INTERNATIONAL STANDARD

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Water quality — Determination of the genotoxicity of water and waste water — *Salmonella*/microsome test (Ames test)

Qualité de l'eau — Détermination de la génotoxicité des eaux et des eaux résiduaires — Essai de Salmonella*/microsome (essai d'Ames)*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 16240 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

It should be decided on a case-by-case basis whether, and to what extent, additional instructions may be necessary for the application of this International Standard.

Water quality — Determination of the genotoxicity of water and waste water — *Salmonella*/microsome test (Ames test)

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted according to this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the determination of the genotoxic potential of water and wastewater using the bacterial strains *Salmonella typhimurium* TA 100 and TA 98. This method includes sterile filtration of water and wastewater prior to the test.

This International Standard is applicable only to the detection of genotoxic substances which are in the filtered aqueous phase. It is not applicable to the detection of genotoxic substances adsorbed by the retained particles.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes*

ISO 5667-2, *Water quality — Sampling — Part 2: Guidance on sampling techniques*

ISO 5667-3, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of water samples*

ISO 5667-14, *Water quality — Sampling — Part 14: Guidance on quality assurance of environmental water sampling and handling*

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
number of revertants
number of mutants
number of visible mutant colonies per plate at the termination of the test

3.2
dilution level
D
denominator of the dilution coefficient (using the numerator 1) of a mixture of water or wastewater with **dilution water** (3.16) as integral number

NOTE For undiluted water or wastewater, the dilution coefficient is by definition 1:1. The corresponding and smallest possible D value is 1.

3.3
dose-response relationship
reduction of the number of visible mutant colonies per plate with increasing D level

3.4
D_{min} **value**
smallest value of D at which, under the conditions of this International Standard, no positive increase in the number of visible mutant colonies per plate is detected

NOTE In the case of more than one D_{min} value (a maximum of four are possible), the highest D value is decisive.

3.5
stock culture
frozen culture for the preservation of the characteristics (e.g. genotype) of *Salmonella typhimurium* TA 100 and TA 98

3.6
inoculum
part of a thawed stock culture used to inoculate culture medium

3.7
culture medium
aqueous solution of nutrients which are required for the cultivation of the bacteria

3.8
overnight culture
mixture of inoculum and culture medium, incubated for about 18 h at 37 °C ± 1 °C and gentle agitation (e.g. shaken at 100 r/min to 150 r/min)

3.9
plate
solidified mixture of water, agar and other possible constituents (e.g. inorganic salts) in Petri dishes

3.10
softagar
mixture of agar, sodium chloride, histidine, biotin and water

NOTE Minimal softagar contains only traces of histidine and is used for the determination of mutants. Maximal softagar contains histidine in excess and is used for the determination of titres.

3.11
S9 fraction
9 000 g supernatant of a tissue homogenate in 0,15 mol/l KCl, obtained from livers of male rats (200 g to 300 g) pretreated with an appropriate substance or substance combination for enzyme induction

3.12
cofactor solution
aqueous solution of chemicals needed for the activity of the enzymes in the S9 fraction

NOTE Examples of chemicals needed are NADP, glucose-6-phosphate and inorganic salts.

3.13
S9 mix
mixture of S9 fraction and cofactor solution

3.14
titre determination
method for the determination of the number of bacteria (colony-forming units) in an overnight culture and for the determination of possible bacteriotoxic effects of the test sample

3.15
test sample
sample to be used as test item after all preparative steps (e.g. sterile filtration) have been carried out

3.16
dilution water
sterile water of a conductivity of $\leqslant$ 5 µS/cm used for the stepwise dilution of the test sample or used as negative control

3.17
negative control
dilution water (3.16) without test sample

3.18
positive control
known mutagen used to verify the sensitivity of the method or the activity of the S9 mix

NOTE The positive controls are dissolved in DMSO prior to use.

3.19
test mixture
mixture of test sample [pure or diluted with **dilution water** (3.16)], negative or positive control, bacterial suspension, softagar and S9 mix or buffer

3.20
induction rate
I
difference between the mean value of mutant colonies counted on the plates treated with a dose of the test sample or with a positive control and the mean value of the corresponding plates treated with the negative control using the same strain under identical activation conditions

3.21
background growth
bacterial lawn formed by microcolonies of non-mutated bacteria on a plate with minimal softagar due to the traces of histidine contained in this softagar

4 Interferences

A strong bacteriotoxic effect of the test sample can lead to a reduction of viable bacteria and to a reduction of mutant colonies compared to the corresponding negative control counts.

In an extreme case of bacteriotoxicity, the number of surviving bacteria may be reduced to such an extent (to several hundred) that the traces of histidine in the minimal softagar are sufficient to allow these bacteria to grow up to visible colonies mimicking the growth of mutant colonies. This may lead to false positive results.

5 Principle

The bacteria are exposed under defined conditions to various doses of the test sample and incubated for 48 h to 72 h at 37 °C ± 1 °C. Due to this exposure, genotoxic agents contained in the test water or wastewater may be able to induce mutations in one or both marker genes (hisG46 for TA 100 and hisD3052 for TA 98) in correlation to the used doses. Such induction of mutations causes a dose-related increase in the numbers of mutant colonies.

The possible mutagenic activity of the test sample is detected by comparing, for the used bacterial strain and the respective activation condition [± **S9 mix** (3.13); Annex B)], the number of mutant colonies on plates treated with the negative control with those treated with undiluted and diluted test sample.

The lowest dilution (1:N) of the test sample inducing, according to the criteria of this International Standard, no mutagenic effect under all experimental conditions (if any mutagenic effect is induced by the test sample) is the parameter relevant for the assessment of the test sample according to this International Standard. Dilutions above this (1:A, A<N) shall induce a mutagenic effect according to the criteria of this International Standard in at least one strain under at least one activation condition. The respective D_{min}-value is N. If no mutagenic effect is observed under all experimental conditions, this dilution is 1:1 and the respective D_{min}-value is 1.

The test facility is qualified for the conduct of this International Standard if the *Salmonella*/microsome test is established in this facility according to the following criteria:

— several independent experiments are performed;

— several known mutagenic and non-mutagenic reagents are tested;

— the mutagenic compounds are included in the positive controls of this International Standard (6.18);

— the results are reproducible;

— the results are in compliance with literature data.

6 Reagents and media

As far as possible, use chemicals of reagent grade. Prepare all aqueous solutions with water of a conductivity of ⩽ 5 µS/cm.

If chemicals with different amounts of crystallisation water are used, recalculate the needed amounts.

Always autoclave for 20 min at 121 °C ± 2 °C. Seal vessels loosely (e.g. with aluminium foil). Sealing should never be air-tight.

All compositions are given for specific final amounts. Other final amounts (N-fold) may be reached by multiplying the amounts all single components of the respective composition by N.

Compositions may be subdivided under appropriate conditions into appropriate amounts.

6.1 Hydrochloric acid, c(HCl) = 1 mol/l.

6.2 Sodium hydroxide solution, c(NaOH) = 1 mol/l.

6.3 Dimethyl sulfoxide (DMSO), $C_2H_6O_4S$.

6.4 Nutrient broth

For each 1 l of water, add 3 g of beef extract, 5 g of peptone and 5 g of sodium chloride (or alternatively, 10 g of beef extract, 10 g of peptone and 5 g of sodium chloride). Warm up and stir to dissolve the compounds. Adjust the pH to 7,4 ± 0,2 and autoclave in appropriate portions. Store under sterile conditions at 4 °C ± 2 °C for not longer than one month.

For use of commercial products, see A.1.

6.5 Ampicillin solution

Under sterile conditions at room temperature, dissolve 80 mg of ampicillin in 10 ml of sterile sodium hydroxide solution (0,02 mol/l). Use immediately.

6.6 Nutrient broth with ampicillin

Under sterile conditions, add 3,15 ml of ampicillin solution (6.5) to 1 l of nutrient broth (6.4). Store under sterile conditions at 4 °C ± 2 °C for not longer than one week.

6.7 Sodium hydrogen phosphate buffer

The following solutions are needed to prepare the buffer:

— **solution 1**: 13,8 g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) dissolved in 1 l water;

— **solution 2**: 14,2 g of disodium hydrogen phosphate (Na_2HPO_4) dissolved in 1 l water.

Stir solution 2 (e.g. with a magnetic stirrer) and add solution 1 until a pH of 7,4 is reached and remains stable. Subdivide this solution in appropriate amounts and autoclave to sterilize. Store at 4 °C ± 2 °C for not longer than one month.

6.8 Cofactor solution

Dissolve the following compounds, in the amounts given, in 70 ml of sodium hydrogen phosphate buffer (6.7):

— 162,6 mg of magnesium chloride hexahydrate ($MgCl_2 \cdot 6\ H_2O$);

— 246,0 mg of potassium chloride (KCl);

— 179,1 mg of glucose-6-phosphate, disodium salt (G6P);

— 315,0 mg of NADP[1)], disodium salt.

Filter sterile through appropriate membrane filters. A volume of 70 ml of cofactor solution is needed for the preparation of 100 ml of S9 mix, sufficient for approximately 200 plates.

6.9 S9 fraction

The preparation of S9 fraction and the treatment for enzyme induction are described in Annex B. If S9 fraction is purchased commercially, it should also be prepared (including enzyme induction) according to Annex B.

6.10 S9 mix

Prepare the needed amount of S9 fraction (6.9) freshly on the day of test or, if stored frozen, thaw it at room temperature. Immediately thereafter, prepare S9 mix by mixing the following under sterile conditions:

— 10 ml of S9 fraction;

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1) Nicotinamide adenine dinucleotide phosphate.