(2) *Evaluation of Results.* (i) Results shall be evaluated in terms of the extent of change in the amount of GFAP as a function of treatment and dose. GFAP assays (of any brain region) from a minimum of 6 samples typically will result in a standard error of the mean of ±5 percent. In this case, a chemically-induced increase in GFAP of 115 percent of control is likely to be statistically significant.

(ii) The results of this assay shall be compared to and evaluated with any relevant behavioral and histopathological data.

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

(1) Brock, T.O and O'Callaghan, J.P. 1987. Quantitative changes in the synaptic vesicle proteins, synapsin I and p38 and the astrocyte specific protein, glial fibrillary acidic protein, are associated with chemical-induced injury to the rat central nervous system, J. Neurosci. 7:931–942.

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(3) O'Callaghan, J.P. 1988. Neurotypic and gliotypic protein as biochemical markers of neurotoxicity. Neurotoxicol. Teratol. 10:445–452.

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(7) Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Annal. Biochem. 150:76–85.

§ 79.68 *Salmonella typhimurium* reverse mutation assay.

(a) *Purpose.* The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay which measures $his^- \rightarrow his^+$ reversion induced by chemicals which cause base changes or frameshift mutations in the genome of the microorganism *Salmonella typhimurium.*

(b) *Definitions.* For the purposes of this section, the following definitions apply:

Base pair mutagen means an agent which causes a base change in DNA. In a reversion assay, this change may occur at the site of the original mutation or at a second site in the chromosome.

Frameshift mutagen is an agent which causes the addition or deletion of single or multiple base pairs in the DNA molecule.

Salmonella typhimurium reverse mutation assay detects mutation in a gene of a histidine-requiring strain to produce a histidine independent strain of this organism.

(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.* Motor vehicle combustion emissions from fuel or additive/base fuel mixtures are, first, filtered to trap particulate matter and, then, passed through a sorbent resin to trap semi-volatile gases. Bacteria are separately exposed to the extract from both the filtered particulates and the resin-trapped organics. Assays are conducted using both test mixtures with and without a metabolic activation system and exposed cells are plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted in test cultures and compared to the number of spontaneous revertants in unexposed control cultures.

(2) *Description.* Several methods for performing the test have been described. The procedures described here are for the direct plate incorporation

method and the azo-reduction method. Among those used are:

(i) Direct plate incorporation method;

(ii) Preincubation method;

(iii) Azo-reduction method;

(iv) Microsuspension method; and

(v) Spiral assay.

(3) *Strain selection*—(i) *Designation.* Five tester strains shall be used in the assay. At the present time, TA1535, TA1537, TA98, and TA100 are designated as tester strains. The fifth strain will be chosen from the pool of Salmonella strains commonly used to determine the degree to which nitrated organic compounds, *i.e.,* nitroarenes, contribute to the overall mutagenic activity of a test substance. TA98/1,8–DNP$_6$ or other suitable Rosenkranz nitro-reductase resistant strains will be considered acceptable. The choice of the particular strain is left to the discretion of the researcher. However, the researcher shall justify the use of the selected bacterial tester strains.

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

(iii) *Bacterial growth.* Fresh cultures of bacteria shall be grown up to the late exponential or early stationary phase of growth (approximately 108–109 cells per ml).

(4) *Exogenous metabolic activation.* Bacteria shall be exposed to the test substance both in the presence and absence of an appropriate exogenous 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 (S-9) prepared from the livers of untreated hamsters is preferred. For this method, the cofactor supplement shall 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) 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 shall be included in the assay. Examples of strain specific positive controls are as follows:

(A) Strain TA1535, TA100: sodium azide;

(B) TA98: 2-nitrofluorene (without activation), 2-anthramine (with activation);

(C) TA1537: 9-aminoacridine; and

(D) TA98/1,8–DNP$_6$: benzo(a)pyrene (with activation).

The papers by Claxton *et al.,* 1991 and 1992 in paragraph (g) in this section will provide helpful information for the selection of positive controls.

(iii) *Positive controls to ensure the efficacy of the activation system.* The positive control reference substances for tests including a metabolic activation system shall 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 shall include positive controls from the same class of compounds as the test agent wherever possible.

(6) *Sampling the test atmosphere.*(i) Extracts of test emissions are collected on Teflon®-coated glass fiber filters using an exhaust dilution setup. The particulates are extracted with dichloromethane (DCM) using Soxhlet extraction techniques. Extracts in DCM can be stored at dry ice temperatures until use.

(ii) Gaseous hydrocarbons passing through the filter are trapped by a porous, polymer resin, like XAD–2/styrene-divinylbenzene, or an equivalent product. Methylene chloride is used to extract the resin and the sample is evaporated to dryness before storage or use.

(iii) Samples taken from this material are then used to expose the cells in this assay. Final concentration of extracts in solvent/vehicle, or after solvent exchange, shall not interfere with cell viability or growth rate. The paper by Stump (1982) in paragraph (g) of this section is useful for preparing extracts of particulate and semi-volatile organic compounds from diesel and gasoline exhaust stream.

(iv) 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 substance 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 samples shall be tested up to the limits of solubility. The upper test chemical concentration shall 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 concentrations of test substance shall be used 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.* All data developed within this study shall be in accordance with good laboratory practice provisions under § 79.60.

(1) Direct plate incorporation method. When testing with metabolic activation, test solution, bacteria, and 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction shall be added to the liquid overlay agar and mixed. This mixture is 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. When testing without metabolic activation, the test sample and 0.1 ml of a fresh bacterial culture shall be added to 2.0 ml of overlay agar.

(2) Azo-reduction method. When testing with metabolic activation, 0.5 ml of activation mixture containing 150 µl of postmitochondrial fraction and 0.1 ml of bacterial culture shall be added to a test tube kept on ice. 0.1 ml of test solution shall be added, and the tubes shall be incubated with shaking at 30 °C for 30 minutes. At the end of the incubation period, 2.0 ml of agar shall 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. For tests without metabolic activation, 0.5 ml of buffer shall be used in place of the 0.5 ml of activation mixture. All other procedures shall be the same as those used for the test with metabolic activation.

(3) Other methods/modifications 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 shall 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, revertants per kilogram (or liter) of fuel, and as revertants per kilometer (or mile, or brake-horsepower/hour, as appropriate) for each replicate and dose. These same measures shall be recorded on both the negative and positive control plates. The mean number of revertant colonies per plate, revertants per kilogram (or liter) of fuel, and revertants per kilometer (or mile, or brake-horsepower/hour), as well as individual plate counts and standard deviations shall be presented

for the test substance, positive control, and negative control plates.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods. Those methods shall include, at a minimum, means and standard deviations of the reversion data.

(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 shall be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results from the *Salmonella 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 *Salmonella typhimurium.*

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR 79.60, the following specific information shall be reported:

(i) Sampling method(s) used and manner in which cells are exposed to sample solution;

(ii) Bacterial strains used;

(iii) Metabolic activation system used (source, amount and cofactor); details of preparation of postmitochondrial fraction;

(iv) Concentration levels and rationale for selection of concentration range;

(v) Description of positive and negative controls, and concentrations used, if appropriate;

(vi) Individual plate counts, mean number of revertant colonies per plate, number of revertants per kilometer (or mile, or brake-horsepower/hour), and standard deviation; and

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

(1) 40 CFR 798.5265, The *Salmonella typhimurium* reverse mutation asay.

(2) Ames, B.N., McCann, J., Yamasaki, E. ''Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test,'' Mutation Research 31:347–364 (1975).

(3) Huisingh, J.L., et al.,''Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: Study Design, Sample Generation, Collection, and Preparation''. In: Health Effects of Diesel Engine Emissions, Vol. II, W.E. Pepelko, R., M., Danner and N. A. Clarke (Eds.), US EPA, Cincinnati, EPA–600/9–80–057b, pp. 788–800 (1980).

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(7) Claxton, L., Houk, V.S., Monteith, L.G., Myers, L.E., Hughes, T.J., ''Assessing the use of known mutagens to calibrate the *Salmonella typhimurium* mutagenicity assay: I. Without exogenous activation.'' Mutation Research 253:137–147 (1991).

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H., Nylund, L., Tokiwa, H., Vink, I.G.R., Wang, Y., Warshawsky, D., ''Results of the IPCS Collaborative Study on Complex Mixtures'' Mutation Research 276:23–32 (1992).

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(13) Maron, D., and Ames, B. N., Revised methods for the Salmonella mutagenicity test, Mutation Research, 113:173–212 (1983).

(14) Prival, M.J., and Mitchell, V.D. ''Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S-9,'' Mutation Research 97:103–116 (1982).

(15) Rosenkranz, H.S., et.al. ''Nitropyrenes: Isolation, identification, and reduction of mutagenic impurities in carbon black and toners'' Science 209:1039–43 (1980).

(16) Stump, F., Snow, R., et.al., ''Trapping gaseous hydrocarbons for mutagenic testing'' SAE Technical Paper Series, No. 820776 (1982).

(17) Vogel, H.J., Bonner, D.M. ''Acetylornithinase of E. coli: partial purification and some properties,'' Journal of Biological Chemistry. 218:97–106 (1956).

[59 FR 33093, June 27, 1994, as amended at 61 FR 36513, July 11, 1996]

PART 80—REGULATION OF FUELS AND FUEL ADDITIVES

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