BS ISO 11350:2012

BSI Standards Publication

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

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee EH/3/5, Biological Methods.

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

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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.

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

INTERNATIONAL STANDARD ISO 11350:2012(E)

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 000g 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, $c(\text{NADP}) = 0{,}04$ 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

be used. Use a shaking frequency of about 150 r/min. From the inoculation of test bacteria to the beginning of incubation at 37 °C, ensure that the temperature of the incubation bath is 19 °C ± 4 °C.

9.2 Preparation of S9 mix

Treatment for enzyme induction and preparation of the S9 fraction are described in Annex D. If the S9 fraction is purchased commercially, it shall also be prepared in accordance with Annex D.

Prepare the S9 mix freshly on the day of testing. Mix:

— 66 µl KCl solution (7.33);

— 64 µl $MgCl_2 \bullet 6H_2O$ solution (7.34);

— 50 µl D-glucose-6-phosphate solution (7.31);

— 200 µl NADP solution (7.32);

— 1 000 µl phosphate buffer (7.28);

— 20 µl water (sterile);

— 600 µl S9 fraction (Annex D).

This mixture is sufficient for two exposure plates. If more than two plates are required, increase the amount of S9 mix proportionally.

Keep the S9 mix permanently on ice for not more than 1 h and use it on the same day. Discard remaining S9 mix at the end of this day.

9.3 Testing of water samples

9.3.1 Preparation of tester strains

In the test cultures a cell density of 180 FAU[3)] for TA 98 and 45 FAU for TA 100 is recommended. Laboratory-specific adaptation of tester strain density may be necessary to achieve the number of negative control revertant wells as defined in Clause 10.

In the test culture, the tester strain inoculum is diluted 10-fold (Table 2 and Table 3). Therefore, adjust the cell density in the overnight culture inoculum to 1 800 FAU for TA 98 and 450 FAU for TA 100. Calculate the required dilution factor and the volume of 1× exposure medium that must be added to the overnight culture in order to adjust the cell density according to Formulae (1), (2), and (3).

For the determination of the actual cell density of the overnight culture, dilute 100 µl of the overnight culture with 900 µl growth medium (7.37), otherwise the FAU is out of range. Measure cell densities ($X_{595\ nm}$) of this dilution of the tester strains TA 98 and TA 100 immediately before exposure and calculate FAU values of this dilution using a FAU calibration curve in accordance with ISO 7027. Set up the calibration curve using $X_{595\ nm}$. Calculate the dilution factor according to Formulae (1) and (2):

In the case of strain TA 98:

$$d = \frac{X_{595\,nm}[\text{FAU}]}{180} \quad (1)$$

where

3) Formazine attenuation units (see ISO 7027).

d is the dilution factor;

$X_{595\ nm}$[FAU] is the FAU value of the 10-fold diluted overnight culture of strain TA 98 that is determined as described above.

In the case of strain TA 100:

$$d = \frac{X_{595\ nm}[\text{FAU}]}{45} \tag{2}$$

where

d is the dilution factor;

$X_{595\ nm}$[FAU] is the FAU value of the 10-fold diluted overnight culture of strain TA 100 that is determined as described above.

Use the calculated dilution factor to determine the required volume of 1× exposure medium that shall be added to the overnight culture in order to adjust the cell density according to Formula (3).

$$V_{add} = (V_{culture} d) - V_{culture} \tag{3}$$

where

d is the dilution factor according to Formula (1) or (2);

V_{add} is the volume of 1× exposure medium to be added to the overnight culture, in millilitres (ml);

$V_{culture}$ is the volume of the undiluted overnight culture, in millilitres (ml).

Table 1 shows a typical example for the adjustment of the cell density for a test with the strain TA 98.

Table 1 — Dilution of the overnight culture (TA 98) as an example

FAU of the 10-fold diluted overnight culture	**Dilution factor [according to Formula (1)]** d	**Volume of the overnight culture (*undiluted*)** $V_{culture}$	**Volume of the exposure medium to be added to the overnight culture** V_{add}	**Volume of the adjusted overnight culture for exposure (1 800 FAU)**
240	1,33	20 ml	6,6 ml	26,6 ml

9.3.2 Test culture without S9 mix

Prepare test cultures according to Table 2 using sterile 24-well plates (plate A) (6.19). For each culture, incubate at least three replicates. Perform under sterile conditions. An example for the configuration of plate A is given in Annex E.

Table 2 — Preparation of test culture plate A without S9 mix

Component	Negative control batch	Dilution level 1[a]	Dilution level 2	Dilution level 4	Positive control batch (see 7.41.2)
	µl	µl	µl	µl	µl
Exposure medium concentrate (7.39)	100	100	100	100	100
Dilution water (7.2)	800	0	300	550	780
Sample[b]	0	800	500	250	20
Adjusted overnight culture of tester strain TA 98 or TA 100 (9.3.1)	100	100	100	100	100
Total volume	1 000	1 000	1 000	1 000	1 000

[a] Dilution level 1: sample concentration 80 %; dilution level 2: sample concentration 50 %; dilution level 3: sample concentration 25 %. If more than three dilution levels are required, extend the dilution series by means of a graduated dilution (e.g. $D = 8, 16, 32$)

[b] As positive control substances, use 4-NOPD for strain TA 98 and NF for strain TA 100.

If calculation of cytotoxicity is performed (9.5), measure initial $X_{595\ nm}$ ($t = 0$ min) and final $X_{595\ nm}$ ($t = 100$ min) of plate A using a microplate photometer (6.20). It is recommended that only tester strain TA 98 be used for measurement of growth since cell density of TA 100 remains low.

Incubate plate A in the dark at 37 °C ± 1 °C for 100 min while shaking (150 r/min).

Pour 2,5 ml of reversion indicator medium (7.40) into each well of another 24 well plate (plate B) (6.19).

Immediately thereafter, transfer 500 µl of test culture from plate A into plate B by using a multistepper pipette (6.16). Mix thoroughly.

Subsequently, transfer the content of one well of plate B to 48 wells of a 384 well plate (plate C) (6.19) in 50 µl aliquots using a multistepper pipette (6.16). An example of this procedure is given in Annex E.

Incubate the 384 well plate in the dark at 37 °C ± 1 °C for 48 h without shaking. Avoid evaporation-promoting conditions (e.g. ventilation). For a low number of wells with revertant growth in the positive controls, extend the incubation time in 384 well plates to 72 h.

If calculation of cytotoxicity is not performed, fill plate A with half the volume of each ingredient (Table 2). After 100 min of incubation, directly add the reversion indicator medium (7.40) to plate A and mix thoroughly. Transfer the cultures to the 384-well plate and incubate as specified in the previous paragraph.

9.3.3 Test culture with S9 mix

Prepare test cultures according to Table 3 using sterile 24 well microplates (plate A) (6.19). For each culture, incubate at least three replicates. Perform under sterile conditions. An example for the configuration of plate A is given in Annex E.

Table 3 — Preparation of test culture plate A with S9 mix

Component	Negative control batch	Dilution level 1[a]	Dilution level 2	Dilution level 4	Positive control batch (7.41.2)
	µl	µl	µl	µl	µl
Exposure medium concentrate (7.39)	100	100	100	100	100
Dilution water (7.2)	800	0	300	550	780
Sample[b]	0	800	500	250	20
Adjusted overnight culture of tester strain TA 98 or TA 100 (9.3.1)	100	100	100	100	100
S9 mix (9.2)	34	34	34	34	34
Total volume	1 034	1 034	1 034	1 034	1 034

[a] Dilution level 1: sample concentration 80 %; dilution level 2: sample concentration 50 %; dilution level 3: sample concentration 25 %. If more than three dilution levels are required extend the dilution series by means of a graduated dilution (e.g. $D = 8, 16, 32$)

[b] As positive control substances, use 2-AA for strain TA 98 and TA 100.

If calculation of cytotoxicity is performed (9.5), measure initial $X_{595\ nm}$ ($t = 0$ min) and final $X_{595\ nm}$ ($t = 100$ min) of plate A using a microplate photometer (6.20). It is recommended that only tester strain TA 98 be used for measurement of growth since cell density of TA 100 remains low.

Incubate the plates in the dark at 37 °C ± 1 °C for 100 min while shaking (approximately 150 r/min).

Pour 2,5 ml reversion indicator medium (7.40) into each well of another 24 well plate (plate B) (6.19).

Immediately thereafter, transfer 500 µl of test culture from plate A into plate B by using a multistepper pipette (6.16). Mix thoroughly.

Then, transfer the content of one well of plate B to 48 wells of a 384 well plate (plate C) (6.19) in 50 µl aliquots using a multistepper pipette (6.16). An example of this procedure is given in Annex E.

Incubate the 384 well plate in the dark at 37 °C ± 1 °C for 48 h without shaking. Avoid evaporation-promoting conditions (e.g. ventilation). For a low number of wells with revertant growth in the positive controls, extend the incubation time in 384 well plates to 72 h.

If calculation of cytotoxicity is not performed, fill plate A with half of the volume of each ingredient (Table 3). After 100 min of incubation, directly add reversion indicator medium (7.40) to plate A and mix thoroughly. Transfer to the 384 well plate and incubate as specified in the previous paragraph.

9.4 Measurement of revertant growth

Score each 384 well plate for the number of positive (yellow) and negative (purple) wells in each 48 well area. Plate scoring may be performed manually or by using a 384 well plate photometer (420 nm ± 10 nm).

9.5 Calculation of cytotoxicity

For calculation of cytotoxicity, use $X_{595\ nm}$ values as measured in 9.3.2 and 9.3.3. Calculate mean values $X_{595\ nm} \pm s$, where s is the standard deviation (SD), for sample wells and negative control wells. Calculate cytotoxicity, C, expressed as a percentage, %, according to Formula (4).

$$C = 100 - 100\left(\frac{X_{S100} - X_{S0}}{X_{NC100} - X_{NC0}}\right) \quad (4)$$

where

X_{S0} is the $X_{595\ nm}$ value of the sample at $t = 0$ min;

X_{S100} is the $X_{595\ nm}$ value of the sample at $t = 100$ min;

X_{NC0} is the $X_{595\ nm}$ value of the negative control batch at $t = 0$ min;

X_{NC100} is the $X_{595\ nm}$ value of the negative control batch at $t = 100$ min.

10 Validity criteria

The test is valid if

a) the mean value for negative controls is ≥0 and ≤10 wells with revertant growth per 48 well area at all testing conditions (±S9 mix, tester strains TA 98 and TA 100);

b) the mean value for positive controls is ≥25 wells with revertant growth per 48 well area at all testing conditions (±S9 mix, tester strains TA 98 and TA 100).

If one or both of these criteria are not met, a part of the test (e.g. only one testing condition) or the entire test is invalid.

11 Assessment criteria

The test sample is regarded as mutagenic in accordance with this International Standard if a significant concentration-related increase of revertant wells over the concentration range tested and/or a reproducible increase at one or more concentrations in the number of revertant wells per 48 well area in at least one strain with or without S9 mix occurs.

An example of statistical assessment is given in Annex I.

12 Test report

This test report shall contain at least the following information:

a) the test method used, together with a reference to this International Standard (ISO 11350:2012);

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

c) negative and positive control substances (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 g and time), filtration (including filter material and pore size) and other manipulations);

e) test strains [strain, source, date of arrival in the laboratory, storage conditions, date of stock culture preparation, and date of genotype checking (if this date deviates from stock culture preparation)], obtained $X_{595\ nm}$ of the overnight culture, adjusted $X_{595\ nm}$ of the inoculum);

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

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

h) incubation time and test results [individual numbers of wells with revertant growth per treatment (Annex F), induction rate (Annex F), indication of cytotoxicity (if any), statistical evaluation, LID values, other observations (e.g. precipitation, bacterial growth without colour shift].

Annex A
(normative)

Nutrient broth and agar

A.1 Nutrient broth

Nutrient broth is commercially available. For reasons of better standardization, the use of commercial products is recommended. Make sure that the commercial product contains sodium chloride.

A.2 Agar

Agar is commercially available in different qualities. The use of Difco[4)] agar or a product of equal quality is recommended.

A.3 Nutrient agar

Nutrient agar is commercially available in different qualities. The use of Difco[4)] nutrient agar or a product of equal quality is recommended.

4) Difco agar is the trade name of a product supplied by Becton Dickinson. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

Annex B
(normative)

Preparation of ampicillin agar plates and stock cultures

B.1 Preparation of ampicillin agar plates

Perform the preparation of ampicillin agar plates under sterile conditions.

Dissolve 1,5 g Bacto[5)] agar, 1,88 g nutrient broth powder (7.37), and 0,124 g NaCl (7.18) in 100 ml water, stir and autoclave. Cool in a controlled way to 38 °C ± 2 °C and add 100 µl of ampicillin solution (7.36). Fill Petri dishes (6.22) with 25 ml of nutrient ampicillin agar. Remove lid immediately before pouring into the plate and close lid immediately thereafter. After solidification of agar, incubate plates at 37 °C ± 2 °C for an appropriate time to remove excess water and avoid condensation during the test.

B.2 Preparation of stock cultures

Prepare stock cultures under sterile conditions.

Immediately upon receipt, spread aliquots of the respective bacterial strain on to the surface of ampicillin agar plates (B.1) to get single colonies. Incubate the plates 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 (Annex A).

Incubate the bacterial suspension overnight at 37 °C ± 1 °C.

Spread samples of these cultures on to the surface of ampicillin nutrient agar plates (B.1) to get single colonies. Incubate the plates for 24 h at 37 °C ± 1 °C.

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

Take a small sample of the bacterial suspension to check the genotype (Annex C).

Stock cultures shall fulfil the requirements of Annex C. Otherwise they shall be discarded.

Mix the remaining parts of the cultures with DMSO (1,8 ml per 20 ml of culture volume) or sterile glycerol (4 ml per 20 ml of culture volume) to protect against freezing effects, and freeze immediately below –70 °C in small aliquots (0,5 ml to 1 ml).

Whenever new stock cultures need to be prepared, repeat the steps as described in the preceding by using a frozen stock culture from a former preparation.

5) Bacto agar is the trade name of a product supplied by Becton Dickinson. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

Annex C
(normative)

Checking of genotype

C.1 Ampicillin resistance (pKM101)

Spread parallel lines of bacterial suspension of the respective strain on two ampicillin plates (B.1). 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 98 and TA 100 (Annex B).

C.2 Crystal-violet sensitivity (deep rough)

Spread 0,1 ml of individual stock cultures on to nutrient agar plates (B.1, without ampicillin). Use four plates per strain. After a few minutes, place filter papers (diameter 9 mm), spiked with 10 µl of aqueous crystal-violet solution (concentration of 1 mg/ml), in the middle of the plates. Incubate the plates overnight at 37 °C ± 2 °C. Measure the diameters of the inhibition zones formed. For an acceptable stock batch, the mean value of the inhibition zone should be at least 14 mm.

C.3 UV sensitivity (*uvrB*)

Spread 0,1 ml of individual stock cultures on to nutrient agar plates (B.1, without ampicillin). Use four plates per strain. Cover half of each plate with an aluminium foil and irradiate without a lid for 8 s with UV light (30 W) of a wavelength of 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 should show growth inhibition on the irradiated half of the plate.

C.4 Histidine requirement

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

Annex D
(normative)

S9 fraction[6)]

D.1 Induction of liver enzymes

For enzyme induction, at least six male rats (e.g. Sprague-Dawley rats), of body mass about 200 g to 300 g, receive 80 mg/kg body mass phenobarbital intraperitoneally and 80 mg/kg body mass β-naphthoflavone orally on three consecutive days simultaneously in appropriate vehicles. The livers are prepared 24 h after the last treatment. In case of polychlorinated biphenyl induction, the rats receive a single intraperitoneal injection of a polychlorinated biphenyl[7)] dissolved in an appropriate vehicle at a dose of 500 mg/kg body mass 5 d prior to liver preparation.

Animals should be housed on absorbent softwood bedding in adequately marked cages, which are only used for this purpose. Animal husbandry should be standardized. Animal room should be maintained at 20 °C to 23 °C and approximately 60 % relative humidity. Lighting should consist of a controlled 12 h light/dark cycle, and air should be exchanged at least 10 times per hour. The animals should be given free access to an appropriate standard diet and water of drinking quality.

D.2 Preparation of S9 fraction

Livers are removed under sterile conditions immediately after euthanasia and kept at 4 °C ± 1 °C until all animals have been prepared. All other steps are carried out under sterile conditions at 4 °C ± 1 °C.

Wash the livers with cold (4 °C ± 1 °C) 0,15 mol/l KCl solution (approximately 1 ml KCl per 1 g liver). Homogenize the livers in fresh, cold (4 °C ± 1 °C) 0,15 mol/l KCl (approximately 3 ml KCl per 1 g liver). Centrifuge the homogenate in a refrigerated centrifuge at 4 °C ± 1 °C and 9 000g for 10 min. Store small aliquots (e.g. 1 ml) of the supernatant (the S9 fraction) in sterile cryogenic vials below −70 °C.

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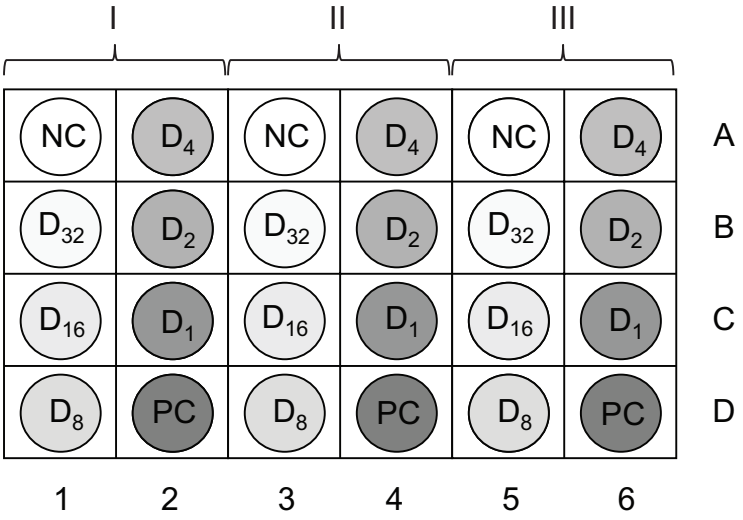
6) This reagent is commercially available. This information is given for the convenience of users of this International Standard and not not constitute an endorsement by ISO of these products.

7) 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 E
(informative)

Example for application of samples on a 24 well plate

NOTE D_1 to D_{32} are different samples or different dilution levels of one or more samples; NC: negative control; PC: positive control.



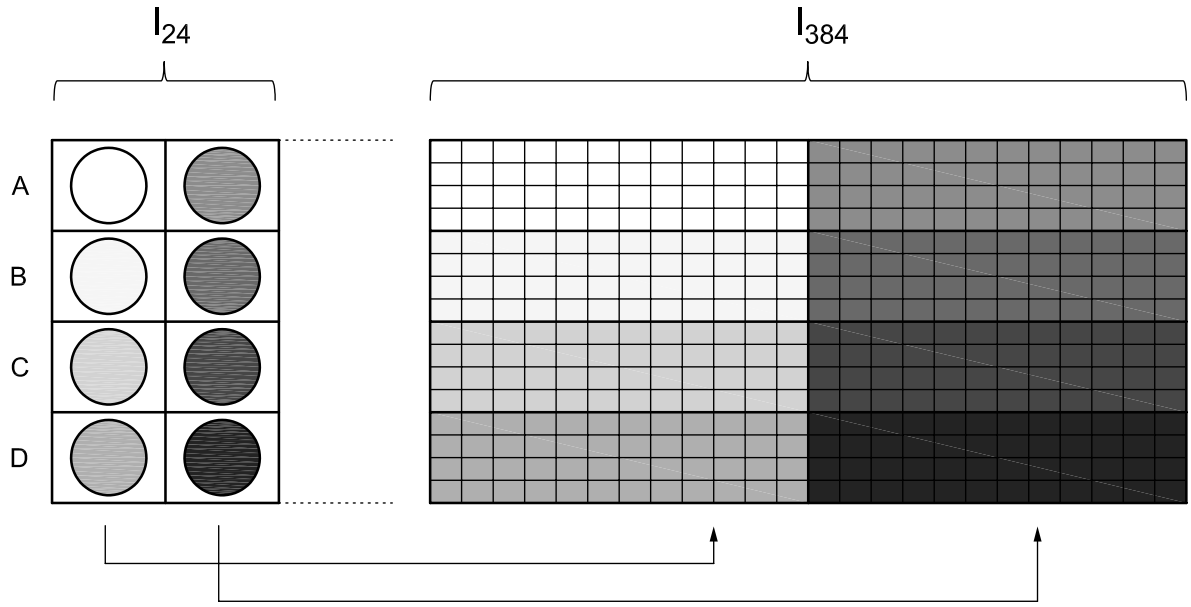
Key

I 1st replicate

II 2nd replicate

III 3rd replicate

Figure E.1 — Incubation plate A (24 wells)



Key

I_{24} 24 well plate, 1st replicate

I_{384} 384 well revertant selection plate, 1st replicate

Figure E.2 — Example of transfer from a 24 well plate (plate A or B) to a 384 well plate (with six dilution levels)

Annex F
(informative)

Example for reporting

Furhter details for Table F.1:

Strain: TA 100

Testing condition: Incubation with addition of S9 mix

Sample: NAME

Table F.1 — Example of a table for reporting

Sample	Dilution level	Number of wells with revertant growth			Mean	Mutant induction rate (3.8)	Mutant induction factor
	D	Replicate 1	Replicate 2	Replicate 3			
NC[a] water		8	7	4	6,3		1
Sample 1	4	12	10	14	12	5,7	1,9
	2	26	22	21	23	16,7	3,7
	1	39	41	35	38,3	32	6,1
Sample 2	4	2	7	9	6,0	−0,3	0,95
	2	8	8	9	8,3	2,0	1,3
	1	12	5	7	8,0	1,7	1,3
PC[b] 2-AA[c]		32	27	25	28	21,7	4,4

[a] Negative control batch.

[b] Positive control batch.

[c] 2-Aminoanthracene.

Annex G
(informative)

Testing of chemicals

G.1 General

Perform overnight culture according to 9.1.

G.2 Preparation of tester strains

In the test cultures, cell densities of 180 FAU for TA 98 and 45 FAU for TA 100 are recommended. Laboratory-specific adaptation of tester strain density may be necessary to achieve the number of negative control wells with revertant growth as defined in Clause 10.

Calculate the required dilution factor and the volume of 1× exposure medium required for addition to the overnight culture in order to adjust the cell density according to Equations (G.1), (G.2) and (G.3).

For the determination of the actual cell density of the overnight culture, dilute 100 µl of the overnight culture with 900 µl growth medium (7.37), otherwise the FAU is out of range. Measure cell densities ($X_{595\ nm}$) of this dilution of the tester strains TA 98 and TA 100 immediately before exposure and calculate FAU values of this dilution using a FAU calibration curve in accordance with ISO 7027. Calculate the dilution factor according to Equations (G.1) and (G.2):

In the case of strain TA 98:

$$d = \frac{X_{595\,nm}[\text{FAU}]}{18} \tag{G.1}$$

where

d is the dilution factor;

$X_{595\,nm}$[FAU] is the FAU value of the 10-fold diluted overnight culture of strain TA 98 that is determined as described above.

In the case of strain TA 100:

$$d = \frac{X_{595\,nm}[\text{FAU}]}{4{,}5} \tag{G.2}$$

where

d is the dilution factor;

$X_{595\,nm}$[FAU] is the FAU value of the 10-fold diluted overnight culture of strain TA 100 that is determined as described above.

Use the calculated dilution factor to determine the required volume of 1× exposure medium required for addition to the overnight culture in order to adjust the cell density according to Equation (G.3).

$$V_{add} = (V_{culture} d) - V_{culture} \tag{G.3}$$

where

d is the dilution factor according to Equation (G.1) or (G.2);

V_{add} is the volume of 1× exposure medium to be added to the overnight culture, in millilitres (ml);

$V_{culture}$ is the volume of the undiluted overnight culture, in millilitres (ml).

Table G.1 — Preparation of tester strain TA 98 (example)

FAU of the 10-fold diluted overnight culture	**Dilution factor** [according to Equation (G.1)] d	**Volume of the overnight culture (undiluted)** $V_{culture}$ ml	**Volume of the adjusted overnight culture for exposure (180 FAU)** ml	**Volume of the exposure medium** (7.38) **for dilution to be added to the overnight culture** V_{add} ml
240	13,3	4	53,3	49,3

G.3 Test culture without S9 mix for testing of chemicals

Prepare test cultures according to Table G.2. Use sterile 24-well microplates (plate A) (6.19). Perform under sterile conditions. An example for configuration on the plate is given in Annex E.

Table G.2 — Preparation of test culture plate A without S9 mix

Component	**Negative control batch** µl	**Chemicals dilutions** µl	**Positive control batch** (7.41.2) µl
Adjusted overnight culture of tester strain TA 98 or TA 100 (9.3.2)	980	980	980
Negative control[a]	20	—	—
Chemical dissolved in an appropriate solvent[b]	—	20	—
Positive control[c]	—	—	20
Total volume	1 000	1 000	1 000

[a] As negative control, use the solvent. Usually, DMSO is used as the solvent for chemicals.

[b] Ensure that each dilution contains the same solvent concentration.

[c] As positive control substance, use 4-NOPD for strain TA 98 and NF for strain TA 100.

If calculation of cytotoxicity is performed (9.5), measure initial $X_{595\ nm}$ ($t = 0$ min) and final $X_{595\ nm}$ ($t = 100$ min) of plate A using a microplate photometer (6.20). It is recommended that only tester strain TA 98 be used for measurement of growth, since cell density of TA 100 remains low.

Incubate plate A in the dark at 37 °C ± 1 °C for 100 min while shaking (about 150 r/min).

Pour 2,5 ml of reversion indicator medium (7.40) into each well of another 24 well plate (plate B) (6.19).

Proceed according to 9.3.

G.4 Test culture with S9 mix for testing of chemicals

Prepare test cultures according to Table G.3. Use sterile 24 well microplates (6.19). Proceed under sterile conditions. An example for configuration on the plate is given in Annex E.

Table G.3 — Preparation of test culture plate A with S9 mix

Component	Negative control batch µl	Chemicals dilutions µl	Positive control batch (7.41.2) µl
Adjusted overnight culture of tester strain TA 98 or TA 100 (9.3.2)	980	980	980
S9 mix (9.2)	34	34	34
Negative control[a]	20	—	—
Chemical dissolved in an appropriate solvent[b]	—	20	—
Positive control[c]	—	—	20
Total volume	1 034	1 034	1 034

[a] As negative control, use the solvent. Usually DMSO is used as the solvent for chemicals.

[b] Ensure that each dilution contains the same solvent concentration.

[c] As positive control substance, use 2-AA for both strains.

If calculation of cytotoxicity is performed (9.5), measure initial $X_{595\ nm}$ ($t = 0$ min) and final $X_{595\ nm}$ ($t = 100$ min) of plate A using a microplate photometer (6.20). It is recommended that only tester strain TA 98 be used for measurement of growth since cell density of TA 100 remains low.

Incubate plate A in the dark at 37 °C ± 1 °C for 100 min while shaking (about 150 r/min).

Pour 2,5 ml of reversion indicator medium (7.40) into each well of another 24 well plate (plate B) (6.19).

Proceed according to 9.3.

Annex H
(informative)

Precision data

An international round-robin study based on the procedure described in this International Standard was carried out in 2010. A total of 18 laboratories from six countries participated in the round-robin test. Four water samples were analysed (sample 1: river water; sample 2: river water prepared by adding two different genotoxicants; sample 3: river water prepared by adding one genotoxicant; sample 4: waste water effluent, not spiked). The results are shown in Tables H.1 to H.3.

Table H.1 — Valid test results from all laboratories with satisfactory repeatability

Strain	TA98		TA100	
S9 mix	–	+	–	+
Positive control	100 %	93,8 %	100 %	100 %
Negative control	100 %	100 %	91,7 %	95 %

Table H.2 — Sensitivity and specificity based on test results of samples one to three

Strain	S9 mix	No. individual test results	Sensitivity	Specificity	No. individual test results after outlier rejection n	Percentage of outliers o %	Sensitivity after outlier rejection %	Specificity after outlier rejection %
TA98	–	32	N/A	94	30	6,25	N/A	100
	+	45	100	100	45	0	100	100
TA100	–	42	100	86	38	9,5	100	100
	+	43	100	80	40	7,0	100	100
overall		162	100	90	153		100	100

The sensitivity and specificity of the test was calculated based on the samples 1 to 3. The mutagenicity of these samples was known based on added mutagenic compounds. Therefore, the test results of the samples 1 to 3 were classified as true positive, n_{+t}, false positive, n_{+f}, true negative, n_{-t}, and false negative, n_{-f}. The sensitivity was calculated according to $100 \times n_{+t}/(n_{+t} + n_{-f})$. The specificity was calculated according to $100 \times n_{-t}/(n_{-t} + n_{+f})$. The sensitivities and specificities were calculated both, for each condition (strain, with or without metabolic activation by S9 mix) separately and for the overall performance of the Ames fluctuation test to classify a sample as mutagenic (mutagenic in at least one condition) or non-mutagenic.

Table H.3 — Median, minimal and maximal Ib(LID) values in terms of dilution factor for samples 1 to 4 of all valid test results

Strain	S9 mix	Sample																			
		1					2					3					4				
		No. laboratories after outlier rejection	Percentage of outliers	Median	Minimum Ib(LID)	Maximum Ib(LID)	No. laboratrories after outlier rejection	Percentage of outliers	Median	Minimum Ib(LID)	Maximum Ib(LID)	No. laboratories after outlier rejection	Percentage of outliers	Median	Minimum Ib(LID)	Maximum Ib(LID)	No. laboratories after outlier rejection	Percentage of outliers	Median	Minimum Ib(LID)	Maximum Ib(LID)
		l	*o* %				*l*	*o* %				*l*	*o* %				*l*	*o* %			
TA98	–	16	0	0	0	0	16	0	2	0	5	14	12,5	0	0	0	16	0	1,5	0	2
	+	16	0	0	0	0	15	0	5	3	6	16	0	4	3	6	15	0	1	0	2
TA100	–	11	15,4	0	0	0	14	0	5	5	6	13	13,3	0	0	0	12	7,8	2	2	2
	+	12	20	0	0	0	14	0	6	4	6	14	0	3	2	5	10	28,5	2	2	2

Annex I
(informative)

Statistical assessment

I.1 General

The output of the Ames fluctuation test is a nominal (quantal) data set containing the number of revertant wells per 48 well area for each replicate of k samples (dilutions) (e.g. Table I.1). Statistical differences between test samples and the negative control (NC) can be derived by performing Fisher's exact test (2×2 table with Bonferroni adjustment). Since the frequency (sum) of observations within one sample can be smaller than or equal to four (as in Table I.1; example 1: sum NC = 1), it is not possible to use the approximation of Fisher's exact test, i.e. the χ^2-test. In order to avoid numerical problems (Fisher's exact test) and to have access to powerful ANOVA methods, it is recommended that the data be transformed (nominal to metric). To obtain an approximate normal distribution and to equalize variances, the arcsine–square-root transformation is applied as in Formula (I.1):

$$y = \arcsin\left(\sqrt{\frac{n_{\text{rev}}}{n_{\text{w}}}}\right) \tag{I.1}$$

where

n_{rev} is the number of revertant wells;

n_{w} is the number of wells.

Afterwards, the transformed data are checked for variance homogeneity (and normality). If these requirements are fulfilled, and provided that the dose–response relationship increases monotonically, a William's t-test can be performed. If the dose–response relationship is non-monotonic (e.g. decreasing revertant growth after a maximum at higher concentrations due to cytotoxic effects), Dunnett's test is appropriate. If the data are variance heterogeneous, pairwise comparisons should be made using the Welch t-test with Bonferroni adjustment. However, William's or Dunnett's test are still suitable since the transformation step involves the creation of variance homogeneity.

Table I.1 — Examples of the output of Ames fluctuation tests — The data sets consist of six dilution levels and a negative and positive control (NC and PC), i.e. eight samples ($k = 8$)

Example 1	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Plate 1	0	2	4	12	16	32	41	37
Plate 2	1	2	1	8	9	30	37	37
Plate 3	0	2	3	5	18	22	39	39
Sum	**1**	**4**	**8**	**25**	**43**	**84**	**117**	**113**
Example 2	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Plate 1	0	0	0	2	2	1	2	47
Plate 2	0	1	1	1	3	2	0	47
Plate 3	0	1	1	1	1	1	1	48
Sum	**0**	**2**	**2**	**4**	**6**	**4**	**3**	**142**
NC	negative control							
PC	positive control							

According to the validity criteria (Clause 10), up to 10 revertant wells are allowed in the NC. If the total number of revertant wells in the NC is rather low, it is possible that samples are considered mutagenic, although the

number of revertant wells in those samples is also below 10, and might therefore give rise to a spontaneously positive response. A clear example is the case where no revertant wells are detected in all three NC replicates (Table I.1; example 2). In that case, dilutions with only a few revertant wells might be regarded positive, due to the recommended use of powerful statistical tests (Table I.1 and I.4; example 2). Since these methods are required for protective assessment and in order to overcome false positives, a threshold value, n_{TH}, should be introduced and is calculated as follows:

$$n_{TH} = \bar{n}_{NC} + \frac{2s_{sam}}{\sqrt{n_{rep\ sam}}} \quad \text{(I.2)}$$

where

$\bar{n}_{NC}$ is the mean number of the basic population of revertant wells appearing in the NCs, i.e. one of:

- the historical negative control of interlaboratory testing [mean of all values of an international round-robin study (Table I.2)];
- the historical negative control of intralaboratory testing (mean value of all NCs determined by one laboratory in the past);
- the mean of 12 values of separately determined negative controls.

NOTE Reference [21] shows, with the help of the data of an international round-robin study, that at least 12×48 wells are required to reach the value of the historical NCs.

s_{sam} is the residual SD of the ANOVA;

$s_{sam}/\sqrt{n_{rep\ sam}}$ is the estimation of the SD of the basic population from the sample according to the central limit theorem.

Hence: n_{TH} = 95 % confidence interval around the historical mean of all NCs (Figure I.1)

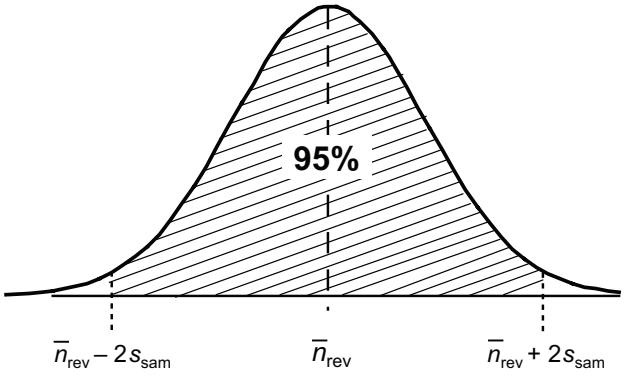


Figure I.1 — Statistical meaning of the threshold value, n_{TH}, which is represented by the hatched area under the curve

The value of n_{TH} is the 95 % confidence interval around the mean number of revertant wells, $\bar{n}_{rev}$, in the negative control (NC) and hence $\bar{n}_{rev} \pm 2$ times the residual SD of the ANOVA of the sample, s_{sam}.

As was already mentioned above, the numbers of revertant wells in the NCs of all tests performed in an international round-robin study were gathered and the mean was calculated to define a historical NC for each individual test strain (Table I.2). The arcsine–square-root transformed values presented can be universally used for calculations of sample-specific values of n_{TH}.

Table I.2 shows the results of an international round-robin study in which 18 laboratories in seven different countries tested four samples with the Ames fluctuation test using two test strains (TA 98 and TA 100) with and without metabolic activation by S9 mix (+S9 and −S9). The total numbers of revertant wells observed in the

negative controls (NCs) are listed and used to derive a historical mean, which represents an estimation of the mean of the basic population of NCs.

Table I.2 — Results of an international round-robin study

Test strain	No. valid replicates n_{val}	Sum of revertant wells $\sum n_{rev}$	Mean No. of revertant wells $\overline{n}_{rev}$	n_{rev}/n_w	$\arcsin\left[\frac{n_{rev}}{n_w}\right]^{1/2}$
TA 98 –S9	179	504	2,8	= 504/(179*48)	0,245
TA 98 +S9	180	333	1,9	= 333/(180*48)	0,198
TA 100 –S9	171	824	4,8	= 824/(171*48)	0,322
TA 100 +S9	171	825	4,8	= 825/(171*48)	0,323

I.2 Summary of the recommended assessment

The recommended assessment can be summarized as:

a) arcsine–square-root transformation of the data;

b) test on variance homogeneity (e.g. Levene's test);

c) ANOVA:

- William's: at variance homogeneity and if the dose–response relationship increases monotonically;
- Dunnett's: at variance homogeneity and if the dose–response relationship is non-monotonic;
- Welsh: at variance heterogeneity;

d) calculation of the threshold value;

e) determination of the least inhibitory dilution (LID).

As mentioned in I.1, this assessment of the results allows the selection of powerful, sensitive statistical tests, and therefore presents a protective approach. Despite the output of nominal data, the deviation between replicates can be involved because of the proposed transformation and subsequent possibility to use ANOVA methods. After pairwise comparison of the number of revertants in samples with the number in the NCs, applied to the same plate, the introduction of m_{TH} avoids considering actual negative responses falsely positive. The value of m_{TH} depends on the number of replicates in the samples and involves scatter between those replicates. Moreover, it is based on the overall population of NCs. In this way, the final evaluation is very close to expert judgement. This was previously derived from an international round-robin study, where it was shown that this approach resulted in little scatter in interlaboratory LID values determined for one sample (Reference [21]). Hence, the data analysis presented delivers a realistic result.

I.3 Example of calculations

After arcsine–square-root transformation of the nominal data of both examples presented in Table I.1, the metric data set obtained allows the mean of the three replicates to be calculated (Table I.3).

Table I.3 — Examples of arcsine–square-root transformed data of Ames fluctuation tests

Example 1	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Plate 1	0,00	0,21	0,29	0,52	0,62	0,96	1,18	1,07
Plate 2	0,15	0,21	0,15	0,42	0,45	0,91	1,07	1,07
Plate 3	0,00	0,21	0,25	0,33	0,66	0,74	1,12	1,12
Sum	**0,05**	**0,21**	**0,23**	**0,42**	**0,57**	**0,87**	**1,12**	**1,09**
Example 2	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Plate 1	0,00	0,00	0,00	0,21	0,21	0,14	0,21	1,43
Plate 2	0,00	0,14	0,14	0,14	0,25	0,21	0,00	1,43
Plate 3	0,00	0,14	0,14	0,14	0,14	0,14	0,14	1,57
Sum	**0,00**	**0,10**	**0,10**	**0,17**	**0,20**	**0,17**	**0,12**	**1,47**
NC	negative control							
PC	positive control							

Levene's test ($\alpha = 0{,}05$) indicates variance homogeneity in both data sets for examples 1 and 2 (Table I.1; calculations not shown).

Since the dose–response relationship is monotonic in both examples 1 and 2 (Table I.1), William's multiple sequential t-test procedure was performed with a significance level of 0,05 (α) to evaluate whether the number of revertant wells in the samples (dilutions) was higher than in the NCs (one-sided greater) (Table I.4). With a total of eight treatments (including PC, $n = 8$) and a sample size of three ($k = 3$), the number of degrees of freedom, ν, amounts to 16, i.e. $\nu = n(k - 1) = 24 - 8$.

Table I.4 — Results of the statistical assessment of data obtained from Ames fluctuation

Example 1	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Mean	0,05	0,21	0,23	0,42	0,57	0,87	1,12	1,09
s	0,08	0,08	0,08	0,08	0,08	0,08	0,08	
t		2,41	2,79	5,76	8,06	12,60	16,50	
t^*		1,75	1,83	1,86	1,87	1,88	1,89	
Significance		+	+	+	+	+	+	
Example 2	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Mean	0,00	0,10	0,10	0,17	0,20	0,17	0,12	1,47
s	0,07	0,07	0,07	0,07	0,07	0,07	0,07	
t		1,73	1,73	2,90	2,90	2,90	2,90	
t^*		1,75	1,83	1,86	1,87	1,88	1,89	
Significance		–	–	+	+	+	+	
NC	negative control							
PC	positive control							
s	SD							
t	t-value derived from William's test							
t^*	critical t-value with a one-side significance level of 0,5							
+	sample shows significantly more revertant wells compared to the negative control							
–	no significant difference with the negative control							

I.4 Calculation of the threshold value

The n_{TH} is determined for both examples (1 and 2; Table I.1) according to Equation (I.1) using the listed historical means, $\bar{n}_{NC}$; then transformed into arcsine–square-root values (Table I.3). Both examples are derived from testing with strain TA 98, whereby an S9 mix was used in the first example only. The SD can be retrieved from

the ANOVA (Table I.4). The number of replicates amounted to three. The calculations of the specific n_{TH} for both examples are presented below:

EXAMPLE 1 $$n_{TH} = 0{,}198 + \frac{2\times 0{,}08}{\sqrt{3}} = 0{,}29$$

EXAMPLE 2 $$n_{TH} = 0{,}245 + \frac{2\times 0{,}07}{\sqrt{3}} = 0{,}32$$

The mean of the replicated arcsine–square-root transformed number of revertant wells in the samples is finally compared with n_{TH}. When the mean is lower than n_{TH}, and a significant effect was observed by means of the ANOVA, the result is changed from positive to negative. In other words, the response is only considered significant if n_{TH} is exceeded (Table I.5).

Table I.5 — Implementation of the threshold value, $+n_{TH}$, after statistical assessment of data obtained from Ames fluctuation tests

Example 1	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Mean	0,05	0,21	0,23	0,42	0,57	0,87	1,12	1,09
Mean vs. n_{TH}		< 0,29	< 0,29	> 0,29	> 0,29	> 0,29	> 0,29	
Significance $-n_{TH}$		+	+	+	+	+	+	
Significance $+n_{TH}$		–	–	+	+	+	+	
Example 2	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Mean	0,00	0,10	0,10	0,17	0,20	0,17	0,12	1,47
Mean vs. n_{TH}		< 0,32	< 0,32	> 0,32	> 0,32	> 0,32	> 0,32	
Significance $-n_{TH}$		–	–	+	+	+	+	
Significance $+n_{TH}$		–	–	–	–	–	–	
NC	negative control							
PC	positive control							
+	sample shows significantly more revertant wells compared to the negative control							
–	no significant difference with the negative control							

I.5 Determination of the final LID

Evaluation of the LID obtained by the recommended data assessment of examples 1 and 2 (Table I.1) shows that the method indicates significant effects from the NC in a conservative way (Table I.6). However, the introduction of the historical mean of NCs avoids the identification of a difference when it most probably concerns spontaneous mutagenicity (e.g. all dilutions in example 2; Table I.6).

Table I.6 — Examples of output and statistical evaluation of Ames fluctuation tests

Example 1	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Plate 1	0	2	4	12	16	32	41	37
Plate 2	1	2	1	8	9	30	37	37
Plate 3	0	2	3	5	18	22	39	39
Significance + n_{TH}		−	LID	+	+	+	+	
Example 2	**NC**	**1→32**	**1→16**	**1→8**	**1→4**	**1→2**	**1→1**	**PC**
Plate 1	0	0	0	2	2	1	2	47
Plate 2	0	1	1	1	3	2	0	47
Plate 3	0	1	1	1	1	1	1	48
Significance + n_{TH}	−	−	−	−	−	−	LID	142

NC	negative control
PC	positive control
n_{TH}	threshold value
LID	least-inhibitory dilution
+	sample shows significantly more revertant wells compared to the negative control
−	no significant difference with the negative control

Annex J
(informative)

Measurement of the lowest ineffective dilution (LID) of a waste water — A simplified evaluation for testing of waste water

J.1 General

In genotoxicity testing of waste water by means of defined dilutions, D, the lowest ineffective dilution (LID) expresses the most concentrated test batch at which no significant induction of revertant growth, or only effects not exceeding the test specific variability, are observed.

J.2 Definitions

J.2.1 Defined dilution

The value of D is expressed as the reciprocal value of the volume fraction of waste water in the test batch. If only the exposure medium concentrate and the adjusted overnight culture is added to the test sample, this dilution is defined as $D = 1$.

EXAMPLE A volume fraction of 25 %, i.e. 1/4 waste water is defined as dilution level $D = 4$.

J.2.2 Lowest ineffective dilution (LID)

In genotoxicity testing of waste water by means of defined dilutions, D, following this International Standard, the lowest ineffective dilution (LID) expresses the most concentrated test batch at which no statistical significant induction of revertants is observed.

J.3 Principle

See also Clause 5.

For the determination of the non-genotoxic effect of waste water, the assessment of D values is prescribed. Waste water is diluted according to a defined scheme of dilutions. The evaluation of results should be based on the average induction of revertants.

J.4 Preparation of dilutions for LID assessment

Add exposure medium concentrate, dilution water, water sample and adjusted overnight culture as shown in Tables 2 and 3.

In the same way, prepare negative and positive control batches.

The lowest ineffective dilution (LID) that does not exceed statistical significance is reported as the D value.

J.5 Test for LID

Use at least three replicates for the treatments and three replicates for the controls at testing.

J.6 Assessment of results — LID value, effluents

From a series of defined testing conditions (strains TA 98 and TA 100, ±S9 mix), the LID is determined by the most sensitive testing condition.

J.7 Documentation of results

Report the test result as D_A (the subscript “A” indicates that the results are from the Ames fluctuation test).

Report only whole numbers of D-values, e.g. $D_A = 2$.

Annex K
(informative)

Use of additional tester strains

Following the recommendations of ISO 16240[9], a minimal set of bacterial tester strains, namely TA 98 for the detection of frameshift mutagens and TA 100 for the detection of base-pair substitution mutagens, is used. Several other tester strains are available like TA 1535, TA 1538, TA 97, TA 102, and base-specific tester strains. However, it is not recommended that the genetically modified tester strain TA 102 be used with this International Standard due to its high spontaneous reversion rate. Strains TA 1535, TA 1538 and TA 97 may be used. Base-specific tester strains may be used as single strains or as mixtures (TAMix). In any case, when applying additional tester strains, adjust the bacterial density of the respective test culture in such a way that the number of wells with revertant growth (yellow) in the negative controls is on average $\leq$10. Include appropriate positive control substances and concentrations. Review the literature to determine which antibiotics have to be applied, if any.

Genetically modified tester strains of the YG series (e.g. frameshift tester strains YG 1021, YG 1024, YG 1041 and base-pair substitution strains YG 1026, YG 1029 and YG 1042) may be used in this International Standard. These strains carry plasmids with genes coding for *O*-acetyltransferase and/or nitroreductase which are involved in the intracellular metabolic activation of nitroarenes and/or aromatic amines (Reference [12]). In any case, ensure the presence of the plasmids by cultivating the strains with antibiotics. Strains YG 1021, YG 1024, YG 1026, and YG 1029 are incubated in the presence of ampicillin and tetracycline, strains YG 1041 and YG 1042 in the presence of ampicillin and kanamycin. Include appropriate positive control substances that indicate the activity of the metabolizing enzymes. When using YG strains, it may be advantageous to prolong the incubation time in 384 well plates up to 72 h.

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