INTERNATIONAL STANDARD

ISO 21427-2

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Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei —

Part 2: Mixed population method using the cell line V79

Qualité de l'eau — Évaluation de la génotoxicité par le mesurage de l'induction de micronoyaux —

Partie 2: Méthode de la population mélangée à l'aide de la lignée de cellules V79

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Foreword

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

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

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 21427-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 21427 consists of the following parts, under the general title *Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei*:

— *Part 1: Evaluation of genotoxicity using amphibian larvae*

— *Part 2: Mixed population method using the cell line V79*

Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei —

Part 2:
Mixed population method using the cell line V79

WARNING — Persons using this part of ISO 21427 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 part of ISO 21427 be carried out by suitably trained staff.

1 Scope

This part of ISO 21427 specifies a method for the determination of genotoxicity of water and waste water using a mammalian *in vitro* test which detects damage, induced by water-soluble substances, to the chromosomes or the mitotic apparatus of V79 cells from the Chinese hamster.

The micronucleus test allows the identification of substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments and/or whole chromosomes.

The assay is based on the increase in the frequency of micronucleated cells after incubation with and without metabolic activation.

2 Normative references

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

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

3 Terms and definitions

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

3.1
cell lines
distinct families of cells grown in culture originated from a single clone

3.2
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

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

3.4
D value
smallest value of D at which, under the conditions of this part of ISO 21427, no increase in the number of micronuclei per culture is detected

NOTE In the case of more than one D value (at maximum two are possible, see 9.2), the highest D value is decisive.

3.5
karyotype
characteristic of the nucleus of a cell, including its size, form and chromosome number

3.6
micronuclei
small particles consisting of acentric fragments of chromosomes and/or entire chromosomes which lag behind at anaphase stage of cell division and form, after telophase, single or multiple micronuclei in the cytoplasm

3.7
mitotic index
percentage of cells of a cell population under division at a particular time of observation

3.8
plating efficiency
measure of the number of colonies originated from single cells

3.9
proliferation index
rate at which cells are dividing within the culture

3.10
proliferation rate
rate with which cells replicate, calculated by a formula which takes into account 1, 2, 4 and 8 cell stages of clones

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

3.12
S9 mix
mixture of the S9 fraction and the cofactor solution

3.13
stock culture
frozen culture for the preservation of the characteristics of V79 cells

3.14
survival index
percentage of surviving cells compared to all cells, used as index of toxicity

3.15
test culture
culture of cells used for the study

4 Principle

The possible clastogenic and/or aneugenic activity of the test sample is detected by comparing, for the respective activation condition, the number of micronucleated cells in cultures treated with the negative control and the number in cultures treated with undiluted and diluted test samples, respectively.

During cell division, chromatid fragments without centromers will not move to the nuclei of the daughter cells and will stay within the cytoplasm. Part of the chromosome aberrations induced by the test item will be chromatid fragments without centromers and will, therefore, not be incorporated in the nuclei of the daughter cells. In addition, spindle disorders may lead to chromosomes which are not incorporated into the nucleus. These particles will form micronuclei in the plasma.

V79 cells are exposed for 24 h (4 h with the S9 mix) to a range of concentrations of a test sample. Thereafter, slides are prepared, and cells are stained and evaluated for the presence of micronucleated cells. An increased incidence of these micronucleated cells in comparison to the negative control indicates that the test item may cause chromosome breaks or spindle disorders in V79 cells *in vitro*.

5 Interferences

Biologically relevant alterations of the culture conditions may induce chromosome aberration due to secondary mechanisms resulting in artificial positive and, therefore, irrelevant results [16]. Those factors are, e.g. stronger changes in osmolality or pH, precipitation of test sample and phagocytosis thereof, and strong cytotoxic effects of the test sample. Therefore, test samples should be monitored at least for changes in pH or osmolality of the cultures using the same proportion of test item per culture as will be used later under test conditions. If there is a shift in pH in the culture, the test item should be adjusted to pH 7,0 ± 0,2. If there is a change in osmolality, the highest concentration used in the test has to be reduced so that no relevant alteration of osmolality occurs in the cultures. To avoid artifacts based on phagocytosis or severe cytotoxicity, limitations are given for the highest concentration, which should be used for testing (see 9.1 and 9.2).

6 Reagents and media

As far as possible use “reagent grade” chemicals.

If chemicals with different amounts of water of crystallization 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.

6.1 Water.

Prepare all aqueous solutions with water of a conductivity of ⩽ 5 µS/cm.

6.2 Reagents.

6.2.1 Glucose-6-phosphate dihydrate, $C_6H_{11}O_9PNa_2 \cdot 2\ H_2O$.

6.2.2 Nicotinamide adenine dinucleotide phosphate disodium salt, NADP, $C_{21}H_{26}N_7Na_2O_{17}P_3$.

6.2.3 Magnesium chloride hexahydrate, $MgCl_2 \cdot 6\ H_2O$.

6.2.4 Potassium dihydrogenphosphate, KH_2PO_4.

6.2.5 di-Sodium hydrogenphosphate dihydrate, $Na_2HPO_4 \cdot 2\ H_2O$.

6.2.6 Ethanol (absolute), C_2H_5OH.

6.2.7 Glacial acetic acid, CH_3COOH.

6.2.8 Formaldehyde, HCHO, 37 % volume fraction.

6.2.9 tri-Sodium citrate dihydrate, $HOC(COONa)(CH_2COONa)_2 \cdot 2\ H_2O$.

6.2.10 di-Sodium hydrogenphosphate, Na_2HPO_4.

6.2.11 Sodium dihydrogenphosphate, NaH_2PO_4.

6.2.12 May-Grünwald-solution, modified [1].

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

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

6.2.15 Dimethyl sulfoxide (DMSO), $C_2H_6SO_4$.

6.2.16 Positive controls.

6.2.16.1 Cyclophosphamide, monohydrate, $C_7H_{15}Cl_2N_2O_2P \cdot H_2O$.

CAS Registration No: 6055-19-2.

6.2.16.2 Ethyl-methane sulfonate (EMS), $CH_3SO_3CH_2CH_3$.

CAS Registration No: 62-50-0.

6.2.17 Sodium citrate solution for hypotonic treatment.

Prepare a 1,5 % solution of tri-sodium citrate in water.

6.2.18 Fixation solution.

Mix 50 ml of glacial acetic acid with 150 ml of ethanol, add 2,5 ml of a 37 % formaldehyde solution.

6.2.19 Buffer solution according to WEISE (pH 7,2) [1].

This solution is commercially available in ampoules. Dilute the contents of one ampoule in water and, using a 1 000 ml measuring flask, bring to volume with water.

6.2.20 Giemsa solution [1].

Prepare a 2,6 % Giemsa solution in buffer according to WEISE (pH 7,2) (6.2.19). Filter prior to use.

6.2.21 Phosphate buffer.

Dissolve 2,13 g of Na_2HPO_4 in 1 l water. Dissolve 1,8 g of NaH_2PO_4 in 1 l water. Mix both solutions at the ratio of 4:1 and adjust to a final pH of 7,4.

6.2.22 MEM-medium (= Minimal Essential Medium) with stabilized glutamine [1].

6.2.23 Fetal bovine serum (= FCS) [1].

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1) This reagent is commercially available. This information is given for the convenience of users of this part of ISO 21427 and does not constitute an endorsement by ISO of these products.

6.2.24 Penicillin/Streptomycin solution, 10 000 E/10 000 µg/ml [1].

6.2.25 Amphotericin-B solution, 250 µg/ml [1].

6.2.26 Trypsin/EDTA solution, 0,25 % [1].

6.2.27 Hanks' Balanced Salt Solution (= HBSS) [1].

6.2.28 Hanks' Balanced Salt Solution (= HBSS) without Ca^{2+} and Mg^{2+} [1].

6.2.29 Potassium chloride solution.

Dissolve 4 g of potassium chloride, in 1 l water.

6.3 Preparation of culture media

6.3.1 Culture medium with FCS.

This medium is used as general culture medium and for treatment of cells without the S9 mix.

Mix 500 ml of MEM-medium, 50 ml of FCS, 5 ml of Penicillin/Streptomycin solution and 5 ml of Amphotericin-B solution.

The medium is stable for up to 4 weeks if stored in a refrigerator at 4 °C ± 2 °C.

6.3.2 Culture medium without FCS.

This medium is used only for the treatment period of cells under activation condition (S9 mix).

Mix 500 ml of MEM-medium, 5 ml of Penicillin/Streptomycin solution and 5 ml of Amphotericin-B solution.

The medium is stable for up to 4 weeks if stored in a refrigerator at 4 °C ± 2 °C.

6.4 Cell system.

6.4.1 Cell line, storage

The V79 cell line is a permanent cell line of Chinese hamster lung cells with

— a high proliferation rate (cell cycle length about 12 h to 16 h);

— a high plating efficiency (⩾ 90 %);

— a stable karyotype (modal number of chromosomes = 22).

Store permanent cultures (1 ml samples including 7 % DMSO) in liquid nitrogen at about −196 °C. Prior to freezing, check each batch for mycoplasma contamination. Karyotype and plating efficiency (colony-forming ability) should be determined at least prior to the first use of a thawed culture.

6.4.2 Cultivation

To start a culture, thaw a permanent culture in a water bath at 37 °C and add 0,5 ml of this sample to a 25 cm^2 culture flask filled already with approximately 5 ml of MEM (minimal essential medium; composed of medium, glutamine and antibiotics) including 10 % FCS (fetal calf serum). Cultivate the cells at 37 °C, using 5 % carbon dioxide and a humidity of at least 90 %. Subcultivate the cells twice a week.

Withdraw the flasks (25 cm^2) from the incubator and place them on a clean bench. Open the flasks singly and remove the medium by suction. Wash the cells once with 5 ml Hanks Balanced Salt Solution (HBBS, without Ca^{2+} and Mg^{2+}) for about 5 min. Thereafter, remove the medium again.

Trypsinize the cells for about 5 min using about 1,0 ml of trypsine (0,25 %) and approximately 1,0 ml HBBS (without Ca^{2+} and Mg^{2+}) to separate the cells from the bottom of the culture flask.

Stop this reaction by adding approximately 3 ml of MEM including 10 % FCS.

Pipette this mixture several times to separate the cells from the flask and to obtain homogenous single cell suspensions.

Count the number of cells in a 10 µl sample in a hemocytometer [2].

Dilute the suspension to the required cell density (30 000 to 80 000 per culture) using MEM including 10 % of FCS.

6.4.3 Duration of cell cycle

The cell cycle length of the V79 cells is normally about 12 h to 16 h. Determine its laboratory specific length using the BrdU[3] method (see Annex A).

6.5 Metabolic activation

6.5.1 S9 fraction

For the treatment of enzyme induction and preparation of the S9 fraction, see Annex C. If the S9 fraction is commercially purchased, it shall have been prepared (including enzyme induction) according to Annex C.

6.5.2 S9 mix

Prepare the needed amount of the S9 fraction freshly on the day of test or, if stored frozen, thaw at room temperature. Immediately thereafter, prepare the S9 mix by mixing the following compounds under sterile conditions:

a) 1 aliquot of S9 fraction;

b) 9 aliquots of S9 supplement (cofactor solution).

Keep the S9 mix permanently on ice (e.g. in a double-walled separator funnel containing iced water in between these walls) and use it only on the same day. At the end of this day, discard the remaining S9 mix. The concentrations of cofactors in the S9 mix are as follows:

$MgCl_2$	8 mmol/l
KCl	33 mmol/l
Glucose-6-phosphate	5 mmol/l
NADP	4 mmol/l
Phosphate buffer (pH 7,4)	15 mmol/l

2) Alternatively, an automatic cell or particle counter device may be used.

3) BrdU stands for bromodeoxyuridine.

7 Apparatus

7.1 Cryo-vials, 1 ml, 2 ml, 5 ml.

7.2 Cell culture flasks, 25 cm^2, 75 cm^2.

7.3 Culture chambers, appropriate for 4 microscope slides, approx. 9 cm × 13 cm.

7.4 Microscope slides with frosted part.

7.5 CO_2 incubator.

7.6 Laminar-airflow work bench.

7.7 Water bath.

7.8 Vacuum pump.

7.9 Inverse microscope.

7.10 Light microscope.

7.11 Bunsen burner.

7.12 Centrifuge.

7.13 Freezer (–80 °C).

7.14 Analytical balance.

7.15 Glass pipettes, 1 ml, 5 ml, 10 ml.

7.16 Adjustable volume pipettes.

7.17 Neubauer counting chamber.

7.18 Photographic clips.

7.19 Dewar tank for storage of the cells in liquid nitrogen.

7.20 Tweezers.

7.21 Cuvettes including holders for staining.

7.22 Sterile filters, 0,22 µm.

8 Test facility criteria

The test facility is qualified for the execution of this part of ISO 21427 if the *in vitro* micronucleus test is established in this facility according to the following criteria:

— a couple of independent experiments shall already have been performed;

— a couple of known mutagenic and non-mutagenic chemicals shall already have been tested.

It should be decided case by case whether and to what extent additional instructions may be necessary for the application of this part of ISO 21427.

9 Procedure

9.1 Sampling and samples

Test samples as soon as possible after sampling, i.e. on the day of collection. Divide large samples into appropriate portions in advance, since thawed samples may only be used on the same day as they were thawed.

High or low pH-values of the test sample may trigger cell toxic effects lowering the possible maximum testable concentration. Therefore, pH of the test sample should be monitored and adjusted, if necessary, to pH 7,0 ± 0,2 using either HCl or NaOH solution (6.2.13 and 6.2.14). Select the concentrations of acid or alkali such that the added volumes are as small as possible. Avoid over-titration. The change of the sample pH and the resulting effects shall be taken into consideration (see ISO 5667-16).

Shake test samples thoroughly before use.

Centrifuge the samples containing solids and use only the liquid supernatant for further processing.

Sterilize all the samples by filtration through sterile filters (7.22). Do not extract or concentrate the samples.

NOTE 1 If necessary, keep the samples at 0 °C to 5 °C for up to 48 h and below −18 °C for up to two months, respectively.

NOTE 2 See ISO 5667-16.

If dilutions are necessary, perform these with sterilized water (6.1).

9.2 Experimental size

Use two cultures per experimental group. Evaluate 1 000 cells per culture for micronuclei. See Table 1.

Table 1 — Experimental size

Treatment group	S9 mix	Treatment time h	Time of harvest h
negative control dilution water	-	24	24
undiluted test item [a]	-	24	24
1:2	-	24	24
1:4	-	24	24
1:8	-	24	24
1:16	-	24	24
1:32	-	24	24
positive control EMS (6.2.16.2) 350 µg/ml	-	24	24
negative control dilution water	+	4	24
undiluted test item [a]	+	4	24
1:2	+	4	24
1:4	+	4	24
1:8	+	4	24
1:16	+	4	24
1:32	+	4	24
positive control cyclophosphamide (6.2.16.1) 2,5 µg/ml	+	4	24

[a] If osmolality is relevantly altered, the highest dilution which does not alter osmolality of the culture.

9.3 Negative controls

Treat the cultures of the negative controls with the dilution water in the same volume as other cultures are treated with test item.

9.4 Positive controls

Dissolve the positive controls in MEM to result in a concentration of EMS of 1,75 mg/ml and in a concentration of cyclophosphamide of 12,5 µg/ml. Apply a volume of 1 ml per culture.

Store the stock solutions of the positive controls at −80 °C. In this case, they should be thawed shortly prior to treatment.

9.5 Time schedule

− 6 h	Seeding of cells
0 h	Treatment
+ 4 h	Washing of cultures (only those with S9 mix)
+ 24 h	Harvest and slide preparation

9.6 Preparatory steps

Thaw permanent cultures in a water bath at 37 °C. Add 0,5 ml of a thawed culture to a 25 cm^2 flask which already contains 5 ml of MEM (including 10 % FCS). Incubate cultures at 37 °C, using an atmosphere containing a volume fraction of 5 % CO_2 and at least 90 % humidity, to reach a confluency (contact inhibition) of 50 % to maximally 100 % (about 2 d to 4 d). To remove dead cells, replace the medium about 30 h after culture initiation. Use the remaining cells for experiments. (They may also be used for further subculturing.) Discard cells which have undergone more than 15 passages because they cannot be used for further experiments.

9.7 Preparation of test cultures

9.7.1 General

Wash the cells once with 5 ml HBBS (without Ca^{2+} and Mg^{2+}) for about 5 min and then remove the medium by suction.

Trypsinize the cells for about 5 min using about 1,0 ml of trypsin (0,25 %) and approximately 1,0 ml of HBBS (without Ca^{2+} and Mg^{2+}) to separate the cells from the bottom of the culture flask.

Stop this reaction by adding approximately 3 ml of MEM including 10 % FCS.

Pipette this mixture several times to separate cells from the flask and to obtain homogenous, single cell suspensions.

Count the number of cells in a 10 µl sample in a hemocytometer [4].

Dilute the suspension to the required cell density (30 000 to 80 000 per 5 ml culture) using MEM including 10 % FCS.

9.7.2 Seeding

Add 5 ml of cell suspension to each chamber of a culture dish (7.3), of which each chamber contains a slide. Each chamber represents one culture.

Incubate the dishes for 6 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO_2 and at least 90 % humidity.

Prior to treatment, check the dishes visually for cell attachment. (See Annex B.)

9.7.3 Preparation of S9 mix

Prepare the S9 mix according to 6.5.2.

9.8 Treatment of test cultures

9.8.1 Treatment without S9 mix

Remove the medium from the culture chambers approximately 6 h after seeding. Add 4 ml of fresh medium (with FCS). Thereafter, add the test sample, then either the positive control or the dilution water for the negative control in a volume of 1 ml (final volume of 5 ml). Incubate the cultures for 24 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO_2 volume fraction and at least 90 % humidity. Higher concentrations of test sample can be applied as long as the validity and cytotoxicity criteria (3.14, 9.8.5, 10.2) are met. If higher amounts of sample are used, the concentration of the medium has to be adjusted.

4) Alternatively, an automatic cell or particle counter device may be used.

9.8.2 Treatment with S9 mix

Remove the medium from the chambers approximately 6 h after seeding. Add to each chamber 1 ml of the S9 mix and 3 ml of fresh medium (without FCS). Thereafter, add the test sample, then either the positive control or the dilution water for the negative control in a volume of 1 ml per culture, resulting in a final volume of 5 ml. Incubate cultures for 4 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO_2 and at least 90 % humidity. Higher concentrations of test sample can be applied as long as the validity and cytotoxicity criteria (3.14, 9.8.5, 10.2) are met. If higher amounts of sample are used, the concentration of the medium has to be adjusted.

After 4 h, remove the medium and wash the cultures twice with 5 ml HBSS. Add 5 ml of MEM (incl. 10 % FCS) and incubate for further 20 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO_2 and at least 90 % humidity.

9.8.3 Processing of cell cultures

Prepare cells as follows [13]:

— remove the culture medium completely;

— treat each culture for 3 min with 5 ml of sodium citrate-solution (6.2.17) at a temperature of 37 °C (hypotonic treatment);

— remove the sodium citrate-solution and replace it by approximately 5 ml of fixation solution (6.2.18) at a temperature of about 4 °C for about 5 min; remove and replace the fixation solution by the same amount of fresh fixation solution (4 °C) for about 5 min;

— remove the slides from the culture chambers. Allow the fixation solution to drip off and dry the slides at ambient temperature.

9.8.4 Staining

— After the slides have dried, stain for 3 min in May-Grünwald-solution, modified (6.2.12);

— rinse the slides with WEISE-buffer (6.2.19) and place the slides for approximately 20 min in a Giemsa-solution (6.2.20);

— rinse twice with WEISE-buffer and remove surplus of staining material in xylene. Thereafter, cover the slides with coverslips and examine the slides as in 10.3.

9.8.5 Treatment for determination of the survival index

Seed approximately 250 000 cells into 25 cm^2 flasks already containing 5 ml of MEM (including 10 % FCS) per negative control or test sample concentration. Cultivate the cells for 30 h at 37 °C in an atmosphere containing a volume fraction of 5 % CO_2 and at least 90 % humidity (9.6). Thereafter, treat the cells with dilution water or test sample concentrations (9.8).

At the end of the respective treatment period, harvest the cells of all cultures as follows:

— remove the medium and wash once with 5 ml of HBBS (without Ca^{2+} and Mg^{2+});

— trypsinize with about 1,0 ml of trypsin (0,25 %) and about 1,0 ml of HBBS (without Ca^{2+} and Mg^{2+}) to remove cells from bottom of flasks;

— stop the process by adding 3 ml of MEM (including 10 % FCS);

— pipette this mixture several times to separate cells from the flask and to obtain homogeneous single cell suspensions;

— dilute the cells to an appropriate amount in negative controls. Dilute all other cultures as cell suspensions of the negative controls were diluted. The same amount is taken from each culture and counted using a haemocytometer [5)] to determine cell survival.

10 Evaluation and assessment

10.1 Cell morphology

At the end of the treatment, check all the cultures by an inverse microscope for changes in cell morphology and attachment of cells at a magnification of about 200-fold with the exception of positive controls. (See Annex B.)

10.2 Selection of concentrations to be analysed

For non-toxic samples, read only the highest tested concentration. For toxic samples, the lowest concentration which induces more than 50 % cytotoxicity in at least one of the indices (10.4, 10.5, 10.6) shall be chosen for reading of slides. Independently, no relevant changes should be induced in cell morphology (10.1) or attachment of cells. If those effects are observed for this concentration, the concentration for reading shall be reduced to the highest concentration which does not induce changes in cell morphology or cell attachment. For toxic samples, the next two lower dilutions of the test sample shall also be read. If a relevant increase in micronucleated cells is observed, lower concentrations are read until the first concentration without clastogenic effect is reached.

Prior to reading slides, check them for their quality. Use only slides of good quality for reading to avoid interference of slide quality with the assessment.

10.3 Reading of slides

Read 1 000 cells per culture and note the number of micronucleated cells amongst them.

Take into account the following criteria:

— the maximum size of a micronucleus is about 30 % of the size of a normal nucleus;

— concerning their staining micronucleus and nucleus shall have the same appearance;

— micronuclei shall be clearly separated from the nucleus;

— only cells with good cytoplasmic outlines are used for reading.

In addition to micronucleated cells, record fragmentations (cells with fragmented nuclei, cells with several nuclei of the same size, and cells with more than six micronuclei) amongst the evaluated 1 000 cells.

10.4 Mitotic index

Determine the mitotic index (3.7) for all cultures microscopically on the slides. The number of mitotic cells among a total of 1 000 cells per culture is determined. All cells which were not in interphase are defined as mitotic.

5) Alternatively, an automatic cell or particle counter device may be used.

10.5 Survival index

Determine the survival index (3.14 and 9.8.5) for all cultures except for positive controls. The number of cells observed for negative controls is set to 100 %. On this basis, the number of living cells in the test sample-treated cultures is converted to a percentage.

10.6 Proliferation index

Determine the proliferation index (3.9) for all cultures except for positive controls.

Evaluate 1 000 cells (counting all cells of each clone) per culture, separated into different clone sizes (1, 2, 4 or 8 cells). Calculate the proliferation index following Equation (1):

$$I_P = \frac{(n_{CL1} \times 1) + (n_{CL2} \times 2) + (n_{CL4} \times 3) + (n_{CL8} \times 4)}{n_{CL}} \quad (1)$$

where

I_P is the proliferation index;

n_{CL} is the number of clones counted;

n_{CL1} is the number of clones with 1 cell;

n_{CL2} is the number of clones with 2 cells;

n_{CL4} is the number of clones with 3 or 4 cells;

n_{CL8} is the number of clones with 5, 6, 7 or 8 cells.

Due to clone disintegration in experiments with the S9 mix, this index is most frequently smaller if compared to experiments without the S9 mix.

10.7 Validity criteria

The mean of the ratio of micronucleated cells in negative control cultures shall not exceed 3 %.

Positive controls shall have induced statistically significant increases in the ratio of micronucleated cells.

10.8 Assessment criteria

The following criteria shall be met for a positive result:

— a significant increase according to an appropriate statistical test (chi-squared test corrected for continuity according to Yates) [15] in micronucleated cells in treated cultures as compared to the respective negative controls;

— the number of micronucleated cells exceed the range of the historical negative control data.

If neither criteria is met, the test sample shall be evaluated as non-genotoxic according to this part of ISO 21427. If only one criterion is met, the test shall be repeated as the result is ambiguous.

10.9 Determination of the decisive D value

The “decisive D value” means the lowest D value (3.4) at which no detectable genotoxic effects are found for cultures treated with the test sample or dilutions thereof according to the criteria given in 10.8.

11 Precision

Results of an interlaboratory trial are given in Annex D.

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 reference to this part of ISO 21427 (ISO 21427-2);

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

— result, according to Clause 10;

— if appropriate, any deviation from this procedure or circumstances that may have affected the result.

The documentation will include the following information:

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

b) 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 diameter of pores) and other manipulations);

c) cell line (cell line, source, date of arrival, storage conditions, check for karyotype stability, plating efficiency and mycoplasma contamination);

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

e) test environment (address of performing laboratory, date of test);

f) results:

   1) survival index, mitotic index, proliferation index, number of cells analysed, micronculei per culture including means, criteria for considering results positive, negative or equivocal, signs of toxicity, dose-response relationship, and

   2) where possible, statistical analysis and method applied, concurrent and historical negative-control data, concurrent and historical positive-control data, conclusions, D values, other observations (e.g. precipitation).

Annex A
(informative)

Bromodeoxyuridine (BrdU) method

A.1 General

Incubate cells in a surplus of bromodeoxyuridine (BrdU) for about 14 h. Bromodeoxyuridine (BrdU) is incorporated into DNA as replacement for thymidine and results in an altered chromosome staining of affected chromatid parts. Using a special staining procedure, it is possible to differentiate between metaphases, which have undergone 1st, 2nd or 3rd mitosis since co-culturing with BrdU.

A.2 BrdU treatment

Perform subculturing in culture dishes by seeding approximately 80 000 cells of a single cell suspension per culture. Use four harvest times and four cultures per harvest time. Dissolve the BrdU freshly prior to use under light protection at a concentration of 5 µg of BrdU per millilitre in culture medium (including 10 % FCS) and store it under light protection at 4 °C until use.

24 h after passaging, remove the medium from the cultures and replace it under light protection by the "BrdU-medium". Incubate the cultures for 14 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO_2 and at least 90 % humidity.

Thereafter, remove the medium under light protection and rinse twice with 5 ml HBSS. Add 5 ml of MEM (including 10 % FCS) per culture and incubate at 37 °C, in an atmosphere containing a volume fraction of 5 % CO_2 and a humidity of at least 90 % until harvest. Harvest the cultures 16 h, 18 h, 20 h and 22 h after the addition of BrdU.

A.3 Preparation of slides

Remove the culture medium completely.

Treat each culture for approximately 20 min with approximately 5 ml of a potassium chloride solution (6.2.29) at a temperature of approximately 37 °C (hypotonic treatment).

Add approximately 5 ml fixation solution (4 °C) (6.2.18) for about 15 min. Remove and replace the fixation solution by the same amount of fresh fixation solution (4 °C) for about 10 min. Remove and replace the fixation solution by the same amount of fresh fixation solution (4 °C) for about 5 min.

Remove the slides from the culture chambers. Allow the fixation solution to drip off and move the slide for a short period through the blue part of the flame of a Bunsen burner.

A.4 Staining of slides

A.4.1 Preparation of Soerensen-buffer

Dissolve 45,36 g KH_2PO_4 in 1 000 ml water (solution A) and dissolve 64,33 g Na_2HPO_4 in 1 000 ml water (solution B). Mix one part A, one part B and 8 parts of deionized water. This results in a solution with a pH of 6.8.

A.4.2 Staining

After storage for 2 d to 4 d in the dark, transfer the slides with the coated side up into dishes and cover with 1 cm of Soerensen-buffer. Thereafter, irradiate with UV-light (wavelength 254 nm) for 10 min from a distance of about 15 cm.

Transfer the slides into holders and immerse them in cuvettes which have previously been filled with “SSC twice” (a solution containing 0,3 mol/l NaCl and 0,03 mol/l of sodium citrate in deionized water). Incubate the slides in “SSC twice” for about 30 min at about 60 °C.

Thereafter, remove the slides and allow them to cool. Afterwards, place them for 10 min in their holders into cuvettes which had already been filled with a Giemsa solution, prepared by adding 15 ml of Giemsa to 185 ml Soerensen-buffer (pH 6.8).

Remove from staining and allow to dry. Remove the surplus of staining material in xylene and cover the slides. Protect slides against light.

A.5 Evaluation

Evaluate 200 metaphases per harvest time and note the numbers of the 1st, 2nd and 3rd metaphases.

The following criteria shall be met:

— in the 1st metaphases, both chromatids of all chromosomes are stained blue-violet;

— in the 2nd metaphases, one chromatid of all chromosomes is stained blue-violet, whereas the corresponding part of its sister chromatid is stained light-violet;

— in the 3rd metaphases, chromatids are stained predominantly light-violet.

A.6 Calculation of cell cycle length

Calculate the cell cycle length, l_{CC}, of the respective cell line according to Equation (A.1):

$$l_{CC} = \frac{t_h}{(1 \times n_1) + (2 \times n_2) + (3 \times n_3)} \times 100 \qquad \text{(A.1)}$$

where

t_h is the harvest time in hours, h;

n_1 is the number of 1st mitosis in percent, %;

n_2 is the number of 2nd mitosis in percent, %;

n_3 is the number of 3rd mitosis in percent, %.

The mean of all four harvest times is used as mean cell cycle length for the respective cell line.