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Biological evaluation of medical devices —

Part 12: Sample preparation and reference materials

Évaluation biologique des dispositifs médicaux —

Partie 12: Préparation des échantillons et matériaux de référence

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 194, *Biological and clinical evaluation of medical devices*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 206, *Biological and clinical evaluation of medical devices,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This fifth edition cancels and replaces the fourth edition (ISO 10993-12:2012), which has been technically revised.

The main changes compared to the previous edition are as follows:

— change of scope to cover extractions only for biological evaluation tests;

— harmonization of definitions with ISO 10993-18;

— revision of 10.3.1 extraction condition table and Annex D regarding exhaustive extraction.

A list of all parts in the ISO 10993 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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Introduction

It is important that sample preparation methods be appropriate for both the biological evaluation methods and the materials being evaluated. Each biological test method requires the selection of materials, extraction solvents and conditions.

This document is based on existing national and international standards and regulations, wherever possible.

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Biological evaluation of medical devices —

Part 12:
Sample preparation and reference materials

1 Scope

This document specifies requirements and gives guidance on the procedures in the preparation of samples and the selection of reference materials for medical device testing primarily in biological test systems primarily in accordance with one or more parts of the ISO 10993 series.

Specifically, this document addresses the following:

— test sample selection;

— selection of representative portions from a medical device;

— test sample preparation;

— experimental controls;

— selection of, and requirements for, reference materials;

— preparation of extracts.

This document is not applicable to live cells but can be relevant to the material or medical device components of combination products containing live cells.

Extractions for chemical characterization are covered in ISO 10993-18. Clause 7, 8, 9, 10 [with the exception of 10.3.5 and 10.3.11 b)], and 11 can apply to extractions for chemical characterization. Information given in C.1 to C.4 can also be relevant.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
blank
extraction vehicle not containing the test material, which is exposed to identical vessels and conditions as the test sample during extraction

Note 1 to entry: The purpose of the blank is to evaluate possible confounding effects due to the extraction vessel, extraction vehicle and extraction process.

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3.2
CRM
certified reference material
reference material (RM) characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability

Note 1 to entry: The concept of value includes a nominal property or a qualitative attribute such as identity or sequence. Uncertainties for such attributes may be expressed as probabilities or levels of confidence.

Note 2 to entry: Metrologically valid procedures for the production and certification of RMs are given in, among others, ISO 17034 and ISO Guide 35.

Note 3 to entry: ISO Guide 31 gives guidance on the contents of RM certificates.

Note 4 to entry: ISO/IEC Guide 99:2007 has an analogous definition (5.14).

[SOURCE: ISO Guide 30:2015, 2.1.2]

3.3
exaggerated extraction
extraction that is intended to result in a greater amount of a chemical constituent being released as compared to the amount generated under the simulated conditions of use

Note 1 to entry: It is important to ensure that the exaggerated extraction does not result in a chemical change of the material.

3.4
exhaustive extraction
extraction conducted until the amount of extractable material in a subsequent extraction is less than 10 % by gravimetric analysis (or that achieved by other means) of that detected in the initial extraction

Note 1 to entry: As it is not possible to demonstrate the exhaustive nature of residual recovery, the definition of exhaustive extraction adopted is as above. See also Annex C.

3.5
experimental control
substance with well-characterized responses, which is used in a specific test system to assist in evaluating if the test system has responded in a reproducible and appropriate manner

3.6
extract
liquid that results from extraction of the test sample or control

3.7
extractable substance
substance that can be released from a medical device or material using either extraction solvents or extraction conditions, or both, that are expected to be at least as aggressive as the conditions of clinical use

3.8
homogeneity
consistency of a material's chemical and physical compositions, and uniformity in response to a biological endpoint

Note 1 to entry: A reference material is said to be homogeneous if the biological response in a specific test is found to lie within the specified uncertainty limits of the test, irrespective of the batch or lot of material from which the test sample is extracted.

3.9
leachable substance
substance that can be released from a medical device or material during clinical use

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3.10
negative control
well-characterized material and/or substance, which, when tested by a specific procedure, demonstrates the suitability of the procedure to yield a reproducible, appropriately negative, non-reactive or minimal response in the test system

Note 1 to entry: In practice, negative controls are reference materials but can include blanks and extraction vehicles/solvents.

3.11
positive control
well-characterized material and/or substance, which, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately positive or reactive response in the test system

3.12
reference material
RM
material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

Note 1 to entry: RM is a generic term.

Note 2 to entry: Properties can be quantitative or qualitative, e.g. identity of substances or species.

Note 3 to entry: Uses may include the calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control.

Note 4 to entry: ISO/IEC Guide 99:2007 has an analogous definition (5.13), but restricts the term "measurement" to apply to quantitative values. However, Note 3 of ISO/IEC Guide 99:2007, 5.13 (VIM), specifically includes qualitative properties, called "nominal properties".

Note 5 to entry: The laboratory is to demonstrate that the simulated-use extraction is carried out under conditions that provide an appropriate representation of intended use. Product-use simulation is carried out assuming the medical device is assigned to the most stringent category possible for the duration of exposure and takes into consideration both the tissue(s) exposed and the temperature of exposure.

[SOURCE: ISO Guide 30:2015, 2.1.1 — Note 5 to entry has been added.]

3.13
stability
characteristic of a material, when stored under specified conditions, to maintain a specified property value within specified limits for a specified period of time

Note 1 to entry: See also the IUPAC Compendium of Analytical Nomenclature[5].

3.14
test sample
medical device, component or material (or a representative sample thereof, manufactured and processed by equivalent methods), or an extract or portion thereof that is subjected to biological evaluation testing

4 General requirements

When identifying hazards and estimating risk in relation to medical devices, hazards that arise from changes in the manufacturing process, or insufficient control of the manufacturing process, shall be considered in the design and preparation of test samples, as described in ISO 14971. Particular attention shall be given to material additives, unintentional base material impurities and manufacturing process residues, e.g. trace elements and cleaning and disinfection agents.

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The ISO 10993 series describes many different biological assay systems. Therefore, the individual parts shall be consulted to ascertain whether these are appropriate for specific test systems.

Experimental controls shall be used in biological evaluations carried out in order to either validate a test procedure or compare the results between materials, or both. Depending on the specifications of a particular test, either negative controls, blanks or positive controls, or all three, shall be used.

NOTE The same type of control can be applicable to different tests and can allow cross-reference to other established materials and test methods. Additional guidance on the selection of experimental controls is given in Annex A. Use of positive controls for *in vivo* testing might be affected by animal welfare regulations.

5 Reference materials (RMs)

5.1 General

RMs are established by individual laboratories. The extent of chemical, physical and biological characterization is determined by the individual laboratory. Commercially available articles may be used as RMs.

NOTE See also ISO Guide 35.

CRMs are selected for their high purity, critical characteristics, suitability for the intended purpose and general availability. The critical chemical, physical and biological characteristics shall be determined by collaborative testing in three or more laboratories and made available to the investigator by the distributor.

It is desirable for users to obtain a commitment from suppliers of RMs or CRMs stating that these materials will be available to the user for at least five years. A second but less desirable option is for the source of the RM or CRM to publish an "open formulation" for the material, i.e. publication of the source materials and details of the processing needed to ensure uniform batches of the RM.

5.2 Certification of RMs for biological safety testing

Qualification of an RM is a procedure that establishes the numerical or qualitative value of the biological response of the material under specified test conditions, ensuring reproducibility of the response either within laboratories or between laboratories, or both. The range of biological responses associated with the material shall be established through laboratory tests.

NOTE See also ISO 17034.

Suppliers of RMs shall certify the materials. The supplier determines the extent of chemical and physical characterization that is performed. The individual laboratories that use the RM shall identify the biological characterization necessary to qualify an RM for a specific test or procedure. Commercially available materials may be used as RMs, provided they are certified and qualified.

Certification of a RM is a procedure that establishes the numerical or qualitative value of the biological response of the material under the specified test conditions. This process serves to validate the testing of the material for that particular response and results in the issuance of a certificate. The biological response of the material shall be established through interlaboratory tests.

6 Use of RMs as experimental controls

RMs or CRMs shall be used in biological tests as control materials to demonstrate the suitability of a procedure to yield a reproducible response, i.e. positive or negative, or both. Any material used in this way shall be characterized with each biological test procedure for which the use of the material is desired. A material characterized and then certified for one reference test method or response, for

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example, delayed-type hypersensitivity, shall not be used as an RM for another, for example, cytotoxicity, without additional validation.

NOTE The use of an RM will facilitate the comparability of the response between laboratories and help assess reproducibility of the test performance within individual laboratories. For comparison of the biological response, it is desirable to use RMs having a range of responses, e.g. minimum, intermediate or severe.

RMs used as experimental controls shall meet the established quality assurance procedures of the manufacturer and test laboratory. They shall be identified in relation to source, manufacturer, grade and type. RMs are processed as described in Clause 8.

When RMs are used as experimental controls, they shall be in the same material class as the test sample, i.e. polymer, ceramic, metal, colloid. However, pure chemicals may be used as experimental controls for mechanistically-based test procedures, for example, genotoxicity and immune delayed-type hypersensitivity assays.

7 Test sample selection

Testing shall be performed on the final product, representative samples from the final product, materials processed in the same manner as the final product (see ISO 10993-1), or on appropriate extracts of any of these. The choice of test sample shall be justified.

NOTE In the case of materials that cure *in situ*, different test samples representative of the cured material versus the uncured state of the material might be needed.

For absorbable materials that could potentially have toxic degradants and residuals, testing of intermediate products should be considered.

The same test sample selection procedure applies when an extract is required.

8 Test sample and RM preparation

Test samples and RMs shall be handled with care to prevent contamination. Any residue from the manufacturing processes, intentional or unintentional additives or contaminants, shall be considered integral to the medical device, medical device portion or component, or representative sample.

NOTE For additional guidance on preparation, see Annex B.

— Test samples from sterilized medical devices and RMs shall be handled aseptically, if appropriate to the test procedure.

— Test samples which are clean, sterile and disinfected, shall be processed by the method recommended by the manufacturer and handled aseptically, if appropriate to the test procedure.

— The influence of the cleaning process and cleaning agent shall be considered in the selection and handling of the test sample.

Test samples from medical devices not required to be sterile in use shall be used as supplied and handled aseptically throughout the test sample preparation. If sterile test samples are required for a test procedure, e.g. for cytotoxicity testing, the effect of the sterilization or resterilization process on the test sample and RM shall be considered.

When test samples and RMs need to be cut into pieces, as described in 10.3.3, the influence of previously unexposed surfaces, e.g. lumens or cut surfaces shall be considered. Tools used for cutting medical devices into representative portions for testing shall be cleaned between uses to prevent contamination. Furthermore, care shall be taken that the tool itself doesn't contaminate the device.

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9 Selection of representative portions from a medical device

9.1 If a medical device cannot be tested as a whole, each individual material in the final product that is required to be tested shall be represented proportionally in the test sample.

— The test sample of the medical devices with surface coatings shall include both the coating material and the substrate, even if the substrate has no tissue contact.

— The test sample shall include a representative portion of the joint or seal, or both, if adhesives, radiofrequency (RF) seals or solvent seals are used in the manufacture of a portion of the medical device which comes into contact with patients.

9.2 Composite materials shall be tested as finished materials.

9.3 When different materials are present in a single medical device, the potential for synergies and interactions shall be considered in the choice of test sample.

9.4 The test sample shall be chosen to maximize the exposure of the test system to the components of a medical device that are known to have potential for a biological response.

9.5 Non-patient contacting portions of the medical device should, if possible, be excluded either physically from test sample extracts or by exclusion of the surface area in the calculation of the extraction ratio. When this is not possible, the extraction ratio shall be justified. Ensure that all contacting portions are covered by the selected extraction vehicle volume.

Clinician and user surface contact with materials other than those in common use in consumer products with a similar nature of contact, should be considered [see ISO 10993-1:2018, 5.2.2, a)].

9.6 Medical device components with different type or duration of tissue contact might need to be extracted and tested separately.

10 Preparation of extracts of samples

10.1 General

If extracts of the medical device are required for a test procedure, the extraction vehicles and conditions of extraction used shall be appropriate to the nature and use of the final product and to the purpose of the test, for example, hazard identification, risk estimation or risk assessment. The physico-chemical properties of the medical device materials, leachable substances or residues shall be considered when choosing the extraction conditions (see ISO 10993-18 and ISO/TS 10993-19). For more information on sample preparation for testing of nanomaterials or nanostructured materials, see ISO/TR 10993-22.

NOTE For additional guidance on the extraction of samples, see Annex C.

10.2 Containers for extraction

The extraction shall be performed in clean, chemically inert, closed containers with minimum dead space.

To ensure that the extraction vessels do not adulterate the extract of the test sample, the extraction vessels shall be either borosilicate glass tubes with caps having an inert liner, for example, polytetrafluoroethylene or other inert extraction vessels, as required for either specific materials or extraction procedures, or both.

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10.3 Extraction conditions and methods

10.3.1 Extraction conditions are based on common practice and are justified on the basis of providing a standardized approach that is, in many ways, an appropriate exaggeration of product use. Extraction shall be conducted under one of the following conditions (see also C.5):

a) (37 ± 1) °C for (24 ± 2) h;

b) (37 ± 1) °C for (72 ± 2) h;

c) (50 ± 2) °C for (72 ± 2) h;

d) (70 ± 2) °C for (24 ± 2) h;

e) (121 ± 2) °C for (1 ± 0,1) h.

Extraction at (37 ± 1) °C for (24 ± 2) h is acceptable for cytotoxicity testing of limited-contact medical devices. For medical devices which are in limited contact with intact skin or mucosa and which are not implanted, extraction times of less than 24 h, but not less than 4 h, are acceptable for cytotoxicity testing (see ISO 10993-5). For medical devices which are in prolonged (>24 h to 30 d) or long-term contact (>30 d), extraction times of 72 h are recommended for cytotoxicity testing because extraction for 24 h may not be sufficient to obtain an extract that represents the chemicals released beyond 24 h of device use. In such cases, all controls including the negative, positive, and the reagent (i.e. cell culture medium alone) controls shall be incubated for 72 h. However, if there are data available for the prolonged or long-term tissue-contacting devices which demonstrate that 24 h extraction is sufficient to release extractables/leachables from the device and extending the extraction time to 72 h does not result in release of additional chemicals from the device, the 24 h extraction is sufficient. For extraction in tissue culture medium with serum, extraction temperatures greater than (37 ± 1) °C can adversely impact either the chemistry of the serum or the stability of the serum, or both, and other constituents in the culture medium.

The extraction conditions described above, which have been used to provide a measure of the hazard potential for the risk estimation of the medical device or material, are based on historical precedent. Other conditions that simulate the leachables occurring during clinical use, or that provide an adequate measure of the hazard potential may be used but shall be described and justified.

Extraction is a complex process influenced by many factors e.g. time, temperature, surface-area-to-volume ratio, the extraction vehicle and the phase equilibrium of the material. For specific products, other factors can also have an influence. The effects of higher temperatures or other conditions on extraction kinetics and the identity of the extraction vehicle(s) should be considered carefully if exaggerated extraction is used.

The phase equilibrium of a material during extraction controls the relative amounts of amorphous and crystalline phases present. For the amorphous phase, the glass transition temperature, T_g, dictates the polymer chain mobility and the diffusion rate in the phase. Usually, at temperatures higher than T_g, the diffusion rate is considerably higher compared with that below T_g. The diffusion rate is lowest in the crystalline phase. The extraction conditions should not alter the phase equilibrium of the material. Phase alteration can affect the amount and type of extractables.

For example, two possibilities exist when elevated temperatures are used:

— the energy of the increased temperature can cause either increased cross-linking or polymerization of the polymer, or both, and, therefore, decrease the amount of free monomer that is available to migrate from the polymer;

— the increased temperature can cause degradation products to form that are not typically found in the finished medical device under conditions of use.

10.3.2 For materials that are intended to dissolve or absorb under conditions of use, the selection of extraction conditions described in 10.3 might need to consider the thermal properties (e.g. glass transition

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temperature of polymers) of device materials of construction and the relevant clinical use conditions. For these materials, the extracts prepared based on 10.3 may have changes either in osmolarities or in the pH that may not be appropriate for the test system to be dosed. Any adjustment applied to the extracts prior to biocompatibility testing should be justified.

NOTE For more information on sample preparation for testing of absorbable medical devices, see ISO 10993-3, ISO 10993-6, ISO 10993-13, ISO 10993-14, ISO 10993-15, ISO 10993-18 and ISO/TS 37137-1.

Perform extraction using the appropriate extraction vehicle and the conditions of time and temperature to simulate exaggerated exposure wherever possible. Complete dissolution using the extraction vehicles and conditions recommended by this document can be appropriate, if justified; however, caution should be taken since complete device dissolution can create challenges for subsequent biological testing (e.g. difficulty in dosing animals with neat test extract if viscosity is increased, difficulty in interpreting *in vitro* cell-based test failure data in case of increased osmolality or pH change). For chemical characterization and hazard assessment of potential intermediate degradants that cannot otherwise be evaluated under these testing conditions, see ISO 10993-17 and ISO 10993-18.

10.3.3 The standard surface area can be used to determine the volume of extraction vehicle needed. This area includes the combined area of all tissue contacting surfaces of the sample and ignores the contribution of indeterminate surface irregularities. When the surface area cannot be determined due to configuration of the sample, a mass/volume of extracting fluid shall be used. See Table 1.

Other surface-area-to-volume extraction ratios, for example, those related to evaluation of porous materials can be used if they simulate the conditions during clinical use or result in a measure of the hazard potential (ISO/TS 10993-19 describes the tests for the morphological characterization of porous materials.)

Materials may be cut into small pieces before the extraction to enhance submersion in the extract media, except when otherwise inappropriate (see 10.3.4). For example, for polymers, pieces approximately 10 mm × 50 mm or 5 mm × 25 mm are appropriate.

Table 1 — Standard surface areas and extract liquid volumes

Thickness[a] mm	**Extraction ratio** (surface area or mass/volume) ±10 %	**Examples of forms of materials**
<0,5	6 cm^2/ml	film, sheet, tubing wall
0,5 to 1,0	3 cm^2/ml	tubing wall, slab, small moulded items
>1,0	3 cm^2/ml	larger moulded items
irregularly shaped solid devices	0,2 g/ml	powder, pellets, foam, non-absorbent moulded items, porous high-density materials
irregularly shaped porous devices (low-density materials)	0,1 g/ml	membranes, textiles

[a] If the medical device includes multiple tissue contacting components with different thicknesses, the extraction ratio should be justified. One way to do this is to base the ratio on the thinnest material layer of that component.

NOTE While there are no standardized methods available at present for testing solvent absorbing polymer materials (e.g. absorbents and hydrocolloids), a suggested protocol is as follows:

— determine the volume of extraction vehicle that each 0,1 g or 1,0 cm^2 of material absorbs;

— then, in performing the material extraction, add this additional volume to each 0,1 g or 1,0 cm^2 in an extraction mixture.

10.3.4 Elastomers, coated materials, composites, laminates shall be tested intact whenever possible because of potential differences in extraction characteristics between the intact and cut surfaces. For materials such as elastomers and latex, it may be appropriate to use an extraction ratio of 1,25 cm^2/ml, if justified.

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10.3.5 Extraction using both polar and non-polar extraction vehicles shall be performed. In some device specific circumstances, it may be appropriate to extract in only one extraction vehicle, either polar or non-polar. Where extraction is in one vehicle only, rationale shall be provided. The following are examples of extraction vehicles:

EXAMPLE 1 For polar extraction vehicle are water, physiological saline, culture media without serum.

EXAMPLE 2 For non-polar extraction vehicle are freshly refined vegetable oil (e.g. cottonseed or sesame oil) of the quality defined in various pharmacopoeias.

NOTE 1 Additional or alternative extraction vehicles are ethanol/water, ethanol/saline, polyethylene glycol 400 (diluted to a physiological osmotic pressure), dimethyl-sulfoxide and culture media with serum can be considered, if justified.

NOTE 2 Other extraction vehicles appropriate to the nature and use of the medical device or to the methods for hazard identification can also be used if their effects on the material and the biological system are known (see Annex D).

NOTE 3 An example of a device only requiring a single (polar) extract is a syringe pre-filled only with saline.

The use of a culture medium with serum is preferred for extraction in testing for cytotoxicity because of its ability to support cellular growth as well as extract both polar and non-polar substances

10.3.6 Extractions should be performed with continuous, mechanical agitation or circulation. When extraction under static conditions or intermittent agitation is considered to be appropriate, the method shall be justified, specified and reported. Care should be taken to not to damage the sample or the container.

10.3.7 Liquid extracts should, if possible, be used immediately after preparation to prevent sorption on to the extraction container or other changes in composition. If an extract is stored for longer than 24 h (e.g. refrigerated at 2 °C to 8 °C) then the stability and the homogeneity of the extract under the storage conditions shall be verified.

10.3.8 Extract pH shall not be adjusted unless a rationale is provided.

10.3.9 The extract shall not routinely be processed by filtration, centrifugation or other methods to remove suspended particulates. However, if such processing is necessary, the rationale shall be documented.

10.3.10 For materials or medical devices not expected to dissolve or absorb under conditions of use, any solvents used in the extraction of a polymeric material or medical device shall not cause dissolution of the polymer formulation. No softening or deformation of the polymeric material shall occur in the presence of the volatile solvent. Selected solvents should not compromise (e.g. severe swelling, particulate generation and degradation) the medical material or devices. The solvent shall be removed (prior to use in a bioassay) to the extent that any residues do not adversely affect the biological assay (e.g. cause protein denaturation or skin irritation). For materials or medical devices expected to dissolve or absorb under conditions of use, see 10.3.2, 10.3.11, and C.7.

10.3.11 For solution and soluble materials, the standard extraction methods used for insoluble materials might be inappropriate. The following guidance should be considered in addition to information contained in Table 1.

a) Factors such as test system compatibility, route of administration and extent of dissolution or degradation should be considered in the final preparation for testing. Use an appropriate vehicle and conditions to simulate exaggerated exposure wherever possible. A pre-test can help to determine appropriate conditions.

b) If the material completely dissolves, in a vehicle or diluent that is compatible with the material and the test system, the resulting solution can be evaluated neat, provided the solution properties

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are also compatible with the test system, e.g. pH, osmolarity, solute concentrations. If the resulting solution contains all the constituents of the material, a second vehicle would not be needed.

c) If the material is an aqueous solution and used in this form, it shall be tested directly and not extracted, provided the solution properties are compatible with the test system [see also a) and b) above].

d) OECD Guidelines for the Testing of Chemicals[16], or similar chemical testing standards, can be used as guidance in determining maximum concentrations of test substances used for specific test methods.

10.3.12 Where fluids circulate through the medical device under normal conditions of use, e.g. extra-corporeal devices, extraction via re-circulation can be used. When possible, one or more of the conditions shall be exaggerated, for example, temperature, time, volume, flow rate. The rationale for the extraction chosen shall be reported.

10.4 Extraction conditions for materials that polymerize *in situ*

In the case of products that polymerize *in situ*, the samples to be tested shall represent the intended clinical conditions of use in order to provide information on the potential toxicity of the reacting components in the polymer during the curing process. Test extracts prepared at different times, if appropriate, shall be based on the kinetics of polymerization after mixing the components, including an extract prepared at the expected cure time. Testing of the material after curing shall be justified. Solvent selection should be justified with rationale that it is unlikely to impact the polymerization process and chemistry of the extractables.

Where extracts are used in the test methods for evaluation of materials that cure *in situ*, initiation of the extraction shall occur from the point in the cure at which the material is placed *in situ*.

For test methods that use these materials directly, for example, direct contact or agar overlay cytotoxicity, implantation, some genotoxicity tests, and direct contact haemolysis, the material shall be used as in clinical use, with *in situ* cure in the test system.

NOTE Modification of the clinical delivery system can be appropriate so that the designated size or weight of the material is delivered for testing.

11 Records

Records of the sample and its preparation shall include, but not be limited to:

— Type and, if known, composition of material, source of material, medical device, medical device portion or component (a written description, drawing, photograph or other methods can achieve all or part of this requirement):

  — lot or batch number, where appropriate;

  — description of processing, cleaning or sterilization treatments, if appropriate;

  — extraction techniques, as appropriate, including documentation of extraction vehicle, extraction ratios, conditions for extraction, means of agitation, as well as any deviations from the conditions specified in this document, such as filtration of the extract or extraction media. The condition of the test extract (e.g. color, clarity, presence of any particulates) shall also be described, and photographed if applicable.

— Documents shall be provided (e.g. schematic or photo) of the medical device components that are sampled, and those that are not sampled.

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Annex A
(informative)

Experimental controls

A.1 The materials listed in the following paragraphs might meet the criteria for an appropriate experimental control in selected tests. It is the responsibility of the investigator to make the appropriate choices (see Table A.1).

Table A.1 — Examples of available RMs and controls

Test	Negative control[a]	Positive control[a]
implantation	PE	PVC-org. Sn
	silicone	SPU-ZDEC
	alumina	natural rubber latex
	stainless steel	
cytotoxicity	PE	PVC-org. Sn
		SPU-ZDEC
		SPU-ZBEC
		natural rubber latex
		polyurethane
haemolysis	HDPE	Y-3
irritation and haemolysis		Y-4

NOTE Information on RMs and controls is supplied only for those tests in the ISO 10993 series which do not call for specific RMs or controls.

[a] Abbreviations on this table refer to specific materials available from sources designated in A.2 and A.3.

A.2 Materials that have been used as negative controls or RMs are, for example, high-density polyethylene[1)] low-density polyethylene[2)], silica-free polydimethylsiloxane[3),4)], polypropylene[5)], aluminium oxide ceramic rods, stainless steel and commercially pure (cp) titanium alloys.

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1) High-density polyethylene (negative control plastic RS), HDPE film, RM-C and HDPE rod, RM-E (are) an example(s) of a suitable product(s) available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

2) PE 140 tubing: RAUMEDIC AG, is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

3) Biomaterials Program, Devices and Technology Branch, National Heart, Lung and Blood Institute, NIH Building, 7550 Wisconsin Ave., Bethesda, MD 20892, USA.

4) SIK 8363 tubing: RAUMEDIC AG is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5) PP 146 tubing: RAUMEDIC AG is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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A.3 Materials that have been used as positive controls are, for example, polyvinylchloride-containing organotin additives[6)], segmented polyurethane rod[7)] or films containing zinc diethyldithiocarbamate (SPU-ZDEC)[8)] or dibutyldithiocarbamate (SPU-ZDBC), and PVC with a known irritant substance (Genapol X-080)[9)], as well as certain latex formulations, solutions of zinc salts, and copper. Substances that have been used as positive controls for extract samples are dilutions of phenol and water.

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6) Positive Control Material, code 499-300-000-000: Portex Limited is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

7) Polyurethane rod — ZDEC: RM-F, is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

8) Polyurethane film — ZDEC: RM-A and Polyurethane film — ZDBC (SPU-ZDBC): RM-B are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

9) Genapol X-080 — Y-3 Pellet and Genapol X-080 — Y-4 Pellet are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

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Annex B
(informative)

General principles on, and practices of, test sample preparation and sample selection

The material used in the biological assay should be representative of the composition and surface characteristics of the final product and of the processes used in its manufacture (see Clause 7).

Documentation of the composition of plastic and rubber materials should include identification of the resin, polymer and any additives. The formulation description should specify the history of the material, for example, information on the thermal processing and whether it is virgin or reground and, if reground, the specification for the maximum allowable regrind.

Materials that may be re-sterilized by the same or alternative methods should be tested after treatment by the multiple sterilizations.

For example, a material that is sterilized by radiation and re-sterilized by ethylene oxide should be tested in the final product after radiation and ethylene oxide sterilization.

If a “worst case” exposure can be identified with appropriate justification, testing may be performed after exposure to this treatment.

Ideally, all biological tests which use a material cut from a medical device, the medical device component itself as the test material, or an extract prepared from either, should be performed with the surface of the material exposed to the test system’s biological environment. An alternative method to cutting the surface is the fabrication of miniatures of the medical device, using the same processes (e.g. extrusion, dipping), temperatures, time, atmosphere, release agents, and processes such as annealing, curing, cleaning and sterilization that were used to manufacture the medical device. This assists in evaluating any effects related to surface area, surface characteristics, concentration of leachables and the material’s surface and shape.

Metals used in biological tests should be from the same stock material used to fabricate the medical device and prepared using the same machining, grinding, polishing, cleaning, passivation, surface treatment and sterilization as used in the manufacture of the final product.

Ceramic materials used in biological tests should be manufactured from the same powder stock using the same casting, investing, moulding, sintering, surface finishing and sterilization processes as used to manufacture the medical device.

Medical devices utilizing animal tissues or their derivatives, and which are treated with a fixative, should be tested after they have been preserved under the manufacturer's maximum and minimum allowable fixation times to allow for varying penetration of the fixative.

Instead of extracting metallic materials and then applying the extract to the test systems, testing the solutions at various concentrations of the appropriate salt for the specific metal(s) identified in the medical device should be considered in order to identify the hazard of the specific metal ion(s) and to determine its highest non-effect level(s).

NOTE This principle is also applicable to organic materials when chemicals in the medical device are identified.

Extraction conditions for implant materials that can cause particle generation *in vivo* during clinical use should be considered in the design of tests on the materials. The effect of extraction procedures should be considered when designing tests for materials where particulates are generated by the extraction conditions.

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The amount of material, and surface area thereof, should be appropriate to the biological and physical constraints of the test system. In practice, the use of a standard sample size for a specific assay is recommended.

Users of this document are directed to the discussion of “proper use” and “misuse” of CRMs in the introduction to ISO Guide 33. This discussion points out areas of both potential under- and over-utilization of RMs and CRMs. Users of this document should also note that the use of calibration materials to evaluate the biological response of materials under investigation within a single laboratory is acceptable.

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Annex C
(informative)

Principles of test sample extraction

WARNING — Application of the test methods of this document to medical device materials comprising proteins shall be made with great care to ensure that the extraction procedure has not altered the biological properties of the materials being extracted.

C.1 Extraction of a medical device is carried out to provide a suitable test sample for the biological evaluation testing.

If extracts of the medical device are prepared, the extraction vehicle and conditions of extraction used should be appropriate to the nature and use of the final product, as well as to the predictability (e.g. test purpose, rationale, sensitivity) of the test method. Extraction conditions and application of the extract to test systems should therefore ideally reflect not only actual conditions of use of the products but also the purpose and predictability of the tests.

Under normal conditions of use where fluids circulate through the medical device (e.g. extra-corporeal devices), if a vertical standard exists, it should be consulted for the appropriate extraction techniques.

Biological tests are carried out to identify hazards and estimate the risks of the hazards occurring in exaggerated use or in actual conditions of use, or both.

C.2 This document assumes that the amount of extractable substance(s) is (are) related to the period of extraction, the temperature, the ratio of the surface area of the material to the volume of the extraction vehicle, and the nature of the extraction vehicle.

C.3 The period of extraction should be sufficient to maximize the amount of material extracted. In practice, use of these standard conditions of time and temperature for extraction are recommended in lieu of other unvalidated or non-standard conditions.

C.4 Extraction temperatures can vary for the different materials to be tested. Extraction should not cause significant degradation of the material, unless the material is intended to dissolve or be resorbed during use (see 10.3.2). The extraction temperature is dependent upon the physico-chemical characteristics of the medical device material(s). The extraction temperature chosen for polymers, for example, should be below the glass transition temperature. If the glass transition temperature is below the use temperature, the extraction temperature should be below the melting temperature. Recommended conditions are given in 10.3.1.

The following examples are presented to illustrate the interpretation of 10.3.1.

— Materials that have a melting or softening point of less than (121 ± 2) °C can be extracted at a standard temperature below the melting point (e.g. very-low-density polyethylene).

— Materials that undergo hydrolysis can be extracted at a temperature that minimizes the amount of hydrolysis [e.g. polyamides are extracted at (50 ± 2) °C].

— Materials that are processed by steam sterilization and contain a liquid during storage can be extracted at (121 ± 2) °C (e.g. pre-filled dialysers).

Material should be extracted at temperatures which provide the maximum extractables without material degradation [e.g. fixed tissues can be extracted at (37 ± 1) °C whereas ceramic implants can be extracted at (121 ± 2) °C].

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For extraction of absorbable materials, see ISO/TS 37137-1.

Where absorbable degradation products are known to affect the pH of the test system, it may be appropriate to adjust the pH of the extract to evaluate if maintenance of pH impacts test results. The accompanying biocompatibility risk assessment should include justification for the pH adjustment, the initial extract pH, the final extract pH and the pH adjustment process along with a discussion of the clinical implications of the pH related hypothesis.

NOTE pH is seldom affected by dilution of extracts, because the buffering capacity of (physiological) solutions used for extraction is low. Dilution can effectively reduce osmolality however pH often needs to be adjusted. Since pH adjustment typically leads to higher osmolality it can be done prior to osmolality adjustments.

For polymeric absorbable materials, extraction above *in vivo* temperatures that are near or above the glass transition temperature may lead to changes in the polymer properties (e.g. degradation) that are not representative of clinical conditions and should be avoided. For absorbable metals, elevated extraction temperatures can introduce new and potentially unrepresentative corrosion mechanisms. Thus, for most absorbable polymers and metals, the standard extraction temperatures listed in 10.3.1 may not be applicable. When evaluating absorbable devices, extraction of partially degraded materials and their related intermediate degradation products can be considered.

C.5 The ratio of the surface area of the medical device to the volume of extraction vehicle or solvent should be sufficient to:

— attain the maximum amount of extractable substance(s) in an appropriate dosage volume for biological testing (i.e. dosage volume within physiological limits);

— demonstrate the hazard potential of using the medical device in humans;

— cover the material in the solvent volume.

In practice, the use of a standard area and solvent volume is recommended (described in 10.3.3) in lieu of device-specific parameters. Some test methods require concentration of extracts to increase the sensitivity of the test.

NOTE Concentration of extracts can result in the loss of volatile materials such as ethylene oxide.

C.6 The solvent(s) selected as the extraction vehicle should:

— be suitable for use in the specific biological test systems;

— simulate the extraction which occurs during clinical use of the medical device;

— maximize the amount of extractable substances

In practice, the use of standard polar and non-polar solvents is recommended. Subclause 10.3.5 recommends these in lieu of device-specific solvents.

By standardizing the parameters given in C.5 and C.6, data obtained from biological tests of medical devices for other types of application, for example, for the estimation of risk and to develop standardized databases, can be used.

C.7 For materials that dissolve or absorb in the body:

— follow the conditions given in Table 1;

— follow the temperature and times given in 10.3.1;

— follow 10.3.9 regarding filtration or centrifugation.

C.8 A standard prescription cannot be constructed to address the specialized needs of preparing extracts of polymerized *in situ* products. The individual components, time to polymerization, intended

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use and the extraction vehicles should be taken into consideration when developing a relevant extract. Language should include the recommendation that the polymerization kinetics be used in the design of the correct methodology to develop a relevant extract for testing. The uncured components should be considered when selecting an appropriate solvent for extracting the sample.

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Annex D
(informative)

Exhaustive extraction of polymeric materials for biological evaluation

D.1 General

Polymeric materials often contain a small amount of low-molecular-weight chemical substances (LMWCs) such as catalysts, processing aids, or other additives[19], residual monomers, or oligomers. A major toxicological concern during the biological evaluation of polymeric materials is the toxicity of any leachables that can migrate from the polymer to the human body during use. This concept is derived from the agreement of the OECD polymer group regarding health concerns and the exemption of polymers from testing (see Reference [15]). The report pointed out the following four parameters, which are important for judging the health risk of polymers:

— number-average molecular weight of the polymer;

— content of low-molecular-weight chemical species;

— presence of reactive functional groups (see Reference [17]);

— presence of bioavailable metals.

NOTE LMWCs are defined as low-molecular-weight chemical substances with a molecular weight not exceeding 1 000 Da.

When performing a biological evaluation of polymeric medical devices, extraction practices are needed for preparing test samples (except in the case of implantation, direct-contact haemocompatibility and direct contact cytotoxicity tests). Annex C points out that exaggerated extraction is appropriate for hazard identification. Exhaustive extraction using organic solvents is recommended by some regulatory agencies for hazard identification of polymeric medical devices, especially those for long-term use.

The rationale for this practice is based on the following considerations:

— for hazard identification, it is recommended that, as far as possible, the total amount of extractable substance be obtained from the polymeric medical device and applied appropriately to each test system;

— several papers showed that body fluids such as serum have a potency comparable with those of organic solvents, such as ethanol and methanol, when extracting chemicals (phthalates, 4,4'-methylenedianiline, bisphenol-A) from polymeric materials[21][29][22];

— organic solvent extraction is routinely used in the field of polymer analysis for either identifying or quantifying LMWCs of polymers, or both.

D.2 Points to be considered for exhaustive extraction of polymeric medical devices for biological testing

There are a wide variety of polymers and their additives. Therefore, no single solvent is universally applicable to exhaustive extraction for all polymers. For example, acetone-chloroform mixture was chosen for the exhaustive extraction of rubber samples for a round robin study by ISO/TC 194 that compared the efficacy of traditional and exhaustive extraction procedures for preparing the test extracts for sensitization testing of rubber samples. However, this solvent mixture is not generally applicable for exhaustive extraction of all polymeric materials and it is recommended that appropriate

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solvents for exhaustive extraction of polymeric medical devices be chosen on a case-by-case basis (see Reference [25]).

The following are important points to be considered for exhaustive extraction of polymeric medical devices for biological testing.

— For biocompatibility evaluation of medical devices, the extraction of the medical device in both polar and non-polar solvents should be considered for hazard identification purposes. The selected solvent(s) should not compromise (e.g. severe swelling, particulate generation and degradation) the medical devices. Also, a rationale should be provided for the chosen extraction temperature.

— For exhaustive extractions, the duration of the extraction cannot be prescribed in advance but can be established in the following manner. A series of successive extractions is carried out by extracting the test sample in the selected solvent for a fixed period (e.g. 24 h), then replacing the extract with fresh solvent and extracting the test sample for another fixed period. This process is repeated until the level of the residue for *n*th successive extraction is one tenth (0,1) of the level in the first extraction, so the extraction can be deemed complete or exhaustive. Gravimetric analysis or other analytical methods can be used to determine if the exhaustive endpoint is achieved.

— The extract residue is obtained by evaporating solvents or another drying process following exhaustive extraction. Data should be provided to demonstrate that the evaporation or drying process and the subsequent reconstitution step (as described below) do not cause loss of either volatiles or semi-volatile compounds, or both, from the extract.

— The reconstitution of the extract residue after exhaustive extraction is a critical step of sample preparation for biological testing. Typically, the extraction solvents for exhaustive extraction are different from the solvents or media used for biocompatibility testing. If the exhaustive extraction is conducted in organic solvents, the obtained extractable/leachable of the polymeric medical device or samples are hydrophobic in nature. The extract residue after solvent evaporation usually has miscibility issues in the cell culture medium and might not be completely soluble in the solvent used for biocompatibility testing. Consequently, the test system dosed with such reconstituted solution of the extract residue can be exposed to a significantly lower amount of extractables/leachables. For this scenario, the biological testing results could be justified by demonstrating that the final extract dose following exhaustive extraction (including sample drying and reconstitution) contains the same or more types and quantities of the extractables/leachables as compared to the extractables/leachables that would be present from exaggerated biological extraction conditions (see 10.3 and Annex C).

— In some cases, dilution of the reconstituted residue solution can be necessary to avoid incompatibility with the test system. For example, DMSO is cytotoxic to cells at greater than 1 % concentration. For cell-based genotoxicity assays such as the mouse lymphoma assay or the *in vitro* chromosomal aberration assay (according to ISO 10993-3), a reconstituted residue solution in DMSO needs to be diluted 100 times before exposing the cells to this solution. If dilution of the reconstituted residue solution is performed before biological testing, the impact of this dilution needs to be justified by assessing the amounts of extractables/leachables being presented to the test system. In addition, the impact of the dilution on test sensitivity should be addressed.

— The final test extract prepared by reconstitution of the residue should not cause any interference with biological testing.

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