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Biological evaluation of medical devices —

Part 5: Tests for *in vitro* cytotoxicity

Évaluation biologique des dispositifs médicaux —

Partie 5: Essais concernant la cytotoxicité in vitro

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Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 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 10993-5 was prepared by Technical Committee ISO/TC 194, *Biological evaluation of medical devices*.

This third edition cancels and replaces the second edition (ISO 10993-5:1999), which has been technically revised.

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

— *Part 1: Evaluation and testing within a risk management process*

— *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* in vitro *cytotoxicity*

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

— *Part 7: Ethylene oxide sterilization residuals*

— *Part 9: Framework for identification and quantification of potential degradation products*

— *Part 10: Tests for irritation and skin sensitization*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

— *Part 13: Identification and quantification of degradation products from polymeric medical devices*

— *Part 14: Identification and quantification of degradation products from ceramics*

— *Part 15: Identification and quantification of degradation products from metals and alloys*

— *Part 16: Toxicokinetic study design for degradation products and leachables*

— *Part 17: Establishment of allowable limits for leachable substances*

— *Part 18: Chemical characterization of materials*

— *Part 19: Physico-chemical, morphological and topographical characterization of materials* [Technical Specification]

— *Part 20: Principles and methods for immunotoxicology testing of medical devices* [Technical Specification]

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 test 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 the 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 that advocate *in vitro* biological tests.

The numerous methods used and endpoints measured in cytotoxicity determination can be grouped into the following categories of evaluation:

— assessments of cell damage by morphological means;

— measurements of cell damage;

— measurements of cell growth;

— measurements of specific aspects of cellular metabolism.

There are several means of producing results in each of these four categories. The investigator should be aware of the test categories and into which category a particular technique fits, in order that comparisons be able to be made with other results on similar devices or materials both at the intra- and interlaboratory level. Examples of quantitative test protocols are given in annexes. Guidance for the interpretation of the results is given in this part of ISO 10993.

Biological evaluation of medical devices —

Part 5:
Tests for *in vitro* cytotoxicity

1 Scope

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

These methods specify the incubation of cultured cells in contact with a device and/or extracts of a device either directly or through diffusion.

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

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 10993-1, *Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management system*

ISO 10993-12, *Biological evaluation of medical devices — Part 12: Sample preparation and reference materials*

3 Terms and definitions

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

3.1
culture vessels
vessels appropriate for cell culture including glass petri dishes, plastic culture flasks or plastic multiwells and microtitre plates

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

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

NOTE The purpose of the positive control is to demonstrate an appropriate test system response. For example, an organotin-stabilized polyurethane[1)] has been used as positive control for solid materials and extracts. Dilutions of phenol, for example, have been used as a positive control for extracts. In addition to a material, pure chemicals can also be used to demonstrate the performance of the test system.

3.3
blank
extraction vehicle not containing the test sample, retained in a vessel identical to that which holds the test sample and subjected to conditions identical to those to which the test sample is subjected during its extraction

NOTE The purpose of the blank is to evaluate the possible confounding effects due to the extraction vessel, vehicle and extraction process.

3.4
negative control material
material which, when tested in accordance with this part of ISO 10993, does not produce a cytotoxic response

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

3.5
test sample
material, device, device portion, component, extract or portion thereof that is subjected to biological or chemical testing or evaluation

3.6
subconfluency
approximately 80 % confluency, i.e. the end of the logarithmic phase of growth

4 Sample and control preparation

4.1 General

The test shall be performed on

a) an extract of the test sample

and/or

b) the test sample itself.

Sample preparation shall be in accordance with ISO 10993-12.

Negative and positive controls shall be included in each assay.

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1) The ZDEC and ZDBC polyurethanes are available from the Food and Drug Safety Center, Hatano Research Institute, Ochiai 729-5, Hadanoshi, Kanagawa 257, Japan.

2) High-density polyethylene can be obtained from the U.S. Pharmacopeia (Rockville, MD, USA) and from the Food and Drug Safety Center, Hatano Research Institute (Ochiai 729-5, Hadanoshi, Kanagawa 257, Japan).

The information given in 1) and 2) is for the convenience of the user of this part of ISO 10993 and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

4.2 Preparation of liquid extracts of material

4.2.1 Principles of extraction

Extracting conditions should attempt to simulate or exaggerate the clinical use conditions so as to determine the potential toxicological hazard without causing significant changes in the test sample, such as fusion, melting or any alteration of the chemical structure, unless this is expected during clinical application. Due to the nature of certain materials (e.g. biodegradable materials), alteration of the chemical structure can occur during the extraction procedure.

NOTE 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, diffusion rate, osmolarity, agitation, temperature, time and other factors.

For devices that involve mixing two or more components in the patient to arrive at the final device (for example bone cement), the final device should not be washed prior to extraction. Washing the test sample can reduce or remove residuals present on the device. If the test sample is to be used in a sterile environment, a sterilized test sample should be used to extract chemical constituents.

4.2.2 Extraction vehicle

The choice of the extraction vehicle(s) taking into account the chemical characteristics of the test sample shall be justified and documented. For mammalian cell assays one or more of the following vehicles shall be used:

a) culture medium with serum;

b) physiological saline solution;

c) other suitable vehicle.

The choice of vehicle should reflect the aim of the extraction. Consideration shall be given to the use of both a polar and a non-polar vehicle. Culture medium with serum is the preferred extraction vehicle. The use of culture medium with serum is preferred for extraction because of its ability to support cellular growth as well as extract both polar and non-polar substances. In addition to culture medium with serum, use of medium without serum should be considered in order to specifically extract polar substances (e.g. ionic compounds). Other suitable vehicles include purified water and dimethyl sulfoxide (DMSO). DMSO is cytotoxic in selected assay systems at greater than 0,5 % (volume fraction). The cellular exposure concentration of extractables in DMSO will be lower due to the greater dilution as compared to extraction in culture medium with serum.

NOTE 1 Different types of serum (e.g. foetal, bovine/calf serum, newborn calf serum) might be used and the choice of the serum is dependent on the cell type.

NOTE 2 It is important to recognise that serum/proteins are known to bind, to some extent, extractables.

4.2.3 Extraction conditions

4.2.3.1 The extraction shall be performed in sterile, chemically inert, closed containers by using aseptic techniques, in accordance with ISO 10993-12.

4.2.3.2 With the exception of circumstances given below, the extraction shall be conducted under one of the following conditions and shall be applied according to the device characteristics and specific conditions for use:

a) (24 ± 2) h at (37 ± 1) °C;

b) (72 ± 2) h at (50 ± 2) °C;

c) (24 ± 2) h at (70 ± 2) °C;

d) (1 ± 0,2) h at (121 ± 2) °C.

Extraction conditions described above, which have been used to provide a measure of the hazard potential for risk estimation of the device or material, are based on historical precedent. Other conditions, e.g. prolonged or shortened extraction times at 37 °C, which simulate the extraction that occurs during clinical use or provide an adequate measure of the hazard potential, may be used, but shall be justified and documented. For medical devices that are in short-term contact (no greater than 4 h cumulative contact duration) with intact skin or mucosa and that are not implanted, this may include extraction times of less than 24 h but no less than 4 h, as given in a) to c).

Cell culture medium with serum should only be used in accordance with a) because extraction temperatures greater than (37 ± 1) °C can adversely impact chemistry and/or stability of the serum and other constituents in the culture medium.

For polymeric test samples, the extraction temperature should not exceed the glass transition temperature as the higher temperature can change the extractant composition.

4.2.3.3 If the extract is filtered, centrifuged or processed by other methods prior to being applied to the cells, these details shall be recorded in the final report along with a rationale for the additional steps (see Clause 9). Any pH adjustment of the extract shall be reported. Manipulation of the extract, such as by pH adjustment, should be avoided because it could influence the result.

4.3 Preparation of material for direct-contact tests

4.3.1 Form of test samples

Materials that have various shapes, sizes or physical states (i.e. liquid, gels, solids, etc.) may be tested without modification in the cytotoxicity assays.

The preferred test sample of a solid material should have at least one flat surface. If not, adjustments shall be made to achieve flat surfaces.

4.3.2 Sterility of test samples

4.3.2.1 Sterility of the test sample shall be taken into account.

4.3.2.2 Test samples from sterilized devices shall be handled aseptically throughout the test procedure.

4.3.2.3 Test samples from devices that 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 test procedure.

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

4.3.2.4 Test samples from devices not required to be sterile in use shall be used as supplied and handled aseptically throughout the test procedure. It may be justifiable to sterilize the test material in order to avoid microbial contamination of the cell culture; however, the sterilization process shall not alter the properties of the test material.

If non-sterile test samples are used, they should be checked for bacterial contamination because the contamination can lead to a false assessment of cytotoxicity.

4.3.3 Liquid test samples

Liquid test samples shall be tested by either

a) direct deposition

or

b) deposition on a biologically inert absorbent matrix.

Filter discs have been found to be suitable for use as inert absorbent matrices.

4.3.4 Absorbent test samples

If appropriate, test samples that are absorbent shall be soaked with culture medium prior to testing to prevent adsorption of the culture medium in the testing vessel.

4.4 Preparation of controls

Controls should be selected so that they can be prepared by the same procedure as the test sample.

5 Cell lines

Established cell lines are preferred and where used shall be obtained from recognised repositories[3)].

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

If a stock culture of a cell line is stored, storage shall be at −80 °C or below in the corresponding culture medium but containing a cryoprotectant, e.g. dimethylsulfoxide or glycerol. Long-term storage (several months up to many years) is only possible at −130 °C or below.

Only cells free from mycoplasma shall be used for the test. Before use, stock cultures should be tested for the absence of mycoplasma.

It is important to check cells regularly (e.g. morphology, doubling time, modal chromosome number) because sensitivity in tests can vary with passage number.

Good cell culture practices should be used. See Reference [5].

6 Culture medium

The culture medium shall be sterile.

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

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

Storage conditions shall be validated.

NOTE The stability of the culture medium varies with the composition and storage conditions.

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

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3) For example, cell lines American Type Culture Collection CCL 1 (NCTC clone 929), CCL 163 (Balb/3T3 clone A31), CCL 171 (MRC-5) and CCL 75 (WI-38), CCL 81 (Vero) and CCL 10 [BHK-21 (C-13)] and V-79 379A are endorsed by ISO experts to be suitable.

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 or more relevant results.

7 Preparation of cell stock culture

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.

When subculturing cells, remove and resuspend the cells 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 This test allows both qualitative and quantitative assessment of cytotoxicity.

8.2.2 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.

8.2.3 Incubate the cultures at (37 ± 1) °C in air with or without carbon dioxide as appropriate for the buffer system chosen for the culture medium.

The test should 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.

8.2.4 Verify the subconfluency and the morphology of the cultures with a microscope before starting the test.

In exceptional cases, exponentially growing cells (e.g. primary cells, high proliferating cells) may be seeded at the starting point of the test.

8.2.5 Perform the test on

a) the original extract

and/or

b) the original extract and a dilution series of the extracts using the extract vehicle as diluent.

Alternatively, where materials of limited solubility are known or suspected to be present, dilution should be achieved by varying the original extraction ratio of test sample to extraction medium.

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.

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

8.2.7 Add known aliquots of the blank and the negative and positive controls to additional replicate vessels.

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

8.2.8 Incubate the vessels using the same conditions as described in 8.2.3 for an appropriate interval corresponding to the selected specific assay.

8.2.9 After an incubation period of at least 24 h, determine the cytotoxic effects in accordance with 8.5.

8.3 Test by direct contact

8.3.1 This test allows both qualitative and quantitative assessment of cytotoxicity.

8.3.2 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.

8.3.3 Incubate the culture at (37 ± 1) °C in air, with or without carbon dioxide as appropriate for the buffer system chosen for the culture medium, until the cultures have grown to subconfluency.

8.3.4 Verify the subconfluency and the morphology of the cultures with a microscope before starting the test.

In exceptional cases, exponentially growing cells (e.g. primary cells, high proliferating cells) may be seeded at the starting point of the test.

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

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

Other ratios of specimen surface to cell layer surface may be used if justified.

Exercise care to prevent unnecessary movement of the specimens, as this could cause physical trauma to the cells. For example, patches of dislodged cells can result from unnecessary movement.

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

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

8.3.8 Incubate the vessels under the same conditions as described in 8.3.3 for an appropriate interval (a minimum of 24 h) corresponding to the selected specific assay.

8.3.9 Discard the supernatant culture medium before adding chemicals/dyes in order to determine the cytotoxic effects in accordance with 8.5.

8.4 Test by indirect contact

8.4.1 Agar diffusion

8.4.1.1 This test allows a qualitative assessment of cytotoxicity. This assay is not appropriate for leachables that cannot diffuse through the agar layer, or that may react with agar. The use of the agar diffusion assay for the assessment of cytotoxicity shall be justified.

8.4.1.2 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.