OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Proposal for updating Test Guideline 487

In Vitro Mammalian Cell Micronucleus Test

INTRODUCTION

1. The *in vitro* micronucleus (MNvit) assay is a genotoxicity test for the detection of micronuclei (MN) in the cytoplasm of interphase cells. Micronuclei may originate from acentric chromosome fragments (*i.e.* lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The assay detects the activity of clastogenic and aneugenic chemicals (1) (2) in cells that have undergone cell division during or after exposure to the test substance. This Test Guideline allows the use of protocols with and without the actin polymerisation inhibitor cytochalasin B (cytoB). The addition of cytoB prior to the targeted mitosis allows for the identification and selective analysis of micronucleus frequency in cells that have completed one mitosis because such cells are binucleate (3) (4). This Test Guideline also allows the use of protocols without cytokinesis block, provided there is evidence that the cell population analysed has undergone mitosis.

2. In addition to using the MNvit assay to identify chemicals that induce micronuclei, the use of a cytokinesis block, immunochemical labelling of kinetochores, or hybridisation with centromeric/telomeric probes (fluorescence *in situ* hybridisation (FISH)), also can provide information on the mechanisms of chromosome damage and micronucleus formation (5) (6) (7) (8) (9) (10) (11) (12) (13) (14) (15) (16). The labelling and hybridisation procedures can be used when there is an increase in micronucleus formation and the investigator wishes to determine if the increase was the result of clastogenic and/or aneugenic events.

3. Micronuclei represent damage that has been transmitted to daughter cells, whereas chromosome aberrations scored in metaphase cells may not be transmitted. In either case, the changes may not be compatible with cell survival. Because micronuclei in interphase cells can be assessed relatively objectively, laboratory personnel need only determine whether or not the cells have undergone division and how many cells contain a micronucleus. As a result, the preparations can be scored relatively quickly and analysis can be automated. This makes it practical to score thousands instead of hundreds of cells per treatment, increasing the power of the assay. Finally, as micronuclei may arise from lagging chromosomes, there is the potential to detect aneuploidy-inducing agents that are difficult to study in conventional chromosomal aberration tests, *e.g.* OECD Test Guideline 473 (17). However, the MNvit assay does not allow for the differentiation of chemicals inducing changes in chromosome number and/or ploidy from those inducing clastogenicity without special techniques such as FISH described under paragraph 2.

4. The MNvit assay is an *in vitro* method that typically uses cultured human or rodent cells. It provides a comprehensive basis for investigating chromosome damaging potential *in vitro* because both aneugens and clastogens can be detected.

5. The MNvit assay is robust and effective in a variety of cell types, and in the presence or absence of cytoB. There are extensive data to support the validity of the MNvit assay using various rodent cell lines (CHO, V79, CHL/IU, and L5178Y), human cell line TK6 and human lymphocytes (18) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) (29) (30) (31) (35) (B2) (B3) (B4). These include, in particular, the international validation studies co-ordinated by the Société Française de Toxicologie Génétique

(SFTG) (18) (19) (20) (21) (22) and the reports of the International Workshop on Genotoxicity Testing (4) (16). The available data have also been re-evaluated in a weight-of-evidence retrospective validation study by the European Centre for the Validation of Alternative Methods (ECVAM) of the European Commission (EC), and the test method has been endorsed as scientifically valid by the ECVAM Scientific Advisory Committee (ESAC) (32) (33) (34). HepaRG (B5)(B6), HepG2 cells (36) (37) and primary Syrian Hamster Embryo cells (38) has been described, although they have not been used in validation studies. At the present time, the available data suggest it is important to consider the *p53* status, genetic (karyotype) stability, DNA repair capacity and origin (rodent *versus* human) of the cells chosen for testing (Pfuhler *et al.,* 2011). These characteristics may be considered relevant for demonstration of chemical safety in human population.

6. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS

7. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation unless the cells are metabolically competent with respect to the substances being tested. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions. Care should also be taken to avoid conditions that would lead to artifactual positive results which do not reflect intrinsic mutagenicity, and may arise from such factors as marked changes in pH or osmolality, or by high levels of cytotoxicity (39) (40) (41).

8. To analyse the induction of micronuclei, it is essential that mitosis has occurred in both treated and untreated cultures. The most informative stage for scoring micronuclei is in cells that have completed one mitosis during or after treatment with the test substance.

PRINCIPLE OF THE TEST

9. Cell cultures of human or mammalian origin are exposed to the test substance both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used.

10. During or after exposure to the test substance, the cells are grown for a period sufficient to allow chromosome or spindle damage to lead to the formation of micronuclei in interphase cells. For induction of aneuploidy, the test substance should ordinarily be present during mitosis. Harvested and stained interphase cells are analysed for the presence of micronuclei. Ideally, micronuclei should only be scored in those cells that have completed mitosis during exposure to the test substance or during the post-exposure period, if one is used. In cultures that have been treated with a cytokinesis blocker, this is achieved by scoring only binucleated cells. In the absence of a cytokinesis blocker, it is important to demonstrate that the cells analysed are likely to have undergone cell division during or after exposure to the test substance. For all protocols, it is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test substance-induced cytotoxicity or cytostasis should be assessed in the cultures (or in parallel cultures) that are scored for micronuclei.

DESCRIPTION OF THE METHOD

Preparations

11. Cultured primary human peripheral blood lymphocytes (5) (19) (42) (43) and a number of rodent cell lines such as CHO, V79, CHL/IU, and L5178Y cells or human cell lines such as TK6 may be used (18) (19) (20) (21) (22) (25) (26) (27) (28) (30) (35)) (B2) (B3) (B4) (see §5.). The use of other cell

lines and types should be justified based on their demonstrated performance in the assay, as described in the Acceptability Criteria section. Because the background frequency of micronuclei will influence the sensitivity of the assay, it is recommended that cell types with a low, stable background frequency of micronucleus formation and a stable karyotype be used.

12. Human peripheral blood lymphocytes should be obtained from young (approximately 18-35 years of age), healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation. [*If cells from more than one donor are pooled for use, the number of donors should be specified. The micronucleus frequency increases with age and this trend is more marked in females than in males (44) and this should be taken into account in the selection of donor cells for pooling*] [Alternative: *It should be considered in the selection of donor cells for pooling that the baseline incidence of micronucleus frequencies increases with age and that this trend is more marked in females than in males (44)*]. Cell cultures are maintained in an exponential cell growth and no more synchronized during exposure to the test substance.

Media and culture conditions

13. Appropriate culture medium and incubation conditions (culture vessels, CO_2 concentration, temperature, and humidity) should be used for maintaining cultures. Cell lines should be checked routinely for the stability of the modal chromosome number and the absence of *Mycoplasma* contamination, and should not be used if contaminated or if the modal chromosome number has changed. The normal cell cycle time for the culture conditions used in the testing laboratory should be established and appropriate to the cell line. If the cytokinesis-block method is used then the concentration of the cytokinesis inhibitor should be optimised for the particular cell type and should be shown to produce a good yield of binucleate cells for scoring.

Preparation of cultures

14. Cell lines: cells are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency in monolayers, and suspension cultures will not reach excessive density before the time of harvest, and incubated at 37°C.

15. Lymphocytes: whole blood treated with an anti-coagulant (*e.g.* heparin), or separated lymphocytes, are cultured at 37°C in the presence of a mitogen *e.g.* phytohaemagglutinin (PHA) prior to exposure to the test substance and cytoB.

Metabolic activation

16. Exogenous metabolising systems should be used when employing cells with inadequate endogenous metabolic capacity. The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (45) (46) or a combination of phenobarbitone and β-naphthoflavone (46) (47) (48) (49). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (50) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (46) (47) (48) (49). The S9 fraction typically is used at concentrations ranging from 1-10% (v/v) in the final test medium. The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of chemical being tested. For a more detailed discussion on this, please see Section 4.1.4 of the Introduction chapter.

Test substance preparation

17. Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to treatment of the cells. Where this is not possible with compatible solvents, suspensions may need to be used. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Gases or volatile substances should be tested by appropriate modifications to the standard protocols, such as treatment in sealed vessels (52) (53). Fresh preparations of the test substance should be used unless stability data demonstrate the acceptability of storage.

Test Conditions

Solvents/vehicles

18. The solvent/vehicle should be chosen to optimize the solubility of the test agent without adversely impacting the assay conduct, i.e., cell growth, integrity of the test material, reaction with culture vessels, metabolic activation system, etc. (B8). It is recommended that, wherever possible, the use of an aqueous solvent should be considered first. Well established solvent/vehicles are for example water, cell culture medium, dimethyl sulfoxide. Generally organic solvents should not exceed 1% (v/v) and aqueous solvents should not exceed 10% (v/v) in the final treatment medium. If other than well established solvents are used, their use should be supported by data indicating their compatibility with the test substance and their lack of genetic toxicity. In the absence of that supporting data, it is important to include untreated controls (see Glossary) to demonstrate that no deleterious or mutagenic effects are induced by the chosen solvent.

Use of cytoB as a cytokinesis blocker

19. One of the most important considerations in the performance of the MNvit assay is ensuring that the cells being scored have completed mitosis during the treatment or the post-treatment incubation period, if one is used. CytoB is the agent that has been most widely used to block cytokinesis because it inhibits actin assembly, and thus prevents separation of daughter cells after mitosis, leading to the formation of binucleated cells (5) (54) (55). Micronucleus scoring, therefore, can be limited to cells that have gone through mitosis during or after treatment. The effect of the test substance on cell proliferation kinetics can be measured simultaneously. CytoB should be used of as a cytokinesis blocker when human lymphocytes are used because cell cycle times will be variable within cultures and among donors and because not all lymphocytes will respond to PHA. Other methods have been used when testing cell lines to determine if the cells being scored have divided; these are addressed below (see Paragraph 25).

20. The appropriate concentration of cytoB should be determined by the laboratory for each cell type to achieve the optimal frequency of binucleated cells in the solvent/vehicle control cultures. The appropriate concentration of cytoB is usually between 3 and 6 μg/ml.

Measuring cell proliferation and cytotoxicity and choosing exposure concentrations

21. When determining the highest test substance concentration to be tested, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity (see Paragraph 27), precipitation in the culture medium (see Paragraph 28), and marked changes in pH or osmolality (see Paragraph 7), should be avoided. If the test chemical causes a marked change in the pH of the medium at the time of addition, the pH might be adjusted by buffering the final treatment medium so as to avoid artifactual positive results and to maintain good cell growth.

22. Measurements of cell proliferation are made to assure that the treated cells have undergone mitosis during the assay and that the treatments are conducted at appropriate levels of cytotoxicity (see Paragraph 28). Cytotoxicity should be determined with and without metabolic activation in cells that do not require metabolic activation using the relative increase in cell counts (RICC) or relative population doubling (RPD) (see Annex 2 for formulas) unless cytoB is used (B2). When cytoB is used, cytotoxicity can be determined using the replication index (RI) (see Annex 2 for formula).

23. Treatment of cultures with cytoB, and measurement of the relative frequencies of mononucleate, binucleate, and multi-nucleate cells in the culture, provides an accurate method of quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity of a treatment (5), and ensures that only cells that divided during or after treatment are scored.

24. In studies with cytoB, cytostasis/cytotoxicity can be quantified from the cytokinesis-block proliferation index (CBPI) (5) (26) (56) or may be derived from the RI from at least 500 cells per culture (see Annex 2 for formulas). When cytoB is used to assess cell proliferation, a CBPI or RI should be determined from at least 500 cells per culture. These measurements among others can be used to estimate cytotoxicity by comparing values in the treated and control cultures. Assessment of other indicators of cytotoxicity (*e.g.* confluency, cell number, apoptosis, necrosis, metaphase counting) can also provide useful information for assessing cytotoxicity.

25. In studies without cytoB, it is necessary to demonstrate that the cells scored in the culture have undergone division during or following treatment with the test substance, otherwise false negative responses may be produced. Methods that have been used for ensuring that divided cells are being scored include incorporation and subsequent detection of bromodeoxyuridine (BrdU) to identify cells that have replicated (57), the formation of clones when cells from permanent cell lines are treated and scored *in situ* on a microscope slide (Proliferation Index (PI)) (25) (26) (27) (28), or the measurement of Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) or other proven methods (16) (56) (58) (59) (see Annex 2 for formulas). At late sampling times (e.g. option B treatment for 1.5-2 normal cell cycles and harvest after an additional 1.5-2 normal cell cycles, i.e. 3-4 normal cell cycles in total), RPD might underestimate cytotoxicity (B7). Under these circumstances RICC could be a better measure. Alternatively, the evaluation of cytotoxicity after a 1.5-2 normal cell cycles would be a helpful estimate. Assessment of other markers for cytotoxicity or cytostasis (*e.g.* confluency, cell number, apoptosis, necrosis, metaphase counting) can also provide useful information for assessing cytotoxicity.

26. At least three analysable test concentrations from duplicate cultures should be evaluated. For substances demonstrating little or no toxicity, concentration intervals of approximately 2 to 3 fold will usually be appropriate. However, many substances exhibit steep concentration response curves and in order to obtain data at low and moderate toxicity, it will be necessary to use more closely spaced concentrations. When it is desirable to study the dose response relationship in detail, more than three concentrations will be needed. In these cases a larger number of concentrations (single cultures or duplicates) will be necessary. If single cultures are used then the negative control should be in duplicate. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing cytotoxicity as described in paragraph 27 and including concentrations at which there is moderate and little or no cytotoxicity.

27. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to produce 55 ± 5% cytotoxicity. Care should be taken not to markedly exceed 50% cytotoxicity because higher levels may induce chromosome damage as a secondary effect of cytotoxicity (60).

28. For poorly soluble compounds that are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration should produce turbidity or a precipitate visible by

eye or with the aid of an inverted microscope at the end of the treatment. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration inducing turbidity or with visible precipitate because artifactual effects may result from the precipitate. For suspension cultures, care should be taken to assure that the precipitate does not interfere with the conduct of the assay (e.g. staining or scoring).

29. If no cytotoxicity or precipitate is observed, the highest test concentration should correspond to [0.01 M, 5 mg/mL or 5 μl/mL, whichever is the lowest]. For mixtures (no one component is more than 50% of the total by weight or volume), the top concentration should be 5 mg/ml. In some circumstances, for mixtures, higher concentrations might be advisable.

Controls

30. Concurrent negative vehicle controls should be included in each experiment conducted either with or without metabolic.

31. Positive controls are needed to demonstrate the ability of the cells used, and the test protocol, to identify clastogens and aneugens under the conditions of the test protocol used. A mutagen that requires metabolic activation (see table in annex) should be used to affirm the metabolic capability of the metabolic activation system preparation

32. Positive controls should be used at concentrations expected to give a reproducible and detectable increase over background which demonstrates the sensitivity of the test system *i.e.* the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Because *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardized the use of positive controls may be confined to a chemical requiring metabolic activation (provided it is done concurrently with the non-activated test using the same treatment duration) to demonstrate the activity of the metabolic activation system and the responsiveness of the test system.

33. At the present time, no aneugens are known that require metabolic activation for their genotoxic activity (16). Currently accepted positive controls for aneugenic activity are, for example, colchicine and vinblastine. Other substances may be used if they induce micronuclei solely, or primarily, through aneugenic activity. To avoid the need for two positive controls (for clastogenicity and aneugenicity) without metabolic activation, the aneugenicity control can serve as the positive control without S9, and the clastogenicity control can be used to test the adequacy of the metabolic activation system used. Positive controls for both clastogenicity and aneugenicity should be used in cells that do not require S9. Suggested positive control chemicals are included in Annex 3.

PROCEDURE

Treatment Schedule

34. In order to maximise the probability of detecting an aneugen or clastogen acting at a specific stage in the cell cycle, it is important that sufficient numbers of cells are treated with the test substance during all stages of their cell cycles. The treatment schedule for cell lines and primary cell cultures may, therefore, differ somewhat from that for lymphocytes which require mitogenic stimulation to begin their cell cycle and these are considered in Paragraphs 38-40 (16).

35. Theoretical considerations, together with published data (18) indicate that most aneugens and clastogens will be detected by a short term treatment period of 3 to 6 hrs in the presence and absence of

S9, followed by removal of the test substance and a growth period of 1.5 – 2.0 cell cycles (6). Cells are sampled at a time equivalent to about 1.5 – 2.0 times the normal (*i.e.* untreated) cell cycle length either after the beginning or at the end of treatment (See Table 1). Sampling or recovery times may be extended if it is known or suspected that the test substance affects the cell cycling time (*e.g.* when testing nucleoside analogues).

36. Because of the potential cytotoxicity of S9 preparations for cultured mammalian cells, an extended exposure treatment of 1.5 – 2.0 normal cell cycles is used only in the absence of S9. In the extended treatment, options are offered to allow treatment of the cells with the test chemical in the absence or presence of cytoB. These options address situations where there may be concern regarding possible interactions between the test substance and cytoB.

37. The suggested cell treatment schedules are presented in Table 1. These general treatment schedules may be modified depending on the stability or reactivity of the test substance or the particular growth characteristics of the cells being used. All treatments should commence and end while the cells are growing exponentially. These schedules are presented in more details in paragraphs 38-44 following.

Table 1. Cell treatment and harvest times for the MNvit assay

Lymphocytes, primary cells and cell lines treated with cytoB	+ S9	Treat for 3-6 hrs in the presence of S9; remove the S9 and treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles later.
	– S9 Short exposure	Treat for 3-6 hrs; remove the treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles later.
	– S9 Extended exposure	*Option A:* Treat for 1.5 – 2 normal cell cycles in the presence of cytoB; harvest at the end of the exposure period. *Option B:* Treat for 1.5 – 2.0 normal cell cycles; remove the test substance; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles later.
Cell lines treated without cytoB (Identical to the treatment schedules outlined above with the exception that no cytoB is added)		

Lymphocytes, primary cells, and cell lines with cytoB

38. For lymphocytes, the most efficient approach is to start the exposure to the test substance at 44-48 hrs after PHA stimulation, when cycle synchronisation will have disappeared (5). In the initial assay, cells are treated for 3 to 6 hrs with the test substance in the absence and presence of S9. The

treatment medium is removed and replaced with fresh medium containing cytoB, and the cells are harvested 1.5 – 2.0 normal cell cycles later.

39. If both initial tests of the short (3-6 hrs) treatment are negative or equivocal, a subsequent, extended exposure treatment without S9 is used. Two treatment options are available and are equally acceptable. However, It might be more appropriate to follow Option A for stimulated lymphocytes where exponential growth may be declining at 96 hrs following stimulation. Also, cultures of cells should not have reached confluence by the final sampling time in Option B.

- Option A: The cells are treated with the test substance for 1.5 – 2.0 normal cell cycles, and harvested at the end of the treatment time.

- Option B: The cells are treated with the test substance for 1.5 – 2.0 normal cell cycles. The treatment medium is removed and replaced with fresh medium, and the cells are harvested after additional 1.5 - 2.0 normal cell cycles.

40. Primary cells and cell lines should be treated in a similar manner to lymphocytes except that it is not necessary to stimulate them with PHA for 44-48 hrs. Cells other than lymphocytes should be exposed such that at the time of study termination, the cells are still in log-phase growth.

Cell lines without cytoB

41. Cells should be treated for 3-6 hrs in the presence and absence of S9. The treatment medium is removed and replaced with fresh medium, and the cells are harvested 1.5 – 2.0 normal cell cycles later.

42. If both initial tests of the short (3-6 hrs) treatment are negative or equivocal, a subsequent, extended exposure treatment (without S9) is used. Two treatment options are available, both of which are equally acceptable:

- Option A: The cells are treated with the test substance for 1.5 – 2.0 normal cell cycles, and harvested at the end of the treatment time.

- Option B: The cells are treated with the test substance for 1.5 – 2.0 normal cell cycles. The treatment medium is removed and replaced with fresh medium, and the cells are harvested after additional 1.5 - 2.0 normal cell cycles.

43. In monolayers, mitotic cells (identifiable as being round and detaching from the surface) may be present at the end of the 3-6 hr treatment. Because these mitotic cells are easily detached, they can be lost when the medium containing the test substance is removed. Care should be taken to collect these when cultures are washed, and to return them to the cultures, to avoid losing cells that are in mitosis, and at risk for micronuclei, at the time of harvest.

Number of cultures

44. Duplicate cultures should normally be used for each test substance concentration and for the negative (vehicle or untreated) control cultures. Where single cultures are used, e.g. for study of the shape of the dose response relationship (see § on number of test concentrations), an increased number of concentrations has to be analysed but negative controls should be done in duplicate.

Cell harvest and slide preparation

45. Each culture is harvested and processed separately. Cell preparation may involve hypotonic treatment, but this step is not necessary if adequate cell spreading is otherwise achieved. Different techniques can be used in slide preparation provided that high-quality cell preparations for scoring are obtained. Cell cytoplasm should be retained to allow the detection of micronuclei and (in the cytokinesis-block method) reliable identification of binucleate cells.

46. The slides can be stained using various methods, such as Giemsa or fluorescent DNA specific dyes (59). The use of a DNA specific stain (*e.g.* acridine orange (61) or Hoechst 33258 plus pyronin-Y (62)) can eliminate some of the artifacts associated with using a non-DNA specific stain. Anti-kinetochore antibodies, FISH with pancentromeric DNA probes, or primed *in situ* labelling with pancentromere-specific primers, together with appropriate DNA counterstaining, can be used to identify the contents (chromosome fragment) of micronuclei if mechanistic information of their formation is of interest (15)(16). Other methods for differentiation between clastogens and aneugens may be used if they have been shown to be effective.

Analysis

47. All slides, including those of the solvent/vehicle and the controls, should be independently coded before the microscopic analysis. Alternatively, coded samples can be analysed using a validated, automated flow cytometric or image analysis system.

48. In cytoB-treated cultures, micronucleus frequencies should be analysed in at least 2000 binucleated cells per concentration (at least 1000 binucleated cells per culture; two cultures per concentration). If single cultures are used, at least 2000 binucleated cells per concentration should be scored from that culture. If substantially fewer than 1000 binucleate cells per culture, or 2000 if a single culture is used, are available for scoring at each concentration, and if a significant increase in micronuclei is not detected, the test should be repeated using more cells, or at less toxic concentrations, whichever is appropriate. Care should be taken not to score binucleate cells with irregular shapes or where the two nuclei differ greatly in size; neither should binucleate cells be confused with poorly spread multi-nucleate cells. Cells containing more than two main nuclei should not be analysed for micronuclei, as the baseline micronucleus frequency may be higher in these cells (63) (64) Scoring of mononucleate cells is acceptable if the test substance is shown to interfere with cytoB activity.

49. In cell lines assayed without cytoB treatment, micronuclei should be scored in at least 2000 cells per concentration (at least 1000 cells per culture; two cultures per concentration). Where only one culture per concentration is used, at least 2000 cells should be scored from that culture.

50. When cytoB is used, a CBPI or an RI should be determined to assess cell proliferation (see Annex 2) using at least 500 cells per culture. When treatments are performed in the absence of cytoB, it is essential to provide evidence that the cells being scored have proliferated, as discussed in Paragraphs 23-26.

Proficiency of the laboratory

51. In order to demonstrate proficiency, the laboratory should perform a series of experiments with reference positive chemicals acting via different mechanisms (Annex 2) and various solvents. These positive and negative control responses should be consistent with the published literature. During the course of these investigations, the laboratory should establish:

- A historical positive control range and distribution
- A historical negative (untreated, vehicle) control range and distribution

Re-evaluation of laboratory proficiency is recommended if major changes to the experimental conditions (e.g. use of automated instead of manual scoring techniques; use of a new cell type) are proposed for the assay.

DATA AND REPORTING

Treatment of results

52. If the cytokinesis-block technique is used, only the frequencies of binucleate cells with micronuclei (independent of the number of micronuclei per cell) are used in the evaluation of micronucleus induction. Scoring of the numbers of cells with one, two, or more micronuclei could provide useful information, but is not mandatory.

53. Concurrent measures of cytotoxicity and/or cytostasis for all treated and solvent/vehicle control cultures should be determined (58). The CBPI or the RI should be calculated for all treated and control cultures as measurements of cell cycle delay when the cytokinesis-block method is used. In the absence of cytoB, the RPD or the RICC or PI should be used (see Annex 2).

54. Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

55. Chemicals that induce micronuclei in the MNvit assay may do so because they induce chromosome breakage, chromosome loss, or a combination of the two. Further analysis using anti-kinetochore antibodies, centromere specific *in situ* probes, or other methods may be used to determine whether the mechanism of micronucleus induction is due to clastogenic and/or aneugenic activity.

Evaluation and interpretation of results

[this section needs to be further discussed by the Expert group]

56. There is no requirement for verification by additional testing of a clear positive or negative response. Equivocal results may be clarified by analysis of another 1000 cells from all the cultures to avoid loss of blinding. If this approach does not resolve the result, further testing should be performed. Modification of study parameters over an extended or narrowed range of conditions, as appropriate, should be considered in follow-up experiments. Study parameters that might be modified include the test concentration spacing, the timing of treatment and cell harvest, and/or the metabolic activation conditions.

57. Although there are several criteria for a positive result, biological relevance of the results should be considered first. Appropriate statistical methods may be used as an aid in evaluating the test results. However, the results of statistical testing should be assessed with respect to dose-response relationship and a statistically significant increase alone is not sufficient for the determination of a positive result. A result can be considered clearly biologically relevant if the following criteria are all satisfied:

(1) the increase is dose-related,

(2) at least one of the measure points is statistically significant higher than the concurrent negative control,

(3) the positive result is reproducible (e.g. between duplicates or between independent experiments),

(4) the positive result is outside the range of the historical negative control data.

The positive and negative controls are within the historical positive range for the test within the laboratory.

58. Although most experiments will give clearly positive or negative results, in some cases the data set will preclude making a definite judgement about the activity of the test substance. These equivocal or questionable responses may occur regardless of the number of times the experiment is repeated.

59. Positive results from the MNvit assay indicate that the test substance induces chromosome breakage loss, in cultured mammalian cells. Negative results indicate that, under the test conditions used, the test substance does not induce chromosome breaks and/or gain or loss in cultured mammalian cells.

Test Report

60. The test report should include the following information:

Test substance:

- identification data and Chemical Abstract Services Registry Number (CASRN);
- physical nature and purity;
- physico-chemical properties relevant to the conduct of the study;
- reactivity of the test substance with the solvent/vehicle or cell culture media;

Solvent/Vehicle:

- justification for choice of solvent/vehicle;
- solubility and stability of the test substance in solvent/vehicle;

Cells:

- type and source of cells used;
- suitability of the cell type used;
- absence of mycoplasma, if applicable;
- for cell lines, information on cell cycle length, doubling time or proliferation index;
- where lymphocytes are used, sex, age and number of blood donors, if applicable;
- where lymphocytes are used, whether whole blood or separated lymphocytes are exposed;
- number of passages, if applicable;
- methods for maintenance of cell cultures, if applicable;
- modal number of chromosomes;
- normal (negative control) cell cycle time;

Test Conditions:

- identity of cytokinesis blocking substance (*e.g.* cytoB), if used, and its concentration and duration of cell exposure;
- rationale for selection of concentrations and number of cultures, including cytotoxicity data and solubility limitations, if available;
- composition of media, CO_2 concentration, if applicable;

- concentrations of test substance;
- concentration (and/or volume) of vehicle and test substance added;
- incubation temperature and time;
- duration of treatment;
- harvest time after treatment;
- cell density at seeding, if applicable;
- type and composition of metabolic activation system, including acceptability criteria;
- positive and negative controls;
- methods of slide preparation and staining technique used;
- criteria for micronucleus identification;
- numbers of cells analysed;
- methods for the measurements of cytotoxicity;
- any supplementary information relevant to cytotoxicity;
- criteria for considering studies as positive, negative, or equivocal;
- method(s) of statistical analysis used;
- methods, such as use of kinetochore antibody, to characterise whether micronuclei contain whole or fragmented chromosomes, if applicable;

Results (individual data):

- the number of cells plated (or treated) and the number of cells harvested for each culture;
- measurement of cytotoxicity used, *e.g.* CBPI or RI in the case of cytokinesis-block method; RICC, RPD or PI when cytokinesis-block methods are not used; other observations when applicable, *e.g.* cell confluency, apoptosis, necrosis, metaphase counting, frequency of binucleated cells;
- signs of precipitation;
- data on pH and osmolality of the treatment medium, if determined;
- definition of acceptable cells for analysis;
- distribution of mono-, bi-, and multi-nucleated cells if a cytokinesis block method is used;
- number of cells with micronuclei given separately for each treated and control culture, and defining whether from binucleate or mononucleate cells, where appropriate;
- concentration-response relationship, where possible;
- concurrent negative (solvent/vehicle) and positive control data (concentrations and solvents);
- historical negative (solvent/vehicle) and positive control data, with ranges, means and standard deviation and confidence interval (*e.g.* 95%);
- statistical analysis; p-values if any;

Discussion of the results:

Conclusions.

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Annex 1

DEFINITIONS

Aneugen: any substance or process that, by interacting with the components of the mitotic and meiotic cell division cycle, leads to aneuploidy in cells or organisms.

Aneuploidy: any deviation from the normal diploid (or haploid) number of chromosomes by a single chromosome or more than one, but not by entire set(s) of chromosomes (polyploidy).

Apoptosis: programmed cell death characterized by a series of steps leading to a disintegration of cells into membrane-bound particles that are then eliminated by phagocytosis or by shedding.

Cell proliferation: increase in cell number as a result of mitotic cell division.

Centromere: DNA region of a chromosome where both chromatids are held together and on which both kinetochores are attached side-to-side.

Clastogen: any substance or process which causes structural chromosomal aberrations in populations of cells or organisms.

Cytokinesis: the process of cell division immediately following mitosis to form two daughter cells, each containing a single nucleus.

Cytokinesis-Block Proliferation index (CBPI): the proportion of second-division cells in the treated population relative to the untreated control (see Annex 2 for formula).

Cytostasis: inhibition of cell growth (see Annex 2 for formula).

Cytotoxicity: harmful effects to cell structure or function ultimately causing cell death.

Genotoxic: a general term encompassing all types of DNA or chromosome damage, including breaks, adducts rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosome damage.

Interphase cells: cells not in the mitotic stage.

Kinetochore: a protein-containing structure that assembles at the centromere of a chromosome to which spindle fibres associate during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Micronuclei: small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis or meiosis by lagging chromosome fragments or whole chromosomes.

Mitosis: division of the cell nucleus usually divided into prophase, prometaphase, metaphase, anaphase and telophase.

Mitotic index: the ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of cell proliferation of that population.

Mutagenic: produces a heritable change of DNA base-pair sequences(s) in genes or of the structure of chromosomes (chromosome aberrations).

Non-disjunction: failure of paired chromatids to disjoin and properly segregate to the developing daughter cells, resulting in daughter cells with abnormal numbers of chromosomes.

Polyploidy: numerical chromosome aberrations in cells or organisms involving entire set(s) of chromosomes, as opposed to an individual chromosome or chromosomes (aneuploidy).

Proliferation Index (PI): method for cytotoxicity measurement when cytoB is not used (see Annex 2 for formula).

Relative Increase in Cell Count (RICC): method for cytotoxicity measurement when cytoB is not used (see Annex 2 for formula).

Relative Population Doubling (RPD): method for cytotoxicity measurement when cytoB is not used (see Annex 2 for formula).

Replication Index (RI): the proportion of cell division cycles completed in a treated culture, relative to the untreated control, during the exposure period and recovery (see annex 2 for formula).

Solvent/vehicle:

Untreated control:

Annex 2

FORMULAS FOR CYTOTOXICITY ASSESSMENT

1. ***When cytoB is used***, evaluation of cytotoxicity should be based on the **Cytokinesis-Block Proliferation Index (CBPI)** or **Replicative Index (RI)** (16) (58). The CBPI indicates the average number of cell cycles per cell during the period of exposure to cytoB, and may be used to calculate cell proliferation. The RI indicates the relative number of nuclei in treated cultures compared to control cultures and can be used to calculate the % cytostasis:

$$\% \text{ Cytostasis} = 100\text{-}100\{(CBPI_T - 1) \div (CBPI_C - 1)\}$$

And:

T = test chemical treatment culture
C = vehicle control culture

Where:

$$\textbf{CBPI} = \frac{((\text{No. mononucleate cells}) + (2 \times \text{No. binucleate cells}) + (3 \times \text{No. multinucleate cells}))}{(\text{Total number of cells})}$$

Thus, a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

$$\text{Cytostasis} = 100\text{-RI}$$

$$\textbf{RI} = \frac{((\text{No. binucleated cells}) + (2 \times \text{No. multinucleate cells})) \div (\text{Total number of cells})_T}{((\text{No. binucleated cells}) + (2 \times \text{No. multinucleate cells})) \div (\text{Total number of cells})_C} \times 100$$

T= treated cultures
C= control cultures

2. Thus, an RI of 53% means that, compared to the numbers of cells that have divided to form binucleate and multinucleate cells in the control culture, only 53% of this number divided in the treated culture, *i.e.* 47% cytostasis.

3. ***When cytoB is not used***, evaluation of cytotoxicity based on **Relative Increase in Cell Counts (RICC)** or on **Relative Population Doubling (RPD)** is recommended (58), as both take into account the proportion of the cell population which has divided.

$$\textbf{RICC} = \frac{(\text{Increase in number of cells in treated cultures (final – starting)})}{(\text{Increase in number of cells in control cultures (final – starting)})} \times 100$$

$$\textbf{RPD} = \frac{(\text{No. of Population doublings in treated cultures})}{(\text{No. of Population doublings in control cultures})} \times 100$$

where:

Population Doubling = [log (Post-treatment cell number ÷ Initial cell number)] ÷ log 2

4. Thus, a RICC, or a RPD of 53% indicates 47% cytotoxicity/cytostasis.

5. By using a **Proliferation Index (PI)**, cytotoxicity may be assessed via counting the number of clones consisting of 1 cell (cl1), 2 cells (cl2), 3 to 4 cells (cl4) and 5 to 8 cells (cl8)

$$\mathbf{PI} = \frac{((1x\ cl1) + (2x\ cl2) + (3x\ cl4) + (4x\ cl8))}{(cl1 + cl2 + cl4 + cl8)}$$

6. The PI has been used as a valuable and reliable cytotoxicity parameter also for cell lines cultured *in situ* in the absence of cytoB (25)(26)(27)(28).

Annex 3

REFERENCE CHEMICALS RECOMMENDED FOR ASSESSING LABORATORY PERFORMANCE AND FOR SELECTION OF POSITIVE CONTROLS

Category	Chemical	CASRN
1. Clastogens active without metabolic activation		
	Cytosine arabinoside	147-94-4
	Mitomycin C	50-07-7
2. Clastogens requiring metabolic activation		
	Benzo(a)pyrene	50-32-8
	Cyclophosphamide	50-18-0
3. Aneugens		
	Colchicine	64-86-8
	Vinblastine	143-67-9

Positive control chemicals should be able to provide appropriate demonstration of metabolic activation and detection of relevant endpoints or mechanisms covered by the test (clastogenicity and aneuploidy).