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Biological evaluation of medical devices —

Part 5:
Tests for cytotoxicity: *in vitro* methods

Évaluation biologique des dispositifs médicaux —
Partie 5: Essais concernant la cytotoxicité — Méthodes in vitro

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Contents

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Foreword

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

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.

International Standard ISO 10993-5 was prepared by Technical Committee ISO/TC 194, *Biological evaluation of medical devices*.

ISO 10993 consists of the following parts, under the general title *Biological evaluation of medical devices*:

— *Part 1: Guidance on selection of tests*

— *Part 2: Animal welfare requirements*

— *Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity*

— *Part 4: Selection of tests for interactions with blood*

— *Part 5: Tests for cytotoxicity: in vitro methods*

— *Part 6: Tests for local effects after implantation*

— *Part 7: Ethylene oxide sterilization residuals*

— *Part 8: Clinical investigation*

— *Part 9: Degradation of materials related to biological testing*

— *Part 10: Tests for irritation and sensitization*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

Future parts will deal with other relevant aspects of biological testing.

Annex A of this part of ISO 10993 is for information only.

Introduction

Due to the general applicability of *in vitro* cytotoxicity tests and their widespread use in evaluating a large range of devices and materials, it is the purpose of this part of ISO 10993, rather than to specify a single test, to define a scheme for testing which requires decisions to be made in a series of steps. This should lead to the selection of the most appropriate test.

Three categories of tests are listed: extract test, direct contact test, indirect contact test.

The choice of one or more of these categories depends upon the nature of the sample to be evaluated, the potential site of use and the nature of the use.

This choice then determines the details of the preparation of the samples to be tested, the preparation of the cultured cells, and the way in which the cells are exposed to the samples or their extracts.

At the end of the exposure time, the evaluation of the presence and extent of a cytotoxic effect is undertaken. It is the intention of this part of ISO 10993 to leave open the choice of type of evaluation. Such a strategy makes available a battery of tests, which reflects the approach of many groups which advocate *in vitro* biological tests.

The numerous methods used and end-points measured in cytotoxicity determination can be grouped into categories of evaluation type:

a) assessments of cell damage by morphological means;

b) measurements of cell damage;

c) measurements of cell growth;

d) measurements of specific aspects of cellular metabolism.

There are, therefore, several alternative means of producing results in each of these four categories. The investigator should be aware of the categories of test and into which a particular technique fits, in order that comparisons may be made with other results on similar devices or materials, and in order that interlaboratory tests may be conducted.

Biological evaluation of medical devices —

Part 5:
Tests for cytotoxicity: *in vitro* methods

1 Scope

This part of ISO 10993 describes test methods to assess the *in vitro* cytotoxicity of medical devices.

NOTE 1 The term medical devices corresponds to the definition given in ISO 10993-1 and covers medical materials as well as dental material and devices. The definition is in accordance with the CEN standard document.

These methods specify the incubation of cultured cells either directly or through diffusion

a) with extracts of the device, and/or

b) in contact with a device.

These methods are designed to determine the biological response of mammalian cells *in vitro* using appropriate biological parameters.

2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this part of ISO 10993. At the time of publication, the edition indicated was valid. All standards are subject to revision, and parties to agreements based on this part of ISO 10993 are encouraged to investigate the possibility of applying the most recent edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 10993-1:1992, *Biological evaluation of medical devices — Part 1: Guidance on selection of tests.*

3 Definitions

For the purposes of this part of ISO 10993, the definitions given in ISO 10993-1 and the following definitions apply.

3.1 negative control material: Material which, when tested in accordance with this part of ISO 10993, does not produce a cytotoxic response.

NOTE 2 The purpose of the negative control is to demonstrate background response of the cells. For example high density polyethylene (see below) for synthetic polymers, and aluminium oxide ceramic rods for dental material, have been used as negative controls.

High density polyethylene can be obtained from the U.S. Pharmacopeia (Rockville — Maryland — USA). This information is given for the convenience of the user of this part of ISO 10993 and does not constitute an endorsement by ISO of the product.

3.2 positive control material: Material which, when tested in accordance with this part of ISO 10993 provides a reproducible cytotoxic response.

NOTE 3 The purpose of the positive control is to demonstrate appropriate test system response. For example an organo-tin stabilized poly(vinylchloride) has been used as a positive control for solid materials and extracts. Dilutions of phenol, for example, have been used as a positive control for extracts.

3.3 reagent control: Extraction vehicle without test material subjected to extraction conditions and test procedures.

3.4 culture vessels: Vessels including glass petri dishes, plastics culture dishes, plastics culture flasks or plastics multi-wells and microtiter plates.

NOTE 4 These may be used interchangeably in these methods provided that they meet the requirements of tissue culture grade and are suitable for use with mammalian cells.

4 Sample preparation

4.1 General

The test shall be performed on either

a) an extract of the material; and/or

b) the material itself.

For both methods the test material shall be representative of either

c) the final product; or

d) a component of the final product that is to be tested.

4.2 Preparation of liquid extracts of material

4.2.1 Principles of extraction

Extracting conditions shall attempt to exaggerate the clinical use conditions so as to define the potential toxicological hazard without causing significant changes such as fusion or melting of the material pieces or alter the chemical structure.

NOTES

5 The results derived from tests where the conditions of extraction were exaggerated need to be viewed in light of these exaggerations. Judgement needs to be used in interpreting the results as to their appropriateness to the actual use conditions and device potential toxicity.

6 The concentration of any endogenous or extraneous substances in the extract, and hence the amount exposed to the test cells, depends on the interfacial area, the extraction volume, pH, chemical solubility, osmolarity, agitation, temperature and other factors.

4.2.2 Extraction vehicle

For mammalian cell assays one or more of the following solvents shall be used:

a) culture medium with serum;

b) culture medium without serum;

c) 9 g/l sodium chloride in deionized water;

d) other suitable solvent.

NOTE 7 The list given is in order of preferred use. Other suitable solvents include water, vegetable oil and dimethyl sulfoxide (DMSO). DMSO is known to be cytotoxic in selected assay systems at greater than 0,5 % (*V/V*) concentrations.

4.2.3 Extraction conditions

4.2.3.1 The extraction shall be performed in sterile, chemically inert closed containers by using aseptic techniques.

4.2.3.2 The extraction time and temperature are dependent on the physicochemical characteristics of the material and extraction vehicle. Recommended conditions are:

a) not less than 24 h at 37 °C;

b) 72 h at 50 °C;

c) 24 h at 70 °C;

d) 1 h at 121 °C.

Extraction conditions should simulate as closely as possible the conditions under which the device will normally be used. Therefore item a) gives the preferred conditions for extraction.

The recommended conditions may be applied according to the device characteristics and specific conditions of use.

Extraction procedures using culture medium with serum can only be used under the conditions specified in 4.2.3.2 a).

4.2.3.3 When agitation is considered to be appropriate, the method should be specified and reported.

4.2.3.4 When appropriate, cut the material into small pieces before extraction. For polymers, 10 mm × 50 mm pieces have been used. Moulded elastomer closures are tested intact.

4.2.3.5 The ratio between the surface area of the material and the volume of extraction vehicle shall be no more than 6 cm^2/ml and no less than 0,5 cm^2/ml. The surface area shall be calculated on the basis of the overall sample dimensions, not taking into account surface irregularity and porosity. However, the actual surface characteristics should be considered in the interpretation of the test results. If the surface area is indeterminate, then 0,1 g/ml to 0,2 g/ml shall be used.

4.2.3.6 Liquid extracts shall, if possible, be used immediately after preparation.

If an extract is stored, then the stability of the extract under the conditions of storage should be verified with appropriate methods.

If the extract is filtered, centrifuged or processed by other methods prior to being applied to the cells, this shall be included in the final report (see clause 9).

4.3 Preparation of material for direct contact tests

4.3.1 Materials which have various shapes, sizes or physical states (i.e. liquid or solid) may be tested without modification in the cytotoxicity assays.

The preferred sample of a solid specimen should have at least one flat surface. Adjustments shall be made for other shapes and physical states.

4.3.2 The sterility of the test specimen shall conform to the requirements in 4.3.2.1 to 4.3.2.3.

4.3.2.1 Test materials from sterilized devices shall be handled aseptically throughout the extraction and test procedure.

4.3.2.2 Test materials from devices which are normally supplied non-sterile but are sterilized before use shall be sterilized by the method recommended by the manufacturer and handled aseptically throughout the extraction and test procedure.

The effect of sterilization methods or agents on the device should be considered in defining the preparation of the test material prior to use in the test system.

4.3.2.3 Test materials from devices not required to be sterile in use shall be used as supplied and handled aseptically throughout the extraction and test procedure.

4.3.3 Liquids shall be tested by either

a) direct deposition; or

b) deposition on to a biologically inert absorbent matrix.

NOTE 8 Filter discs have been found to be suitable.

4.3.4 If appropriate, materials classed as superabsorbent shall be wetted with culture medium prior to testing.

5 Cell lines

5.1 Established cell lines are preferred and where used shall be obtained from recognized repositories.

NOTE 9 For example, recommended cell lines are American Type Culture Collection CCL1 (NCTC clone 929), CCL 163 (Balb/3T3 clone A31), CCL 171 (MRC-5) and CCL 75 (WI-38), CCL 81 (Vero) and CCL10 [BHK-21 (C-13)] and V-79 379A.

This information is given for the convenience of the user of this part of ISO 10993 and does not constitute an endorsement by ISO of the products named. Other cell lines may be used if they can be shown to lead to the same results.

5.2 Where specific sensitivity is required, primary cell cultures and cell lines obtained directly from living tissues shall only be used if reproducibility and accuracy of the response can be demonstrated.

5.3 If a stock culture of a cell line is stored, storage shall be at − 100 °C or below in the corresponding culture medium, but containing a cryoprotectant, e.g. dimethylsulfoxide or glycerol.

5.4 Only cells free from mycoplasma should be used for the test. Before use, stock cultures should be tested by a well established method for the absence of mycoplasma.

6 Culture medium

6.1 The culture medium shall be sterile.

6.2 The culture medium with or without serum shall meet the growth requirements of the selected cell line.

Antibiotics may be included in the media provided that they do not adversely affect the assays.

The stability of the culture medium varies with the composition and storage conditions. Media containing serum and glutamine may be stored at 2 °C to 8 °C for no more than one week. Glutamine containing media without serum may be stored at 2 °C to 8 °C for no more than one month.

6.3 The culture medium shall be maintained at a pH of between 7,2 and 7,4.

7 Preparation of cell stock culture

7.1 Using the chosen cell line and culture medium, prepare sufficient cells to complete the test. If the cells are to be grown from cultures taken from storage, remove the cryoprotectant if present. Subculture the cells at least once before use.

7.2 Cells are removed and resuspended by enzymatic and/or mechanical disaggregation using a method appropriate for the cell line.

8 Test procedures

8.1 Number of replicates

A minimum of three replicates shall be used for test samples and controls.

8.2 Test on extracts

8.2.1 Pipette an aliquot of the continuously stirred cell suspension into each of a sufficient number of vessels for exposure to the extracts. Distribute the cells evenly over the surface of each vessel by gentle rotation.

8.2.2 Incubate the cultures at (37 ± 2) °C in air with or without 5 % (*V/V*) carbon dioxide as appropriate for the buffer system chosen for the culture medium.

The test may be performed on a subconfluent monolayer or on freshly suspended cells.

In the colony-forming assay only, an appropriate low cell density shall be used.

8.2.3 Examine the cultures with a microscope.

8.2.4 Perform the test on either

a) the original extract; or

b) a dilution series of the extract using the culture medium as diluent.

If monolayers are used for the test, remove and discard the culture medium from the cultures and add an aliquot of the extract or dilution thereof into each of the vessels.

If suspended cells are used for the test, add the extract or dilution thereof into each of the replicate vessels, immediately after preparation of the cell suspension.

8.2.5 When a non-physiological extract is used, e.g. water, the extract shall be tested at the highest physiologically compatible concentration after dilution in culture medium.

NOTE 10 Concentrated culture medium, e.g. 2 ×, 5 × is recommended for use in diluting aqueous extracts.

8.2.6 Add known aliquots of the reagent blank and the negative and positive controls into additional replicate vessels.

NOTE 11 A fresh culture medium control may also be tested, if appropriate.

8.2.7 Incubate the vessels using the same conditions as described in 8.2.1 for an appropriate interval corresponding to the selected specific assay.

8.2.8 Determine the cytotoxicity in accordance with 8.5.

8.3 Test by direct contact

8.3.1 Pipette a known aliquot of the continuously stirred cell suspension into each of a sufficient number of vessels for direct exposure to the test sample. Distribute the cells evenly over the surface of each vessel by gentle horizontal rotation.

8.3.2 Incubate the culture at (37 ± 2) °C in air with or without 5 % (*V/V*) carbon dioxide as appropriate for the buffer system chosen for the culture medium, until the cultures have grown to approximate confluence at the end of the log phase of the growth curve.

8.3.3 Examine the culture with a microscope.

8.3.4 Remove and discard the culture medium. Then add fresh culture medium to each vessel.

8.3.5 Carefully place individual specimens of the test sample on the cell layer in the centre of each of replicate vessels. Ensure that the specimen covers approximately one-tenth of the cell layer surface.

Exercise care to prevent unnecessary movement of the specimens as this could cause physical trauma to the cells that is evidenced by patches of dislodged cells.

NOTE 12 When appropriate, the specimen can be placed in the culture vessel prior to the addition of the cells.

8.3.6 Prepare replicate vessels for both the negative control and the positive control material.

8.3.7 Incubate the vessels under the same conditions as in 8.3.2 for an appropriate interval corresponding to the selected specific assay.

8.3.8 Discard the supernatant culture medium and determine the cytotoxicity in accordance with 8.5.

8.4 Test by indirect contact

8.4.1 Agar diffusion

8.4.1.1 Pipette a known aliquot of the continuously stirred cell suspension into each of a sufficient number of replicate vessels for the test. Distribute the cells evenly over the surface of each vessel by gentle horizontal rotation.

8.4.1.2 Incubate the cultures at (37 ± 2) °C in air with or without 5 % (*V/V*) carbon dioxide as appropriate for the buffer system chosen for the culture medium until the cultures have grown to approximate

confluence at the end of the log phase of the growth curve.

8.4.1.3 Examine the cultures with a microscope.

8.4.1.4 Remove and discard the culture medium from the vessel. Then mix fresh culture medium containing serum with melted agar to obtain a final agar concentration of 0,5 g/l to 2 g/l and pipette an appropriate volume into each vessel. Use only agar which is suitable for the growth of mammalian cells in culture. The agar culture medium mixture should be in a liquid state and at a temperature which is compatible with mammalian cells.

NOTE 13 Agar is available in various molecular weight ranges and purities.

8.4.1.5 Carefully place replicate specimens of the test sample on the solidified agar layer in each vessel. Ensure that the specimen covers approximately one-tenth of the cell layer surface.

Pre-wet any absorbent material with the culture medium before placing it on the agar to prevent dehydration of the agar.

8.4.1.6 Prepare replicate vessels with both the negative control and positive control specimens.

8.4.1.7 Incubate the vessels using the same conditions as described in 8.4.1.2 for between 24 h and 72 h.

8.4.1.8 Examine the cells for cytotoxicity before and after carefully removing the specimens from the agar. Use of a vital stain, e.g. neutral red, may aid in the detection of cytotoxicity. The vital stain may be added before or after the incubation with the specimen. If the stain is added before the incubation, protect the cultures from light to prevent cell damage elicited by photoactivation of the stain.

8.4.2 Filter diffusion

8.4.2.1 Place a surfactant-free filter with 0,45 µm pore size into each vessel and add a known aliquot of the continuously stirred cell suspension into each of a sufficient number of replicate vessels for the test. Distribute the cells evenly over the surface of each filter by gentle rotation.

8.4.2.2 Incubate the cultures at (37 ± 2) °C in air with or without 5 % (*V/V*) carbon dioxide as appropriate for the buffer system chosen for the culture medium until the cultures have grown to approximate confluence at the end of the log phase of the growth curve.

8.4.2.3 Examine the cultures with a microscope.

8.4.2.4 Remove and discard the culture medium from the vessels. Then transfer the filters, cell side down, on to a layer of solidified agar (see 8.4.1.5).

8.4.2.5 Carefully place the replicate specimens of the test sample on the acellular (top) side of the filter. Retain liquid extracts and freshly mixed compounds in non-reactive rings placed on the filter.

8.4.2.6 Prepare replicate filters with both the negative control and positive control specimens.

8.4.2.7 Incubate the vessels using the same conditions described in 8.4.2.2 for 2 h ± 10 min.

8.4.2.8 Carefully remove the specimens from the filter and carefully separate the filter from the agar surface.

8.4.2.9 Determine the cytotoxicity using an appropriate stain procedure.

8.5 Determination of cytotoxicity

8.5.1 Determine cytotoxicity by either qualitative or quantitative means.

a) **Qualitative evaluation:** examine the cells microscopically to assess for changes in for example general morphology, vacuolization, detachment, cell lysis and membrane. The change from normal morphology may be recorded in the test report descriptively (e.g. none, slight, moderate and severe) or numerically (e.g. 0, 1, 2, 3). Describe the method of evaluation in the report.

b) **Quantitative evaluation:** measure cell death, inhibition of cell growth, cell proliferation or colony formation. The number of cells, amount of protein, release of enzymes, release of vital dye, reduction of vital dye or other measurable parameter may be quantified by objective means. The objective measure and response is recorded in the test report.

NOTE 14 For particular methods of determining cytotoxicity, a zero time or baseline cell culture control may be necessary.

8.5.2 Ensure that care is taken in the choice of evaluation methods as the test results may be invalid if the test specimen releases substances which interfere with the test system or measurement.

NOTE 15 Materials which release formaldehyde can only be reliably tested if the assay measurement evaluates cell viability.

8.5.3 If there are evident differences in the test result for replicate culture vessels, then the test is either inappropriate or invalid.