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

Qualité de l'eau — Évaluation de la génotoxicité des eaux résiduaires — Essai de Salmonella/microsome (essai d'Ames-fluctuation)

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Foreword

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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.

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ISO 11350 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

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

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This 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 waste water using the bacterial strains *Salmonella enterica* subsp. *enterica* serotype Typhimurium TA 98 and TA 100 in a fluctuation assay. This combination of strains is able to measure the genotoxicity of chemicals that induce point mutations (base pair substitutions and frameshift mutations) in genes coding for enzymes that are involved in the biosynthesis of the amino acid, histidine.

NOTE 1 ISO 13829[8] applies for the measurement of genotoxicity of samples containing DNA-crosslinking agents.

This method is applicable to:

— fresh water;

— waste water;

— aqueous extracts and leachates;

— eluates of sediments (fresh water);

— pore water;

— aqueous solutions of single substances or of chemical mixtures;

— drinking water.

NOTE 2 When testing drinking water, extraction and pre-concentration of water samples can prove necessary.

2 Normative references

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

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 7027, *Water quality — Determination of turbidity*

3 Terms and definitions

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

3.1
cofactor solution
aqueous solution of chemicals (e.g. NADP, glucose-6-phosphate, and inorganic salts) needed for the activity of the enzymes in the S9 fraction

[Source: ISO 21427-2:2006,[10] definition 3.2]

3.2
culture medium
nutrients presented in a form and phase (liquid or solidified) which support microbiological growth

[Source: ISO 6107-6:2004,[6] definition 24]

3.3
dilution level
D
denominator of the dilution coefficient (using the numerator 1) of a mixture of water or waste water with dilution water as integral number

NOTE 1 to entry: For undiluted water or waste water, this coefficient per definition is 1→1. [In this International Standard, the arrow indicates the transition from initial total volume to final total volume.] The corresponding and smallest possible value of *D* is 1.

[Source: ISO 6107-6:2004,[6] definition 28]

3.4
lowest ineffective dilution
LID
lowest dilution within a test batch which does not show any effect, i.e. no statistically significant increase in the number of revertant wells compared with the negative control

NOTE 1 to entry: LID is determined for each incubation condition (strain, ±S9 mix). The highest LID value is decisive for the overall assessment.

3.5
induction rate
difference between the mean value of wells with revertant growth counted on the plates treated with a dose of the test sample or with a positive control, and the mean value of the corresponding wells treated with the negative control using the same strain under identical conditions

[Source: ISO 6107-6:2004,[6] definition 43, modified: “wells with revertant growth” replaces “mutant colonies”; “corresponding wells” replaces “corresponding plates”]

3.6
inoculum
fraction of a culture of microorganisms used to start a new culture, or an exponentially growing preculture, in fresh medium

[Source: ISO 6107-6:2004,[6] definition 44]

3.7
negative control
dilution water without test sample

[Source: ISO 6107-6:2004,[6] definition 51]

3.8
revertant growth
visible mutant colonies on the microplate at the end of the respective test

3.9
overnight culture
culture started late in the afternoon and incubated overnight (usually about 16 h) to be ready during the following morning for purposes such as the inoculation of a preculture

[Source: ISO 6107-6:2004,[6] definition 54]

NOTE 1 to entry For specification, see 9.1.

3.10
positive control
any well characterized material and/or substance, which, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriate positive or negative response in the test system

[Source: ISO 10993-12:—,[7] definition 3.12]

NOTE 1 to entry The positive controls mentioned in this International Standard are dissolved in dimethyl sulfoxide (DMSO) prior to use. For the purposes of this International Standard, the positive controls are known mutagens which are suitable for the verification of the sensitivity of the method and/or the activity of the S9 mix.

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

[Source: ISO 6107-6:2004,[6] definition 74]

3.12
S9 mix
mixture of S9 fraction and cofactor solution

[Source: ISO 6107-6:2004,[6] definition 75]

3.13
stock culture
culture of a strain of organisms maintained under conditions to preserve original features such as nucleotide sequences

[Source: ISO 6107-6:2004,[6] definition 87]

3.14
test sample
undiluted, diluted or otherwise prepared portion of a sample to be tested, after completion of all preparation steps such as centrifugation, filtration, homogenization, pH adjustment and determination of ionic strength

[Source: ISO 6107-6:2004,[6] definition 92]

4 Interferences

Bacteriotoxic effects of the test sample can lead to a reduction of viable bacteria and to a reduction of wells with revertants due to a repression of revertant growth.

This method includes sterile filtration of water and waste water prior to the test. Due to this filtration, solid particles are separated from the test sample. Thus, there is a possibility that genotoxic substances adsorbed on particles are not detected.

5 Principle

The bacteria are exposed under defined conditions to various concentrations of the test sample and incubated for 100 min at 37 °C ± 1 °C in 24 well plates. Due to this exposure, genotoxic agents enclosed in the test sample can induce mutations in one or both marker genes of the bacterial strains used (hisG46 for TA 100 and hisD3052 for TA 98) in correlation with the applied concentrations. Induction of mutations causes a concentration-related increase in the number of mutant colonies.

After exposure of the bacteria, reversion indicator medium (7.40), containing the pH indicator dye bromocresol purple (7.7), is added to the wells. Subsequently, the batches are distributed to 384 well plates (48 wells for each parallel) and incubated for 48 h to 72 h (9.3.2, 9.3.3).

Mutagenic activity of the test sample is determined by counting the number of purple to yellow shifted wells (per 48 wells of each parallel), treated with the undiluted or the diluted test sample, compared to the negative control.

The lowest dilution ($1 \rightarrow N$) of the test sample which induces no mutagenic effect under all experimental conditions (if any mutagenic effect is induced by the test sample) is the criterion for evaluating the mutagenic potential. Sample dilutions above this ($1 \rightarrow A$, $A < N$) shall induce a mutagenic effect according to the criteria of this International Standard in at least one strain under at least one activation condition (with or without addition of S9 mix). The respective LID value is N. If no mutagenic effect is observed under all experimental conditions, this dilution is $1 \rightarrow 1$ and the respective LID value is 1.

6 Apparatus and materials

6.1 Temperature- and time-controlled incubator, 37 °C ± 1 °C.

6.2 pH meter.

6.3 Analytical balance.

6.4 Steam sterilizer.

6.5 Dry sterilizer.

6.6 Magnetic stirrer.

6.7 Rotary mixer.

6.8 Freezer, capable of being maintained at ≤ –18 °C and at ≤ –70 °C.

6.9 Pipettes, 0,1 ml, 0,5 ml, 1 ml, 2 ml, 5 ml, 10 ml and 25 ml, of glass or plastics.

6.10 Storage bottles, 250 ml and 1 000 ml.

6.11 Measuring cylinders, 100 ml and 200 ml.

6.12 Volumetric flasks, 20 ml, 200 ml and 500 ml.

6.13 Sterile filters, 0,2 µm and 0,45 µm.

6.14 Erlenmeyer flasks, 50 ml, 100 ml and 250 ml.

6.15 Inoculating loops.

6.16 Eight-channel multistepper pipette (repeater pipette).

6.17 Eight-channel pipettes, 5 µl to 50 µl and 50 µl to 300 µl.

6.18 Spectrophotometer.

6.19 Transparent sterile polystyrene 24 well and 384 well plates with flat bottom and lid.

6.20 Microplate photometer for 24 well plates and optionally for 384 well plates, filters: 420 nm ± 15 nm and 595 nm ± 10 nm.

6.21 Clean bench.

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

6.23 Cryogenic vials, sterile, 1 ml, 10 ml.

7 Reagents, media and dilutions

7.1 General. As far as possible, use "reagent grade" chemicals. If hydrates of anhydrous compounds or hydrates different from those specified are used, ensure that the appropriate mass of the main compound is employed.

When necessary, autoclave for 20 min at 121 °C ± 2 °C. Cover vessels loosely (e.g. with aluminium foil). Never seal air-tight.

7.2 Water, grade 1, as defined in ISO 3696, or water with a conductivity of ≤5 µS/cm.

If sterile water is needed, sterilize by sterile filtration (0,2 µm) or autoclaving. Water as specified here is also used for the stepwise dilution of the test sample.

7.3 Tester strains. Use mutant strains of *Salmonella* Typhimurium LT2, which enable detection of point mutations, to determine the mutagenic potential of a test sample. Since point mutations can be subdivided into two classes (frameshift mutations and base pair substitutions), the two tester strains TA 98 and TA 100 are used. TA 98 contains as a marker the frameshift mutation (+2 type) hisD3052, whereas TA 100 bears the base pair substitution hisG46.

In addition, both strains shall have the following genetic properties:

— they contain the plasmid pKM101, coding for ampicillin resistance;

— they are all deep rough, e.g. partly deficient in lipopolysaccharide side chains, enabling also larger molecules to penetrate the bacterial cell wall and to cause mutations;

— due to a mutation in *uvrB*, the capability of the tester strains to repair DNA-damage is limited and the likelihood that DNA-damage results in mutations is increased.

NOTE The use of additional tester strains is described in Annex K.

7.4 2-Aminoanthracene (2-AA), $C_{14}H_{11}N$, CAS No: 613-13-8.

7.5 Ampicillin sodium salt, $C_{16}H_{18}N_3NaO_4S$, CAS No: 69-52-3.

7.6 D-Biotin, $C_{10}H_{16}N_2O_3S$, CAS No: 58-85-5.

7.7 Bromocresol purple, sodium salt, CAS No: 62625-30-3.

7.8 Citric acid monohydrate, $C_6H_8O_7 \bullet H_2O$, CAS No: 5949-29-1.

7.9 Dimethylsulfoxide, DMSO, C_2H_6SO, CAS No: 67-68-5.

7.10 D-Glucose, anhydrous, $C_6H_{12}O_6$, CAS No: 50-99-7.

7.11 D-Glucose-6-phosphate disodium salt hydrate, G-6-P-Na_2, $C_6H_{11}Na_2O_9P \bullet 2H_2O$ CAS No: 3671-99-6.

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

7.13 Magnesium chloride hexahydrate, $MgCl_2 \bullet 6H_2O$, CAS No: 7791-18-6.

7.14 Magnesium sulfate heptahydrate, $MgSO_4 \bullet 7H_2O$, CAS No: 10034-99-8.

7.15 Potassium chloride, KCl, CAS No: 7447-40-7.

7.16 Dipotassium hydrogenphosphate, K_2HPO_4, CAS No: 7758-11-4.

7.17 Sodium ammonium hydrogenphosphate tetrahydrate, $NaNH_4HPO_4 \bullet 4H_2O$, CAS No: 7583-13-3.

7.18 Sodium chloride, NaCl, CAS No: 7647-14-5.

7.19 Sodium dihydrogenphosphate, anhydrous, NaH_2PO_4, CAS No: 7558-80-7.

7.20 Disodium hydrogenphosphate, anhydrous, Na_2HPO_4, CAS No: 7558-79-4.

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

7.22 β-Nicotinamide adenine dinucleotide phosphate sodium salt, $NADP \cdot H_2O$, $C_{21}H_{27}N_7NaO_{17}P_3 \cdot H_2O$, CAS No: 698999-85-8.

7.23 Nitrofurantoin (NF), CAS No: 67-20-9.

7.24 4-Nitro-*o*-phenylenediamine (4-NOPD), CAS No: 99-56-9.

7.25 Nutrient broth powder.[1)]

7.26 S9 fraction (liver homogenate; induced by phenobarbital/β-naphthoflavone).[1)]

7.27 L-Histidine, $C_6H_9N_3O_2$, CAS No: 71-00-1.

7.28 Phosphate buffer.

7.28.1 Sodium dihydrogenphosphate buffer, c(NaH_2PO_4) = 0,2 mol/l.

Dissolve 14,39 g NaH_2PO_4 (or 16,55 g $NaH_2PO_4 \bullet H_2O$) in 600 ml of water (7.2).

1) This reagent is commercially available. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

7.28.2 Disodium hydrogenphosphate buffer, $c(Na_2HPO_4) = 0{,}2$ mol/l. Dissolve 28,39 g Na_2HPO_4 in 1 000 ml of water (7.2).

Add sodium dihydrogenphosphate buffer (7.28.1) to disodium hydrogenphosphate buffer (7.28.2) until a pH value of 7,4 is reached and autoclave. Store at room temperature in the dark. The solution is stable for at least 1 year.

7.29 D-Biotin solution. Dissolve 12,2 mg D-biotin (7.6) in 100 ml of water (7.2) by boiling up. After cooling, sterilize by filtration (0,2 µm filter). Store 10 ml aliquots at −18 °C or below in sterile cryogenic vials (6.23). Aqueous solutions stored as frozen aliquots are stable for at least 1 year.

7.30 L-Histidine solution. Dissolve 50 mg of L-histidine (7.27) in 50 ml of water (7.2) and sterilize by filtration (0,2 µm filter). Store 1,5 ml aliquots at −18 °C or below in sterile cryogenic vials (6.23). Aqueous solutions stored as frozen aliquots are stable for at least 1 year.

7.31 Glucose-6-phosphate solution. Dissolve 0,68 g of D-glucose-6-phosphate (7.11) in 10 ml of water (7.2) and sterilize by filtration (0,2 µm). Store aliquots (e.g. 200 µl) at −18 °C or below in sterile cryogenic vials (6.23). Aqueous solutions stored as frozen aliquots are stable for at least 1 year.

7.32 NADP solution, $c(NADP) = 0{,}04$ mol/l. Dissolve the appropriate mass of NADP in 10 ml of water (7.2) to obtain a final concentration of 0,04 mol/l and sterilize by filtration (0,2 µm). Store aliquots (e.g. 700 µl) at −18 °C or below in sterile cryogenic vials (6.23). Aqueous solutions stored as frozen aliquots are stable for at least 1 year.

Various hydrates of NADP are available. The actual molecular weight is specified in the product data sheet. Calculate the amount of NADP required according to the molecular weight given.

7.33 Potassium chloride solution. Dissolve 74,56 g of KCl (7.15) in 1 000 ml of water (7.2) and autoclave. Store at room temperature. The solution is stable for at least 1 year.

7.34 $MgCl_2 \bullet 6H_2O$ solution. Dissolve 50,83 g of $MgCl_2 \bullet 6H_2O$ (7.13) in 1 000 ml of water (7.2) and autoclave the solution. Store at room temperature. The solution is stable for at least 1 year.

7.35 Bromocresol purple solution. Dissolve 51 mg of bromocresol purple sodium salt (7.7) in 30 ml of water (7.2). Prepare this solution freshly before addition to the reversion indicator medium (7.40).

7.36 Ampicillin solution. Dissolve 500 mg of ampicillin (7.5) in 10 ml of water (7.2) and sterilize by filtration (0,2 µm filter). Store 500 µl aliquots at −18 °C or below in sterile cryogenic vials (6.23). The solution is stable for at least 6 months.

7.37 Growth medium. Dissolve 4,7 g of nutrient broth powder[2)] and 0,31 g of sodium chloride (7.18) in 200 ml of water (7.2). Adjust the pH to 7,5 ± 0,1. Add water (7.2) to 250 ml and autoclave the solution.

The following final concentrations in the growth medium shall result:

— 7,5 g/l meat extract;

— 7,5 g/l peptone;

— 5,0 g/l sodium chloride.

Solutions stored under sterile conditions as frozen aliquots are stable for at least 1 year.

2) Use nutrient broth powder containing 40 % meat extract, 40 % peptone, and 20 % sodium chloride.

7.38 Exposure medium. Prepare a medium for incubation of bacteria with the sample containing a low amount of L-histidine to support a few cell divisions.

Dissolve *consecutively* the following ingredients in 900 ml water:

— 0,2 g magnesium sulfate heptahydrate (7.14);

— 2,0 g citric acid (7.8);

— 10,0 g dipotassium hydrogenphosphate (7.16);

— 3,5 g sodium ammonium hydrogenphosphate tetrahydrate (7.17);

— 4,0 g D-glucose (7.10).

Add water (7.2) to 1 000 ml, adjust the pH to 7,0 ± 0,2, if necessary, and sterilize by filtration (0,2 µm filter). Store the medium at 2 °C to 8 °C.

Add, per 100 ml, 0,6 ml of D-biotin solution (7.29) and 0,1 ml of L-histidine solution (7.30) under sterile conditions. Prepare only the amount of medium necessary for the next 2 weeks. Store the medium at 2 °C to 8 °C.

7.39 Exposure medium concentrate. Dissolve *consecutively* the following ingredients in 70 ml water:

— 0,2 g magnesium sulfate heptahydrate (7.14);

— 2,0 g citric acid (7.8);

— 10,0 g dipotassium hydrogenphosphate (7.16);

— 3,5 g sodium ammonium hydrogenphosphate tetrahydrate (7.17);

— 4,0 g D-glucose (7.10).

Add water (7.2) to 93 ml, adjust the pH, if necessary, and sterilize by filtration (0,2 µm filter). Store the medium concentrate at 2 °C to 8 °C.

Add 6 ml of D-biotin solution (7.29) and 1 ml of L-histidine solution (7.30) under sterile conditions. Prepare only the amount of medium necessary for the next 2 weeks. Store the medium concentrate at 2 °C to 8 °C.

7.40 Reversion indicator medium. Prepare a pH indicator medium without L-histidine.

7.40.1 Solution I. Dissolve the following ingredients in 950 ml water in the given order:

— 0,4 g magnesium sulfate heptahydrate (7.14);

— 4,0 g citric acid (7.8);

— 20,0 g dipotassium hydrogenphosphate (7.16);

— 7,0 g sodium ammonium hydrogenphosphate tetrahydrate (7.17).

Add water (7.2) to 1 000 ml and add 30,0 ml of bromocresol purple solution (7.35). Adjust the pH to 7,3 ± 0,1. Transfer the solution one half each into two 1 000 ml flasks and autoclave.

7.40.2 Solution II. Dissolve 8,0 g of D-glucose (7.10) in 800 ml of water (7.2). Adjust the pH to 7,3 ± 0,1. Transfer both halves of the solution into two 1 000 ml flasks and autoclave.

7.40.3 Mixing and storage. After cooling to ambient temperature, mix 515 ml of solution I (7.40.1) with 400 ml of solution II (7.40.2) under sterile conditions. Add 20 ml of D-biotin solution (7.29) under sterile conditions to each flask.

Store the medium at room temperature in the dark. The medium is stable for at least 1 month.

7.41 Control solutions.

7.41.1 Negative controls. For preparation of the negative controls, always use the same solvent as for the samples to be tested. This is usually water (7.2) when testing water samples and DMSO (7.9) when testing chemicals.

7.41.2 Positive controls. In general, dissolve 10 mg of each positive control substance in 10 ml of DMSO (7.9). Prepare 50 µl aliquots as stock solutions in sterile cryogenic vials and store them at −18 °C or below. Under these conditions stock solutions are stable for at least 1 year. On the day of the test, unfreeze one aliquot.

7.41.3 Strain TA 98 without S9 mix. Use 4-nitro-*o*-phenylenediamine (4-NOPD) (7.24) as positive control substance for strain TA 98 without S9 mix.

Dilute the stock solution 1→2 with DMSO (7.9). This dilution is used in the test.

7.41.4 Strain TA 100 without S9 mix. Use nitrofurantoin (NF) (7.23) as a positive control substance for strain TA 100 without S9 mix.

Dilute the stock solution 1→80 with DMSO. This dilution is used in the test.

7.41.5 Strain TA 98 with S9 mix. Use 2-aminoanthracene (2-AA) (7.4) as a positive control substance for strain TA 98 with S9 mix.

Dissolve the stock solution 1→200 with DMSO. This dilution is used in the test.

7.41.6 Strain TA 100 with S9 mix. Use 2-aminoanthracene (2-AA) (7.4) as positive control substance for strain TA 100 with S9 mix.

Dissolve the stock solution 1→50 with DMSO. This dilution is used in the test.

8 Sampling and samples

Test the samples immediately after sampling. If this is not possible, keep water samples at 0 °C to 5 °C (for <48 h) or below −18 °C (for up to 2 months). For multiple testing divide larger 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 them. In this case, only the supernatant is processed further.

Sterilize all samples using sterile filters (0,45 µm). Homogenize test samples by thoroughly shaking before use.

Adjust the sample to a pH of 7,2 ± 0,2 using either HCl (7.12) or NaOH solution (7.21). Select the acid or alkali concentrations such that the added volumes are as small as possible. Avoid overtitration. Take into account the change in the sample's pH and resulting effects (see ISO 5667-16[5]).

Perform dilutions of the test sample as specified in Tables 2 and 3 with sterilized water (7.2).

9 Procedure

9.1 Overnight culture

Under sterile conditions, pipette 20 ml of growth medium (7.37) supplemented with 20 µl of ampicillin solution (7.36) into a 100 ml Erlenmeyer flask (6.14) hermetically sealed with caps or aluminium foil and mix by gentle agitation.

Add 20 µl of the respective test strain (TA 98 or TA 100) immediately after thawing. Incubate the culture at 37 °C ± 1 °C for 10 h. If the required cell density (9.3.1, G.1.1) is not reached, extend incubation time to 12 h. If the required cell density is still not reached after 12 h, inoculate a fresh overnight culture. A clock timer may