(vii) Females showing signs of abortion or premature delivery shall be sacrificed and subjected to a thorough macroscopic examination.

(8) Gross necropsy. (i) At the time of sacrifice or death during the study, the dam shall be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

(ii) Immediately after sacrifice or as soon as possible after death, the uterus shall be removed and the contents examined for embryonic or fetal deaths and the number of viable fetuses. The degree of resorption shall be described in order to help estimate the relative time of death of the conceptus. The weight of the gravid uterus should be recorded for dams that are sacrificed. Gravid uterine weights should not be obtained from dead animals if autolysis or decomposition has occurred.

(iii) The number of corpora lutea shall be determined for all species except mice.

(iv) The sex of the fetuses shall be determined and they shall be weighed individually, the weights recorded, and the mean fetal weight derived.

(v) Following removal, each fetus shall be examined externally.

(vi) For rats, mice and hamsters, one-third to one-half of each litter shall be prepared and examined for skeletal anomalies, and the remaining part of each litter shall be prepared and examined for soft tissue anomalies using appropriate methods.

(vii) For rabbits, each fetus shall be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

(f) Data and reporting—(1) Treatment of results. Data shall be summarized in tabular form, showing for each test group: the number of animals at the start of the test, the number of pregnant animals, the number and percentages of live fetuses and the number of fetuses with any soft tissue or skeletal abnormalities.

(2) Evaluation of results. The findings of a developmental toxicity study shall be evaluated in terms of the observed effects and the exposure levels producing effects. It is necessary to consider the historical developmental toxicity data on the species/strain tested. A properly conducted developmental toxicity study should provide a satisfactory estimation of a no-effect level.

(3) Test report. In addition to the reporting requirements as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Toxic response data by concentration.

(ii) Species and strain.

(iii) Date of death during the study or whether animals survived to termination.

(iv) Date of onset and duration of each abnormal sign and its subsequent course.

(v) Food, body weight and uterine weight data.

(vi) Pregnancy and litter data.

(vii) Fetal data (live/dead, sex, soft tissue and skeletal defects, resorptions).

(g) References. For additional background information on this test guideline the following references should be consulted:


[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19077, May 20, 1987]
originating in the germ line of a mammalian species.

(b) Definitions. (1) A biochemical specific locus mutation is a genetic change resulting from a DNA lesion causing alterations in proteins that can be detected by electrophoretic methods.

(2) The germ line is comprised of the cells in the gonads of higher eukaryotes, which are the carriers of the genetic information for the species.

(c) Reference substances. Not applicable.

(d) Test method—(1) Principle. The principle of the MBSL is that heritable damage to the genome can be detected by electrophoretic analysis of proteins in the tissues of the progeny of mice treated with germ cell mutagens.

(2) Description. For technical reasons, males rather than females are generally treated with the test chemical. Treated males are then mated to untreated females to produce F1 progeny. Both blood and kidney samples are taken from progeny for electrophoretic analysis. Up to 33 loci can be examined by starch-gel electrophoresis and broad-range isoelectric focussing. Mutants are identified by variations from the normal electrophoretic pattern. Presumed mutants are bred to confirm the genetic nature of the change.

(3) Animal selection—(i) Species and strain. Mice shall be used as the test species. Although the biochemical specific locus test could be performed in a number of inbred strains, in the most frequently used cross, C57BL/6 females are mated to DBA/2 males to produce (C57BL/6 x DBA/2) F1 progeny for screening.

(ii) Age. Healthy, sexually-mature (at least 8 weeks old) animals shall be used for treatment and breeding.

(iii) Number. A decision on the minimum number of treated animals should take into account possible effects of the test chemical on the fertility of the treated animals. Other considerations should include:

(A) The production of concurrent spontaneous controls.

(B) The use of positive controls.

(C) The power of the test.

(4) Control groups—(i) Concurrent controls. An appropriate number of concurrent control loci shall be analyzed in each experiment. These should be partly derived from matings of untreated animals (from 5 to 20 percent of the treated matings), although some data on control loci can be taken from the study of the alleles transmitted from the untreated parent in the experimental cross. However, any laboratory which has had no prior experience with the test shall produce a spontaneous control sample of about 5,000 progeny animals and a positive control (using 100 mg/kg ethylnitrosourea) sample of at least 1,200 offspring.

(ii) Historical controls. Long-term, accumulated spontaneous control data (currently, 1 mutation in 1,200,000 control loci screened) are available for comparative purposes.

(5) Test chemicals—(i) Vehicle. When possible, test chemicals shall be dissolved or suspended in distilled water or buffered isotonic saline. Water-insoluble chemicals shall be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce major toxic effects. Fresh preparations of the test chemical should be employed.

(ii) Dose levels. Usually, only one dose need be tested. This should be the maximum tolerated dose (MTD), the highest dose tolerated without toxic effects. Any temporary sterility induced due to elimination of spermatogonia at this dose must be of only moderate duration, as determined by the return of males to fertility within 80 days after treatment. For evaluation of dose-response, it is recommended that at least two dose levels be tested.

(iii) Route of administration. Acceptable routes of administration include, but are not limited to, gavage, inhalation, and mixture with food or water, and intraperitoneal or intravenous injections.

(e) Test performance—(1) Treatment and mating. Male DBA/2 mice shall be treated with the test chemical and mated to virgin C57BL/6 females immediately after cessation of treatment. Each treated male shall be mated to new virgin C57BL/6 females each week. Each pairing will continue for a week until the next week’s mating is to begin. This mating schedule permits sampling of all post-spermatogonial
stages of germ-cell development during the first 7 weeks after exposure. Spermatogonial stem cells are studied thereafter. Repeated mating cycles should be conducted until sufficient offspring have been obtained to meet the power criterion of the assay for spermatogonial stem cells.

(2) Examination of offspring—(i) Birth and weaning. Offspring shall be examined at birth and at weaning for externally detectable changes in morphology and behavior; these could be due to dominant mutations. Such characteristics may include, but are not limited to, variations in coat color, appearance of eyes, size (in which case weighing of variant animals and littermates should be carried out), fur texture, etc. Gross changes in external form and behavior shall also be sought. Scrutiny of such visible characteristics of all animals shall be made during all subsequent manipulations of the animals.

(ii) Tissue sampling. Blood (about 0.1 mL) and one kidney shall be removed from progeny mice under anesthesia. Both tissues are then prepared for analysis by electrophoresis.

(iii) Electrophoresis. The gene products of 6 loci shall be analyzed in the blood sample by broad-range isoelectric focusing and of 27 loci in the kidney sample by starch-gel electrophoresis and enzyme-specific staining. Details on these procedures are included in paragraphs (g)(1) through (g)(3) of this section.

(iv) Mutant identification. Presumptive electrophoretic mutants shall be identified by variation from the normal electrophoretic banding patterns. Reruns of all variant samples shall be performed to confirm the presence of altered banding patterns. Samples from parents of progeny exhibiting banding pattern variations shall be assayed to determine whether the variant was induced by the experimental treatment or was pre-existing. All treatment-induced variants are bred to determine the genetic nature of the change.

(f) Data and reports—(1) Treatment of results. Data shall be presented in tabular form and shall permit independent analysis of cell stage-specific effects, and dose-dependent phenomena. The data shall be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected shall be thoroughly described. In addition, concurrent positive control data (if employed) and spontaneous control data shall also be tabulated. These concurrent controls shall be added to, as well as compared with, the historical control data.

(2) Statistical evaluation. Data shall be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the frequency of electrophoretic mutations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of these test points.

(ii) A test chemical which does not produce a statistically significant increase in the frequency of electrophoretic mutations over the spontaneous frequency, or a statistically significant and reproducible positive response for at least one of the test points, is considered nonmutagenic in this system, provided that the sample size is sufficient to exclude a biologically significant increase in mutation frequency.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results in the MBSL indicate that, under the test conditions, the test chemical induces heritable gene mutations in a mammalian species.

(ii) Negative results indicate that, under the test conditions, the test chemical does not induce heritable gene mutations in a mammalian species.

(5) Test report. In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, and paragraph (h) of this section, the following specific information shall be reported:

(i) Strain, age and weight of animals used; numbers of animals of each sex in experimental and control groups.

(ii) Test chemical vehicle, doses used, rationale for dose selection, and toxicity data, if available.

(iii) Route and duration of exposure.

(iv) Mating schedule.
§ 798.5200 Mouse visible specific locus test.

(a) Purpose. The mouse visible specific locus test (MSLT) may be used to detect and quantitate mutations in the germ line of a mammalian species.

(b) Definitions. (1) A visible specific locus mutation is a genetic change that alters factors responsible for coat color and other visible characteristics of certain mouse strains.

(2) The germ line is the cells in the gonads of higher eukaryotes which are the carriers of the genetic information for the species.

(c) Reference substances. Not applicable.

(d) Test method—(1) Principle. (i) The principle of the MSLT is to cross individuals who differ with respect to the genes present at certain specific loci, so that a genetic alteration involving the standard gene at any one of these loci will produce an offspring detectably different from the standard heterozygote. The genetic change may be detectable by various means, depending on the loci chosen to be marked.

(ii) Three variations of the method currently exist for detecting newly arising point mutations in mouse germ cells:

(A) The visible specific locus test using either 5 or 7 loci.

(B) The biochemical specific locus test using up to 20 enzymes.

(C) The test for mutations at histocompatibility loci.

(iii) Of the three tests, the visible specific locus test has been most widely used in assessing genetic hazard due to environmental agents. It is the method described in this guideline.

(2) Description. For technical reasons, males rather than females are generally treated with the test agent. Treated males are then mated to females which are genetically homozygous for certain specific visible marker loci. Offspring are examined in the next generation for evidence that a new mutation has arisen.

(3) Animal selection—(i) Species and strain. Mice shall be used as the test species. Male mice shall be either (C3-H × 101)F1 or (101 × C3-H)F1 hybrids. Females shall be T stock virgins.
(ii) Age. Healthy sexually mature animals shall be used.

(iii) Number. A decision on the minimum number of treated animals should take into account the spontaneous variation of the biological characterization being evaluated. Other considerations should include:

(A) The use of either historical or concurrent controls.
(B) The power of the test.
(C) The minimal rate of induction required.
(D) The use of positive controls.
(E) The level of significance desired.
(iv) Assignment to groups. Animals shall be randomized and assigned to treatment and control groups.

(4) Control groups—(i) Concurrent controls. The use of positive or spontaneous controls is left to the discretion of the investigator. However, any laboratory which has had no prior experience with the test shall, at its first attempt, produce a negative control sample of 20,000 and a positive control, using 100 mg/kg 1-ethyl-nitrosourea, in a sample of 5,000 offspring.

(ii) Historical controls. Long term, accumulated spontaneous control data of 43/801,406 are available for comparative purposes.

(5) Test chemicals—(i) Vehicle. When possible, test chemicals should be dissolved or suspended in distilled water or isotonic saline buffered appropriately, if needed, for stability. Water-insoluble chemicals shall be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test compound nor produce major toxic effects. Fresh preparations of the test chemical should be employed.

(ii) Dose levels. Usually, only one dose level need be tested. This should be the highest dose tolerated without toxic effects, provided that any temporary sterility induced due to elimination of spermatagonia is of only moderate duration, as determined by a return of males to fertility within 80 days after treatment. For evaluation of dose-response, it is recommended that at least two dose levels be tested.

(iii) Route of administration. Acceptable routes of administration include gavage, inhalation, admixture with food or water, and IP or IV injections.

(e) Test performance—(1) Treatment and mating. Hybrid F, (C₃H × 101 or 101 × C₃H) male mice shall be treated with the test substance and immediately mated to virgin T stock females. Each treated male shall be mated to a fresh group of 2 to 4 virgin females each week for 7 weeks, after which he shall be returned to the first group of females and rotated through the seven sets of females repeatedly. This mating schedule generally permits sampling of all post spermatogonial stages of germ cell development during the first 7 weeks and rapid accumulation of data for exposed spermatogonial stem cells thereafter. Repeated mating cycles should be conducted until the entire spermatogonial cycle has been evaluated and enough offspring have been obtained to meet the power criterion of the assay.

(2) Examination of offspring. (i) Offspring may be examined at (or soon after) birth but must be examined at about 3 weeks of age at which time the numbers of mutant and nonmutant offspring in each litter shall be recorded.

(ii) Nonmutant progeny should be discarded. Mutant progeny shall be subjected to genetic tests for verification.

(f) Data and report—(1) Treatment of results. Data shall be presented in tabular form and shall permit independent analysis of cell stage specific effects and dose dependent phenomena. The data shall be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected shall be thoroughly described. In addition, concurrent positive and negative control data, if they are available, shall be tabulated so that it is possible to differentiate between concurrent (when available) and long-term accumulated mutation frequencies.

(2) Statistical evaluation. Data shall be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of specific locus mutations. Another criterion may be based upon detection of a reproducible and statistically significant positive
response for at least one of the test points.
(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of specific locus mutations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.
(iii) Both biological and statistical significance should be considered together in the evaluation.
(4) Test evaluation.
(i) Positive results in the MSLT indicate that under the test conditions the test substance induces heritable gene mutations in the test species.
(ii) Negative results indicate that under the test conditions the test substance does not induce heritable gene mutations in the test species.
(5) Test report. In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, and paragraph (h) of this section, the following specific information shall be reported:
(i) Strain, age and weight of animals used, number of animals of each sex in experimental and control groups.
(ii) Test chemical vehicle, doses used and rationale for dose selection, toxicity data.
(iii) Route and duration of exposure.
(iv) Mating schedule.
(v) Time of examination for mutant progeny.
(vi) Criteria for scoring mutants.
(vii) Use of concurrent or negative controls.
(viii) Dose response relationship, if applicable.
(g) References. For additional background information on this test guideline the following references should be consulted:
(2) [Reserved]
(h) Additional requirements. Testing facilities conducting the mouse visible specific locus test in accordance with this section shall, in addition to adhering to the provisions of §§ 792.190 and 792.195 of this chapter, obtain, and retain for at least 10 years, acceptable 35-mm color photographs (and their negatives) demonstrating the visible mutations observed in mutant animals and the lack of such mutations in their siblings and parents.

§ 798.5265 The salmonella typhimurium reverse mutation assay.

(a) Purpose. The Salmonella typhimurium histidine (his) reversion system is a microbial assay which measures his⁻ his⁺ reversion induced by chemicals which cause base changes or frameshift mutations in the genome of this organism.
(b) Definitions.
(1) A reverse mutation assay in Salmonella typhimurium detects mutation in a gene of a histidine requiring strain of this organism.
(2) Base pair mutagens are agents which cause a base change in the DNA. In a reversion assay, this change may occur at the site of the original mutation or at a second site in the chromosome.
(3) Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.
(c) Reference substances. These may include, but need not be limited to, sodium azide, 2-nitrofluorene, 9-aminoacridine, 2-aminoanthracene, congo red, benzopurpurin 4B, trypan blue or direct blue 1.
(d) Test method—(1) Principle. Bacteria are exposed to test chemical with and without a metabolic activation system and plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or vehicle control culture.
(2) Description. Several methods for performing the test have been described. Among those used are:
(i) The direct plate incorporation method.
(ii) The preincubation method.
(iii) The azo-reduction method.
The procedures described here are for the direct plate incorporation method and the azo-reduction method.

(3) Strain selection—(i) Designation. At the present time four strains, TA 1535, TA 1537, TA 98 and TA 100 should be used. The use of other strains in addition to these four is left to the discretion of the investigator.

(ii) Preparation and storage. Recognized methods of stock culture preparation and storage should be used. The requirement of histidine for growth should be demonstrated for each strain. Other phenotypic characteristics should be checked using such methods as crystal violet sensitivity and resistance to ampicillin. Spontaneous reversion frequency should be in the range expected either as reported in the literature or as established in the laboratory by historical control values.

(iii) Bacterial growth. Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^8 - 10^9 cells per ml).

(4) Metabolic activation. Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. For the direct plate incorporation method, the most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents such as Aroclor 1254. For the azo-reduction method, a cofactor supplemented postmitochondrial fraction prepared from the livers of untreated hamsters is preferred. For this method, the cofactor supplement should contain flavin mononucleotide, exogenous glucose 6-phosphate dehydrogenase, NADH and excess of glucose-6-phosphate.

(5) Control groups—(i) Concurrent controls. Concurrent positive and negative (untreated and/or vehicle) controls shall be included in each experiment. Positive controls shall ensure both strain responsiveness and efficacy of the metabolic activation system.

(ii) Strain specific positive controls. Strain specific positive controls shall be included in the assay. Examples of strain specific positive controls are as follows:

(A) Strain TA 1535, TA 100, sodium azide.
(B) TA 98, 2-nitrofluorene.
(C) TA 1537, 9-aminoacridine.

(iii) Positive controls to ensure the efficacy of the activation system. The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test. 2-Aminoanthracene is an example of a positive control compound in plate-incorporation tests using postmitochondrial fractions from the livers of rodents treated with enzyme inducing agents such as Aroclor 1254. Congo red is an example of a positive control compound in the azo-reduction method. Other positive control reference substances may be used.

(iv) Class-specific positive controls. The azo-reduction method should include positive controls from the same class of compounds as the test agent wherever possible.

(B) Test chemicals—(i) Vehicle. Test chemicals and positive control reference substances should be dissolved or suspended in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) Exposure concentrations. (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(B) Generally, a maximum of 5 mg/plate for pure substances is considered acceptable. At least 5 different amounts of test substance shall be tested with adequate intervals between test points.

(C) When appropriate, a single positive response shall be confirmed by
testing over a narrow range of concentrations.

(e) Test performance—(1) Direct plate incorporation method. For this test without metabolic activation, test chemical and 0.1 ml of a fresh bacterial culture should be added to 2.0 ml of overlay agar. For tests with metabolic activation, 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction should be added to the agar overlay after the addition of test chemical and bacteria. Contents of each tube shall be mixed and poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted.

(2) Azo-reduction method. (i) For this test with metabolic activation, 0.5 ml of S±9 mix containing 150 ul of S±9 and 0.1 ml of bacterial culture should be added to a test tube kept on ice. One-tenth milliliter of chemical should be added, and the tubes should be incubated with shaking at 30°C for 30 min. At the end of the incubation period, 2.0 ml of agar should be added to each tube, the contents mixed and poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted.

(ii) For tests without metabolic activation, 0.5 ml of buffer should be used in place of the 0.5 ml of S±9 mix. All other procedures shall be the same as those used for the test with metabolic activation.

(3) Other methods. Other methods may also be appropriate.

(4) Media. An appropriate selective medium with an adequate overlay agar shall be used.

(5) Incubation conditions. All plates within a given experiment shall be incubated for the same time period. This incubation period shall be for 48-72 hours at 37°C.

(6) Number of cultures. All plating should be done at least in triplicate.

(f) Data and report—(1) Treatment of results. Data shall be presented as number of revertant colonies per plate for each replicate and dose. The numbers of revertant colonies on both negative (untreated and/or vehicle) and positive control plates shall also be presented. Individual plate counts, the mean number of revertant colonies per plate and standard deviation shall be presented for test chemical and positive and negative (untreated and/or vehicle) controls.

(2) Statistical evaluation. Data should be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of revertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of revertants or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results from the S. typhimurium reverse mutation assay indicate that, under the test conditions, the test substance induces point mutations by base changes or frameshifts in the genome of this organism.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in S. typhimurium.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) Bacterial strain used.

(ii) Metabolic activation system used (source, amount and cofactor); details of preparations of S±9 mix.

(iii) Dose levels and rationale for selection of dose.

(iv) Positive and negative controls.

(v) Individual plate counts, mean number of revertant colonies per plate, standard deviation.
(vi) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline the following references should be consulted:


§ 798.5275 Sex-linked recessive lethal test in drosophila melanogaster.

(a) Purpose. The sex-linked recessive lethal (SLRL) test using Drosophila melanogaster detects the occurrence of mutations, both point mutations and small deletions, in the germ line of the insect. This test is a forward mutation assay capable of screening for mutations at about 800 loci on the X-chromosome. This represents about 80 percent of all X-chromosome loci. The X-chromosome represents approximately one-fifth of the entire haploid genome.

(b) Definitions. (1) Lethal mutation is a change in the genome which, when expressed, causes death to the carrier.

(2) Recessive mutation is a change in the genome which is expressed in the homozygous or hemizygous condition.

(3) Sex-Linked genes are present on the sex (X or Y) chromosomes. Sex-linked genes in the context of this guideline refer only to those located on the X-chromosome.

(c) Reference substances. These may include, but need not be limited to, ethyl methanesulfonate or N-nitroso-dimethylamine.

(d) Test method—(1) Principle. Mutations in the X-chromosome of D. melanogaster are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The SLRL test takes advantage of these facts by means of specially marked and arranged chromosomes.

(2) Description. Wild-type males are treated and mated to appropriate females. Female offspring are mated individually to their brothers, and in the next generation the progeny from each separate dose are scored for phenotypically wild-type males. Absence of these males indicates that a sex-linked recessive lethal mutation has occurred in a germ cell of the P1 male.

(3) Drosophila stocks. Males of a well-defined wild type stock and females of the Muller-5 stock may be used. Other appropriately marked female stocks with multiple inverted X-chromosomes may also be used.

(4) Control groups—(i) Concurrent controls. Concurrent positive and negative (vehicle) controls shall be included in each experiment.

(ii) Positive controls. Examples of positive controls include ethyl methanesulfonate and N-nitroso-dimethylamine.

(iii) Other positive controls. Other positive control reference substances may be used.

(iv) Negative controls. Negative (vehicle) controls shall be included. The size of the negative (vehicle) control group shall be determined by the availability of appropriate laboratory historical control data.

(5) Test chemicals—(i) Vehicle. Test chemicals should be dissolved in water. Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g., a mixture of ethanol and Tween-60 or 80) and then diluted in water or saline prior to administration. Dimethylsulfoxide should be avoided as a vehicle.

(ii) Dose levels. For the initial assessment of mutagenicity, it is sufficient
to test a single dose of the test substance for screening purposes. This dose should be the maximum tolerated dose, or that which produces some indication of toxicity, or shall be the highest dose attainable. For dose-response purposes, at least three additional dose levels should be used.

(iii) Route of administration. Exposure may be oral, by injection or by exposure to gases or vapors. Feeding of the test compound may be done in sugar solution. When necessary, substances may be dissolved in 0.7 percent NaCl solution and injected into the thorax or abdomen.

(e) Test performance—(1) Treatment and mating. Wild-type males (3 to 5 days old) shall be treated with the test substance and mated individually to an appropriate number of virgin females from the Muller-5 stock or females from another appropriately marked (with multiply-inverted X-chromosomes) stock. The females shall be replaced with fresh virgins every 2 to 3 days to cover the entire germ cell cycle. The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late stage spermatids, early spermatids, spermatocytes, and spermatogonia at the time of treatment.

(2) F1 matings. Heterozygous F1 females from the above crosses shall be allowed to mate individually (i.e., one female per vial) with their brothers. In the F2 generation, each culture shall be scored for the absence of wild-type males. If a culture appears to have arisen from an F1 female carrying a lethal in the parental X-chromosome (i.e., no males with the treated chromosome are observed), daughters of that female with the same genotype shall be tested to ascertain if the lethality is repeated in the next generation.

(3) Number of matings. (i) The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these defined parameters. The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analysed to detect substances which show mutation rates close to those of the controls.

(ii) Test results should be confirmed in a separate experiment.

(f) Data and report—(1) Treatment of results. Data shall be tabulated to show the number of chromosomes tested, the number of nonfertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated. Numbers of clusters of different size per male shall be reported.

(2) Statistical evaluation. Data shall be evaluated by appropriate statistical techniques.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of sex-linked recessive lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of sex-linked recessive lethals or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results in the SLRL test in D. melanogaster indicate that under the test conditions the test agent causes mutations in germ cells of this insect.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in D. melanogaster.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported.

(i) Drosophila stock used in the assay, age of insects, number of males treated, number of sterile males, number of F2 cultures established, number of F2 cultures without progeny.

(ii) Test chemical vehicle, treatment and sampling schedule, exposure levels,


§ 798.5300 Detection of gene mutations in somatic cells in culture.

(a) Purpose. Mammalian cell culture systems may be used to detect mutations induced by chemical substances. Widely used cell lines include L5178Y mouse lymphoma cells and the CHO and V-79 lines of Chinese hamster cells. In these cell lines the most commonly used systems measure mutation at the thymidine kinase (TK), hypoxanthine-guanine-phosphoribosyl transferase (HPRT) and Na\(^{+}\)/K\(^{+}\) ATPase loci. The TK and HPRT mutational systems detect base pair mutations, frameshift mutations, and small deletions; the Na\(^{+}\)/K\(^{+}\) ATPase system detects base pair mutations only.

(b) Definitions. (1) A forward mutation assay detects a gene mutation from the parental type to the mutant form which gives rise to a change in an enzymatic or functional protein.

(2) Base pair mutagens are agents which cause a base change in the DNA.

(3) Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

(4) Phenotypic expression time is a period during which unaltered gene products are depleted from newly mutated cells.

(c) Reference substances. These may include, but need not be limited to, ethyl methanesulfonate, N-nitroso-dimethylamine, 2-acetylaminofluorene, 7,12-dimethylbenzanthracene or hycanthone.

(d) Test method—(1) Principle. Cells are exposed to test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cells deficient in thymidine kinase (TK) due to the forward mutation TK\(^{-}\)TK\(^{+}\) are resistant to the cytotoxic effects of pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). The deficiency of the "salvage" enzyme thymidine kinase means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained solely from de novo synthesis. However, in the presence of thymidine kinase, BrdU, FdU or TFT are incorporated into the nucleotides, resulting in inhibition of cellular metabolism and cytotoxicity. Thus mutant cells are able to proliferate in the presence of BrdU, FdU or TFT whereas normal cells, which contain thymidine kinase, are not. Similarly cells deficient in HPRT are selected by resistance to 6-azaguanine (AG) or 6-thioguanine (TG) and cells with altered Na\(^{+}\)/K\(^{+}\) ATPase are selected by resistance to ouabain.

(2) Description. Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a defined period of time. Cytotoxicity is determined by measuring the colony forming ability or growth rate of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time—characteristic of each selected locus—to allow near-optimal phenotypic expression of induced...
mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency. After a suitable incubation time, cell colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in nonselective medium to derive the mutant frequency.

3 Cells—(i) Type of cells used in the assay. A variety of cell lines are available for use in this assay including subclones of L5178Y, CHO cells or V-79 cells. Cell types used in this assay should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a low spontaneous mutation frequency. Cells should be checked for Mycoplasma contamination and may be periodically checked for karyotype stability.

(ii) Cell growth and maintenance. Appropriate culture media and incubation conditions (culture vessels, CO₂ concentrations, temperature and humidity) shall be used.

4 Metabolic activation. Cells shall be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

5 Control groups. Positive and negative (untreated and/or vehicle) controls shall be included in each experiment. When metabolic activation is used, the positive control substance shall be known to require such activation.

6 Test chemicals—(i) Vehicle. Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle shall not interfere with cell viability or growth rate. Treatment vessels should be chosen to ensure that there is no visible interaction, such as etching, between the solvent, the test chemical, and the vessel.

(ii) Exposure concentrations. (A) The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures, and concentrations of test substance used should reflect these defined parameters. The number of cells per culture is based on the expected background mutant frequency; a general guide is to use a number which is 10 times the inverse of this frequency.

(B) Several concentrations (usually at least 4) of the test substance shall be used. Generally, these shall yield a concentration-related toxic effect. The highest concentration shall produce a low level of survival (approximately 10 percent), and the survival in the lowest concentration shall approximate the negative control. Cytotoxicity shall be determined after treatment with the test substance both in the presence and in the absence of an exogenous metabolic activation system. Relatively insoluble substances should be tested up to their limit of solubility under culture conditions. For freely-soluble nontoxic substances the highest concentration used should be determined on a case-by-case basis.

(e) Test performance. (1) Cells shall be exposed to the test substance both with and without exogenous metabolic activation. Exposure shall be for a suitable period of time, in most cases 1 to 5 hours is effective; exposure time may be extended over one or more cell cycles.

(2) At the end of the exposure period, cells shall be washed and cultured to determine viability and to allow for expression of the mutant phenotype.

(3) At the end of the expression period, which shall be sufficient to allow near optimal phenotypic expression of induced mutants, cells should be grown in medium with and without selective agent(s) for determination of number of mutants and cloning efficiency, respectively.

(4) Results shall be confirmed in an independent experiment. When appropriate, a single positive response should be confirmed by testing over a narrow range of concentrations.

(f) Data and report—(1) Treatment of results. Data shall be presented in tabular form. Individual colony counts for the treated and control groups shall be presented for both mutation induction and survival. Survival and cloning efficiencies shall be given as a percentage of the controls. Mutant frequency shall be expressed as number of mutants per number of surviving cells.

(2) Statistical evaluation. Data should be evaluated by appropriate statistical methods.
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(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant concentration-related increase in the mutant frequency. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant concentration-related increase in the mutant frequency or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results for an in vitro mammalian cell gene mutation test indicate that, under the test conditions, a substance induces gene mutations in the cultured mammalian cells used.

(ii) Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Cell type used, number of cell cultures, methods used for maintenance of cell cultures.

(ii) Rationale for selection of concentrations and number of cultures.

(iii) Test conditions: composition of media, CO₂ concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period (including number of cells seeded and subculture and feeding schedules, if appropriate), selective agent(s).

(iv) Methods used to enumerate numbers of viable and mutant cells.

(v) Dose-response relationship, where possible.

(g) References. For additional background information on this test guide the following references should be consulted:


routine cytogenetics assay. Structural aberrations may be of two types, chromosome or chromatid.

(b) Definitions. (1) Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

(2) Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

(c) Reference substances. Not applicable.

(d) Test method—(1) Principle. In vitro cytogenetics assays may employ cultures of established cell lines, cell strains or primary cell cultures. Cell cultures are exposed to the test substance both with and without metabolic activation. Following exposure of cell cultures to test substances, they are treated with a spindle inhibitor (e.g., colchicine or Colcemid®) to arrest cells in a metaphase-like stage of mitosis (c-metaphase). Cells are then harvested and chromosome preparations made. Preparations are stained and metaphase cells are analyzed for chromosomal aberrations.

(2) Description. Cell cultures are exposed to test compounds and harvested at various intervals after treatment. Prior to harvesting, cells are treated with a spindle inhibitor (e.g., colchicine or Colcemid®) to accumulate cells in c-metaphase. Chromosome preparations from cells are made, stained and scored for chromosomal aberrations.

(3) Cells—(i) Type of cells used in the assay. There are a variety of cell lines or primary cell cultures, including human cells, which may be used in the assay. Established cell lines and strains should be checked for Mycoplasma contamination and may be periodically checked for karyotype stability.

(ii) Cell growth and maintenance. Appropriate culture media, and incubation conditions (culture vessels CO₂ concentrations, temperature and humidity) shall be used.

(4) Metabolic activation. Cells shall be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(5) Control groups. Positive and negative (untreated and/or vehicle) controls both with and without metabolic activation shall be included in each experiment. When metabolic activation is used, the positive control substance shall be known to require such activation.

(e) Test chemicals—(i) Vehicle. Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle shall not interfere with cell viability or growth rate. Treatment vessels should be chosen to ensure that there is no visible interaction, such as etching, between the solvent, the test chemical, and the vessel.

(ii) Exposure concentrations. Multiple concentrations of the test substance over a range adequate to define the response should be tested. Generally the highest test substance concentrations tested with and without metabolic activation should show evidence of cytotoxicity or reduced mitotic activity. Relatively insoluble substances should be tested up to the limit of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(f) Test performance—(1) Established cell lines and strains. Prior to use in the assay, cells should be generated from stock cultures, seeded in culture vessels at the appropriate density and incubated at 37°C.

(2) Human lymphocyte cultures. Heparinized or acid-citrate-dextrose whole blood should be added to culture medium containing a mitogen, e.g., phytohemagglutinin (PHA) and incubated at 37°C. White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient may also be utilized.

(3) Treatment with test substance. For established cell lines and strains, cells in the exponential phase of growth shall be treated with test substances in the presence and absence of an exogenous metabolic activation system. Mitogen-stimulated human lymphocyte cultures may be treated with the test substance in a similar manner.

(4) Number of cultures. At least two independent cultures shall be used for each experimental point.
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(5) Culture harvest time. (i) For established cell lines and strains, multiple harvest times are recommended. However, for screening purposes, a single harvest time may be appropriate. If the test chemical changes the cell cycle length, the fixation intervals should be changed accordingly. If a single harvest time is selected, supporting data for the harvest time should be presented in such a study.

(ii) For human lymphocyte cultures, the substance to be tested may be added to the cultures at various times after mitogen stimulation so that there is a single harvest time after the initiation of the cell culture. Alternatively, a single treatment may be followed by multiple harvest times. Harvest time should be extended for those chemicals which induce an apparent cell cycle delay. Because the population of human lymphocytes is only partially synchronized, a single treatment, at, or close to, the time when metaphase stages first appear in the culture will include cells in all phases of the division cycle. Therefore, a single harvest at the time of second mitosis may be carried out for screening purposes.

(iii) Cell cultures shall be treated with a spindle inhibitor, (e.g., colchicine or Colcemid®), 1 or 2 hours prior to harvesting. Each culture shall be harvested and processed separately for the preparation of chromosomes.


(7) Analysis. Slides shall be coded before analysis. In human lymphocytes, only cells containing 46 centromeres shall be analyzed. In established cell lines and strains, only metaphases containing ±2 centromeres of the modal number shall be analyzed. Uniform criteria for scoring aberrations shall be used.

(f) Data and report—(1) Treatment of results. Data shall be presented in a tabular form. Different types of structural chromosomal aberrations shall be listed with their numbers and frequencies for experimental and control groups. Data should be evaluated by appropriate statistical methods. Gaps or achromatic lesions are recorded separately and not included in the total aberration frequency.

(2) Statistical evaluation. Data should be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structural chromosomal aberrations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of structural chromosomal aberrations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results in the in vitro cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in cultured mammalian somatic cells.

(ii) Negative results indicate that under the test conditions the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Cells used, density and passage number at time of treatment, number of cell cultures.

(ii) Methods used for maintenance of cell cultures including medium, temperature and CO₂ concentration.

(iii) Test chemical vehicle, concentration and rationale for the selection of the concentrations used in the assay, duration of treatment.

(iv) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay.
(v) Identity of spindle inhibitor, its concentration and duration of treatment.
(vi) Date of cell harvest.
(vii) Positive and negative controls.
(viii) Methods used for preparation of slides for microscopic examination.
(ix) Number of metaphases analysed.
(x) Mitotic index where applicable.
(xi) Criteria for scoring aberrations.
(xii) Type and number of aberrations, given separately for each treated and control culture, total number of aberrations per group; frequency distribution of number of chromosomes in established cell lines and strains.
(xiii) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline the following references should be consulted.

§ 798.5385 In vivo mammalian bone marrow cytogenetics tests: Chromosomal analysis.

(a) Purpose. The in vivo bone marrow cytogenetic test is a mutagenicity test for the detection of structural chromosomal aberrations. Chromosomal aberrations are generally evaluated in first post-treatment mitoses. With the majority of chemical mutagens, induced aberrations are of the chromatid type but chromosome type aberrations also occur.

(b) Definitions. (1) Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time. (2) Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

(c) Reference substances. Not applicable.

(d) Test method—(1) Principle. Animals are exposed to test chemicals by appropriate routes and are sacrificed at sequential intervals. Chromosome preparations are made from bone marrow cells. The stained preparations are examined and metaphase cells are scored for chromosomal aberrations.

(2) Description. The method employs bone marrow of laboratory rodents which have been exposed to test chemicals. Prior to sacrifice, animals are further treated with a spindle inhibitor, (e.g., colchicine or Colcemid) to arrest the cells in G2-metaphase. Chromosome preparations from the cells are stained and scored for chromosomal aberrations.

(3) Animal selection—(i) Species and strain. Any appropriate mammalian species may be used. Examples of commonly used rodent species are rats, mice, and hamsters.
(ii) Age. Healthy young adult animals shall be used.
(iii) Number and sex. At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group treated with the test compound if several test times
after treatment are included in the experimental schedule. The use of a single sex or smaller number of animals should be justified.

(iv) Assignment to groups. Animals shall be randomized and assigned to treatment and control groups.

(4) Control groups—(1) Concurrent controls. (i) Concurrent positive and negative (vehicle) controls shall be included in the assay.

(ii) Positive controls. A single dose positive control showing a significant response at any one time point is adequate. A compound known to produce chromosomal aberrations in vivo shall be employed as the positive control.

(5) Test chemicals—(i) Vehicle. When possible, test chemicals shall be dissolved in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicles used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test compound should be employed.

(ii) Dose levels. For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity (e.g., partial inhibition of mitosis) or shall be the highest dose attainable (to a maximum of 5,000 mg/kg). Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

(iii) Route of administration. The usual routes are oral or by intraperitoneal injection. Other routes may be appropriate.

(iv) Treatment schedule. In general, test substances shall be administered once only. However, based on toxicological information a repeated treatment schedule may be employed.

(e) Test performance—(1) Generally the test may be performed in two assays. (i) Animals should be treated with the test substance once at the selected dose(s). Samples should be taken at three times after treatment. For rodents, the central sampling interval is 24 hours. Since cell cycle kinetics can be influenced by the test substance, one earlier and one later sampling interval adequately spaced within the range of 6 to 48 hours shall be applied. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at the central sampling time. If the most sensitive interval is known and documented with data, only this one time point shall be sampled.

(ii) If a repeated treatment schedule is used at the selected dose(s), samples shall be taken 6 and 24 hours after the last treatment; other sampling times may be used if justified. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at 6 hours after the last treatment.

(2) Administration of spindle inhibitor. Prior to sacrifice, animals shall be injected IP with an appropriate dose of a spindle inhibitor (e.g., colchicine or Colcemid®) to arrest cells in G/metaphase.

(3) Preparation of slides. Immediately after sacrifice, the bone marrow shall be obtained, exposed to hypotonic solution, and fixed. The cells shall then be spread on slides and stained. Chromosome preparations shall be made following standard procedures.

(4) Analysis. The number of cells to be analyzed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity, and the power chosen for the test. Slides shall be coded before microscopic analysis.

(f) Data and report—(1) Treatment of results. Data should be presented in tabular form for both cells and animals. Different types of structural chromosomal aberrations should be listed with their numbers and a mean frequency per cell for each animal in all treated and control groups. Gaps (achromatic lesions) should be recorded separately and not included in the total aberration frequency. Differences among animals within each group should be considered before making comparisons between treated and control groups.

(2) Statistical evaluation. Data should be evaluated by appropriate statistical methods.
(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structural chromosomal aberrations or abnormal metaphase figures. Another criterion may be based upon detection of a reproducible and statistically significant positive response for a least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of chromosomal aberrations or abnormal metaphase figures or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results in the in vivo bone marrow cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in the bone marrow of the test species.

(ii) Negative results indicate that under the test conditions, the test substance does not induce chromosomal aberrations in the bone marrow of the test species.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Species, strain, age, weight, number and sex of animals in each treatment and control group.

(ii) Test chemical, vehicle, dose levels used, rationale for dose selection.

(iii) Route of administration, treatment and sampling schedules, toxicity data, negative and positive controls.

(iv) Identity of spindle-inhibitor, its concentration and duration of treatment.

(v) Details of the protocol used for chromosome preparation, number of cells scored per animal, type and number of aberrations given separately for each treated and control animal.

(vi) Mitotic index, where applicable.

(vii) Criteria for scoring aberrations.

(viii) Number and frequency of aberrant cells per animal in each treatment and control group.

(ix) Total number of aberrations per group.

(x) Number of cells with aberrations per group.

(xi) Dose-response relationship, if applicable.

(6) References. For additional background information on this test guideline the following references should be consulted:


(5) [50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19080, May 20, 1987]
Micronuclei form under normal conditions. The assay is based on an increase in the frequency of micronucleated polychromatric erythrocytes in bone marrow of treated animals.

(b) Definition. Micronuclei are small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm.

(c) Reference substances. Not applicable.

(d) Test method—(1) Principle. (i) Animals are exposed to test substance by an appropriate route. They are sacrificed, the bone marrow extracted and smear preparations made and stained. Polychromatic erythrocytes are scored for micronuclei under the microscope. (ii) Micronuclei may also be detected in other test systems:
(A) Tissue culture.
(B) Plants.
(C) Blood smears.
(D) Fetal tissues.
(E) Meiotic cells.
(F) Hepatic cells.
(iii) The present guideline is based on the mammalian bone marrow assay.
(2) Description. The method employs bone marrow of laboratory mammals which are exposed to test substances.
(3) Animal selection—(i) Species and strain. Mice are recommended. However, any appropriate mammalian species may be used.
(ii) Age. Young adult animals shall be used.
(iii) Number and sex. At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group if several test times after treatment were included in the experimental schedule. The use of a single sex or a smaller number of animals should be justified.
(iv) Assignment to groups. Animals shall be randomized and assigned to treatment and control groups.
(4) Control groups—(i) Concurrent controls. Concurrent positive and negative (vehicle) controls shall be included in each assay.
(ii) Positive controls. A compound known to produce micronuclei in vivo shall be employed as the positive control.
(5) Test chemicals—(i) Vehicle. When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test compound nor produce toxic effects. Fresh preparations of the test compound should be employed.
(ii) Dose levels. For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity, e.g., a change in the ratio of polychromatic to normochromatic erythrocytes. Additional dose levels may be used. For determination of dose response, at least three dose levels shall be used.
(iii) Route of administration. The usual routes of administration are IP or oral. Other routes may be appropriate.
(iv) Treatment schedule. Test substances should generally be administered only once. However, based upon toxicological information a repeated treatment schedule may be employed.
(e) Test performance—(1) Treatment and sampling times. (i) Animals shall be treated with the test substance once at the highest tolerated dose. Sampling times should coincide with the maximum responses of the assay which varies with the test substance. Therefore, using the highest dose, bone marrow samples should be taken at least three times, starting not earlier than 12 hours after treatment, with appropriate intervals following the first sample but not extending beyond 72 hours. When other doses are used sampling shall be at the maximum sensitive period, or, if that is not known, approximately 24 hours after treatment. Other appropriate sampling times may be used in addition. If the most sensitive interval is known and documented with data, only this one time point need be sampled.
(ii) If a repeated treatment schedule is used, samples shall be taken at least
three times, starting not earlier than 12 hours after the last treatment and at appropriate intervals following the first sample, but not extending beyond 72 hours.

(iii) Bone marrow shall be obtained immediately after sacrifice. Cells shall be prepared, put on slides, spread as a smear and stained.

(2) Analysis. Slides shall be coded before microscopic analysis. At least 1,000 polychromatic erythrocytes per animal shall be scored for the incidence of micronuclei. The ratio of polychromatic to normochromatic erythrocytes should be determined for each animal by counting a total of 200 erythrocytes. To ensure consistency with OECD and other guidelines, 1,000 polychromatic erythrocytes are recommended. Additional information may be obtained by scoring normochromatic erythrocytes for micronuclei.

(f) Data and report—(1) Treatment of results. Criteria for scoring micronuclei shall be given. Individual data shall be presented in a tabular form including positive and negative (vehicle) controls and experimental groups. The number of polychromatic erythrocytes scored, the number of micronucleated cells, the number of micronucleated polychromatic erythrocytes, the percentage of micronucleated cells, the number of micronucleated normochromatic erythrocytes, and, if applicable, the percentage of micronucleated erythrocytes and the ratio of normochromatic to polychromatic erythrocytes shall be listed separately for each experimental and control animal. Absolute numbers shall be included if percentages are reported.

(2) Statistical evaluation. Data should be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(ii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) The results of the micronucleus test provide information on the ability of a chemical to induce micronuclei in polychromatic erythrocytes of the test species under the conditions of the test. This damage may have been the result of chromosomal damage or damage to the mitotic apparatus.

(ii) Negative results indicate that under the test conditions the test substance does not produce micronuclei in the bone marrow of the test species.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) Species, strain, age, weight, number and sex of animals in each treatment and control group.

(ii) Test chemical, vehicle, dose levels used, rationale for dose selection.

(iii) Rationale for and description of treatment and sampling schedules, toxicity data, negative and positive controls.

(iv) Details of the protocol used for slide preparation.

(v) Criteria for identifying micronucleated erythrocytes.

(vi) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline the following references should be consulted:


§ 798.5450 Rodent dominant lethal assay.

(a) Purpose. Dominant lethal (DL) effects cause embryonic or fetal death. Induction of a dominant lethal event after exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. Dominant lethals are generally accepted to be the result of chromosomal damage (structural and numerical anomalies) but gene mutations and toxic effects cannot be excluded.

(b) Definition. A dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo.

(c) Reference substances. These may include, but need not be limited to, triethylenemelamine, cyclophosphamide or ethyl methanesulfonate.

(d) Test method—(1) Principle. Generally, male animals are exposed to the test substance and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The females are sacrificed after an appropriate period of time and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos. The calculation of the dominant lethal effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the control group. Pre-implantation loss can be estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and control groups.

(2) Description. (i) Several treatment schedules are available. The most widely used requires single administration of the test substance. Other treatment schedules, such as treatment on five consecutive days, may be used if justified by the investigator.

(ii) Individual males are mated sequentially to virgin females at appropriate intervals. The number of matings following treatment is governed by the treatment schedule and should ensure that germ cell maturation is adequately covered. Females are sacrificed in the second half of pregnancy and the uterine contents examined to determine the total number of implants and the number of live and dead embryos.

(3) Animal selection—(i) Species. Rats or mice are generally used as the test species. Strains with low background dominant lethality, high pregnancy frequency and high implant numbers are recommended.

(ii) Age. Healthy, sexually mature animals shall be used.

(iii) Number. An adequate number of animals shall be used taking into account the spontaneous variation of the biological characteristics being evaluated. The number chosen should be based on the predetermined sensitivity of detection and power of significance. For example, in a typical experiment, the number of males in each group shall be sufficient to provide between 30 and 50 pregnant females per mating interval.
(iv) Assignment to groups. Animals shall be randomized and assigned to treatment and control groups.

(4) Control groups—(i) Concurrent controls. Generally concurrent positive and negative (vehicle) controls shall be included in each experiment. When acceptable positive control results are available from experiments conducted recently (within the last 12 months) in the same laboratory these results can be used instead of a concurrent positive control.

(ii) Positive controls. Positive control substances shall be used at a dose which demonstrates the test sensitivity.

(5) Test chemicals—(i) Vehicle. When possible, test substances shall be dissolved or suspended in isotonic saline or distilled water. Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

(ii) Dose levels. Normally, three dose levels shall be used. The highest dose shall produce signs of toxicity (e.g., slightly reduced fertility and slightly reduced body weight). However, in an initial assessment of dominant lethality a single high dose may be sufficient. Nontoxic substances shall be tested at 5g/kg or, if this is not practicable, then as the highest dose attainable.

(iii) Route of administration. The usual routes of administration are oral or by IP injection. Other routes may be appropriate.

(e) Test performance. (1) Individual males are mated sequentially at appropriate predetermined intervals to one or two virgin females. Females should be left with the males for at least the duration of one estrus cycle or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

(2) The number of matings following treatment should be governed by the treatment schedule and should ensure that germ cell maturation is adequately covered.

(3) Females should be sacrificed in the second half of pregnancy and uterine contents examined to determine the number of implants and live and dead embryos. The ovaries may be examined to determine the number of corpora lutea.

(f) Data and report—(1) Treatment of results. Data shall be tabulated to show the number of males, the number of pregnant females, and the number of nonpregnant females. Results of each mating, including the identity of each male and female, shall be reported individually. For each female, the dose level and week of mating and the frequencies of live implants and of dead implants shall be enumerated. If the data are recorded as early and late deaths, the tables shall make that clear. If preimplantation loss is estimated, it shall be reported. Preimplantation loss can be calculated as the difference between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per female in comparison with control matings.

(2) Statistical evaluation. Data shall be evaluated by appropriate statistical methods. Differences among animals within the control and treatment groups shall be considered before making comparisons between treated and control groups.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of dominant lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of dominant lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) A positive DL assay suggests that under the test conditions the test substance may be genotoxic in the germ cells of the treated sex of the test species.
(ii) A negative result suggests that under the conditions of the test the test substance may not be genotoxic in the germ cells of the treated sex of the test species.

(5) Test Report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) Species, strain, age and weights of animals used, number of animals of each sex in experimental and control groups.

(ii) Test substance, vehicle used, dose levels and rationale for dosage selection, negative (vehicle) and positive controls, experimental observations, including signs of toxicity.

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Methods used to determine that mating has occurred (where applicable).

(vi) Criteria for scoring dominant lethals including the number of early and late embryonic deaths.

(vii) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline the following references should be consulted:


§ 798.5460 Rodent heritable translocation assays.

(a)目的。本试验检测遗传物质损伤，这些损伤形成平衡或不平衡的连锁。
(2) Description. Essentially, two methods have been used to screen for translocation heterozygosity; one method uses a mating sequence to identify sterile and semisterile males followed by cytological examination of suspect male individuals; the other method deletes the mating sequence altogether and all F1 male progeny are examined cytologically for presence of translocation. In the former approach, the mating sequence serves as a screen which eliminates most fully fertile animals for cytological confirmation as translocation heterozygotes.

(3) Animal selection—(i) Species. The mouse is the species generally used, and is recommended.

(ii) Age. Healthy sexually mature animals shall be used.

(iii) Number. (A) The number of male animals necessary is determined by the following factors:

(1) The use of either historical or concurrent controls.

(2) The power of the test.

(3) The minimal rate of induction required.

(4) Whether positive controls are used.

(B) [Reserved]

(iv) Assignment to groups. Animals shall be randomized and assigned to treatment and control groups.

(4) Control groups—(i) Concurrent controls. No concurrent positive or negative (vehicle) controls are recommended as routine parts of the heritable translocation assay. However, investigators not experienced in performing translocation testing shall include a substance known to produce translocations in the assay as a positive control reference chemical.

(ii) Historical controls. At the present time, historical control data must be used in tests for significance. When statistically reliable historical controls are not available, negative (vehicle) controls shall be used.

(5) Test chemicals—(i) Vehicle. When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

(ii) Dose levels. At least two dose levels shall be used. The highest dose level shall result in toxic effects (which shall not produce an incidence of fatalities which would prevent a meaningful evaluation) or shall be the highest dose attainable or 5g/kg body weight.

(iii) Route of administration. Acceptable routes of administration include oral, inhalation, admixture with food or water, and IP or IV injection.

(e) Test performance—(1) Treatment and mating. The animals shall be dosed with the test substances 7 days per week over a period of 35 days. After treatment, each male shall be caged with 2 untreated females for a period of 1 week. At the end of 1 week, females shall be separated from males and caged individually. When females give birth, the day of birth, litter size, and sex of progeny shall be recorded. All male progeny should be weaned, and all female progeny should be discarded.

(2) Testing for translocation heterozygosity. When males are sexually mature, testing for translocation heterozygosity shall begin. One of two methods shall be used; the first method involves mating, determining those F1 progeny which are sterile or semisterile and subsequent cytological analysis of suspect progeny; the other method does not involve mating and determining sterility or semisterility; all progeny are examined cytologically.

(i) Determination of sterility or semisterility—(A) Conventional method. Females are mated, usually three females for each male, and each female is killed at midpregnancy. Living and dead implantations are counted. Criteria for determining normal and semisterile males are usually established for each new strain because the number of dead implantations varies considerably among strains.

(B) Sequential method. Males to be tested are caged individually with females and the majority of the presumably normal males are identified on the basis of a predetermined size of 1 or 2 litters. Breeding pens are examined daily on weekdays beginning 18 days
after pairing. Young are discarded immediately after they are scored. Males that sire a litter whose size is the same as or greater than the minimum set for a translocation-free condition are discarded with their litter. If the litter size is smaller than the predetermined number, a second litter is produced with the same rule applying. Males that cannot be classified as normal after production of a second litter are tested further by the conventional method or by cytological confirmation of translocation.

(ii) Cytological analysis. For cytological analysis of suspected semisteriles, the air-drying technique is used. Observation of at least 2 diakinesis-metaphase 1 cells with multivalent association constitutes the required evidence for the presence of a translocation. Sterile males are examined by one of two methods, those with testes of normal size and sperm in the epididymis are examined by the same techniques used for semisteriles. Animals with small testes are examined by squash preparations or, alternatively, by examination of mitotic metaphase preparations. If squash preparations do not yield diakinesis-metaphase 1 cells, analysis of spermatogonia or bone marrow for the presence of unusually long or short chromosomes should be performed.

(f) Data and report—(1) Treatment of results. (i) Data shall be presented in tabular form and shall include the number of animals at risk, the germ cell stage treated, the number of partial steriles and semisteriles (if the fertility test is used), the number of cytogenetically confirmed translocation heterozygotes (if the fertility test is used), report the number of confirmed steriles and confirmed partial steriles), the translocation rate, and either the standard error of the rate or the upper 95 percent confidence limit on the rate.

(ii) These data shall be presented for both treated and control groups. Historical or concurrent controls shall be specified, as well as the randomization procedure used for concurrent controls.

(2) Statistical evaluation. Data shall be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results in the heritable translocation assay indicate that under the test conditions the test substance causes heritable chromosomal damage in the test species.

(ii) Negative results indicate that under the test conditions the test substance does not cause heritable chromosomal damage in the test species.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) Species, strain, age, weight and number of animals of each sex in each group.

(ii) Test chemical vehicle, route and schedule of administration, toxicity data.

(iii) Dosing regimen, doses tested and rationale for dosage selection.

(iv) Mating schedule, number of females mated to each male.

(v) The use of historical or concurrent controls.

(vi) Screening procedure including the decision criteria used and the method by which they were determined.

(vii) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline the following references should be consulted:

(1) Generoso, W.M., Bishop, J.B., Goslee, D.G., Newell, G.W., Sheu, G.J., von Halle, E. “Heritable translocation
§ 798.5500 Differential growth inhibition of repair proficient and repair deficient bacteria: “Bacterial DNA damage or repair tests.”

(a) Purpose. Bacterial DNA damage or repair tests measure DNA damage which is expressed as differential cell killing or growth inhibition of repair deficient bacteria in a set of repair proficient and deficient strains. These tests do not measure mutagenic events per se. They are used as an indication of the interaction of a chemical with genetic material implying the potential for genotoxicity.

(b) Definition. Test for differential growth inhibition of repair proficient and repair deficient bacteria measure differences in chemically induced cell killing between wild-type strains with full repair capacity and mutant strains deficient in one or more of the enzymes which govern repair of damaged DNA.

(c) Reference substances. These may include, but need not be limited to, chloramphenicol or methyl methanesulfonate.

(d) Test method—(1) Principle. The tests detect agents that interact with cellular DNA to produce growth inhibition or killing. This interaction is recognized by specific cellular repair systems. The assays are based upon the use of paired bacterial strains that differ by the presence of absence of specific DNA repair genes. The response is expressed in the preferential inhibition of growth or the preferential killing of the DNA repair deficient strain since it is incapable of removing certain chemical lesions from its DNA.

(2) Description. Several methods for performing the test have been described. Those described here are:

(i) Tests performed on solid medium (diffusion tests).

(ii) Tests performed in liquid culture (suspension tests).

(3) Strain selection—(i) Designation. At the present time, Escherichia coli polA (W3110/p3478) or Bacillus subtilis rec (H17/M45) pairs are recommended. Other pairs may be utilized when appropriate.

(ii) Preparation and storage. Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

(4) Bacterial growth. Good microbiological techniques should be used to grow fresh cultures of bacteria. The phase of growth and cell density should be documented and should be adequate for the experimental design.

(5) Metabolic activation. Bacteria 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 postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents. The use of other species, tissues or techniques may also be appropriate.

(i) Concurrent controls. Concurrent positive, negative, and vehicle controls should be included in each assay.

(ii) Negative controls. The negative control should show nonpreferential growth inhibition (i.e., should affect both strains equally). Chloramphenicol is an example of a negative control.

(iii) Genotype specific controls. Examples of genotype specific positive controls are methyl methanesulfonate for polA strains and mitomycin C for rec strains.

(iv) Positive controls to ensure the efficacy of the activation system. The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test.

(v) Other positive controls. Other positive control reference substances may be used.

(7) Test chemicals—(i) Vehicle. Test chemicals and positive and negative control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) Exposure concentrations. The test should initially be performed over a broad range of concentrations. Among
the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. Because results are expressed as diameters of zones of growth inhibition in the diffusion test, it is most important that the amounts of chemical on the disc (or in the wells) are exact replicates. When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

(e) Test performance—(1) Diffusion assay—Disc diffusion assays may be performed in two ways:

(A) A single strain of bacteria may be added to an agar overlay or spread on the surface of the agar and the test chemical placed on a filter disc on the surface of the agar or;

(B) DNA repair proficient and DNA repair deficient bacteria may be streaked in a line on the surface of the agar of the same plate and a disc saturated with test chemical placed on the surface of the agar in contact with the streaks.

(ii) Well diffusion assays. In well diffusion assays, bacteria may be either added to the agar overlay or spread onto the surface of the agar. A solution of the test chemical is then placed into a well in the agar.

(2) Suspension assays. (i) A bacterial suspension may be exposed to the test chemical and the number of surviving bacteria determined (as colony-forming units) either as a function of time of treatment or as a function of the concentration of test agent.

(ii) Nonturbid suspensions of bacteria may be exposed to serial dilutions of the test chemical and a minimal inhibitory concentration for each strain determined, as evidenced by the presence or absence of visible growth after a period of incubation.

(iii) Paired bacterial suspensions (usually with some initial turbidity) may be treated with a single dose of the chemical. Positive results are indicated by a differential inhibition in the rate of increase of turbidity of the paired cultures.

(3) Number of cultures. When using a plate diffusion procedure, at least two independent plates should be used at each dilution. In liquid suspension assays, at least two independent specimens for determination of the number of viable cells should be plated.

(4) Incubation conditions. All plates in a given test should be incubated for the same time period. This incubation period should be for 18 to 24 hrs at 37 °C.

(f) Data and report—(1) Treatment of results—Diffusion assays. Results should be expressed in diameters of zones of growth inhibition in millimeters or as areas derived therefrom as mm². Dose-response data, if available, should be presented using the same units.

(ii) Liquid suspension assays. (A) Survival data can be presented as dose responses, preferably as percentage of survivors or fractional survival of each strain or as a relative survival (ratio) of the two strains.

(B) Results can also be expressed as the concentrations required to effect a predetermined survival rate (e.g., D₀₇₀, the dose permitting 37 percent survival). These data are derived from the survival curve. The concentration should be expressed as weight per volume, as moles, or as molarity.

(C) Similarly, results can be expressed as minimal inhibitory concentration or as minimal lethal dose. The former is determined by the absence of visible growth in liquid medium and the latter is determined by plating dilutions onto semisolid media.

(iii) In all tests, concentrations must be given as the final concentrations during the treatment. Raw data, prior to transformation, should be provided. These should include actual quantities measured, e.g., neat numbers. For measurement of diffusion, the diameters of the discs and/or well should be indicated and the measurements should indicate whether the diameter of the discs and/or well was subtracted. Moreover, mention should be made as to whether the test chemical gave a sharp, diffuse, or double-zone of growth.
inhibition. If it is the latter, the investigator should indicate whether the inner or the outer zone was measured.

(iv) Viability data should be given as the actual plate counts with an indication of the dilution used and the volume plated or as derived titers (cells per ml). Transformed data alone in the absence of experimental data are not acceptable (i.e., ratios, differences, survival fraction).

(2) Statistical evaluation. Data should be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related preferential inhibition or killing of the repair deficient strain. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related preferential inhibition or killing of the repair deficient strain or a statistically significant and reproducible positive response at any one of the test points is considered not to interact with the genetic material of the organisms used in assay.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. DNA damage tests in bacteria do not measure DNA repair per se nor do they measure mutations. They measure DNA damage which is expressed as cell killing or growth inhibition. A positive result in a DNA damage test in the absence of a positive result in another system is difficult to evaluate in the absence of a better data base.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792 subpart J the following specific information should be reported:

(i) Bacterial strains used.

(ii) Phase of bacterial cell growth at time of use in the assay.

(iii) Media composition.

(iv) Details of both the protocol used to prepare the metabolic activation system and its use in the assay.

(v) Treatment protocol, including doses used and rationale for dose selection, positive and negative controls.

(vi) Method used for determination of degree of cell kill.

(vii) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline the following references should be consulted:


(2) Kada, T., Sadie, Y., Tukikawa, K. "In vitro and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected," Mutation Research, 16:165-174 (1972).


§ 798.5955 Heritable translocation test in drosophila melanogaster.

(a) Purpose. The heritable translocation test in Drosophila measures the induction of chromosomal translocations in germ cells of insects. Stocks carrying genetic markers on two or more chromosomes are used to follow the assortment of chromosomes in meiosis. The F₁ male progeny of treated parents are individually mated to females and the F₂ progeny phenotypes are scored. The observed spectrum of phenotypes is used to determine the presence or absence of a translocation. This is usually indicated by a lack of independent assortment of genes on different chromosomes.

(b) Definitions—(1) Chromosome mutations are chromosomal changes resulting from breakage and reunion of chromosomes. Chromosomal mutations
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are also produced through nondisjunction of chromosomes during cell division.

(2) Reciprocal translocations are chromosomal translocations resulting from reciprocal exchanges between two or more chromosomes.

(3) Heritable translocations are reciprocal translocations transmitted from parent to the succeeding progeny.

(c) Reference substances. These may include, but need not be limited to, ethyl methanesulfonate or N-dimethylnitrosamine.

(d) Test method—(1) Principle. The method is based on the principle that balanced reciprocal chromosomal translocations can be induced by chemicals in the germ cells of treated flies and that these translocations are detected in the F_2 progeny using genetic markers (mutations). Different mutations may be used as genetic markers and two or more of the four chromosomes may be genetically marked for inclusion in this test.

(2) Description. Wild-type males are treated with chemical and bred with females of known genetic markers. The F_1 males are collected and individually bred with virgin females of the female parental stock. The resulting F_2 progeny are scored. Putative translocation carriers are confirmed with an F_3 cross.

(i) Illustrative example. The following example serves to illustrate the method. Males carrying genes for red eye color on chromosomes II and III are bred with females of white eye color carrying alleles for brown (bw) on the second chromosome and scarlet (st) and pink (pp) on the third chromosome. The F_1 male progeny are bred with virgin females of the female parental stock and the resulting F_2 progeny are examined for eye color phenotypes. If there is no translocation in the F_1 male, then the resulting F_2 progeny will have four eye color phenotypes: red, white, orange, and brown. If the F_1 male carries a translocation between chromosomes II and III, only red and white eye phenotypes are obtained in the F_2 generation. This happens because the F_1 translocation heterozygote produces two balanced (carrying either the parental or the translocated configuration of markers) and two unbalanced gametes. The unbalanced gametes (carrying one normal and one translocated chromosome) are unable to develop into normal individuals in the F_3 generation.

(ii) [Reserved]

(3) Drosophila stocks. Wild-type males and females of the genotype bw:st:pp (white eyes) may be used in the heritable translocation test. Other appropriately marked Drosophila stocks may also be used.

(e) Test performance—(1) P_1 mating. (i) In the primary screen of a chemical, it is enough to sample one germ cell stage, either mature sperm or spermatids (for indirect acting mutagens). Other stages may be sampled if needed, i.e., when mature germ cells give a positive result and data from earlier germ cells are needed for the purpose of risk assessment. Thus,
(21) Environmental Protection Agency § 798.5955

the treated males may be mated only once for a period of 3 days to sample sperm or transferred every 2 to 3 days to cover the entire germ cell cycle.

(ii) Mass matings may be performed because the control rate for translocations in the available literature is very low (near 0) and clustered events are extremely rare. Mated females may be aged for 2 weeks in order to recover an enhanced incidence of translocation due to the storage effect. The females are then allowed to lay eggs and F₁ males are collected for test mating.

(2) F₁ mating. F₁ males should be bred with virgin females of the parental female stock. Since each F₁ male represents one treated gamete of the male parent, the F₁ males have to be mated individually to virgin females. Each F₁ male should be mated to three females to ensure sufficient progeny.

(3) Scoring the F₂ generation. F₂ cultures (each representing 1 F₁ male tested) should be scored for the presence or absence of phenotype variations (linkage of markers) from the expected types. The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these defined parameters. The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analyzed to detect substances which show mutation rates close to those of the controls. A positive test should be confirmed by F₃ mating trials.

(4) Number of replicate experiments. Replicate experiments are usually performed for each dose of the compound tested. If a chemical is a potent inducer of translocations, one experiment may be sufficient. Otherwise two or three replicate experiments should be done.

(f) Data and report—(1) Treatment of results. Data should be tabulated to show the number of translocations and the number of fertile F₁ males at each exposure for each germ cell stage sampled.

(2) Statistical evaluation. Data should be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) Test evaluation. (i) Positive results in the heritable translocation test in Drosophila indicate that under the test conditions the test substance causes chromosome damage in germ cells of this insect.

(ii) Negative results indicate that under the test conditions the test substance does not cause chromosomal damage in D. melanogaster.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

(i) Drosophila stock used in the assay, age of insects, number of males treated, number of F₂ cultures established, number of replicate experiments.

(ii) Test chemical vehicle, treatment and mating schedule, exposure levels, toxicity data, dose and route of exposure.

(iii) Positive and negative (vehicle) controls.

(iv) Historical control data, if available.

(v) Number of chromosomes scored.

(vi) Criteria for scoring mutant chromosomes.

(vii) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline the following references should be consulted:
§ 798.6050

(a) Purpose. In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for neurotoxic effects. Substances that have been observed to cause neurotoxic signs (e.g., convulsions, tremors, ataxia) in other toxicity tests, as well as those having a structural similarity to known neurotoxicants, should be evaluated for neurotoxicity. The functional observational battery is a noninvasive procedure designed to detect gross functional deficits in young adults resulting from exposure to chemicals and to better quantify neurotoxic effects detected in other studies. This battery of tests is not intended to provide a detailed evaluation of neurotoxicity. It is designed to be used in conjunction with neuropathologic evaluation and/or general toxicity testing. Additional functional tests may be necessary to assess completely the neurotoxic potential of a chemical.

(b) Definitions. (1) Neurotoxicity is any adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

(2) A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) Principle of the test method. The material is administered by an appropriate route to laboratory rodents. The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period.

(d) Test procedures—(1) Animal selection—(i) Species and strain. The laboratory rat or mouse is recommended. Although information will generally be lacking, whenever possible the choice of species should take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies. The potential for combined studies should also be considered. Standard strains should be used.

(ii) Age. Young adult animals (at least 42 days old for the rat or mouse) shall be used.

(iii) Sex. (A) Equal numbers of animals of each sex are required for each dose level.

(B) The females shall be nulliparous and nonpregnant.

(2) Number of animals. At least eight animals of each sex should be used at each dose level and should be designated for behavioral testing. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the end of the study. Animals shall be randomly assigned to treatment and control groups.

(3) Control groups. (i) A concurrent (“sham” exposure or vehicle) control group is required. Subjects shall be treated in the same way as for an exposure group except that administration of the test substance is omitted.

(ii) Concurrent or historic data from the laboratory performing the testing shall provide evidence of the ability of the procedures used to detect major neurotoxic endpoints such as limb weakness or paralysis (e.g., acrylamide), CNS stimulation (e.g., β, β′-iminodipropionitrile) autonomic signs (e.g., physostigmine).

(iii) A satellite group may be treated with the high dose level for the duration of exposure and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate duration, normally not less than 28 days.

(4) Dose levels and dose selection. At least 3 doses, equally spaced on a log scale (e.g., ½ log units) over a range of