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Water quality — Determination of the genotoxicity of water and waste water using the umu-test

Qualité de l'eau — Détermination de la génotoxicité des eaux et des eaux résiduaires à l'aide de l'essai umu

Reference number
ISO 13829:2000(E)

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Contents

Page

Foreword iv
Introduction v
1 Scope 1
2 Normative reference 1
3 Terms and definitions 1
4 Principle 3
5 Test organism and reagents 3
6 Apparatus 5
7 Sample preparation and preservation 5
8 Interferences 6
9 Test performance 6
10 Measurements 8
11 Calculation and expression of results 9
12 Precision 10
13 Validity criteria 10
14 Test report 10
Annex A (informative) **General definitions and terms regarding genotoxicity** 11
Annex B (informative) ***Salmonella typhimurium* TA1535/pSK1002** 12
Annex C (informative) **Composition of the sample and control wells** 13
Annex D (informative) **Lowest value of the dilution series** 14
Annex E (informative) **Checking the genotype** 15
Annex F (informative) **Precision data from an interlaboratory study** 16
Annex G (informative) **Final protocol for the umu-test** 17
Bibliography 18

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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 3.

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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 13829 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Annexes A to G of this International Standard are for information only.

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Introduction

The genetically engineered bacterium *Salmonella typhimurium* TA1535/pSK1002 serves as a test organism.

The bacteria are exposed under controlled conditions to different concentrations of the samples to be tested. The test is based on the capability of genotoxic agents to induce the umuC-gene in the *Salmonella* strain in response to genotoxic lesions in the DNA.

Due to its capability to respond to different types of genotoxic lesions, only one single strain is necessary to detect different kinds of genotoxic substances.

The induction of the umuC-gene is thus a measure for the genotoxic potential of the sample. Since the umuC-gene is fused with the lacZ-gene for β-galactosidase, the induction of the umuC-gene can be easily assessed by determination of the β-galactosidase activity.

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Water quality — Determination of the genotoxicity of water and waste water using the umu-test

WARNING — This test involves the use of genetically modified organisms. National or international licensing may restrict the use of these organisms.

Test conducted according to this International Standard should be carried out by qualified experts or by a qualified testing laboratory.

When applying this International Standard it is necessary in each case, depending on the range to be tested, to determine if and to which extent additional criteria should be established.

1 Scope

This International Standard specifies a procedure which can be used to determine the genotoxicity[1] of water and waste water using the umu-test.

This assay is based on the detection of genotoxicity of a test sample which increases the expression of the SOS-repair system[2] associated with the umuC-gene[3].

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the normative document indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

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

3 Terms and definitions

For the purposes of this International Standard, the following terms and definitions apply. Other related terms and definitions have been included in annex A for information.

3.1
stock culture
culture of a bacterial strain to preserve the original test strain and to prepare the inoculation material for the overnight culture or the pre-culture

1) Toxicity which specifically affects the genome (genetic material).

2) SOS repair occurs when cells are overwhelmed by genotoxins allowing the cell to survive at the cost of mutagenesis.

3) umuC-gene is the acronym for UV mutagenesis gene C. The induction of the umuC-gene is part of the specific response of the bacterial cell to DNA-damage.

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3.2
overnight culture
culture of test bacteria for the preparation of the pre-culture

3.3
pre-culture
culture for adaptation of the overnight culture to the test conditions and to prepare the inoculum for the assay

3.4
inoculum
inoculation material
aliquot of a bacterial suspension used for inoculation in the assay

3.5
concentration effect relationship
induction of the umuC-gene depending on the concentration of genotoxic agents in the test sample

3.6
culture medium
aqueous solution of nutrients required for bacterial growth

3.7
test sample
the sample to be tested, after finishing all preparations

EXAMPLES Preparations may include centrifugation, filtration, homogenization, pH adjustment and measurement of conductivity.

3.8
dilution series
mixture of the test sample and dilution water in varying proportions

3.9
test mixture
mixture of culture medium, inoculum and dilution series

3.10
negative control
culture medium

3.10.1
blank
culture medium without bacteria

3.10.2
negative control for test samples
mixture of culture medium, inoculum and distilled water

3.10.3
solvent control
mixture of culture medium, inoculum and dimethyl sulfoxide

3.11
positive control
mixture of culture medium, inoculum and a dissolved genotoxic substance

EXAMPLES Typical genotoxic substances are 4-nitroquinoline-*N*-oxide or 2-aminoanthracene in the case of metabolic activation.

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3.12
S9 fraction
⟨metabolic activation system⟩ 9 000 *g* centrifugation supernatant prepared from the livers of male rats pretreated with enzyme-inducing agents

NOTE Bacteria are exposed to the test sample both with and without an appropriate metabolic activation system.

4 Principle

The test organisms are exposed to the test sample with and without metabolic activation system using microplates. After 4 h of incubation, the genotoxin-dependent induction of the umuC-gene is compared to the spontaneous activation of the untreated, control culture.

5 Test organism and reagents

5.1 Test organism and stock culture

5.1.1 Test organism

Salmonella typhimurium is a gram-negative, facultative, anaerobic bacterium from the *Enterobacteriaceae* family. *Salmonella typhimurium* TA1535 is the original strain. The test organism carries the plasmid pSK1002 with the umuC-lacZ gene and a gene for ampicillin resistance. The designation of this *Salmonella* strain is "TA1535/ pSK1002" (see annex B). This bacterial strain can be easily selected due to its ampicillin resistance.

5.1.2 Stock culture preparation and preservation

Preserve *Salmonella typhimurium* TA1535/pSK1002 in 150 µl culture medium with 10 % dimethyl sulfoxide (DMSO) or 20 % glycerol in 2 ml ampoules at a temperature not above − 80 °C. For the preparation of an overnight culture only one ampoule is used.

5.2 Reagents

Chemicals shall be of analytical grade. Prepare all solutions with purified deionized water or water of equivalent purity.

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

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

5.2.3 Dimethyl sulfoxide (DMSO), $C_2H_6SO_4$.

WARNING — DMSO forms mutagenic products over a period of time.

5.2.4 TGA-culture medium, consisting of tryptone, glucose and ampicillin, prepared as follows.

Dissolve 10 g of tryptone, 5 g of sodium chloride (NaCl) and 11,9 g of 4-(2-hydroxyethyl)-*l*-piperazineethanesulphonic acid (HEPES) in water, adjust the pH-value to 7,0 ± 0,2, dilute to 980 ml and autoclave for 20 min at 121 °C. Dissolve 2 g of *D*(+)-glucose (anhydrous) in 20 ml distilled water and autoclave separately. After autoclaving, mix the two solutions in equal proportions and add 50 mg of ampicillin to 1 000 ml of cooled TGA medium under sterile conditions. The solution can be stored in portions at − 20 °C for up to 4 weeks.

5.2.5 Concentrated 10× TGA-culture medium, consisting of a tenfold concentrated TGA (5.2.4) solution, which can be stored for 14 days at 4 °C.

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5.2.5.1 For incubation without S9, prepared as follows.

Dissolve 10 g of tryptone, 5 g of sodium chloride (NaCl) and 11,9 g of HEPES in 80 ml water. Adjust the pH-value to 7,0 ± 0,2. Dissolve 2 g of *D*(+)-glucose (anhydrous) in 20 ml of water. Autoclave the solutions separately for 20 min at 121 °C, mix the solutions and add 50 mg of ampicillin to 100 ml of the mixed solution under sterile conditions.

5.2.5.2 For incubation with S9, prepared as follows.

Dissolve 10 g of tryptone, 5 g of sodium chloride (NaCl), 2,46 g of potassium chloride (KCl), 1,63 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6\ H_2O$), and 11,9 g of HEPES in 80 ml of water. Adjust the pH to 7,0 ± 0,2. Dissolve 2 g of *D*(+)-glucose (anhydrous) in 20 ml of distilled water. Autoclave both solutions separately for 20 min at 121 °C, mix the solutions and add 50 mg of ampicillin to 100 ml mixed solution under sterile conditions.

5.2.6 B-buffer [4], consisting of a cell-lysis and reaction buffer, prepared as follows.

Dissolve 20,18 g of disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2\ H_20$), 5,5 g of sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), 0,75 g of potassium chloride (KCl), 0,25 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_20$) in 900 ml water. Adjust the pH to 7,0 ± 0,2. Then add 1,0 g of sodiumdodecylsulfate (SDS) and dilute to 1 000 ml. Before use, add 0,27 ml of 2-mercaptoethanol to 100 ml of B-buffer and mix.

5.2.7 Phosphate buffer pH (7,0 ± 0,2) [4].

Dissolve 1,086 g disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2\ H_20$) and 0,538 g sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) in 100 ml water.

If necessary adjust pH value to 7,0 ± 0,2. Autoclave the solution at 121 °C for 20 min.

5.2.8 Stop reagent [4].

Dissolve 105,99 g of sodium carbonate (Na_2CO_3) in 900 ml water and dilute to 1 000 ml.

5.2.9 *o*-Nitrophenol-*β*-*D*-galactopyranoside (ONPG) solution.

Dissolve 45 mg ONPG in 10 ml phosphate buffer (5.2.7).

Due to the poor solubility of ONPG, prepare this solution in advance and stir at room temperature in the dark until completely dissolved (approximately 2 h). Keep the solution in the dark.

5.2.10 S9 fraction, the required quantity being taken from the freezer.

Immediately after thawing, the S9 fraction is to be cooled on ice until use. Shake briefly before adding to the pre-culture.

NOTE The S9 fraction is available commercially.

5.2.11 Cofactor solution, consisting of a freshly prepared solution, kept on ice during the test and prepared as follows.

Dissolve 148 mg NADP (sodium salt) and 76 mg glucose-6-phosphate (disodium salt) in 5 ml 10× TGA (5.2.5).

5.2.12 Positive-control substances in dimethysulfoxide (DMSO), prepared as follows.

Warning — 2-AA can be rapidly photooxidized. Avoid prolonged exposure to light.

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4) This solution can be stored at room temperature.

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Dissolve 5 mg of 4-nitroquinoline-*N*-oxide (4-NQO) in 5 ml of DMSO. This stock solution can be stored in aliquots at − 20 °C.

Prior to the test dilute 2 000-fold using a 30 % by volume DMSO solution (DMSO/distilled water: 3/7).

Dissolve 5 mg of aminoanthracene (2-AA) in 5 ml of DMSO. This stock solution can be stored in aliquots at − 20 °C.

Prior to the test, dilute 500-fold using a 30 % by volume DMSO solution (DMSO/distilled water: 3/7).

6 Apparatus

The apparatus shall consist of the following:

— storage bottles: 250 ml and 500 ml;

— pipettes with rated volume: 1 ml, 10 ml, 25 ml;

— multi-channel pipettes (8 channel) having volumes: 5 µl to 50 µl, 50 µl to 200 µl, 50 µl to 300 µl;

— reservoirs for charging the multi-channel pipettes having volumes: 17 ml and 34 ml;

— measuring cylinders: 500 ml;

— culture vessels, conical flasks: 100 ml;

— sterile preservation ampoules: 2 ml (cryotubes);

— thermometer capable of measuring in the range 25 °C to 30 °C (± 1 °C);

— temperature- and time-controlled water bath;

— microplate-incubator for (28 ± 1) °C and (37 ± 1) °C with shaker (frequency of 125 r/min to 150 r/min);

— autoclave;

— centrifuge;

— 96-well microplates with lids and flat transparent bottoms (well capacity 380 µl);

— pH-meter;

— UV/Vis photometer (1 cm cuvettes);

— photometer for the microplates.

7 Sample preparation and preservation

Test water and waste water samples as soon as possible after sampling.

If immediate testing is not possible, store the sample at 4 °C. In this case, analyse the sample within 48 h after collection. Otherwise, keep the sample at a temperature below − 18 °C in accordance with ISO 5667-16.

In exceptional cases, additional preparation measures, for example centrifugation and filtration, can be carried out. This can lead to the elimination of genotoxic material (ISO 5667-16). Thus, avoid solvent extraction and concentration.

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Adjust the pH of the sample to 7,0 ± 0,2 before incubation by the addition of hydrochloric acid (5.2.1) or sodium hydroxide solution (5.2.2). Select the concentrations of acid or alkali in such a manner that the added volumes are as small as possible. Avoid over titration. The change of the sample pH and the resulting effects have to be taken into consideration (ISO 5667-16).

8 Interferences

Undissolved substances can falsify the test result and/or affect reproducibility.

In heavily coloured and/or turbid samples, light loss due to absorption can occur during photometric measurement. In this case, take the uninoculated sample as the blank.

If a sample contains high levels of cytotoxic materials, these may impede cell division and can even lead to cell death.

9 Test performance

9.1 Preparation of the overnight culture

Prepare and incubate the overnight culture under sterile conditions as follows:

- add 20 ml of TGA-culture medium (5.2.4) into a sterilized conical flask and close it with an air porous sterile stopper;
- thaw the frozen stock culture (5.1.2);
- add 1 ml TGA-culture medium (5.2.4) to the ampoule;
- centrifuge the test bacteria in the ampoule (10 min, 3 000 g);
- decant the culture supernatant;
- resuspend the test bacteria in 1 ml TGA-culture medium (5.2.4);
- inoculate the TGA medium in the conical flask with 0,5 ml of the test bacteria suspension;
- incubate overnight (no longer than 12 h, use a timer) by shaking at (37 ± 1) °C. Following incubation, an optical density of ⩾ 800 FNU (formazine nephelometric units) shall be achieved otherwise the culture has to be discarded;

9.2 Preparation of the inoculum

Prepare a tenfold dilution of the overnight culture with fresh warm TGA-culture medium (37 °C) (5.2.4).

Continue incubation for approximately 1,5 h by shaking at (37 ± 1) °C. Measure the optical density at (600 ± 20) nm in a 1 cm cuvette (see ISO 7027, reference [1] in the Bibliography) using TGA-culture medium as the blank and adjust using TGA-culture medium to 340 FNU to 350 FNU.

At this stage, the test organisms should be in the exponential growth phase and shall be ready to be used in the test, which has to be started within 10 min.

9.3 Test procedure without addition of S9 mixture

During the incubation of the pre-culture (9.2), dilute the samples and prepare the test plates. Carry out the test in triplicate for each concentration using the pipetting scheme given in Table 1 as follows:

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a) add 180 µl of distilled water to all wells, omitting A to F, 1 to 3 and H, 1 to 6;

b) add 360 µl of the test sample to the first three wells (three replicates determination) of the same microplate (A to F, 1 to 3: sample 1 in wells A, 1 to 3; sample 2 in wells B, 1 to 3 etc.);

c) at this point prepare a series of 1:2 dilutions of the test sample, (see pipetting scheme in Table 1). Make sure to resuspend the preparation each time to obtain proper mixing;

d) discard 180 µl from the last three wells (A to F, 10 to 12);

e) add 153 µl water to the positive-control and solvent-control wells (H1 to H6);

f) add 27 µl of a 30 % water/DMSO solution as solvent control to the wells H4 to H6;

g) add 27 µl of the 2 000-fold diluted NQO stock solution (5.2.12) as a positive control (H1 to H3). Final concentration: 50 ng/ml of 4-NQO;

h) add 20 µl of 10× TGA-culture medium (5.2.5) to all wells (A to H, 1 to 12);

i) pipette 70 µl of the inoculum (340 FNU to 350 FNU) (9.2) to all wells (A to F, 1 to 12) and mix (from right to left, i.e. from lower to higher concentration);

j) pipette 70 µl of the inoculum (9.2) to the negative-, solvent- and positive-controls (G, 1 to 12 and H, 1 to 6) and mix;

k) add 70 µl TGA-culture medium (5.2.4) to the blank (H7 to H12) and mix.

Table 1 — Pipetting scheme (96-well microplate with flat and transparent bottoms)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S1 1:1,5	S1 1:1,5	S1 1:1,5	S1 1:3	S1 1:3	S1 1:3	S1 1:6	S1 1:6	S1 1:6	S1 1:12	S1 1:12	S1 1:12
B	S2 1:1,5	S2 1:1,5	S2 1:1,5	S2 1:3	S2 1:3	S2 1:3	S2 1:6	S2 1:6	S2 1:6	S2 1:12	S2 1:12	S2 1:12
C	S3 1:1,5	S3 1:1,5	S3 1:1,5	S3 1:3	S3 1:3	S3 1:3	S3 1:6	S3 1:6	S3 1:6	S3 1:12	S3 1:12	S3 1:12
D	S4 1:1,5	S4 1:1,5	S4 1:1,5	S4 1:3	S4 1:3	S4 1:3	S4 1:6	S4 1:6	S4 1:6	S4 1:12	S4 1:12	S4 1:12
E	S5 1:1,5	S5 1:1,5	S5 1:1,5	S5 1:3	S5 1:3	S5 1:3	S5 1:6	S5 1:6	S5 1:6	S5 1:12	S5 1:12	S5 1:12
F	S6 1:1,5	S6 1:1,5	S6 1:1,5	S6 1:3	S6 1:3	S6 1:3	S6 1:6	S6 1:6	S6 1:6	S6 1:12	S6 1:12	S6 1:12
G	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
H	PC	PC	PC	SC	SC	SC	BL	BL	BL	BL	BL	BL

S1 = Sample No. 1, dilutions 1:1,5, 1:3, 1:6, 1:12

S6 = Sample No. 6, dilutions 1:1,5, 1:3, 1:6, 1:12

NC = Negative control

PC = Positive control

SC = Solvent control

BL = Blank

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9.4 Test procedure with addition of S9

The preparation of the dilution series takes place as described above [9.3 a) to 9.3 g)] up to the addition of 10× TGA-culture.

Instead of 4-NQO as a positive control [see 9.3 g); H1 to H3], use 27 µl of the 500-fold diluted 2-AA stock solution in 3 % DMSO (5.2.12). Final concentration: 200 ng/ml 2-AA.

Then, proceed as follows:

a) thaw S9 slowly (5.2.10);

b) add 20 µl cofactor solution (5.2.11) to each well;

c) add 450 µl S9 (shake briefly before adding) to 15 ml of the inoculum (340 FNU to 350 FNU) and mix the suspension;

d) pipette 70 µl of this suspension with S9 to the wells A1 to F12 and to the control wells and mix;

e) add 45 µl S9 (shake briefly before adding) to 1,5 ml of the TGA-culture medium to prepare the blanks + S9 and add 70 µl of this mixture into the blank wells.

9.5 Incubation

Microplates A, B, and C are to be used for cultures without S9 (9.3) and another three microplates separately, for those with S9 (9.4). Proceed as follows:

— cover the microplates with lids;

— incubate the microplate A for 2 h at (37 ± 1) °C, on a shaker (120 r/min to 150 r/min) to prevent sedimentation of the bacteria. Avoid cross-contamination;

— towards the end of the first incubation phase (2 h) load a new microplate B with TGA-culture medium (270 µl each well) and place it in an incubator to adjust temperature (37 ± 1) °C. Use lids to avoid evaporation;

— pipette 30 µl from each well of microplate A into a corresponding well of microplate B (tenfold dilution);

— incubate microplate B for 2 h in the same way as microplate A.

NOTE A recapitulative table of the composition of the sample and control wells is given for information in annex C.

10 Measurements

10.1 Measurement of the optical density

Measure the bacterial growth in plate B[$A_{(600 \text{ nm} \pm 20 \text{ nm})}$] with a photometer for microplates after 2 h of incubation.

10.2 Determination of the induction of the umuC-gene

Proceed as follows:

— place 120 µl B-buffer (5.2.6) in each well of a new microplate (C);

— adjust the temperature of the plate to (28 ± 1) °C in a microplate incubator (use a lid to avoid evaporation);

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— place 30 µl from each well of microplate B (from right to left, i.e. from the lower to the higher concentration) in the corresponding wells of microplate C. Immediately add 30 µl ONPG solution (5.2.9) and mix;

— place the microplate immediately into the incubator and incubate on the shaker for 30 min at (28 ± 1) °C;

— stop the reaction by adding 120 µl of stop reagent (5.2.8) to each well of microplate C;

— mix and remove bubbles by means of a cold air stream;

— measure the absorption of the solution in each well immediately at (420 ± 20) nm with a photometer for microplates;

— decontaminate all microplates by autoclaving.

11 Calculation and expression of results

Prepare a table which shows the individual concentrations (dilution levels), the measured optical density and absorption values, the induction ratio and β-galactosidase units calculated and the growth factors.

Determination of the growth factor G:

$$G = \frac{A_{600,\mathrm{T}} - A_{600,\mathrm{B}}}{A_{600,\mathrm{N}} - A_{600,\mathrm{B}}}$$

where

$A_{600,\mathrm{T}}$ is the absorption of the sample well at (600 ± 20) nm.

$A_{600,\mathrm{N}}$ is the absorption (mean value) of the negative control at (600 ± 20) nm.

$A_{600,\mathrm{B}}$ is the absorption (mean value) of the blank at (600 ± 20) nm.

Calculation of the activity of the β-galactosidase in relative units (U_T):

$$U_\mathrm{T} = \frac{A_{420,\mathrm{T}} - A_{420,\mathrm{B}}}{A_{600,\mathrm{T}} - A_{600,\mathrm{B}}}$$

The units of the negative controls (U_N) shall be calculated in an analogous manner.

NOTE 1 The wells G1 to G12 contain the negative controls for the water samples according to the pipetting scheme (Table 1).

Determination of the induction ratio I_R:

$$I_\mathrm{R} = \frac{1}{G} \times \frac{A_{420,\mathrm{T}} - A_{420,\mathrm{B}}}{A_{420,\mathrm{N}} - A_{420,\mathrm{B}}}$$

where

$A_{420,\mathrm{T}}$ is the extinction of the sample well at (420 ± 20) nm;

$A_{420,\mathrm{N}}$ is the extinction (mean value) of the negative control respectively solvent control at (420 ± 20) nm;

$A_{420,\mathrm{B}}$ is the extinction (mean value) of the blank at (420 ± 20) nm.

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The smallest value of D (dilution level), at which $I_R < 1,5$ is measured, shall be taken as the result (see also informative annex D).

If different induction ratios are measured with or without S9, then the higher of the two values shall be taken as the result.

NOTE 2 If a typical results are obtained, check the genotype (see informative annex E).

12 Precision

Precision data taken from an interlaboratory study are presented in Tables F.1 and F.2 of informative annex F.

13 Validity criteria

The test is considered valid if the positive controls reach an induction ratio of at least 2 under the given test conditions.

The results cannot be evaluated if $G < 0,5$.

Minimum growth of the negative controls (G, 1 to 12) on plate B is 140 FNU.

14 Test report

The test protocol (see annex G for an example) shall refer to this International Standard and contain the following points:

a) identification of the test material;

b) preparation of the sample:

— pH adjustment;

— storage of the samples;

— centrifugation;

— filtration;

— homogenization;

— solvents used;

c) test organism (type, strain);

d) date of test;

e) statement of the results: growth factors, A_{600} and A_{420} values, β-galactosidase units [(A_{420}/A_{600})-values], standard error;

f) performance characteristics:

— possible deviations from the procedure specified in this International Standard;

— other effects (for example turbidity, solubility, precipitation);

— procedural characteristics;

— quality control criteria (for example level of β-galactosidase units related to historical data).

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Annex A
(informative)

General definitions and terms regarding genotoxicity

A.1
genotoxicity
toxicity which specifically affects the genome (genetic material)

A.2
genotoxicity test
a test system to demonstrate genotoxicity for example DNA-damage and DNA-repair

A.3
DNA damage
modifications of the molecular structure of genetic material caused by chemical or physical agents

EXAMPLES Typical DNA damage is single- and double-strand breaks, DNA crosslinks, oxidative lesions, loss of bases and adduct-formation.

A.4
genome
total genetic material (nucleic acids, DNA) of a cell

A.5
gene mutation
point mutation
changes of DNA at the molecular level in terms of loss (deletion) or addition (insertion) of nucleotides (frameshift mutations) or substitution of nucleotic bases

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Annex B
(informative)

Salmonella typhimurium TA1535/pSK1002

This strain is available from the German Collection for Microorganisms and Cell cultures (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig, Germany: Order No. 9274.

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Annex C
(informative)

Composition of the sample and control wells

The composition of the sample and control wells is given in Table C.1.

Table C.1

Samples						
Dilution	**Dilution level** D	**Sample** (clause 7) µl	**Dilution water** (clause 5) µl	**10× TGA** (5.2.5 or 9.4) µl	**Inoculum** (9.2 or 9.4) µl	
1:1,5	1,5	180	0	20	70	
1:3	3	90	90	20	70	
1:6	6	45	135	20	70	
1:12	12	22,5	157,5	20	70	
Controls						
		Positive control/ solvent control µl	**Dilution water** µl	**10× TGA** µl	**Inoculum** µl	**1× TGA** µl (5.2.4)
Blank		0	180	20	0	70
Negative control		0	180	20	70	0
Positive/solvent control		27	153	20	70	0

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Annex D
(informative)

Lowest value of the dilution series

The lowest D value of the dilution series at which the measured induction rate < 1,5 is the so called D_{LI} or [$D(I_R < 1,5)$].

D_{LI} = lowest ineffective dilution

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Annex E
(informative)

Checking the genotype

If atypical test results are obtained (poor growth, induction ratio of the positive controls less than 2) the presence of the genetic markers pSK1002, deep rough mutation and UVrB should be checked. This should also be done with fresh stocks received from the DSMZ.

Ampicillin resistance (pSK1002)

Spread parallel lines of the bacterial suspension (overnight culture) on two ampicillin agar plates. This should also be done in parallel with a strain which does not contain the plasmid pSK1002 (for example TA1535). Incubate the plates overnight at (37 ± 2) °C. No growth should be observed for TA1535, whereas full growth should be observed for the tester strain *Salmonella typhimurium* TA1535/pSK1002.

Crystal-violet sensitivity (deep rough)

A quantity of 0,1 ml bacterial solution should be taken from the overnight culture and spread onto four nutrient agar plates. After a few minutes filter papers (diameter 9 mm) soaked with 10 µl of an aqueous crystal-violet solution (concentration 1 mg/ml) should be placed in the middle of the plates. Incubate the plates overnight at (37 ± 2) °C. The diameters of the resulting growth inhibition zones around the filter paper should be measured. The mean of the inhibition zone should be at least 14 mm.

UV sensitivity (UVrB)

A quantity of 0,1 ml bacterial solution should be taken from the overnight culture and spread onto four nutrient agar plates. Cover one half of each plate with aluminium foil and irradiate without a lid for 8 s with UV light (30 W) at a wavelength of 254 nm and at a distance of 33 cm. Incubate the irradiated plates overnight at (37 ± 2) °C. In the case of adequate sensitivity, the bacteria should show growth inhibition over the irradiated area.

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Annex F
(informative)

Precision data from an interlaboratory study

Precision data taken from an interlaboratory study carried out in accordance with the DIN[5] method are presented in Tables F.1 and F.2.

Table F.1 — Data taken from a German interlaboratory study

umu-Test	l	n	o %	CV %
Sample 1 to 8 (mean) without S9	10	140	5	17,5
Sample 1 to 8 (mean) with S9	10	134	2,1	24,8

l number of laboratories (outliers eliminated)
n number of measured values (outliers eliminated)
o percentage of outliers
CV coefficient of variation

Table F.2 — Data taken from a German interlaboratory study

umu-Test	$\bar{x}$	σ	CV %
4-NQO	2,404	0,358	14,91
2-AA with S9	2,512	0,445	17,73
Effluent 1	3,174	0,636	20,03
Effluent 2	7,422	0,826	11,13
Effluent 3	6,006	0,753	12,54

$\bar{x}$ mean value of the induction ratio
σ standard deviation
CV coefficient of variation

5) DIN 38415-3, *Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung — SuborganismischeTestverfahren (Gruppe T) — Teil 3: Bestimmung des erbgutverändernden Potentials von Wasser mit dem umu-Test (T 3).*

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Annex G
(informative)

Final protocol for the umu-test

Sample / dilution step (A to F, 1 to 12) D	**S9**	**Growth factor** G	**Induction ratio** I_R
1,5 (original sample)	– S9:..		
3	– S9:..		
6	– S9:..		
12	– S9:..		
1,5 (original sample)	+ S9:..		
3	+ S9:..		
6	+ S9:..		
12	+ S9:..		

Positive controls:

4-NQO (4-NQO: 50 ng/ml) – S9:..

2-AA (2-AA: 200 ng/ml) + S9:..

NOTE In dilution step 1 (original sample) the sample is already diluted by the medium and the reagents 1:1,5.

Therefore the smallest value of D (original sample) is 1,5.

The smallest value of D, at which an induction ratio $< 1,5$ is measured under the given test conditions, counts as the result $[D(I_R < 1,5)]$.

If by the addition of S9 differing induction ratios are measured, then the higher of the two values counts as the results $[D(I_R < 1,5)]$.

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