreast of more recent developments in our understanding of MN, NPB and NBUD formation and events that may alter expression of these genotoxicity end points. Furthermore, scoring criteria in the CBMN Cyt assay are continually being reviewed as part of the HUMN project activity and protocols for validation of automated systems are also currently under consideration by the HUMN project and will be developed in the near future.

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COMPETING INTERESTS STATEMENT The author declares no competing financial interests.

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Corrigendum: Cytokinesis-block micronucleus cytome assay

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The version of this article initially published stated that the amount of FBS required to make up the culture medium was 10 ml of a 10% solution. The actual amount required is 10 ml of a 100% solution. The error has been corrected in the HTML and PDF versions of the article.