Section 4
Health effects

Test Guideline No. 487

In Vitro Mammalian Cell Micronucleus Test

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Mammalian Cell Micronucleus Test

INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed in light of scientific progress, changing regulatory needs and animal welfare considerations. The original Test Guideline 487 was adopted in 2010; it has been revised in the context of an overall review of the OECD Test Guidelines on genotoxicity and to reflect several years of experience with this test and the interpretation of the data. This Test Guideline is part of a series of Test Guidelines on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to these Test Guidelines has been developed (1).

2. The *in vitro* micronucleus (MNvit) test 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. Therefore the MNvit test is an *in vitro* method that provides a comprehensive basis for investigating chromosome damaging potential *in vitro* because both aneugens and clastogens can be detected (2) (3) in cells that have undergone cell division during or after exposure to the test chemical (see paragraph 13 for more details). 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.

3. This Test Guideline allows the use of protocols with and without the actin polymerisation inhibitor cytochalasin B (cytoB). The addition of cytoB prior to mitosis results in cells that are binucleate and therefore allows for the identification and analysis of micronuclei in only those cells that have completed one mitosis (4) (5). This Test Guideline also allows for the use of protocols without cytokinesis block, provided there is evidence that the cell population analysed has undergone mitosis.

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4. In addition to using the MNvit test to identify substances that induce micronuclei, the use of immunochemical labelling of kinetochores, or hybridisation with centromeric/telomeric probes (fluorescence *in situ* hybridisation (FISH)), also can provide additional information on the mechanisms of chromosome damage and micronucleus formation (6) (7) (8) (9) (10) (11) (12) (13) (14) (15) (16) (17). Those 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.

5. Because micronuclei in interphase cells can be assessed relatively objectively, laboratory personnel need only determine the number of binucleate cells when cytoB is used and the incidence of micronucleate cells in all cases. As a result, the slides 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 test. 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 (18). However, the MNvit test as described in this Test Guideline does not allow for the differentiation of substances inducing changes in chromosome number and/or ploidy from those inducing clastogenicity without special techniques such as FISH mentioned under paragraph 4.

6. The MNvit test is robust and can be conducted 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 test using various cell types (cultures of cell lines or primary cell cultures) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) (29) (30) (31) (32) (33) (34) (35) (36). These include, in particular, the international validation studies co-ordinated by the Société Française de Toxicologie Génétique (SFTG) (19) (20) (21) (22) (23) and the reports of the International Workshop on Genotoxicity Testing (5) (17). 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) (37) (38) (39).

7. The mammalian cell MNvit test may employ cultures of cell lines or primary cell cultures, of human or rodent origin. Because the background frequency of micronuclei will influence the sensitivity of the test, it is recommended that cell types with a stable and defined background frequency of micronucleus formation be used. The cells used are selected on the basis of their ability to grow well in culture, stability of their karyotype (including chromosome number) and spontaneous frequency of micronuclei (40). At the present time, the available data do not allow firm recommendations to be made but suggest it is important, when evaluating chemical hazards to consider the *p53* status, genetic (karyotype) stability, DNA repair capacity and origin (rodent *versus* human) of the cells chosen for testing. The users of this Test Guideline are thus encouraged to consider the influence of these and other cell characteristics on the performance of a cell line in detecting the induction of micronuclei, as knowledge evolves in this area.

8. Definitions used are provided in Annex 1.

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INITIAL CONSIDERATIONS AND LIMITATIONS

9. 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 test substances. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions. Care should be taken to avoid conditions that could lead to artifactual positive results which do not reflect the genotoxicity of the test chemicals. Such conditions include changes in pH (41) (42) (43) or osmolality, interaction with the cell culture medium (44) (45) or excessive levels of cytotoxicity see paragraph 29.

10. 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 chemical. For Manufactured Nanomaterials, specific adaptations of this Test Guideline are needed but they are not described in this Test Guideline.

11. *Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.*

PRINCIPLE OF THE TEST

12. Cell cultures of human or other mammalian origin are exposed to the test chemical both with and without an exogenous source of metabolic activation unless cells with an adequate metabolising capability are used (see paragraph19).

13. During or after exposure to the test chemical, the cells are grown for a period sufficient to allow chromosome damage or other effects on cell cycle/cell division to lead to the formation of micronuclei in interphase cells. For induction of aneuploidy, the test chemical 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 chemical or during the post-treatment period, if one is used. In cultures that have been treated with a cytokinesis blocker, this is easily 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, based on an increase in the cell population, during or after exposure to the test chemical. 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 chemical-induced cytotoxicity should be assessed in all of the cultures that are scored for micronuclei.

DESCRIPTION OF THE METHOD

Cells

14. Cultured primary human or other mammalian peripheral blood lymphocytes (7) (20) (46) (47) and a number of rodent cell lines such as CHO, V79, CHL/IU, and L5178Y cells or human

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cell lines such as TK6 can be used (19) (20) (21) (22) (23) (26) (27) (28) (29) (31) (33) (34) (35) (36) (see paragraph 6). Other cell lines such as HT29 (48), Caco-2 (49), HepaRG (50) (51), HepG2 cells (52) (53), A549 and primary Syrian Hamster Embryo cells (54) have been used for micronucleus testing but at this time have not been extensively validated. Therefore the use of those cell lines and types should be justified based on their demonstrated performance in the test, as described in the Acceptability Criteria section. Cyto B was reported to potentially impact L5178Y cell growth and therefore is not recommended with this cell line (23). When primary cell are used, for animal welfare reasons, the use of cells from human origin should be considered where feasible and sampled in accordance with the human ethical principles and regulations.

15. Human peripheral blood lymphocytes should be obtained from young (approximately 18-35 years of age), non-smoking individuals with no known illness or recent exposures to genotoxic agents (*e.g.* chemicals, ionising radiation) at levels that would increase the background incidence of micronucleate cells. This would ensure the background incidence of micronucleate cells to be low and consistent. The baseline incidence of micronucleate cells increases with age and this trend is more marked in females than in males (55). If cells from more than one donor are pooled for use, the number of donors should be specified. It is necessary to demonstrate that the cells have divided from the beginning of treatment with the test chemical to cell sampling. Cell cultures are maintained in an exponential growth phase (cell lines) or stimulated to divide (primary cultures of lymphocytes) to expose the cells at different stages of the cell cycle, since the sensitivity of cell stages to the test substances may not be known. The primary cells that need to be stimulated with mitogenic agents in order to divide are generally no longer synchronised during exposure to the test chemical (*e.g.* human lymphocytes after a 48-hour mitogenic stimulation). The use of synchronised cells during treatment with the test chemical is not recommended, but can be acceptable if justified.

Media and culture conditions

16. Appropriate culture medium and incubation conditions (culture vessels, humidified atmosphere of 5% CO_2 if appropriate, temperature of 37°C) 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 cells should not be used if contaminated or if the modal chromosome number has changed. The normal cell cycle time of cell lines or primary cultures used in the testing laboratory should be established and should be consistent with the published cell characteristics.

Preparation of cultures

17. Cell lines: cells are propagated from stock cultures, seeded in culture medium at a density such that the cells in suspensions or in monolayers will continue to grow exponentially until harvest time (*e.g.* confluence should be avoided for cells growing in monolayers).

18. Lymphocytes: whole blood treated with an anti-coagulant (*e.g.* heparin), or separated lymphocytes, are cultured (*e.g.* for 48 hours for human lymphocytes) in the presence of a mitogen (*e.g.* phytohaemagglutinin (PHA) for human lymphocytes) in order to induce cell division prior to exposure to the test chemical and cytoB.

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Metabolic activation

19. Exogenous metabolising systems should be used when employing cells with inadequate endogenous metabolic capacity. The most commonly used system that is recommended by default, unless another system is justified is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (generally rats) treated with enzyme-inducing agents such as Aroclor 1254 (56) (57) or a combination of phenobarbital and β-naphthoflavone (58) (59) (60). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (61) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (58) (59) (60). The S9 fraction typically is used at concentrations ranging from 1 to 2% (v/v) but may be increased to 10% (v/v) in the final test medium. The use of products that reduce the mitotic index, especially calcium complexing products (62), should be avoided during treatment. The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of substances being tested.

Test chemical preparation

20. Solid test chemicals should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells. Liquid test chemicals may be added directly to the test system and/or diluted prior to treatment of the test system. Gaseous or volatile test chemicals should be tested by appropriate modifications to the standard protocols, such as treatment in sealed vessels (63) (64) (65). Preparations of the test chemical should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

Test Conditions

Solvents

21. The solvent should be chosen to optimise the solubility of the test chemicals without adversely impacting the conduct of the assay, *i.e.* changing cell growth, affecting integrity of the test chemical, reacting with culture vessels, impairing the metabolic activation system. It is recommended that, wherever possible, the use of an aqueous solvent (or culture medium) should be considered first. Well established solvents are water or dimethyl sulfoxide (DMSO). Generally organic solvents should not exceed 1% (v/v). If cytoB is dissolved in DMSO, the total amount of organic solvent used for both the test chemical and cytoB should not exceed 1% (v/v); otherwise, untreated controls should be used to ensure that the percentage of organic solvent has no adverse effect. Aqueous solvents (saline or water) should not exceed 10% (v/v) in the final treatment medium. If other than well-established solvents are used (*e.g.* ethanol or acetone), their use should be supported by data indicating their compatibility with the test chemical, the test system and their lack of genetic toxicity at the concentration used. In the absence of that supporting data, it is important to include untreated controls (see Annex 1), as well as solvent controls to demonstrate that no deleterious or chromosomal effects (*e.g.* aneuploidy or clastogenicity) are induced by the chosen solvent.

Use of cytoB as a cytokinesis blocker

22. One of the most important considerations in the performance of the MNvit test is ensuring that the cells being scored have completed mitosis during the treatment or the post-treatment incubation period, if one is used. Micronucleus scoring, therefore, should be limited to cells that have gone through mitosis during or after treatment. CytoB is the agent that has been

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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 binucleate cells (6) (66) (67). The effect of the test chemical on cell proliferation kinetics can be measured simultaneously, when cytoB is used. CytoB should be used as a cytokinesis blocker when human lymphocytes are used because cell cycle times will be variable among donors and because not all lymphocytes will respond to PHA stimulation. CytoB is not mandatory for other cell types if it can be established they have undergone division as described in paragraph 27. Moreover CytoB is not generally used when samples are evaluated for micronuclei using flow cytometric methods.

23. The appropriate concentration of cytoB should be determined by the laboratory for each cell type to achieve the optimal frequency of binucleate cells in the solvent control cultures and should be shown to produce a good yield of binucleate cells for scoring. The appropriate concentration of cytoB is usually between 3 and 6 μg/ml (19).

Measuring cell proliferation and cytotoxicity and choosing treatment concentrations

24. When determining the highest test chemical concentration, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity (see paragraph 29), precipitation in the culture medium (see paragraph 30), or marked changes in pH or osmolality (see paragraph 9), 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 appropriate culture conditions.

25. Measurements of cell proliferation are made to assure that sufficient treated cells have undergone mitosis during the test and that the treatments are conducted at appropriate levels of cytotoxicity (see paragraph 29). Cytotoxicity should be determined in the main experiment with and without metabolic activation using an appropriate indication of cell death and growth (see paragraphs 26 and 27). While the evaluation of cytotoxicity in an initial preliminary test may be useful to better define the concentrations to be used in the main experiment, an initial test is not mandatory. If performed, it should not replace the measurement of cytotoxicity in the main experiment.

26. 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 (6), and ensures that only cells that divided during or after treatment with the test chemical are microscopically scored. The cytokinesis-block proliferation index (CBPI) (6) (27) (68) or the Replication Index (RI) from at least 500 cells per culture (see Annex 2 for formulas) are the recommended parameters to estimate cytotoxicity of a treatment by comparing values in the treated and control cultures. Assessment of other indicators of cytotoxicity (*e.g.* cell integrity, apoptosis, necrosis, metaphase counting, cell cycle) could provide useful information, but should not be used in place of CBPI or RI.

27. In studies without cytoB, it is necessary to demonstrate that the cells in culture have divided, so that a substantial proportion of the cells scored have undergone division during or following treatment with the test chemical, otherwise false negative responses may be produced. The measurement of Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) are the recommended parameters to estimate cytotoxicity of a treatment (17) (68) (69)

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(70) (71) (see Annex 2 for formulas). At extended sampling times (*e.g.* treatment for 1.5-2 normal cell cycle lengths and harvest after an additional 1.5-2 normal cell cycle lengths, leading to sampling times longer than 3-4 normal cell cycle lengths in total as described in paragraphs 38 and 39), RPD might underestimate cytotoxicity (71). Under these circumstances RICC might be a better measure or the evaluation of cytotoxicity after a 1.5-2 normal cell cycle lengths would be a helpful estimate. Assessment of other markers for cytotoxicity (*e.g.* cell integrity, apoptosis, necrosis, metaphase counting, Proliferation index (PI), cell cycle, nucleoplasmic bridges or nuclear buds) could provide useful additional information, but should not be used in place of either the RPD or RICC.

28. At least three test concentrations (not including the solvent and positive controls) that meet the acceptability criteria (appropriate cytotoxicity, number of cells, etc) should be evaluated. Whatever the types of cells (cell lines or primary cultures of lymphocytes), either replicate or single treated cultures may be used at each concentration tested. While the use of duplicate cultures is advisable, single cultures are also acceptable provided that the same total number of cells are scored for either single or duplicate cultures. The use of single cultures is particularly relevant when more than 3 concentrations are assessed (see paragraphs 44-45). The results obtained from the independent replicate cultures at a given concentration can be pooled for the data analysis. For test chemicals demonstrating little or no cytotoxicity, concentration intervals of approximately 2 to 3 fold will usually be appropriate. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing cytotoxicity as described in paragraph 29 and including concentrations at which there is moderate and little or no cytotoxicity. Many test chemicals exhibit steep concentration response curves and in order to obtain data at low and moderate cytotoxicity or to study the dose response relationship in detail, it will be necessary to use more closely spaced concentrations and/or more than three concentrations (single cultures or replicates) in particular in situations where a repeat experiment is required (see paragraph 60).

29. If the maximum test concentration is based on cytotoxicity, the highest test concentration should aim to achieve 55 ± 5% cytotoxicity using the recommended parameters to estimate cytotoxicity (*i.e.* RICC and RPD for cell lines when cytoB is not used, and CBPI or RI when cytoB is used). Percent cytotoxicity should be determined using the formulas in Annex 2 (72). Positive results found only at the higher end of the above-mentioned cytotoxicity range should be interpreted with caution (71).

30. For poorly soluble test chemicals that are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. 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. At the concentration producing a precipitate, care should be taken to assure that the precipitate does not interfere with the conduct of the test (*e.g.* staining or scoring). The determination of solubility in the culture medium prior to the experiment may be useful.

31. If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 μl/mL, whichever is the lowest (73) (74) (75). When the test chemical is not of defined composition, *e.g.* substance of unknown or variable composition, complex reaction products or biological materials (*i.e.* UVCBs) (76), environmental extracts, etc., the top concentration may need to be higher (*e.g.* 5 mg/ml) in the absence of sufficient

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cytotoxicity, to increase the concentration of each of the components. It should be noted however that these requirements may differ for human pharmaceuticals (93).

Controls

32. Concurrent negative controls (see paragraph 21), consisting of solvent alone in the treatment medium and processed in the same way as the treatment cultures, should be included for every harvest time.

33. Concurrent positive controls are needed to demonstrate the ability of the laboratory to identify clastogens and aneugens under the conditions of the test protocol used and the effectiveness of the exogenous metabolic activation system (when applicable). Examples of positive controls are given in Table 1 below. Alternative positive control substances can be used, if justified.

34. At the present time, no aneugens are known that require metabolic activation for their genotoxic activity (17). Because *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardised for the short-term treatments done concurrently with and without metabolic activation using the same treatment duration, the use of positive controls may be confined to a clastogen requiring metabolic activation. In this case a single clastogenic positive control response will demonstrate both the activity of the metabolic activation system and the responsiveness of the test system. However, long term treatment (without S9) should have its own positive control, as the treatment duration will differ from the test using metabolic activation. If a clastogen is selected as the single positive control for short-term treatment with and without metabolic activation, an aneugen should be selected for the long-term treatment without metabolic activation. Positive controls for both clastogenicity and aneugenicity should be used in metabolically competent cells that do not require S9.

35. Each positive control should be used at one or more concentrations expected to give reproducible and detectable increases over background in order to demonstrate 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), and the response should not be compromised by cytotoxicity exceeding the limits specified in this TG.

Table 1. Reference substances recommended for assessing laboratory proficiency and for the selection of positive controls

Category	Substance	CASRN
1. Clastogens active without metabolic activation		
	Methyl methanesulphonate	66-27-3
	Mitomycin C	50-07-7
	4-Nitroquinoline-N-Oxide	56-57-5
	Cytosine arabinoside	147-94-4
2. Clastogens requiring metabolic activation		

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	Benzo(a)pyrene	50-32-8
	Cyclophosphamide	50-18-0
3. Aneugens		
	Colchicine	64-86-8
	Vinblastine	143-67-9

PROCEDURE

Treatment Schedule

36. 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 representing all of the various stages of their cell cycles are treated with the test chemical. All treatments should commence and end while the cells are growing exponentially and the cells should continue to grow up to the time of sampling. 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 (17). For lymphocytes, the most efficient approach is to start the treatment with the test chemical at 44-48 hours after PHA stimulation, when cells will be dividing asynchronously (6).

37. Published data (19) indicate that most aneugens and clastogens will be detected by a short term treatment period of 3 to 6 hours in the presence and absence of S9, followed by removal of the test chemical and sampling at a time equivalent to about 1.5 – 2.0 normal cell cycle lengths after the beginning of treatment (7).

38. However, for thorough evaluation, which would be needed to conclude a negative outcome, all three following experimental conditions should be conducted using a short term treatment with and without metabolic activation and long term treatment without metabolic activation (see paragraphs 56, 57 and 58):

- Cells should be exposed to the test chemical without metabolic activation for 3-6 hours, and sampled at a time equivalent to about 1.5 – 2.0 normal cell cycle lengths after the beginning of treatment (19),
- Cells should be exposed to the test chemical with metabolic activation for 3-6 hours, and sampled at a time equivalent to about 1.5 – 2.0 normal cell cycle lengths after the beginning of treatment (19),
- Cells should be continuously exposed without metabolic activation until sampling at a time equivalent to about 1.5 – 2.0 normal cell cycle lengths.

In the event that any of the above experimental conditions lead to a positive response, it may not be necessary to investigate any of the other treatment regimens.

If it is known or suspected that the test chemical affects the cell cycling time (*e.g.* when testing nucleoside analogues), especially for p53 competent cells (35) (36) (77), sampling or recovery times may be extended by up to a further 1.5 – 2.0 normal cell cycle lengths (*i.e.* total 3.0 to 4.0 cell cycle lengths after the beginning of short-term and long-term treatments). These options address situations where there may be concern regarding possible interactions between the test chemical and cytoB. When using extended sampling times (*i.e.* when total 3.0 to 4.0 cell cycle lengths culture time), care should be taken to ensure that the cells are still actively dividing. For

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example, for lymphocytes exponential growth may be declining at 96 hours following stimulation and monolayer cultures of cells may become confluent.

39. The suggested cell treatment schedules are summarised in Table 2. These general treatment schedules may be modified (and should be justified) depending on the stability or reactivity of the test chemical or the particular growth characteristics of the cells being used.

Table 2. Cell treatment and harvest times for the MNvit test

Lymphocytes, primary cells and cell lines treated with cytoB	+ S9 Short treatment	Treat for 3-6 hours in the presence of S9; remove the S9 and treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycle lengths after the beginning of treatment.
	– S9 Short treatment	Treat for 3-6 hours; remove the treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycle lengths after the beginning of treatment.
	– S9 Extended treatment	Treat for 1.5 – 2 normal cell cycle lengths in the presence of cytoB; harvest at the end of the treatment period.
Cell lines treated without cytoB (Identical to the treatment schedules outlined above with the exception that no cytoB is added)		

40. For monolayer cultures, mitotic cells (identifiable as being round and detaching from the surface) may be present at the end of the 3-6 hour treatment. Because these mitotic cells are easily detached, they can be lost when the medium containing the test chemical is removed. If there is evidence for a substantial increase in the number of mitotic cells compared with controls, indicating likely mitotic arrest, then the cells should be collected by centrifugation and the cells added back to the culture, to avoid losing cells that are in mitosis, and at risk for micronuclei/chromosome aberration, at the time of harvest.

Cell harvest and slide preparation

41. Each culture should be 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. Cells with intact cell membrane and intact cytoplasm should be retained to allow the detection of micronuclei and (in the cytokinesis-block method) reliable identification of binucleate cells.

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42. The slides can be stained using various methods, such as Giemsa or fluorescent DNA specific dyes. The use of appropriate fluorescent stains (*e.g.* acridine orange (78) or Hoechst 33258 plus pyronin-Y (79)) 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 (whole chromosomes will be stained while acentric chromosome fragments will not) of micronuclei if mechanistic information of their formation is of interest (16) (17). Other methods for differentiation between clastogens and aneugens may be used if they have been shown to be effective and validated. For example, for certain cell lines the measurements of sub-2N nuclei as hypodiploid events using techniques such as image analysis, laser scanning cytometry or flow cytometry could also provide useful information (80) (81) (82). Morphological observations of nuclei could also give indications of possible aneuploidy. Moreover, a test for metaphase chromosome aberrations, preferably in the same cell type and protocol with comparable sensitivity, could also be a useful way to determine whether micronuclei are due to chromosome breakage (knowing that chromosome loss would not be detected in the chromosome aberration test).

Analysis

43. All slides, including those of the solvent and the untreated (if used) and positive controls, should be independently coded before the microscopic analysis of micronucleus frequencies. Appropriate techniques should be used to control any bias or drift when using an automated scoring system, for instance, flow cytometry, laser scanning cytometry or image analysis. Regardless of the automated platform is used to enumerate micronuclei, CBPI, RI, RPD, or RICC should be assessed concurrently.

44. In cytoB-treated cultures, micronucleus frequencies should be analysed in at least 2000 binucleate cells per concentration and control (83), equally divided among the replicates, if replicates are used. In the case of single cultures per dose (see paragraph 28), at least 2000 binucleate cells per culture (83) should be scored in this single culture. If substantially fewer than 1000 binucleate cells per culture (for duplicate cultures), or 2000 (for single culture), 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 cytotoxic 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. In addition, binucleate cells should not 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 (84). Scoring of mononucleate cells is acceptable if the test chemical is shown to interfere with cytoB activity. A repeat test without CytoB might be useful in such cases. Scoring mononucleate cells in addition to binucleate cells could provide useful information (85) (86), but is not mandatory.

45. In cell lines tested without cytoB treatment, micronuclei should be scored in at least 2000 cells per test concentration and control (83), equally divided among the replicates, if replicates are used. When single cultures per concentration are used (see paragraph 28), at least 2000 cells per culture should be scored in this single culture. If substantially fewer than 1000 cells per culture (for duplicate cultures), or 2000 (for single culture), 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 cytotoxic concentrations, whichever is appropriate.

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46. 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 in culture have divided, as discussed in paragraphs 24-28.

Proficiency of the laboratory

47. In order to establish sufficient experience with the assay prior to using it for routine testing, the laboratory should have performed a series of experiments with reference positive substances acting via different mechanisms (at least one with and one without metabolic activation, and one acting via an aneugenic mechanism, and selected from the substances listed in Table 1) and various negative controls (including untreated cultures and various solvents/vehicle). These positive and negative control responses should be consistent with the literature. This is not applicable to laboratories that have experience, *i.e.* that have an historical data base available as defined in paragraphs 49 to 52.

48. A selection of positive control substances (see Table 1) should be investigated with short and long treatments in the absence of metabolic activation, and also with short treatment in the presence of metabolic activation, in order to demonstrate proficiency to detect clastogenic and aneugenic substances, determine the effectiveness of the metabolic activation system and demonstrate the appropriateness of the scoring procedures (microscopic visual analysis, flow cytometry, laser scanning cytometry or image analysis). A range of concentrations of the selected substances should be chosen so as to give reproducible and concentration-related increases above the background in order to demonstrate the sensitivity and dynamic range of the test system.

Historical control data

49. The laboratory should establish:
- A historical positive control range and distribution,
- A historical negative (untreated, solvent) control range and distribution.

50. When first acquiring data for an historical negative control distribution, concurrent negative controls should be consistent with published negative control data where they exist. As more experimental data are added to the control distribution, concurrent negative controls should ideally be within the 95% control limits of that distribution (87) (88). The laboratory's historical negative control database, should initially be built with a minimum of 10 experiments but would preferably consist of at least 20 experiments conducted under comparable experimental conditions. Laboratories should use quality control methods, such as control charts (*e.g.* C-charts or X-bar charts (88)), to identify how variable their positive and negative control data are, and to show that the methodology is 'under control' in their laboratory (83). Further recommendations on how to build and use the historical data (*i.e.* criteria for inclusion and exclusion of data in historical data and the acceptability criteria for a given experiment) can be found in the literature (87).

51. Any changes to the experimental protocol should be considered in terms of the consistency of the data with the laboratory's existing historical control databases. Any major inconsistencies should result in the establishment of a new historical control database.

52. Negative control data should consist of the incidence of micronucleated cells from a single culture or the sum of replicate cultures as described in paragraph 28. Concurrent negative

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controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database (87) (88). Where concurrent negative control data fall outside the 95% control limits, they may be acceptable for inclusion in the historical control distribution as long as these data are not extreme outliers and there is evidence that the test system is 'under control' (see paragraph 50) and there is evidence of absence of technical or human failure.

DATA AND REPORTING

Presentation of the results

53. 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. The scoring of the numbers of cells with one, two, or more micronuclei can be reported separately and could provide useful information, but is not mandatory.

54. Concurrent measures of cytotoxicity for all treated, negative and positive control cultures should be determined (16). 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 should be used (see Annex 2).

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

Acceptability Criteria

56. Acceptance of a test is based on the following criteria:

- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database as described in paragraph 50;
- Concurrent positive controls (see paragraph 50) should induce responses that are compatible with those generated in the laboratory's historical positive control data base and produce a statistically significant increase compared with the concurrent negative control;
- Cell proliferation criteria in the solvent control should be fulfilled (paragraph 25-27);
- All experimental conditions were tested unless one resulted in positive results (paragraphs 36-40);
- Adequate number of cells and concentrations are analysable (paragraphs 28 and 44-46);
- The criteria for the selection of top concentration are consistent with those described in paragraphs 24-31.

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Evaluation and interpretation of results

57. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined (see paragraphs 36-39):

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control (89);
- the increase is dose-related in at least one experimental condition when evaluated with an appropriate trend test (see paragraph 28);
- any of the results are outside the distribution of the historical negative control data (*e.g.* Poisson-based 95% control limits; see paragraph 52).

When all of these criteria are met, the test chemical is then considered able to induce chromosome breaks and/or gain or loss in this test system. Recommendations for the most appropriate statistical methods can also be found in the literature (90) (91) (92).

58. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined (see paragraphs 36-39):

- none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control;
- there is no concentration-related increase when evaluated with an appropriate trend test;
- all results are inside the distribution of the historical negative control data (*e.g.* Poisson-based 95% control limits; see paragraph 52).

The test chemical is then considered unable to induce chromosome breaks and/or gain or loss in this test system. Recommendations for the most appropriate statistical methods can also be found in the literature (90) (91) (92).

59. There is no requirement for verification of a clear positive or negative response.

60. In case the response is neither clearly negative or clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (*e.g.* concentration spacing, other metabolic activation conditions [*i.e.* S9 concentration or S9 origin]) could be useful.

61. In rare cases, even after further investigations, the data set will not allow a conclusion of positive or negative, and will therefore be concluded as equivocal.

62. Test chemicals that induce micronuclei in the MNvit test 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.

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Test Report

63. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- reactivity of the test chemicals with the solvent/vehicle or cell culture media;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality, and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent:

- justification for choice of solvent;
- percentage of solvent in the final culture medium

Cells:

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

Test Conditions:

- identity of the cytokinesis blocking substance (*e.g.* cytoB), if used, and its concentration and duration of cell exposure;
- concentration of the test chemical expressed as a final concentration the culture medium (*e.g.* µg or mg/mL, or mM of culture medium);
- rationale for the selection of concentrations and the number of cultures, including cytotoxicity data and solubility limitations;
- composition of media, CO_2 concentration, if applicable, humidity level;
- concentration (and/or volume) of the solvent and test chemical added in the culture

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medium;
- incubation temperature and time;
- duration of treatment;
- harvest time after treatment;
- cell density at seeding, if applicable;
- type and composition of metabolic activation system, (source of S9, method of preparation of the S9 mix, the concentration or volume of S9 mix and S9 in the final culture medium, quality controls of S9 (*e.g.* enzymatic activity, sterility, metabolic capability);
- positive and negative control substances, final concentrations, conditions and durations of treatment and recovery periods;
- methods of slide preparation and the staining technique used;
- criteria for scoring micronucleate cells (selection of analysable cells and identification of micronucleus);
- numbers of cells analysed;
- methods for the measurements of cytotoxicity;
- any supplementary information relevant to cytotoxicity and method used;
- criteria for considering studies as positive, negative, or equivocal;
- method(s) of statistical analysis used;
- methods, such as use of kinetochore antibody or pan-centromeric specific probes, to characterise whether micronuclei contain whole or fragmented chromosomes, if applicable;
- methods used to determine pH, osmolality and precipitation.

Results:

- definition of acceptable cells for analysis;
- in the absence of cyto B, the number of cells treated and the number of cells harvested for each culture in case of cell lines;
- parameters to estimate cytotoxicity, *e.g.* CBPI or RI in the case of cytokinesis-block method; RICC or RPD when cytokinesis-block methods are not used; other observations if any (*e.g.* cell confluency, apoptosis, necrosis, metaphase counting, frequency of binucleated cells);
- signs of precipitation and time of the determination;
- data on pH and osmolality of the treatment medium, if determined;
- distribution of mono-, bi-, and multi-nucleate 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) and positive control data (concentrations and solvents);
- historical negative (solvent) and positive control data, with ranges, means and standard deviation and 95% control limits for the distribution, as well as the number of data;
- statistical analysis; p-values if any.

Discussion of the results.

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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 apparatus, 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 the disintegration of cells into membrane-bound particles that are then eliminated by phagocytosis or by shedding.

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

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

Concentrations: refer to final concentrations of the test chemical in the culture medium.

Clastogen: any substance or event which causes structural chromosomal aberrations in populations of cells or eukaryotic 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 average number of nuclei per cell, which may be used to estimate cytotoxicity (see Annex 2 for formula).

Cytostasis: inhibition of cell proliferation.

Cytotoxicity: a general term for describing adverse effects to cells e.g. cell death, inhibition of cell proliferation (cytostasis). The formulas for estimating cytotoxicity are described in Annex 2.

Genotoxic: a general term encompassing all types of DNA or chromosome damage, including breaks, deletions, adducts, nucleotides modifications and linkages, rearrangements, gene 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

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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.

p53 status: p53 protein is involved in cell cycle regulation, apoptosis and DNA repair. Cells deficient in functional p53 protein, unable to arrest cell cycle or to eliminate damaged cells via apoptosis or other mechanisms (e.g. induction of DNA repair) related to p53 functions in response to DNA damage, should be theoretically more prone to gene mutations or chromosomal aberrations.

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): an indication of cell proliferation determined by counting the number of clones consisting of one, two, three to four or five to eight cells. It is a useful additional parameter for confirming cell proliferation has occurred in cell lines tested in the absence of cytoB.

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

Relative Population Doubling (RPD): parameter to estimate cytotoxicity 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).

S9 liver fraction: supernatant of liver homogenate after 9000g centrifugation, *i.e.* raw liver extract.

S9 mix: mix of the S9 liver fraction and cofactors necessary for metabolic enzyme activity.

Solvent control: General term to define the control cultures receiving the solvent alone used to dissolve the test chemical.

Untreated control: cultures that receive no treatment (*i.e.* no test chemical nor solvent) but are processed concurrently in the same way as the cultures receiving the test chemical.

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ANNEX 2

FORMULAS TO ESTIMATE CYTOTOXICITY

Parameters of cell proliferation, cytostasis and cytotoxicity are all interrelated. Changes in cell proliferation parameters in treated cultures relative to concurrent negative controls can be used as surrogate estimates for cytotoxicity using the formulas described below. Each of these parameters is different in terms of the contribution made by cytostasis or other forms of cytotoxicity to the calculations. However, for consistency with the rest of TG487 and for ease of understanding, the term cytotoxicity has been used to describe the final calculation, irrespective of the starting parameter.

1. **With cyto B**

When cytoB is used, estimates of cytotoxicity should be based on the **Cytokinesis-Block Proliferation Index (CBPI)** or **Replication Index (RI)** (17) (69). The result obtained from the final calculation of cytotoxicity is the same, regardless of whether CBPI or RI are used.

- The **CBPI** indicates the average number of nuclei per cell. The inclusion of mononucleates in the calculation accounts for cytostasis and therefore, CBPI gives an estimate of cell proliferation. CBPI for the Treated cells and CBPI for the Controls are calculated separately.

Formula:

$$\mathrm{CBPI} = \frac{[\text{Number of mononucleate cells}] + 2\times[\text{Number of binucleate cells}] + 3\times[\text{Number of multinucleate cells}]}{[\text{Total number of cells}]}$$

Cytotoxicity estimate:

$$\%\ \text{Cytotoxicity} = 100 - 100 \times \frac{\mathrm{CBPI_T} - 1}{\mathrm{CBPI_C} - 1}$$

where
T = test chemical treated cultures
C = control cultures

- The **RI** indicates the relative number of cell cycles per cell during the period of exposure to cytoB in treated cultures compared to control cultures:

Formula:

$$\mathrm{RI} = \frac{\dfrac{[\text{Number of binucleate cells}]_\mathrm{T} + 2\times[\text{Number of multinucleate cells}]_\mathrm{T}}{[\text{Total number of cells}]_\mathrm{T}}}{\dfrac{[\text{Number of binucleate cells}]_\mathrm{C} + 2\times[\text{Number of multinucleate cells}]_\mathrm{C}}{[\text{Total number of cells}]_\mathrm{C}}} \times 100$$

where
T= test chemical treated cultures
C= control cultures

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Cytotoxicity estimate:

$$\%\ \text{Cytotoxicity} = 100\text{-RI}$$

2. Without cytoB

When cytoB is not used, estimates of cytotoxicity should be based on **Relative Increase in Cell Counts (RICC)** or on **Relative Population Doubling (RPD)** as both take into account the proportion of the cell population which has divided (69). The result obtained from the final calculation of cytotoxicity is not necessarily the same, depending on whether RICC or RPD is used.

- **Relative Increase in Cell Counts (RICC)**

Formula:

$$\text{RICC} = \frac{[\text{Final number of cells}]_{\text{T}} - [\text{Starting number of cells}]_{\text{T}}}{[\text{Final number of cells}]_{\text{C}} - [\text{Starting number of cells}]_{\text{C}}} \times 100$$

Cytotoxicity estimate:

$$\%\ \text{Cytotoxicity} = 100 - \text{RICC}$$

- **Relative Population Doubling (RPD)**

Formula:

$$\text{RPD} = \frac{[\text{Number of population doublings}]_{\text{T}}}{[\text{Number of population doublings}]_{\text{C}}} \times 100$$

where:

$$\text{Number of population doublings} = \frac{1}{\log_{10} 2} \times \log_{10} \frac{[\text{Final number of cells}]}{[\text{Starting number of cells}]}$$

Cytotoxicity estimate:

$$\%\ \text{Cytotoxicity} = 100 - \text{RPD}$$

Additional comments

In all cases, the number of cells before treatment should be the same for treated and negative control cultures.

When using automated scoring systems, for instance flow cytometry, laser scanning cytometry or image analysis, the number of cells in the formula can be substituted by the number of nuclei.

In the negative control cultures, the cell proliferation parameter(s)should be compatible with the requirement to sample cells after treatment at a time equivalent to about 1.5 – 2.0 normal cell cycle.

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