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Third edition
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Biological evaluation of medical devices —

Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity

Évaluation biologique des dispositifs médicaux —

Partie 3: Essais concernant la génotoxicité, la cancérogénicité et la toxicité sur la reproduction

Reference number
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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. 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. www.iso.org/patents

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

The committee responsible for this document is ISO/TC 194.

This third edition of ISO 10993-3 cancels and replaces the second edition (ISO 10993-3:2003), which has been technically revised.

The major technical changes are the following:

a) test strategy changed by inclusion of a *in vivo* test and a follow-up evaluation;

b) new Annex A " Guidance on selecting an appropriate sample preparation procedure in genotoxicity testing" included;

c) Inclusion of further *in vitro* and *in vivo* test for evaluating the genotoxic potential of medical devices;

d) new Annex B "Flowchart for follow-up evaluation" included;

e) Annex E changed to "Considerations for carcinogenicity studies performed as implantation studies" and made normative;

f) new Annex F "*In vitro* tests for embryo toxicity" included.

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]

The following part is under preparation:

— *Part 33: Supplement to* ISO 10993-3:— *Guidance on tests to evaluate genotoxicity* [Technical Report]

The following definitions apply in understanding how to implement an ISO International Standard and other normative ISO deliverables (TS, PAS, IWA):

— "shall" indicates a requirement;

— "should" indicates a recommendation;

— "may" is used to indicate that something is permitted;

— "can" is used to indicate that something is possible, for example, that an organization or individual is able to do something.

ISO/IEC Directives, Part 2 (sixth edition, 2011), 3.3.1, defines a requirement as an "expression in the content of a document conveying criteria to be fulfilled if compliance with the document is to be claimed and from which no deviation is permitted."

ISO/IEC Directives, Part 2 (sixth edition, 2011), 3.3.2, defines a recommendation as an "expression in the content of a document conveying that among several possibilities one is recommended as particularly suitable, without mentioning or excluding others, or that a certain course of action is preferred but not necessarily required, or that (in the negative form) a certain possibility or course of action is deprecated but not prohibited."

Introduction

The basis for biological evaluation of medical devices is often empirical and driven by the relevant concerns for human safety. The risk of serious and irreversible effects, such as cancer or second generation abnormalities, is of particular public concern. It is inherent in the provision of safe medical devices that such risks be minimised to the greatest extent feasible. The assessment of mutagenic, carcinogenic and reproductive hazards is an essential component of the control of these risks. Not all test methods for the assessment of genotoxicity, carcinogenicity or reproductive toxicity are equally well developed, nor is their validity well established for the testing of medical devices.

Significant issues with test sample size and preparation, scientific understanding of disease processes and test validation can be cited as limitations of available methods. For example, the biological significance of solid state carcinogenesis is poorly understood. It is expected that on-going scientific and medical advances will improve our understanding of and approaches to these important toxicological effects. At the time this document was prepared, the test methods proposed were those most acceptable. Scientifically sound alternatives to the proposed testing may be acceptable insofar as they address relevant matters of safety assessment.

In the selection of tests needed to evaluate a particular medical device, there is no substitute for a careful assessment of expected human uses and potential interactions of the medical device with various biological systems. These considerations will be particularly important in such areas as reproductive and developmental toxicology.

This part of ISO 10993 presents test methods for the detection of specific biological hazards, and strategies for the selection of tests, where appropriate, that will assist in hazard identification. Testing is not always necessary or helpful in managing toxicological risks associated with exposure to medical device materials but, where it is appropriate, it is important that maximum test sensitivity is achieved.

In view of the multitude of possible outcomes and the importance of factors such as extent of exposure, species differences and mechanical or physical considerations, risk assessment have to be performed on a case-by-case basis.

Biological evaluation of medical devices —

Part 3:
Tests for genotoxicity, carcinogenicity and reproductive toxicity

1 Scope

This part of ISO 10993 specifies strategies for risk estimation, selection of hazard identification tests and risk management, with respect to the possibility of the following potentially irreversible biological effects arising as a result of exposure to medical devices:

— genotoxicity;

— carcinogenicity;

— reproductive and developmental toxicity.

This part of ISO 10993 is applicable when the need to evaluate a medical device for potential genotoxicity, carcinogenicity, or reproductive toxicity has been established.

NOTE Guidance on selection of tests is provided in ISO 10993-1.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 process*

ISO 10993-2, *Biological evaluation of medical devices — Part 2: Animal welfare requirements*

ISO 10993-6, *Biological evaluation of medical devices — Part 6: Tests for local effects after implantation*

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

ISO 10993-18, *Biological evaluation of medical devices — Part 18: Chemical characterization of materials*

OECD 414, *Prenatal Development Toxicity Study*

OECD 415, *One-Generation Reproduction Toxicity Study*

OECD 416, *Two-generation Reproduction Toxicity*

OECD 421, *Reproduction/Developmental Toxicity Screening Test*

OECD 451, *Carcinogenicity Studies*

OECD 453, *Combined Chronic Toxicity/Carcinogenicity Studies*

OECD 471, *Bacterial Reverse Mutation Test*

OECD 473, *In vitro Mammalian Chromosome Aberration Test*

OECD 476, *In vitro Mammalian Cell Gene Mutation Test*

OECD 487, *In Vitro Mammalian Cell Micronucleus Test*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 10993-1, ISO 10993-12 and the following apply.

3.1
carcinogenicity test
test to determine the carcinogenic potential of medical devices, materials, and/or extracts using multiple exposures for a major portion of the life span of the test animal

3.2
energy-depositing medical device
device intended to exert its therapeutic or diagnostic effect by the delivery of electromagnetic radiation, ionising radiation or ultrasound

Note 1 to entry: This does not include medical devices that deliver simple electrical current, such as electrocautery medical devices, pacemakers or functional electrical stimulators.

3.3
genotoxicity test
test using mammalian or non-mammalian cells, bacteria, yeasts, fungi or whole animals to determine whether gene mutations, changes in chromosome structure, or other DNA or gene changes are caused by the test samples

3.4
maximum tolerated dose
MTD
maximum dose that a test animal can tolerate without any adverse effects

3.5
reproductive and developmental toxicity test
test to evaluate the potential effects of test samples on reproductive function, embryonic morphology (teratogenicity), and prenatal and early postnatal development

3.6
test sample preparation
residual, extractables, leachables or biodegradable device materials that are resuspended in a vehicle compatible with the test system

4 Requirements for test strategies

4.1 General

ISO 10993-1 indicates circumstances where the potential for genotoxicity, carcinogenicity and reproductive toxicity is a relevant hazard for consideration in an overall biological safety evaluation. Testing to investigate these hazards shall be justified on the basis of a risk assessment. In determining if genotoxicity, carcinogenicity and reproductive toxicity testing of the device is warranted an assessment of risk shall address the following factors

— an analysis of the chemical constituents of the device material(s), including manufacturing process residues and degradation products or metabolites, to identify causes of concern on the basis of structure-activity relationships or previous demonstration of relevant toxicity in the chemical class,

— the mechanistic basis of the toxic response under consideration, if available,

— existing information relevant to the genotoxicity, carcinogenicity and reproductive toxicity evaluation of the medical device,

— the extent of previous use of comparable materials in relevant applications,

— consideration of residuals from the final finished device with respect to how well they are characterized and their potential biological activity (e.g. structure-activity relationships, or previous demonstration of relevant outcomes).

— exposure route,

— patient population,

— extent and duration of localized (at the site of implantation or use) and systemic exposure,

— the anticipated impact of test results (or lack of testing) on risk management judgements, and

— changes in the type or amount of residuals that the patient will be exposed to, either through an increase in device exposure, or an increase in devices size when compared to an equivalent device.

Commonly used risk assessment tools (e.g. TTC) may be helpful in evaluating these factors.

Where an analysis of the composition of device materials reveals the presence of chemical constituents that are of concern but for which inadequate toxicity data are available, consideration shall be given to testing individual chemical. Individual chemicals shall be tested in preference to compounded materials or extracts, where this would improve the risk estimate. Where testing of a device material is indicated testing shall be conducted on the final product (including sterilization if applicable), or representatives from the final products, or materials processed in the same manner as the final product (including sterilization if applicable). The decision to test, and the nature of the test sample, shall be justified and documented.

Testing may be warranted for additional states of the device such as, wear debris generated from the device or materials that cure *in situ* (e.g. cements, adhesives and pre-polymer mixtures) unless toxicological risk assessment determines no cause for concern from additional device/material states. For guidance on *in situ* curing devices see ISO 10993-12.

4.2 Additional requirements for carcinogenicity testing

For carcinogenicity testing, in addition to 4.1, the following factors shall be addressed:

— physical characteristics (e.g. particle size and shape, pore size, surface continuity, surface condition, device thickness),

— results from genotoxicity, implantation and other studies.

4.3 Additional requirements for reproductive toxicity testing

For reproductive testing, in addition to 4.1, the total direct or indirect cumulative contact duration with reproductive tissue, the embryo/foetus or the germ cells shall be addressed.

Any information from published literature on the effect of device materials on male/female reproductive organs or from subacute/chronic study on the histopathology of reproductive system should also form the basis before a full scale reproductive toxicity testing is performed.

5 Genotoxicity tests

5.1 General

Before a decision to perform a genotoxicity test is made, ISO 10993-1 shall be taken into account. The rationale for a test programme, taking into consideration all relevant factors given in 4.1 to 4.3, shall be justified and documented.

Genotoxicity tests are designed to detect the two major classes of genetic damage:

— Gene mutations (point mutations);

— Chromosomal damage [structural aberrations such as translocations, small or large deletions and insertions, and numerical chromosomal aberrations (aneuploidy)].

5.2 Test strategy

5.2.1 General

No single test is capable of detecting all relevant genotoxic agents. Therefore, the usual approach is to conduct a battery of *in vitro* and under certain circumstances also *in vivo* tests.

Bacterial reverse mutation assays have been shown to detect relevant genetic changes produced by the majority of genotoxic carcinogens detected by rodent assays. Certain classes of genotoxin, e.g. alkyl halides, are not detected.

The potential of test materials to produce DNA damage in bacterial systems might not be relevant to their likely effects in eukaryotic cells, and therefore, testing in mammalian cell test systems shall be performed unless otherwise justified. Several mammalian cell systems are in use: systems that detect gross chromosomal damage (*in vitro* tests for structural and numerical chromosomal aberrations), systems that detect primarily gene mutations (HPRT mutation assay), and a system that detects gene mutations and clastogenic effects [mouse lymphoma thymidine kinase (tk) assay with both colony number and size determination]. *In vitro* tests for chromosomal damage and the mouse lymphoma tk assay yield results that are equivalent. Results from both tests have a relatively high level of congruence for compounds that are regarded as genotoxic but yield negative results in the bacterial reverse mutation assay. Therefore, the chromosome aberration test and the mouse lymphoma tk assay are currently considered equally acceptable when either is used with the bacterial reverse mutation assay in a standard battery for genotoxicity testing.

5.2.2 Test battery

When genotoxicity testing is performed, the test battery shall include

a) a test for gene mutations in bacteria (OECD 471), modified for medical devices to allow, for example, testing with extracts from devices, see ISO/TR 10993-33:—, Clause 6, and either

b) an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells (OECD 473), modified for medical devices, see ISO/TR 10993-33:—, Clause 7, or

c) an *in vitro* mouse lymphoma tk assay (OECD 476), modified for medical devices, including detection of small (slow growing) and large colonies, see ISO/TR 10993-33:—, Clause 9, or

d) an *in vitro* mammalian cell micronucleus test for chromosomal damage and aneugenicity (OECD 487), modified for medical devices, see ISO/TR 10993-33:—, Clause 8.

When additional relevant factors (such as genotoxic mechanism and pharmacokinetics) that can influence the genotoxic activity of a compound, need to be considered an *in vivo* test may be performed if justified. An *in vivo* test for chromosomal damage in rodent haematopoietic cells could be either an analysis of chromosomal aberrations in bone marrow cells or an analysis of micronuclei in bone marrow

or peripheral blood erythrocytes [see ISO/TR 10993-33:—, Clause 10 (OECD 474) or ISO/TR 10993-33:—, Clause 11 (OECD 475)].

Where applicable, the *in vivo* test for chromosomal damage in rodent haemapoietic cells shall be performed using two extracts (see ISO 10993-12 or Annex A). The preferred application route of polar vehicles is intravenously. The preferred application route for the non-polar vehicles is intraperitoneally.

An *in vivo* assay is not necessary, if the user can demonstrate that the quantities of extractables from the test article are less than the amount of material that would induce a positive response with a potent well-characterized *in vivo* micronucleus genotoxin.

An example is cisplatin (CAS no. 15663-27-1), which was shown a positive response at 0,3 mg/kg, see Reference.[35]

5.2.3 Follow-up evaluation

If genotoxicity testing is performed in accordance with 5.2.2 and if the results of the two *in vitro* tests are negative, further genotoxicity testing in animals is unnecessary.

If any test is positive, the following step-wise procedure is applicable (see also Annex B).

Step 1: Identification of confounding factors in results from the initial set of genotoxicity tests, if available.

a) Identification of confounding factors (e.g. non-physiological conditions, interaction of test article with culture medium, auto-oxidation and cytotoxicity).

b) Identification of metabolic effects (e.g. nature of the exogenous metabolic system, nature of the metabolic profile, unique metabolites).

c) Identification of impurities by chemical characterization (i.e. materials ingredient research or analytical testing).

Step 2: Weight of evidence (WOE) assessment with mechanism and mode of action (MOA) to be considered.

a) Direct DNA reactive versus non direct DNA reactive mode of action.

b) Aneuploidy and polyploidy issues. Is an aneuploidy mechanism involved?

Step 3: Decision point.

Determine whether the extract from the medical device or chemical of concern is a genotoxin and if,

a) the interpretation of results and WOE/MOA analysis within a toxicological risk assessment framework present a low/negligible concern for patients under the expected usage, or

b) the interpretation of results and WOE/MOA analysis within a toxicological risk assessment framework suggest there may be potential risks for patients under expected usage.

If the determination is a) no further additional tests or evaluation are needed.

If the decision is b), then continue to step 4.

Step 4: Perform risk management.

Either manage risks assuming a genotoxic hazard or select the appropriate *in vitro* and/or *in vivo* follow-up testing.

Step 5: Select and run additional *in vitro* and/or *in vivo* test.

Any *in vivo* test shall be chosen on the basis of the most appropriate end point identified by the *in vitro* tests.

in vivo tests commonly used are

— micronucleus test in rodents (OECD 474),

— metaphase analysis in rodent bone marrow (OECD 475),

— transgenic mutagenicity tests (OECD 488).

The decision as to the most appropriate test system shall be justified and documented.

NOTE Recently, a draft OECD guideline for the testing of chemicals on rodent alkaline single cell gel electrophoresis (Comet) assay is under development for genotoxicity testing. This test might prove valuable for medical device testing, but at the time of publication of this International Standard the OECD Guideline was not published.[1)]

An attempt shall be made to demonstrate that the test substance has reached the target organ. For micronucleus test in rodents or metaphase analysis in rodent bone marrow, the bioavailability can be proved by one of the following approaches

— analytical quantification of specific extract compounds in the blood or serum,

— test extract induced cytotoxicity to the bone marrow cells,

— intravenous route of exposure (for polar vehicles).

If the target organ exposure cannot be demonstrated, a second *in vivo* test in another target organ shall be performed to verify the lack of *in vivo* genotoxicity.

Step 6: Reinterpret all of the accumulated data and determine if the test article is genotoxic.

In some cases, positive *in vitro* tests may not be relevant. The following should be considered in determining the overall relevance of the *in vitro* results. This list is not exhaustive but is given as an aid for decision making process.

a) Only one of the original two *in vitro* tests performed had a positive result.

b) Further *in vitro* investigation using similar mechanistic end points do not confirm the positive result.

c) Mechanistic information indicates that positive *in vitro* results are not relevant to *in vivo* situations (e.g. high cytotoxicity, osmolality, etc).

d) *In vivo* testing including evidence that the test sample reached the target organ did not demonstrate a genotoxic effect.

The overall WOE and interpretation of the entire data set shall be documented with the final conclusion. In some cases, site-specific or genetic end point specific tests might be necessary. In most cases, these tests do not have internationally recognized protocols.

5.3 Sample preparation

Unless the sample can be dissolved in a solvent compatible with the test system, appropriate extraction solvents shall be chosen on the basis of its ability to maximize extraction of the material or medical device to a level at which the concentration of genotoxic residues would be sufficient to produce a positive response in the test system, but without degradation of the device or the test sample. The test system vehicle(s) shall be chosen on the basis of its compatibility with the genotoxicity test system. Tests shall be performed on solutions, suspensions (e.g. Method A in Annex A), extracts (e.g. Method C in Annex A) or exaggerated extracts (e.g. Method B in Annex A) of the finished device (including sterilization if applicable), device material, device component or the individual chemicals of the device.

1) OECD Draft-Guideline for the testing of chemicals – In vivo Mammalian Alkaline Comet Assay, available at: http://www.oecd.org/

Device materials should include all final formulation and processing, unless otherwise justified. It is generally not appropriate to conduct testing on raw materials, as formulation and processing could change the potential for toxicity of the final device.

The rationale for choosing to test individual chemicals shall be justified and documented. The rationale shall include considerations of interactions and synergistic effects.

Where relevant, the test material should be extracted with the two solvents (see ISO 10993-12 or Annex A).

Any decision to omit testing with one class of solvent shall be justified and documented.

6 Carcinogenicity tests

6.1 General

Before a decision to perform a carcinogenicity test is made, ISO 10993-1 shall be taken into account. The decision to perform a test shall be justified on the basis of an assessment of the risk of carcinogenesis arising from the use of the medical device. Carcinogenicity testing shall not be performed when risks can be adequately assessed or managed without generating new carcinogenicity test data.

These tests may be designed to examine simultaneously in a single study both chronic toxicity and carcinogenicity. When chronic toxicity and carcinogenicity are to be evaluated in a single study, particular care needs to be taken at the study design stage to ensure the dose groups are appropriate. This helps to prevent or minimize premature mortality from chronic/cumulative systemic toxicity compromising the statistical evaluation of data derived from animals surviving to the end of the study period (i.e. normal life-span).

NOTE *In vitro* cell transformation systems are available for carcinogenicity pre-screening [e.g. Syrian hamster embryo (SHE) cell transformation assay and Balb3T3 cell transformation assay]. At the time of publication of this International Standard an OECD Guideline was not published. Additional information on cell transformation test systems is given in Annex D.

6.2 Evaluation strategy

Carcinogenicity testing of genotoxic materials shall be scientifically justified. In most instances for genotoxic materials, a carcinogenic hazard can be presumed and the risk managed accordingly.

In the absence of evidence to rule out carcinogenic risks for non-genotoxic materials, situations in which the need for carcinogenicity testing shall be considered can include the following:

— materials for which the degradation time is greater than 30 days;

— materials introduced in the body and/or its cavities with a cumulative contact of greater than 30 days.

Circumstances where testing cannot be justified include:

— materials with significant and adequate data on human use or exposure;

— materials that are expected to give rise to solid state carcinogenesis (see Annex E)

— methodological constraints or other circumstances that would limit the predictive value of a test.

To determine if a device has significant human-use history, the assessment should include an evaluation that addresses if the device undergoes a similar manufacturing process, is used to treat a similar patient population, at a similar treatment site and with a smaller or similar accumulated exposure. Human use history should document whether information is available from monitoring for adverse events, particularly cancer risk, in the human use population.

When considering whether a carcinogenicity study should be performed, the role of the study in the evaluation of human risk shall be described, and the need for the study and the study design shall be justified. This justification shall take into account the uncertain role implantation carcinogenicity studies play in the evaluation of biological safety along with the significant number of animals.

If according to ISO 10993-1, chronic toxicity and carcinogenicity is considered relevant, and it is determined that testing is necessary, testing shall be performed in accordance with OECD 453, if possible.

If according to ISO 10993-1, only a carcinogenicity study is considered relevant, and it is determined that testing is necessary, testing shall be performed in accordance with OECD 451.

One animal species is typically sufficient for testing medical devices. The choice of species shall be in accordance with ISO 10993-2 and shall be justified and rationale documented.

6.3 Sample preparation

When carcinogenicity tests are necessary as part of an evaluation of biological safety these studies shall be performed with either materials, defined chemicals or characterized extracts of medical devices.

The medical device shall be tested in a form representative of the finished device state. Additional testing may be warranted for additional states of the device such as, wear debris generated from the device or materials that cure *in situ* (e.g. cements, adhesives and pre-polymer mixtures). For guidance on *in situ* curing devices, see 10993-12.

The selection of the test sample (device material, device material extracts or defined chemical) shall be justified and documented.

The highest dose used in the animals is either the maximum tolerated dose or that limited by the physical constraints of the animals model. This dose shall be expressed as a multiple of the estimated maximum human exposure (in weight and/or surface area per kilogram).

6.4 Test methods

If testing of an extract is considered relevant, the carcinogenicity tests shall be performed in accordance with OECD 451 or OECD 453.

Tissues evaluated shall include relevant tissues from the list indicated in OECD 451 or OECD 453, as well as the implantation site and adjacent tissues.

For studies using device material extracts or defined chemicals, an explanation shall address why the material's surface properties are not a carcinogenic consideration. The considerations for the performance of implantation studies (see Annex E) shall be taken into account and the role of surface properties in the evaluation of human risk shall be described and documented.

For implantation studies to evaluate carcinogenicity, the amount of material implanted shall be representative of an exaggerated human dose which provides a sufficient margin-of-safety. The highest dose will be limited by the physical constraints of the animal model. This dose shall be expressed as a multiple of the estimated maximum human exposure (in weight and/or surface area per kilogram).

A 100 times safety factor is applied to the estimated maximum human exposure (in weight and/or surface area per kilogram), however, the dose should be physiologically compatible with the model. The negative control group will generally receive a comparable shape and form of a clinically acceptable material or reference control material whose lack of carcinogenic potential has been documented (e.g. polyethylene).

When appropriate, a suitably formed implant in accordance with ISO 10993-6 shall be made of the test material(s), with appropriate consideration being given to the possibility of inducing solid state carcinogenicity, (see Reference[33]).

EXAMPLE Test article: Polymer

Exposure scenario: A typical patient will be exposed to a maximum of 11 g of polymer in a device.

Human dose: 0,19 g/kg (based on average female body weight of 58 kg). If device will be used in children, 10 kg for the body weight is recommended.

Considering a 100 times safety factor, the mouse dose is equal to 19 g/kg. Thus, a typical 25 g mouse will receive 0,475 g.

The polymer can be tested in disc form. A disc not exceeding approx. Fifteen mm in diameter and 2 mm to 3 mm in thickness is recommended. However, for materials with high density, these dimensions may need to be reduced to avoid tissue damage related to weight of the sample. Multiple samples may be implanted to obtain the desired dose. Thus, in the above example, two disc-shaped implants containing 0,2 g of polymer per implant would be implanted in each mouse.

Tissues evaluated shall include relevant tissues from the list indicated in OECD 451 or OECD 453, as well as the implantation and adjacent tissues.

In recent years, the use of transgenic animals for carcinogenicity testing has gained some acceptance, but has not been validated for medical devices. The carcinogenicity assay in transgenic models is shorter in duration (usually six months) and requires fewer animals than the two-year life time carcinogenicity assays in rodents. In addition to their shorter duration, these studies offer an additional benefit of not being complicated by the solid state tumorogenesis phenomenon. Since the transgenic model studies last only six months, and 8 to 9 months are required for solid state tumours to develop, this phenomenon is not a confounding factor. The *ras*H2 transgenic mouse model has been the primary transgenic model used to assess the carcinogenicity risk of medical devices.

Due to limited available data, and the lack of formal validation studies with the *ras*H2 transgenic mouse model, the study design with this model frequently includes a positive control along with the negative control.

7 Reproductive and developmental toxicity tests

7.1 General

Before a decision to perform reproductive and developmental toxicity tests is made, ISO 10993-1 shall be taken into account. The decision to perform a test shall be justified on the basis of an assessment reproductive status of the subject population of the potential for exposure of reproductive tissues, the embryo/foetus or nursing child to test material or leachable substances.

There is no need for reproductive toxicity testing of biodegradable medical devices or medical devices containing leachable substances where there are adequate and reassuring data from absorption, metabolism, distribution and excretion studies demonstrating a lack of exposure to relevant tissues or from reproductive and developmental toxicity studies.

Reproductive and developmental toxicity testing is not required where an acceptable toxicological risk assessment of the medical device takes into account the fact that the risk of reproductive and developmental toxicity has been adequately mitigated.

7.2 Test strategy

Reproductive and developmental testing shall be considered for the following devices:

— prolonged or permanent contact devices with materials, or degradation byproducts or leachable substances, likely to come into direct contact with reproductive tissues,the embryo/foetus or the germ cells;

— energy-depositing medical devices.

In determining if reproductive toxicity testing of the device is warranted an assessment of risk shall address the following factors:

— extent of systemic exposure from leachable compounds (if device does not directly contact the reproductive tissue);

— device's physical characteristics;

— metabolites of the device material;

— genotoxicity results.

Testing is indicated when there is inadequate information about one or more of the above factors and this risk cannot be mitigated through other risk control measures (e.g. information about the lack of reproductive toxicity data).

Testing shall be conducted on the finished device or test material.

The use of specific test material other than finished device shall be justified and rationale documented.

If testing is required, this may start with OECD 421 in order to provide initial information on possible effects on reproduction and/or development. Positive results with these tests are useful for initial hazard assessment and contribute to decisions with respect to the necessity for and timing of additional tests.

If additional tests are considered necessary, they shall be performed in accordance with OECD 414, OECD 415 or OECD 416, as appropriate.

It may be possible to start with appropriate test systems which clearly demonstrate absence or presence of reproductive toxicity according to OECD 414, OECD 415 or OECD 416.

7.3 Sample preparation

Sample preparation shall be in accordance with ISO 10993-12. Whenever possible the medical device shall be tested in a form representative of its final state. Additional testing may be warranted for additional states of the device such as, device or materials that cure *in situ* (e.g. cements, adhesives and pre-polymer mixtures).

In the case of energy-depositing medical devices, whole-body exposure of the animals is appropriate. A multiple of the predicted human exposure to the reproductive organs shall be applied.

The highest dose used in the animals is either the maximum tolerated dose or that limited by the physical constraints of the animals model. This dose shall be expressed as a multiple of the estimated maximum human exposure (in weight and/or surface area per kilogram).

In vivo testing shall be performed in accordance with ISO 10993-2.

7.4 Test methods

Assessment of effects on the first generation (F1) or even second generation (F2) shall be made in accordance with OECD 414, OECD 415 or OECD 416 and OECD 421. As the OECD guidelines were not intended for medical devices the following modifications shall be considered:

— dose (in the case of energy-depositing medical devices);

— route of application (implant, parenteral, other);

— extraction media;

— exposure time (elevated blood levels during organogenesis when possible).

NOTE Depending on intended human use and material characteristics, peri-/post-natal studies can be indicated.

If information derived from other tests indicates potential effects on the male reproduction system, then appropriate tests for male reproductive toxicity shall be conducted.

Recently, *in vitro* reproductive test systems have been developed. They can be useful as a pre-screening test method for reproductive and developmental toxicity.

8 Test report

If relevant, the test report shall include at least the following details:

a) description of material and/or medical device (e.g. chemical composition, processing, conditioning and surface treatment) including its intended use;

b) description and rationale/justification of test methods, test conditions, test materials, test dose and test procedures;

c) description of analytical methods including quantification limits;

d) statement of compliance to appropriate current/valid best laboratory/quality practices, for example Good Laboratory Practices (GLP) or ISO/IEC 17025, where applicable;

e) test results including summary;

f) statistical methods;

g) interpretation and discussion of results;

h) further details as specified in the relevant OECD guidelines or Annex C and Annex D, and ISO/TR 10993-33;

i) name and certifications of the testing laboratory;

j) date of the test;

k) name and signature of the responsible person.

Annex A
(informative)

Guidance on selecting an appropriate sample preparation procedure in genotoxicity testing

A.1 General

This annex gives guidance for selecting an appropriate sample preparation procedure in genotoxicity testing of medical devices. In selecting the method, the user should consider the medical device material physicochemical properties and the medical device manufacturing process. For example, many polymers in medical devices contain, in addition to the relatively inert, high molecular weight polymer, other components such as residual monomers, oligomers, catalysts, processing aids, etc. These are present at varying levels depending on the raw material sources, the manufacturing processes, and intended function of additives. Also, additional chemical species can be generated during manufacturing processes such as heat sealing, welding, or sterilization of the device. All of these can migrate from the device into the human body and should be the subject to a risk assessment.

Information relevant to the biological risk analysis may be available from the literature and/or the manufacturer(s) or supplier(s).

If sufficient information is available on the qualitative and quantitative characteristics of the finished device or facsimile, including the materials and processing aides used in the device manufacturing, there is no need for testing.

In evaluating if sufficient information is available the user should include in their analysis the following.

— Does the finished device undergo an equivalent manufacturing process (including sterilization, if applicable)?

— Does the device contain the same additives and contaminants (such as processing aids, unreacted monomers, catalysts)?

In order to carry out a risk-based evaluation in accordance with ISO 14971, the risk analysis procedure should include the following three steps.

— Material/device characterization.

— Hazard identification.

— Risk estimation.

However, if some necessary information is lacking, testing should be conducted. Biological test methods including sample preparation procedure should be designed appropriately for the purposes of identifying biological hazards and estimating their risks.

The selection of the appropriate sample preparation is critical to obtain relevant results from genotoxicity tests. Inappropriate sample preparation can result in an underestimation of genotoxicity risk. For example, the extraction of polymers with water was once generally considered to simulate the *in situ* migration of leachates from polymers into blood. However, by Tsuji et al.[76] it was demonstrated that di(ethylhexyl)phthalate (DEHP) was not extracted from polyvinyl chloride blood circuit tubing when water was used as an extracting solvent. They demonstrated that human plasma extracted significant quantities of DEHP and the percentage of DEHP extractable with human plasma was similar to that observed with 40 % ethanol. Based on this study, Oba et al.[77] succeeded in reproducing eye lesions,

which occurs among dialysis patients treated with a specific brand of acetate dialyzer by infusing the rabbit with extracts obtained from 40 % ethanol.

A.2 Device materials

A.2.1 Low Molecular Weight Chemicals (LMWC)

LMWC, non-polymeric substances contained in medical devices, can penetrate cell membranes, to react with DNA, genes, or chromosomes so that they can give rise to genotoxic reactions (e.g. cyanoacrylate adhesive), see A.2.2.1.

A.2.2 Polymers (including naturally occurring polymers)

A polymer is a chemical substance consisting of molecules characterized by the sequence of one or more types of monomer units and comprising a simple weight majority of molecules containing at least 3 monomer units. These units are covalently bound to at least one other monomer unit or other reactant, and consist of less than a simple weight majority of molecules of the same molecular weight. Such molecules must be distributed over a range of molecular weights wherein differences in the molecular weight are primarily attributable to differences in the number of monomer units.

Common polymer groups are: non-degradable synthetic polymers (e.g. polyethylene, polymethylmethacrylate, silicone); naturally occurring polymers (e.g. cellulose, alginate, gelatin, collagen); and biodegradable polymers (e.g. poly(L-lactic acid) (PLLA), polyglycolic acid).

A.2.2.1 LMWC contained in polymers

Polymeric materials often contain a small amount of LMWC such as additives, catalyst, processing aids, and radiation products. These LMWC may be potentially genotoxic. In cases of an invasive contact, LMWC can migrate out of the polymer and into the patient. Therefore, the LMWC in polymers should be evaluated for their genotoxic risk.

Migration of LMWC from the polymeric device to body fluid is considered a phenomenon similar to the migration of LMWC from the food container into the food. In the food container, the migration rate is expressed as

— a function of the total content of LMWC in the polymer,

— the diffusion coefficient of LMWC in the polymer, and

— the partition equilibrium constant of LMWC between polymer and food (see Reference[82]).

The assumptions and equations made for food containers can therefore be used in risk evaluations of LMWCs in medical devices.

A.2.2.2 Oligomers

An oligomer is a polymer molecule consisting of only a few monomer units (dimer, trimer, tetramer). Oligomers may be present in the polymers and can migrate out of the polymer. Oligomers with reactive functional chemical groups in their structure are a health concern because of their potential genotoxicity. In 1993, the 3rd OECD Experts meeting on polymers[78] concluded that the following parameters should be considered for their effects on patient health when making a decision on polymers,

— number-average MW,

— low MW content,

— presence of reactive functional groups, and

— presence of bioavailable metals, which were part of the polymer structure.

Reactive functional groups are defined e.g. as follows: acid halides, acid anhydrides, aldehydes, hemiacetals, methylolamides, -amines or –ureas, alkoxysilanes (>C2), allylethers, conjugated olefins, cyanates, epoxides, imines, unsubstituted ortho or para positions to phenolic hydroxyl, pendant acrylates and methacrylates, aziridines, carbodiimides, halosilanes, hydrosilanes, hydrazines, isocyanates, isothiocyanates, alpha or beta lactones, methoxy or ethoxy silanes, vinylsulfones or analogous compounds (see Reference[79]).

A.2.2.3 Biodegradable polymers

A biodegradable polymer is a polymer designed or is reasonably anticipated to substantially degrade, decompose, or depolymerize, including those polymers that could substantially decompose after manufacture and use, even though they are not actually intended to do so. For biodegradable polymers, the total amount of LMWC in the polymer is released into the body. Most biodegradable polymers are similar to polyester, and generally do not have reactive functional groups as defined in the OECD report. The LMWC contained in or added to the biodegradable polymer should be evaluated for their genotoxic risk.

A.2.3 Inorganic materials: Wear debris from metals, alloys and ceramics

The quantity and genotoxicity of metallic ions released from inorganic materials (e.g. stainless steel, titanium alloy, hydroxyapatite, tricalcium phosphate, alumina and zirconia) are a health concern. For example, *in vivo* genotoxic effects have been seen in periprosthetic lymphocytes of patient implanted with metal on metal hip implants. Many metal ions play an important regulatory role in the body and this role is dependent on their chemical characteristics and valence state. Metal ions bind to proteins in the physiological fluids (such as blood, lymphatics and urine) and may partition to different fractions for biodistribution. Metal ions are internalized into the cell where they may bind to nuclear material and alter cell signalling locally, systemically or both. Therefore, the metal ions' genotoxicity profile should be evaluated and assessed as far as possible based on literature data.

A.3 Sample Preparation Methods

A.3.1 General

The ideal sample preparation design for estimating the genotoxicity risk of a device is to apply the total amount of the extractables from the whole device to the test system. However, this approach is not practical for larger devices. For larger devices, a portion of the test sample is extracted with an appropriate sample preparation procedure and applied to the test system.

The selection of a sample preparation procedure for any material or device intended for use in humans requires a structured approach that takes the chemical composition and physicochemical properties of the material or device into consideration. The sample preparation should follow the decision tree in Figure A.1. This figure diagrams the decision process used to select the extraction method (Method A, B, or C) for a device material unless the medical device or medical device material has to be evaluated according to a special sample preparation procedure as described in A.4.

The solvents or extraction solvents used should not be suspected of causing a chemical reaction with the test sample.

Method A requires direct application of the test sample to the test system and is used when the test sample can be dissolved or suspended in an appropriate solvent compatible with the test system.

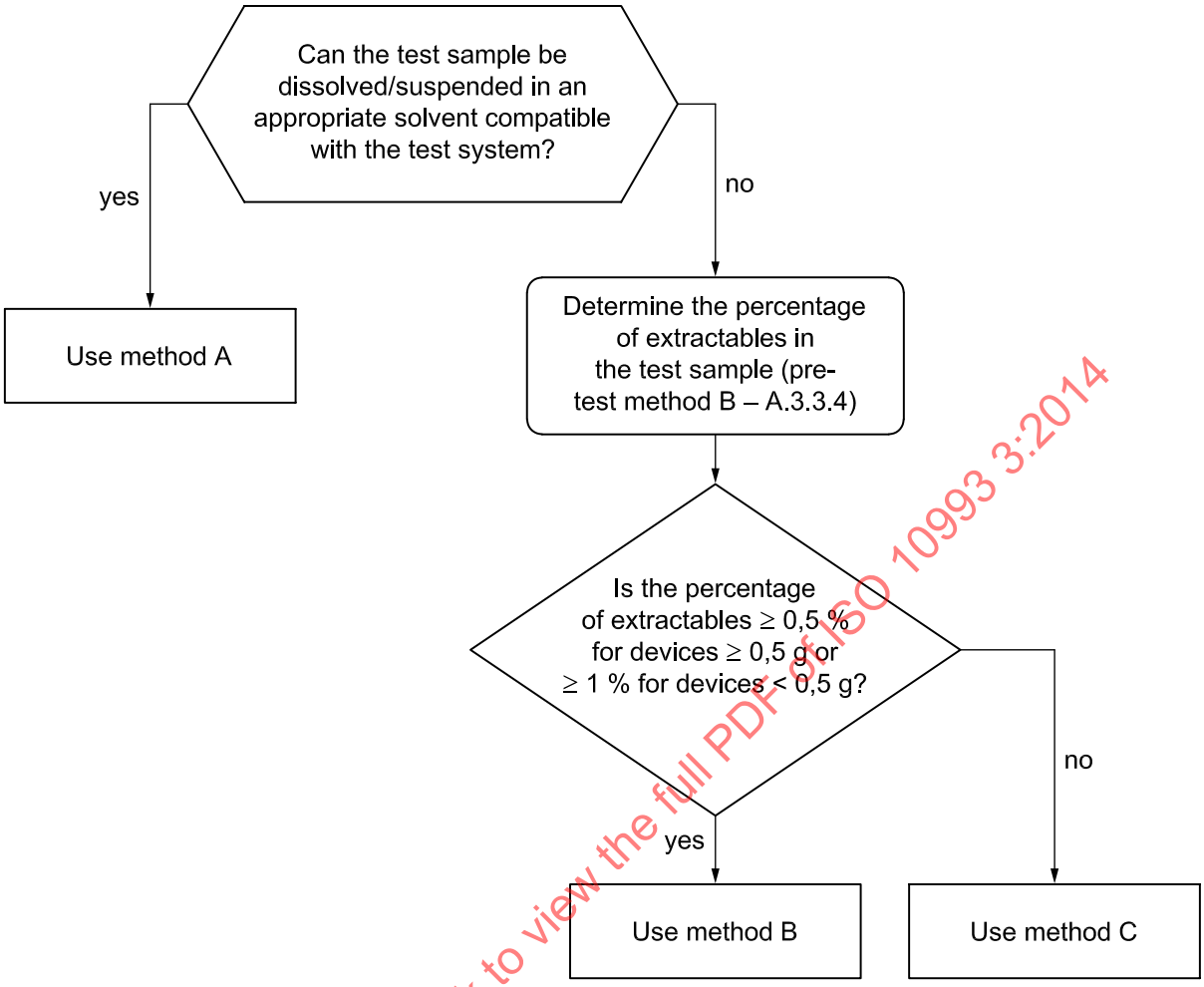

Figure A.1 — Structured approach to select a sample preparation procedure

When the test sample is neither soluble nor suspendable in either polar or non-polar solvent, Methods B and C, extraction methods, are chosen based on the physical characteristics of the materials comprising the device. The selection of Method B or C depends on the percentage of extractables removed from the test sample.

The percentage of extractable (as reported as a percentage of the amount of residue versus total device weight, see also A.3.3.4) with each solvent should be reported. Common extraction solvents are methanol and acetone.

Methanol is better at extracting water soluble substances while acetone is better at extracting fat-soluble substances. The methanol and acetone extracts are separately evaporated to dryness to determine the percentage of extractables drawn out of the test system by each solvent. The percentage of extractables with each solvent should be reported.

Additional solvents can be used in preliminary tests for materials if an appropriate rationale is provided. If using volatile solvents, these may degrade the test material or may not extract the residual effectively from the test material.

Table A.1 can be useful in choosing an appropriate solvent for extraction of medical devices. This table lists common extraction solvents and their octanol-water partition coefficient, log K_{ow}. A solvent with more negative log K_{ow} partitions in water more efficiently than octanol. A solvent with a more positive log K_{ow} dissolves in octanol more readily than water. The selection of solvent should be justified.

Table A.1 — Common extraction solvents

Solvent	log K_{ow}
Dimethyl formamide	−1,01
Methanol	−0,74
Ethanol	−0,30
Acetone/2-propanone	−0,24
Dichloromethane[a]	+1,25
Chloroform[a]	+1,97
Hexane[a]	+3,90
[a] These chemicals may be subject to controls to address safety concerns	

A.3.2 Method A

The test sample is either dissolved or suspended (or partially dissolved) in the solvent. The final volume of test sample preparation in the *in vitro* mammalian test system should not exceed 10 %, if the test article is dissolved in an aqueous solvent such as water or saline. The maximum concentration tested on an *in vitro* mammalian test system is 5 mg/ml. In the bacterial reverse mutation test, 100 µl of the test sample preparation should be applied to an agar plate. The maximum concentration for the bacterial reverse mutation test is 5 mg/plate.

Dose selection should be based on the toxicity profile within the context of the genotoxicology assay. In some cases preliminary dose range assays may be useful to achieve the proper dose selection. It may also be appropriate to dose at a single, maximum level if toxicity is not expected. See instructions for dose evaluation within each assay in ISO/TR 10993-33.

For the *in vivo* test, the maximum test sample preparation volume that can be administered by injection at one time usually is 20 ml/kg body weight for mice and 10 ml/kg for rats. For nontoxic test sample preparations, the maximum dose level is 2 000 mg/kg body weight. For toxic test sample preparations, a dose range finding study should be conducted prior to the main *in vivo* study in order to determine the toxicity of the test sample preparation and set dose levels for the main study.

If applicable, data from acute toxicity studies may be used due to animal welfare reason.

Users are referred to ISO/TR 10993-33 for details.

The principles set forth in Reference[107] can be used to guide the top dose solutions.

A.3.3 Method B

A.3.3.1 General

A pre-test in accordance with test sample preparation (see A.3.3.2) and extraction procedure (see A.3.3.3) should be carried out in order to select either method B or method C.

Method B is selected if the percentage of extractables obtained in a pre-test meets the following criteria:

a) For devices with a mass < 0,5 g, such as contact or intraocular lenses, method B should be used if the percentage of extractables in the test sample is ≥ 1 %.

b) For devices with a mass ≥ 0,5 g, Method B should be used if the percentage of extractables in the test sample is ≥ 0,5 %.

If the percentage of extractables does not meet the criteria above, the extract should be prepared using Method C.

A.3.3.2 Test sample preparation

The test sample is immersed in a volatile organic extraction vehicle that extracts residuals from the test sample but does not dissolve the test sample. If test sample appearance or weight confirms partial degradation, use Method C. Two or more solvents are tested to determine which solvent extracts the highest percentage of extractables from the test sample (see A.3.3.3 and A.3.3.4). Common extraction solvents are methanol and acetone. The extracted, evaporated test sample residue is dissolved or suspended in a solvent compatible with the genotoxicity test system. The final volume of organic or aqueous solvent in the culture should not exceed 1 % (organic) and 10 % (aqueous), in the chromosome aberration test or the mouse lymphoma test. In the bacterial mutation test, 100 µl of the residual solution/suspension should be applied to an agar plate. The maximum concentration tested in the *in vitro* chromosome aberration test or *in vitro* mouse lymphoma test is 5 mg/ml. The maximum concentration for the bacteria reverse mutation test is 5 mg/plate.

The sample extraction procedure is given in A.3.3.3. See Reference.[86]

For the *in vivo* test, the extracted and evaporated test sample residue is dissolved or suspended in vehicles compatible with the test system. The selection of the highest dose and administration route are the same as those in Method A.

The principles set forth in Reference[107] can be used to guide the top dose solutions.

A.3.3.3 Procedure

— Chop an appropriate amount of test sample into small pieces and place them into a glass container along with the extraction vehicle. A ratio of 1 g to 10 ml or a sufficient volume to immerse the test sample should be used. If the test sample cannot be cut, use sufficient volume to cover the test sample, preferably using a ratio of 1 g to 10 ml.

NOTE Details on the extraction of absorbent materials are included in ISO 10993-12.

— Extract the test sample for (24 ± 2) h at room temperature with constant agitation.

— After extraction, filter the extracts through a low binding and chemically resistant filter to remove the test sample.

— Pour the extract into a pear-shaped flask of known constant mass m_1 and evaporate the extraction solvent in the extract to dryness or to constant weight with a reduced-pressure concentrator apparatus at ≤ 30 °C. Determine the mass of the flask after evaporation m_2.

— Calculate the percentage of extractables.

— A portion of the residue can be used to check the solubility/uniformity in compatible solvents with the test system.

— Neither the extract nor the dosing solutions may be heated to avoid chemical changes of residues or loss of volatile compounds.

NOTE The Soxhlet exhaustive extraction method can be considered as an alternative method.

— Evaporation of the extract following extraction is not applicable for cases when the suspected residue in the device is highly volatile (e.g. ethylene oxide, low molecular weight acrylate monomers).

— For sample preparation according to method B, both extracts from the test sample should be used individually if the criteria for method B are fulfilled. If only one extract from the test sample meets the criteria only this extract is used for genotoxicity testing. The other extract is not used.

— Dissolve or suspend the residue of extractables in a solvent on the basis of maximising the test concentration for the appropriate test system. This solvent can be identified e.g. using the residue obtained in the pre-test of method B. Use this solution within 24 h.

A.3.3.4 Expression of results

Calculate the mass of the extracted residuals, W_R, in the pear-shaped flask by determining the change in mass of the flask using Formula (A.1).

$$W_R = m_2 - m_1 \quad \text{(A.1)}$$

where

m_1 is the mass of the empty flask;

m_2 is the mass of the flask after evaporation of the extract.

Calculate the percentage of extractables by determining the ratio of the mass of extractable materials to the mass of the test sample and multiplying by 100 by using Formula (A.2).

$$\%_e = \frac{W_R}{m_3} \times 100 \quad \text{(A.2)}$$

where

$\%_e$ is the percentage of extractables;

m_3 is the mass of the test sample prior to extraction.

Record and report the percentage of extractables for each solvent.

The study report should include the rationale for selection of the extracting solvent and the percentage of residue for each tested solvent.

A.3.4 Method C

A.3.4.1 General

Method C is a simulated-use extraction method similar to that described in ISO 10993-12.

The test sample is extracted in a solvent/vehicle compatible with the test system. The test sample preparation is applied to the test system. Use this extract within 24 h.

A.3.4.2 Procedure

A.3.4.2.1 For the bacterial reverse mutation assay, the test sample is chopped into small pieces, if possible, and extracted as specified in ISO 10993-12.

A.3.4.2.2 For the *in vitro* mammalian cell tests, the test sample is chopped into small pieces, if possible, and extracted as specified in ISO 10993-12.

A.3.4.2.3 If the culture medium without serum is used as a polar solvent for extraction, the test extract is tested neat following supplementation with serum before dosing the cells. The test extract in the cell culture medium with serum (as a non-polar solvent) is tested as neat extract. If saline is used as polar solvent for extraction, the test extract should be diluted to 10 % with serum-supplemented cell culture medium before dosing the cells. The DMSO or Ethanol test extract should be tested at 1 % following dilution with serum supplemented cell culture medium. For cytotoxic test extracts, acceptable cytotoxicity limit for the assays should be considered for selecting adequate test extract dose.

NOTE 1 (37 ± 1) °C for (48 ± 2) h can also accepted if cell culture medium with or without serum is used for extraction.

A.3.4.2.4 For the *in vivo* test, the test sample is chopped into small pieces, if possible, and extracted as specified in this part of ISO 10993.

A.3.4.2.5 The test extract is administered intravenously (saline) or intraperitoneally (hydrophobic) to animals depending on the solvent used. The volume should not exceed 20 ml/kg body weight for mice and 10 ml/kg for rats.

A.4 Additional guidance on special sample preparation procedures

A.4.1 Biodegradable polymers

For devices made with biodegradable polymers, a modified Method B can be used to prepare the test material because of the concern that the total LMWC is released in the patient. Using solvents that are suitable for dissolution and re-precipitation filter the resultant solution to remove precipitates. Report any precipitate in the filter paper after filtration. Evaporate the solvents from the filtrate (e.g. rotary evaporator might be used). Record the amount of residue generated.

Dissolve or suspend the residue in a solvent/vehicle compatible with the test system and apply it to the test system.

A.4.2 Inorganic materials: Wear debris from metals, alloys and ceramics

In assessing genotoxicity of devices made of inorganic materials such as hip joint prostheses, major concern is the genotoxic potential of metal ions released from wear debris and/or by corrosion during clinical exposure and their amounts. As the assays in this document are designed to measure the genotoxic potential of final device extracts (in solution) and not particulates, alternative approaches to assess the genotoxic potential of wear debris or particles will be necessary.

A.4.3 LMWC

When the test sample is composed of a single or multiple LMWC, its genotoxic risk can be evaluated by applying its solution/suspension in a vehicle compatible with the test system. Method A is applicable.

Annex B
(informative)

Flowchart for follow-up evaluation

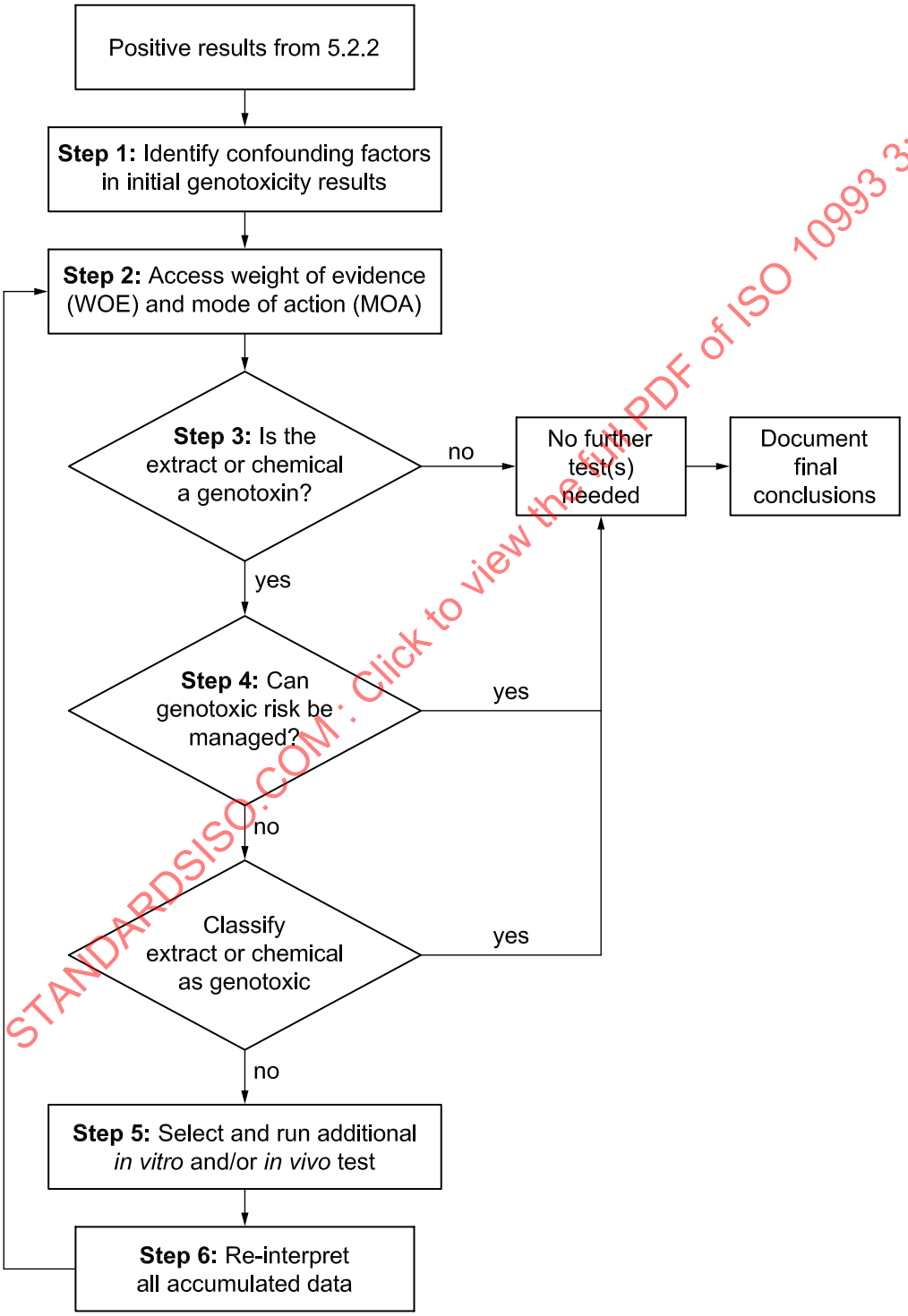

Figure B.1 — Flowchart for follow-up evaluation

Annex C
(informative)

Rationale of test systems

C.1 Genotoxicity tests

The primary function of genotoxicity testing is to investigate, using test cells or organisms, the risks of germ cell and somatic cell genetic changes. Scientific data generally support the hypothesis that DNA damage in somatic cells is a critical event in the initiation of cancer. Thus, some tests for DNA damage are useful for the investigation of putative carcinogenic activity.

While in classical toxicology testing, several pertinent parameters or end points can be observed within one experimental design, the same is not true for genetic toxicology. The diversity of the genetic end points usually precludes the detection of more than one of them in a single test system.

Approximately 15 different tests are cited in test guidelines. The selection of the most appropriate of these to meet a particular requirement is governed by a number of factors. These include the type of genetic change the test is required to detect, or the metabolic capability of the test system.

It must be emphasized that there is no international agreement on the best combination of tests for a particular purpose, though there have been attempts to harmonize the selection of the most appropriate tests. It may also be helpful to note that there are other mutagenicity tests in use or in development, which, although without an OECD Guideline, can also be useful. The existence of the ICH/S2B agreement for pharmaceuticals should be noted.

Chemicals that interact with DNA produce lesions that, after the influence of various repair processes, may lead to genetic changes at the gene level, e.g. gene or point mutations, small deletions, mitotic recombination, or various microscopically visible chromosome changes, and tests are available to investigate each of these events.

Current short-term tests cannot, of course, mimic all the stages in the carcinogenic process and are frequently assumed to detect only the event leading to the initiation phase, i.e. the ability to induce a mutagenic or clastogenic lesion. The main value of these procedures, therefore, lies in their ability to identify substances that may, under certain exposure conditions, either cause cancer by a predominantly genotoxic mechanism or induce the initial phase of the carcinogenic process. It is apparent, from the complexity of the carcinogenic process compared with the relative simplicity of short-term tests, that, although they provide useful qualitative information, considerable caution is required in their interpretation in terms of carcinogenic activity.

Since no single test has proved capable of detecting mammalian mutagens and carcinogens with an acceptable level of precision and reproducibility, it is usual scientific practice to apply these tests in "batteries". Initial information on the mutagenicity of a substance can be obtained using tests that measure gene mutations and chromosomal damage. Because separate procedures are required to investigate these end points, a battery of tests is needed.

If additional relevant information is available, such as Absorption Distribution Metabolism and Excretion (ADME) which indicates there are leachables at specific organ sites, an *in vivo* test for genotoxicity should be considered. The choice of which *in vivo* test to perform will be influenced by the sites where leachables are accumulating. In many cases, an *in vivo* test for chromosomal damage in rodent haematopoietic cells will be appropriate. In some cases, site-specific or genetic end-point specific tests can be indicated. In most cases, these tests do not have internationally recognized protocols. For the majority of medical devices and/or materials for which genotoxicity testing is considered necessary a standard *in vitro* test battery is sufficient to provide evidence for genotoxic potential of the test sample.

C.2 Carcinogenicity tests

The objective of a long-term carcinogenicity study is to observe test animals, for a major portion of their life span, for the development of neoplastic lesions, during or after exposure to various doses of a test sample by an appropriate route. Such a test requires careful planning and documentation of the experimental design (see Annex E), a high quality of pathology and unbiased statistical analysis.

C.3 Reproductive/developmental toxicity tests

Reproductive toxicity tests cover the areas of reproduction, fertility and embryo-foetus development. Fertility can be affected in males and females and effects can range from slight decrease in reproductive capability to sterility. Toxic effects on the developing embryo or foetus can affect the health of the offspring.

Teratogenicity deals with the adverse effects of a substance on the developing embryo and foetus. Reproductive toxicity is important as it has an important bearing on the health of mankind. Testing techniques are developing and the concept of combined tests, covering all aspects of reproductive toxicology, appears promising.

Annex D
(informative)

Cell transformation test systems

In vivo rodent models are utilized for experimental investigation of the carcinogenic risk of chemicals to human. However, the rodent carcinogenicity assay is expensive and time-consuming. Several *in vitro* alternatives to animal-based methods have been developed. Among these methods, cell transformation assays which mimic some stages of *in vivo* multistep carcinogenesis, have been proposed for predicting carcinogenic potential of chemicals.

Compared to *in vivo* based-assays, *in vitro* cell transformation tests are fast, cost efficient and provide a means for initial screening of carcinogenic potential. Cell transformation has been defined as the induction of certain phenotypic alterations in cultured cells that are characteristics of carcinogenic cells. These phenotypic alterations can be induced by exposing mammalian cells to carcinogens. Transformed cells that have acquired the characteristics of malignant cells have the ability to induce tumours in susceptible animals. *In vitro* transformed cells exhibit morphological changes related to neoplasia. This phenomenon of morphological cell transformation involves changes in behaviour and growth control of cultured cells, such as alteration of cell morphology, disorganized pattern of colony growth, and acquisition of anchorage-independent growth.

The Syrian hamster embryo (SHE) cell transformation assay has since been described as the most predictive short-term test for rodent carcinogens. Schectman[34] describes how the methodology for rodent cell transformation testing has changed over time to result in an SHE cell assay that is more reproducible than earlier methods. Cellular transformation assays are able to also detect some non genotoxic carcinogens which is a particular advantage in comparison to genotoxicity assays. However, the cellular transformation assays are technically difficult and not well understood mechanistically.

Also a two-stage cell transformation assay in Balb/c 3T3 cells or in SHE cells seems potentially useful not only for detecting compounds inducing cellular transformation but also tumour promoters. Morphological cell transformation has been shown to arise from point mutation, chromosomal damage, aneuploidy and other effects associated with cellular proliferation. However, because of the wide range of mechanisms by which non-genotoxic carcinogens might act, and especially as some effects are tissue specific, it is unlikely that a combination of this assay and one for aneugenic agents would be sufficient to detect all types of non-genotoxic carcinogens. Therefore, to increase the spectrum of non-genotoxic carcinogens that can be detected *in vitro,* it will be necessary to develop a battery of assays that involve the detection of the principal end points by which these agents act.

Guidance and review for the *in vitro* cell transformation test are given in References[13] and.[14]

Annex E
(normative)

Considerations for carcinogenicity studies performed as implantation studies

E.1 Foreign body carcinogenesis

Tumours induced by implants are well known in experiments using rats and this phenomenon is called "foreign body carcinogenesis" or "solid state carcinogenesis." The phenomenon is summarized as follows.

Tumours usually develop around or near an implant with a frequency that is dependent on several factors.

— The size of the implant (large implants generally produce more sarcoma than small ones).

— Their shape.

— Their smoothness (those with rough surfaces are less carcinogenic than those with smooth surfaces).

— The continuity of the surface area (the larger the holes or pores in the implant, the lower the tumour incidence).

— For certain materials, their thickness (thicker implants produce more sarcomas).

— The length of time the implant remains in the tissue.

The same material that produces tumours as a film or sheet will, for the most part, produce fewer or no tumours when implanted as a powder, a thread or a porous material. See References[37] and.[38]

Tumours due to this phenomenon first begin to be detected after 8 months to 9 months following implantation. The incidence continues to increase after this latency period.

On the other hand, many reports indicate a difference in the incidence of tumour formation among different materials of similar shape and size using the same animal experimental protocol.

Mechanistic understandings were summarized in an IARC Monograph. See References[36][37] and.[38]

E.2 Animal welfare considerations

The conduct of carcinogenicity studies by implantation requires surgically invasive procedures on a large number of both test and control (sham operated) animals.

Annex F
(informative)

In vitro tests for embryo toxicity

In the field of developmental toxicity, a variety of alternatives to whole animal testing are available. Over the past 30 years, a wide spectrum of *in vitro* tests have been developed ranging from cell, tissue and organ cultures to whole-embryo cultures. In line with the recommendations of an ECVAM workshop on reproductive toxicity, see Reference,[87] ECVAM initiated and funded a validation study of three *in vitro* embryo toxicity tests. In two of these assays, pregnant laboratory animals are used to obtain embryonic tissue — either primary embryonic cells in the mouse micro mass (MM), see Reference[88] test or embryos in the rat whole embryo culture (WEC) test, see Reference[89][90] and [91][92] In contrast, in the embryonic stem cell test, see References[92][93][94] and[95] a permanent mouse embryonic stem cell (EST) line is used. The main objectives of the validation study were to assess the performance of the three *in vitro* tests in discriminating between non-embryo toxic, weakly embryo toxic and strongly embryo toxic compounds. All three *in vitro* embryo toxicity tests proved to be applicable to testing a diverse group of chemicals with different embryo toxic potentials, see Reference.[96] The results obtained in the blind trial of the definitive phase of the ECVAM validation study were reproducible, both within and among laboratories, and the concordances between the embryo toxic potentials derived from the *in vitro* data and from the *in vivo* data were good for the EST and the WEC test (with one prediction model [PM]), and sufficient for the MM test and the WEC test (with another PM), according to the performance criteria (Table F.1) defined by the Management Team before the formal validation study, see Reference.[96] A summary of the comparison of *in vitro* classifications to the *in vivo* classifications based on 14 chemicals used in the validation study is given in Table F.2. A summarizing report on the results of the ECVAM validation study and, in addition, one report on the selection of test chemicals and three detailed studies on the bio statistical performance of each *in vitro* embryo toxicity test in the validation study have been published, see References[96][97][98][99] and.[100]

The ECVAM Scientific Advisory Committee (ESAC) concluded from the results that the three *in vitro* embryo toxicity tests had been sufficiently validated and could be applied to the assessment of the embryo toxic potential of drugs and other chemicals, see Reference.[101] Furthermore, ESAC recommended setting up a workshop with the aim of producing a guidance document on the applicability of the three scientifically valid test methods in the context of reproductive toxicity testing. ECVAM and ZEBET (Centre for the Documentation and Evaluation of Alternatives to Animal Experiments), therefore, held a second embryo toxicity workshop to further evaluate the practical applications of the three *in vitro* embryo toxicity tests. The results of this workshop have been published, see Reference.[102]

Table F.1 — Criteria defined by the management team of the study to evaluate test performance

Criteria	Performance
By chance	33 %
Sufficient	≥ 65 %
Good	≥ 75 %
Excellent	≥ 85 %

Table F.2 — Summary of the classification results (all data[88])

Classification	EST	MM	WEC	
			PM1	PM2
Predictivity (non-embryo toxic)	72 %	57 %	56 %	70 %
Predictivity (weakly embryo toxic)	70 %	71 %	75 %	76 %
Predictivity (strongly embryo toxic)	100 %	100 %	79 %	100 %
Precision (non-embryo toxic)	70 %	80 %	70 %	80 %
Precision (weakly embryo toxic)	83 %	60 %	45 %	65 %
Precision (strongly embryo toxic)	81 %	69 %	94 %	100 %
Accuracy	78 %	70 %	68 %	80 %

Bibliography

General

[1] OECD 474, *Mammalian Erythrocyte Micronucleus Test*

[2] OECD 475, *Mammalian Bone Marrow Chromosome Aberration Test*

[3] OECD 478, *Genetic Toxicology — Rodent Dominant Lethal Test*

[4] OECD 479, *Genetic Toxicology — In vitro Sister Chromatid Exchange Assay in Mammalian Cells*

[5] OECD 480, *Genetic Toxicology — Saccharomyces cerevisiae — Gene Mutation Assay*

[6] OECD 481, *Genetic Toxicology — Saccharomyces cerevisiae —Miotic Recombination Assay*

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