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[65 FR 78802, Dec. 15, 2000]

§ 799.9510 TSCA bacterial reverse mutation test.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA.

(1) The bacterial reverse mutation test uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain.

(2) Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumor suppressor genes of somatic cells are involved in tumor formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of

mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds.

(b) *Source.* The source material used in developing this TSCA test guideline are the OECD replacement guidelines for 471 and 472 (February 1997). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section:

A reverse mutation test in either *Salmonella typhimurium* or *Escherichia coli* detects mutation in an amino-acid requiring strain (histidine or tryptophan, respectively) to produce a strain independent of an outside supply of amino-acid.

Base pair substitution mutagens are agents that cause a base change in DNA. In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome.

Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA

(d) *Initial considerations.* (1) The bacterial reverse mutation test utilizes prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals.

(2) The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests. There are examples of mutagenic agents which are not detected by this

test; reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. On the other hand, factors which enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity.

(3) The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g. certain antibiotics) and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate.

(4) Although many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute. It is dependent on chemical class and there are carcinogens that are not detected by this test because they act through other, non-genotoxic mechanisms or mechanisms absent in bacterial cells.

(e) *Test method*—(1) *Principle*. (i) Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after 2 or 3 days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

(ii) Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method, the preincubation method, the fluctuation method, and the suspension method. Suggestions for modifications for the testing of gases or vapors are described in the reference in paragraph (g)(12) of this section.

(iii) The procedures described in this section pertain primarily to the plate

incorporation and preincubation methods. Either of them is acceptable for conducting experiments both with and without metabolic activation. Some compounds may be detected more efficiently using the preincubation method. These compounds belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo compounds, pyrrolizidine alkaloids, allyl compounds and nitro compounds. It is also recognized that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as “special cases” and it is strongly recommended that alternative procedures should be used for their detection. The following “special cases” could be identified (together with examples of procedures that could be used for their detection): azo-dyes and diazo compounds (alternative procedures are described in the references in paragraphs (g)(3), (g)(5), (g)(6), and (g)(13) of this section), gases and volatile chemicals (alternative procedures are described in the references in paragraphs (g)(12), (g)(14), (g)(15), and (g)(16) of this section), and glycosides (alternative procedures are described in the references in paragraphs (g)(17) and (g)(18) of this section). A deviation from the standard procedure needs to be scientifically justified.

(2) *Description*—(i) *Preparations*—(A) *Bacteria*. (1) Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^9 cells per ml). Cultures in late stationary phase should not be used. The cultures used in the experiment shall contain a high titre of viable bacteria. The titre may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

(2) The culture temperature shall be 37 °C.

(3) At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98; and

TA100) that have been shown to be reliable and reproducibly responsive between laboratories. These four *S. typhimurium* strains have GC base pairs at the primary reversion site and it is known that they may not detect certain oxidizing mutagens, cross-linking agents, and hydrazines. Such substances may be detected by *E. coli* WP2 strains or *S. typhimurium* TA102 (see reference in paragraph (g)(19) of this section) which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

- (i) *S. typhimurium* TA1535.
- (ii) *S. typhimurium* TA1537 or TA97 or TA97a.
- (iii) *S. typhimurium* TA98.
- (iv) *S. typhimurium* TA100.
- (v) *E. coli* WP2 *uvrA*, or *E. coli* WP2 *uvrA* (pKM101), or *S. typhimurium* TA102. In order to detect cross-linking mutagens it may be preferable to include TA102 or to add a DNA repair-proficient strain of *E. coli* [e.g. *E. coli* WP2 or *E. coli* WP2 (pKM101).]

(4) Established procedures for stock culture preparation, marker verification and storage should be used. The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains, and tryptophan for *E. coli* strains). Other phenotypic characteristics should be similarly checked, namely: the presence or absence of R-factor plasmids where appropriate [i.e. ampicillin resistance in strains TA98, TA100 and TA97a or TA97, WP2 *uvrA* and WP2 *uvrA* (pKM101), and ampicillin = tetracycline resistance in strain TA102]; the presence of characteristic mutations (i.e. *rfa* mutation in *S. typhimurium* through sensitivity to crystal violet, and *uvrA* mutation in *E. coli* or *uvrB* mutation in *S. typhimurium*, through sensitivity to ultra-violet light). The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

(B) *Medium*. An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and

biotin or tryptophan, to allow for a few cell divisions, shall be used. The procedures described in the references under paragraphs (g)(1), (g)(2), and (g)(9) of this section may be used for this analysis.

(C) *Metabolic activation*. Bacteria shall be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (the system described in the references under paragraphs (g)(1) and (g)(2) of this section may be used) or a combination of phenobarbitone and β -naphthoflavone (the system described in the references under paragraphs (g)(18), (g)(20), and (g)(21) of this section may be used). The post-mitochondrial fraction is usually used at concentrations in the range from 5 to 30% v/v in the S9-mix. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. For azo-dyes and diazo-compounds, using a reductive metabolic activation system may be more appropriate (the system described in the references under paragraphs (g)(6) and (g)(13) of this section may be used).

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

(ii) *Test conditions*—(A) *Solvent/vehicle*. The solvent/vehicle should not be suspected of chemical reaction with the test substance and shall be compatible with the survival of the bacteria and the S9 activity (for further information see the reference in paragraph (g)(22) of this section). If other than well-known

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solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used be free of water.

(B) *Exposure concentrations.* (1) Amongst the criteria to be taken into consideration when determining the highest amount of test substance to be used are cytotoxicity and solubility in the final treatment mixture. It may be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity may be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a substance may be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye. The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate or 5 µl/plate. For non-cytotoxic substances that are not soluble at 5 mg/plate or 5 µl/plate, one or more concentrations tested should be insoluble in the final treatment mixture. Test substances that are cytotoxic already below 5mg/plate or 5 µl/plate should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

(2) At least five different analyzable concentrations of the test substance shall be used with approximately half log (i.e. √10) intervals between test points for an initial experiment. Smaller intervals may be appropriate when a

concentration-response is being investigated.

(3) Testing above the concentration of 5 mg/plate or 5 µl/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities.

(C) *Controls.* (1) Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and without metabolic activation, shall be included in each assay. Positive control concentrations that demonstrate the effective performance of each assay should be selected.

(2)(i) For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used. The following chemicals are examples of suitable positive controls for assays with metabolic activation:

Chemical	CAS No.
9,10-Dimethylanthracene	[CAS no. 781-43-1]
7,12-Dimethylbenzanthracene	[CAS no. 57-97-6]
Congo Red (for the reductive metabolic activation method).	[CAS no. 573-58-0]
Benzo(a)pyrene	[CAS no. 50-32-8]
Cyclophosphamide (monohydrate)	[CAS no. 50-18-0]
	[CAS no. 6055-19-2]
2-Aminoanthracene	[CAS no. 613-13-8]

(ii) 2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. If 2-aminoanthracene is used, each batch of S9 should also be characterized with a mutagen that requires metabolic activation by microsomal enzymes, e.g., benzo(a)pyrene, dimethylbenzanthracene.

(3) For assays performed without metabolic activation system, examples of strain-specific positive controls are:

Chemical	CAS No.	Strain
(a) Sodium azide	[CAS no. 26628-22-8]	TA1535 and TA100
(b) 2-Nitrofluorene	[CAS no. 607-57-8]	TA 98
(c) 9-Aminoacridine or ICR 191	[CAS no. 90-45-9] or	TA1537, TA97 and TA97a
	[CAS no. 17070-45-0]	
(d) Cumene hydroperoxide	[CAS no. 80-15-9]	TA102
(e) Mitomycin C	[CAS no. 50-07-7]	WP2 <i>uvrA</i> and TA102
(f) N-Ethyl-N-nitro-N-nitrosoguanidine or	[CAS no. 70-25-7] or	WP2, WP2 <i>uvrA</i> and WP2 <i>uvrA</i>
4-nitroquinoline 1-oxide	[CAS no. 56-57-5]	(pKM101)
(g) Furfurylamide (AF-2)	[CAS no. 3688-53-7]	Plasmid-containing strains

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

(5) Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, shall be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

(3) *Procedure*—(i) *Treatment with test substance*. (A) For the plate incorporation method, without metabolic activation, usually 0.05 ml or 0.1 ml of the test solutions, 0.1 ml of fresh bacterial culture (containing approximately 10^8 viable cells) and 0.5 ml of sterile buffer are mixed with 2.0 ml of overlay agar. For the assay with metabolic activation, usually 0.5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 5 to 30% v/v in the metabolic activation mixture) are mixed with the overlay agar (2.0 ml), together with the bacteria and test substance/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

(B) For the preincubation method the test substance/test solution is preincubated with the test strain (containing approximately 10^8 viable cells) and sterile buffer or the metabolic activation system (0.5 ml) usually for 20 min. or more at 30–37 °C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Usually, 0.05 or 0.1 ml of test substance/test solution, 0.1 ml of bacteria, and 0.5 ml of S9-mix or sterile buffer, are mixed with 2.0 ml of overlay agar. Tubes should be aerated during preincubation by using a shaker.

(C) For an adequate estimate of variation, triplicate plating should be used at each dose level. The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

(D) Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels (methods described in the references under paragraphs (g)(12), (g)(14), (g)(15), and (g)(16) of this section may be used).

(ii) *Incubation*. All plates in a given assay shall be incubated at 37 °C for 48–72 hrs. After the incubation period, the number of revertant colonies per plate is counted.

(f) *Data and reporting*—(1) *Treatment of results*. (i) Data shall be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control, and untreated control if used) and positive control plates shall also be given.

(ii) Individual plate counts, the mean number of revertant colonies per plate and the standard deviation shall be presented for the test substance and positive and negative (untreated and/or solvent) controls.

(iii) There is no requirement for verification of a clear positive response. Equivocal results shall be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing, the method of treatment (plate incorporation or liquid preincubation), and metabolic activation conditions.

(2) *Evaluation and interpretation of results*. (i) There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response.

(ii) A test substance for which the results do not meet the criteria described under paragraph (f)(2)(i) of this section is considered non-mutagenic in this test

(iii) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

(iv) Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

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

(i) Test substance:

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

(B) Physical nature and purity.

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

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

(ii) Solvent/vehicle:

(A) Justification for choice of solvent/vehicle.

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

(iii) Strains:

(A) Strains used.

(B) Number of cells per culture.

(C) Strain characteristics.

(iv) Test conditions:

(A) Amount of test substance per plate (mg/plate or ml/plate) with rationale for selection of dose and number of plates per concentration.

(B) Media used.

(C) Type and composition of metabolic activation system, including acceptability criteria.

(D) Treatment procedures.

(v) Results:

(A) Signs of toxicity.

(B) Signs of precipitation.

(C) Individual plate counts.

(D) The mean number of revertant colonies per plate and standard deviation.

(E) Dose-response relationship, where possible.

(F) Statistical analyses, if any.

(G) Concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.

(H) Historical negative (solvent/vehicle) and positive control data, with e.g. ranges, means and standard deviations.

(vi) Discussion of the results.

(vii) Conclusion.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Non-confidential Information Center, Rm. NE-B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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[62 FR 43824, Aug. 15, 1997, as amended at 64 FR 35079, June 30, 1999]

§ 799.9530 TSCA *in vitro* mammalian cell gene mutation test.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA. The *in vitro* mammalian cell gene mutation test can be used to detect gene mutations induced by chemical substances. Suitable cell lines include L5178Y mouse lymphoma cells, the CHO, AS52 and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells under paragraph (g)(1) of this section. In these cell lines the most commonly-used genetic endpoints measure mutation at thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), and a transgene of xanthine-guanine phosphoribosyl transferase (XPRT). The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK and XPRT may allow the detection of genetic events (e.g. large deletions) not detected at the HPRT locus on X-chromosomes (For a discussion see the references in paragraphs (g)(2), (g)(3), (g)(4), (g)(5), and (g)(6) of this section).

(b) *Source.* The source material used in developing this TSCA test guideline is the OECD guideline 476 (February 1997). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section:

Base pair substitution mutagens are substances which cause substitution of one or several base pairs in the DNA.

Forward mutation is a gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity or the function of the encoded protein.

Frameshift mutagens are substances which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Mutant frequency is the number of mutant cells observed divided by the number of viable cells.

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

Relative suspension growth is an increase in cell number over the expression period relative to the negative control.

Relative total growth is an increase in cell number over time compared to a control population of cells; calculated as the product of suspension growth relative to the negative control times cloning efficiency relative to negative control.

Survival is the cloning efficiency of the treated cells when plated at the end of the treatment period; survival is usually expressed in relation to the survival of the control cell population.

Viability is the cloning efficiency of the treated cells at the time of plating in selective conditions after the expression period.

(d) *Initial considerations.* (1) In the *in vitro* mammalian cell gene mutation test, cultures of established cell lines or cell strains can be used. The cells used are selected on the basis of growth ability in culture and stability of the spontaneous mutation frequency. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot entirely mimic the mammalian *in vivo* conditions. Care should be taken to avoid conditions which would lead to results not reflecting intrinsic mutagenicity. Positive results which do not reflect intrinsic mutagenicity may arise from changes in pH, osmolality or high levels of cytotoxicity.

(2) This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through other, non-genotoxic mechanisms or mechanisms absent in bacterial cells.

(e) *Test method*—(1) *Principle.* (i) Cells deficient in thymidine kinase (TK) due