OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE 487:

In Vitro Micronucleus Test

INTRODUCTION

1. The *in vitro* micronucleus assay is a genotoxicity test system used for the detection of micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. The assay detects the activity of both clastogenic and aneugenic chemicals (1, 2) in cells that have undergone cell division after exposure to the test substance. Development of the cytokinesis-block methodology, by addition of the actin polymerisation inhibitor cytochalasin B during the targeted mitosis, allows the identification and selective analysis of micronucleus frequency in cells that have completed one cell division as such cells are binucleate (3, 4). However, the Test Guideline also allows the use of protocols without cytokinesis block, provided there is evidence that the majority of cells analysed are likely to have undergone cell division.

2. The immunochemical labelling (FISH) of kinetochores, or hybridisation with general or chromosome specific centromeric/telomeric probes can provide useful information on the mechanism of micronucleus formation (5). Use of cytokinesis block facilitates the acquisition of the additional mechanistic information (*e.g.*, chromosome non-disjunction) that can be obtained by FISH-techniques (6-15).

3. The micronucleus assay has a number of advantages over metaphase analysis performed to measure chromosome aberrations in the OECD Guidelines 473 and 475 (16, 17). Because micronuclei in interphase cells can be assessed more objectively than chromosomal aberrations in metaphase cells, a less detailed training for testing personnel to achieve competence in this assay is required. Also, there is no requirement to count the chromosomes in a metaphase preparation and to evaluate subtle chromatid and chromosome damage, but only to determine whether or not a cell contains a micronucleus. As a result, the preparations can be scored much more quickly and analysis can be automated. This makes it practical to score thousands instead of hundreds of cells per treatment, and this increases the reliability of the assay. Finally, as micronuclei may contain whole (lagging) chromosomes there is the potential to detect aneuploidy-inducing agents that are difficult to study in conventional chromosomal aberration tests.

4. The assay is an *in vitro* method, where cultured mammalian cells may be used. However, the test method does not replace the OECD Test Guideline 474 “Mammalian Erythrocyte Micronucleus Test” (18). Rather it provides a more comprehensive basis for investigating mutagenic potential *in vitro* because both aneugens and clastogens are detected. This is of particular value in testing strategies in which *in vivo* testing for genotoxic activity is not included in the initial screen, because current OECD Test Guidelines do not include a method that can detect aneugens in an *in vitro* test.

5. There is now an extensive amount of data to support the validation of the *in vitro* micronucleus assay using various cell lines or human lymphocytes (19-29). These include, in particular, the international validation studies co-ordinated by the French Society of Genetic Toxicology (SFGT) (19-23) and the reports of the international *in vitro* micronucleus assay working group (4, 5, 30). The available data has also been re-evaluated in a weight-of-evidence retrospective validation study by the European Centre for the Validation of Alternative Methods (ECVAM) and endorsed as scientifically valid by the ECVAM

Scientific Advisory Committee (ESAC) as an alternative to the *in vitro* chromosome aberration assay for genotoxicity testing (TG 473, 31).

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. This metabolic activation system cannot entirely mimic *in vivo* conditions. Care should also be taken to avoid conditions that would lead to artefactual positive results which do not reflect intrinsic mutagenicity and may arise from e.g. changes in pH, osmolality or high levels of cytotoxicity (32, 33).

8. In order to analyse the induction of micronuclei it is essential that nuclear division has occurred in both treated and untreated cultures. The most convenient stage to score micronuclei is when the cells have completed one cell division after chemical treatment and are, therefore, capable of expressing micronuclei. This was clearly demonstrated by applying the cytokinesis-block method to human lymphocytes and evaluating the micronuclei at the binucleate interphase stage (33, 34).

9. Treatment of cultures with a cytokinesis blocker and measurement of the relative frequencies of binucleate to mononucleate cells within a culture also provides a simple method of measuring the cytostatic or toxic activity of a treatment (35).

PRINCIPLE OF THE TEST

10. Cell cultures are exposed to the test substances both with and without an exogenous source of metabolic activation unless primary cells with metabolizing capability are used. After exposure to the test substance, cell cultures are grown for a period sufficient to allow chromosome or spindle damage to lead to the formation of micronuclei in interphase cells and to trigger the aneuploidy sensitive cell stage (G2/M). Harvested and stained interphase cells are then analysed microscopically for the presence of micronuclei. Ideally, micronuclei should only be scored in those cells that have completed nuclear division following exposure to the test chemical. In cultures that have been treated with a cytokinesis blocker, this is achieved by scoring only binucleate cells. In the absence of a blocker, it is important to demonstrate that the majority of mononucleate cells are likely to have undergone at least one cell division since exposure to the test substance. For all protocols, it is important that cell proliferation is demonstrated in both control and treated cells, together with an assessment of cytotoxicity in the treated cells scored for micronuklei.

DESCRIPTION OF THE METHOD

Preparations

Cells

11. Cultured cells from human peripheral blood lymphocytes or from Syrian Hamster Embryo (SHE) may be used. Cell lines CHO, V79, CHL/IU and L5178\Y are also suitable (20, 26, 28, 36), although there is some concern about the possible interactions when using the cytokinesis blocker, cytochalasin B, with L5178/Y cells (19, 37). The use of other cell types, such as human derived hepatoma (HepG2) cells (38, 39) should be justified. Since the background frequency of micronuclei will influence the sensitivity of the assay, it is recommended that cell types with stable background frequency of micronuclei are used in these studies. The frequency of micronuclei in the negative control cultures should be within the historic negative control range for the laboratory.

12. When human peripheral lymphocytes are used, blood from healthy, non-smoking donors should be used. Micronucleus frequency increases with age and this trend is more marked in females than in males. However, many factors can affect not only the micronucleus frequency but also the response to mitogens and xenobiotics. Evidence for large variations in the human population (40) derives largely from biomonitoring studies and relates to background frequencies rather than the response to xenobiotics. However, the latter variability cannot be entirely discounted (41). It is therefore recommended that peripheral lymphocytes from a panel of donors with a known background micronucleus frequency and response to mitogen stimulation and positive control genotoxins should be used. The range of responses within this Panel should be narrow to allow comparison with historical values and pooling of samples from 2 or more donors. Blood from male and female donors should not be pooled and comparisons with historic ranges should be gender-specific.

Media and culture conditions

13. Appropriate culture medium and incubation conditions (culture vessels, CO_2 concentration, temperature and humidity) should be used in 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 cultures should not be used if contaminated. The normal cell cycle time for the cell and culture conditions used should be known. If the cytokinesis-block method is used then the concentrations of the cytokinesis inhibitor used must give an adequate yield of binucleate cells.

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 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 mitogen (*e.g.*, phytohemagglutinin, PHA) prior to exposure to the test chemical.

Metabolic Activation

16. Exogenous metabolising systems are required when using cell cultures 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 (41, 42) or preferably a combination of phenobarbitone and β-naphthoflavone (44, 45). The latter, safer, combination is in keeping with the Stockholm POPs convention 2001 (46) and has been shown to be as effective as Araclor 1254 induced S-9 (47) The post-mitochondrial fraction is usually used at concentrations ranging from 1-10% (v/v) in the final test medium. The selection of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilise more than one concentration of post-mitochondrial fraction.

17. A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. In such cases the choice of the cell lines used should be scientifically justified (*e.g.*, by relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance) (48).

Test substance/Preparation

18. Solid test substances should be dissolved or suspended 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. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

Test Conditions

Solvents/vehicle

19. The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells used and with the maintenance of S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility with the test. It is recommended that, wherever possible, the use of an aqueous solvent/vehicle should be considered first. When testing water-unstable substances, the organic solvents used should be free of water.

Use of cytochalasin B as a cytokinesis blocker

20. Cytochalasin B is the agent that has been most widely used to block cytokinesis but other agents can be used with justification. Cytochalasin B inhibits actin assembly and cytokinesis and thus prevents separation of daughter cells after mitosis and leads to binucleated cells (49, 50). The evaluation can thus be limited to proliferating cells and a reduction of cell proliferation can be measured simultaneously. The use of a cytokinesis blocker is mandatory when human lymphocytes are used because cell cycle lengths will be variable within and between cultures. This is not the case with cell lines and cytochalasin B need not be used provided that cell proliferation is demonstrated to ensure that the majority of cells scored have progressed through mitosis.

21. The appropriate concentration of cytochalasin B is usually between 3 and 6 μg/ml and should be tested for each cell line to achieve the maximum binucleated cells in the solvent/vehicle control cultures. Cytochalasin B should be added after the test substance is removed.

22. Parallel cultures of human lymphocytes or cell lines, exposed simultaneously to the test substance and cytochalasin B, may be run to check for effects on cell cycling

Exposure concentrations

23. Among the criteria to be considered when determining the highest concentration to be tested are cytotoxicity, solubility in the test system and any changes in pH or osmolality (32, 33).

24. Cytotoxicity should be determined with and without metabolic activation concurrently in the main experiment. Since micronucleus expression is dependent on cell proliferation, quantification of cell proliferation and cell death should be carried out to obtain a sound evaluation of cell kinetics and micronucleus frequencies. Assessing cytotoxicity as measured by mitotic index is therefore a sub-optimal choice as mitotic figures may result from mitotic block.

25. Assessment of cytostasis indicates a reduction in cell proliferation in treated cultures as compared to the control/untreated cultures. In the case of studies without cytochalasin B, cell proliferation should be measured by the cell counts or the population doubling, combined with an assessment of cytotoxicity. In the case of studies with cytochalasin B, cytostasis is quantified as the cytokinesis-block proliferation index (CBPI) (29, 51) or the replication index (RI) (30). CBPI indicates the number of cell cycles per cell during the period of exposure to cytochalasin B. It may be used to calculate cytostasis by the following formula;

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

Where:

$$CBPI = \frac{\text{N° mononucleate cells} + 2 \times \text{N° binucleate cells} + 3 \times \text{N° multinucleate cells}}{\text{Total number of cells}}$$

And:

T = test chemical treatment culture

C = vehicle control culture

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

The RI indicates the relative number of nuclei in treated cultures compared to control cultures and it may be used to calculate the % cytostasis by the following formula;

Cytotostasis = RI

$$RI = \frac{(\text{N° binucleated cells} + 2 \times \text{N° multinucleate cells})/ \text{Total number of cells treated cultures}}{(\text{N° binucleated cells} + 2 \times \text{N° multinucleate cells})/ \text{Total number of cells control cultures}} \times 100$$

Assessment of other markers for cytotoxicity (*e.g.*, confluency, apoptosis, necrosis and, metaphase counting) can provide useful additional information.

26. At least 3 analysable test concentrations should be used. These concentrations should be chosen with care using data from preliminary cytotoxicity/cytostasis studies. Where cytotoxicity/cytostasis occurs, these concentrations should cover a range from the maximum to little or no cytotoxicity/cytostasis. The highest concentration should aim to produce 50 - 70% cytotoxicity/cytostasis (5). This level is recommended to avoid false negatives when very steep toxicity curves are observed, in particular with aneugens (5). Other concentrations should, in general, be separated by spacing of no more than the square root of 10.

27. If no cytotoxicity/cytostasis is observed, the highest concentration should correspond to 0.01 M, 5 mg/ml or 5 µl/ml, whichever is the lowest.

28. For poorly soluble compounds that are not cytotoxic at concentrations lower than the insoluble concentration, the highest concentration should produce a precipitate visible by the unaided eye or with the aid of an inverted microscope at the end of the treatment. In some cases (e.g. when toxicity occurs only above the solubility limit) it is advisable to test at more than one concentration with visible precipitate). A total of at least three concentrations should be used with spacing as described in paragraph 26. The precipitate should not interfere with scoring.

29. Gases or volatile substances should be tested by appropriate modifications to the standard protocols, such as treatment in sealed vessels (52, 53).

Controls

30. Concurrent positive and negative (solvent or vehicle) controls should be included in each experiment. One of the positive control chemicals should require activation to give a mutagenic response. This provides a control for the activity of the metabolising system, whether endogenous or exogenous.

31. Positive controls should employ a known inducer of micronucleus formation at exposure levels expected to give a reproducible and detectable increase over background, which demonstrates 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.

32. Since to date no aneugens requiring metabolic activation are commonly recognised (5), a clastogen that requires metabolic activation (*e.g.*, cyclophosphamide or benzo[a]pyrene) should be used to demonstrate both the metabolic competence and the ability of the test system to detect clastogens. Since cyclophosphamide can be activated directly by some cell lines by an atypical route of metabolism (5) it is not an appropriate positive control for metabolism in such cell lines. Benzo[a]pyrene is recommended as the positive control compound for a clastogen requiring metabolic activation and colchicine or vinblastine as the positive controls for aneugenic activity.

33. Other appropriate positive control reference substances may be used if justified. The use of chemical class-related positive control chemicals may be considered, when available. All positive control substances used should be demonstrated to be appropriate for the cell line used.

34. Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment cultures, should be included for every harvest time. In addition, untreated control (lacking solvent) should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

PROCEDURE

Treatment Schedule

35. In lymphocyte cultures, mitogenic stimulation results in a wave of the actively dividing cells that are initially synchronised and this synchrony will gradually decrease over time of the cell cycle. This is not the case with cell lines. In order to maximise the probability of detecting an aneugen or clastogen acting at an unknown stage in the cell cycle, it is important that cells are exposed to the test substance at all stages. The treatment schedule for cell lines therefore differs somewhat from that for lymphocytes, and these are considered in turn (5).

Cell Lines

36. Theoretical considerations based on the non-synchronised cycling of cell lines in culture, together with data (ref) indicate that most aneugens and clastogens will be detected by a short term treatment (3 - 6 hours) in the presence and absence of S9 followed by a recovery period, if required (5). Cells are sampled at a time equivalent to about 2 times the normal (i.e. untreated) cell cycle lengths after the beginning of treatment. In some instances a longer recovery period (employing sampling times of about 3 cell cycles) may be appropriate.

37. If negative or equivocal results are obtained, they should be confirmed using continuous treatment, or modified conditions as appropriate. In the study without exogenous metabolic activation, cells are exposed continuously for about 2 times the normal cell cycle and then sampled. If the test chemical is known to prolong the cell cycle considerably (e.g. nucleoside analogues) then a longer period (about 3 cell cycles) may be appropriate. In the study in the presence of exogenous metabolic activation, a confirmatory study should be done by repeating the first study or by employing modified conditions (such as an increased concentration of S9), with justification for the modified conditions being given.

38. When cytochalasin B is used in the test cultures, it should be added after the test substance is removed and at least one cell cycle before harvest.

39. When cytochalasin B is used in parallel cultures to detect cell cycle effects, it is recommended that it be added during the first cell cycle following the start of the treatment and that the cells are harvested prior to the second mitosis.

Lymphocytes

40. Theoretical considerations together with limited experimental data (54) indicate that the most efficient approach is to test lymphocytes 48 h after PHA stimulation, when cycle synchronisation will have dissipated, with a pulse (3-6h) of exposure to the test substance in the presence and absence of S9. This should be accompanied by a prolonged (20h) exposure to the test substance in the absence of S9. Cytochalasin B is added to all cultures after removal of the test substance and 28 h prior to harvest.

41. If the protocols give negative or equivocal results confirmation should be considered by varying the conditions, such as commencing exposure at 24 hours after PHA stimulation, and/or by varying the S9 concentration. Cytochalasin B is added after removal of the test compound and 28 h prior to harvest unless the results of the initial protocols indicated that the period of exposure to cytochalasin should be reduced (if there were excess numbers of polynucleate cells) or increased (if there were insufficient binucleate cells) .

42. If it is known or suspected that the test substance acts a specific, identified phase of the cell cycle, the protocol should be modified to target exposure to this phase.

Number of cultures

43. Duplicate cultures should be performed at each concentration and are strongly recommended for negative/solvent control cultures. Where minimal variation between duplicate cultures can be demonstrated, from historical data, it may be acceptable for single cultures to be used at each concentration.

Preparation of cells

44. 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 preparations are obtained. Cell cytoplasm should be retained but well-spread to allow the detection of micronuclei and (in the cytokinesis-block method) reliable identification of binucleate cells.

45. The slides can be stained using various methods. Fluorescent DNA-specific dyes are preferred to less specific stains (such as Giemsa), as they will facilitate the detection of even very small micronuclei (54). Antikinetochore antibodies, fluorescence *in situ* hybridization 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 chromosome/chromosomal fragment) of micronuclei if mechanistic information is of interest (14, 15). . Other methods for differentiation between clastogens and aneugens may be used.

Analysis

46. All slides, including those of positive and negative controls, should be independently coded before the microscopic analysis.

47. In cytochalasin B-treated cultures, micronucleus frequencies should be analysed in 2000 binucleated cells per concentration (1000 binucleated cells per culture, two cultures per concentration).

With cytochalasin B, a parallel scoring of micronuclei in mononucleated cells is optional (1000 cells/culture). In cell lines analysed without cytochalasin B micronuclei should be scored in 2000 cells per concentration (1000 cells per culture: two cultures per concentration).

48. When cytochalasin B is used to assess cell proliferation, a CBPI (see paragraph 25) should be determined from 500 cells per culture.

When treatments are performed in the absence of cytochalasin B, mononucleate cells are analysed for the presence of micronuclei; in such cases it is essential to provide evidence that the majority of the cells in the culture are proliferating as discussed in paragraph 25.

49. When the cytokinesis-block assay is used, it should be kept in mind that some cells may escape the cytokinesis block. Thus, some mononucleate cells may actually have divided in the culture, while some binucleate cells may have divided more than once. Care should be taken not to include binucleate cells with irregular shapes or sizes of the main nuclei, as these cells may represent the latter category; neither should binucleate cells be confused with poorly spread multinucleate cells. Cells containing more than two main nuclei should not be analysed for micronuclei as the baseline micronucleus frequency is higher in these cells owing to non-genotoxic effects such as nuclear disintegration. (55)

50. In the case of test substances that interfere with cell division (*e.g.*, colchicine), there may be very few binucleate cells available for analysis after exposure to concentrations of interest. However, such test substances increase the frequency of micronuclei in mononucleate cells. In such cases analysis of micronuclei in mononucleate cells may be useful (56).

DATA AND REPORTING

Treatment of results

51. The experimental unit is the cell, and the unit for statistical analysis is the culture. Additionally, cells with one, two and more than two micronuclei should be recorded separately. If the cytokinesis-block technique is applied only the frequencies of binucleate cells with micronuclei (independent on the number of micronuclei per cell) should be used in the evaluation of micronucleus induction. In the absence of cytokinesis block, the frequency of mononucleated cells with one or more micronuclei is the key parameter.

52. The CBPI should be provided for all treated and control cultures as a measure of cell cycle delay in the cytokinesis-block method. Concurrent measures of cytotoxicity for all treated and negative control cultures in the main micronuclei induction experiment(s) should also be recorded.

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

54. There is no requirement for verification of a clear-cut positive response. Equivocal results should be clarified by analysis of more cells to improve the power of the test to detect small differences and/or by further testing. Negative results should be confirmed in an independent experiment. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results. Study parameters that might be modified include the test concentration spacing, the timing of treatment and cell harvest and the metabolic activation conditions.

Chemicals which induce micronuclei in the *in vitro* assay may do so by a variety of mechanisms such as chromosome breakage and chromosome loss. Further analyis using kinetochore antibodies or centromere specific *in situ* probes may be useful in assessing whether the mechanism is due to clastogenic or aneugenic activity. **Evaluation and interpretation of results**

55. There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells containing micronuclei. The biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (57). Statistical significance should not be the only determinant of a positive response.

56. 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.

57. Positive results from the *in vitro* micronucleus test indicate that the test substance induces chromosome damage and/or damage to the cell division apparatus, in cultured mammalian somatic cells, under the test conditions. Negative results indicate that, under the test conditions, the test substance does not induce chromosome structural and/or numerical aberrations in cultured mammalian somatic cells.

58. When a test substance has been shown to induce micronuclei containing whole chromosomes due to loss of chromosomes from the mitotic spindle further studies can be performed to determine whether the substance induces non-disjunction by producing mal-segregation of chromosomes in binucleate cells. Chromosome specific centromere probes are particularly convenient for this purpose as they can be used to measure the segregation of individual chromosomes.

Test Report

59. The test report should include the following information:

Test substance:

- identification data and Chemical Abstract Services Registry Number, if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance, if known.

Solvent/Vehicle:

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

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;
- sex and age of blood donors and smoking habit, whole blood or separated lymphocytes;
- number of passages, if applicable;
- methods for maintenance of cell cultures, if applicable;
- modal number of chromosomes, if applicable.

Test Conditions:

- identity of cytokinesis blocking substance (*e.g.*, cytochalasin B), if used and its concentration and duration of cell exposure;

- rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available;
- composition of media, CO_2 concentration, if applicable;
- concentrations of test substance;
- volume (and/or final concentration) of vehicle and test substance added;
- incubation temperature;
- incubation time;
- duration of treatment;
- cell density at seeding, if appropriate;
- type and composition of metabolic activation system, including acceptability criteria;
- positive and negative controls;
- methods of slide preparation;
- criteria for micronuclei 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;
- methods, such as use of kinetochore antibody, to characterise whether micronuclei contain whole or fragmented chromosomes.

Results:

- measurement of cytotoxicity by determining cell proliferation, *e.g.*, CBPI in the case of cytokinesis-block method, and cell counts or population doubling 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 what constitutes a micronucleus;
- definition of acceptable cells for analysis;
- distribution of mono-, bi-, tri- and tetra-nucleated cells
- number of cells with micronuclei and number of micronuclei per cell, 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;
- statistical analysis, p-values if any.

Discussion of the results.

Conclusions.

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Annex 1

DEFINITIONS

Aneugenic: any substance (aneugen) or process that by interacting with the components of the mitotic and meiotic cell division cycle may lead to aneuploidy.

Aneuploidy: any deviation from the normal diploid number of chromosomes.

Kinetochore: Region(s) of a chromosome with which spindle fibres are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

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

Clastogenic: any substance or process which causes breaks in chromosomes.

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

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 proliferation of that population.