

U.S. Food and Drug Administration
Protecting and Promoting *Your* Health

Redbook 2000: IV.C.1.a Bacterial Reverse Mutation Test

Toxicological Principles for the Safety Assessment of Food Ingredients

Redbook 2000

Chapter IV.C.1.a. Bacterial Reverse Mutation Test

Return to [Redbook 2000 table of contents \(/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm2006826.htm#TOC\)](http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm2006826.htm#TOC)

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I. Purpose

- A. The bacterial reverse mutation test uses amino acid-requiring strains of *Salmonella typhimurium* (*S. typhimurium*) and *Escherichia coli* (*E. coli*) to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs.⁽³⁾⁽⁹⁾⁽¹⁶⁾ The principle of this bacterial reverse mutation test is that it detects chemicals that induce mutations which revert mutations present in the tester 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 tester strain.
- B. 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 tester 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 tester 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 chemical structures is available for bacterial reverse mutation tests and well-established procedures have been developed for testing chemicals with different physicochemical properties, including volatile compounds.

II. Definitions

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 whose growth is independent of an outside supply of the amino acid.

Point mutations are changes in one or a small number of base pairs in a DNA sequence. Point mutations may result from base pair substitutions or from small insertions or deletions.

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.

III. Initial Considerations

- A. 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.
- B. 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 are genotoxic in other tests. There are examples of mutagenic agents which are not detected by this test; reasons for this shortcoming can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability.
- C. There are circumstances in which the bacterial reverse mutation test may not provide sufficient information for the assessment of genotoxicity. This may be the case for compounds that are excessively toxic to bacteria (*e.g.*, some antibiotics) and compounds thought or known to interfere with the mammalian cell replication system (*e.g.*, topoisomerase inhibitors, nucleoside analogues, or inhibitors of DNA metabolism). For these cases, usually two *in vitro* mammalian cell tests should be performed using two different cell types and two different endpoints, *i.e.*, gene mutation and chromosomal damage (as discussed in [section a. under "Modifications of Test Battery," in IV.C.1. \(/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078321.htm\)](#)). Nevertheless, it is still important to perform the bacterial reverse mutation test.
- D. Although most compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute; it varies with chemical class. There are carcinogens that are not detected by this test because they act through other, presumably nongenotoxic mechanisms or mechanisms absent in bacterial cells or fail because of inadequate metabolic activation.

IV. Test Method

A. Principle

1. Bacterial mutagenicity tests are generally conducted using one of two basic methods. In both of these procedures, bacterial cultures are exposed to the test substance in the presence and in the absence of an exogenous metabolic

activation system. In the plate incorporation method,^{(3),(9),(14),(16)} these components are combined in molten overlay agar and plated immediately onto minimal agar medium. In the preincubation method,^{(2),(8),(9),(16),(18),(34)} the treatment mixture is incubated and then mixed with the overlay agar before plating onto minimal agar 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.

2. Several procedures for performing the bacterial reverse mutation test have been described in addition to the plate incorporation method and the preincubation method. These additional procedures include the fluctuation method,^{(10),(12)} and the suspension method.⁽³¹⁾ Suggestions for procedures for the testing of gases or vapors have also been described.⁽⁴⁾
3. The procedures described in this document pertain primarily to the plate incorporation and preincubation methods. Either method is acceptable for conducting experiments both with and without metabolic activation, although 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.⁽⁹⁾ 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 be used for their detection. The following "special cases" could be identified (together with literature citations describing examples of procedures that could be used for their detection): azo dyes and diazo compounds,^{(9),(18),(26),(34)} gases and volatile chemicals,^{(4),(13),(21),(28),(35)} and glycosides.^{(5),(20),(23),(30)} Deviations from standard procedures need to be scientifically justified. In the cases of azo compounds (which are reduced in the intestine to free aromatic amines) and glycosides (which are hydrolyzed in the intestine to a sugar and an aglycone), it is preferable to test the free aromatic amine or aglycone metabolites, if available, by standard techniques rather than using the modified methods in the references cited above.
4. There are cases in which test substances derived from plant or animal tissues may contain amino acids (histidine in the case of the *S. typhimurium* tester strains and tryptophan for the *E. coli* WP2 strains), or peptides that can serve as a source of these amino acids, at levels that interfere with the conduct of these standard mutation assay procedures.^{(1),(27)} While there are alternative bacterial mutagenicity testing procedures that are not affected by the presence of amino acids in test samples (*e.g.*, see references^{(11),(22),(24),(29)}), such procedures have not been standardized, widely used, and well validated. If a test substance derived from biological material causes an increase in mutant colonies in a bacterial mutagenicity test, the possibility that such an increase may be due solely to the presence of histidine or tryptophan in the test substance should be evaluated. Experiments designed for such an evaluation might involve, for example, the testing of amino acid-free extracts of the test substance, with appropriate controls to show that the procedures used are capable of detecting mutagens added to the test substance.

B. Description

1. Preparations

a. Bacteria

- i. 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. Excessive aeration of overnight cultures should be avoided. It has been recommended that overnight

shaking of cultures in flasks not exceed 120 rpm.⁽¹⁶⁾ The cultures used in the experiment should contain a high titer of viable bacteria. The titer 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.

- ii. The culture temperature should be 37°C.
- iii. 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 among 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, crosslinking agents and hydrazines. Such substances may be detected by *E. coli* WP2 strains or *S. typhimurium* TA102⁽³⁾⁽¹⁰⁾⁽¹⁶⁾ which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

- *S. typhimurium* TA1535
- *S. typhimurium* TA1537 or TA97 or TA97a
- *S. typhimurium* TA98
- *S. typhimurium* TA100
- *E. coli* WP2 *uvrA*, or *E. coli* WP2 *uvrA* (pKM101), or *S. typhimurium* TA102.

If there is reason to believe that the test substance may be a crosslinking mutagen, then the test battery should include strain TA102, or a DNA repair-proficient strain of *E. coli* (*e.g.*, *E. coli* WP2 or *E. coli* WP2 (pKM101)) should be added.

- iv. 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, TA97a, TA97, 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 ultraviolet light)⁽⁹⁾⁽¹⁶⁾. The strains should also yield spontaneous revertant colony 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 (for *S. typhimurium*) or tryptophan (for *E. coli*), to allow for a few cell divisions, should be used.⁽³⁾⁽¹⁰⁾⁽¹⁶⁾

c. 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 post-mitochondrial fraction (S9) prepared from the livers of rodents (usually rats) treated with enzyme-inducing agents such as Aroclor 1254⁽³⁾⁽¹⁹⁾ or a combination of phenobarbitone and beta-naphthoflavone.⁽⁷⁾⁽¹⁹⁾⁽²⁵⁾⁽³⁰⁾ The post-mitochondrial supernatant fraction is usually used at concentrations in the range from 10 to 30 percent v/v in the S9 mix. The choice and concentration 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.⁽¹⁸⁾⁽²⁶⁾

Liver S9 should be prepared using aseptic techniques so that subsequent filter-sterilization is not required. Filtration of the S9 or S9 mix may lead to loss of enzyme activity.⁽¹⁶⁾ Each batch of S9, whether produced by the testing laboratory or obtained commercially, should be tested for sterility and discarded if contaminated.

d. Test Substance/Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted as 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.

2. Test Conditions

a. Solvent/Vehicle

The solvent/vehicle should not be suspected of chemical reaction with the test substance and the concentration used should be compatible with the survival of the bacteria and the S9 activity.⁽¹⁷⁾ If other than well-established solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever appropriate, an aqueous solvent/vehicle be used. When testing water-unstable substances, the organic solvents used should be free of water.

b. Exposure Concentrations

- i. Among 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 or by a clearing or diminution of the background lawn. However, preliminary toxicity tests in which survival of cells in diluted cultures is determined may give erroneous results.⁽³²⁾ The cytotoxicity of a substance may be altered in the presence of metabolic activation systems.

If the doses of the test substance are limited by toxicity, then toxicity should be evident in all preliminary and final assays at one or more doses, and no toxicity should be evident at three or more doses in each assay, in each bacterial strain, both with and without metabolic activation. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye in the tube or on the plate. The recommended maximum test concentration for soluble noncytotoxic substances is 5 mg/plate or 5 µl/plate. For noncytotoxic 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 below 5 mg/plate or 5 µl/plate should be tested up to a cytotoxic concentration. If precipitate is present on any of the plates, it may interfere with automatic counting of the colonies. In such a situation, all plates in that series of doses and controls should be counted by hand.

In some cases, toxic levels of a test chemical may kill almost all the cells but permit those that survive to utilize the histidine in the medium and to grow into visible colonies, even though they have not undergone mutations from histidine-requiring (His⁻) to histidine-independent (His⁺) or, in the case of *E. coli*, from tryptophan-requiring (Trp⁻) to tryptophan independent (Trp⁺). This phenomenon may result in an increase in colony counts at one or more toxic doses although the chemical may not be mutagenic. In such cases, careful

observation of the plates will usually reveal a clear or almost clear background lawn and unusually small "pinpoint" colonies resulting from toxicity. When the nature of such colonies remains in question, representative colonies from the plates of interest can be streaked onto minimal agar plates (supplemented with biotin (for *Salmonella*) but not histidine or tryptophan); colonies from solvent control plates are also streaked as controls. If the cells streaked from the questionable plates do not grow into colonies and those streaked from the solvent control plates do grow, then it can be concluded that the questionable colonies seen were made up of His⁻ (or Trp⁻) cells and that the increase in colony counts is not an indication of mutagenicity of the test chemical. If the cells do grow, this demonstrates that they were mutants and that the chemical is mutagenic.

- ii. At least five different analyzable concentrations of the test substance should 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.
- iii. 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

- i. Concurrent negative (solvent or vehicle) and strain-specific positive controls, both with and without metabolic activation, should be included in each assay. Positive control chemicals and concentrations that demonstrate the effective performance of each assay should be selected.
- ii. 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 Number
9,10-Dimethylanthracene	781-43-1
7,12-Dimethylbenzanthracene	57-97-6
Congo Red (for the reductive metabolic activation method)	573-58-0
Benzo(a)pyrene	50-32-8
2-Acetamidofluorene	53-96-3
Cyclophosphamide (monohydrate)	50-18-0 (6055-19-2)
2-Aminoanthracene*	613-13-8
*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, <i>e.g.</i> , benzo(a)pyrene, dimethylbenzanthracene.	

- iii. For assays performed without metabolic activation system, examples of strain-specific positive controls are:

Chemical	CAS Number	Strain
Sodium azide	26628-22-8	TA1535 and TA100
Nitrofurantoin	67-20-9	TA100
2-Nitrofluorene or 4-nitro-1,2-phenylenediamine	607-57-8 or 99-56-9	TA 98
9-Aminoacridine or ICR 191	90-45-9 or 17070-45-0	TA1537, TA97 and TA97a
Cumene hydroperoxide	80-15-9	TA102

Mitomycin C	50-07-7	WP2 <i>uvrA</i> and TA102
N-Methyl-N'-nitro- N-nitrosoguanidine or 4-nitroquinoline 1-oxide	70-25-7 or 56-57-5	WP2, WP2 <i>uvrA</i> and WP2 <i>uvrA</i> (pKM101)
Furylfuramide (AF-2)	3688-53-7	Plasmid-containing strains

- iv. Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals may be considered, when available.
- v. Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, should 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.

C. Procedure

1. Treatment with Test Substance

- a. For the plate incorporation method,^{(3), (9), (14), (16)} without metabolic activation, usually 0.05 ml or 0.1 ml of the test solutions and 0.1 ml of fresh bacterial culture (containing approximately 10⁸ viable cells) are mixed with 2.0 ml of overlay agar (0.5 ml of sterile buffer may also be included). 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 10 to 30 percent 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,^{(9), (16), (18), (34)} the test substance/test solution (usually 0.05 ml or 0.1 ml) is preincubated with the tester strain (0.1 ml, containing approximately 10⁸ viable cells) and sterile buffer (0.5 ml) 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 (2.0 ml) and pouring onto the surface of a minimal agar plate. Tubes are usually 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.^{(4), (13), (28), (35)}

2. Incubation

All plates in a given assay should be incubated at 37°C for 2 or 3 days. After the incubation period, the number of revertant colonies per plate is counted.

V. Data and Reporting

A. Treatment of Results

1. Data should 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 should also be given.

2. Individual plate counts, the mean number of revertant colonies per plate and the standard deviation should be presented for each dose of the test substance and positive and negative (untreated and/or solvent) controls.
3. There is no need to verify a clear positive response. Marginally or weakly positive results should be verified by additional testing. An attempt should be made to clarify repeatedly equivocal results by further testing using a modification of experimental conditions. Study parameters that might be modified include the concentration spacing, the method of treatment (plate incorporation or liquid preincubation), and metabolic activation conditions such as the mammalian source species for the S9 or the concentration of S9 in the S9 mix. Nevertheless, it is recognized that results may remain equivocal or questionable even after repeat testing with modified protocols.

The results of a range-finding test may supply sufficient data to provide reassurance that a reported clearly negative result is correct. Preliminary range-finding tests performed on all bacterial strains, with and without metabolic activation, with appropriate positive and negative controls, and with quantification of mutants, may be considered a sufficient replication of a subsequent complete test whose results are clearly negative. Alternatively, if negative results are to be confirmed by additional complete testing, modification of protocols, as described above for repeats of equivocal tests, is recommended.

B. Evaluation and Interpretation of Results

1. 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.⁽⁹⁾ Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results.⁽¹⁵⁾ However, statistical significance should not be the only determining factor for a positive response.
2. A test substance for which the results do not meet the above criteria is considered nonmutagenic in this test.
3. 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.
4. Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions and/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.

C. Test Report

The test report should include the following information:

1. Test Substance

- Identification data, including name and CAS no., if known.
- Physical nature and purity.
- Physicochemical properties relevant to the conduct of the study.
- Stability of the test substance, if known.

2. Solvent/Vehicle

- Justification for choice of solvent/vehicle.
- Solubility and stability of the test substance in solvent/vehicle, if known.

3. Dosing Solutions

- Times dosing solutions were prepared and used (or interval between preparation and usage), and storage conditions.
- Data that verify the concentration of the dosing solution, if available.

4. Strains

- Strains used.
- Number of cells per culture.
- Strain characteristics.

5. Test Conditions

- Amount of test substance per plate ($\mu\text{g}/\text{plate}$, mg/plate , or $\mu\text{l}/\text{plate}$) with rationale for selection of dose and number of plates per concentration.
- Media used.
- Source, type and composition of metabolic activation system, including concentration of S9 in S9 mix and acceptability criteria.
- Treatment procedures.

6. Results

- Signs of toxicity.
- Signs of precipitation.
- Individual plate counts.
- The mean number of revertant colonies per plate and standard deviation.
- Dose-response relationship, where possible.
- Statistical analyses, if any.
- Concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.
- Historical negative (solvent/vehicle) and positive control data, with *e.g.*, ranges, means and standard deviations.

7. Discussion of the results.

8. Conclusion.

VI. References

The following references should be consulted for additional background information on this test guideline.

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