BRITISH STANDARD

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Paper and board — Determination of the cytotoxicity of aqueous extracts

ICS 13.060.20; 67.250; 85.060

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British Standards

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee PAI/11, Methods of test for paper, board and pulps.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Papier et carton - Détermination de la cytotoxicité des extraits aqueux

Papier und Pappe - Bestimmung der Zytotoxizität von wässrigen Extrakten

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Foreword

This document (EN 15845:2010) has been prepared by Technical Committee CEN/TC 172 “Pulp, paper and board”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2010, and conflicting national standards shall be withdrawn at the latest by July 2010.

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1 Scope

This European Standard specifies a test method for the laboratory assessment of the potential cytotoxic effect of paper and board materials. This test method is intended to assess wet contact with food simulant.

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.

EN 645, *Paper and board intended to come into contact with foodstuffs — Preparation of a cold water extract*

EN 647, *Paper and board intended to come into contact with foodstuffs — Preparation of a hot water extract*

3 Terms and definitions

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

3.1
reference water
purified water or pyrodistilled water used as the reference in the cytotoxicity assay

3.2
purified water
produced starting with tap water, which then undergoes the following treatment sequence: pre-filtration, reverse osmosis, filtering through activated carbon powder (adsorption) then through cartridges of mixed-bed ion exchange microresins (demineralisation), ultrafiltration (molecular weight cut-off at 10 kDa), and UV photo-oxidation

NOTE Alternatively, any other purification regime, which produces HPLC-quality water (resistance > 18,0 MΩ/cm, Total organic carbon < 3 ppb, no micro-organisms) or waters of grade 1 or 2 (see EN ISO 3696), can be used.

3.3
pyrodistilled water
water prepared as described in Annex A, that is used to maintain the cell line, and which can also be used for cleaning laboratory glassware

3.4
water extract
reference water that has been exposed to contact with paper or board

NOTE See EN 645 or EN 647 with necessary modifications.

3.5
control water
reference water that has been treated according to the same conditions as the water extract but without being exposed to contact with paper or board

3.6
positive control
potassium dichromate (CAS 7778-50-9) 5 mM solution freshly made in reference water

3.7
sample
culture medium prepared with water extract as specified in 3.4

3.8
test material
one or more components, or a specified quantity of paper or board, that is randomly sampled from a batch

4 Principle

The test protocol specified in this document is intended to evaluate the cytotoxic effect of substances migrating from food contact paper and board into wet foods intended for human consumption. The food simulant used is water as described in Clauses 7 and 10. The test evaluates the impact of the water extract on the rate of RNA (Ribonucleic Acid) synthesis by measuring the incorporation of a radioactive tracer (tritiated uridine) in human cells (HeLa S3).

The food contact paper and board samples to be tested are exposed to the extraction water as described in Clause 10.

The extracts then undergo cytotoxicity assessment, and the results obtained are compared to the results for a non-cytotoxic control (a purified water for which the rate of RNA synthesis is considered optimal and is therefore arbitrarily set at 100 %). Potassium dichromate, as described in 11.4.3, is used as a positive control.

5 Reagents

5.1 Liquid scintillant for tritium counts on dry filters

5.2 Culture media

The pH of all culture media used shall be 7,4 ± 0,1: pH to be adjusted using a sterile NaOH (or HCl) solution.

5.2.1 Culture media – quality and storage

All culture media, foetal serum and solutions used for cell culture shall be sterile and of sufficiently high quality to guarantee optimal cell growth (see 12.2).

They shall be stored in compliance with manufacturer's instructions, where given.

5.2.2 Medium for maintaining HeLa S3 cells in monolayer culture

Composition:

a)	minimum Essential Medium Eagle[1)] (10x)[2)]	100 ml
b)	sodium bicarbonate solution[1)], 7,5 % (*m*/*V*)	30 ml
c)	glutamine solution, 200 mM (or Glutamax I®)[1)] (100x)[2)]	10 ml
d)	non-essential amino acids solution[1)] (100x)[2)]	10 ml
e)	foetal calf serum[3)]	50 ml

1) Commercially available, the composition is based on the used of Earle's salts in the minimum essential medium. Glutamax I® is an example of a suitable product available commercially. This information is given for the convenience of this European Standard and does not constitute an endorsement by CEN of this product.

2) 10x or 100x imply tenfold or hundredfold concentrated media or solutions.

3) Heat inactivated (56 °C for 40 min) before use.

f) reference water 800 ml

5.2.3 Concentrated culture medium for cytotoxicology testing

A 5-fold concentrated culture medium is prepared by successively mixing:

a) minimum Essential Medium Eagle[4] (10x)[5] 100 ml

b) sodium bicarbonate solution, 7,5% (*m*/*V*) 30 ml

c) glutamine solution, 200 mmol (or Glutamax I®)[4] (100x)[5] 10 ml

d) non-essential amino acids solution[4] (100x)[5] 10 ml

e) foetal calf serum[6] 50 ml

5.3 Solution for rinsing cell lawns

Composition:

a) Dulbecco's PBS (Phosphate buffered saline) solution, (10x)[5] 100 ml

b) reference water added up to 1 000 ml

5.4 Cell dissociation reagent

Cells are detached using a solution of Versene[7] 1/5 000.

5.5 Analytical-grade dimethyl sulfoxide or glycerol

5.6 Sodium dodecyl sulphate (SDS) for analysis, at 3 % (*m/v*)

5.7 Trichloroacetic acid (TCA) for analysis, at 5 % (*m/V*)

5.8 Ethanol, 95 % to 98 % (*v/v*)

5.9 [5,6-^{3}H] uridine (35Ci/mmol to 50 Ci/mmol; 1 m Ci/ml): a sterile and non-cytotoxic aqueous solution.

NOTE The products and materials referred to in the present document are considered non-cytotoxic if they do not trigger a cytotoxic response, i.e. if the linear regression line generated by measuring rate of RNA synthesis meets the conditions set out in 12.2.

6 Cell line

6.1 Generating the cell strain

The cell line used is HeLa S3, a human cell line.

4) Commercially available, the composition is based on the used of Earle's salts in the minimum essential medium. Glutamax I® is an example of a suitable product available commercially. This information is given for the convenience of this European Standard and does not constitute an endorsement by CEN of this product.

5) 10x or 100x imply tenfold or hundredfold concentrated media or solutions.

6) Heat inactivated (56 °C for 40 min) before use.

7) Or equivalent amount of aqueous solution of the tetrasodium salt of ethylenediaminetetraacetic acid (NA_4EDTA).

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This line shall be generated from recognized sources, such as the CCL2.2 HeLa S3 line of the American Type Culture Collection.

6.2 Maintaining the cell strain

Cells should be cultured without antibiotics, and checks should be run at frequent intervals to screen for mycoplasma:

a) seed the HeLa S3 cells in the culture medium (5.2.2) in a flask as defined in 9.2.1 (75 cm^3 or 150 cm^3) and incubate at (37 ± 1) °C, 5 % CO_2 and 95 % humidity until a confluent lawn of growth is formed;

b) remove the culture medium, and rinse the cell lawn at least twice with around 10 ml of the rinse medium (5.3);

c) cover the cell lawn in a film of the Versene solution or equivalent (5.4). Leave the flask at (37 ± 1) °C for a few minutes. Detach the cells by gently shaking:

   1) add a few millilitres of the culture medium (5.2.2);

   2) disperse the cells by repeat pipetting;

d) redistribute the cell suspension obtained into the required number of flasks, and add the appropriate volume of culture medium.

6.3 Storing the cell strain

If a stock of the cell line culture is stored, then it shall be stored in liquid nitrogen, with the cells preserved in the culture medium with added dimethyl sulfoxide (10 % *v/v*, final concentration) or glycerol (10 % *v/v*, final concentration), as per 5.5. The cells should be propagated through the minimum of three passages after the storage, before they can be used for testing.

7 Food simulants used for testing

7.1 Test water: Reference water (3.1) that will be used directly for contact with paper and board intended for wet foodstuffs.

8 Cleaning laboratory glassware

8.1 Cleaning fluids for laboratory glassware

8.1.1 Laboratory detergent: RBS 25®[8)] 5 % (*v*/*v*) or Aquet®[8)], 1 % (*v*/*v*) or any equivalent alkaline detergent prepared in reference water (3.1).

8.1.2 Nitric acid: in solution, at 5 % (*v/v*), prepared by diluting 65 % to 70 % analysis-grade nitric acid in the reference water (3.1).

8.1.3 Rinsing water

8.1.3.1 Reference water (3.1).

8.1.3.2 Water prepared by mixing 3,30 g of analytical-grade $CaCl_2$ x $2H_2O$ in 20 l of reference water (3.1).

8) RBS 25 and Aquet are examples of suitable products available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products.

8.2 Cleaning procedure for laboratory glassware

Cleanliness of the laboratory glassware is a very important factor, since it affects the quality of the results. Glassware cleanliness is therefore checked by measuring the rate of RNA synthesis with control water (3.5).

The cleaning procedure consists of the following steps:

— soaking in the laboratory detergent (8.1.1) for at least 12 h;

— washing and copiously rinsing using the rinsing water (8.1.3.2);

— soaking in analytical grade nitric acid (8.1.2) for about 2 h;

— copiously rinsing using the rinsing water (8.1.3.1);

— air-drying in a dust-free area away from toxic vapour;

— sterilisation by autoclaving (at 120 °C for 30 min) of laboratory glassware intended for cell culturing.

NOTE This procedure can be automated.

Any traces of organic materials left on the incubation tubes (9.2.2) after the last tube rinsing cycle should be eliminated by sterilising in an oven at 550 °C for 2 h.

8.3 Alternative cleaning procedure

— Standard washing programme for laboratory glassware is applied, but the utensils intended for cell culture work are washed separated from other glassware;

— the glassware is subsequently dried at 100 °C for 2 h;

— sterilisation by autoclaving (at 120 °C for 30 min) of laboratory glassware intended for cell culturing;

— all cell culture glassware is subsequently heat sterilised in an oven at 550 °C for 2 h.

NOTE This alternative procedure can be applied, if it can be demonstrated that the cytotoxicity assay and its results as described in Clauses 11 and 12 are not affected by the treatment

9 Equipment

9.1 Equipment for the migration test

9.1.1 Equipment or clean room able to maintain the temperature required for the test within a tolerance of ± 2 °C.

9.1.2 Borosilicate wide-necked (about 40 mm) **glass flasks** into which test material (paper and board) can be introduced, and which are fitted with a stopper in a material that does not affect the migration testing (borosilicate glass).

9.2 Cell culture equipment

9.2.1 Flasks for cell culture.

9.2.2 Round-bottom, sterile, borosilicate glass **cell culture tubes**, with a preferably polypropylene or otherwise a bakelite stopper with a PTFE-seal, and fitted with magnetic stir bars (9.3.11), for cell incubation with the water extract.

Polypropylene tubes can be used as a workaround solution.

NOTE 16 ml culture tubes should be used for a 5 ml culture volume.

9.2.3 Round-bottom, sterile, disposable polypropylene **culture tubes**, with stoppers, for kinetics runs.

NOTE 5 ml culture tubes should be used.

9.2.4 50 ml sterile, disposable, polypropylene **centrifuge tubes**.

9.2.5 **Sterile** disposable **pipettes**.

9.2.6 **Work area with laminar air flow.**

9.2.7 **Incubator** able to maintain a temperature of (37 ± 1) °C, 95 % humidity and 5 % CO_2 atmosphere.

9.2.8 **Inverted microscope.**

9.2.9 Routinely-used laboratory cell culture equipment.

9.3 Equipment used for cytotoxicity testing

9.3.1 **Centrifuge** able to centrifuge at up to 1 000 g.

9.3.2 **Water bath** working at a temperature of (37,0 ± 0,5) °C.

9.3.3 **Multipoint magnetic stirrer.**

9.3.4 **0,22 µm filters adaptable on syringes**, either in cellulose acetate or else nylon, shall be non-cytotoxic, and in particular, they shall be free of surfactants.

9.3.5 Sterile disposable polypropylene **syringes** for the media filtration, and long-needle syringes for cell homogenisation before sampling for kinetics analysis (or any other appropriate apparatus).

9.3.6 **Micropipettes** with sterilised cones.

9.3.7 **Chamber for descending chromatography.**

9.3.8 **Chromatography paper**: in pure cellulose (thickness: 0,34 mm; capillary rise rate: 130 mm/30 min [water]).

NOTE Whatman 3MM®[9] sheets or equivalent should be used

9.3.9 **Vortex stirrer.**

9.3.10 **Liquid scintillation counter** for tritium measurements.

9.3.11 Sterile, **magnetic stir bars** adapted to fit the incubation tubes.

NOTE Cylindrical stir bars (10 mm x 5 mm) should be used for 16-ml tubes.

9.3.12 Borosilicate **glassware** set aside exclusively for the cytotoxicity test.

Any scratched glassware shall not be used.

9.3.13 **Scintillation flasks.**

9.3.14 **Infrared radiation heater** (optional)

9) Whatman 3MM is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.

9.3.15 Cell counting system (Thoma chamber®[10)] or equivalent)**.**

10 Preparation of specimens

10.1 General

10.1.1 The sample preparation procedures are designed to mimic contact with aqueous foods by applying hot and cold water extraction.

10.1.2 In cases where the selected extraction temperature is higher than (20 ± 2) °C, the water extract (3.4) is to be chilled to room temperature before the cytotoxicology assessment.

10.1.3 The paper and board materials to be tested shall be clean and without any external contamination. They may be tested after different storage times and conditions (temperature, humidity, light, etc.) but it should be noted that these factors may affect the results and should be known when interpreting the outcome of the assays. The samples should be provided enclosed in non-transparent inert cover material (i.e. aluminium foil) stored at ambient temperature and extracted within a week after arrival.

10.2 Paper and board intended for wet contact

The paper and board extracts shall be prepared according to EN 645 and EN 647 procedures, with the exception that reference water (as specified in 3.1) shall be used. The extracts can be stored frozen (- 20 °C) for a limited time (up to three months). The extracts should be melted slowly at ambient temperature. Ultrasound can be used to disperse any precipitations. Before the testing the extracts are sterilised by filtration (using filters of 0,22 µm pore size).

11 Cytotoxicicity assessment

11.1 Principle

HeLa S3 cells are incubated in a culture medium prepared with either water extract from paper and board intended for wet food contact, reference water, control water or positive control. After a (19 ± 2) h incubation period, the rate of incorporation of tritiated uridine in cell RNA is measured. The rate of cell RNA synthesis remains linear for at least 30 min after an initial lag phase of about 2 min to 3 min, which is the time-lapse required for the uridine to penetrate the cell.

RNA synthesis rate is determined by calculating the slope of the regression line plotted using experimental values obtained for each kinetics run. This rate is then compared to the rate obtained for the reference sample, which is arbitrarily set at 100 %. The result is expressed as percentage RNA synthesis compared to the reference sample, where 100 % represents zero cytotoxicity. The value obtained with the positive control should be < 60 % of that of the reference sample

11.2 General

11.2.1 Using the cell line (6.1) and the culture medium (5.2.2), prepare a sufficient quantity of cells to conduct the test.

11.2.2 If the cells to be grown are taken from stock cell culture, eliminate any cryoprotectant (5.5) that may be present. Before the cells can be used, they shall be sub-cultured at least three times, as specified in 6.3, but no more than ten times.

10) Thoma Chamber is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.

11.2.3 The test is therefore performed using cells in monolayer culture.

11.2.4 All operations up to measurement of the rate of RNA synthesis shall be conducted in asepsis, preferably in laminar air flow facilities.

11.3 Preparation of samples

Prepare cell culture media from the water extracts obtained from 10.2: add one volume of concentrated culture medium (5.2.3) to four volumes of each water extract to be tested.

11.4 Preparation of the reference sample, control sample and the positive control sample

11.4.1 Each reference sample defined shall be prepared by adding one volume of concentrated culture medium (5.2.3) to four volumes of reference water (3.1).

11.4.2 The control sample shall be prepared by adding one volume of the concentrated culture medium (5.2.3) to four volumes of control water (3.5).

11.4.3 The positive control sample (3.6) shall be prepared from a 5 mmol/l freshly made stock solution of potassium dichromate (in reference water as defined in 3.1). This stock solution is further diluted into the culture medium prepared as defined in 11.4.1 to get a 5 µmmol/l positive control sample.

11.5 Incubation of cells obtained from monolayer culture

— Use cells in monolayer culture, as described in 6.2. Eliminate the culture medium and rinse the cell lawn at least twice, using about 10 ml of rinsing solution (5.3), then detach the cells using a Versene solution or equivalent (5.4) brought to (37 ± 1) °C for a few minutes;

— Quickly disperse the cells by repeated pipetting in about 10 ml of culture medium (5.2.2). If several different flasks were used, all the cells shall be mixed together in a single flask;

— Dilute the cell suspension with culture medium (5.2) in order to determine cell concentration (using a cell counter, as defined in 9.3.15);

— Redistribute this suspension into centrifuge tubes (9.2.4), at about 12×10^6 cells per tube. Prepare at least one tube for the reference water, one tube for each control and as many tubes for water extract as there are extracts to test;

— After centrifugation (at 300 g to 400 g at the bottom of the centrifuge tube, for 5 min, at room temperature) and once the supernatants have been removed, take up each pellet with 20 ml of each culture medium extemporaneously reproduced and prepared as defined in 11.3 and 11.4. This will result in a cell concentration of about 6×10^5 cells/ml;

— Disperse the cell aggregates by repeated pipetting;

— Redistribute the cell suspension from each centrifuge tube into three incubation tubes (9.2.2) fitted with a sterile magnetic stir bar (9.3.11), at about 5 ml per tube.

NOTE Prepare at least three incubation tubes from each sample, each reference sample and each control, so that the cytotoxicity assessment can be conducted at least in triplicate.

The figures cited in this paragraph for cell numbers, volumes, etc. are given to illustrate the example of a cytotoxicity assessment performed in triplicate.

— Incubate the cell suspension at (37 ± 1) °C under gentle magnetic stirring for (19 ± 2) h at 37 °C and 5 % CO_2 atmosphere.

Initial cell concentrations should be checked by cell counts (9.3.15) performed on the remaining cell suspension in the centrifuge tubes.

11.6 Preparation of the chromatography sheet

The chromatography sheet is to be prepared before the uridine incorporation kinetics analysis described in 11.7.

— Trim the chromatography paper (9.3.8) to a suitable format for the chromatography chamber;

— Position the paper sheets from top to bottom;

— Take a pencil, and outline spot areas at a sufficient distance from the top of the sheet. Outline six rectangles per incubation tube;

NOTE The surface area and number of spot rectangles will depend on the sample volume to be spotted and the number of samples to be chromatographed. For example, for a 40 µl spot, outline rectangles 3 cm high and 1,5 cm wide.

— Prime the outlined spot area with SDS (5.6);

— Also prepare another chromatography sheet (9.3.8), that is not primed with SDS and that presents the following characteristics: rectangles: h = 3 cm and w = 2 cm for a 40 µl spot. Outline two rectangles per incubation tube.

11.7 Kinetics of uridine incorporation in the cell RNA

11.7.1 Homogenise the cell suspensions contained in the incubation tubes. Withdraw and transfer one volume V_1 from each suspension into as many culture tubes (9.2.3), and then place these tubes in a heated water bath set at (37 ± 1) °C.

11.7.2 Introduce a volume V_2 of tritiated uridine (5.9) into each tube. This step represents time-point zero in the kinetics run.

For V_1 = 500 µl, introduce 2 µl to 6 µl of uridine (V_2).

11.7.3 Proceed as follows for each of these prepared tubes:

— Leave for a brief cycle in the vortex stirrer. Take an aliquot of V_3 µl at time-points 5 min, 10 min, 15 min, 20 min, 25 min and 30 min, and spot each aliquot on the chromatography sheet prepared according to 11.6, which is then left to dry.

For V_1 = 500 µl, V_3 = 40 µl.

Independently of the operations performed for the kinetics analysis, a sample should also be taken from one of the tubes containing the reference sample at less than 10 s after the tritiated uridine has been added. This sample can then be used to check the quality of the uridine.

— Eliminate the acid-soluble fraction by descending chromatography in the trichloroacetic acid (5.7);

— Cut away the spot area, rinse it in ethanol (5.8) for 10 min, and then leave it to dry;

— Place each spotted rectangle in a scintillation flask (9.3.13) and add the volume of scintillation mixture required. Measure the activity of the uridine incorporated by liquid scintillation counting, the results being expressed in cpm (counts per minute).

This measurement represents the radioactivity incorporated at time t.

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— At the end of each kinetic run, take two volumes of V_3 µl and spot them on the chromatography paper (9.3.8) not primed with SDS, and leave to dry. Place each spotted rectangle in a scintillation flask and add the volume of scintillation mixture required. Measure by liquid scintillation counting. Then calculate the mean value in order to obtain the total initial radioactivity introduced into each tube, expressed in cpm (counts per minute).

NOTE The confidence interval for the counts should be at least 95,5 %, which gives a 2–sigma uncertainty.

12 Expression of results

12.1 Graphic representation of the results (to be generated for each tube)

12.1.1 Each kinetic run includes six experimental time-points (ranging from time 5 min to time 30 min).

12.1.2 For each experimental time-point, calculate the percentage (*Pt*) radioactivity incorporated into the RNA at a given time-point *t* in relation to uridine according to the following formula:

$$Pt = \frac{radioactivity\ incorporated\ at\ time\ t}{total\ \ initial\ \ radioactivity\ introduced\ \ \ into\ each\ \ tube} \times 100$$

12.1.3 For each kinetic run, plot the curve $Pt = f(t)$, where *t* is expressed in minutes (min).

12.1.4 Based on the six experimental time-points, or on at least five if one of the time-points yields an outlier, plot the linear regression line for each kinetic run. Determine the equation for this linear regression $y = at + b$, as well as its coefficient of correlation *R*.

12.1.5 Since the uridine is not incorporated instantaneously, do not include the time point corresponding to time zero of the kinetic run in the regression line plotted. It should nevertheless be recorded, since excessive variation generally indicates that the uridine is substandard.

12.2 Calculation of percentage RNA synthesis and the validity of the test

NOTE The test may only be deemed valid if it meets the conditions according to 12.2.1 to 12.2.5.

12.2.1 General

— The reference sample and the control sample shall be incubated at the same time as the test sample, and with cells from the same batch;

— the number of cells used for incubation in each tube has to be more or less identical for each reference sample, each control sample and each test sample analysed at the same time, i.e. between 550 000 cells/ml and 700 000 cells/ml.

12.2.2 Reference sample

Given that at least three kinetic runs are to be carried out for the reference sample:

a) Each of the three linear regression lines shall meet all three of the following conditions:

1) $b < 0$;

2) $R \geq 0{,}980$;

3) $a \geq 1{,}75$.

NOTE $a < 1,75$ indicates that the cells are not fully viable, which distorts the results. Toxicity is in fact expressed differently according to different levels of cell health.

b) At least two out of the three slope values shall fall within the same precision interval.

If any of the abovementioned conditions is not met, the test shall be repeated.

c) Assign a 100 % value to the average of the three slope values obtained.

If one of these slopes values lies clearly outside this precision range, it should be rejected, and only the other two values should be retained. It is in fact highly possible that the outlier stems from a substandard tube.

12.2.3 Control sample

Given that at least three kinetic runs are to be carried out for the control sample:

a) Each of the three linear regression lines shall meet all three of the following conditions:

   1) $b < 0$;

   2) $R \geq 0,980$;

   3) $a \geq 1,75$;

b) At least two out of the three slope values shall fall within the same precision interval.

If any of the above mentioned conditions is not met, the test shall be repeated.

c) Calculate the average of the three slope values obtained, rejecting any outlier obtained;

d) Compare the average value obtained for the control sample with the average value obtained for the reference sample, expressing the result as percentage RNA synthesis compared to the reference sample.

12.2.4 Positive control sample

Given that at least three kinetic runs are to be carried out for the test sample:

a) Each of the three linear regression lines shall meet all three of the following conditions:

   1) at least two slope values are within the same precision interval;

   2) $b < 0$;

   3) $R \geq 0,980$.

If any of the above mentioned conditions is not met, the test shall be repeated.

b) Calculate the average of the three slope values obtained, rejecting any outlier obtained;

c) Compare the average value obtained for the test sample with the average value obtained for the reference sample, expressing the result as percentage RNA synthesis compared to the reference sample.

12.2.5 Test sample

Given that at least three kinetic runs are to be carried out for the test sample:

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a) Each of the three linear regression lines shall meet all three of the following conditions:

   1) at least two slope values are within the same precision interval;

   2) $b < 0$;

   3) $R \geq 0{,}980$.

If any of the abovementioned conditions is not met, the test shall be repeated.

b) Calculate the average of the three slope values obtained, rejecting any outlier obtained;

c) Compare the average value obtained for the test sample with the average value obtained for the reference sample, expressing the result as percentage RNA synthesis compared to the reference sample.

13 Interpretation of the results

13.1 Results for the reference sample

If the percentage RNA synthesis of the control sample is not in the same order of magnitude as for the reference sample (the tolearance ≤ 10 %), then the test shall be repeated. This measurement in fact acts as a control of the level of cleanliness of the laboratory glassware used: any clear deviation between these two values would invalidate the test results.

13.2 Results of the positive control sample

The RNA-synthesis rate of the positive control sample should be less than 60 % of that of the reference sample.

13.3 Results for the test sample

If, after subtracting the RNA synthesis rate of the positive sample from that of the test sample, the difference is less than ten units, the test indicates a decrease in the RNA synthesis requiring confirmatory tests (repeating the experiment with diluted samples, if necessary)

14 Precision

From an interlaboratory test (n = 3) with water extracts of two different samples the test method described above gave repeatability (r) and reproducibility (R) results as shown in table 1. The repeatability (r) has been calculated as the coefficient of variation (CV) from the total five tests performed (two of the laboratories provided replicates, the third a single experiment). The reproducibility (R) is given as the coefficient of variation (CV) between the means from the three laboratories.

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Table 1 — Repeatability and reproducibility found in an interlaboratory test

Sample	The difference between the test sample and the positive control sample[a]	Repeatability (*r*) *CV* %	Reproducibility (*R*) *CV* %
A1 (paper)	44,2	17,4	14,8
A2 (board)[b]	- 3,8	65,6	85,2

[a] This value is obtained by subtracting the RNA-synhesis rate of the positive control sample from that of the sample.

[b] The range of the values obtained for the sample A2 was between - 10,4 and 1,0 indicating that in each laboratory and in each test the value was practically equal to that of the positive control sample. The high CVs result from the small percentages giving an apparent high relative variability.

15 Test report

The test report shall include the following information:

a) reference to this European Standard;

b) date and place of testing;

c) complete identification of the sample tested;

d) result, expressed as percentage RNA synthesis;

e) any deviation from the specified procedure, or other circumstances that may have affected the results.

Annex A
(informative)

Pyrodistilled water

Pyrodistilled water is produced by pyrolysis of the purified water (3.2) at 650 °C, in a quartz pyrodistillation unit: the result is a water that has absolutely no mineral or organic trace impurities.

Pyrodistilled water used for washing laboratory glassware can be stored in carboys or flasks with no cytotoxic effect, i.e. borosilicate glass or polycarbonate glass for pyrodistilled water and polycarbonate glass for purified water.

Bibliography

[1] EN ISO 3696, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

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