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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

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. 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 polyploidy 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) and human lymphocytes (18) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) (29) (30) (31). 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). The use of the human TK6 lymphoblastoid cell line (35), HepG2 cells (36) (37) and primary Syrian Hamster Embryo cells (38) has been described, although they have not been used in validation studies.

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). If the test chemical causes a change in the pH of the medium at the time of addition, the pH should be adjusted, preferably by buffering the stock solution so that all the volumes at all test concentrations, and for all controls, remain the same.

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. Concurrent solvent/vehicle and positive controls are included in all tests.

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 binucleate 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 may be used (18) (19) (20) (21) (22) (25) (26) (27) (28) (30). 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 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.

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. Established cell lines and strains 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 known. 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. Established cell lines and strains: 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 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 using 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 condition of a metabolic activation system may depend upon the class of chemical being tested and in some cases it may be appropriate to utilise more than one S9 concentration.

17. Genetically engineered cell lines expressing specific human or rodent activating enzymes may eliminate the need for an exogenous metabolic activation system, and may be used as the test cells. In such cases the choice of the cell lines used should be scientifically justified, *e.g.* by relevance of the mixed function oxidases for the metabolism of the test substance (51), and their responsiveness to known clastogens and aneugens (see separate section on Acceptability Criteria). It should be recognized that the substance being tested may not be metabolised by the expressed mixed function oxidase(s); in this case, the negative results would not indicate that the substance cannot induce micronuclei.

Test substance preparation

18. Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to treatment of the cells. 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

19. The solvent/vehicle should not react with the test substance, or be incompatible with the survival of the cells or with the maintenance of S9 activity at the concentration used. If other than well established solvent/vehicles (*e.g.* water, cell culture medium, dimethyl sulfoxide) are used, their use should be supported by data indicating their compatibility with the test substance and their lack of genetic toxicity. It is recommended that, wherever possible, the use of an aqueous solvent/vehicle should be considered first.

Use of cytoB as a cytokinesis blocker

20. 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 26).

21. 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

22. 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, precipitation in the culture medium, and marked changes in pH or osmolality (39) (40) (41), should be avoided.

23. 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 29). 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. When cytoB is used, cytotoxicity can be determined using the replication index (RI) (see Annex 2 for formula).

24. 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.

25. 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 markers of cytotoxicity (*e.g.* confluency, cell number, apoptosis, necrosis, metaphase counting) can provide useful information.

26. 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). Assessment of other markers for cytotoxicity or cytostasis (*e.g.* confluency, cell number, apoptosis, necrosis, metaphase counting) can provide useful information.

27. At least three analysable test concentrations should be evaluated. In order to achieve this, it may be necessary to perform the experiment using a larger number of closely spaced concentrations and analyse micronucleus formation in those concentrations providing the appropriate range of cytotoxicities. An alternative strategy is to perform a preliminary cytotoxicity test to narrow the range for the definitive test.

28. The highest concentration should aim to produce 55 ± 5% cytotoxicity. Higher levels may induce chromosome damage as a secondary effect of cytotoxicity (60). Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing 55 ± 5% cytotoxicity, to little or no cytotoxicity.

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. The concentrations selected for analysis should, in general, be separated by a spacing of no more than $\sqrt{10}$. For test substances that exhibit a steep concentration-response curve, it may be necessary to more closely space the test substance concentrations so that cultures in the moderate and low toxicity ranges also will be scored.

30. When solubility is a limiting factor, the maximum concentration, if not limited by cytotoxicity, should be the lowest concentration at which minimal precipitate is visible in cultures, provided there is no interference with scoring. Evaluation of precipitation should be done by methods such as light microscopy, noting precipitate that persists, or appears during culture (by the end of treatment).

Controls

31. Concurrent positive and solvent/vehicle controls both with and without metabolic activation should be included in each experiment.

32. Positive controls are needed to demonstrate the ability of the cells used, and the test protocol, to identify clastogens and aneugens, and to affirm the metabolic capability of the S9 preparation. The positive controls should employ known inducers of micronucleus formation at concentrations expected to give small, but reproducible increases over background, and demonstrate the sensitivity of the test system.

Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader.

33. A clastogen that requires metabolic activation (*e.g.* cyclophosphamide; benzo[a]pyrene) should be used to demonstrate both the metabolic competence and the ability of the test system to detect clastogens. Other positive control substances may be used if justified. Because some positive controls that need metabolic activation may be active without exogenous metabolic activation under certain treatment conditions or in certain cell lines, the need for metabolic activation, and the activity of the S9 preparation, should be tested in the selected cell line and at the selected concentrations.

34. 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.

35. The use of chemical class-related positive control chemicals may be considered, when suitable substances are available. All positive control substances used should be appropriate for the cell type and activation conditions.

36. Solvent/vehicle controls should be included for every harvest time. In addition, untreated negative controls (lacking solvent/vehicle) should also be used unless there are published or laboratory historical control data demonstrating that no genotoxic or other deleterious effects are induced by the chosen solvent at the concentrations used.

PROCEDURE

Treatment Schedule

37. 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 41-43 (16).

38. 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).

39. 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.

40. 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 41-47 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

41. 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.

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 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.

43. 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

44. 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.

45. 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.

46. 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

47. Duplicate cultures should be used for each test substance concentration and for the vehicle/solvent and negative control cultures. Where minimal variation between duplicate cultures can be demonstrated from historical laboratory data, it may be acceptable for single cultures to be used. If single cultures are used, it is recommended that an increased number of concentrations be analysed.

Cell harvest and slide preparation

48. 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.

49. 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/chromosomal 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

50. 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.

51. 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.

52. 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.

53. 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 24-27.

Acceptability criteria

54. A laboratory proposing to use the MNvit assay described in this Test Guideline should demonstrate its ability to reliably and accurately detect substances of known aneugenic and clastogenic activity, with and without metabolic activation, as well as known negative substances, using the reference substances in Annex 3. As evidence of its ability to perform this test method correctly, the laboratory should provide evidence that the cells being scored for micronucleus formation have completed one nuclear division if the test is performed without the use of cytoB.

55. The chemicals in Annex 3 are recommended for use as reference chemicals. Substitute or additional chemicals can be included if their activity is known and if they induce micronuclei by the same mechanisms of action, and if they are shown to be relevant to the chemicals that will be tested using the MNvit procedure. Justification could include a validation study employing a broad variety of substances or focused on a narrower spectrum based on the chemical class of the test substance or the mechanism of damage being studied.

56. Solvent/vehicle control and untreated cultures should give reproducibly low and consistent micronuclei frequencies (typically 5-25 micronuclei/1000 cells for the cell types identified in paragraph

11). Other cell types may have different ranges of responses which should be determined when validating them for use in the MNvit assay. Data from negative, solvent, and positive controls should be used to establish historical control ranges. These values should be used in deciding the adequacy of the concurrent negative/positive controls for an experiment

57. If minor changes to the protocol (*e.g.* use of automated instead of manual scoring techniques; use of a new cell type) are proposed for the assay, then the effectiveness of the change should be demonstrated before the modified protocol can be considered acceptable for use. Demonstration of effectiveness includes demonstration that the major mechanisms of chromosome breakage and gain or loss can be detected, and that appropriate positive and negative results can be achieved for the class of the individual substance, or the broad range of substances, to be tested.

DATA AND REPORTING

Treatment of results

58. 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.

59. 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).

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

61. 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

62. 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.

63. There are several criteria for determining a positive result, such as a concentration-related increase or a statistically significant increase in the number of cells containing micronuclei. The biological relevance of the results should be considered first. Consideration of whether the observed values are within or outside of the historical control range can provide guidance when evaluating the biological significance of the response. Appropriate statistical methods may be used as an aid in evaluating the test results (65). However, the results of statistical testing should be assessed with respect to dose-response relationship. Reproducibility and historical data should also be taken into consideration."

64. 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.

65. 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

66. 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;
- 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:

- 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.

LITERATURE

(1) Kirsch-Volders, M. (1997), Towards a validation of the micronucleus test. *Mutation Res.*, 392, 1-4.

(2) Parry, J.M. and Sors, A. (1993), The detection and assessment of the aneugenic potential of environmental chemicals: the European Community aneuploidy project, *Mutation Res.*, 287, 3-15.

(3) Fenech, M. and Morley, A.A. (1985), Solutions to the kinetic problem in the micronucleus assay, *Cytobios.*, 43, 233-246.

(4) Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate, M. Jr, Lorge, E., Norppa, H., Surralles, J., von der Hude, W. and Wakata, A. (2000), Report from the *In Vitro* Micronucleus Assay Working Group, *Environ. Mol. Mutagen*., 35, 167-172.

(5) Fenech, M. (2007), Cytokinesis-block micronucleus cytome assay, *Nature Protocols, 2*(5), 1084-1104.

(6) Fenech, M. and Morley, A.A. (1986), Cytokinesis-block micronucleus method in human lymphocytes: effect of *in-vivo* ageing and low dose X-irradiation, *Mutation Res.*, 161, 193-198.

(7) Eastmond, D.A. and Tucker, J.D. (1989), Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody, *Environ. Mol. Mutagen.*, 13, 34-43.

(8) Eastmond, D.A. and Pinkel, D. (1990), Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence *in-situ* hybridisation with chromosome-specific DNA probes, *Mutation Res.*, 234, 9-20.

(9) Miller, B.M., Zitzelsberger, H.F., Weier, H.U. and Adler, I.D. (1991), Classification of micronuclei in murine erythrocytes: immunofluorescent staining using CREST antibodies compared to *in situ* hybridization with biotinylated gamma satellite DNA, *Mutagenesis*, 6, 297-302.

(10) Farooqi, Z., Darroudi, F. and Natarajan, A.T. (1993), The use of fluorescence *in-situ* hybridisation for the detection of aneugens in cytokinesis-blocked mouse splenocytes, *Mutagenesis*, 8, 329-334.

(11) Migliore, L., Bocciardi, R., Macri, C. and Lo Jacono, F. (1993), Cytogenetic damage induced in human lymphocytes by four vanadium compounds and micronucleus analysis by fluorescence *in situ* hybridization with a centromeric probe, *Mutation Res.*, 319, 205-213.

(12) Norppa, H., Renzi, L. and Lindholm, C. (1993), Detection of whole chromosomes in micronuclei of cytokinesis-blocked human lymphocytes by antikinetochore staining and *in situ* hybridization, *Mutagenesis*, 8, 519-525.

(13) Eastmond, D.A, Rupa, D.S. and Hasegawa, L.S. (1994), Detection of hyperdiploidy and chromosome breakage in interphase human lymphocytes following exposure to the benzene metabolite

hydroquinone using multicolor fluorescence *in situ* hybridization with DNA probes, *Mutation Res.*, 322, 9-20.

(14) Marshall, R.R., Murphy, M., Kirkland, D.J. and Bentley, K.S. (1996), Fluorescence *in situ* hybridisation (FISH) with chromosome-specific centromeric probes: a sensitive method to detect aneuploidy, *Mutation Res.*, 372, 233-245.

(15) Zijno, P., Leopardi, F., Marcon, R. and Crebelli, R. (1996), Analysis of chromosome segregation by means of fluorescence *in situ* hybridization: application to cytokinesis-blocked human lymphocytes, *Mutation Res.*, 372, 211-219.

(16) Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate Jr., M., Lorge, E., Norppa, H., Surrallés, J., von der Hude, W. and Wakata, A. (2003), Report from the *in vitro* micronucleus assay working group. *Mutation Res.*, 540, 153-163.

(17) OECD (1997), *In Vitro Mammalian Chromosome Aberration Test*, Test Guideline No. 473, OECD Guidelines for Testing of Chemicals, OECD, Paris. Available at: [www.oecd.org/env/testguidelines]

(18) Lorge, E., Thybaud, V., Aardema, M.J., Oliver, J., Wakata, A., Lorenzon G. and Marzin, D. (2006), SFTG International collaborative Study on *in vitro* micronucleus test. I. General conditions and overall conclusions of the study, *Mutation Res.*, 607, 13-36.

(19) Clare, G., Lorenzon, G., Akhurst, L.C., Marzin, D., van Delft, J., Montero, R., Botta, A., Bertens, A., Cinelli, S., Thybaud, V. and Lorge, E. (2006), SFTG International collaborative study on the *in vitro* micronucleus test. II. Using human lymphocytes, *Mutation Res.*, 607, 37-60.

(20) Aardema, M.J., Snyder, R.D., Spicer, C., Divi, K., Morita, T., Mauthe, R.J., Gibson, D.P., Soelter, S., Curry, P.T., Thybaud, V., Lorenzon, G., Marzin, D. and Lorge, E. (2006), SFTG International collaborative study on the *in vitro* micronucleus test, III. Using CHO cells, *Mutation Res.*, 607, 61-87.

(21) Wakata, A., Matsuoka, A., Yamakage, K., Yoshida, J., Kubo, K., Kobayashi, K., Senjyu, N., Itoh, S., Miyajima, H., Hamada, S., Nishida, S., Araki, H., Yamamura, E., Matsui, A., Thybaud, V., Lorenzon, G., Marzin, D. and Lorge, E. (2006), SFTG International collaborative study on the *in vitro* micronucleus test, IV. Using CHO/IU cells, *Mutation Res.*, 607, 88-124.

(22) Oliver, J., Meunier, J.-R., Awogi, T., Elhajouji, A., Ouldelhkim, M.-C., Bichet, N., Thybaud, V., Lorenzon, G., Marzin, D. and Lorge, E. (2006), SFTG International collaborative study on the *in vitro* micronucleus test, V. Using L5178Y cells, *Mutation Res.*, 607, 125-152.

(23) Albertini, S., Miller, B., Chetelat, A.A. and Locher, F. (1997), Detailed data on *in vitro* MNT and *in vitro* CA: industrial experience, *Mutation Res.*, 392, 187-208.

(24) Miller, B., Albertini, S., Locher, F., Thybaud, V. and Lorge, E. (1997), Comparative evaluation of the *in vitro* micronucleus test and the *in vitro* chromosome aberration test: industrial experience, *Mutation Res.*, 392, 45-59.

(25) Miller, B., Potter-Locher, F., Seelbach, A., Stopper, H., Utesch, D. and Madle, S. (1998), Evaluation of the *in vitro* micronucleus test as an alternative to the *in vitro* chromosomal aberration assay: position of the GUM Working Group on the *in vitro* micronucleus test. Gesellschaft fur Umwelt-Mutations-forschung, *Mutation Res.*, 410, 81-116.

(26) Kalweit, S., Utesch, U., von der Hude, W. and Madle, S. (1999), Chemically induced micronucleus formation in V79 cells – comparison of three different test approaches, *Mutation Res.* 439, 183-190.

(27) Kersten, B., Zhang, J., Brendler Schwaab, S.Y., Kasper, P. and Müller, L. (1999), The application of the micronucleus test in Chinese hamster V79 cells to detect drug-induced photogenotoxicity, *Mutation Res.* 445, 55-71.

(28) von der Hude, W., Kalweit, S., Engelhardt, G., McKiernan, S., Kasper, P., Slacik-Erben, R., Miltenburger, H.G., Honarvar, N., Fahrig, R., Gorlitz, B., Albertini, S., Kirchner, S., Utesch, D., Potter-Locher, F., Stopper, H. and Madle, S. (2000), *In vitro* micronucleus assay with Chinese hamster V79 cells - results of a collaborative study with *in situ* exposure to 26 chemical substances, *Mutation Res*., 468, 137-163.

(29) Garriott, M.L., Phelps, J.B. and Hoffman, W.P. (2002), A protocol for the *in vitro* micronucleus test, I. Contributions to the development of a protocol suitable for regulatory submissions from an examination of 16 chemicals with different mechanisms of action and different levels of activity, *Mutation Res.*, 517, 123-134.

(30) Matsushima, T., Hayashi, M., Matsuoka, A., Ishidate, M. Jr., Miura, K.F., Shimizu, H., Suzuki, Y., Morimoto, K., Ogura, H., Mure, K., Koshi, K. and Sofuni, T. (1999), Validation study of the *in vitro* micronucleus test in a Chinese hamster lung cell line (CHL/IU), *Mutagenesis*, 14, 569-580.

(31) Elhajouji, A., and Lorge, E. (2006), Special Issue: SFTG International collaborative study on *in vitro* micronucleus test, *Mutation Res.*, 607, 1-152.

(32) ECVAM (2006), Statement by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) on the scientific validity of the *in vitro* micronucleus test as an alternative to the *in vitro* chromosome aberration assay for genotoxicity testing. ESAC 25th meeting, 16-17 November, 2006, Available at: [http://ecvam.jrc.it/index.htm]

(33) ESAC (2006), ECVAM Scientific Advisory Committee (ESAC) Peer Review, Retrospective Validation of the *In Vitro* Micronucleus Test, Summary and Conclusions of the Peer Review Panel, Available at: [http://ecvam.jrc.it/index.htm]

(34) Corvi, R., Albertini, S., Hartung, T., Hoffmann, S., Maurici, D., Pfuhler, S, van Benthem, J., Vanparys P. (2008), ECVAM Retrospective Validation of *in vitro* Micronucleus Test (MNT), *Mutagenesis*, 23, 271-283.

(35) Zhang, L.S., Honma, M., Hayashi, M., Suzuki, T., Matsuoka, A. and Sofuni, T. (1995), A comparative study of TK6 human lymphoblastoid and L5178Y mouse lymphoma cell lines in the *in vitro* micronucleus test, *Mutation Res.*, 347, 105-115.

(36) Ehrlich, V., Darroudi, F., Uhl, M., Steinkellner, S., Zsivkovits, M. and Knasmeuller, S. (2002), Fumonisin B_1 is genotoxic in human derived hepatoma (HepG2) cells, *Mutagenesis*, 17, 257-260.

(37) Knasmüller, S., Mersch-Sundermann, V., Kevekordes, S., Darroudi, F., Huber, W.W., Hoelzl, C., Bichler, J. and Majer, B.J. (2004), Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge, *Toxicol.*, 198, 315-328. .

(38) Gibson, D.P., Brauninger, R., Shaffi, H.S., Kerckaert, G.A., LeBoeuf, R.A., Isfort, R.J. and Aardema, M.J. (1997), Induction of micronuclei in Syrian hamster embryo cells: comparison to results in the SHE cell transformation assay for National Toxicology Program test chemicals, *Mutation Res.*, 392, 61-70.

(39) Scott, D., Galloway, S.M., Marshall, R.R., Ishidate, M. Jr., Brusick, D., Ashby, J. and Myhr, B.C. (1991), International Commission for Protection Against Environmental Mutagens and Carcinogens, Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9, *Mutation Res.*, 257, 147-205.

(40) Morita, T., Nagaki, T., Fukuda, I. and Okumura, K. (1992), Clastogenicity of low pH to various cultured mammalian cells, *Mutation Res.*, 268, 297-305.

(41) Brusick, D. (1986), Genotoxic effects in cultured mammalian cells produced by low pH treatment conditions and increased ion concentrations, *Environ. Mutagen.*, 8, 789-886.

(42) Fenech, M. and Morley, A.A. (1985), Measurement of micronuclei in lymphocytes, *Mutation Res.*, 147, 29-36.

(43) Fenech, M. (1997), The advantages and disadvantages of cytokinesis-blood micronucleus method, *Mutation Res.*, 392, 11-18.

(44) Bonassi, S., Fenech, M., Lando, C., Lin, Y.P., Ceppi, M., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Jia, C., Di Giorgio, M., Ferguson, L.R., Fucic, A., Lima, O.G., Hrelia, P., Krishnaja, A.P., Lee, T.K., Migliore, L., Mikhalevich, L., Mirkova, E., Mosesso, P., Muller, W.U., Odagiri, Y., Scarffi, M.R., Szabova, E., Vorobtsova, I., Vral, A. and Zijno, A. (2001), HUman MicroNucleus Project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes, I. Effect of laboratory protocol, scoring criteria and host factors on the frequency of micronuclei, *Environ. Mol. Mutagen.* 37, 31-45.

(45) Maron, D.M. and Ames, B.N. (1983), Revised methods for the Salmonella mutagenicity test, *Mutation Res.*, 113, 173-215.

(46) Ong, T.-m., Mukhtar, M., Wolf, C.R. and Zeiger, E. (1980), Differential effects of cytochrome P450-inducers on promutagen activation capabilities and enzymatic activities of S-9 from rat liver, *J. Environ. Pathol. Toxicol.*, 4, 55-65.

(47) Elliott, B.M., Combes, R.D., Elcombe, C.R., Gatehouse, D.G., Gibson, G.G., Mackay, J.M. and Wolf, R.C. (1992), Alternatives to Aroclor 1254-induced S9 in *in-vitro* genotoxicity assays. *Mutagenesis*, 7, 175-177.

(48) Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T. (1976), A safe substitute for Polychlorinated Biphenyls as an Inducer of Metabolic Activation Systems, *In:* de Serres, F.J., Fouts, J. R., Bend, J.R. and Philpot, R.M. (eds), *In Vitro* Metabolic Activation in Mutagenesis Testing, Elsevier, North-Holland, pp. 85-88.

(49) Johnson, T.E., Umbenhauer, D.R. and Galloway, S.M. (1996), Human liver S-9 metabolic activation: proficiency in cytogenetic assays and comparison with phenobarbital/beta-naphthoflavone or Aroclor 1254 induced rat S-9, *Environ. Mol. Mutagen.*, 28, 51-59.

(50) UNEP (2001), Stockholm Convention on Persistent Organic Pollutants, United Nations Environment Programme (UNEP). Available at: [http://www.pops.int/]

(51) Doherty, A.T., Ellard, S., Parry, E.M. and Parry, J.M. (1996), An investigation into the activation and deactivation of chlorinated hydrocarbons to genotoxins in metabolically competent human cells, *Mutagenesis*, 11, 247-274.

(52) Krahn, D.F., Barsky, F.C. and McCooey, K.T. (1982), CHO/HGPRT Mutation Assay: Evaluation of Gases and Volatile Liquids, *In:* Tice, R.R., Costa, D.L. and Schaich, K.M. (eds), Genotoxic Effects of Airborne Agents. New York, Plenum, pp. 91-103.

(53) Zamora, P.O., Benson, J.M., Li, A.P. and Brooks, A.L. (1983), Evaluation of an exposure system using cells grown on collagen gels for detecting highly volatile mutagens in the CHO/HGPRT mutation assay, *Environ. Mutagenesis* 5, 795-801.

(54) Fenech, M. (1993), The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations, *Mutation Res.*, 285, 35-44.

(55) Phelps, J.B., Garriott, M.L., and Hoffman, W.P. (2002), A protocol for the *in vitro* micronucleus test. II. Contributions to the validation of a protocol suitable for regulatory submissions from an examination of 10 chemicals with different mechanisms of action and different levels of activity, *Mutation Res.*, 521, 103-112.

(56) Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate, M. Jr., Kirchner, S., Lorge, E., Morita, T., Norppa, H., Surralles, J., Vanhauwaert, A. and Wakata, A. (2004), Corrigendum to "Report from the *in vitro* micronucleus assay working group", *Mutation Res.*, 564, 97-100.

(57) Pincu, M., Bass, D. and Norman, A. (1984), An improved micronuclear assay in lymphocytes, *Mutation Res.*, 139, 61-65.

(58) Lorge, E., Hayashi, M., Albertini, S. and Kirkland, D. (2008), Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test. I. Theoretical aspects, *Mutation Res.*, 655, 1-3.

(59) Surralles, J., Xamena, N., Creus, A., Catalan, J., Norppa, H. and Marcos, R. (1995), Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures, *Mutation Res.*, 341, 169-184.

(60) Galloway, S. (2000), Cytotoxicity and chromosome aberrations *in vitro*: Experience in industry and the case for an upper limit on toxicity in the aberration assay, Environ. Molec. *Mutagenesis* 35, 191-201.

(61) Hayashi, M., Sofuni, T., and Ishidate, M. Jr. (1983), An Application of Acridine Orange Fluorescent Staining to the Micronucleus Test, *Mutation Res.*, 120, 241-247.

(62) MacGregor, J. T., Wehr, C. M., and Langlois, R. G. (1983), A Simple Fluorescent Staining Procedure for Micronuclei and RNA in Erythrocytes Using Hoechst 33258 and Pyronin Y, *Mutation Res.*, 120, 269-275.

(63) Hayashi, M., Sofuni, T. and Ishidate, M. Jr. (1983), An application of acridine orange fluorescent staining to the micronucleus test, *Mutation Res.,* 120, 241-247.

(64) Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S. and Zeiger, E. (2003), HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, *Mutation Res.,* 534, 65-75.

(65) Hoffman, W.P., Garriott, M.L. and Lee, C. (2003), *In vitro* micronucleus test, *In:* Encyclopedia of Biopharmaceutical Statistics, Second edition. S. Chow (ed.), Marcel Dekker, Inc. New York, NY, pp. 463-467.

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).

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 PERFORMANCE[1]

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
4. Negative substances		
	Di(2-ethylhexyl)phthalate	117-81-7
	Nalidixic acid	389-08-2
	Pyrene	129-00-0
	Sodium chloride	7647-14-5

[1.] The reference chemicals are the recommended chemicals for use. Substitution or adding of chemicals to the list of reference chemicals can be done if their activity is known and if they induce micronuclei by the same mechanisms of action, and if they are shown to be relevant to the chemicals that will be tested using the MNvit procedure. Depending on the purpose, justification could also include a validation study employing a broad variety of substances or focused on a narrower spectrum based on the chemical class of the test substance or the mechanism of damage being studied.