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OECD GUIDELINE FOR TESTING OF CHEMICALS

**"Genetic Toxicology: *Escherichia coli*,
Reverse Mutation Assay"**

1. INTRODUCTORY INFORMATION

• Prerequisites

- Solid, liquid, gaseous or vapour test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Solubility characteristics
- Melting point/boiling point
- pH (where appropriate)

• Standard documents

There are no relevant international standards.

2. METHOD

**A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE,
APPLICATION AND LIMITS OF TEST**

The *Escherichia coli* tryptophan (trp) reversion system is a microbial assay which measures $\text{trp}^- \rightarrow \text{trp}^+$ reversion induced by chemicals which cause mutations in the genome of this organism.

• Definitions

A reverse mutation assay in *Escherichia coli* detects mutation in a gene of a tryptophan-requiring bacterial strain to produce a tryptophan-independent strain.

• Reference substances

Examples of substances which might be used as positive controls are:

- 4-nitroquinoline oxide
- N-ethyl N-nitro nitrosoguanidine
- methyl methane sulfonate
- 2-aminoanthracene

*Users of this Test Guideline should consult the Preface,
in particular paragraphs 3, 4, 7 and 8.*

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• Principle of the test method

Bacteria are exposed to the test substance with and without metabolic activation system and plated on 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 solvent control culture.

B. DESCRIPTION OF THE TEST PROCEDURE

Several methods for performing the test have been described. Among those used are the direct plate incorporation method (6), the pre-incubation method (5), and the fluctuation method (6). The procedure described in this Test Guideline is that for the direct plate incorporation method.

• Preparations

Bacteria

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). The recommended incubation temperature is 37°C. At the present time, three strains – WP2, WP2 uvrA and WP2 uvrA pKM 101 – should be used; other strains may be utilised when appropriate.

Recognised methods of stock culture preparation and storage should be used. The requirement of tryptophan for growth should be demonstrated for each strain and the other phenotypic characteristics should be checked using such methods as sensitivity to Mitomycin C and resistance to ampicillin (1) (2). The strains should also yield spontaneous revertants within the frequency ranges expected.

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 prepared from the livers of rodents treated with enzyme-inducing agents (1) (3).

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Medium

An appropriate selective medium is used with an adequate overlay agar.

- Test conditions

Amount of test substance

Among the criteria to be taken into consideration when determining the highest amount of the test substance used are cytotoxicity and solubility. The cytotoxicity of a test substance 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. For freely soluble, non-toxic substances the highest amount of test substance used should be determined on a case-by-case basis. Generally, a maximum of 5 mg/plate is considered acceptable. At least five different amounts of test substance should be tested with adequate intervals between test points.

Controls

Positive and negative (untreated and/or solvent) controls should be included in each experiment.

Positive controls should ensure both strain responsiveness and efficacy of the metabolising mixture. Methyl methane sulfonate, 4-nitroquinoline oxide or N-ethyl N-nitro nitrosoguanidine may be used as positive controls for assays performed without metabolic activation.

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 may be used as a positive control in tests performed with metabolic activation.

Other appropriate positive control reference substances may be used.

- Performance of the test

For the direct plate incorporation method without metabolic activation, the test substance and 0.1 ml of fresh bacterial culture are added to 2.0 ml of overlay agar. For tests with metabolic activation, 0.5 ml of metabolic activation mixture containing an adequate amount of post-

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mitochondrial fraction is added to the overlay agar after the addition of the bacteria and test substance. The contents of each tube are mixed and poured over the surface of a selective agar plate. Overlay agar is allowed to solidify. All plates in a given test should be incubated for the same time period. This incubation period may be from 48 to 72 hours at 37° C. At the end of the incubation period, revertant colonies per plate are counted. In general, all plating should be done at least in triplicate; the use of plating in duplicate may be acceptable where scientifically justified.

3. DATA AND REPORTING

- **Treatment of results**

Data should be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (untreated and/or solvent) and positive control plates should also be given.

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

All results should be confirmed in an independent experiment. Statistical methods may be used as an aid in evaluating the test results.

- **Evaluation of results**

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 the detection of a reproducible and statistically significant positive response for at least one of the test points.

A test substance producing neither a statistically significant dose-related increase in the number of revertants nor a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

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- Test report

The test report should also include the following information:

- bacteria: strains used
- test conditions: dose levels and rationale for selection of dose and number of plates per test point, toxicity, composition of media, type and composition of metabolic activation system, treatment procedures, positive and negative controls
- individual plate counts
- the mean number of revertant colonies per plate
- standard deviation
- dose/response relationship, where possible

- Interpretation of results

Positive results from the *E. coli* reverse mutation assay indicate that a substance induces mutations in the genome of this organism. Negative results indicate that under the test conditions the test substance is not mutagenic in *E. coli*.

The *E. coli* reverse mutation assay may be especially suited to testing some classes of chemicals such as hydrazines, nitrofurans and nitrosamines.

4. LITERATURE

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