INTERNATIONAL STANDARD

ISO 10993-10

Fourth edition
2021-11

Biological evaluation of medical devices —

Part 10: Tests for skin sensitization

Évaluation biologique des dispositifs médicaux —
Partie 10: Essais de sensibilisation cutanée

Reference number
ISO 10993-10:2021(E)

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

Published in Switzerland

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Foreword

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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 on 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 the following URL: 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 fourth edition cancels and replaces the third edition (ISO 10993-10:2010), which has been technically revised.

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

— this document now contains a description of skin sensitization testing only;

— Annex C on non-animal methods for skin sensitization (formerly Annex D) has been updated;

— the testing for irritation is now described in ISO 10993-23.

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.

Introduction

This document assesses possible contact hazards from chemicals released from medical devices, which may produce skin sensitization.

Some materials that are included in medical devices have been tested, and their skin sensitization potential has been documented. Especially for dental materials, sensitizing properties were reported —see Reference [51]. Other materials and their chemical components have not been tested and may induce adverse effects when in contact with human tissue. The manufacturer is thus obliged to evaluate each device for potential adverse effects prior to marketing.

Traditionally, small animal tests are performed prior to testing on humans to help predict human response (background information is provided in Annex D). Since 2015, several in chemico and in vitro assays have been validated and Organization for Economic Co-operation and Development (OECD) test guidelines released to assess the skin sentization potential of chemicals.[75][79][104] An overview of available alternative skin sensitization tests for neat chemicals is given in Annex C. These test methods, each developed to address a specific key event, can possibly not be sufficient alone to conclude on the presence or absence of skin sensitization potential of chemicals and should be considered in the context of integrated approaches such as integrated approaches to testing and assessment (IATA), combining them with other complementary information. Note that the in vitro and in chemico tests for skin sensitization in Annex C have thus far been validated only for neat chemicals and not for medical devices. To confirm that they are applicable for evaluation of the skin sensitization potential of medical devices, their assays need to be assessed and validated.

Where appropriate, the preliminary use of in vitro methods is encouraged for screening purposes prior to animal testing. To reduce the number of animals used, this document presents a step-wise approach, with review and analysis of test results at each stage. It is intended that, for regulatory submission, skin sensitization studies be conducted using GLP or ISO/IEC 17025 as applicable to the respective country and comply with regulations related to animal welfare. Statistical analyses of data are recommended and used whenever appropriate. This document includes important tools for the development of safe products and is intended for use by professionals, appropriately qualified by training and experience, who can interpret its requirements and judge the outcomes of the evaluation for each medical device, taking into consideration all the factors relevant to the device, its intended use and the current knowledge of the medical device provided by review of the scientific literature and previous clinical experience.

This document is based on numerous standards and guidelines, including OECD Guidelines, US Pharmacopoeia and the European Pharmacopoeia. It is intended to be the basic document for the selection and conduct of tests enabling the evaluation of dermal sensitization responses relevant to the safety of medical materials and devices.

Biological evaluation of medical devices —

Part 10:
Tests for skin sensitization

1 Scope

This document specifies the procedure for the assessment of medical devices and their constituent materials with regard to their potential to induce skin sensitization.

This document includes:

— details of in vivo skin sensitization test procedures;

— key factors for the interpretation of the results.

NOTE Instructions for the preparation of materials specifically in relation to the above tests are given in Annex A.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

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

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 medical device materials within a risk management process*

3 Terms and definitions

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

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

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

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

3.1
allergen
sensitizer
substance or material that is capable of inducing a specific hypersensitivity reaction upon repeated contact with that substance or material

3.2
allergic contact dermatitis
clinical diagnosis based on an observed immunologically-mediated cutaneous reaction to a substance

3.3
blank
extraction *vehicle* (3.17) not containing the *test material* (3.15), retained in a vessel identical to that which holds the test material and subjected to identical conditions to which the test material is subjected during its extraction

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

3.4
challenge
process following the *induction* (3.8) phase, in which the immunological effects of subsequent exposures in an individual to the inducing material are examined

3.5
elicitation
immunological reaction to exposure to a sensitizer in a previously sensitized individual

3.6
erythema
reddening of the skin or mucous membrane

3.7
extract
liquid that results from extraction of the *test sample* (3.16) or control

[SOURCE: ISO 10993-12:2021, 3.6]

3.8
induction
process that leads to the *de novo* generation of an enhanced state of immunological activity in an individual, after initial exposure to a specific material

3.9
irritant
agent that produces *irritation* (3.10)

3.10
irritation
localized non-specific inflammatory response to single, repeated or continuous application of a substance/material

Note 1 to entry: Skin irritation is a reversible reaction and is mainly characterized by symptoms like local *erythema* (3.6) (redness), swelling, itching, peeling, cracking and scaling of the skin.

3.11
negative control
well-characterized material or substance that, when evaluated by a specific test method, 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 include *blanks* (3.3), *vehicles* (3.17)/solvents and reference materials.

[SOURCE: ISO 10993-12:2021, 3.10, modified — Note 1 to entry has been replaced.]

3.12
oedema
swelling due to abnormal infiltration of fluid into the tissues

3.13
positive control
well-characterized material or substance that, 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

3.14
skin sensitization
T-cell mediated delayed-type hypersensitivity reaction induced by low molecular weight reactive chemicals (allergens) comprising two phases, induction and elicitation

Note 1 to entry: In humans, the responses can be characterized by pruritis, *erythema* (3.6), *oedema* (3.12), papules, vesicles, bullae or a combination of these. In other species, the reactions can differ and only erythema and oedema can be seen.

3.15
test material
material, device, device portion or component thereof that is sampled for biological or chemical testing

3.16
test sample
material, device, device portion, component, *extract* (3.7) or portion thereof that is subjected to biological or chemical testing or evaluation

3.17
vehicle
liquid used to moisten, dilute, suspend, *extract* (3.7) or dissolve the test substance/material

4 General principles — Step-wise approach

The available methods for testing sensitization were developed specifically to detect skin sensitization potential. Other types of adverse effects are generally not predicted by these tests.

This document requires a step-wise approach, considering that any stage can result in the conclusion that further testing for skin sensitization is not necessary:

a) literature and supplier information review, including chemical and physical properties, and information on the skin sensitization potential of any medical device constituent as well as structurally-related chemicals and materials; refer to ISO 10993-1 for details; conduct risk assessment based on existing information to determine whether skin sensitization risk is acceptable or whether further testing is necessary;

b) additional characterization and risk assessment, if needed, of the device material, involving chemical characterization and analysis of the test sample according to the general principles described in ISO 10993-18;

c) in vitro tests shall be considered in preference to in vivo tests in accordance with ISO 10993-2, and the replacement of the latter as new in vitro tests are scientifically validated and become reasonably and practicably available;

NOTE There are currently a number of internationally validated and accepted in vitro tests to detect the skin sensitization potential of chemicals; however, these in vitro tests are not yet validated for medical devices. Work is ongoing for some of these tests to qualify them for use with medical devices.

d) in vivo animal tests are only appropriate when test materials cannot be characterized and risk assessments cannot be undertaken using information obtained by the means set out in a), b) and c).

5 Pretest considerations

5.1 General

It is important to emphasize that pretest considerations can result in the conclusion that testing for skin sensitization is not necessary.

The requirements given in ISO 10993-1:2018, Clause 5, and the following apply.

In vivo, non-sterile samples shall be investigated by topical investigation only, as the possibility of microbial contamination of the test sample can confound the final assay interpretation. In cases where the sterility of a test sample cannot be guaranteed, but the sample is still considered to be free from microbial contamination, intradermal administration may be justified.

5.2 Types of material

5.2.1 Initial considerations

It shall be taken into consideration that during manufacture and assembly of medical devices, additional chemical components may be used as processing aids, e.g. lubricants or mould-release agents. In addition to the chemical components of the starting material and manufacturing process aids, adhesive/solvent residues from assembly and also sterilant residues or reaction products resulting from the sterilization process may be present in a finished product. Whether these components pose a risk depends on the leaching or degradation characteristics of the finished products. Those chemical components which have skin sensitization potential shall be identified.

5.2.2 Ceramics, metals and alloys

These materials are normally less complex than polymers and biologically derived materials in terms of the number of chemical constituents.

5.2.3 Polymers

The chemical composition of these materials is typically more complex than those in 5.2.2. A number of reaction products/impurities/additives/residual catalyst can be present and the degree or extent of polymerization can vary.

5.2.4 Biologically derived materials

These materials are inherently complex in their composition. They often also contain process residues, for example, cross-linkers and anti-microbial agents. Biological materials can be inconsistent from sample to sample.

The methods in this document have not been designed for testing of biologically derived materials and can therefore be less adequate. For example, the tests in this document do not consider cross-species sensitization.

5.3 Information on chemical composition

5.3.1 General

Full qualitative data on the chemical constituents of the material shall be established. Quantitative data on the chemical composition shall also be obtained. If quantitative data are not obtained, the rationale shall be documented and justified.

5.3.2 Existing data sources

Qualitative and quantitative information on the composition shall be obtained where possible from the supplier of the starting material.

For polymers, this often requires access to proprietary information; provision should be made for the transfer and use of such confidential information.

Qualitative information about any additional processing additives (e.g. mould-release agents) shall also be obtained from appropriate members of the manufacturing chain, including converters and component manufacturers.

If information on composition is incomplete, a literature study to establish the likely nature of the starting material and any additives is recommended, so as to assist in the selection of the most appropriate methods of analysis for the material concerned.

The chemical composition of finalized products shall be determined in accordance with ISO 10993-18.

NOTE The composition of ceramics, metals and alloys can be specified in accordance with ISO or ASTM international standards and/or can be specified by the user. However, in order to obtain full qualitative and quantitative details on composition, it can be necessary to request these from the supplier or manufacturer of the starting material and also from component manufacturers to ensure that processing aids are also identified. Material master files held by regulatory authorities are another source of data, where they are accessible.

6 Skin sensitization tests

6.1 Choice of test methods

In vitro and in chemico alternative approaches have been developed for neat chemicals using a combination of different assays to identify skin sensitizers. Several of these methods have been included in the OECD test guidelines (TG 442C[75], TG 442D[79] and TG 442E[104]) or in the OECD test guideline program[121] (see Annex C).

Together, the assays described in these test guidelines cover three key events of the now identified adverse outcome pathway (AOP) for skin sensitization, including the molecular initiating event (protein binding), induction of inflammation, and activation of dendritic cells. These test methods developed to address a specific key event can possibly not alone be sufficient to conclude on the presence or absence of skin sensitization potential of chemicals and should be considered in the context of integrated approaches such as IATA, combining them with other complementary information.

In accordance with ISO 10993-2, such integrated approaches shall be taken into consideration for assessing skin sensitization potential of neat chemicals. Whether these approaches are also applicable for medical devices or medical device extracts is not yet known. An overview of available alternative skin sensitization tests for neat chemicals is presented in Annex C.

There are currently three animal assays available for the determination of the skin sensitizing potential of chemicals. These include two guinea pig assays and one murine assay. The two guinea pig assays are the guinea pig maximization test (GPMT) and the closed-patch test (Buehler test). Of these two assays, the maximization test is the most sensitive method. See Reference [9]. The closed-patch test is suitable for topical products.

The murine local lymph node assay (LLNA) was internationally accepted as an OECD test guideline in 2010[33] for testing single chemicals as a stand-alone alternative to the guinea pig assays, and is now the preferred in vivo assay for chemicals. See References [19] and [32]. In some instances, guinea pig assays can be necessary for the evaluation of the sensitizing potential of certain test samples. Such can be true for certain metals (see Reference [44]) that can give false negative findings in the LLNA or skin

irritants that can give false positive findings, as well as high molecular weight substances, which do not penetrate the skin or substances that are not soluble in the recommended vehicles.

NOTE All three animal assays were developed for the detection of skin sensitizing potential of chemicals, i.e. contact dermatitis, delayed type (type IV) hypersensitivity.

In view of the provisions laid down in ISO 10993-2 on animal welfare requirements, when an in vivo assay is performed, the LLNA shall be taken into consideration. In addition to animal welfare considerations, the LLNA has the advantage of providing objective quantitative data.

6.2 Murine local lymph node assay

6.2.1 Principle

Following topical treatment of a test sample on the dorsum of the ears, the extent of lymphocyte proliferation is measured in the lymph nodes that drain the sites of application (ears). A response in cellular proliferation of threefold or more compared with the activity of the controls is the threshold for designating a test material as a sensitizer.

The LLNA shall be performed using a dose response approach when substances are used. For final products/medical devices, it can be sufficient to test only the undiluted extract.

NOTE References [15] to [44] contain representative LLNA publications. Laboratories conducting this assay are encouraged to review these and other relevant publications available.

6.2.2 Test sample preparation

The test sample shall be a liquid, suspension, gel or paste such that it can be applied to the ears of the mice. Where possible, a series of doses (dilutions) shall be investigated. Otherwise, the highest concentration prepared as a chemical solution or suspension or as an extract should be used. When a strong response in the LLNA is detected with an extract, a follow-up study evaluating multiple doses may be necessary to evaluate the possible skin sensitization potency of the extract. Systemic toxicity and excessive local skin irritation can invalidate the test results; these reactions should therefore be avoided. In certain circumstances, pre-testing can be necessary.

A commonly used vehicle for substances/chemicals is an acetone olive oil (AOO) 4:1 mixture. Liquid samples that are hydrophilic and/or do not adequately adhere to the skin of the ear should be modified to adhere to the test site. This can be obtained by adding a thickening agent like carboxy methyl cellulose or hydroxyethyl cellulose (with a density of 0,5 %) or by a surfactant such as Pluronic® L92[1)] with a volume fraction of 1 %. For water soluble chemicals, dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF) are preferred above the surfactant Pluronic® L92. See Reference [34]. Alternatively, other extract vehicles can be used, as mentioned. See Reference [33]. The effect of additions to the extract media and/or changes in vehicle composition shall be validated and documented. This can be done by experiments using weak to moderate skin sensitizers as commonly used as positive controls. In addition, spiking of the test sample with a positive control can be performed in order to demonstrate that the LLNA is still able to detect the presence of potential skin sensitizers in the prepared extract. Other fundamental aspects of test article extraction are specified in ISO 10993-12.

A separate extract shall be prepared for each daily application.

NOTE For polymeric materials, an optional extraction method is given in Annex B.

6.2.3 Animals and husbandry

Healthy female non-pregnant mice of the CBA/Ca, CBA/J or BALB/c strain shall be used, unless another strain is validated. See References [33], [41] and [42]. Several mouse strains have been reported as

1) Pluronic® L92 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.

acceptable (DBA/2, B6C3F1). See Reference [35]. The mice shall be 7 weeks to 12 weeks of age; the mice in each study shall be matched in age (within a one-week age range).

Husbandry and selection of animals shall be in accordance with ISO 10993-2. The mice, routinely acclimatized to the laboratory, shall be individually identified. For certain test samples, individual housing can be necessary. This shall be justified and documented.

Animals shall be uniquely identified by methods not to include ear punches or ear tags.

When group housing is performed, cross contamination and unwanted oral intake should be taken into consideration.

6.2.4 Test procedure

For chemicals, the LLNA is generally performed in a dose-response manner. For solid medical devices, samples to be tested shall be extracts. In these cases, only a single dose is available for testing. In general, the extract can be investigated undiluted. However, when the extract contains highly toxic components, this can result in a negative response in the LLNA due to toxicity. It is therefore recommended, to perform the LLNA in a dose-response manner and to dilute the extract when investigating cytotoxic extracts (see ISO 10993-5). In addition, when a strong response is detected in the LLNA, a dose response follow up can be conducted to evaluate the possible sensitization potency of the extract.

To ensure reproducibility and sensitivity, a test of a positive-control substance for skin sensitization shall be included by the testing laboratory in order to validate the test system and demonstrate a positive response. Well-known weak to moderate contact allergens (e.g. mercaptobenzothiazole, hexyl cinnamic aldehyde, or benzocaine), shall be used as positive control. The examples mentioned can possibly not be suitable for each vehicle used for sample preparation (e.g. water-based vehicle); in such cases, another positive control can be selected. ASTM F2148 indicates that in such circumstances formalin and 2,4-dinitrochlorobenzene (DNCB) should be used as positive controls. This shall be justified and documented.

While inclusion of a concurrent positive control group is recommended, there may be situations in which only periodic testing (i.e. at intervals ≤ 6 months) of the positive control test substance can be adequate. This is the case for laboratories that conduct the LLNA regularly (i.e. conduct the LLNA at a frequency of no less than once per month) and have an established historical positive control database that demonstrates the laboratory's ability to obtain reproducible and accurate results with positive controls. Adequate proficiency with the LLNA can be successfully demonstrated by generating consistent positive results with the positive control in at least 10 independent tests conducted within a reasonable period of time (i.e. less than one year).

The individual body weights shall be recorded at initiation and at the end of the study. In order to detect potential toxicity of the test sample, clinical observation shall be performed and recorded during the study.

Using a positive control only once every six months can have consequences for the results obtained in the previous six months period when this positive control shows a negative outcome. Reference [33] states that periodic testing (i.e. at intervals ≤ 6 months) of the positive control substance can be considered in laboratories that conduct the LLNA regularly (i.e. conduct the LLNA at a frequency of no less than once per month), and that have a history and a documented proficiency for obtaining consistent results with positive controls. It is important to realize that the decision to only include a positive control periodically instead of concurrently can have ramifications on the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false-negative result is obtained in the periodic positive control test, all negative test substance results obtained in the interval between the last acceptable periodic positive control test and the unacceptable periodic positive control test can be questioned. In order to demonstrate that the prior negative test substance results are acceptable, a laboratory can be expected to repeat all negative tests, which requires additional expenses and increased animal use.

6.2.5 Treatment groups

When the LLNA is performed, the data of a minimum of five mice per group shall be available for evaluation. Lymph node responses may be determined either by individual measurement or by measurement of pooled lymph node samples. For statistical analysis, individual measurement is preferred.

When only a single dose is available for evaluation, for example, an extract, a minimum of five mice shall be used for each group, when individual responses are measured.

Treatment groups shall be assigned to:

— blank of each type of vehicle employed (see Annex A);

— when appropriate, positive control for each vehicle employed;

— test groups for each extract vehicle employed.

When testing a single chemical or substance, the LLNA shall be performed in a dose-response manner. For other types of test and sample-like extracts, a dose-response evaluation can possibly not be feasible. When only one test group is employed, this shall be justified and documented.

NOTE When sufficient data have been collected to demonstrate consistency for the dose response of the positive control, a single dose can be included to demonstrate the sensitivity of the assay. See Reference [32].

The appropriate sample shall be applied to the dorsal side of both ears of designated mice at a dose of 25 µl/d for three consecutive days. Each day, observe the ears for signs of irritation that can interfere with interpreting results. See References [23], [27] and [29].

6.2.6 Determination of cellular proliferation and tissue preparation

The proliferating cells in the draining lymph nodes can be labelled by either a radioactive or fluorescent label. Radiolabels commonly used are ^{3}H-methyl thymidine and ^{125}I-iododeoxyuridine, while for fluorescence, fluorodeoxyuridine can be used.

At (72 ± 2) h after the last treatment, record individual mouse weights and administer intravenously the label for cell proliferation. Inject 0,25 ml of phosphate buffered saline (PBS) containing 740 KBq (20 µCi) units of radioactivity of ^{3}H-methyl thymidine into all test and control mice via the tail vein. For ^{125}I-iododexyuridine, inject 0,25 ml PBS containing 74 KBq (2 µCi), and for fluorodeoxyuridine inject 0,25 ml containing 10^{-5} mol/l into the tail vein. See Reference [33].

Other alternative procedures not requiring radiolabelling are available and should be considered [e.g. adenosine triphosphate (ATP) (OECD TG 442A [122]) determination (DA method), bromodeoxyuridide BrdU (OECD TG 442B [123]) determination (ELISA or FCM method)].

NOTE 1 For more information, see References [33], [36], [42], [43] and [49].

Euthanize the mice (5 ± 0,75) h after the administration of the labelling solution according to ISO 10993-2. Remove the draining auricular lymph node. Care shall be taken to avoid cross contamination of the tissue samples. The lymph nodes of each group may be pooled, or pairs of lymph nodes of each individual animal may be pooled. Data from each individual animal is preferred as it provides the variability between each animal in a group. Single cell preparations are prepared by gently pressing the lymph nodes through a 200 µm stainless steel wire mesh or nylon mesh over a container. Rinse the strainer with chilled PBS into the container to remove cells from the mesh filter. The container now contains the cell preparation. Cell preparations are washed twice by centrifugation and resuspended in PBS. Cells are precipitated with 5 % trichloroacetic acid (TCA) at (4 ± 2) °C for (18 ± 1) h. After a final centrifugation step, pellets are resuspended in 1 ml of TCA and transferred to scintillation vials

containing 10 ml of scintillation fluid for ^{3}H-counting, or transferred directly to a gamma counter for ^{125}I-counting. See References [21], [35] and [36].

NOTE 2 Alternatively, labelling and determination of cellular proliferation can be performed ex vivo. See References [37] and [38].

6.2.7 Results and interpretation

Measure the level of radioactivity in the lymph node cells in counts per minute per mouse (cpm/mouse). Convert counts per minute (cpm) to disintegration per minute (dpm). Calculate the mean and standard deviation of dpm for at least three counts for each animal or each group of mice. Subtract the background value from each result.

When using the individual sampling method continue to calculate the mean and standard deviation of the dpm for each group of five mice. Determine the stimulation index (SI) by dividing the mean test dpm by the blank dpm. An SI of three or more (≥3,0) shall be considered positive for designating a test sample as a sensitizer. See Reference [16].

Positive control samples shall produce an SI that is greater than or equal to 3,0.

For a valid study, the positive control shall be conducted either concurrently or within the previous six months. See Reference [33].

6.2.8 Test report

The test report shall include:

a) a description of the test material(s) or device;

b) the intended use/application of the test sample or material;

c) the International Standard used (including its year of publication);

d) a detailed description of the method employed in preparing the test sample or test material or device;

e) a description of the test animals;

f) the method of application to the ears;

g) a description of the method for determining cellular proliferation;

h) any deviations from the procedure;

i) records of the observations, including clinical and body weight observations;

j) an assessment of the results, including positive control;

k) the date of the test.

6.3 Guinea pig assays for the detection of skin sensitization

6.3.1 Principle

The two guinea pig assays currently used for the detection of sensitizing activity of chemicals and medical devices are the Buehler assay and the GPMT. Both assays consist of an induction and challenge phase, thus covering all stages of hypersensitivity.

6.3.2 Choice of test sample concentrations

Current guidelines for testing the sensitizing potential of single chemicals recommend using only one concentration for the test.

NOTE For polymeric materials, an optional extraction method is given in Annex B.

6.3.3 Induction

The sensitization rate is highly dependent on the induction dose, which in guinea pig assays shall be mildly to moderately irritating, where possible. If the irritation threshold is not reached, then the highest possible concentration shall be used. However, it shall not interfere with the health of the animals. The induction dose in the guinea pig assays is normally selected based on preliminary tests as described for the individual guinea pig tests (see 6.5.4.2). Undiluted extracts with the usual solvents need not be subjected to a preliminary test.

6.3.4 Challenge

The challenge concentration in the guinea pig assays is also based on preliminary tests on animals previously not exposed to the test material. The highest non-irritant dose, as determined in the pre-test evaluations, shall be used. The use of more than one concentration is advised for the challenge procedure, in order to facilitate the evaluation of the results.

6.4 Important factors affecting the outcome of the test

The biochemical and physical characteristics of the test sample can influence the choice of test, since the maximization test requires intradermal injections. If the test sample cannot be injected intradermally, an alternative method shall be used (i.e. topical application). The extract solutions shall be prepared under aseptic conditions. Non-sterile samples shall be investigated by topical investigation only, as the possibility of microbial contamination of the test sample can confound the final assay interpretation. In cases where the sterility of a test sample cannot be guaranteed, but the sample is still considered to be non-contaminated, intradermal administration may be justified.

The bioavailability of the test material is influenced by the choice of vehicle. Although there is no vehicle that is optimal for all materials, a vehicle that optimizes exposure by solubilization and penetration should be selected. The concentration of test material should be the highest possible without affecting the interpretation of results. Most investigators prefer the test sample as a solution because dispersions are prone to form a sediment, making exact dosing difficult. Examples of vehicles for intradermal injection include saline, propylene glycol and vegetable oils.

Variation among results from different laboratories can have several sources. The following factors in the test procedure are important:

— ambient test conditions;

— test site on the animal;

— method of hair removal (clipping/shaving or chemical depilation);

— type of patch design;

— quantity of test material;

— quality of occlusion;

— exposure time and reading of the animals.

Animal responsiveness also varies according to genetic factors and husbandry.

Comparison of the number of test animals having a positive response at challenge with the appropriate controls is essential for indication of a positive test result, though the severity of reaction will aid in the

interpretation. Borderline reactions at challenge are best clarified by rechallenge. Histopathology has not been shown to be of help in the evaluation of test results.

To ensure reproducibility and sensitivity, a test of a positive-control substance for skin sensitization shall be included by the testing laboratory in order to validate the test system and demonstrate a positive response. Positive controls should preferably be weak to moderate contact allergens (e.g. mercaptobenzothiazole, hexyl cinnamic aldehyde and benzocaine). However, when consistency has been demonstrated over a six-month or more extended period, a positive control does not need to be included in every assay; but may be run at regular intervals which shall not exceed six months. Ten animals are normally used as positive controls in Guinea pig assays. Fewer uinea pigs may be used when an assay with a positive control substance is performed more frequently than once every six months. At least five test animals with a positive substance and five control animals should be used. See Reference [1].

NOTE In order to get a positive response, dilutions of moderate to strong skin sensitizers (e.g. formaldehyde and DNCB) can be used. However, this does not guarantee that the assay can also identify responses of weak sensitizers in extracts of medical devices.

6.5 Guinea pig maximization test

6.5.1 Principle

An assessment is made of the potential of the material under test to produce skin sensitization in the guinea pig using the technique applied for single chemicals in the guinea pig maximization test.

6.5.2 Test sample preparation

The test sample shall be prepared as specified in Annex A. The concentration of the test sample shall be the highest possible without affecting interpretation of the results (see 6.5.4.2).

NOTE For polymeric materials, an optional extraction method is given in Annex B.

6.5.3 Animals and husbandry

Healthy young adult albino guinea pigs of either sex from a single outbred strain, weighing 300 g to 500 g at the start of the test, shall be used. If female animals are used, they shall be nulliparous and not pregnant.

The animals shall be acclimatized and cared for as specified in ISO 10993-2. Preliminary tests, when necessary, should be carried out on one set of animals to determine the optimal test concentrations (see 6.5.4.2).

If the test material is powder or liquid, a minimum of 10 animals shall be treated with the test sample and a minimum of five animals shall act as a control group. If a preliminary test is needed, it shall be carried out on additional animals.

When testing extracts, a minimum of 10 animals shall be treated with each extract, and a minimum of five animals shall act as a solvent control group. If a preliminary test is needed, it shall be carried out on additional animals.

If testing on 10 test animals and five control animals produces completely negative results, it is unlikely that testing of a further 10 plus five animals will give positive results. However, if any equivocal responses develop, a rechallenge (see 6.5.6) shall be carried out. If equivocal responses remain, conduct a new study on a minimum of 20 test animals and 10 control animals.

6.5.4 Test procedure

6.5.4.1 Preparation

Clip and shave the fur on all treatment sites prior to all steps in the test procedure.

6.5.4.2 Preliminary tests

The preliminary tests are intended to determine the concentration of the test sample to be used in the main test given in 6.5.4.3.

Undiluted extracts using the usual solvents (e.g. saline or vegetable oil) need not be subjected to preliminary testing.

For topical application, saturate an appropriate filter paper or absorbent gauze patch (4 cm^2 to 8 cm^2) or chamber with the test sample and apply the patch to the clipped skin under an occlusive dressing secured by a wrap and/or jacket around the torso of the animal.

When wrapping an animal for securing an occlusive dressing, care should be taken to allow for normal breathing of the animal. A flexible wrapping is preferred, which should be applied by well-trained personnel.

Topically apply a range of dilutions of the test sample to the flanks of at least two animals. Remove the occlusive dressings and patches after 24 h and assess the application sites for erythema and oedema using the Magnusson and Kligman grading scale given in Table 1. It can also be appropriate to explore dilution of the article by intradermal injection when using non-usual solvents.

For the topical induction phase in the main test, select the highest concentration that causes mild to moderate erythema but does not otherwise adversely affect the animal in accordance with ISO 10993-2. It should be recognized that for extracts of medical devices, the irritating threshold can possibly not be obtained. In such cases, the highest concentration possible shall be used (e.g. the undiluted extract). For final products/medical devices, it may be sufficient to test only the undiluted extract.

For the challenge phase in the main test, select the highest concentration that produces no erythema (see Table 1).

Table 1 — Magnusson and Kligman scale

Patch test reaction	Grading scale
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and/or swelling	3

Consideration shall be given to the pre-treatment of all animals by injection with Freund's complete adjuvant (FCA).

6.5.4.3 Main test

6.5.4.3.1 Intradermal induction phase

Make a pair of 0,1 ml intradermal injections of each of the following, into each animal, at the injection sites (e.g. sites A, B and C), as shown in Figure 1, in the clipped intrascapular region.

— Site A: A 50:50 volume ratio stable emulsion of Freund's complete adjuvant mixed with the chosen solvent. Use physiological saline (BP, USP or equivalent) or extraction vehicle/solvent.

— Site B: The test sample (undiluted extract); inject the control animals with the extraction vehicle/solvent alone.

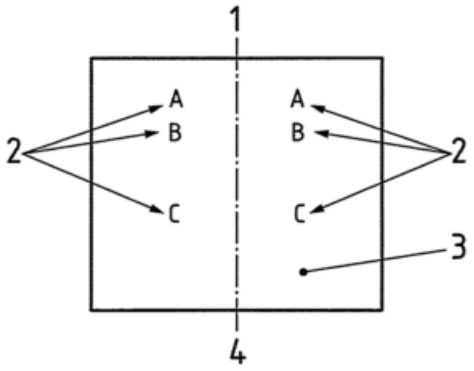
— Site C: The test sample at the concentration used at site B, emulsified in a 50:50 volume ratio stable emulsion of Freund's complete adjuvant and the solvent/extraction vehicle (the solution used at site A); inject the control animals with an emulsion of the blank liquid with adjuvant.

6.5.4.3.2 Topical induction phase

At (7 ± 1) d after the intradermal induction phase, administer the test sample by topical application to the intrascapular region of each animal, using a patch of area approximately 8 cm^2 (filter paper or absorbent gauze), so as to cover the intradermal injection sites (see Figure 1). Use the concentration selected in the preliminary test by topical application (if done; see 6.5.4.2). If the maximum concentration that can be achieved in 6.5.4.3.1 does not produce irritation, pretreat the area with 10 % sodium dodecyl sulfate (SDS) massaged into the skin (24 ± 2) h before the patch is applied. Any remaining SDS should be removed prior to application of the patches for the topical induction phase, as the remaining SDS can affect absorption of the extract. Secure the patches with an occlusive dressing. Remove the dressings and patches after (48 ± 2) h.

Freshly prepared extracts are preferred. If an extract is stored longer than (24 ± 2) h, then the stability of the extract under the conditions of storage should be verified.

Treat the control animals similarly, using the blank liquid alone.



Key

1 cranial end

2 0,1 ml intradermal injections (see 6.5.4.3.1)

3 clipped intrascapular region

4 caudal end

A, B, C injection sites

Figure 1 — Location of intradermal injection sites

6.5.4.3.3 Challenge phase

For the challenge phase testing the procedure described below shall be followed:

a) All test and control animals shall be challenged at (14 ± 1) d after completion of the topical induction phase

b) For undiluted test extracts using standard solvents (e.g. saline or vegetable oil):

   1) both test and control animals shall be dosed with both test and control extracts; or

2) control animals shall be dosed with undiluted vehicle and test animals with undiluted test extract.

For the challenge phase, the use of highest non-irritating concentration of the test sample is recommended. For testing with medical device extracts, preliminary testing to determine the highest non-irritating concentration of the extracts is not generally conducted. It is stated in 6.5.4.2 that undiluted extracts using the usual solvents need not be subjected to preliminary testing.

The use of option b),1) allows one to determine if any skin reaction observed in the test animals is due to irritation rather than sensitization. Per option b),1), both test and control animals are dosed with both test and control extracts. If any skin reactions to the test extracts are observed in the control animals, the reaction(s) can be due to irritation rather than sensitization as the control animals have not been previously exposed to the test extracts.

Per option b),2), the control animals are not dosed with the test extract and test animals are not dosed with the control extract. In the event of skin reactions observed in the test animals where no preliminary testing was conducted to determine the highest non-irritating concentration of the extracts, additional testing can be needed to rule out a false positive.

c) When non-standard solvents are used, all test and control animals shall be dosed with both the test and control extracts.

d) The extracts shall be administered by topical application to shaved sites that were not treated during the induction stage, such as the upper flank of each animal, using appropriate patches or chambers soaked in the test sample at the concentration selected in 6.5.4.3.1 for site B. In case the concentration selected in 6.5.4.3.1 for site B is not the highest non-irritating concentration, the highest non-irritating concentration determined in the preliminary test (see 6.5.4.2) shall be used. The extract volume used for saturation of patches/chambers shall be specified and justified.

e) The patches/chambers shall be secured with an occlusive dressing.

f) Dressings and patches shall be removed after (24 ± 2) h.

6.5.5 Observation of animals

Observe the appearance of the challenge skin sites of the test and control animals (24 ± 2) h and (48 ± 2) h after removal of the dressings. Use of natural or full-spectrum lighting is highly recommended in order to visualize the skin reactions. Describe and grade the skin reactions for erythema and oedema according to the Magnusson and Kligman grading scale given in Table 1 for each challenge site and at each time interval. It is highly recommended that reading be done without knowledge of the treatment, in order to minimize bias in the evaluation of the results.

Clipping and shaving should be done before each step in the test procedure (see 6.5.4.1). However, reshaving may not be necessary after the challenge or re-challenge when an animal is shaved the day before.

6.5.6 Evaluation of results

Magnusson and Kligman grades of 1 or greater in the test group generally indicate sensitization, provided grades of less than 1 are seen in control animals. If grades of 1 or greater are noted in control animals, then the reactions of test animals which exceed the most severe reaction in control animals are presumed to be due to sensitization. If the response is equivocal, rechallenge is recommended to confirm the results from the first challenge. The outcome of the test is presented as the frequency of positive challenge results in test and control animals.

Occasionally, the test group has a greater number of animals showing a response than the controls, although the intensity of the reaction is not greater than that exhibited by the controls. In these instances, a rechallenge can be necessary to define the response clearly. A rechallenge shall be carried

out 1 week to 2 weeks after the first challenge. The method used shall be as described for the first challenge, using a naïve side on the animal.

6.5.7 Test report

The test report shall include:

a) a description of the test material(s) or device;

b) the intended use/application of the test sample or material;

c) the International Standard used (including its year of publication);

d) a detailed description of the method employed in preparing the test sample or test material or device;

e) a description of the test animals;

f) the method of application to the test sites;

g) any deviations from the procedure;

h) how the sites were marked, and the readings performed;

i) records of the observations;

j) assessment of the results;

k) the date of the test.

6.6 Closed-patch test (Buehler test)

6.6.1 Principle

An assessment is made of the potential of the material under test to produce skin sensitization in guinea pigs.

6.6.2 Test sample preparation

The test sample shall be prepared as specified in Annex A. The concentration of test sample shall be the highest possible without affecting interpretation of the results (see 6.6.4.2). Where shape and size permit, topical devices (e.g. electrodes) can be used as they are.

6.6.3 Animals and husbandry

Healthy young adult albino guinea pigs of either sex from a single outbred strain, weighing 300 g to 500 g at the start of the test, shall be used. If female animals are used, they shall be nulliparous and not pregnant.

The animals shall be acclimatized and cared for as specified in ISO 10993-2. Preliminary tests should be carried out on one set of animals to determine concentrations of test sample (see 6.5.4.2).

For testing powders or liquids, a minimum of 10 animals shall be treated with the test material and a minimum of five animals shall act as a control group. If a preliminary test is needed, it shall be carried out on additional animals.

When testing extracts, a minimum of 10 animals shall be treated with each extract and a minimum of five animals shall act as a control for each solvent. If a preliminary test is needed, it shall be carried out on additional animals.

If testing in 10 test and five control animals produces completely negative results, it is unlikely that testing of a further 10 plus five animals will give positive results. However, if any equivocal responses develop, a rechallenge (see 6.5.6) shall be carried out. If equivocal responses remain, conduct a new study in a minimum of 20 tests and 10 control animals.

6.6.4 Test procedure

6.6.4.1 Preparation

Closely clip or shave the fur on all treatment sites prior to all steps in the test procedure.

6.6.4.2 Preliminary tests

The preliminary tests are intended to determine the concentrations of the test sample to be used in the main test described in 6.6.4.3.

Medical devices intended for topical use and undiluted extracts using the usual solvents need not be subjected to preliminary testing.

For all topical applications, saturate a patch (filter paper or absorbent gauze) of the appropriate dimensions with the test material or extract and apply the patch to the clipped area under an occlusive dressing for (6 ± 0,5) h. Restraint for each animal can be used to ensure occlusion of the test sites. If wrapping is used, its adequacy should be evaluated in every experiment. Assess the application sites for erythema and oedema using the Magnusson and Kligman grading given in Table 1 at (24 ± 2) h and (48 ± 2) h after patch removal.

Topically apply four concentrations of the test sample to the flanks of each of at least two animals using appropriate patches. Remove the occlusive dressings and patches after (6 ± 0,5) h. Assess the application sites for erythema and oedema using the Magnusson and Kligman grading given in Table 1 at (24 ± 2) h and (48 ± 2) h after patch removal.

Select:

a) for the induction phase in the main test, the highest concentration that causes no more than slight erythema but does not otherwise adversely affect the animals;

b) for the challenge phase in the main test, the highest concentration that produces no erythema.

6.6.4.3 Main test

6.6.4.3.1 Induction phase

Administer the test sample by topical application to the clipped left upper back region of each animal using appropriate patches soaked in the test sample at the concentration selected in 6.6.4.2 a). Remove the restrainer of any occlusive dressings and patches after (6 ± 0,5) h. Perform this procedure three days a week for three weeks. Treat the control animals similarly, using the blank liquid alone.

6.6.4.3.2 Challenge phase

At (14 ± 1) d after the last induction application, challenge all test and control animals with the test sample. Administer the test sample by a single topical application to a clipped untested area of each animal using appropriate patches soaked in the test sample at the concentration selected in 6.6.4.2 b). Remove the restrainer and occlusive dressings and patches after (6 ± 0,5) h.

6.6.5 Observation of animals

If necessary, at (24 ± 2) h after the start of the primary challenge or rechallenge exposure, either

a) depilate all of the animals with a commercial depilatory by placing the material on the test site and surrounding areas according to the manufacturer's instructions or

b) shave all of the animals on the challenge sites and surrounding areas.

Thoroughly wash the depilated area with warm water and dry the animals with a towel before returning them to their cages. At (24 ± 2) h after removal of the challenge patches and a minimum of 2 h after removal of the hair, grade the test sites using the scale given in Table 1. Repeat the grading (48 ± 2) h after removal of the challenge patch. Use of natural or full-spectrum lighting is highly recommended in order to visualize the skin reactions. It is highly recommended that reading be done without knowledge of the treatment, in order to minimize bias in the evaluation of the results.

6.6.6 Evaluation of results

The Magnusson and Kligman grading scale given in Table 1 shall be applied.

Grades of 1 or greater in the test group generally indicate sensitization, provided grades of less than one are seen on control animals. If grades of 1 or greater are noted on control animals, then the reactions of test animals which exceed the most severe control reaction are presumed to be due to sensitization. Rechallenge is recommended to confirm the results from the first challenge. The outcome of the test is presented as the frequency of positive challenge results in test and control animals.

Occasionally, the test group has a greater number of animals showing a response than the controls, although the intensity of the reaction is not greater than that exhibited by the controls. In these instances, a rechallenge can be necessary to define the response clearly. A rechallenge shall be carried out 1 week to 2 weeks after the first challenge. The method used shall be as described for the first challenge, using an untested area on the flank of the animal.

In these situations, a new negative control group is recommended.

6.6.7 Test report

The test report shall include:

a) a description of the test material(s) or device;

b) the International Standard used (including its year of publication);

c) the intended use/application of the test material(s) or device;

d) a detailed description of the method employed in preparing the test samples and materials;

e) a description of the test animals;

f) the method of application to the test sites;

g) any deviations from the procedure;

h) a description of how the sites were marked, and the readings performed;

i) records of the observations;

j) assessment of the results, including statistical methods;

k) the date of the test.

7 Key factors in interpretation of test results

The tests included in this document are important tools for development of safe products and shall be executed in accordance with appropriate quality assurance measures (e.g. ISO/IEC 17025 or GLP) and interpreted by trained personnel. Evidence shall be provided that those planning, conducting and interpreting the tests are appropriately qualified by training and experience for the tasks undertaken.

Evidence of skin sensitization by any method does not necessarily exclude the test material or device from use because the amount of test material in the test procedure can be exaggerated compared with actual conditions of use. An adverse finding using any of the described procedures indicates the need for further analysis that can allow risk assessment of intended human exposure.

Predictive test results generated by the procedures described in this document cannot stand alone and need to be interpreted alongside other information to assess the risk of a hypersensitivity reaction or other forms of immunotoxicity. A negative test result does not exclude the possibility that a product can cause allergic skin reactions. The results should be compared with other sources of information, such as:

— industry and consumer complaint data;

— experience with devices containing similar components;

— diagnostic test results in dermatologic clinics;

— retrospective epidemiologic data.

Annex A
(normative)

Preparation of materials for skin sensitization testing

A.1 General

The conduct of the tests and interpretation of the data from skin sensitization tests shall take into account the nature, degree, frequency, duration and conditions of exposure of the medical device in humans. One of the parameters critical to these tests is the preparation of the test material.

A.2 Materials for direct-contact exposure

A.2.1 Solid test materials

Solid materials that have appropriate physical states (e.g. sheets, films) shall be tested without modification. Prepare samples 2,5 cm × 2,5 cm with a thickness that approximates normal use but is not greater than 0,5 cm. Prepare suitable negative control samples in the same way. The negative control shall physically resemble the test material closely and should be non-irritating. Absorbent gauze may be used as a substitute if a more suitable control cannot be identified.

The solid can be pulverized, care being taken to ensure no contamination occurs during this process, or moistened sufficiently with water or a suitable non-irritant solvent to ensure good contact with the tissues. In the case of ceramics where pulverization is required, remember that the physico-chemical properties of the ceramic can be altered by reducing the ceramic to a powder, with potentially marked effects on biological activity.

Powders (e.g. super-absorbents) shall be tested by direct deposition or by making a paste in an appropriate solvent. A control using the same solvent shall be evaluated in parallel with the moistened, diluted or suspended test material.

NOTE Either surface area or particle size, or both, are important factors in biological responses such as phagocytosis, which plays an important role in inflammatory and immune responses.

A.2.2 Liquid test materials

Liquids shall be tested undiluted by direct deposition or, if impractical, diluted with an appropriate solvent. A control using the same solvent shall be evaluated in parallel with the diluted test liquid.

A.3 Extracts of test materials

A solid can be tested by preparing extracts from the solid. If extracts are tested, they shall be prepared as specified in ISO 10993-12, using polar, non-polar and/or additional solvents when appropriate. A rationale shall be provided for the adequacy of an extraction method.

A blank sample, using the extracting solvent, shall be evaluated in parallel with the extract of the test material.

NOTE For polymeric materials, an optional extraction method is described in Annex B.

A.4 Solvents

If the test material has to be extracted, diluted, suspended or moistened, a suitable non-irritant and non-sensitizing solvent shall be used. ISO 10993-12 provides a list of appropriate solvents.

A.5 Sterile test materials

If the final product is supplied in a sterile condition, then the test material shall be sterilized using the same process prior to testing. Products sterilized by ethylene oxide present a technical difficulty in that ethylene oxide and its reaction products can produce a biological response in the tests described in this document.

To enable differentiation between effects produced by the test material and those produced by ethylene oxide residuals when an irritant reaction is observed, consideration shall be given to evaluations of this response to the device pre- and post-ethylene oxide sterilization.