B. 13/14. MUTAGENICITY : REVERSE MUTATION TEST USING BACTERIA

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal.
This method can be found in Dir 2000/32/EC (OJ L 136 2000).
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

1. METHOD

This method is a replicate of the OECD TG 471, Bacterial Reverse Mutation Test (1997).

1.1. INTRODUCTION

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 (1)(2)(3). 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 synthesise 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.

Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour 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.

See also General Introduction Part B.

1.2. DEFINITIONS

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.

1.3. INITIAL CONSIDERATIONS

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic.
This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal.
This method can be found in Dir 2000/32/EC (OJ L 136 2000).
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

The bacterial reverse mutation test utilises 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.

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.

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.

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.

1.4. PRINCIPLE OF THE TEST METHOD

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 two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method (1)(2)(3)(4), the preincubation method (2)(3)(5)(6)(7)(8), the fluctuation method (9)(10), and the suspension method (11). Modifications for the testing of gases or vapours have been described (12).

The procedures described in the method pertain primarily to the plate incorporation and preincubation methods. Either of them is acceptable for conducting experiments both with and without metabolic activation. Some substances may be detected more efficiently using the preincubation method. These substances belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds (3). It is also recognised 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 (3)(5)(6)(13), gases and volatile chemicals (12)(14)(15)(16) and glycosides (17)(18). A deviation from the standard procedure needs to be scientifically justified.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal. This method can be found in Dir 2000/32/EC (OJ L 136 2000). A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Preparations**

1.5.1.1. Bacteria

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. It is essential that the cultures used in the experiment 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.

The recommended incubation temperature is 37°C.

At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA 1535; TA 1537 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 may not detect certain oxidising mutagens, cross-linking agents and hydrazines. Such substances may be detected by *E. coli* WP2 strains or *S. typhimurium* TA102 (19) which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

- *S. typhimurium* TA1535, and
- *S. typhimurium* TA1537 or TA97 or TA97a, and
- *S. typhimurium* TA98, and
- *S. typhimurium* TA100, and
- *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)]

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal.
This method can be found in Dir 2000/32/EC (OJ L 136 2000).
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

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) (2)(3). 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.

1.5.1.2. 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, is used (1)(2)(9).

1.5.1.3. 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 treated with enzyme-inducing agents such as Aroclor 1254 (1)(2) or a combination of Phenobarbitone and ß–naphthoflavone (18)(20)(21). 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 (6)(13).

1.5.1.4. 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.

The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the bacteria and the S9 activity (22). If other than well-known 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 should be free of water.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal.
This method can be found in Dir 2000/32/EC (OJ L 136 2000).
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

1.5.2. **Test conditions**

1.5.2.1. Test strains (see 1.5.1.1)

1.5.2.2. Exposure concentration

Amongst the criteria to be taken into consideration when determining the highest amount of the test substance to be used are the cytotoxicity and the 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 5 mg/plate or 5 µl/plate should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

At least five different analysable concentrations of the test substance should be used with approximately half log (i.e. $\sqrt{10}$) intervals between test points for an initial experiment. Smaller intervals may be appropriate when a concentration-response is being investigated. 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.

1.5.2.3. Negative and positive controls

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

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 substances are examples of suitable positive controls for assays with metabolic activation:

CA numbers	EINECS numbers	Names
781-43-1	212-308-4	9,10-dimethylanthracene
57-97-6	200-359-5	7,12-dimethylbenz[*a*]anthracene
50-32-8	200-028-5	benzo[a]pyrene
613-13-8	210-330-9	2-aminoanthracene
50-18-0	200-015-4	cyclophosphamide
6055-19-2		cyclophosphamide monohydrate

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal.
This method can be found in Dir 2000/32/EC (OJ L 136 2000).
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

The following substance is a suitable positive control for the reductive metabolic activation method:

573-58-0	209-358-4	Congo Red

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 characterised with a mutagen that requires metabolic activation by microsomal enzymes, e.g., benzo[a]pyrene, dimethylbenzanthracene.

The following substances are examples of strain-specific positive controls for assays performed without exogenous metabolic activation system:

CAS numbers	EINECS numbers	Names	Strain
26628-22-8	247-852-1	sodium azide	TA 1535 and TA 100
607-57-8	210-138-5	2-nitrofluorene	TA 98
90-45-9	201-995-6	9-aminoacridine	TA 1537, TA 97 and TA 97a
17070-45-0	241-129-4	ICR 191	TA 1537, TA 97 and TA 97a
80-15-9	201-254-7	cumene hydroperoxide	TA 102
50-07-7	200-008-6	mitomycin C	WP2 uvrA and TA102
70-25-7	200-730-1	N-ethyl-N-nitro-N-nitrosoguanidine	WP2, WP2uvrA and WP2uvrA(pKM101)
56-57-5	200-281-1	4-nitroquinoline-1-oxide	WP2, WP2uvrA and WP2uvrA(pKM101)
3688-53-7		furylfuramide (AF2)	plasmid-containing strains

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

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.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal. This method can be found in Dir 2000/32/EC (OJ L 136 2000). A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

1.5.3. **Procedure**

For the plate incorporation method (1)(2)(3)(4), 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.

For the preincubation method (2)(3)(5)(6), 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 pre-incubation by using a shaker.

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.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels (12)(14)(15)(16).

1.5.4. **Incubation**

All plates in a given assay should be incubated at 37°C for 48-72 hours. After the incubation period, the number of revertant colonies per plate is counted.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal. This method can be found in Dir 2000/32/EC (OJ L 136 2000). A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

2. DATA

2.1. TREATMENT OF RESULTS

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. 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.

There is no requirement for verification of a clear positive response. Equivocal results should 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 pre-incubation), and metabolic activation conditions.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

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 (23). Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (24). However, statistical significance should not be the only determining factor for a positive response.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

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.

Positive results from the bacterial reverse mutation test indicate that the 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.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal. This method can be found in Dir 2000/32/EC (OJ L 136 2000). A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

3. REPORTING

TEST REPORT

The test report must include the following information :

Solvent/Vehicle

? justification for choice of solvent/vehicle;

? solubility and stability of the test substance in solvent/vehicle, if known.

Strains :

? strains used;

? number of cells per culture;

? strain characteristics.

Test conditions :

? amount of test substance per plate (mg/plate or µl/plate) with rationale for selection of dose and number of plates per concentration;

? media used;

? type and composition of metabolic activation system, including acceptability criteria;

? treatment procedures.

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 ranges, means and standard deviations.

Discussion of results.

Conclusions.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal.
This method can be found in Dir 2000/32/EC (OJ L 136 2000).
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

4. REFERENCES

1? Ames, B.N., McCann, J. and Yamasaki E. (1975). Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Res., 31, 347-364.

2? Maron, D.M. and Ames, B.N. (1983). Revised Methods for the Salmonella Mutagenicity Test. Mutation Res., 113, 173-215.

3? Gatehouse, D., Haworth, S., Cebula, T., Gocke, E., Kier, L., Matsushima, T., Melcion, C., Nohmi, T., Venitt, S. and Zeiger, E. (1994). Recommendations for the Performance of Bacterial Mutation Assays. Mutation Res., 312, 217-233.

4? Kier, L.D., Brusick D.J., Auletta, A.E., Von Halle, E.S., Brown, M.M., Simmon, V.F., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T.K. and Ray V. (1986). The *Salmonella typhimurium*/Mammalian Microsomal Assay: A Report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res., 168, 69-240.

5? Yahagi, T., Degawa, M., Seino, Y.Y., Matsushima, T., Nagao, M., Sugimura, T. and Hashimoto, Y. (1975). Mutagenicity of Carcinogen Azo Dyes and their Derivatives, Cancer Letters, 1, 91-96.

6? Matsushima, M., Sugimura, T., Nagao, M., Yahagi, T., Shirai, A. and Sawamura, M. (1980). Factors Modulating Mutagenicity Microbial Tests. In: Short-term Test Systems for Detecting Carcinogens. Ed. Norpoth K.H. and Garner, R.C., Springer, Berlin-Heidelberg-New York. pp. 273-285.

7? Gatehouse, D.G., Rowland, I.R., Wilcox, P., Callender, R.D. and Foster, R. (1980). Bacterial Mutation Assays. In: Basic Mutagenicity Tests: UKEMS Part 1 Revised. Ed. D.J. Kirkland, Cambridge University Press, pp. 13-61.

8? Aeschacher, H.U., Wolleb, U. and Porchet, L. (1987). Liquid Preincubation Mutagenicity Test for Foods. J. Food Safety, 8, 167-177.

9? Green, M.H.L., Muriel, W.J. and Bridges, B.A. (1976). Use of a simplified fluctuation test to detect low levels of mutagens. Mutation Res., 38, 33-42.

10? Hubbard, S.A., Green, M.H.L., Gatehouse, D. and J.W. Bridges (1984). The Fluctuation Test in Bacteria. In: Handbook of Mutagenicity Test Procedures. 2nd Edition. Ed. Kilbey, B.J., Legator, M., Nichols, W. and Ramel, C., Elsevier, Amsterdam-New York-Oxford, pp. 141-161.

11? Thompson, E.D. and Melampy, P.J. (1981). An Examination of the Quantitative Suspension Assay for Mutagenesis with Strains of *Salmonella typhimurium*. Environmental Mutagenesis, 3, 453-465.

12? Araki, A., Noguchi, T., Kato, F. and T. Matsushima (1994). Improved Method for Mutagenicity Testing of Gaseous Compounds by Using a Gas Sampling Bag. Mutation Res., 307, 335-344.

13? Prival, M.J., Bell, S.J., Mitchell, V.D., Reipert, M.D. and Vaughn, V.L. (1984). Mutagenicity of Benzidine and Benzidine-Congener Dyes and Selected Monoazo Dyes in a Modified Salmonella Assay. Mutation Res., 136, 33-47.

14? Zeiger, E., Anderson, B.E., Haworth, S., Lawlor, T. and Mortelmans, K. (1992). Salmonella Mutagenicity Tests. V. Results from the Testing of 311 Chemicals. Environ. Mol. Mutagen., 19, 2-141.

15? Simmon, V., Kauhanen, K. and Tardiff, R.G. (1977). Mutagenic Activity of Chemicals Identified in Drinking Water. In Progress in Genetic Toxicology, D. Scott, B. Bridges and F. Sobels (Eds.) Elsevier, Amsterdam, pp. 249-258.

16? Hughes, T.J., Simmons, D.M., Monteith, I.G. and Claxton, L.D. (1987). Vaporization Technique to Measure Mutagenic Activity of Volatile Organic Chemicals in the Ames/Salmonella Assay. Environmental Mutagenesis, 9, 421-441.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal. This method can be found in Dir 2000/32/EC (OJ L 136 2000). A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

17? Matsushima, T., Matsumoto, A., Shirai, M., Sawamura, M., and Sugimura, T. (1979). Mutagenicity of the Naturally Occurring Carcinogen Cycasin and Synthetic Methylazoxy Methane Conjugates in *Salmonella typhimurium*. Cancer Res., 39, 3780-3782.

18? Tamura, G., Gold, C., Ferro-Luzzi, A. and Ames, B.N. (1980). Fecalase: A Model for Activation of Dietary Glycosides to Mutagens by Intestinal Flora. Proc. Natl. Acad. Sci. USA, 77, 4961-4965.

19? Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990). Comparison of *Salmonella typhimurium* TA 102 with *Escherichia coli* WP2 Tester strains. Mutagenesis, 5, 285-291.

20? Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T. (1976). A Safe Substitute for Polychlorinated Biphenyls as an Inducer or Metabolic Activation Systems. In: "*In Vitro* metabolic Activation in Mutagenesis Testing" Eds. F.J. de Serres et al. Elsevier, North Holland, pp. 85-88.

21? Elliot, B.M., Combes, R.D., Elcombe, C.R., Gatehouse, D.G., Gibson, G.G., Mackay, J.M. and Wolf, R.C. (1992). Alternatives to Aroclor 1254-induced S9 in *in vitro* Genotoxicity Assays. Mutagenesis, 7, 175-177.

22? Maron, D., Katzenellenbogen, J. and Ames, B.N. (1981). Compatibility of Organic Solvents with the Salmonella/Microsome Test. Mutation Res., 88 343-350.

23? Claxton, L.D., Allen, J., Auletta, A., Mortelmans, K., Nestmann, E. and Zeiger, E. (1987). Guide for the *Salmonella typhimurium*/Mammalian Microsome Tests for Bacterial Mutagenicity. Mutation Res. 189, 83-91.

24? Mahon, G.A.T., Green, M.H.L., Middleton, B., Mitchell, I., Robinson, W.D. and Tweats, D.J. (1989). Analysis of Data from Microbial Colony Assays. In: UKEMS Sub-Committee on Guidelines for Mutagenicity Testing. Part II. Statistical Evaluation of Mutagenicity Test Data. Ed. Kirkland, D.J., Cambridge University Press, pp. 28-65.