OECD GUIDELINE FOR THE TESTING OF CHEMICALS
PROPOSAL FOR UPDATING TEST GUIDELINE 473
In Vitro Mammalian Chromosomal Aberration Test

INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed in the 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. Modifications within the current version reflect nearly thirty years of experience with this test. This Test Guideline is part of a series of Test Guidelines on genetic toxicology. A document presented as an Introduction to the Test Guidelines on genetic toxicology (34) can also be referred to and provides succinct and useful guidance to users of these Test Guidelines.

2. The purpose of the in vitro chromosomal aberration test is to identify agents that cause structural chromosomal aberrations in cultured mammalian cells (1)(2)(3). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosomal aberrations and related events are the cause of many human genetic diseases. There is substantial evidence that these events cause alterations in oncogenes and tumour suppressor genes of cells which in turn lead to cancer in human and experimental animals. An increase in polyploidy or endoreduplications (see (34) Introduction document) may give an indication of aneuploidy inducing potential. However, this test is not designed to measure numerical aberrations and is not routinely used for that purpose. An in vitro micronucleus test (35) would be recommended for the detection of aneuploidy.

3. The in vitro chromosomal aberration test may employ cultures of cell lines or primary cell cultures of human or rodent origin. For animal welfare reasons, the use of primary cells from human origin is preferable (e.g. blood cells). The cells used are selected on the basis of growth ability in culture, stability of the karyotype (including chromosome number), chromosome diversity and spontaneous frequency of chromosomal aberrations (39). 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 (4) (34). 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 substances being tested (34). The exogenous metabolic activation system does not entirely mimic in vivo conditions. Care should also be taken to avoid conditions that could lead to artifactual positive results, i.e. chromosome damage not caused by direct interaction between the test article and chromosomes. Such conditions include changes in pH or osmolality, interaction with the medium components (5) (49) or excessive levels of cytotoxicity( 6) (7) (8).
6. This test is used to detect chromosomal aberrations that may result from clastogenic events. The analysis of chromosomal aberration induction should be done on cells in metaphase initiated since the beginning of the treatment. It is thus essential that mitosis has occurred in both treated and untreated cultures. For insoluble or particulate materials specific adaptation of this Test Guideline may be needed but this is not within the scope of this Test Guideline as written.

**PRINCIPLE OF THE TEST**

7. 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 12 and Introduction document (34) for more information). At 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**

8. A variety of cell lines (e.g. Chinese Hamster Ovary (CHO), Chinese Hamster lung V79, Chinese Hamster Lung (CHL)/IU) or primary cell cultures, including human cells or other mammalian peripheral blood lymphocytes, can be used (39). When primary cells are used, for animal welfare reasons, the use of primary cells from human origin should be considered where feasible. 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 (9)(10). 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 are dividing 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 chemical 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 chemicals (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**

9. Appropriate culture medium and incubation conditions (culture vessels, humidified atmosphere of 5% CO₂, 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 (39)(40), and cells should not be used if contaminated or if the modal chromosome number
has changed. The normal cell cycle time of cell lines for the culture conditions used in the testing laboratory should be established and should be consistent with the published cell characteristics.

**Preparation of cultures**

10. 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. confluency should be avoided for cells growing in monolayers).

11. 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**

12. 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 (11) (12) (13) or a combination of phenobarbital and β-naphthoflavone (14) (15) (16) (17) (18) (19). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (20) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (14) (15) (16) (18). 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 (e.g. anticoagulants, phosphate buffer), should be avoided (42). The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of chemical being tested. For a more detailed discussion on the use of metabolising systems, see the Introduction document (34).

**Test chemical preparation**

13. Solid test chemicals should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells (see paragraph 22). Liquid test chemicals may be added directly to the test system and/or diluted prior to treatment of the test system. Gaseous or volatile chemicals should be tested by appropriate modifications to the standard protocols, such as treatment in sealed culture vessels (21) (22) (23). Preparations of the test chemical should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

**Test conditions**

**Solvents**

14. The solvent should be chosen to optimize the solubility of the test chemical without adversely impacting the conduct of the assay, e.g. changing cell growth, affecting the integrity of the test material, 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 other than well-established solvents are used, 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) to demonstrate that no deleterious or clastogenic effects are induced by the chosen solvent.

Measuring cell proliferation and cytotoxicity and choosing treatment concentrations

15. When determining the highest test chemical concentration, concentrations that have the capability of producing artificial positive responses, such as those producing excessive cytotoxicity (see paragraph 21), precipitation in the culture medium (see paragraph 22), 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 artificial positive results and to maintain appropriate culture conditions.

16. Measurements of cell proliferation are made to assure that the treated cells have undergone mitosis during the test and that the treatments are conducted at appropriate levels of cytotoxicity (see paragraphs 17 and 21). 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, it is not mandatory and should not replace the measurement of cytotoxicity in the main experiment.

17. For cell lines, it is necessary to demonstrate that the cells scored in the culture have undergone division during or following treatment with the test chemical, or else false negative responses may be produced. Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) are appropriate methods for the assessment of cytotoxicity in cytogenetic tests (24) (25) (26) (see Annex 2 for formulas). In case of long-term treatment and sampling times after the end of treatment longer than 1.5 normal cell cycle length (i.e. longer than 3 cell cycle length in total), RPD might underestimate cytotoxicity (27). Under these circumstances RICC could be a better measure. Alternatively, the evaluation of cytotoxicity after 1.5 normal cell cycle length would be a helpful estimate.

18. For lymphocytes in primary cultures, while the mitotic index (MI) is only an indirect measure of cytotoxic/cytostatic effects and depends on the time after treatment, the MI is acceptable because other cytotoxicity measurements may be cumbersome and impractical.

19. 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) could provide useful additional information.

20. 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. Either replicate or single treated cultures may be used at each concentration tested. The results obtained in the independent replicate cultures at a given concentration can be pooled for the data analysis (28). The total number of cells analysed per concentration should remain the same either using replicates or single cultures, to provide confidence in the evaluation (see paragraph 29). For 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 21 and including concentrations at which there is moderate and little or no cytotoxicity. Many test substances 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 43).

21. 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.* RICC and RPD for cell lines and MI for primary cultures of lymphocytes). Care should be taken in interpreting positive results only to be found in the higher end of this range (24).

22. For poorly soluble compounds 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. 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 also be useful.

23. If no cytotoxicity or precipitate is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest (37), (43), (44). This applies to test chemicals of defined composition. Justification for not testing individual components of the composition should be provided. In other circumstances where the test chemical is not of defined composition *e.g.* substance of unknown or variable composition, complex reaction products or biological materials (UVCBs) (38), environmental extracts etc., the top concentration should be at least 5 mg/ml. It should be noted however that these requirements may differ for pharmaceuticals (36).

### Controls

24. Concurrent negative controls (see paragraph 14), 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.

25. Positive controls are needed to demonstrate the ability of the cells used to identify clastogens under the conditions of the test protocol used and the proficiency of the exogenous metabolic activation system, when applicable (examples of positive controls are given in the table in Annex 3). 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) to demonstrate 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 activated test. Each positive control should be used at a concentration expected to give a reproducible and detectable increase 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). Cytotoxicity for positive controls should not exceed the highest recommended value.
PROCEDURE

Treatment with test chemical

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

Culture harvest time

27. For thorough evaluation, one, two or the 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 39, 40 and 41):

- 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 length 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 length 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 length. Certain chemicals (e.g. nucleoside analogues) may be more readily detected by treatment/sampling times longer than 1.5 normal cell cycle length (24).

For human lymphocytes 1.5 cell cycle length generally consists in 20-24 hours (41).

Chromosome preparation

28. 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.

Analysis

29. All slides, including those of 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 with loss of chromosomes, the cells scored should, therefore, contain a number of centromeres equal to the modal number +/- 2 for all cell types. At least 400 well-spread metaphases should be scored per concentration and control equally divided among the replicates, if applicable. In case of single culture per concentration (see paragraph 20), at least 400 well spread metaphases should be scored in this single culture. Scoring 400 cells has the advantage of increasing the statistical power of the test and in addition, zero values will be rarely observed (see Annex 4).

30. Cells with structural chromosomal aberration(s) including and excluding gaps should be scored. 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.

31. 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 and Introduction document (34) for interpretation).
Proficiency of the laboratory

32. 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 chemicals acting via different mechanisms (at least one active with and one active without metabolic activation among chemicals listed in Annex 3) and various solvents. These positive and negative control responses should be consistent with the literature.

Historical control data

33. During the course of the investigations conducted for the demonstration of proficiency, the laboratory should establish:
   - A historical positive control range and distribution,
   - A historical negative (untreated, solvent) control range and distribution.

34. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (45)), to identify how variable their data are, and to show that the methodology is 'under control' in their laboratory (see Annex 5). 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 20. Concurrent negative controls should ideally be within the Poisson-based 99% control limits of the distribution of the laboratory’s historical control database, measured in at least 10 experiments conducted under the same experimental conditions. In those rare situations (around 1%) where data fall close to the existing historical control range and for which there is no evidence that the test system has gone out of control and no evidence of technical or human failure, the data should be acceptable for evaluation and acceptable for addition to the historical database. 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 (30).

35. Renewal/re-establishment of historical ranges is recommended if major changes to the experimental conditions (e.g. use of a new cell type) are proposed for the test (30).

DATA AND REPORTING

Presentation of the results

36. 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.

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

38. Individual culture data should be provided. Additionally, all data should be summarised in tabular form.
Acceptability Criteria

39. Acceptance of a test is based on the following criteria:
   - Concurrent negative control is consistent with historical negative control data (see paragraph 34).
   - Concurrent positive controls (see paragraph 25) should fulfil the positivity criteria (see paragraph 40).
   - Proliferation criteria should be fulfilled (Paragraph 16 and 17).
   - All three experimental conditions were conducted unless one resulted in positive results (see paragraph 27).
   - Adequate number of cells and concentrations should be analyzable. (Paragraphs 29 and 20).
   - The criteria for the selection of top concentration are consistent with those described in paragraphs 21, 22 and 23.

Evaluation and interpretation of results

40. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear positive if at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control and the increase is dose-related in at least one experimental condition (see paragraph 27). The test chemical is then considered able to induce chromosomal aberrations in cultures mammalian cells in this test system. Recommendations for the most appropriate statistical methods can also be found in the literature (46) (47) (48).

41. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear negative if none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, and there is no concentration-related increase in any experimental condition (see paragraph 27). The test chemical is then considered unable to induce chromosomal aberrations in cultured mammalian cells in this test system. Recommendations for the most appropriate statistical methods can also be found in the literature (46) (47) (48).

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

43. In case the response is not clearly negative or positive and in order to assist in establishing the biological relevance of a result (e.g. weak or borderline increase, or increases observed only at or close to the maximum recommended cytotoxic or precipitating concentrations), the data should be evaluated by expert judgement and/or further investigations using the existing experiments, such as determining if the positive result is outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 33 and Annex 5). Analysing more cells/cultures 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], length of treatment, sampling time) could be useful.

44. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and will therefore be concluded as equivocal.
45. An increase in the number of polyploid cells may indicate that the test chemical has the potential to inhibit mitotic processes and to induce numerical chromosomal aberrations (31). An increase in the number of cells with endoreduplicated chromosomes may indicate that the test chemical has the potential to inhibit cell cycle progress (32)(33), which is a different mechanism of inducing numerical chromosome changes than inhibition of mitotic processes (see paragraph 2). Therefore incidence of polyploid cells and cells with endoreduplicated chromosomes should be recorded separately.

Test report

46. The test report must include the following information:

Test chemical:
- identification data and CAS no., if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test chemical, if known, and reactivity of the test chemical with the solvent or cell culture media.

Solvent:
- justification for choice of solvent.
- solubility and stability of the test chemical in solvent, if known.
- 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, in case of cell lines;
- for cell lines, information on cell cycle length, doubling time or proliferation index;
- sex of blood donors, age and medical status, smoking status, whole blood or separated lymphocytes, mitogen used;
- number of passages, in case of cell lines;
- methods for maintenance of cell cultures, in case of cell lines;
- modal number of chromosomes, in case of cell lines.

Test conditions:
- identity of 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, CO₂ 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 compounds, 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 analyzed;
- 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 (individual data):

- the number of cells treated and the number of cells harvested for each culture in case of cell lines
- 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;
- statistical analyses, if any;
- historical negative (solvent) and positive control data, with ranges, means and standard deviations and confidence interval (e.g. 95%) as well as the number of data.

Discussion of the results.

Conclusions.
LITERATURE


OECD (2013), Draft Introduction to the OECD guidelines on genetic toxicology testing and guidance on the selection and application of assays


International Conference on Harmonisation (ICH) Guidance S2 (R1) on Genotoxicity Testing and Data Interpretation For Pharmaceuticals Intended For Human Use

OECD (2013) 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) *Olis cote:to be added*

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.


Morita T., Honma M., Morikawa K. (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, Mutat Res. 741:32-56.


OECD (2013) Draft report on statistical issues related to OECD in vitro genotoxicity Test Guidelines
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-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.

Chromosome diversity: diversity of chromosome shapes (e.g. metacentrique, acrocentriques, etc….) and sizes

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

Concentrations: refer to final concentrations in the culture medium even if not specified.

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 cell lines 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, adducts, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosome damage.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

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 induction, 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.

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 (ie no test chemical nor solvent) but are processed 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:

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

As an example, a RICC, or a RPD of 53% indicates 47% cytotoxicity/cytostasis.

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

For information relative cell counts (RCC, i.e. Number of cells in treated cultures/ Number of cells in control cultures) used as cytotoxicity parameter in the past is no more recommended, because it is considered to possibly underestimate the cytotoxicity.
Annex 3

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

<table>
<thead>
<tr>
<th>Category</th>
<th>Chemical</th>
<th>CASRN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Clastogens active without metabolic activation</td>
<td>Methyl methanesulphonate</td>
<td>66-27-3</td>
</tr>
<tr>
<td></td>
<td>Mitomycin C</td>
<td>50-07-7</td>
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<tr>
<td></td>
<td>4-Nitroquinoline-N-Oxide</td>
<td>56-57-5</td>
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<td></td>
<td>Cytosine arabinoside</td>
<td>147-94-4</td>
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<tr>
<td>2. Clastogens requiring metabolic activation</td>
<td>Benzo(a)pyrene</td>
<td>50-32-8</td>
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<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>50-18-0</td>
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</table>

For the demonstration of the proficiency of the laboratory:

Positive control chemicals (at least one with and one without metabolic activation) should be investigated in order to demonstrate proficiency to detect clastogenic compounds and the effectiveness of the metabolic activation system.

The concentrations should be selected to give a reproducible and concentration-related increase above the background in order to demonstrate the performance of the test system. One or more concentrations showing reproducible increases above the background should be selected as concurrent positive controls used to show the performance of the test system in each experiment.

A sufficient number of experiments should be conducted under each experimental condition, to allow historical negative and positive control data base to be built (see paragraph 33).
Annex 4

STATISTICAL CONSIDERATIONS ON THE NUMBER OF CELLS TO SCORE

A statistical analysis was performed to derive recommendations on these statistical issues (50).

Scoring 400 cells per concentration is a compromise between achieving an optimal number for the statistical power of the assay and the practicability of scoring. It also takes into account that where the events (cells with a chromosomal aberration) have an incidence of 1% (p=0.01) and are Poisson distributed and 500 cells (n=500) are scored per cultures then cultures with no cells with aberrations will be rare. This is based upon the Poisson distribution of counts with a mean (np) of 5 where less that 0.7% of cultures are expected to be zero. The Poisson distribution also approximates to a normal distribution when np ≥5.
A statistical analysis was performed to derive recommendations on these statistical issues (50).

Historical negative control data are used both for acceptability of the assays and to help with the interpretation of the results in those cases where results are neither clearly negative nor clearly positive. Quality control methods (for example C-charts) should be used to characterise the distribution of the negative control database.

These methods can be used to assess the acceptability of the assay, using the 99% control limits. In order to avoid narrowing the database, all data should be included in the historical database, even when rejected for the evaluation of the results, unless a technical or human failure has been identified. In this case the data will not be added to the database.

In the interpretation of the results, the treated values should be compared with the 95% control levels for the distribution of the historical negative control data base. Results which fall within the 95% control levels for the historical control could be considered broadly consistent with historical negative control data. The limit of 95% is a more conservative approach adequate for the evaluation of the results.