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 $\leqslant$ 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;

1) Nicotinamide adenine dinucleotide phosphate.

— 70 ml of cofactor solution (6.8);

— 20 ml of potassium chloride solution (6.19).

Cool S9 mix permanently (e.g. in a double-walled separator funnel containing ice water between the walls) and use it only on the same day. Discard the remaining S9 mix at the end of this day.

6.11 Vogel-Bonner E-medium (50×)

Dissolve by stirring the following amounts of the substances, in the given order, into 670 ml water. Allow each ingredient to dissolve completely before adding the next.

10 g $MgSO_4 \cdot 7\ H_2O$

100 g citric acid $\cdot\ H_2O$

500 g K_2HPO_4, anhydrous

175 g $NaNH_4HPO_4 \cdot 4\ H_2O$

This results in a final volume of approximately 1 l. Divide in appropriate portions and store at room temperature.

6.12 Vogel-Bonner plates (see also 8.1)

For the preparation of 1 l of agar:

— add 15 g of agar (see A.2) to 880 ml water and dissolve by heating under stirring; seal the container (e.g. with aluminium foil);

— dissolve 20 g of glucose monohydrate in water to a final volume of 100 ml; seal the container (e.g. with aluminium foil);

— transfer 20 ml of Vogel-Bonner E-medium (6.11) into a container and seal container;

— autoclave all components;

— under sterile conditions, add glucose solution and Vogel-Bonner E-medium to the agar solution.

CAUTION — Delayed boiling leading to splashing can occur.

Mix and pour into Petri dishes (25 ml to 30 ml per dish).

6.13 Nutrient agar plates (see also 8.1)

Mix 23 g of nutrient agar (see A.3) with 1 l of water and dissolve by heating under stirring. Seal container (e.g. with aluminium foil) and autoclave. Pour into Petri dishes (25 ml per dish).

Alternatively, dissolve 15 g of agar (see A.2) in 1 l of nutrient broth (see A.1) by warming in an appropriate vessel. Seal vessel loosely (e.g. with aluminium foil) and autoclave the solution. Thereafter pour into Petri dishes (see 8.1).

6.14 Ampicillin agar plates (see also 8.1)

Dissolve agar (see A.2) in 860 ml water. Prepare 100 ml of glucose-solution and 20 ml of Vogel-Bonner E-medium as described in 6.11. In addition, the following ingredients are needed:

— 3,15 ml ampicillin solution (6.5);

— 10,0 ml histidine solution (add 500 mg of L-histidine hydrochloride monohydrate to 100 ml water);

— 6,0 ml biotin solution [add 12,2 mg of (D+)-biotin to 100 ml of water].

Autoclave all components. Under sterile conditions, add all solutions and Vogel-Bonner E-medium to the autoclaved agar solution.

CAUTION — Delayed boiling leading to splashing can occur.

Mix and pour into Petri dishes (25 ml per dish).

If nutrient agar (6.13) is used as an alternative, add 3,15 ml of ampicillin solution (6.5) per litre of agar solution.

6.15 Amino acid solutions for softagar

The only difference between amino acid solutions for mutant and titre determinations is the amount of histidine needed.

6.15.1 Minimal medium

Add 105 mg of L-histidine hydrochloride monohydrate and 122 mg of (D+)-biotin to 1 l water, mix, seal and autoclave. Divide under sterile conditions into appropriate amounts (e.g. 10 ml) in marked vessels, and store at 4 °C ± 2 °C.

6.15.2 Complete medium

Add 1 050 mg of L-histidine hydrochloride monohydrate and 122 mg of (D+)-biotin to 1 l of water, mix, seal and autoclave. Divide under sterile conditions into appropriate amounts (e.g. 10 ml) in marked vessels, and store at 4 °C ± 2 °C.

6.16 Softagar

6.16.1 Softagar for mutant determination

Mix 0,8 g of agar (see A.2) and 0,6 g of sodium chloride with 45 ml water.

Place 10 ml of amino acid solution (minimal medium, 6.15.1) in a separate vessel.

To adjust the volume in experiments without S9 mix, 0,5 ml of sodium hydrogen phosphate buffer (6.7) per 1 ml of softagar may also be needed.

Autoclave all components.

Add minimal medium and, if appropriate, buffer to agar and mix thoroughly. Avoid temperatures below 45 °C. Place tubes in a water bath (45 °C ± 1 °C) and add 1 ml (for experiments with S9 mix) to each tube or 1,5 ml (for experiments without S9 mix) of the respective mixture under sterile conditions. Cover tubes again with aluminium foil. Mark stands in an appropriate form to avoid mistakes. Experience has shown that soft agar may be stored in this form for up to 24 h.

6.16.2 Softagar for titre determination

Mix 0,8 g of agar (see A.2) and 0,6 g of sodium chloride with 45 ml water.

Place 10 ml of amino acid solution (complete medium, 6.15.2) in a separate vessel.

Autoclave all components.

Add complete medium to agar and mix thoroughly. Avoid temperatures below 45 °C. Place tubes in a water bath (45 °C ± 1 °C) and add 1 ml of the mixture to each tube under sterile conditions. Cover tubes again with

aluminium foil. Mark stands in an appropriate form to avoid mistakes. Experience has shown that soft agar may be stored in this form for up to 24 h.

6.17 Physiological NaCl solution

Dissolve 9 g of sodium chloride in 1 l water. Separate in appropriate amounts, autoclave and store under sterile conditions.

6.18 Positive controls

Stock solutions of positive control substances can be stored at – 20 °C as long as validity criteria (Clause 10) are met.

6.18.1 Nitrofurantoin

Dissolve 20 mg of nitrofurantoin (NF) under sterile conditions in 10 ml of dimethyl sulfoxide, DMSO (6.3) resulting in a stock solution of 2 mg/ml. Dilute stock solution three times 1:10 with DMSO. Use 0,1 ml of the final dilution (10^{-3}) (containing 2 µg/ml NF) in the test.

6.18.2 4-Nitro-1,2-phenylene diamine

Dissolve 25 mg of 4-nitro-1,2-phenylene diamine (4-NPDA) under sterile conditions in 5 ml of DMSO (6.3) resulting in a stock solution of 5 mg/ml. Dilute stock solution three times 1:10 with DMSO. Use 0,1 ml of the final dilution (10^{-3}) (containing 5 µg/ml 4-NPDA) in the test.

6.18.3 2-Aminoanthracene

Dissolve 30 mg of 2-aminoanthracene (2-AA) under sterile conditions in 10 ml of DMSO (6.3) resulting in a stock solution of 3 mg/ml. Dilute stock solution twice 1:10 with DMSO. Use 0,1 ml of the final dilution (10^{-2}) (containing 30 µg/ml 2-AA) in the test.

6.19 KCl solution

Dissolve 1,12 g of potassium chloride in 100 ml water and autoclave.

6.20 Tester strains

Use mutants of *Salmonella typhimurium* LT2, which detect point mutations, to determine the mutagenic potential of a test sample. Since point mutations can be subdivided into two classes (base-pair substitutions and frame-shift mutations), two strains are used. These strains are TA 100 and TA 98. TA 100 bears the base-pair substitution hisG46 as marker, whereas TA 98 contains the frame-shift mutation (+2 type) hisD3052.

In addition, both strains have the following genetic properties.

a) They contain the plasmid pKM101, coding for an ampicillin resistance.

b) They are all deep rough, e.g. partly deficient in lipopolysaccharide side chains, enabling larger molecules to penetrate the bacterial cell wall and also produce mutations.

c) Due to the mutation *uvrB,* their capability to repair DNA-damage is limited, as it is induced e.g. by UV-light, which increases the likelihood that such damage results in mutations.

Immediately upon receipt, spread aliquots of the respective bacterial strain onto the surface of plates with ampicillin agar (6.14) thus allowing to grow in single colonies. Incubate for 24 h at 37 °C ± 1 °C.

Take samples from individual colonies with a sterile inoculation loop, and transfer them to 20 ml of ampicillin-containing nutrient broth (6.6).

Incubate again overnight at 37 °C ± 1 °C.

Inoculate samples of these cultures onto nutrient agar plates, which have been provided with ampicillin (6.14), again so as to grow single colonies. Incubate for 24 h at 37 °C ± 1 °C.

Transfer new samples of individual colonies from these plates to flasks containing approximately 20 ml normal nutrient broth (6.4). Incubate this inoculum overnight at 37 °C ± 1 °C.

Take a small sample to check the genotype (see Annex C).

These investigations are performed to check the presence of the genetic markers “deep rough”, *uvrB* and pKM101. In addition, determine the number of spontaneous mutations and the sensitivity to standard mutagens (positive controls, 6.18).

Stock cultures should fulfil the requirements of Annex C, otherwise they should be discarded.

Treat and mix the remaining parts of the cultures with DMSO (1,8 ml per 20 ml of culture volume) or glycerol (4 ml per 20 ml of culture volume) to protect against the effects of freezing, and freeze immediately at $\leqslant$ – 70 °C in small portions (0,5 ml to 1 ml).

Whenever new stock cultures need to be produced, inoculate from a frozen stock culture on ampicillin containing nutrient agar (6.13) so as to grow single colonies. Incubate for 24 h at 37 °C ± 1 °C. Transfer samples of individual colonies to 20 ml of nutrient broth (6.4), incubate, divide, and check the genotype, then freeze the cultures as described above.

6.21 Bacterial suspensions

6.21.1 Bacterial suspension for mutant count

Pre-warm the needed amount of nutrient broth (6.4) to 37 °C ± 1 °C for approximately 1 h.

Mark broth vessels with the respective strain code (e.g. 0 for TA 100, 9 for TA 98). Inoculate broth with the respective strain.

Add per culture 0,2 ml of a thawed stock culture per 10 ml of nutrient broth. Incubate culture for 18 h at 37 °C ± 1 °C and use only on this day for testing.

6.21.2 Bacterial suspension for titre

Dilute a bacterial suspension (prepared according to 6.21.1) 6 times 1:10 with physiological saline solution (6.17). Store vessel with final dilution of 10^{-6} in ice water.

7 Apparatus

7.1 Temperature- and time-controlled water-bath

7.2 Incubator, maintained at 37 °C ± 1 °C.

7.3 pH meter

7.4 Analytical balance

7.5 Steam sterilizer

7.6 Dry sterilizer

7.7 Magnetic stirrer

7.8 Casting machine for pouring agar plates

7.9 Rotary mixer

7.10 Freezer, maintained in the range – 20 °C to – 70 °C.

7.11 Colony-counter system

7.12 Pipettes, to deliver 0,1 ml, 0,5 ml, 1 ml, 2 ml, 5 ml, 10 ml, 25 ml.

7.13 Storage bottles, of capacity 250 ml and 1 000 ml.

7.14 Graduated cylinders, marked to 100 ml and 200 ml.

7.15 Graduated flasks, of capacity 20 ml, 200 ml, 500 ml.

7.16 Sterile filter units, of pore size 0,22 µm or 0,45 µm.

7.17 Metal racks for polypropene tubes, to hold 2 × 6, 4 × 6 and 4 × 12 tubes.

7.18 Erlenmeyer flasks, of capacity 50 ml, 100 ml, 250 ml.

7.19 Petri dishes with venting ribs, diameter approximately 94 mm, height approximately 16 mm.

7.20 Drygalski spatula

7.21 Inoculating loops

7.22 Polypropene tubes with cap, of capacity 5 ml and 10 ml.

7.23 Thermometer

8 Procedure

8.1 Pouring and labelling of plates

Sterilize all tools or parts thereof which come into contact with agar. Keep the liquid agar homogeneous and avoid too low temperatures.

If pouring is performed with an automated device, follow the manufacturer's instructions.

Fill Petri dishes with 25 ml of agar per dish. Remove the lid immediately before pouring agar into the plate and close the lid immediately thereafter. Incubate plates after solidification of agar at 37 °C ± 2 °C for an appropriate time to remove excess water and to avoid condensation during the test.

Short-term storage at room temperature is permissible. Longer storage shall be at 4 °C ± 2 °C. Storage times should be as short as possible and not longer than one month.

Never use plates with rough surface, nor contaminated plates. Dry moist plates before use (see above).

Mark all plates either individually or in appropriate packaging (containing only plates of the same type) to rule out mix-up. The colour of the agar may serve as an additional criterion.

Prior to the start of a test, mark Vogel-Bonner plates so that test sample, dilution step, strain and activation condition are clearly distinguishable. Mark plates for titre determination additionally.

8.2 Test samples and their preparation

Take samples in accordance with ISO 5667-1, ISO 5667-2, ISO 5667-3, ISO 5667-14 and ISO 5667-16.

Test samples immediately after sampling. If this is not possible, samples should be stored at 0 °C to 5 °C (maximum 48 h) or < -18 °C (maximum two months). Divide large samples in advance into appropriate portions, since thawed samples can only be used on the same day.

Samples containing solids should be centrifuged to separate solids. In this case, only the supernatant is processed further.

Sterilize all samples using sterile filters. Do not apply any extraction or concentration procedures to the samples. Shake test samples thoroughly before use to avoid inhomogeneous distribution.

Usually no adjustment to give pH neutrality is needed. However, excessively high or low pH values may trigger bacteriotoxic effects leading to reductions in mutant colonies as compared to the relevant negative control. In this case, repeat the test after adjusting the sample to pH $7{,}0 \pm 0{,}2$ using either HCl or NaOH solution (see 6.1 and 6.2). Select the concentrations of acid or alkali such that the added volumes are as small as possible. Avoid over-titration. Take into account the change in sample pH and the resulting effects (see ISO 5667-16).

Perform dilutions of the test sample (1:2 to 1:16) with sterilized water (3.16).

8.3 Number of test samples

Perform the test with TA 100 and TA 98, without and with S9 mix for both strains. Apply the following treatments:

a) negative control;

b) undiluted test sample D = 1;

c) dilution 1:2 of test sample D = 2;

d) dilution 1:4 of test sample D = 4;

e) dilution 1:8 of test sample D = 8;

f) dilution 1:16 of test sample D = 16;

g) positive controls:

   1) nitrofurantoin: 0,2 µg/plate only TA 100 without S9 mix;

   2) 4-nitro-1,2-phenylene diamine: 0,5 µg/plate only TA 98 without S9 mix;

   3) 2-aminoanthracene: 3,0 µg/plate TA 100 and TA 98 with S9 mix.

Except for the negative control, treat two plates, both with and without S9 mix, for each strain and treatment [b) to g) 3)]. Three plates should be treated, both with and without S9 mix, for each strain with dilution water (3.16), comprising the negative controls [treatment a)].

The application volume for treatments a) to f) is 1 ml. The application volume for the positive controls [treatments g) 1) to g) 3)] is 0,1 ml. Replace the lacking volume of 0,9 ml by dilution water (3.16) which shall be added directly to the tubes.

If two or more test samples are tested in parallel with the same bacterial suspension (6.21.1), use the same dilution water and the same S9 mix. There is no need to perform multiple negative and positive controls.

The density of viable cells per millilitre (titre) is determined at least for each negative control with S9 mix using two plates per strain.

8.4 Preparation

8.4.1 On the day prior to the test:

— weigh the ingredients for the cofactor solution (6.9);

— weigh the positive controls (6.18);

— label the plates (8.1);

— sterilize the glass material;

— sterilize the pipettes and/or pipette tips;

— sterilize the test tubes;

— prepare the softagar (6.16, 6.16.1, 6.16.2);

— inoculate the bacterial suspension for 18 h (6.21.1).

8.4.2 On the day of testing only:

— prepare cofactor solution (6.8);

— prepare S9 mix (6.10);

— test sample preparation and dilution (Clause 8);

— dilution of bacterial suspensions (6.21.2).

8.5 Study conduct

8.5.1 General

Bring all tubes containing softagar in a water bath at 45 °C ± 1 °C. Perform all parts of the test (TA 100 without and with S9 mix as well as TA 98 without and with S9 mix) in parallel, using the same test sample and the same S9 mix. In the experimental parts with one strain, use the same bacterial suspension. For the determination of titre dilutions, use the bacterial suspension for the mutagenicity study.

8.5.2 Mutagenicity study

For the experimental part without S9 mix (6.16.1), add per tube with softagar the respective volume given for the respective treatment [treatments a) to g) 2), see 8.3] and 0,1 ml of bacterial suspension (6.21.1). Remove tubes from the water bath, mix the contents of each tube thoroughly with a rotation mixer and pour on a Vogel-Bonner plate (6.12). Move the plate gently to distribute the softagar over the surface of the plate, and bring the plate to a horizontal position. Plates may be stacked.

For the experimental part with S9 mix (6.16.1), add per tube with softagar the respective volume given for the respective treatment [treatments a) to f) and g) 3), see 8.3] and 0,1 ml bacterial suspension (6.21.1). Remove tubes from the water bath, add 0,5 ml of S9 mix, mix the contents of each tube thoroughly with a rotation mixer and pour on a Vogel-Bonner plate (6.12). Move the plate gently to distribute the softagar over the surface of the plate, and bring the plate to a horizontal position. Plates may be stacked.

The time between pipetting the respective bacterial suspension into a tube and removing the tube from the water bath should not be longer than 30 s. However, less than 15 s is preferable.

After the softagar has sufficiently solidified on the agar surface of the plate (approximately 30 min to 60 min), invert the plates. Place the plates in an incubator and incubate in the dark at 37 °C ± 1 °C for 48 h to 72 h.

8.6 Titre determination

For the experimental part with S9 mix (6.16.2), add per tube with softagar 1 ml of dilution water and 0,1 ml of bacterial suspension for titre determination (6.21.2). Remove tubes from the water bath, add 0,5 ml of S9 mix, mix the contents thoroughly with a rotation mixer and pour on a Vogel-Bonner plate (6.12). Move plate gently to distribute the softagar over the surface of the plate and place the plate on a horizontal surface. Plates may be stacked.

After the softagar has sufficiently solidified on the agar surface of the plate (approximately 30 min to 60 min), invert the plates. Place the plates in an incubator and incubate in the dark at 37 °C ± 1 °C for 48 h to 72 h.

9 Colony-counting, evaluation and assessment

9.1 Colony-counting

It is preferable to count colonies with an automatic counter. If no automatic counter is available or if its use is not adequate for other reasons (e.g. plate heavily stained by the test sample, heavy precipitation on the plate), count manually using an appropriate counting device and a magnifying glass.

Bacteriotoxicity should be assessed on the basis of three parameters:

a) reduction of background growth on the plate;

b) reduction of mutant colonies per plate in comparison to the corresponding negative control;

c) reduction of titre, if determined also for plates treated with test sample.

If reading of the plates is not possible on the same day as termination of incubation, store plates at 4 °C ± 2 °C in the dark for maximum two days.

9.2 Evaluation

Calculate (with and without S9 mix) mean values of mutant colonies per plate for each treatment and strain. Additionally, calculate the induction rate (3.20).

9.3 Assessment criteria

A test sample is evaluated as being mutagenic if an increase in mutant colonies per plate, above the defined induction rate defined per strain, is achieved in relation to dose in at least one strain under at least one activation condition.

For TA 100, this induction rate (3.20) is 80 and for TA 98 it is 20.

An example of dose-related increases is given in Table D.2.

9.4 Determination of the decisive D_{min} value

The “decisive D_{min} value” is the lowest D_{min} value (3.4) at which no genotoxic effects are found for plates treated with the test sample, or dilutions thereof, according to the criteria given in 9.3.

For test samples without genotoxic activity, the “decisive D_{min} value” is 1.

10 Validity criteria

The following criteria shall be met for valid experiments:

a) the mean values of negative controls are within the following range:

   — for TA 100: 80 to 180 mutant colonies per plate;

   — for TA 98: 15 to 40 mutant colonies per plate;

b) the mean values of positive controls show at least the following induction rates:

   — nitrofurantoin TA 100: + 100 colonies;

   — 4-nitro-1,2-phenylenediamine TA 98: + 50 colonies;

   — 2-aminoanthracene TA 100: + 800 colonies;

   TA 98: + 800 colonies;

c) titre determinations shall demonstrate sufficient bacterial density ($> 10^8$ per millilitre).

If one (or more) of these criteria is not met, a part or the entire test may be invalidated. If the strain-specific positive control for a strain (NF for TA 100 and 4-NPDA for TA 98) is below the predefined range, both parts of the test with the respective strain (results without as well as with S9 mix) are invalidated. If the positive control for the activity of the S9 mix (2-AA) is below the predefined range for at least one strain, the parts of the test performed with S9 mix are invalidated for both strains.

Invalid results cannot be used for assessment.

If atypical results are obtained more than once within a short time, a verification of the genotype (see Annex C) of the respective strain shall be carried out.

11 Test report

At least the following information shall be included in the test report:

a) a reference to this International standard (ISO 16240);

b) identity of the test sample (origin and date of sampling, pH value);

c) positive controls [chemical name, source, batch number or comparable data (if available)];

d) storage of sample and preparation of test sample [storage conditions (if not tested directly), adjustment of pH value, centrifugation (including mass and time), filtration (including filter material and pore diameter) and other manipulations];

e) tester strains [strain, source, data of arrival, storage conditions, date of stock culture preparation, and date of genotype verification (if this date deviates from stock culture preparation)];

f) metabolizing system (preparation and origin of S9 fraction, protein content, date of preparation, storage conditions);

g) test environment (address of performing laboratory, date of test, method of counting);

h) results [individual mutant colony number per plate for all treatments a) to g) 3) (8.3), induction rates (3.20), alteration of background growth (if any), $D_{\min}$ values, other observations (e.g. precipitation, staining of the plate].

Annex A
(normative)

Nutrient broth and agar

A.1 Nutrient broth

Nutrient broth is commercially available. To acheive better standardization, the use of commercial products is recommended. Ensure that the commercial product contains sodium chloride.

A.2 Agar

Agar is commercially available in different qualities. It is recommended to use Difco [2)] agar or a product of equal quality.

A.3 Nutrient agar

Nutrient agar is commercially available in different qualities. It is recommended to use Difco [2)] nutrient agar or a product of equal quality.

2) Difco agar is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

Annex B
(normative)

S9 fraction

B.1 Induction of liver enzymes

For enzyme induction, at least 6 male rats (e.g. Sprague-Dawley rats), of body mass about 200 g to 300 g, receive a single intraperitoneal injection of a polychlorinated biphenyl [3)] dissolved in an appropriate vehicle at a dose of 500 mg/kg body mass five days prior to termination. In case of phenobarbital/β-naphthoflavone induction, the rats receive 80 mg/kg body mass phenobarbital intraperitoneally and 80 mg/kg body mass β-naphthoflavone orally on three consecutive days simultaneously, in an appropriate vehicle. The rat livers are analysed 24 h after the last treatment.

Animals should be kept in special adequately marked cages which are only used for this purpose. Husbandry should be standardized, with 12 h of electrical lighting daily, 20 °C to 23 °C room temperature, and about 60 % mean relative humidity, on a bedding of softwood granules. Air change should be about 10 times per h. The animals should receive an appropriate standard diet and water of drinking quality ad libitum.

B.2 Preparation of S9 fraction

Livers are removed under sterile conditions immediately after termination and kept at 4 °C $\pm$ 1 °C until all animals have been prepared. All the remaining steps are carried out under sterile conditions at 4 °C $\pm$ 1 °C.

Wash the livers with cold (4 °C $\pm$ 1 °C) 0,15 mol/l KCl solution (approximately 1 ml KCl per 1 g liver). Homogenize the livers in fresh, cold (4 °C $\pm$ 1 °C) 0,15 mol/l KCl (approximately 3 ml KCl per 1 g liver). Centrifuge the homogenate in a cooling centrifuge at 4 °C $\pm$ 1 °C and 9 000 g for 10 min. Store the supernatant (the S9 fraction) below – 70 °C in small portions. On the day of the test, thaw (if frozen), divide in appropriate portions and store at room temperature until used.

3) Aroclor 1254 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

Annex C
(normative)

Verification of genotype

C.1 Ampicillin resistance (pKM101)

Spread parallel lines of the bacterial suspension of the respective strain on two ampicillin plates (6.14). This should also be done in parallel with a strain which does not contain the plasmid pKM101 (e.g. TA 1535). Incubate the plates overnight at 37 °C ± 2 °C. No growth should be observed for TA 1535, whereas full growth of the stripes should be observed for acceptable stock batches of TA 100 and TA 98 (6.20).

C.2 Crystal-violet sensitivity (deep rough)

Take a quantity of 0,1 ml from the samples of individual stocks and spread onto nutrient agar (6.13), using four plates per strain. After a few minutes, filter papers (diameter 9 mm), to which 10 µl of an aqueous, crystal-violet solution have been added at a concentration of 1 mg/ml, should be placed in the middle of the plates. The plates should be incubated overnight at 37 °C ± 2 °C. Measure the diameters of the inhibition zones that have formed. The mean of the inhibition zones of an acceptable stock batch shall be at least approximately 14 mm.

C.3 UV sensitivity (*uvrB*)

A quantity of 0,1 ml should be taken from the samples of individual stocks and spread onto nutrient agar (6.13), using four plates per strain. Cover one-half of each plate with aluminium foil and irradiate without a lid for 8 s with UV light (30 W) of wavelength 254 nm at a distance of 33 cm. Incubate the irradiated plates overnight at 37 °C ± 2 °C. To demonstrate adequate sensitivity in this test, acceptable stock batches shall show an inhibition of bacteria growth over the irradiated half of their area.

C.4 Histidine requirement

A special test for histidine requirement is not necessary, since the histidine dependence of the cultures is automatically verified by the negative controls in each individual test.

Annex D
(informative)

Examples of results obtained

Table D.1 — Examples for assessment

Strain	Dilution level D	Addition of S9 mix	Induction rate I
TA 100	1	without	324
	2	without	192
	4	without	96
	8 [a]	without	23
	16	without	0
	1 [a]	with	16
	2	with	—
	4	with	8
	8	with	24
	16	with	0
TA 98	1 [a]	without	12
	2	without	18
	4	without	6
	8	without	15
	16	without	0
	1	with	143
	2	with	78
	4	with	63
	8	with	25
	16 [a]	with	0

[a] Lowest D value (3.4) at which no genotoxic effects are found according to the criteria given in 9.3 ($I < 80$ for TA 100 and $I < 20$ for TA 98).

In the present example, the D values for the four parts of the test are as follows:

— TA 100 without S9 mix $D = 8$;

— TA 100 with S9 mix $D = 1$;

— TA 98 without S9 mix $D = 1$;

— TA 98 with S9 mix $D = 16$.

The decisive D value is the lowest D value (3.4) at which no genotoxic effects are found in all four parts of the test according to the criteria given in 9.3. In the present example this decisive D value is 16.

Table D.2 — Example of a table for reporting test data

Name of test sample: NAME		*Salmonella typhimurium* TA 98							
Treatment		Mutant colonies				Titre determination		Induction rate	
						Dilution	Titre	I	
	D value	–S9 mix	Mean	+S9 mix	Mean	10^{-6}	10^{+8}	–S9 mix	+S9 mix
Negative control		25	26	31	33	191	17,7	0	0
	—	26		33		163			
		27		35					
Dilution 1:16		24	26	31	34			0	1
	16	28		37					
Dilution 1:8		27	28	59	58			2	25 [a]
	8	29		57					
Dilution 1:4		34	35	75	76			9	43 [a]
	4	35		77					
Dilution 1:2		44	56	99	102			30 [a]	69 [a]
	2	68		105					
Dilution 1:1		65 [b]	67	165 [b]	168			41 [a]	135 [a]
	1	68 [b]		171 [b]					
4-NPDA 0,5 µg	—	110	115	[c]				89 [a]	
		120							
2-AA 3 µg		[c]		1 194	1 194				1 161 [a]
	—			1 194					

[a] Mutagenic effect.

[b] Background growth reduced.

[c] Not tested.

Bibliography

[1] ISO 5667-10, *Water quality — Sampling — Part 10: Guidance on sampling of waste waters*

[2] ISO 13829, *Water quality — Determination of the genotoxicity of water and waste water using the umu-test*

[3] AMES, B.N.: Identifying environmental chemicals causing mutations and cancer. *Science* **204**, 587-593, 1979

[4] AMES, B.N., DURSTON, W.E., YAMASAKI, E. and LEE, F.D.: Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Nat. Acad. Sci. (USA)* **70**, 2281-2285, 1973a

[5] AMES, B.N., LEE, F.D. and DURSTON, W.E.: An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc. Nat. Acad. Sci. (USA)* **70**, 782-786, 1973b

[6] AMES, B.N., MCCANN, J. and YAMASAKI, E.: Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Res.* **31**, 347-364, 1975

[7] MARON, D.M. and AMES, B.N.: Revised methods for the Salmonella mutagenicity test. *Mutation Res*. **113**, 173-215, 1983

[8] OECD Guidelines for Testing of Chemicals No. 471, *Bacterial Reverse Mutation Test*, Adopted 21st July 1997

[9] Health Effects Test Guidelines; United States Environmental Protection Agency; *Prevention, Pesticides and Toxic Substances* (7101); EPA712-C-98-247, August 1998

[10] Commission Directive 2000/32/EC of 19 May 2000, Official Journal of the European Communities of 8.6.2000, L136/57-L136/64, B.13/14. *Mutagenicity — Reverse Mutation Test Bacteria*

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