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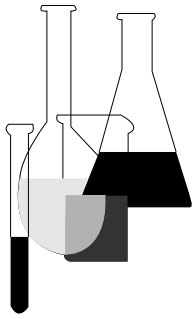
Health Effects Test Guidelines

OPPTS 870.5380 Mammalian Spermatogonial Chromosome Aberration Test



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

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OPPTS 870.5380 Mammalian spermatogonial chromosome aberration test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline is OECD 483, Mammalian Spermatogonial Chromosome Aberration Test.

(b) **Purpose**. (1) The purpose of the *in vivo* mammalian spermatogonial chromosome aberration test is to identify those substances that cause structural aberrations in mammalian spermatogonial cells (see paragraphs (i)(1), (i)(2), (i)(3), (i)(4), and (i)(5) of this guideline). 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. This guideline is not designed to measure numerical aberrations and is not routinely used for this purpose. Chromosome mutations and related events are the cause of many human genetic diseases.

(2) This test measures chromosome events in spermatogonial germ cells and is, therefore, expected to be predictive of induction of inheritable mutations in germ cells.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

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

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

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

Numerical aberration is a change in the number of chromosomes from the normal number characteristic of the animals utilized.

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

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

(d) **Initial considerations**. (1) Rodents are routinely used in this test. This *in vivo* cytogenetic test detects chromosome aberrations in spermatogonial mitoses. Other target cells are not the subject of this guideline.

(2) To detect chromatid-type aberrations in spermatogonial cells, the first mitotic cell division following treatment should be examined before these lesions are lost in subsequent cell divisions. Additional information from treated spermatogonial stem cells can be obtained by meiotic chromosome analysis for chromosome-type aberrations at diakinesis-metaphase I when the treated cells become spermatocytes.

(3) This *in vivo* test is designed to investigate whether somatic cell mutagens are also active in germ cells. In addition, the spermatogonial test is relevant to assessing mutagenicity hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes.

(4) A number of generations of spermatogonia are present in the testis with a spectrum of sensitivity to chemical treatment. Thus, the aberrations detected represent an aggregate response of treated spermatogonial cell populations, with the more numerous differentiated spermatogonial cells predominating. Depending on their position within the testis, different generations of spermatogonia may or may not be exposed to the general circulation, because of the physical and physiological Sertoli cell barrier and the blood-testis barrier.

(5) If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

(e) **Principle of the test method**. Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase-arresting agent (e.g., colchicine or Colcemid®). Chromosome preparations are then made from germ cells and stained, and metaphase cells are analyzed for chromosome aberrations.

(f) **Description of the method**—(1) **Preparations**—(i) **Selection of animal species**. Male Chinese hamsters and mice are commonly used. However, males of other appropriate mammalian species may be used. Commonly used laboratory strains of healthy young-adult animals should be employed. At the commencement of the study the weight variation of animals should be minimal and not exceed ±20 percent of the mean weight.

(ii) **Housing and feeding conditions**. The temperature in the experimental animal room should be 22 °C (±3 °C). Although the relative humidity should be at least 30 percent and preferably not exceed 70 percent

other than during room cleaning, the aim should be 50–60 percent. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups.

(iii) **Preparation of the animals**. Healthy young-adult males should be randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least 5 days prior to the start of the study.

(iv) **Preparation of doses**. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

(2) **Test conditions**—(i) **Solvent/vehicle**. The solvent/vehicle should not produce toxic effects at the dose levels used and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

(ii) **Controls**. (A) Concurrent positive and negative (solvent/vehicle) controls should be included in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals in the treated groups.

(B) Positive controls should produce structural chromosome aberrations *in vivo* in spermatogonial cells when administered at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Chemical	CAS number
Cyclophosphamide (monohydrate)	[50-18-0 (6055-19-2)]
Cyclohexylamine	[108-91-8]
Mitomycin C	[50-07-7]
Monomeric acrylamide	[79-06-1]
Triethylenemelamine	[51-18-3]

(C) Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequency of cells with chromosome aberrations are demonstrated by historical control data. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

(g) **Procedure**—(1) **Number of animals**. Each treated and control group should include at least five analyzable males.

(2) **Treatment schedule**. (i) Test substances are preferably administered once or twice (i.e. as a single treatment or as two treatments). Test substances may also be administered as a split dose, i.e. two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material. Other dose regimens should be scientifically justified.

(ii) In the highest dose group, two sampling times after treatment should be used. Since cell cycle kinetics can be influenced by the test substance, one early and one late sampling time are used around 24 and 48 hours after treatment. For doses other than the highest dose, a sampling time of 24 hours or 1.5 cell cycle length after treatment should be taken, unless another sampling time is known to be more appropriate for detection of effects (see paragraph (i)(6) of this guideline).

(iii) In addition, other sampling times may be used. For example, in the case of chemicals which may induce chromosome lagging, or may exert S-independent effects, earlier sampling times may be appropriate (see paragraph (i)(1) of this guideline).

(iv) The appropriateness of a repeated treatment schedule needs to be identified on a case-by-case basis. Following a repeated treatment schedule the animals should then be sacrificed 24 hours (1.5 cell-cycle length) after the last treatment. Additional sampling times may be used where appropriate.

(v) Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting substance (e.g., Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For

mice this interval is approximately 3–5 hours, for Chinese hamsters this interval is approximately 4–5 hours.

(3) **Dose levels**. If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, and treatment regimen to be used in the main study (see paragraph (i)(7) of this guideline). If there is toxicity, three-dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher-dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the spermatogonial cells (e.g., a reduction in the ratio of spermatogonial mitoses to first and second meiotic metaphases; this reduction should not exceed 50 percent).

(4) **Limit test**. If a test at one dose level of at least 2,000 mg/kg body weight/day using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three-dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

(5) **Administration of doses**. The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

(6) **Chromosome preparation**. Immediately after sacrifice, cell suspensions should be obtained from one or both testes, exposed to hypotonic solution and fixed. The cells should be then spread on slides and stained.

(7) **Analysis**. For each animal at least 100 well-spread metaphases should be analyzed (i.e. a minimum of 500 metaphases per group). This number could be reduced when high numbers of aberrations are observed. 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 metaphases with loss of chromosomes, the cells scored should contain a number of centromeres equal to the number 2n±2.

(h) **Data and reporting**—(1) **Treatment of results**. (i) Individual animal data should be presented in tabular form. The experimental unit is the animal. For each animal the number of cells with structural chromosome aberration(s) and the number of chromosome aberrations per cell should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups. Gaps are recorded separately and reported but generally not included in the total aberration frequency.

(ii) If mitosis as well as meiosis is observed, the ratio of spermatogonial mitoses to first and second meiotic metaphases should be determined as a measure of cytotoxicity for all treated and negative control animals in a total sample of 100 dividing cells per animal to establish a possible cytotoxic effect. If only mitosis is observed, the mitosis index should be determined in at least 1,000 cells for each animal.

(2) **Evaluation and interpretation of results**. (i) There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single-dose group at a single-sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (see paragraph (i)(8) of this guideline). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

(ii) A test substance for which the results do not meet the criteria in paragraph (h)(2)(i) of this guideline is considered nonmutagenic in this test.

(iii) 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.

(iv) Positive results from the *in vivo* spermatogonial chromosome aberration test indicate that a substance induces chromosome aberrations in the germ cells of the species tested. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the germ cells of the species tested.

(v) The likelihood that the test substance or its metabolites reach the target tissue should be discussed.

(3) **Test report**. The test report should include the following information:

(i) Test substance.

(A) Identification data and CAS No., if known.

(B) Physical nature and purity.

(C) Physicochemical properties relevant to the conduct of the study.

(D) Stability of the test substance, if known.

(ii) Solvent/vehicle.

(A) Justification for choice of vehicle.

(B) Solubility and stability of the test substance in solvent/vehicle, if known.

(iii) Test animals.

(A) Species/strain used.

(B) Number and age of animals.

(C) Source, housing conditions, diet, etc.

(D) Individual weight of the animals at the start of the test, including body weight range, mean, and standard deviation for each group.

(iv) Test conditions.

(A) Data from range finding study, if conducted.

(B) Rationale for dose level selection.

(C) Rationale for route of administration.

(D) Details of test substance preparation.

(E) Details of the administration of the test substance.

(F) Rationale for sacrifice times.

(G) Conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable.

(H) Details of food and water quality.

(I) Detailed description of treatment and sampling schedules.

(J) Methods for measurement of toxicity.

(K) Identity of metaphase arresting substance, its concentration and duration of treatment.

(L) Methods of slide preparation.

(M) Criteria for scoring aberrations.

(N) Number of cells analyzed per animal.

(O) Criteria for considering studies as positive, negative, or equivocal.

(v) Results.

(A) Signs of toxicity.

(B) Mitotic index.

(C) Ratio of spermatogonial mitoses cells to first and second meiotic metaphases.

(D) Type and number of aberrations, given separately for each animal.

(E) Total number of aberrations per group.

(F) Number of cells with aberrations per group.

(G) Dose-response relationship, where possible.

(H) Statistical analyses, if any.

(I) Concurrent negative control data.

(J) Historical negative control data with ranges, means, and standard deviations.

(K) Concurrent positive control data.

(L) Changes in ploidy, if seen.

(vi) Discussion of the results.

(vii) Conclusion.

(i) **References.** The following references should be consulted for additional background information on this test guideline.

(1) Adler, I.D. Clastogenic Potential in Mouse Spermatogonia of Chemical Mutagens Related to Their Cell-Cycle Specifications. Genetic Toxicology of Environmental Chemicals, Part B: Genetic Effects and Applied Mutagenesis, Ramel, C., Lambert, B. and Magnusson, J. (Eds.) Liss, New York, pp. 477–484 (1986).

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(3) Evans, E.P., Breckon, G. and Ford, C.E. An Air-Drying Method for Meiotic Preparations from Mammalian Testes. *Cytogenetics and Cell Genetics* 3, 289–294 (1964).

(4) Richold, M. *In Vivo* Cytogenetics Assays. D.J.Kirkland (Ed.) Basic Mutagenicity Tests, UKEMS Recommended Procedures. UKEMS Subcommittee on Guidelines for Mutagenicity Testing. Report. Part I revised. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp. 115–141 (1990).

(5) Yamamoto, K. and Kikuchi, Y. A New Method for Preparation of Mammalian Spermatogonial Chromosomes. *Mutation Research* 52, 207–209 (1978).

(6) Adler I.D. et al. International Workshop on Standardisation of Genotoxicity Test Procedures. Summary Report of the Working Group on Mammalian Germ Cell Tests. *Mutation Research* 312, 313–318 (1994).

(7) Fielder, R. J. et al. Report of British Toxicology Society/UK Environmental Mutagen Society Working Group: Dose setting in *In Vivo* Mutagenicity Assays. *Mutagenesis* 7, 313–319 (1992).

(8) Lovell, D.P. et al. Statistical Analysis of *In Vivo* Cytogenetic Assays. D.J. Kirkland (Ed.) Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Report, Part III. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp. 184–232 (1989).