

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Mammalian Chromosomal Aberration 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 473 was adopted in 1983. In 1997 a revised version was issued, based on scientific progress made to that date. This modified version of Test Guideline reflects nearly thirty 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 purpose of the *in vitro* chromosomal aberration test is to identify substances that cause structural chromosomal aberrations in cultured mammalian cells (2) (3) (4). Structural aberrations may be of two types, chromosome or chromatid. Polyploidy (including endoreduplication) could arise in chromosome aberration assays *in vitro*. While aneugens can induce polyploidy, polyploidy alone does not indicate aneugenic potential and can simply indicate cell cycle perturbation or cytotoxicity (5). This test is not designed to measure aneuploidy. An *in vitro* micronucleus test (6) would be recommended for the detection of aneuploidy.

3. The *in vitro* chromosomal aberration test may employ cultures of established cell lines or primary cell cultures of human or rodent origin. The cells used should be selected on the basis of growth ability in culture, stability of the karyotype (including chromosome number) and spontaneous frequency of chromosomal aberrations (7). 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 chromosomal aberrations, as knowledge evolves in this area.

4. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. 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, *i.e.* chromosome damage not caused by direct interaction between the test chemicals and chromosomes; such conditions include changes in pH or osmolality (8) (9) (10), interaction with the medium components (11) (12) or excessive levels of cytotoxicity (13) (14) (15) (16).

6. This test is used to detect chromosomal aberrations that may result from clastogenic events. The analysis of chromosomal aberration induction should be done using cells in metaphase. It is thus essential that cells should reach mitosis both in treated and in untreated cultures. For manufactured nanomaterials, specific adaptations of this Test Guideline may be needed but are not described in this Test Guideline.

7. 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

8. 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 metabolizing capability are used (see paragraph 13). At an appropriate predetermined intervals after the start of exposure of cell cultures to the test chemical, they are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine), harvested, stained and metaphase cells are analysed microscopically for the presence of chromatid-type and chromosome-type aberrations.

DESCRIPTION OF THE METHOD

Preparations

Cells

9. A variety of cell lines (e.g. Chinese Hamster Ovary (CHO), Chinese Hamster lung V79, Chinese Hamster Lung (CHL)/IU, TK6) or primary cell cultures, including human or other mammalian peripheral blood lymphocytes, can be used (7). The choice of the cell lines used should be scientifically justified. When primary cells are used, for animal welfare reasons, the use of primary cells from human origin should be considered where feasible and sampled in accordance with the human ethical principles and regulations. 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, ionizing radiations) at levels that would increase the background incidence of chromosomal aberrations. This would ensure the background incidence of chromosomal aberrations to be low and consistent. The baseline incidence of chromosomal aberrations increases with age and this trend is more marked in females than in males (17) (18). 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 cell 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 synchronized during exposure to the test chemical (e.g. human lymphocytes after a 48-hour mitogenic stimulation). The use of synchronized cells during treatment is not recommended, but can be acceptable if justified.

Media and culture conditions

10. Appropriate culture medium and incubation conditions (culture vessels, humidified atmosphere of 5% CO₂ if appropriate, incubation 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 (7) (19), 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 (20).

Preparation of cultures

11. 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).

12. 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.

Metabolic activation

13. Exogenous metabolising systems should be used when employing cells which have inadequate endogenous metabolic capacity. The most commonly used system that is recommended by default, unless otherwise 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 (21) (22) (23) or a combination of phenobarbital and β -naphthoflavone (24) (25) (26) (27) (28) (29). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (30) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (24) (25) (26) (28). 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 (31) 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

14. Solid test chemicals should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells (see paragraph 23). 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 culture vessels (32) (33)

(34). Preparations of the test chemical should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

Test conditions

Solvents

15. The solvent should be chosen to optimize the solubility of the test substances without adversely impacting the conduct of the assay, e.g. changing cell growth, affecting the 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 for example water or dimethyl sulfoxide. Generally organic solvents should not exceed 1% (v/v) and aqueous solvents (saline or water) should not exceed 10% (v/v) in the final treatment medium. If not well-established solvents are used (e.g. ethanol or acetone), their use should be supported by data indicating their compatibility with the test chemicals, 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) to demonstrate that no deleterious or clastogenic effects are induced by the chosen solvent.

Measuring cell proliferation and cytotoxicity and choosing treatment concentrations

16. 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 22), precipitation in the culture medium (see paragraph 23), or marked changes in pH or osmolality (see paragraph 5), 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.

17. Measurements of cell proliferation are made to assure that a sufficient number of treated cells have reached mitosis during the test and that the treatments are conducted at appropriate levels of cytotoxicity (see paragraphs 18 and 22). Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell death and growth. While the evaluation of cytotoxicity in an initial 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.

18. Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) are appropriate methods for the assessment of cytotoxicity in cytogenetic tests (13) (15) (35) (36) (55) (see Annex 2 for formulas). In case of long-term treatment and sampling times after the beginning of treatment longer than 1.5 normal cell cycle lengths (i.e. longer than 3 cell cycle lengths in total), RPD might underestimate cytotoxicity (37). Under these circumstances RICC might be a better measure or the evaluation of cytotoxicity after 1.5 normal cell cycle lengths would be a helpful estimate using RPD.

19. For lymphocytes in primary cultures, while the mitotic index (MI) is a measure of cytotoxic/cytostatic effects, it is influenced by the time after treatment it is measured, the mitogen used and possible cell cycle disruption. However, the MI is acceptable because other cytotoxicity measurements may be cumbersome and impractical and may not apply to the target population of lymphocytes growing in response to PHA stimulation.

20. While RICC and RPD for cell lines and MI for primary culture of lymphocytes are the recommended cytotoxicity parameters, other indicators (e.g. cell integrity, apoptosis, necrosis, cell cycle) could provide useful additional information.

21. 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 paragraph 31). The results obtained in the independent replicate cultures at a given concentration can be pooled for the data analysis (38). 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 22 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 47).

22. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve $55 \pm 5\%$ cytotoxicity using the recommended cytotoxicity parameters (*i.e.* reduction in RICC and RPD for cell lines and reduction in MI for primary cultures of lymphocytes to $45 \pm 5\%$ of the concurrent negative control). Care should be taken in interpreting positive results only to be found in the higher end of this $55 \pm 5\%$ cytotoxicity range (13).

23. 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 producing turbidity or with a 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.

24. 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 (39) (40) (41). 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) (42), environmental extracts etc., the top concentration may need to be higher (e.g. 5 mg/ml), in the absence of sufficient cytotoxicity, to increase the concentration of each of the components. It should be noted however that these requirements may differ for human pharmaceuticals (43).

Controls

25. Concurrent negative controls (see paragraph 15), consisting of solvent alone in the treatment medium and treated in the same way as the treatment cultures, should be included for every harvest time.

26. Concurrent positive controls are needed to demonstrate the ability of the laboratory to identify clastogens 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 the table 1 below. Alternative positive control substances can be used, if justified. Because *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardized, the use of positive controls may be confined to a clastogen

requiring metabolic activation. Provided it is done concurrently with the non-activated test using the same treatment duration, this single positive control response will demonstrate both the activity of the metabolic activation system and the responsiveness of the test system. Long term treatment (without S9) should however have its own positive control as the treatment duration will differ from the test using metabolic activation. 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 the Test Guideline.

Table 1. Reference substances recommended for assessing laboratory proficiency and for 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		
	Benzo(a)pyrene	50-32-8
	Cyclophosphamide	50-18-0

PROCEDURE

Treatment with test chemical

27. Proliferating cells are treated with the test chemical in the presence and absence of a metabolic activation system.

Culture harvest time

28. For thorough evaluation, which would be needed to conclude a negative outcome, all three of the 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 43, 44 and 45):

- 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 normal cell cycle lengths after the beginning of treatment (18),
- 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 normal cell cycle lengths after the beginning of treatment (18),
- Cells should be continuously exposed without metabolic activation until sampling at a time equivalent to about 1.5 normal cell cycle lengths. Certain substances (e.g. nucleoside analogues) may be more readily detected by treatment/sampling times longer than 1.5 normal cell cycle lengths (24).

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.

Chromosome preparation

29. Cell cultures are treated with Colcemid® or colchicine usually for one to three hours prior to harvesting. Each cell culture is harvested and processed separately for the preparation of chromosomes. Chromosome preparation involves hypotonic treatment of the cells, fixation and staining. In monolayers, 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 added back to cultures, to avoid losing cells that are in mitosis, and at risk for chromosome aberration, at the time of harvest.

Analysis

30. All slides, including those of the positive and negative controls, should be independently coded before microscopic analysis for chromosomal aberrations. Since fixation procedures often result in a proportion of metaphase cells which have lost chromosomes, the cells scored should, therefore, contain a number of centromeres equal to the modal number +/- 2.

31. At least 300 well-spread metaphases should be scored per concentration and control to conclude a test chemical as clearly negative (see paragraph 45). The 300 cells should be equally divided among the replicates, when replicate cultures are used. When single cultures are used per concentration (see paragraph 21), at least 300 well spread metaphases should be scored in this single culture. Scoring 300 cells has the advantage of increasing the statistical power of the test and in addition, zero values will be rarely observed (expected to be only 5%) (44). The number of metaphases scored can be reduced when high numbers of cells with chromosome aberrations are observed and the test chemical considered as clearly positive.

32. Cells with structural chromosomal aberration(s) including and excluding gaps should be scored. Breaks and gaps are defined in Annex 1 according to (45) (46). Chromatid- and chromosome-type aberrations should be recorded separately and classified by sub-types (breaks, exchanges). Procedures in use in the laboratory should ensure that analysis of chromosomal aberrations is performed by well-trained scorers and peer-reviewed if appropriate.

33. Although the purpose of the test is to detect structural chromosomal aberrations, it is important to record polyploidy and endoreduplication frequencies when these events are seen. (See paragraph 2).

Proficiency of the laboratory

34. In order to establish sufficient experience with the test prior to using it for routine testing, the laboratory should have performed a series of experiments with reference positive substances acting via different mechanisms and various negative controls (using 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 paragraph 37.

35. A selection of positive control substances (see Table 1 in paragraph 26) 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 substances and determine the effectiveness of the metabolic activation system. 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

36. The laboratory should establish:

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

37. When first acquiring data for an historical negative control distribution, concurrent negative controls should be consistent with published 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 (44) (47). 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 (48)), to identify how variable their positive and negative control data are, and to show that the methodology is 'under control' in their laboratory (44). 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 (47).

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

39. Negative control data should consist of the incidence of cells with chromosome aberrations from a single culture or the sum of replicate cultures as described in paragraph 21. Concurrent negative controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database (44) (47). 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 37) and evidence of absence of technical or human failure.

DATA AND REPORTING

Presentation of the results

40. The percentage of cells with structural chromosomal aberration(s) should be evaluated. Chromatid- and chromosome-type aberrations classified by sub-types (breaks, exchanges) should be listed separately with their numbers and frequencies for experimental and control cultures. Gaps are recorded and reported separately but not included in the total aberration frequency. Percentage of polyploidy and/or endoreduplicated cells are reported when seen.

41. Concurrent measures of cytotoxicity for all treated, negative and positive control cultures in the main aberration experiment(s) should be recorded.

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

Acceptability Criteria

43. 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 39.
 - Concurrent positive controls (see paragraph 26) should induce responses that are compatible with those generated in the 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 (paragraphs 17 and 18).
 - All three experimental conditions were tested unless one resulted in positive results (see paragraph 28).
 - Adequate number of cells and concentrations are analysable (paragraphs 31 and 21).
 - The criteria for the selection of top concentration are consistent with those described in paragraphs 22, 23 and 24.

Evaluation and interpretation of results

44. 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 paragraph 28):
- a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
 - b) the increase is dose-related when evaluated with an appropriate trend test,
 - c) any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits; see paragraph 39).

When all of these criteria are met, the test chemical is then considered able to induce chromosomal aberrations in cultured mammalian cells in this test system. Recommendations for the most appropriate statistical methods can be found in the literature (49) (50) (51).

45. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined (see paragraph 28):
- a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
 - b) there is no concentration-related increase when evaluated with an appropriate trend test,
 - c) all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits; see paragraph 39).

The test chemical is then considered unable to induce chromosomal aberrations in cultured mammalian cells in this test system.

46. There is no requirement for verification of a clearly positive or negative response.

47. In case the response is neither clearly negative nor clearly positive as described above 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.

48. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and therefore the test chemical response will be concluded to be equivocal.

49. An increase in the number of polyploid cells may indicate that the test substances have the potential to inhibit mitotic processes and to induce numerical chromosomal aberrations (52). An increase in the number of cells with endoreduplicated chromosomes may indicate that the test substances have the potential to inhibit cell cycle progress (53) (54) (see paragraph 2). Therefore incidence of polyploid cells and cells with endoreduplicated chromosomes should be recorded separately.

Test report

50. 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;
- 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 should also be indicated.

Cells:

- type and source of cells
- karyotype features and suitability of the cell type used;
- absence of mycoplasma, for cell lines;
- for cell lines, information on cell cycle length, doubling time or proliferation index;
- sex of blood donors, age and any relevant information on the donor, whole blood or separated lymphocytes, mitogen used;

- number of passages, if available, for cell lines;
- methods for maintenance of cell cultures, for cell lines;
- modal number of chromosomes, for cell lines.

Test conditions:

- identity of the metaphase-arresting substance, its concentration and duration of cell exposure;
- concentration of test chemical expressed as final concentration in the culture medium (e.g. μg or mg/mL or mM of culture medium).
- rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations;
- composition of media, CO_2 concentration if applicable, humidity level;
- concentration (and/or volume) of solvent and test chemical added in the culture medium;
- incubation temperature;
- incubation time;
- duration of treatment;
- harvest time after treatment;
- cell density at seeding, if appropriate;
- 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);
- positive and negative control substances, final concentrations for each conditions of treatment;
- methods of slide preparation and staining technique used;
- criteria for acceptability of assays;
- criteria for scoring aberrations;
- number of metaphases 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;
- methods used to determine pH, osmolality and precipitation.

Results:

- the number of cells treated and the number of cells harvested for each culture when cell lines are used
- cytotoxicity measurements, e.g. RPD, RICC, MI, other observations if any;
- information on cell cycle length, doubling time or proliferation index in case of cell lines;
- signs of precipitation and time of the determination;
- definition for aberrations, including gaps;
- Number of cells scored, number of cells with chromosomal aberrations and type of chromosomal aberrations given separately for each treated and control culture, including and excluding gaps;
- changes in ploidy (polyploid cells and cells with endoreduplicated chromosomes, given separately) if seen;
- 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 deviations and 95% control limits for the distribution, as well as the number of data;
- statistical analyses, p-values if any.

Discussion of the results.

Conclusions.

LITERATURE

- (1) OECD (2016). Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014-2015. ENV Publications. Series on Testing and Assessment, No. 234, OECD, Paris.
- (2) Evans, H.J. (1976), "Cytological Methods for Detecting Chemical Mutagens", in *Chemical Mutagens, Principles and Methods for their Detection*, Vol. 4, Hollaender, A. (ed.), Plenum Press, New York and London, pp. 1-29
- (3) Ishidate, M. Jr., T. Sofuni (1985), "The In Vitro Chromosomal Aberration Test Using Chinese Hamster Lung (CHL) Fibroblast Cells in Culture" in *Progress in Mutation Research*, Vol. 5, Ashby, J. et al. (eds.), Elsevier Science Publishers, Amsterdam-New York- Oxford, pp. 427-432.
- (4) Galloway, S.M. et al. (1987), Chromosomal aberration and sister chromatid exchanges in Chinese hamster ovary cells: Evaluation of 108 chemicals, *Environmental and Molecular Mutagenesis*, Vol. 10/suppl. 10, pp. 1-175.
- (5) Muehlbauer, P.A. et al. (2008), "Improving dose selection and identification of aneugens in the in vitro chromosome aberration test by integration of flow cytometry-based methods, *Environmental and Molecular Mutagenesis*, Vol. 49/4, pp. 318-327.
- (6) OECD (2014), *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- (7) ILSI paper (draft), Lorge, E., M. Moore, J. Clements, M. O Donovan, F. Darroudi, M. Honma, A. Czich, J van Benthem, S. Galloway, V. Thybaud, B. Gollapudi, M. Aardema, J. Kim, D.J. Kirkland, Recommendations for good cell culture practices in genotoxicity testing.
- (8) Scott, D. et al. (1991), Genotoxicity under Extreme Culture Conditions. A report from ICPEMC Task Group 9, *Mutation Research/Reviews in Genetic Toxicology*, Vol.257/2, pp. 147-204.
- (9) Morita, T. et al. (1992), Clastogenicity of Low pH to Various Cultured Mammalian Cells, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 268/2, pp. 297-305.
- (10) Brusick, D. (1986), Genotoxic effects in cultured mammalian cells produced by low pH treatment conditions and increased ion concentrations, *Environmental and Molecular Mutagenesis*, Vol. 8/6, pp. 789-886.
- (11) Long, L.H. et al. (2007), Different cytotoxic and clastogenic effects of epigallocatechin gallate in various cell-culture media due to variable rates of its oxidation in the culture medium, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 634/1-2, pp. 177-183.
- (12) Nesslany, F. et al. (2008), Characterization of the Genotoxicity of Nitriлотriacetic Acid, *Environmental and Molecular Mutagenesis*, Vol. 49/6, pp. 439-452.

- (13) Galloway, S. (2000), Cytotoxicity and chromosome aberrations *in vitro*: Experience in industry and the case for an upper limit on toxicity in the aberration assay, *Environmental and Molecular Mutagenesis*, Vol. 35/3, pp. 191-201.
- (14) Kirkland, D. et al. (2005), Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I: Sensitivity, specificity and relative predictivity, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 584/1-2, pp. 1-256.
- (15) Greenwood, S. et al. (2004), Population doubling: a simple and more accurate estimation of cell growth suppression in the *in vitro* assay for chromosomal aberrations that reduces irrelevant positive results, *Environmental and Molecular Mutagenesis*, Vol. 43/1, pp. 36-44.
- (16) Hilliard, C.A. et al. (1998), Chromosome aberrations *in vitro* related to cytotoxicity of nonmutagenic chemicals and metabolic poisons, *Environmental and Molecular Mutagenesis*, Vol. 31/4, pp. 316-326.
- (17) Hedner K. et al. (1982), Sister chromatid exchanges and structural chromosomal aberrations in relation to age and sex, *Human Genetics*, Vol. 62, pp. 305-309.
- (18) Ramsey M.J. et al. (1995), The effects of age and lifestyle factors on the accumulation of cytogenetic damage as measured by chromosome painting, *Mutation Research*, Vol. 338, pp. 95-106.
- (19) Coecke S. et al. (2005), Guidance on Good Cell Culture Practice. A Report of the Second ECVAM Task Force on Good Cell Culture Practice, *ATLA*, Vol. 33/3, pp. 261-287.
- (20) Henderson, L. et al. (1997), Industrial Genotoxicology Group collaborative trial to investigate cell cycle parameters in human lymphocyte cytogenetics studies, *Mutagenesis*, Vol.12/3, pp.163-167.
- (21) Ames, B.N., J. McCann, E. Yamasaki (1975), Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian Microsome Mutagenicity Test, *Mutation Research/Environmental Mutagenesis and Related Subjects*, Vol. 31/6, pp. 347-363.
- (22) Maron, D.M., B.N. Ames (1983), Revised Methods for the Salmonella Mutagenicity Test, *Mutation Research/Environmental Mutagenesis and Related Subjects*, Vol. 113/3-4, pp. 173-215.
- (23) Natarajan, A.T. et al. (1976), Cytogenetic Effects of Mutagens/Carcinogens after Activation in a Microsomal System *In Vitro*, I. Induction of Chromosomal Aberrations and Sister Chromatid Exchanges by Diethylnitrosamine (DEN) and Dimethylnitrosamine (DMN) in CHO Cells in the Presence of Rat-Liver Microsomes, *Mutation Research*, Vol. 37/1, pp. 83-90.
- (24) Matsuoka, A., M. Hayashi, M. Jr. Ishidate (1979), Chromosomal Aberration Tests on 29 Chemicals Combined with S9 Mix *In vitro*, *Mutation Research/Genetic Toxicology*, Vol. 66/3, pp. 277-290.
- (25) Ong, T.-m. et al. (1980), Differential effects of cytochrome P450-inducers on promutagen activation capabilities and enzymatic activities of S-9 from rat liver, *Journal of Environmental Pathology and Toxicology*, Vol. 4/1, pp. 55-65.

- (26) Elliot, B.M. et al. (1992), Report of UK Environmental Mutagen Society Working Party. Alternatives to Aroclor 1254-induced S9 in In Vitro Genotoxicity Assays, *Mutagenesis*, Vol. 7/3, pp. 175-177.
- (27) Matsushima, T. et al. (1976), "A Safe Substitute for Polychlorinated Biphenyls as an Inducer of Metabolic Activation Systems", in *In Vitro Metabolic Activation in Mutagenesis Testing*, de Serres, F.J. et al. (eds.), Elsevier, North-Holland, pp. 85-88.
- (28) Galloway, S.M. et al. (1994). Report from Working Group on in Vitro Tests for Chromosomal Aberrations, *Mutation Research/Environmental Mutagenesis and Related Subjects*, Vol. 312/3, pp. 241-261.
- (29) Johnson, T.E., D.R. Umbenhauer, S.M. Galloway (1996), Human liver S-9 metabolic activation: proficiency in cytogenetic assays and comparison with phenobarbital/beta-naphthoflavone or Aroclor 1254 induced rat S-9, *Environmental and Molecular Mutagenesis*, Vol. 28/1, pp. 51-59.
- (30) UNEP (2001), Stockholm Convention on Persistent Organic Pollutants, United Nations Environment Programme (UNEP). Disponible à l'adresse: <http://www.pops.int/>.
- (31) Tucker, J.D., M.L. Christensen (1987), Effects of anticoagulants upon sister-chromatid exchanges, cell-cycle kinetics, and mitotic index in human peripheral lymphocytes, *Mutation Research*, Vol. 190/3, pp. 225-8.
- (32) Krahn, D.F., F.C. Barsky, K.T. McCooley (1982), "CHO/HGPRT Mutation Assay: Evaluation of Gases and Volatile Liquids", in *Genotoxic Effects of Airborne Agents*, Tice, R.R., D.L. Costa, K.M. Schaich (eds.), Plenum, New York, pp. 91-103.
- (33) Zamora, P.O. et al. (1983), Evaluation of an Exposure System Using Cells Grown on Collagen Gels for Detecting Highly Volatile Mutagens in the CHO/HGPRT Mutation Assay, *Environmental and Molecular Mutagenesis*, Vol. 5/6, pp. 795-801.
- (34) Asakura, M. et al. (2008), An improved system for exposure of cultured mammalian cells to gaseous compounds in the chromosomal aberration assay, *Mutation Research*, Vol. 652/2, pp. 122-130.
- (35) Lorge, E. et al. (2008), Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test. I. Theoretical aspects, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 655/1-2, pp. 1-3.
- (36) Galloway, S. et al. (2011), Workshop summary: Top concentration for in vitro mammalian cell genotoxicity assays; and Report from working group on toxicity measures and top concentration for in vitro cytogenetics assays (chromosome aberrations and micronucleus), *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 723/2, pp. 77-83.
- (37) Honma, M. (2011), Cytotoxicity measurement in *in vitro* chromosome aberration test and micronucleus test, *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, Vol. 724/1-2, pp. 86-87.

- (38) Richardson, C. et al. (1989), *Analysis of Data from In Vitro Cytogenetic Assays. In: Statistical Evaluation of Mutagenicity Test Data*, Kirkland, D.J. (ed.) Cambridge University Press, Cambridge, pp. 141-154.
- (39) OECD (2014), Document supporting the WNT decision to implement revised criteria for the selection of the top concentration in the *in vitro* mammalian cell assays on genotoxicity (Test Guidelines 473, 476 and 487) [ENV/JM/TG\(2014\)17](#). Available upon request.
- (40) Morita, T., M. Honma, K. Morikawa (2012), Effect of reducing the top concentration used in the *in vitro* chromosomal aberration test in CHL cells on the evaluation of industrial chemical genotoxicity, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 741/1-2, pp. 32-56.
- (41) Brookmire, L., J.J. Chen, D.D. Levy (2013), Evaluation of the Highest Concentrations Used in the In Vitro Chromosome Aberrations Assay, *Environmental and Molecular Mutagenesis*, Vol. 54/1, pp. 36-43.
- (42) EPA, Office of Chemical Safety and Pollution Prevention (2011), Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials: UVCB Substances, <http://www.epa.gov/opptintr/newchems/pubs/uvcb.txt>.
- (43) USFDA (2012), International Conference on Harmonisation (ICH) Guidance S2 (R1) on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended For Human Use. Disponible à l'adresse: <https://federalregister.gov/a/2012-13774>.
- (44) OECD (2014), "Statistical analysis supporting the revision of the genotoxicity Test Guidelines, OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 198, OECD Publishing, Paris.
- (45) ISCN (2013), *An International System for Human Cytogenetic Nomenclature*, Schaffer, L.G., J. MacGowan-Gordon, M. Schmid (eds.), Karger Publishers Inc., Connecticut.
- (46) Scott, D. et al. (1990), "Metaphase chromosome aberration assays *in vitro*", in *Basic Mutagenicity Tests: UKEMS Recommended Procedures*, Kirkland, D.J. (ed.), Cambridge University Press, Cambridge, pp. 62-86.
- (47) Hayashi, M. et al. (2011), Compilation and use of genetic toxicity historical control Data, *Mutation Research*, Vol. 723/2, pp. 87-90.
- (48) Ryan, T. P. (2000), *Statistical Methods for Quality Improvement*, 2nd Edition, John Wiley and Sons, New York.
- (49) Fleiss, J. L., B. Levin, M.C. Paik (2003), *Statistical Methods for Rates and Proportions*, 3rd ed., John Wiley & Sons, New York.
- (50) Galloway, S.M. et al. (1987), Chromosome aberration and sister chromatid exchanges in Chinese hamster ovary cells: Evaluation of 108 chemicals, *Environmental and Molecular Mutagenesis*, Vol. 10/suppl. 10, pp. 1-175.

- (51) Richardson, C. et al. (1989), "Analysis of Data from *In Vitro* Cytogenetic Assays", in *Statistical Evaluation of Mutagenicity Test Data*, Kirkland, D.J. (ed.), Cambridge University Press, Cambridge, pp. 141-154.
- (52) Warr, T.J., E.M. Parry, J.M. Parry (1993), A comparison of two in vitro mammalian cell cytogenetic assays for the detection of mitotic aneuploidy using 10 known or suspected aneugens, *Mutation Research*, Vol. 287/1, pp. 29-46.
- (53) Locke-Huhle, C. (1983), Endoreduplication in Chinese hamster cells during alpha-radiation induced G2 arrest, *Mutation Research*, Vol. 119/3, pp. 403-413.
- (54) Huang, Y., C. Change, J.E. Trosko (1983), Aphidicolin - induced endoreduplication in Chinese hamster cells, *Cancer Research*, Vol. 43/3, pp. 1362-1364.
- (55) Soper, K.A., S.M. Galloway (1994), Cytotoxicity measurement in *in vitro* chromosome aberration test and micronucleus test, *Mutation Research*, Vol. 312, pp. 139-149.

ANNEX 1

DEFINITIONS

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

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

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

Chromatid break: discontinuity of a single chromatid in which there is a clear misalignment of one of the chromatids.

Chromatid gap: non-staining region (achromatic lesion) of a single chromatid in which there is minimal misalignment of the chromatid.

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

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

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

Cytotoxicity: For the assays covered in this guideline using cell lines, cytotoxicity is identified as a reduction in relative population doubling (RPD) or relative increase in cell count (RICC) of the treated cells as compared to the negative control (see paragraph 17 and Annex 2).

For the assays covered in this guideline using primary cultures of lymphocytes, cytotoxicity is identified as a reduction in mitotic index (MI) of the treated cells as compared to the negative control (see paragraph 18 and Annex 2).

Endoreduplication: a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with 4, 8, 16..., chromatids.

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.

Mitotic index (MI): 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.

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

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

Numerical aberration: a change in the number of chromosomes from the normal number characteristic of the cells utilised.

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

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.

Relative Increase in Cell Counts (RICC): the increase in the number of cells in chemically-exposed cultures versus increase in non-treated cultures, a ratio expressed as a percentage.

Relative Population Doubling (RPD): the increase in the number of population doublings in chemically-exposed cultures versus increase in non-treated cultures, a ratio expressed as a percentage.

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 enzymes activity.

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

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges.

Untreated controls: 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.

ANNEX 2

FORMULAS FOR CYTOTOXICITY ASSESSMENT

Mitotic index (MI):

$$\text{MI (\%)} = \frac{\text{Number of mitotic cells}}{\text{Total number of cells scored}} \times 100$$

Relative Increase in Cell Counts (RICC) or **Relative Population Doubling (RPD)** is recommended, as both take into account the proportion of the cell population which has divided.

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

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

where:

$$\text{Population Doubling} = [\log (\text{Post-treatment cell number} \div \text{Initial cell number})] \div \log 2$$

For example, a RICC, or a RPD of 53% indicates 47% cytotoxicity/cytostasis and 55% cytotoxicity/cytostasis measured by MI means that the actual MI is 45% of control.

In any case, the number of cells before treatment should be measured and the same for treated and negative control cultures.

While RCC (i.e. Number of cells in treated cultures/ Number of cells in control cultures) had been used as cytotoxicity parameter in the past, is no longer recommended because it can underestimate cytotoxicity

In the negative control cultures, population doubling should be compatible with the requirement to sample cells after treatment at a time equivalent to about 1.5 normal cell cycle length and mitotic index should be higher enough to get a sufficient number of cells in mitosis and to reliably calculate a 50% reduction.