

Suitability of non-metallic products for use in contact with water intended for human consumption with regard to their effect on the quality of the water —

Part 2: Methods of test —

Section 2.5: The extraction of substances that may be of concern to public health

ICS 13.060.20

Committees responsible for this British Standard

The preparation of this British Standard was entrusted to Technical Committee EH/6, *Effects of materials on water quality*, upon which the following bodies were represented:

- Automatic Vending Association of Britain
- British Cement Association
- BCF — British Coatings Federation Ltd
- British Malleable Tube Fittings Association
- British Plastics Federation
- British Plumbing Fittings Manufacturers' Association
- British Precast Concrete Federation Ltd
- British Rubber Manufacturers' Association Ltd
- British Water
- DEFRA — Water and Land Directorate
- Galvanizers Association
- Laboratory of the Government Chemist
- Pipeline Industries Guild
- UK Steel Association
- Water Regulations Advisory Scheme
- Water Research Centre plc

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Foreword

Publishing information

This subsection of BS 6920 is published by BSI Standards Limited, under licence from The British Standards Institution and came into effect on 15 May 2000. It was prepared by Technical Committee EH/6, *Effects of materials on water quality*.

Supersession

BS 6920-2.5:2000+A2:2014 supersedes BS 6920-2.5:2000, Incorporating Amendment 1, which is withdrawn.

Relationship with other publications

BS 6920 is published in several parts, namely *Part 1: Specification*, *Part 2: Methods of test*, *Part 3: High temperature tests* and *Part 4: Method for the GCMS identification of water leachable organic substances*.

Part 2 is further subdivided into a number of sections and subsections as follows.

Section 2.1: Samples for testing;

Section 2.2: Odour and flavour of water;

Subsection 2.2.1: General method of test;

Subsection 2.2.2: Method of testing odours and flavours imparted to water by multi-layered hoses and pipes;

Subsection 2.2.3: Method of testing odours and flavours imparted to water by hoses for conveying water for food and drink preparation;

Section 2.3: Appearance of water;

Section 2.4: Growth of aquatic microorganisms test;

Section 2.5: The extraction of substances that may be of concern to public health;

Section 2.6: The extraction of metals.

Information about this document

This edition introduces technical changes but it does not reflect a full review or revision of the standard. The start and finish of text introduced or altered by Amendment No. 2 is indicated in the text by tags **A2** **A2**. Minor editorial changes are not tagged.

Hazard warnings

WARNING. This British Standard calls for the use of substances and/or procedures that can be injurious to the health if adequate precautions are not taken.

As well as observing safe working practices, take particular care in handling continuous cell lines since they can become infected with pathogenic viruses and bacteria during the course of their manipulation. The nutrient media used in this test are capable of supporting microbial growth, and the cell line is capable of being infected by human viruses. It is essential that this test procedure is carried out only by persons with experience of mammalian tissue culture techniques and cell line morphology.

This British Standard refers only to technical suitability and does not absolve the user from legal obligations relating to health and safety at any stage.

Contractual and legal considerations

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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Summary of pages

This document comprises a front cover, an inside front cover, pages i and ii, pages 1 to 9 and a back cover.

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Introduction

A1 Text deleted **A1**

This section of BS 6920 describes a simple cytotoxicity technique to test leachates from materials and articles, used in customer's premises in contact with water for human consumption, for biologically active compounds. Materials and chemicals used by water suppliers are subjected to a fuller assessment using an extraction procedure followed by sophisticated analytical methods.

This method should be regarded as only an initial screening test for substances potentially hazardous to health. A satisfactory result indicates that the leachate probably does not contain significant amounts of acutely toxic substances, but it does not indicate the absence of small quantities of substances which may be harmful on prolonged exposure.

1 Scope

This section of BS 6920 specifies a screening procedure (simple cytotoxicity test) using a mammalian cell line and a leachate from a product. The results of this procedure will assist in the toxicological assessment of the product for use in contact with water intended for human consumption.

The procedure given in this section of BS 6920 is suitable for all non-metallic materials that may be used in contact with water intended for human consumption.

NOTE **A2** Under the relevant National Regulations [1] to [7], the National Regulator may specify additional provisions in some cases and will assess the significance of the results obtained. **A2**

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this section of BS 6920. For dated references, subsequent amendments to or revisions of any of these publications do not apply. For undated references, the latest edition of the publication referred to applies.

BS 748, *Specification for haemocytometer and particle counting chambers*.

BS 6920-2.1:2014, *Suitability of non-metallic products for use in contact with water intended for human consumption with regard to their effect on the quality of the water — Part 2: Methods of test — Section 2.1: Samples for testing*.

BS EN ISO 3696, *Water for analytical laboratory use — Specification and test methods*.

3 Terms and definitions

For the purposes of this section of BS 6920, the following definitions apply.

3.1

cytotoxic

poisonous (toxic) to cells under the conditions of the test

3.2

monolayer

resulting cell layer, only one cell thick, formed when a cell culture grows in contact with a suitable solid surface, normally provided by the walls of the culture vessel. The cells are contact dependent and spread out onto the surface and multiply until adjacent cells are in contact whereupon the growth ceases

NOTE Only certain types of cell culture (including the cell line used in this test) form monolayers.

3.3

tissue culture

technique whereby cells are grown and maintained as a monolayer sheet on the inner surface of a container in a nutrient (culture) medium

3.4

cell line

cells capable of growth under laboratory conditions using a tissue culture technique; a cell line capable of unlimited growth in vitro is described as a continuous cell line

3.5

biologically clean atmosphere

atmosphere in which the numbers of microorganisms are sufficiently low to not have any adverse effect on the test

3.6

morphology

microscopic study of physical form; when applied to a cell line it is the overall appearance of both the cell monolayer and the individual appearance of the constituent cells

3.7

rounding off

abnormal cells which are spherical or oval in shape and exhibit no visible internal structures when using a low-power microscope

3.8

cell suspension

suspension prepared from a monolayer by the application of digestive enzymes and/or chelating agents, thereby loosening the cells from the surface to which they are attached and dispersing them to give a suspension, which can be used to start fresh cultures or to perform tests

4 Principle

The product is immersed in test water for 24 h. This water is used subsequently in the preparation of a nutrient medium.

The morphology of the mammalian cell line grown in this medium is observed.

A blank extract is assessed in parallel with the material.

NOTE A flow diagram showing the sequence of this test procedure is given in Annex A.

5 Test premises

Manipulate the cells and media only in a laboratory with a biologically clean atmosphere that is dust free, and carry out the test in an environment which avoids infection of the cell line.

NOTE For example, the test can be carried out in a laminar flow cabinet conforming to BS 5726-3.

6 Safety

Observe the warning given in the Introduction.

7 Reagents

7.1 Waters

7.1.1 Test water, obtained from a tap connected directly to a service pipe at mains pressure.

Before collection of the water, the tap shall be flushed until the temperature of the flowing water does not vary by more than 1 °C over a period of 1 min and does not exceed 25 °C. Alternatively, glass-distilled, demineralized or reverse osmosis treated water conforming to grade 3 of BS EN ISO 3696 shall be used.

The test water shall be free from substances that are toxic to or inhibit the growth of the cell line.

NOTE For example, this may be verified by growing at least three successive generations of the cell line in a nutrient medium made with the test water and comparing the morphology of the cells with that of cells from the same generations grown in the same medium made with distilled water (see 10.2).

7.1.2 Distilled water (for the preparation of media), glass-distilled water, or water produced by reverse osmosis and conforming to grade 3 of BS EN ISO 3696.

NOTE Deionized water is not suitable.

7.2 Media

7.2.1 General

All reagents shall be of analytical quality.

NOTE 1 Foetal calf serum may be used in place of newborn-calf serum.

NOTE 2 Other established growth and maintenance media may be used in place of those specified provided that healthy growth of the test cell line can be maintained using them.

The use of gentamicin shall be preferred to other antibiotics to control possible bacterial growth/contamination from the non-sterile test samples and test water.

NOTE 3 Commercially available sterile media constituents are available and should be used wherever possible.

7.2.2 Growth medium, prepared from the following.

Sterile distilled water	90 ml
199 concentrate ($\times 10$) with Earle's salts but without sodium hydrogen carbonate buffer	10 ml
Newborn-calf serum	7 ml
Gentamicin solution (4 000 i.u./ml)	1 ml
Sodium hydrogen carbonate buffer solution, 44 g/l, saturated with carbon dioxide (7.2.4)	2 ml

Sterilized ingredients shall be used. The medium shall be prepared aseptically and stored in the absence of light at $(4 \pm 1) ^\circ\text{C}$.

NOTE 199 concentrate ($\times 10$) with Earle's salts but without glutamine and sodium hydrogen carbonate is available commercially. Before use, 0.34 ml of a 200 mmol/l solution of L-glutamine should be added to each 100 ml of concentrate. Newborn-calf serum, mycoplasma screened, is available commercially.

7.2.3 Maintenance medium, prepared from the following.

Sterile distilled water	90 ml
199 concentrate ($\times 10$) with Earle's salts but without sodium hydrogen carbonate buffer	10 ml
Newborn-calf serum	2 ml
Gentamicin solution (4 000 i.u./ml)	1 ml
Sodium hydrogen carbonate buffer solution, 44 g/l, saturated with carbon dioxide (7.2.4)	3 ml

Sterilized ingredients shall be used. The medium shall be prepared aseptically and stored in the absence of light at $(4 \pm 1) ^\circ\text{C}$.

7.2.4 Sodium hydrogen carbonate buffer solution, 44 g/l, saturated with carbon dioxide, prepared from the following.

Sodium hydrogen carbonate	8.8 g
Phenol red (2 g/l aqueous solution)	10 ml
Distilled water	to 200 ml

The ingredients shall be mixed and the solution saturated with carbon dioxide either by bubbling the gas through it, or by the addition of pieces of solid carbon dioxide.

Immediately after preparation, the buffer shall be placed into 10 ml glass bottles, filled to the rim, closed tightly and sterilized at a temperature of 115 °C and at a gauge pressure of 69 kPa for 20 min in a small autoclave. Store at (4 ± 1) °C. Discard bottles showing a bright magenta colouration.

7.2.5 Phosphate-buffered saline solution, prepared from the following.

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen orthophosphate	1.15 g
Potassium dihydrogen orthophosphate	0.2 g
Distilled water	to 1 l

The ingredients shall be mixed and the solution shall be dispensed into glass bottles in 20 ml amounts and sterilized at a temperature of 115 °C and a gauge pressure of 69 kPa for 10 min.

7.2.6 Trypsin-EDTA solution, as commercially available trypsin-EDTA solution or equivalent.

NOTE This should be stored at a temperature lower than -18 °C.

7.2.7 Concentrated growth medium, prepared as described in 7.2.2, but omitting the distilled water.

The medium shall be stored at (4 ± 1) °C in the absence of light.

7.3 Cell line

The established VERO cell line of African green monkey kidney cells (ATCC number CCL 81) shall be used. Establish that the cell line is healthy upon receipt (see 10.2).

NOTE The BGM monkey kidney cell line may be used in place of the VERO cell line for this test, but the VERO cell line is preferred.

8 Apparatus

8.1 Tissue-culture ware, prepared in accordance with the requirements given below.

All glassware used for this test shall be cleaned using an aqueous solution of a proprietary detergent specifically designed for use in tissue culture techniques. Glassware shall be thoroughly rinsed after cleaning, and given two final rinses in distilled water (7.1.2). Glassware shall be sterilized at a temperature of 121 °C and at a gauge pressure of 103 kPa for 15 min.

NOTE An alternative to the above requirements is to use presterilized plastics containers supplied specifically for tissue culture work.

8.2 Haemocytometer counting chamber, conforming to BS 748.

8.3 Extraction containers, consisting of borosilicate glass beakers calibrated for a capacity in accordance with BS 6920-2.1:2014, 4.1.2.

The beakers shall be cleaned using an aqueous solution of a biodegradable laboratory detergent. The beakers shall be rinsed in test water (7.1.1) and then once in distilled water (7.1.2). Following this, the beakers shall be drained and dried in a hot air cabinet. Before use, the beakers shall be rinsed in the test water (7.1.1).

9 Samples

9.1 General

Samples shall conform to the pertinent requirements given in BS 6920-2.1, except in the following respects.

9.2 Reference materials

At present there is no safe and proven reference material or substance that can be used in this test.

9.3 Cementitious samples

Precondition all samples made from cementitious materials by the method described in BS 6920-2.1:2014, 5.2.8, because a rise in the pH of the nutrient medium will affect the growth of the cell line.

9.4 Validation solution

Prepare a solution of zinc sulfate (analytical reagent grade) with a concentration of 800 mg/l as ZnSO_4 . Include this solution with each set of test samples evaluated by this technique.

10 Test procedures

10.1 Introduction

Divide the procedure into three stages as follows:

- establish and maintain the cell line on a continuous basis in the test laboratory;
- submit samples to an extraction procedure, collect the leachate and use this to prepare batches of nutrient media;
- observe the effect of these media on the morphology of the cells.

10.2 Maintenance of the cell line

Grow the cell line (7.3) in sterile containers with airtight closures, using the growth (7.2.2) and maintenance (7.2.3) media. A suitable method for maintaining the cell line follows.

Into a sterile 100 ml tissue-culture container, aseptically place 1 ml to 2 ml of a freshly prepared cell suspension having approximately 1×10^6 cells per millilitre (see 10.3) and containing 9 ml of growth medium (7.2.2). Close the bottle tightly and incubate at $(37 \pm 1)^\circ\text{C}$ for as long as is required to obtain a confluent monolayer. Decant off the growth medium and thoroughly rinse the cell monolayer with 10 ml of the phosphate-buffered saline solution (7.2.5). Discard the rinsing solution and then add 9 ml of maintenance medium (7.2.3). Incubate as above. Replace the maintenance medium (7.2.3) at least every 5 days. After 2 to 3 weeks, prepare a new cell suspension (see 10.3) and transfer 1 ml to 2 ml aliquot volumes to new tissue-culture containers and continue growing the cells by repeating all of the above steps. Each week, examine the cells microscopically and if there are any signs of unusual growth or excessive granular inclusions in the cells, discard the cells but pay due attention to all necessary safe disposal procedures.

NOTE Other methods of cell culture may be used providing that the cell line remains healthy.

10.3 Preparation of the cell suspension

Decant the maintenance medium (7.2.3) from a healthy monolayer of cells (obtained from 7.3 or 10.2), rinse the monolayer with 10 ml of the phosphate-buffered saline solution (7.2.5) and discard. Add 3 ml of the trypsin-EDTA solution (7.2.6) to the bottle so as to completely cover the monolayer. Leave for 30 s at room temperature, and decant off the excess trypsin-EDTA solution. Incubate at $(37 \pm 1)^\circ\text{C}$ for 4 to 5 min. Examine the tissue culture container to ensure that the cells have become detached from the container surface and then add 2.5 ml of growth medium (7.2.2) and very gently shake the container to suspend the cells in the medium.

Determine the number of viable cells per millilitre of the cell suspension using a haemocytometer counting chamber that conforms to BS 748.

10.4 Extraction procedure

On the same day as testing is to start, rinse the sample in the test water (7.1.1) for 10 min. Place each sample (9.1) in a separate clean extraction container (8.3). In addition, with each batch of samples, include one empty container as the blank. Add sufficient test water (7.1.1) to each container to reach the calibration mark given in BS 6920-2.1:2000, 5.1.2 and seal with a fresh piece of aluminium foil. If the density of the sample is less than that of water, ensure the sample is kept totally submerged in the test water for the duration of the test by using glass-encapsulated weights.

Incubate the containers in the absence of light at $(23 \pm 2)^\circ\text{C}$ for 24 h.

10.5 Growth procedure

After incubation (see **10.4**), remove measured portions of each extract from every container (including the blank and validation solution of zinc sulfate) and use to dilute the concentrated growth medium (**7.2.7**) to the equivalent concentration of the constituents of the growth medium (**7.2.2**).

NOTE 1 This can be achieved by placing 0.55 ml of concentrated growth medium (**7.2.7**) into a sterile 30 ml glass bottle together with 2.45 ml of the extract (**10.4**).

To each bottle add sufficient cell suspension (**10.3**) to give a concentration of 10^4 to 10^5 cells per ml, and gently mix the contents. Transfer 1 ml portions of the resulting suspension of cells into three sterile plastics tubes or tissue-culture containers with airtight closures. Cap each container tightly.

NOTE 2 If insufficient numbers of viable cells are included in each tube, only limited growth will occur making comparison of cell confluence and cell medium colour hard to achieve and therefore reported results may be more inaccurate. The presence or absence of a confluent cell layer will be affected not only by the "toxicity" of a leachate but also by the number of cells added to the culture tube (see **10.3**); adding an insufficient number of cells to each tube will not result in a confluent cell layer even if the leachate is not toxic.

NOTE 3 If another established growth medium is being used in place of that specified, then dilute $\times 10$ concentrate with the extract and add the required supplements in the same proportion as used when preparing the growth medium with sterile distilled water.

Incubate at $(37 \pm 1)^\circ\text{C}$ for 24 h. Incline the tubes at an angle of 5° to 15° to the horizontal.

10.6 Microscopic examination

After incubation, microscopically examine the condition of the cells in each container from **10.5**.

NOTE 1 The use of a low-powered optical microscope that gives a magnification of $\times 40$ is recommended.

Record the presence or absence of a confluent cell layer, and the presence of any irregularly shaped cell or cells showing signs of "rounding off". If confluent growth is not observed, record the appearance of any cells floating in the growth medium.

NOTE 2 Comparison of the sample cell confluence against the confluence demonstrated in the blank (negative) control, as well as observation of cell morphology, should be the correct manner to measure toxicity.

11 Expression of results

11.1 Validation

If any tube representing the blank fails to exhibit confluent growth, or exhibits significant rounding off of the cells, or irregularly shaped cells, repeat the procedures given in **10.4**, **10.5** and **10.6** using fresh reagents and samples.

If the 800 mg/l solution of zinc sulfate does not lead to rounding off and death of the cells, repeat the procedures given in **10.4**, **10.5** and **10.6** using fresh reagents and samples.

NOTE It is essential to ensure that any death of the cells is not the result of failure to obtain airtight seals on any tube. This is normally indicated by the formation of a bright magenta colouration in the medium. Therefore, if the tissue culture medium in blank tubes turn bright magenta, the test should be repeated using fresh test samples.

11.2 Interpretation of results

If the extracts collected from a sample affect the morphology of the cell line in any way, examine two further samples using fresh reagents.

If the extracts from more than one of the three samples affect the morphology of the cell line, interpret this as a cytotoxic response.

NOTE Such a result will not necessarily mean that the test material is toxic to man, but indicates rather that there are substances leached into the water from the product which require further investigation before the product may be approved for use in contact with water intended for human consumption.

If the cell line in each of the tubes forms a confluent cell monolayer, and the degree of rounding off or irregular shaping of the cells is no greater than that observed in the tubes from the blank, then interpret this as a non-cytotoxic response.

11.3 Precision

This method does not yield numerical results that can be subjected to statistical treatment for the purposes of calculating reproducibility, R , and repeatability, r . However, in an inter-laboratory trial (conducted in 1985) involving the testing of 12 materials in duplicate in three laboratories, there was 92 % agreement in recording the presence or absence of a cytotoxic response with 24 h sample extracts.

12 Test report

12.1 General

The test report shall include the following particulars:

- a title (e.g. “Test Report”) and the date of issue of the report;
- a reference to this British Standard, i.e. BS 6920-2.5;
- name and address of laboratory, and location where the tests were carried out if different from the address of the laboratory;
- unique identification of the test report (such as serial number), and on each page an identification in order to ensure that the page is recognized as a part of the test report, and a clear identification of the end of the test report;
- name and address of the client placing the order;
- description and unambiguous identification of the item(s) tested – this shall meet the minimum requirements set out in BS 6920-2.1:2014, Clause 9;
- reference to any sampling or sample preparation procedures used by the laboratory, or other bodies and where appropriate, chain of custody details where these are relevant; in the case of site applied products include all the requirements in accordance with BS 6920-2.1:2014, 9.5;
- date of receipt of test item(s) and date(s) of performance of the tests undertaken;
- deviations from, additions to or exclusions from the test method;
- a description of the morphology of the cells in contact with the test extract, the blank and the zinc sulfate solution, together with a statement as to whether the findings indicate a cytotoxic or non-cytotoxic response;
- the name(s), function(s) and signature(s) or equivalent identification of person(s) authorizing the test report;
- a statement to the effect that the results relate only to the items tested, and that the test report shall not be reproduced except in full, without written approval of the laboratory.

When the test report contains results of tests performed by subcontractors, these shall be clearly identified.

When reports are reissued to take into account errors/omissions, results of additional tests or for other reasons one of the following two approaches shall be adopted:

- a) issue an additional test report containing the additional information or corrections only and clearly marked “Supplement to Test Report, Reference.....”;
- b) issue a complete amended report (usually the preferred option) with a statement added below the original date of issue of the report stating “Reissued with correction/additional data/etc. (as appropriate):(date)”.

All previous results for the tested item(s) shall be included in any reissued reports containing the results of additional tests.

When it is necessary to issue a completely new test report, it shall be uniquely identified and a reference to the original that it replaces shall be included.

In reports based upon only some of the sections of BS 6920-2 the statement “NO OTHER TESTS WERE UNDERTAKEN ON THIS PRODUCT” shall be included.

12.2 Retesting using cold water test conditions/failure in BS 6920-3

If a product fails to comply with the high temperature tests and a cold water test is then undertaken and satisfactory results obtained, both sets of results in the final report shall be included.

Annex A (informative)

Test sequence

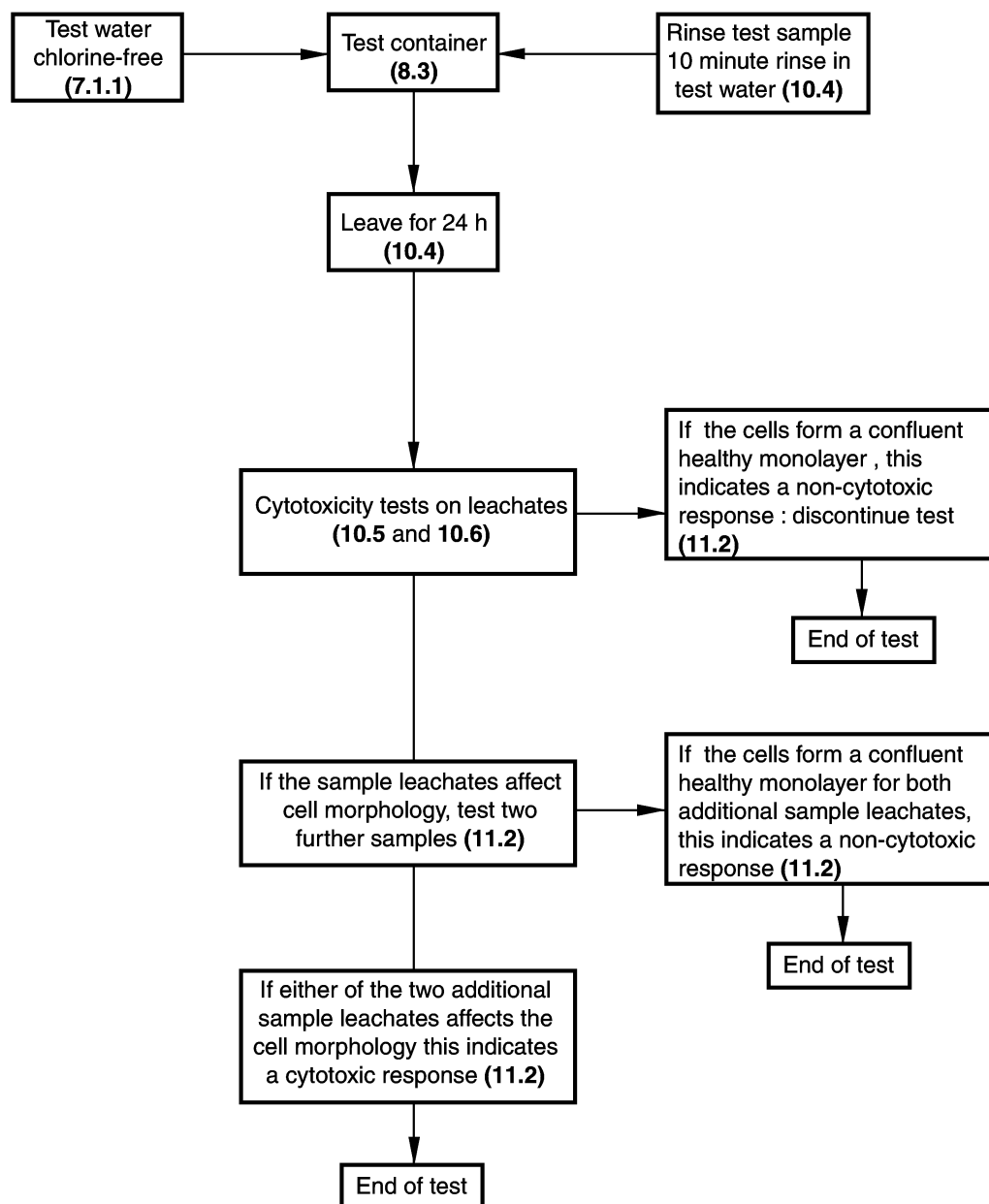


Figure A.1 — Test sequence

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Standards publications

BS 5726-3, *Microbiological safety cabinets — Part 3: Specification for performance after installation*.

Other documents

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