Environmental Protection Agency

§ 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—(1) Designation. At the present time, Escherichia coli polA (W3110/pS478) 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.

(6) Control groups—(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—(i) Disc diffusion assays. 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 agent 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—(i) Diffusion assays. Results should be expressed in diameters of zones of growth inhibition in millimeters or as areas derived therefrom as
(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., Tutikawa, K. “In vitro and host-mediated “rec-
§ 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 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-dimethyl-nitrosamine.

(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 F₂ 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 F₁ males are collected and individually bred with virgin females of the female parental stock. The resulting F₂ progeny are scored. Putative translocation carriers are confirmed with an F₃ 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 F₁ male progeny are bred with virgin females of the female parental stock and the resulting F₂ progeny are examined for eye color phenotypes. If there is no translocation in the F₁ male, then the resulting F₂ progeny will have four eye color phenotypes: red, white, orange, and brown. If the F₁ male carries a translocation between chromosomes II and III, only red and white eye phenotypes are obtained in the F₂ generation. This happens because the F₁ 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 F₂ 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.

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

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