OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR UPDATING GUIDELINE 473

In Vitro Mammalian Chromosome Aberration Test

INTRODUCTION

1. The purpose of the *in vitro* chromosome aberration test is to identify agents that cause structural chromosome 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. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. However, this guideline is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome aberrations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals.

2. The *in vitro* chromosome aberration test may employ cultures of cell lines or primary cell cultures. The cells used are selected on the basis of growth ability in culture, stability of the karyotype, chromosome number, chromosome diversity and spontaneous frequency of chromosome aberrations. At the present time, the available data suggest that it is important to consider the p53 status, genetic (karyotype) stability, DNA repair capacity and origin (rodent versus human) of the cells chosen for testing (Pfuhler et al., 2011). These characteristics may be considered relevant for demonstration of chemical safety in human population.

3. Definitions used are set out in the Annex.

INITIAL CONSIDERATIONS

4. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation unless the cells are metabolically competent with respect to the substances being tested. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions. Care should also be taken to avoid conditions that would lead to artifactual positive results which do not reflect intrinsic mutagenicity, and may arise from such factors as marked changes in pH or osmolality, or by high levels of cytotoxicity (4) (5) (A41).

5. This test is used to detect chromosomal aberrations which may result from clastogenic events. To analyse the induction of chromosomal aberrations, it is essential that mitosis has occurred in both treated and untreated cultures.

PRINCIPLE OF THE TEST METHOD

6. Cell cultures are exposed to the test substance both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used. At predetermined intervals after the start of exposure of cell cultures to the test substance, 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 chromosome aberrations.

DESCRIPTION OF THE METHOD

Preparations

Cells

7. A variety of cell lines, strains or primary cell cultures, including human cells, may be used. Human peripheral blood lymphocytes should be obtained from young (approximately 18-35 years of age), healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation. [*If cells from more than one donor are pooled for use, the number of donors should be specified. The baseline incidence of chromosome aberrations increases with age and this trend is more marked in females than in males (B4)*] [Alternative: *It should be considered in the selection of donor cells for pooling that the baseline incidence of chromosome aberrations increases with age and that this trend is more marked in females than in males (44)*]. Cell cultures are maintained in an exponential cell growth and no more synchronized during exposure to the test substance

Media and culture conditions

8. Appropriate culture medium and incubation conditions (culture vessels, CO_2 concentration, temperature, and humidity) should be used for maintaining cultures. Cell lines should be checked routinely for the stability of the modal chromosome number and the absence of *Mycoplasma* contamination, and should not be used if contaminated or if the modal chromosome number has changed. The normal cell cycle time for the culture conditions used in the testing laboratory should be established and appropriate to the cell line.

Preparation of cultures

9. Cell lines: cells are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency in monolayers, and suspension cultures will not reach excessive density before the time of harvest, and incubated at 37°C.

10. Lymphocytes: whole blood treated with an anti-coagulant (e.g. heparin) or separated lymphocytes are cultured at 37°C in the presence of a mitogen *e.g.* phytohaemagglutinin (PHA).

Metabolic activation

11. Exogenous metabolising systems should be used when employing cells which have inadequate endogenous metabolic capacity. The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (6) (7) (8) (9) (A46) or a combination of phenobarbitone and β-naphthoflavone (A46) (10) (11) (12) (A49). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (A50) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (A46) (10) (11) (A49). The S9 fraction typically is used at concentrations ranging from 1-10% (v/v) in the final test medium. 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 this, please see Section 4.1.4 of the Introduction chapter.

Test substance/Preparation

12. Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to treatment of the cells. Where this is not possible with compatible solvents, suspensions may need to be used. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Gaseous or volatile substances should be tested by appropriate

methods, such as in sealed culture vessels (15) (16). Fresh preparations of the test substance should be used unless stability data demonstrate the acceptability of storage.

<u>Test conditions</u>

Solvents/vehicles

13. The solvent/vehicle should be chosen to optimize the solubility of the test agent without adversely impacting the assay conduct, i.e., cell growth, integrity of the test material, reaction with culture vessels, metabolic activation system, etc. (Ref. to add). It is recommended that, wherever possible, the use of an aqueous solvent should be considered first. Well established solvents/vehicles are for example water, cell culture medium, dimethyl sulfoxide). Generally organic solvents should not exceed 1% (v/v) and aqueous solvents 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 substance and their lack of genetic toxicity. In the absence of that supporting data, it is important to include untreated controls (see Glossary) to demonstrate that no deleterious or mutagenic effects are induced by the chosen solvent.

Measuring cell proliferation and cytotoxicity and choosing exposure concentrations

14. When determining the highest test substance concentration to be tested, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity (see paragraph 20), precipitation (see paragraph 21) in the culture medium, and marked changes in pH or osmolality (see paragraph 4), 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 good cell growth.

15. Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth.

16. 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 substance, 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 cytogenetics (A60) (B1) (B2) (see Annex 2 for formulas). In case of treatment and sampling times longer than 1.5 normal cell cycle, RPD might underestimate cytotoxicity (B7). Under these circumstances RICC could be a better measure. Alternatively, the evaluation of cytotoxicity after a 1.5 normal cell cycles would be a helpful estimate.

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

18. Assessment of other indicators of cytotoxicity (e.g. confluency, cell number, cell integrity, viable cell counts, apoptosis, necrosis, metaphase counting) can also provide useful information for assessing cytotoxicity. It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

19. At least three analysable test concentrations from duplicate cultures should be evaluated. For substances demonstrating little or no toxicity, concentration intervals of approximately 2 to 3 fold will usually be appropriate. However, many substances exhibit steep concentration response curves and in order to obtain data at low and moderate toxicity, it will be necessary to use more closely spaced concentrations. When it is desirable to study the dose response relationship in detail, more than three concentrations will be needed. In these cases a larger number of concentrations (single cultures or duplicates) will be necessary. If single cultures are used then the negative control should be in

duplicate. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing cytotoxicity as described in paragraph 20 and including concentrations at which there is moderate and little or no cytotoxicity.

20. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve a 50% reduction in cell proliferation. Care should be taken not to markedly exceed 50% cytotoxicity because higher levels may induce chromosome damage as a secondary effect of cytotoxicity (60).

21. For poorly soluble compounds that are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration 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. For suspension cultures, care should be taken to assure that the precipitate does not interfere with the conduct of the assay (e.g. staining or scoring).

22. If no cytotoxicity or precipitate is observed, the highest test concentration should correspond to [0.01 M, 5 mg/mL or 5 µl/mL, whichever is the lowest]. For mixtures (no one component is more than 50% of the total by weight or volume), the top concentration should be at least 5 mg/ml. In some circumstances, for mixtures, higher concentrations might be advisable.

Controls

23. Concurrent negative vehicle controls should be included in each experiment conducted either with or without metabolic activation.

24. Positive controls are needed to demonstrate the ability of the cells to identify clastogens and/or aneugens under the conditions of the test protocol used. A clastogen that requires metabolic activation (see table in Annex 3) should be used to affirm the metabolic capability of the metabolic activation system preparation. Positive controls should be used at concentrations expected to give a reproducible and detectable increase over background which demonstrates 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.

25. Because in vitro mammalian cell tests for genetic toxicity are sufficiently standardized the use of positive controls may be confined to a chemical 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.

PROCEDURE

Treatment with test substance

26. Proliferating cells are treated with the test substance in the presence and absence of a metabolic activation system. Treatment of lymphocytes should commence at about 48 hours after mitogenic stimulation.

27. Duplicate cultures should normally be used for each test substance concentration and for the negative (vehicle or untreated) control cultures. Where single cultures are used, e.g. for study of the shape of the dose response relationship (see § on number of test concentrations), an increased number of concentrations has to be analysed but negative controls should be done in duplicate.

Culture harvest time

28. In the first experiment, cells should be exposed to the test substance both with and 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 (12). If this protocol gives negative results both with and without activation, an additional experiment without activation should be done, with continuous treatment until sampling at a time equivalent to about 1.5 normal cell cycle lengths. Certain chemicals may be more readily detected by treatment/sampling times longer than 1.5 normal cell cycle lengths.

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.

Analysis

30. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since fixation procedures often result in the breakage of 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 200 well-spread metaphases should be scored per concentration and control equally divided amongst the duplicates, if applicable. This number can be reduced when high numbers of aberrations are observed.

31. Though the purpose of the test is to detect structural chromosome aberrations, it is important to record polyploidy and endoreduplication when these events are seen.

Proficiency of the laboratory

32. In order to demonstrate proficiency, the laboratory should perform a series of experiments with reference positive chemicals acting via different mechanisms (Annex 3) and various solvents. These positive and negative control responses should be consistent with the published literature. During the course of these investigations, the laboratory should establish:

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

Re-evaluation of laboratory proficiency is recommended if major changes to the experimental conditions (e.g. use of a new cell type) are proposed for the assay.

DATA AND REPORTING

Treatment of results

33. The percentage of cells with structural chromosome aberration(s) should be evaluated. Chromatid- and chromosome-type aberrations 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.

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

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

Evaluation and interpretation of results

[this section needs to be further discussed by the Expert group]

36. There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using modification of experimental conditions. The need to confirm negative results has been discussed in paragraph 28. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

37. Although there are several criteria for a positive result, biological relevance of the results should be considered first. Appropriate statistical methods may be used as an aid in evaluating the test results. However, the results of statistical testing should be assessed with respect to dose-response relationship and a statistically significant increase alone is not sufficient for the determination of a positive result. A result can be considered clearly biologically relevant if the following criteria are all satisfied:

(1) the increase is dose-related,
(2) at least one of the measure points is statistically significant higher than the concurrent negative control,
(3) the positive result is reproducible (e.g. between duplicates or between experiments),
(4) the positive result is outside the range of the historical negative control data.

The positive and negative controls are within the historical positive range for the test within the laboratory.

38. An increase in the number of polyploid cells may indicate that the test substance has the potential to inhibit mitotic processes and to induce numerical chromosome aberrations. An increase in the number of cells with endoreduplicated chromosomes may indicate that the test substance has the potential to inhibit cell cycle progression (17)(18).

39. A test substance for which the results do not meet the above criteria is considered non-mutagenic in this system.

40. Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. . These equivocal or questionable responses may occur regardless of the number of times the experiment is repeated.

41. Positive results from the *in vitro* chromosome aberration test indicate that the test substance induces structural chromosome aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in cultured mammalian somatic cells.

Test report

42. The test report must include the following information:

Test substance:

- identification data and CAS no., if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance, if known.

Solvent/Vehicle:

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

Cells:

- type and source of cells
- karyotype features and suitability of the cell type used;
- absence of mycoplasma, if applicable;
- for cell lines, information on cell cycle length, doubling time or proliferation index;
- sex of blood donors, whole blood or separated lymphocytes, mitogen used;
- number of passages, if applicable;
- methods for maintenance of cell cultures if applicable;
- modal number of chromosomes.

Test conditions:

- identity of metaphase arresting substance, its concentration and duration of cell exposure;
- rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available;
- composition of media, CO_2 concentration if applicable;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature;
- incubation time;
- duration of treatment;
- cell density at seeding, if appropriate;
- type and composition of metabolic activation system, including acceptability criteria;
- positive and negative controls;
- methods of slide preparation;
- criteria for scoring aberrations;
- number of metaphases analyzed;
- methods for the measurements of toxicity;
- criteria for considering studies as positive, negative or equivocal.

Results (individual data):

- the number of cells plated (or treated) and the number of cells harvested for each culture

- cytotoxicity measurements and indications, e.g. RPD, RICC, mitotic index, degree of confluency, cell cycle data, cell counts,;
- information on cell cycle length, doubling time or proliferation index;
- signs of precipitation;
- data on pH and osmolality of the treatment medium, if determined;
- definition for aberrations, including gaps;
- number of cells with chromosome aberrations and type of chromosome aberrations given separately for each treated and control culture;
- changes in ploidy if seen;
- concentration-response relationship, where possible;
- statistical analyses, if any;
- concurrent negative (solvent/vehicle) and positive control data;
- historical negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.

Discussion of the results.

Conclusion.

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Annex 1

DEFINITIONS

Aneugen: any substance that, by interacting with the components of the mitotic and meiotic cell division cycle, leads to aneuploidy in cells or organisms.

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

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 organisms.

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.

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

Mitotic index: the ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of proliferation of that population.

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

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

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

Solvent/vehicle:

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:

Annex 2

FORMULAS FOR CYTOTOXICITY ASSESSMENT

Mitotic index (MI):

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

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

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

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

where:

Population Doubling = [log (Post-treatment cell number ÷ Initial cell number)] ÷ log 2

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

Annex 3

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

Category	Chemical	CASRN
1. Clastogens active without metabolic activation		
	Methyl methanesulphonate	66-27-3
	Mitomycin C	50-07-7
	4-Nitroquinoline-N-Oxide	56-57-5
2. Clastogens requiring metabolic activation		
	Benzo(a)pyrene	50-32-8
	Cyclophosphamide	50-18-0

Positive control chemicals should be able to provide appropriate demonstration of metabolic activation and detection of relevant endpoints or mechanisms covered by the test (clastogenicity).