B. 10. MUTAGENICITY – *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

1. METHOD

This method is a replicate of the OECD TG 473, *In Vitro* Mammalian Chromosome Aberration Test (1997).

1.1 INTRODUCTION

The purpose of the *in vitro* chromosomal 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 method is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome mutations 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.

The *in vitro* chromosome aberration test may employ cultures of established cell lines, cell strains 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.

Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions which would lead to positive results which do not reflect intrinsic mutagenicity and may arise from changes in pH, osmolality or high levels of cytotoxicity (4)(5).

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage.

See also General Introduction Part B.

1.2 DEFINITIONS

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

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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: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e. 3*n*, 4*n* and so on).

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.

1.3 PRINCIPLE OF THE TEST METHOD

Cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals after 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.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 **Preparations**

1.4.1.1 *Cells*

A variety of cell lines, strains or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes).

1.4.1.2 *Media and culture conditions*

Appropriate culture media, and incubation conditions (culture vessels, CO_2 concentration, temperature and humidity) should be used in maintaining cultures. Established cell lines and strains should be checked routinely for stability in the modal chromosome number and the absence of mycoplasma contamination and should not be used if contaminated. The normal cell cycle time for the cells and culture conditions used should be known.

1.4.1.3 *Preparation of cultures*

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

Lymphocytes: whole blood treated with an anti-coagulant (e.g. heparin) or separated lymphocytes obtained from healthy subjects are added to the culture medium containing a mitogen (e.g. phytohaemagglutinin) and incubated at 37°C.

1.4.1.4 *Metabolic activation*

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as: Aroclor 1254 (6)(7)(8)(9), or a mixture of phenobarbitone and ß–naphthoflavone (10)(11)(12).

The post-mitochondrial fraction is usually used at concentrations in the range from 1-10% v/v in the final test medium. The condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilise more than one concentration of post-mitochondrial fraction.

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

1.4.1.5 *Test substance/Preparation*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 **Test conditions**

1.4.2.1 *Solvent/vehicle*

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

1.4.2.2 *Exposure concentrations*

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system and changes in pH or osmolality.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, such as degree of confluency, viable cell counts, or mitotic index. It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

At least three analysable concentrations should be used. Where cytotoxicity occurs, these concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentrations should be separated by no more than a factor between 2 and $\sqrt{10}$. At the time of harvesting, the highest concentration should show a significant reduction in degree of confluency, cell count or mitotic index, (all greater than 50%). The mitotic index is only an indirect measure of cytotoxic/cytostatic effects and depends on the time after treatment. However, the mitotic index is acceptable for suspension cultures in which other toxicity measurements may be cumbersome and impractical. Information on cell cycle kinetics, such as average generation time (AGT), could be used as supplementary information. AGT, however, is an overall average that does not always reveal the existence of delayed subpopulations, and even slight increases in average generation time can be associated with very substantial delay in the time of optimal yield of aberrations.

For relatively non-cytotoxic substances, the maximum test concentration should be 5 µl/ml, 5 mg/ml or 0.01 M, whichever is the lowest.

For relatively insoluble substances that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period. In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration) it is advisable to test at more than one concentration with visible precipitation. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

1.4.2.3 *Negative and positive controls*

Concurrent positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a mutagenic response.

Positive controls should employ a known clastogen at exposure levels expected to give a reproducible and detectable increase over background which demonstrates the sensitivity of the test system.

Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Examples of positive control substances include:

Metabolic Activation condition	Substance	CAS No.	EINECS No.
Absence of Exogenous Metabolic Activation	Methyl methanesulphonate	66-27-3	200-625-0
	Ethyl methanesulphonate	62-50-0	200-536-7
	Ethyl nitrosourea	759-73-9	212-072-2
	Mitomycin C	50-07-7	200-008-6
	4–Nitroquinoline–*N*–oxide	56-57-5	200-281-1
Presence of Exogenous Metabolic Activation	Benzo[*a*]pyrene	50-32-8	200-028-5
	Cyclophosphamide	50-18-0	200-015-4
	Cyclophosphamide monohydrate	6055-19-2	

Other appropriate positive control substances may be used. The use of chemical class-related positive control chemicals should be considered, when available.

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

1.4.3 **Procedure**

1.4.3.1 *Treatment with the test substance*

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.

1.4.3.2 Duplicate cultures should normally be used at each concentration, and are strongly recommended for negative/solvent control cultures. Where minimal variation between duplicate cultures can be demonstrated (13)(14), from historical data, it may be acceptable for single cultures to be used at each concentration.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (15)(16).

1.4.3.3 *Culture harvest time*

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 cycle lengths. Negative results with metabolic activation need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided.

1.4.3.4 *Chromosome preparation*

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.

1.4.3.5 *Analysis*

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 number of aberrations are observed.

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.

2. DATA

2.1 TREATMENT OF RESULTS

The experimental unit is the cell, and therefore the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for experimental and control cultures. Gaps are recorded separately and reported but generally not included in the total aberration frequency.

Concurrent measures of cytotoxicity for all treated and negative control cultures in the main aberration experiments should also be recorded.

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

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 1.4.3.3. 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.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosome aberrations. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (3)(13). Statistical significance should not be the only determining factor for a positive response.

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).

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

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. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

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.

3. **REPORTING**

TEST REPORT

The test report must include the following information:

Solvent/Vehicle

— justification for choice of 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;

— information on cell cycle length;

— sex of blood donors, whole blood or separated lymphocytes, mitogen used;

— number of passages, if applicable;

— methods for maintenance of cell culture, 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 analysed;

— methods for the measurements of toxicity;

— criteria for considering studies as positive, negative or equivocal.

Results :

— signs of toxicity, e.g. degree of confluency, cell cycle data, cell counts, mitotic 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;

— dose-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 results.

Conclusions.

4. REFERENCES

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