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Title

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

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Introductory note

After the positive result of a new work item questionnaire, the project was included in the work programme of SC 5 in April 2008, allocated to WG 9. The working draft distributed with the nwip was revised according to comments received and discussion at the meeting in Ontario, April 2008. A second draft was distributed in December 2008 for further comments, and is now distributed for CD voting.

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ISO/TC 147/SC 5 N **0646**

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ISO/TC 147/SC 5/WG 9 **N 0052**

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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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Contents

Page

Foreword iv
1 Scope 1
2 Normative references 1
3 Terms and definitions 2
4 Interferences 4
5 Principle 4
6 Apparatus and materials 4
7 Reagents, media and dilutions 5
8 Sampling and samples 9
9 Procedure 10
10 Validity criteria 14
11 Assessment criteria 14
12 Test report 15
Annex A (normative) **Nutrient broth and agar** 16
Annex B (normative) **Preparation of stock cultures and ampicillin agar plates** 17
Annex C (normative) **Checking of genotype** 18
Annex D (normative) **S9-fraction** 19
Annex E (informative) **Example for application of samples on a 24-well plate** 20
Annex F (informative) **Example for reporting** 21
Annex G (informative) **Testing of chemicals** 22
Annex H (informative) **Precision data** 25
Annex I (informative) **Use of alternative tester strains** 26
Bibliography 27

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Foreword

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

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

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

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

ISO 11350 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 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)

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 typhimurium* TA 98 and TA 100 in a fluctuation assay. This combination of strains is able to measure 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. For measuring genotoxicity of samples containing DNA crosslinking agents ISO 13829 should be applied.

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, genotoxic substances adsorbed on particles might not be detected.

This method is applicable to:

— fresh water;

— waste water;

— aqueous extracts and leachates;

— eluates of sediment (fresh water);

— pore water;

— aqueous solutions of single substances or of chemical mixtures.

2 Normative references

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

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

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

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

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

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

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

ISO 7027, *Water quality — Determination of turbidity*

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

ISO/TS 20281, *Water quality — Guidance on statistical interpretation of ecotoxicity data*

3 Terms and definitions

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

3.1
concentration response relationship
reduction of the number of revertant wells with increasing dilution level D

3.2
cofactor solution
aqueous solution of chemicals needed for enzyme activity in the S9 fraction

3.3
culture medium
aqueous solution of nutrients, required for cultivation of the bacteria

3.4
dilution level D
denominator of the dilution coefficient (using the numerator 1) of a mixture of water or waste water with dilution water (3.5) as whole number

NOTE For undiluted water or waste water, this coefficient per definition is 1 : 1. The corresponding and smallest possible D value is 1.

3.5
dilution water
sterile water with defined properties (e.g. conductivity) used for the stepwise dilution of the test sample or used as negative control

3.6
D_{min} value
smallest value of D at which, under the conditions of this International Standard, no positive increase in the number of visible mutant colonies per microplate is detected

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

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3.7
exposure medium
medium for incubation of bacteria with the sample containing a low amount of L-histidine to support a few cell divisions

3.8
induction rate
difference of the mean values of revertant wells counted on the plates treated with a dose of the test sample or with a positive control, and of the corresponding wells treated with the negative control using the same strain under identical conditions

3.9
inoculum
aliquot of a unfreezed stock culture used to inoculate culture medium

3.10
negative control
mixture of culture medium, inoculum and dilution water

3.11
number of revertant wells, number of mutant wells
number of wells with colour shift or visible mutant colonies on the microplate at the end of the respective test

3.12
overnight culture
mixture of inoculum and culture medium, incubated for a defined period to prepare the test culture

3.13
positive control
mixture of culture medium, inoculum and a known mutagen used to verify the sensitivity of the method or the activity of the S9-mix

NOTE The positive controls mentioned in this International Standard are dissolved in dimethyl sulfoxide (DMSO) prior to use.

3.14
reversion indicator medium
pH indicator medium without L-histidine

3.15
S9 fraction
9 000 g supernatant of a tissue homogenate, obtained from livers of male rats pretreated with an appropriate substance or substance combination for enzyme induction

3.16
S9 mix
mixture of S9 fraction and cofactor solution

3.17
stock culture
frozen culture for the preservation of the characteristics (e.g. genotype) of *Salmonella typhimurium* TA 100 and TA 98

3.18
test sample
the sample to be tested after all preparative steps (e.g. sterile filtration)

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4 Interferences

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

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 contained in the test sample may be able to induce mutations in one or both marker genes of the bacterial strains used (hisG46 for TA 100 and hisD3052 for TA 98) in correlation to the applied concentrations. Induction of mutations will cause 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.

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 sample, compared to the negative control.

The lowest dilution (1 : 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 : A, A < N) shall induce a mutagenic effect according to the criteria of this standard in at least one strain under at least one activation condition (with or without addition of S9 mix). The respective D_{min}-value is N. If no mutagenic effect is observed under all experimental conditions, this dilution is 1 : 1 and the respective D_{min}-value is 1.

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 steriliser

6.5 Dry steriliser

6.6 Magnetic stirrer

6.7 Rotary mixer

6.8 Freezer, at least -20 °C and at least -70 °C.

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

6.10 Storage bottles, 250 ml and 1 000 ml.

6.11 Graduated cylinders, 100 ml and 200 ml.

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

6.13 Sterile filters, 0,22 µm and 0,45 µm.

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

6.15 Inoculating loops

6.16 8-channel multistepper pipette (repeater pipette).

6.17 8-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 384-well plates, filters: 420 nm ± 10 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.

7 Reagents, media and dilutions

7.1 General

As far as possible, use ”reagent grade“ chemicals. If chemicals with different amounts of water are used, calculate the needed amounts accordingly.

Always perform autoclaving 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, respectively water with a conductivity of ≤ 5 µS/cm.

If sterile water is needed, sterilize by sterile filtration (0,22 µm) or autoclaving.

7.3 Tester strains

Use mutant strains of *Salmonella typhimurium* LT2, which enable to detect 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.

7.4 2-aminoanthracene (2-AA), $C_{14}H_{11}N$, CAS: 613-13-8.

7.5 Ampicillin sodium salt, $C_{16}H_{18}N_3NaO_4S$, CAS: 69-52-3.

7.6 D-biotin, $C_{10}H_{16}N_2O_3S$, CAS: 58-85-5.

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7.7 Bromocresol purple, sodium salt, CAS: 62625-30-3.

7.8 Citric acid, $C_6H_8O_7 \cdot H_2O$, CAS: 5949-29-1.

7.9 Dimethyl sulfoxide, DMSO, C_2H_6SO, CAS: 67-68-5.

7.10 D-glucose, $C_6H_{12}O_6$, CAS: 50-99-7.

7.11 D-glucose 6-phosphate disodium salt hydrate, G-6-P-Na_2, CAS: 3671-99-6.

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

7.13 Magnesium chloride hexahydrate, $MgCl_2 \cdot 6\ H_2O$, CAS: 7791-18-6.

7.14 Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7\ H_2O$, CAS: 10034-99-8.

7.15 Potassium chloride, KCl, CAS: 7447-40-7.

7.16 Di-potassium hydrogen phosphate, K_2HPO_4, CAS: 7758-11-4.

7.17 Sodium ammonium hydrogen phosphate tetrahydrate, $NaNH_4HPO_4 \cdot 4\ H_2O$, CAS: 7583-13-3.

7.18 Sodium chloride, NaCl, CAS: 7647-14-5.

7.19 Sodium dihydrogen phosphate monohydrate, NaH_2PO_4, CAS: 7558-80-7.

7.20 Di-sodium hydrogen phosphate, anhydrous, Na_2HPO_4, CAS: 7558-79-4.

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

7.22 β-Nicotinamide adenine dinucleotide phosphate sodium salt, NADP, $C_{21}H_{27}N_7Na_2O_{17}P_3$, CAS: 698999-85-8.

7.23 Nitrofurantoin (NF), CAS: 67-20-9.

7.24 4-Nitro-o-phenylenediamine (4-NOPD), CAS: 99-56-9.

7.25 Nutrient broth powder[1]**.**

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

7.27 L-histidine, $C_6H_9N_3O_2$, CAS: 71-00-1

7.28 Phosphate buffer

7.28.1 Sodium hydrogen phosphate buffer I, $c(NaHPO_4)$ = 0,2 mol/l.

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

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[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.

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7.28.2 Disodium hydrogen phosphate buffer II

Dissolve 28,39 g Na_2HPO_4 in 1 000 ml of water (7.2).

Add solution 7.28.1 to solution 7.28.2 until a pH value of 7,4 is reached.

7.29 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 (0,22 µm filter). Store 10 ml aliquots at -20 °C in sterile cryogenic vials (6.23).

7.30 Histidine solution

Dissolve 50 mg of L-histidine (7.27) in 50 ml of water (7.2) and sterilize by filtration (0,22 µm filter). Store 1,5 ml aliquots at -20 °C in sterile cryogenic vials (6.23).

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 (0,22 µm). Store aliquots (e.g. 200 µl) at -20 °C in sterile cryogenic vials (6.23).

7.32 NADP solution

Dissolve 0,31 g of NADP in 10 ml of water (7.2) and sterilize by filtration (0,22 µm). Store aliquots (e.g. 700 µl) at -20 °C in sterile cryogenic vials (6.23). Aqueous solutions stored as frozen aliquots are stable for at least one year.

7.33 Potassium chloride solution

Dissolve 74,56 g of KCl (7.15) in 1 000 ml of water (7.2) and autoclave. Store the solution at room temperature.

7.34 $MgCl_2 \cdot 6\ H_2O$ solution

Dissolve 50,83 g of $MgCl_2 \cdot 6\ H_2O$ (7.13) in 1 000 ml of water (7.2) and autoclave the solution. Store the solution at room temperature.

7.35 Bromocresol purple solution

Dissolve 170 mg of bromocresol purple (7.7) in 100 ml of water (7.2). Store the solution at room temperature in the dark.

7.36 Ampicillin solution

Dissolve 500 mg of ampicillin (7.5) in 10 ml of water (7.2) and sterilize by filtration (0,22 µm filter). Store 500 µl aliquots at -20 °C in sterile cryogenic vials (6.23).

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 pH to 7,5 ± 0,1. Add water (7.2) to 250 ml and autoclave the solution.

Per 1 000 ml growth medium the following final concentrations shall result:

— 7,5 g meat extract;

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

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- 7,5 g peptone;
- 5,0 g sodium chloride.

7.38 Exposure medium

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 di-potassium hydrogenphosphate (7.16);
- 3,5 g sodium-ammonium hydrogenphosphate tetrahydrate (7.17);
- 4,0 g D-glucose (7.10).

Add 6 ml of D-biotin solution (7.29) and 1 ml of L-histidine solution (7.30). Add water (7.2) to 1 000 ml, adjust the pH to 7,0 ± 0,2, if necessary, and sterilize by filtration (0,22 µm filter). Store medium at + 4 °C.

7.39 Exposure medium concentrate

Dissolve consecutively the following ingredients in 90 ml water:

- 0,2 g magnesium sulfate heptahydrate (7.14);
- 2,0 g citric acid (7.8);
- 10,0 g di-potassium hydrogen phosphate (7.16);
- 3,5 g sodium-ammonium hydrogen phosphate tetrahydrate (7.17);
- 4,0 g D-glucose (7.10).

Add 6 ml of D-biotin solution (7.29) and 1 ml of L-histidine solution (7.30). Add water (7.2) to 100 ml, adjust the pH, if necessary, and sterilize by filtration (0,22 µm filter). Store medium concentrate at + 4 °C.

7.40 Reversion indicator medium

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 di-potassium hydrogenphosphate (7.16);
- 7,0 g sodium ammonium hydrogen phosphate tetrahydrate (7.17).

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

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Solution II

Dissolve 8,0 g of D-glucose (7.10) in 800 ml of water (7.2). Transfer the solution one half each into two 1 000 ml flasks and autoclave.

After cooling to ambient temperature, mix 515 ml of solution I with 400 ml of solution II 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.

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 -20 °C. 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 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 positive control substance for strain TA 100 without S9-mix.

Dilute stock solution 1 : 160 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 positive control substance for strain TA 98 with S9-mix.

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

8 Sampling and samples

Take samples as specified in ISO 5667-1, ISO 5667-3, ISO 5667-14, and ISO 5667-16.

Test the samples immediately after sampling. If this is not possible, keep water samples at 0 °C to 5 °C (up to < 48 h) or below -18 °C (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.

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Sterilize all samples using sterile filters (0,45 µm). Do not extract or concentrate the samples. 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 over-titration. Take into account the change of the sample's pH and resulting effects (ISO 5667-16).

Perform dilutions of the test sample (1 : 2 to 1 : 16) 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) closed permeable to air with caps or aluminium foil and mix by gentle agitation.

Add 20 µl of the respective tester strain (TA 98 or TA 100) immediately after unfreezing. Incubate the culture at 37 °C ± 1 °C for not more than 10 h. A clock timer may be used. Use a shaking rate of at least 150 rpm.

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 should also be prepared according to Annex D.

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

— 66 µl KCl solution (7.33);

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

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

— 200 µl NADP solution (7.32);

— 1 000 µl sodium hydrogen phosphate buffer (7.28);

— 20 µl water (sterile);

— 600 µl S9-fraction (Annex D).

This mixture is sufficient for 2 exposure plates. In the case of more than 2 plates, increase the amount of S9-mix proportionally.

Keep the S9-mix permanently on ice and use it only 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 cell density of 180 FAU (2) 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.

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In the test culture the tester strain inoculum is diluted 10-fold (Table 2 and Table 3). Therefore adjust the cell density in the over night culture inoculum to 1 800 FAU for TA 98 and 450 FAU for TA 100 according to Equations (1) and (2).

Measure cell densities (OD_{595}) of the over night cultures of the tester strains TA 98 and TA 100 immediately before exposure. Therefore dilute the over night culture 10-fold with exposure medium (7.38) because otherwise FAU will be out of range. Take only a small aliquot of the over night culture.

$$d = \frac{OD_{595}}{180} \qquad (1)$$

where

d is the dilution factor;

OD_{595} is the measured FAU (of the tenfold diluted overnight culture TA 98).

$$d = \frac{OD_{595}}{45} \qquad (2)$$

where

d is the dilution factor;

OD_{595} is the measured FAU (of the tenfold diluted overnight culture TA 100).

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

Measured FAU in the 10-fold diluted overnight culture	d	Volume overnight culture (undiluted)	Adjusted overnight culture for exposure (1 800 FAU)	Volume of exposure medium for dilution to be added
240	1,33	20 ml	26,6 ml	6,6 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 configuration of plate A is given in Annex E.

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

	Negative control µl	Dilution 1[a] µl	Dilution 2 µl	Dilution 3 µl	Positive control (see 7.41.2) 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 1: sample concentration 80 %; dilution 2: sample concentration 50 %; dilution 3: sample concentration 25 %

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

Measure initial OD_{595} (t = 0 min) of plate A using a microplate photometer (6.20). It is recommended to use only tester strain TA 98 for measurement of growth as cell density of TA 100 remains low.

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

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

At the end of incubation of plate A measure OD_{595} using a microplate photometer (6.20).

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. 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).

If cytotoxicity measurement is not applied, fill plate A with half of the volume of each ingredient (Table 2). After 100 min of incubation, directly add the reversion indicator into plate A and mix thoroughly. Transfer to the 384-well plate and incubate for 48 h as described in 9.3.2.

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9.3.3 Test culture with S9-mix

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

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

	Negative control µl	Dilution 1 µl	Dilution 2 µl	Dilution 3 µl	Positive control (see 7.41) µl
Exposure medium concentrate (7.39)	100	100	100	100	100
Dilution water (7.2)	800	0	300	550	780
Sample[a]	0	800	500	250	20
Adjusted overnight culture of tester strains TA 98 and 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] As positive control substance use 2-AA for strain TA 98 and TA 100					

Measure initial OD_{595} (t = 0 min) of plate A using a microplate photometer (6.20). It is recommended to use only tester strain TA 98 for measurement of growth as cell density of TA 100 remains low.

Incubate the plates in the dark at 37 °C ± 1 °C for 100 min with shaking (150 rpm).

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

At the end of incubation of plate A measure OD_{595} using a microplate photometer (6.20).

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. 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).

If cytotoxicity measurement is not applied, fill plate A with half of the volume of each ingredient (Table 3). After 100 min of incubation, directly add reversion indicator medium into plate A and mix thoroughly. Transfer to the 384-well plate and incubate for 48 h as described in 9.3.3.

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).

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9.5 Calculation of cytotoxicity

For calculation of cytotoxicity, use OD_{595} values as measured in 9.3.2 and 9.3.3. Calculate mean OD_{595} values ± sd of sample wells and negative control wells. Calculate cytotoxicity (CT) according to Equation (3).

$$CT = 100 - 100\left(\frac{S_{t=100} - S_{t=0}}{NC_{t=100} - NC_{t=0}}\right) \quad (3)$$

where

CT is the cytotoxicity in percent, %;

S is the OD_{595} of sample;

NC is the OD_{595} of negative control;

$t = 0$ is the initial value at $t = 0$ min;

$t = 100$ is the final value at $t = 100$ min.

10 Validity criteria

The test is valid if

a) the mean value for negative controls is > 0 and ≤ 5 revertant wells per 48-well area for TA 98 and > 0 and ≤ 10 revertant wells per 48-well area for TA 100;

b) the mean value for positive controls is ≥ 25 revertant wells 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 according to 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. For pairwise comparisons use powerful ANOVA methods. For this the proportion of revertant wells is arcsine-sqrt transformed. If normality and variance homogeneity requirements are fulfilled, the Student's t-test is applied otherwise the Welch-t test. If a NOEC/LOEC has to be determined use Dunnett's or Williams-test, if not the Welch-t test (Bonferroni).

Measurement of cell growth (9.3.2) provides information about the cytotoxicity of the sample. Cytotoxicity may lead to false-negative test results by suppression of mutagenesis due to inhibition of cell division. However, in the presence of high cytotoxicity, a positive test result (enhancement of revertant wells compared to the negative control) is regarded as valid.

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12 Test report

This clause specifies which information is to be included in the test report. The clause shall require information to be given on at least the following aspects of the test:

a) a reference to this International Standard (ISO/CD 11350);

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) tester strains (strain, source, date of arrival, storage conditions, date of stock culture preparation, and date of genotype checking (if this date deviates from stock culture preparation)), obtained OD_{595} of the overnight culture, adjusted OD_{595} 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) results (individual numbers of revertant wells per treatment (Annex F), induction rate (Annex F), indication of cytotoxicity (if any), statistical evaluation, D_{min} values, other observations (e.g. precipitation, bacterial growth without colour shift).

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Annex A
(normative)

Nutrient broth and agar

A.1 Nutrient broth

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

A.2 Agar

Agar is commercially available in different qualities. It is recommended to use Difco[3] agar or a product of equal quality.

A.3 Nutrient agar

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

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

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Annex B
(normative)

Preparation of stock cultures and ampicillin agar plates

B.1 Preparation of stock cultures

Perform the preparation of stock cultures under sterile conditions.

Immediately upon receipt, spread aliquots of the respective bacterial strain onto the surface of ampicilline agar plates (B.2) 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 onto the surface of ampicillin nutrient agar plates (B.2) 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 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 should fulfill the requirements of Annex C. Otherwise they should 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 above by using a frozen stock culture from a former preparation.

B.2 Preparation of ampicillin agar plates

Perform the preparation of ampicilline agar plates under sterile conditions.

Dissolve 1,5 g of bacto agar, 1,88 g nutrient broth powder (7.37), and 0,124 g NaCl in 100 ml water, stir and autoclave. Cool down temperature 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 to avoid condensation during the test.

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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.2). 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 onto nutrient agar plates (B.2, 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 onto nutrient agar plates (B.2, without ampicillin). Use four plates per strain. Cover one half of each plate with aluminum 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.

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Annex D
(normative)

S9-fraction

D.1 Induction of liver enzymes

For enzyme induction, at least 6 male rats (e.g. Sprague-Dawley rats), about 200 g to 300 g of body weight, receive 80 mg/kg body weight phenobarbital intraperitoneally and 80 mg/kg body weight ß-naphtoflavone orally on 3 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 (e.g. Aroclor 1254) dissolved in an appropriate vehicle at a dose of 500 mg/kg body weight 5 d prior to liver preparation.

Animals should be housed on absorbent softwood bedding in adequately marked cages, which will only be 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 9 000 g 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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Annex E
(informative)

Example for application of samples on a 24-well plate

1st replicate		2nd replicate		3rd replicate		
NC	D_3	NC	D_3	NC	D_3	A
D_6	D_2	D_6	D_2	D_6	D_2	B
D_5	D_1	D_5	D_1	D_5	D_1	C
D_4	PC	D_4	PC	D_4	PC	D
1	2	3	4	5	6	

NOTE D_1 to D_6 are different samples or different dilutions of one or more samples; NC negative control; PC positive control.

Figure E.1 — 24-well incubation plate A

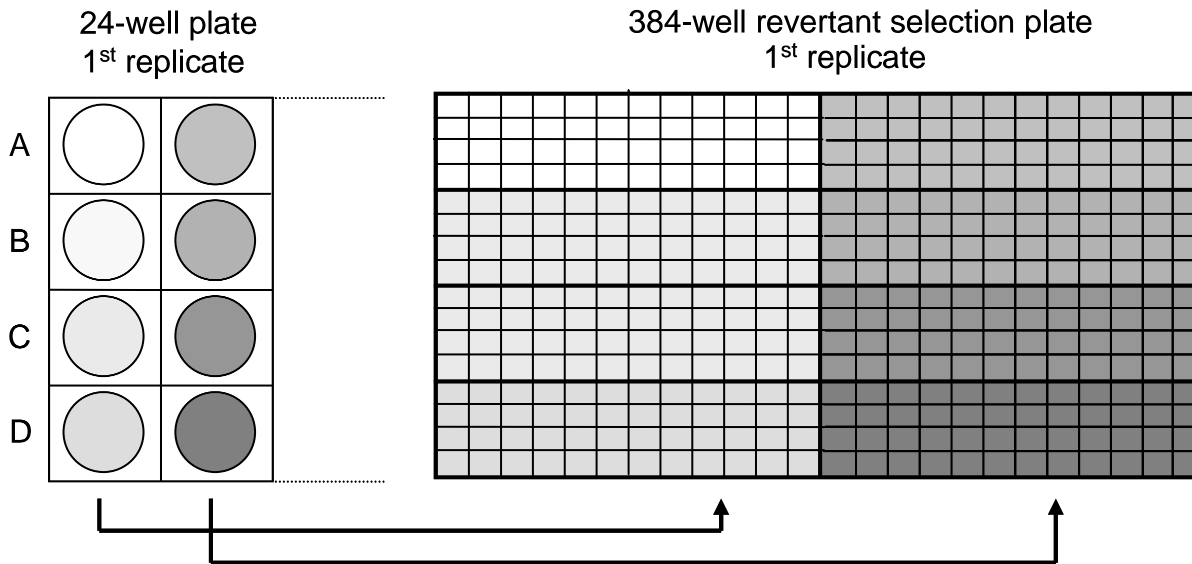

Figure E.2 — Example of transfer from a 24-well plate (plate A or B) to a 384-well plate (with six dilution steps)

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Annex F
(informative)

Example for reporting

Table F.1 — Example of a table for reporting and sample assessment

Explanation to Table F.1:

Strain: TA 100

Testing condition: Incubation with addition of S9-mix

Sample: NAME

Sample	**D value**	**Revertant wells**			**Mean**	**Revertant well**	**Mutant**	
		Replicate 1	Replicate 2	Replicate 3		Induction rate (3.18)	Induction factor	
NC[a] water		8	7	4	6,3		1	
Sample 1	4[d]	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[d]	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

[b] Positive control

[c] 2-aminoanthracene

[d] D-value

(annotation for ISO type setter: please delete last column of Table F.1)

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Annex G
(informative)

Testing of chemicals

G.1 General

Perform overnight culture according to 9.1.

G.1.1 Preparation of tester strains

In the test cultures cell density of 180 FAU (2) 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.

Measure cell densities (OD_{595}) of the over night cultures of the tester strains TA 98 and TA 100 immediately before exposure. For measuring dilute the over night culture 10-fold with exposure medium because otherwise FAU will be out of range. Take only a small aliquot of the over night culture. Calculate the dilution factor for the over night culture according to Equation (G.1) and (G.2) respectively.

$$d = \frac{OD_{595}}{18} \qquad \text{(G.1)}$$

where

d is the dilution factor;

OD_{595} is the measured FAU (of the tenfold diluted overnight culture TA 98).

$$d = \frac{OD_{595}}{4,5} \qquad \text{(G.2)}$$

where

d is the dilution factor;

OD_{595} is the measured FAU (of the tenfold diluted overnight culture TA 100).

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

Measured FAU in the 10-fold diluted overnight culture	Dilution factor	Volume overnight culture (undiluted) ml	Adjusted overnight culture for exposure ml	Volume of exposure medium (7.38) for dilution to be added ml
240	13,3	4	53,3	49,3

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G.1.2 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

	Negative control µl	Chemicals dilutions µl	Positive control (see7.41.1) µl
Adjusted overnight culture of tester strain TA 98 or TA 100 (9.4.2)	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. Normally DMSO is used as 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.

Measure initial OD_{595} (t = 0 min) of plate A using a microplate photometer (6.20). It is recommended to use only tester strain TA 98 for measurement of growth as cell density of TA 100 remains low.

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

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

At the end of incubation of plate A, measure OD_{595} for cytotoxicity assessment using a microplate photometer (6.20).

Proceed according to 9.3.

G.1.3 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.

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

	Negative control µl	Chemicals dilutions µl	Positive control (see 7.41) µl
Adjusted overnight culture of tester strain TA 98 or TA 100 (9.4.2)	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. Normally DMSO is used as solvent for chemicals.

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

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

Measure initial OD_{595} (t = 0 min) of plate A using a microplate photometer (6.20). It is recommended to use only tester strain TA 98 for measurement of growth as cell density of TA 100 remains low.

Incubate plate A in the dark at 37 °C ± 1 °C for 100 min with shaking (150 min^{-1}).

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

At the end of incubation of plate A, measure OD_{595} for assessment of cytotoxicity using a microplate photometer (6.20).

Proceed according to 9.3.

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Annex H
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Precision data

To be completed after the interlaboratory trial.

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Annex I
(informative)

Use of alternative tester strains

Following the recommendations of ISO 16240 in this international standard 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 to use the genetically modified tester strain TA 102 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. In any case, when applying other tester strains than TA 98 and TA 100 adjust the bacterial density of the test culture in such a way that the number of revertant wells (yellow) in the negative controls is on average ≤ 10. Include appropriate positive control substances and concentrations.

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 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 [9]. In any case, assure 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 ampicilline 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 metabolising 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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