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Biological evaluation of medical devices —

Part 10: Tests for irritation and skin sensitization

Évaluation biologique des dispositifs médicaux —

Partie 10: Essais d'irritation et de sensibilisation cutanée

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Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 10993-10 was prepared by Technical Committee ISO/TC 194, *Biological evaluation of medical devices*.

This third edition cancels and replaces the second edition (ISO 10993-10:2002), which has been technically revised.

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

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

— *Part 2: Animal welfare requirements*

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

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

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

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

— *Part 7: Ethylene oxide sterilization residuals*

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

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

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

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

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

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

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

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

— *Part 18: Chemical characterization of materials*

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

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

Introduction

This part of ISO 10993 assesses possible contact hazards from chemicals released from medical devices, which may produce skin and mucosal irritation, eye irritation or skin sensitization.

Some materials that are included in medical devices have been tested, and their skin or mucosal irritation or sensitization potential has been documented. 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. More recently, *in vitro* tests as well as human tests have been added as adjuncts or alternatives. Despite progress and considerable effort in this direction, a review of findings suggests that currently no satisfactory *in vitro* test has been devised to eliminate the requirement for *in vivo* testing. Where appropriate, the preliminary use of *in vitro* methods is encouraged for screening purposes prior to animal testing. In order to reduce the number of animals used, this part of ISO 10993 presents a step-wise approach, with review and analysis of test results at each stage. An animal test is usually required prior to human testing.

It is intended that these studies be conducted using Good Laboratory Practice and comply with regulations related to animal welfare. Statistical analysis of data is recommended and should be used whenever appropriate.

This part of ISO 10993 is intended for use by professionals, appropriately qualified by training and experience, who are able to 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.

The tests included in this part of ISO 10993 are important tools for the development of safe products, provided that these are executed and interpreted by trained personnel.

This part of ISO 10993 is based on numerous standards and guidelines, including OECD Guidelines, U.S. Pharmacopoeia and the European Pharmacopoeia. It is intended to be the basic document for the selection and conduct of tests enabling evaluation of irritation and dermal sensitization responses relevant to safety of medical materials and devices.

Biological evaluation of medical devices —

Part 10:
Tests for irritation and skin sensitization

1 Scope

This part of ISO 10993 describes the procedure for the assessment of medical devices and their constituent materials with regard to their potential to produce irritation and skin sensitization.

This part of ISO 10993 includes:

a) pretest considerations for irritation, including *in silico* and *in vitro* methods for dermal exposure;

b) details of *in vivo* (irritation and sensitization) test procedures;

c) key factors for the interpretation of the results.

Instructions are given in Annex A for the preparation of materials specifically in relation to the above tests. In Annex B several special irritation tests are described for application of medical devices in areas other than skin.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10993-1:2009, *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 10993-9, *Biological evaluation of medical devices — Part 9: Framework for identification and quantification of potential degradation products*

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

ISO 10993-13, *Biological evaluation of medical devices — Part 13: Identification and quantification of degradation products from polymeric medical devices*

ISO 10993-14 *Biological evaluation of medical devices — Part 14: Identification and quantification of degradation products from ceramics*

ISO 10993-15, *Biological evaluation of medical devices — Part 15: Identification and quantification of degradation products from metals and alloys*

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

ISO 14155-1, *Clinical investigation of medical devices for human subjects — Part 1: General requirements*

ISO 14155-2, *Clinical investigation of medical devices for human subjects — Part 2: Clinical investigation plans*

3 Terms and definitions

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

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

3.2
blank
extraction vehicle not containing the test material, 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 The purpose of the blank control is to evaluate possible confounding effects due to the extraction vessel, vehicle and extraction process.

3.3
challenge
elicitation
process following the induction phase, in which the immunological effects of subsequent exposures in an individual to the inducing material are examined

3.4
dose
dosage
amount of test sample administered (e.g. mass, volume) expressed per unit of body weight or surface area

NOTE The terms are often used interchangeably (more commonly dosage).

3.5
erythema
reddening of the skin or mucous membrane

3.6
eschar
scab or discoloured slough of skin

3.7
extract
liquid or suspension that results from exposing a test or control material to a solvent under controlled conditions

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

3.9
irritant
agent that produces irritation

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

NOTE Skin irritation is a reversible reaction and is mainly characterized by local erythema (redness) of the skin.

3.11
necrosis
cell death as a direct result of irreversible changes caused by injury or disease

NOTE One should be aware that tissue repair will occur either resulting in complete functional restoration or resulting in scar formation.

3.12
negative control
any well-characterized material or substance that, when tested by a specific procedure, demonstrates the suitability of the procedure to yield a reproducible, appropriately negative, non-reactive or minimal response in the test system

NOTE In practice, negative controls include blanks, vehicles/solvents and reference materials.

3.13
oedema
swelling due to abnormal infiltration of fluid into the tissues

3.14
positive control
any 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

3.15
skin corrosion
production of irreversible damage to the skin, manifested as visible necrosis through the epidermis and into the dermis, following application of a test sample

EXAMPLE The action of a compound/chemical/test sample resulting in **ulceration** of skin (see 3.19).

3.16
skin sensitization
allergic contact dermatitis
immunologically mediated cutaneous reaction to a substance

NOTE In the human, the responses can be characterized by pruritis, erythema, oedema, papules, vesicles, bullae or a combination of these. In other species the reactions can differ and only erythema and oedema can be seen.

3.17
test material
material, device, device portion or component thereof that is sampled for biological or chemical testing

3.18
test sample
material, device, device portion, component, extract or portion thereof that is subjected to biological or chemical testing or evaluation

3.19
ulceration
open sore representing loss of superficial tissue

3.20
vehicle
liquid used to moisten, dilute, suspend, extract or dissolve the test substance/material

4 General principles — Step-wise approach

The available methods for testing irritation and sensitization were developed specifically to detect skin and mucous membrane irritation and skin sensitization potential. Other types of adverse effect are generally not predicted by these tests. For medical devices that are used as implants or external communicating devices, intradermal testing is more relevant in approaching the application and so for detection of irritation activity, intracutaneous testing shall be used as described in 6.4.

This part of ISO 10993 requires a step-wise approach, which shall include one or more of the following:

a) characterization of test material, involving chemical characterization and analysis of the test sample according to the general principles described in ISO 10993-9, ISO 10993-13, ISO 10993-14, ISO 10993-15 and ISO 10993-18;

b) literature review, including an evaluation of chemical and physical properties, and information on the irritation and sensitization potential of any product constituent as well as structurally-related chemicals and materials;

c) in accordance with ISO 10993-2, *in vitro* tests in preference to *in vivo* tests shall be considered, and replacement of the latter as new *in vitro* tests are scientifically validated and become reasonably and practicably available. For the evaluation of skin irritation and corrosion, *in vitro* alternatives are available for chemicals; there are currently no internationally validated and accepted *in vitro* tests to detect sensitizers;

d) *in vivo* animal tests: in order to ensure reproducibility and sensitivity, a test of a positive-control substance for irritation and skin sensitization shall be included in each assay by the testing laboratory in order to validate the test system and demonstrate a positive response; for guinea pig sensitization assays, 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.

NOTE 1 Sensitization can at the moment only be determined by an *in vivo* assay. This can be accomplished by using the local lymph node assay (LLNA) in mice, the occluded patch test in guinea pigs or the guinea pig maximization test (GPMT). For single chemicals the LLNA is now the preferred assay for determining the sensitizing potential. See References [69] [88] [90].

NOTE 2 *In vivo* animal tests are 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).

NOTE 3 For sensitization assays in guinea pigs, ten animals are normally used for positive control once every six months. Fewer guinea pigs can 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.

e) Non-invasive human tests/clinical trials; if the material has been demonstrated not to be an irritant, a sensitizer or toxic in animals, studies on skin irritation may then be considered in humans.

Clinical studies in accordance with ISO 14155-1, ISO 14155-2 and to ethics principles shall not be performed before the results of the other evaluations in a) to d) are known.

5 Pretest considerations

5.1 General

It is important to emphasise that pretest considerations may result in the conclusion that testing for irritation and/or sensitization is not necessary.

The requirements given in Clause 5 of ISO 10993-1:2009 and the following apply.

Non-sterile samples shall be investigated by topical investigation only, as the possibility of microbial contamination of the test sample could 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.

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 health hazard/risk depends on the leakage or degradation characteristics of the finished products. These components shall be taken into account for their potential irritation/sensitization activity.

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

These materials are normally chemically more complex than those in 5.2.2 in terms of composition. A number of reaction products/impurities/additives may be present and the completeness of polymerization may vary.

5.2.4 Biologically derived materials

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

The methods in this part of ISO 10993 have not been designed for testing of biologically derived materials and can therefore be less adequate. For example, the tests in this part of ISO 10993 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. Where relevant to biological safety, quantitative data 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 (for example, mould-release agents) shall also be obtained from appropriate members of the manufacturing chain, including converters and component manufacturers.

In the absence of any data on composition, 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 American Society of Testing Materials (ASTM) 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 Irritation tests

6.1 *In vitro* irritation tests

In vitro methods, the rat skin Transcutaneous Electrical Resistance (TER) test and the Human skin model test, have been internationally validated and accepted as alternative tests to assess the skin corrosivity of chemicals (OECD Guidelines 430[9] and 431[10]). National and international organizations continue working to develop and validate *in vitro* tests for skin irritancy in parallel with the search for alternative methods; others have been developing methods to quantify the responses of animals and humans in order to better define endpoints using non-invasive techniques (see F.1).

NOTE In 2007 the ECVAM Scientific Advisory Committee (ESAC) evaluated the validation process of an *in vitro* human skin model for the determination of skin irritation of chemicals. See Reference [101]. The use of *in vitro* human skin models for assessing the potential of chemicals to induce skin irritation is described in Annex D.

The *in vitro* test for skin irritation has so far been validated only for neat chemicals and not for medical device extracts. In order to apply these assays for the testing of irritation potential of medical devices, further validation for this specific area is essential.

6.2 *In vivo* irritation tests — Factors to be considered in design and selection of *in vivo* tests

Irritation testing of medical devices can be performed with the finished product and/or extracts thereof.

Factors affecting the results of irritation studies include the following:

a) the nature of the device used in a patch test;

b) the dose of the test material;

c) the method of application of the test material;

d) the degree of occlusion;

e) the application site;

f) the duration and number of exposures;

g) the techniques used in evaluating the test.

Additional background information is provided in Annex F.

Whilst flexibility with respect to the precise protocol followed allows the investigator to enhance the sensitivity of the test to suit conditions of use and population exposure, consistency in procedure contributes to comparability of test results with different materials and from different laboratories.

Provisions have been included in the test procedures for evaluation of devices and materials that will have repeated and/or prolonged exposure. The study shall be designed to exaggerate the anticipated contact (time and/or concentration) in the clinical situation. This shall be borne in mind during interpretation of the result.

If the pH of the test sample is $\leqslant$ 2,0 or $\geqslant$ 11,5, the material shall be considered an irritant and no further testing is required. However, experimental evidence suggests that acidity and alkalinity of the test material are not the only factors to be considered in relation to the capacity of a material to produce severe injury. The concentration of the test material, its period of contact, and many other physical and chemical properties are also important.

In exceptional cases where further risk characterization/assessment is needed, it might be necessary to test materials which are either an irritant or have a pH outside the range mentioned above. These cases shall be justified and documented.

6.3 Animal irritation test

6.3.1 Principle

An assessment is made of the potential of the material under test to produce dermal irritation in a relevant animal model.

The rabbit is the preferred test animal.

6.3.2 Test material

If the test material is a solid or a liquid, it shall be prepared as specified in Annex A.

The sensitivity of the assay shall be demonstrated. This can be done by including a positive control in the assay. However, the use of a positive control to confirm sensitivity is only warranted when the testing laboratory has not within the previous six months produced positive results using the test method.

NOTE A suitable positive control is sodium lauryl sulphate (SLS).

6.3.3 Animals and husbandry

Three healthy young adult albino rabbits of either sex from a single strain, weighing not less than 2 kg, shall be used. If irritation is anticipated, consideration shall be given to testing in one animal first. Unless a well-defined positive response [score greater than 2 for either erythema or oedema (see Table 1)] is observed, a minimum of two additional animals shall be used. If the response in the test using the minimum of three animals is equivocal, further testing shall be considered.

The animals shall be acclimatized and cared for as specified in ISO 10993-2.

Table 1 — Scoring system for skin reaction

Reaction	Irritation score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond exposure area)	4
Maximal possible score for irritation	8
Other adverse changes at the skin sites shall be recorded and reported.	

6.3.4 Test procedure

6.3.4.1 Preparation of animals

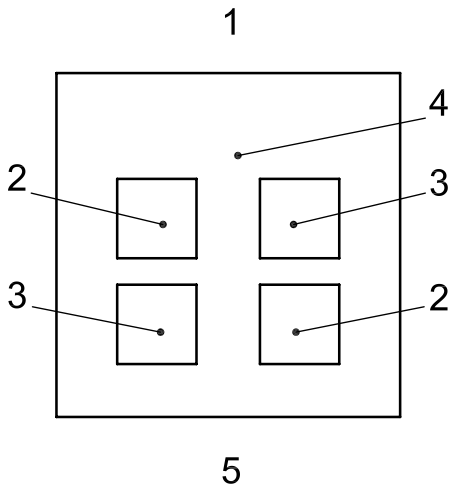
The condition of the skin is a critical factor. Use only animals with healthy intact skin. Fur is generally clipped within 24 h to 4 h of testing on the backs of the animals, a sufficient distance on both sides of the spine for application and observation of all test sites (approximately 10 cm × 15 cm). Fur may be re-clipped to facilitate observation and/or to accommodate repeated exposures. Depilatories may be used by trained technicians, if the process has been validated at the testing facility. If repeated exposure is required, follow the procedures in 6.3.4.2.1, 6.3.4.2.2 or 6.3.4.2.3, repeated for a maximum of 21 d.

6.3.4.2 Application of test sample

6.3.4.2.1 Application of powder or liquid sample

Apply 0,5 g or 0,5 ml of the test material directly to each test skin site as shown in Figure 1. For solid and hydrophobic materials, there is no need for moistening. If the material is a powder, it should be slightly moistened with water or other suitable vehicle before application (see Annex A).

Cover the application sites with a 2,5 cm × 2,5 cm non-occlusive dressing (such as an absorbent gauze patch) and then wrap the application site with a bandage (semi-occlusive or occlusive) for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites with permanent ink. Remove residual test material by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent, and careful drying.



Key

1 cranial end
2 test site
3 control site
4 clipped dorsal region
5 caudal end

Figure 1 — Location of skin application sites

6.3.4.2.2 Application of extracts and extract vehicle

Apply the appropriate extract(s) to the 2,5 cm × 2,5 cm absorbent gauze patches. Use a volume of extract sufficient to saturate the gauze, generally 0,5 ml per patch. Apply one patch on each side of the animal as shown in Figure 1. Apply a control patch of gauze moistened with the extract vehicle as shown in Figure 1.

Cover the application sites with a bandage (semi-occlusive or occlusive) for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites with permanent ink. Remove residual test material by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent and careful drying.

6.3.4.2.3 Application of solid sample

Apply the samples of the test material directly to the skin on each side of each rabbit as shown in Figure 1. Similarly, apply the control samples to each rabbit. When testing solids (which may be pulverized if considered necessary), the test material shall be moistened sufficiently with water or, where necessary, an alternative solvent, to ensure good contact with the skin (see Annex A). When solvents are used, the influence of the solvent on irritation of skin caused by the test material shall be taken into account.

Cover the application sites with 2,5 cm × 2,5 cm non-occlusive dressings (such as a gauze patch) and then wrap the application sites with a bandage (semi-occlusive or occlusive) for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites with permanent ink. Remove residual test material by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent and careful drying.

6.3.5 Observation of animals

6.3.5.1 General

Use of natural or full-spectrum lighting is highly recommended to visualize the skin reactions. Describe and score the skin reactions for erythema and oedema according to the scoring system given in Table 1, for each application site at each time interval, and record the results for the test report.

NOTE Histological or non-invasive techniques of evaluating the skin reaction(s) can assist in certain cases.

6.3.5.2 Single-exposure test

For single-exposure tests, record the appearance of each application site at (1 ± 0,1) h, (24 ± 2) h, (48 ± 2) h and (72 ± 2) h following removal of the patches. Extended observation can be necessary if there are persistent lesions, in order to evaluate the reversibility or irreversibility of the lesions over a period of time not exceeding 14 d.

6.3.5.3 Repeated-exposure test

Repeated exposure shall only be carried out after completion of an acute single-exposure test [after at least (72 ± 2) h of observation].

For repeated-exposure tests, record the appearances of the application site at (1 ± 0,1) h after removal of the patches and immediately prior to the next application. The number of exposures can vary.

After the last exposure, note the appearance of each application site at (1 ± 0,1) h, (24 ± 2) h, (48 ± 2) h and (72 ± 2) h following removal of the patches. Extended observation can be necessary if there are persistent lesions, in order to evaluate the reversibility or irreversibility of the lesions. This need not exceed a period of 14 d.

6.3.6 Evaluation of results

For single exposure tests, determine the primary irritation index (PII) as follows.

Use only (24 ± 2) h, (48 ± 2) h and (72 ± 2) h observations for calculations. Observations made prior to dosing or after 72 h to monitor recovery are not used in the determination.

After the 72 h grading, all erythema grades plus oedema grades (24 ± 2) h, (48 ± 2) h and (72 ± 2) h are totalled separately for each test sample and blank for each animal. The primary irritation score for an animal is calculated by dividing the sum of all the scores by 6 (two test/observation sites, three time points).

To obtain the primary irritation index for the test sample add all the primary irritation scores of the individual animals and divide by the number of animals (generally three).

When blank or negative control is used, calculate the primary irritation score for the controls and subtract that score from the score using the test material to obtain the primary irritation score.

For repeated exposure assays the primary irritation score for each animal shall be calculated according to the principle mentioned above, taking into consideration all evaluation points. For repeated exposure, determine the cumulative irritation index as follows.

Add together the irritation scores of all animals and divide by the total number of animals. This value is the cumulative irritation index.

The cumulative irritation index is compared with the categories of irritation response given in Table 2 and the appropriate response category is recorded for the report.

NOTE The categories of cumulative irritation index are based on the data relating the primary irritation index for chemicals in rabbits to the primary irritation response in humans for a number of chemicals that have been tested on both species.

For any response, record the maximum primary irritation score given in Table 1 for each animal, the time of onset of the response and the time to maximum response.

The primary or cumulative irritation index is characterized by number (score) and description (response category) given in Table 2. In case different extracts have been tested, the one giving the highest PII determines the response category.

Table 2 — Primary or cumulative irritation index categories in a rabbit

Mean score	Response category
0 to 0,4	Negligible
0,5 to 1,9	Slight
2 to 4,9	Moderate
5 to 8	Severe

6.3.7 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 material(s) or device;

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

d) a description of the test animals;

e) the method of application to the test sites and type (semi-occlusive or occlusive) of bandage material;

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

g) records of the observations;

h) number of exposures and intervals between them, when repeated exposures were carried out;

i) evaluation of the results.

6.4 Animal intracutaneous (intradermal) reactivity test

6.4.1 Introduction

For medical devices that are used as an implant, the use of an intracutaneous (intradermal) reactivity test is indicated. An assessment is made of the potential of the material under test to produce irritation following intradermal injection of extracts of the material.

6.4.2 Exclusion from test

Any material shown to be a skin, eye or mucosal tissue irritant or material with a pH $\leqslant$ 2,0 or $\geqslant$ 11,5 shall not be tested intradermally. In exceptional cases where further risk characterization/assessment is needed, it might be necessary to test materials which are either an irritant or have a pH outside the range mentioned above. These cases shall be justified and documented.

6.4.3 Test sample

The test sample shall be an extract prepared in accordance with Annex A. As there are multiple test sites on each animal, several test samples might be applied together with the appropriate negative controls or blank.

6.4.4 Animals and husbandry

Healthy young adult albino rabbits of either sex from a single strain, weighing not less than 2 kg, shall be used. The animals shall be acclimatized and cared for as specified in ISO 10993-2. A minimum of three animals shall initially be used to evaluate the test material. If irritation is anticipated, consideration shall be given to testing in one animal first. Unless a well-defined positive response [score greater than 2 for either erythema or oedema (see Table 1)] is observed, a minimum of two additional animals shall be used. If the response in the test using the minimum of three animals is equivocal, further testing shall be considered.

6.4.5 Test procedure

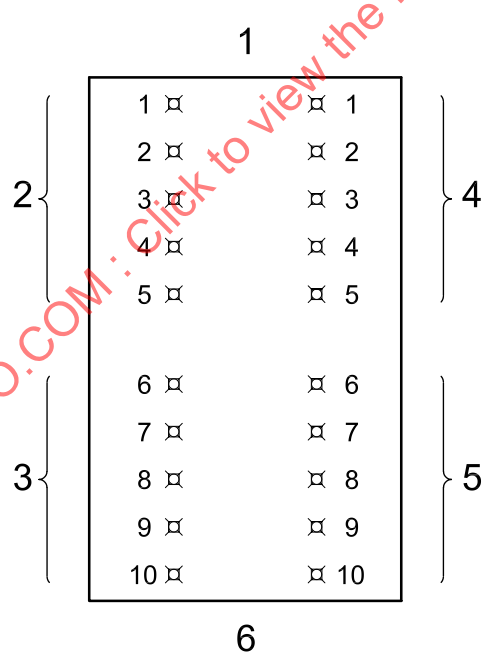
Within a 4 h to 18 h period before testing, closely clip the fur on the backs of the animals, allowing a sufficient distance on both sides of the spine for injection of the extracts.

Inject intracutaneously 0,2 ml of the extract obtained with polar or non-polar solvent at five sites on one side of each rabbit. Use the smallest needle appropriate to the viscosity of the test material for the intradermal injections.

An example of the arrangements of the injection sites is presented in Figure 2.

Similarly, inject 0,2 ml of the polar or non-polar solvent control on five sites of the contralateral side of each rabbit (for example, see Figure 2).

If other solvents are used, repeat the above steps for the extract obtained with the other solvents and the solvent controls.



Key

1 cranial end
2 0,2 ml injections of polar extract
3 0,2 ml injections of non-polar extract
4 0,2 ml injections of polar solvent control
5 0,2 ml injections of non-polar solvent control
6 caudal end

Figure 2 — Arrangement of injection sites

6.4.6 Observation of animals

Note the appearance of each injection site immediately after injection and at (24 ± 2) h, (48 ± 2) h and (72 ± 2) h after injection.

Grade the tissue reaction for erythema and oedema according to the system given in Table 3 for each injection site and at each time interval observed, and record the results.

NOTE Intradermal injection of oil frequently elicits an inflammatory response.

Intravenous injection of an appropriate vital dye, such as Trypan blue or Evans blue, might be undertaken at the (72 ± 2) h reading to assist in evaluation of the response by delineating the area of irritation.

Non-invasive techniques might be used to assist in the evaluation if they are available.

Table 3 — Grading system for intracutaneous (intradermal) reactions

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond exposure area)	4
Maximal possible score for irritation	8
Other adverse changes at the injection sites shall be recorded and reported.	

6.4.7 Evaluation of results

After the (72 ± 2) h grading, all erythema grades plus oedema grades (24 ± 2) h, (48 ± 2) h and (72 ± 2) h are totalled separately for each test sample or blank for each individual animal. To calculate the score of a test sample or blank on each individual animal, divide each of the totals by 15 (3 scoring time points × 5 test or blank sample injection sites). To determine the overall mean score for each test sample and each corresponding blank, add the scores for the three animals and divide by three. The final test sample score can be obtained by subtracting the score of the blank from the test sample score. The requirements of the test are met if the final test sample score is 1,0 or less. If at any observation period the average reaction to the test sample is questionably greater than the average reaction to the blank, repeat the test using three additional rabbits. The requirements of the test are met if the final test sample score is 1,0 or less.

NOTE The blank control sample is either the polar or the non-polar solvent control as mentioned in Figure 2.

6.4.8 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 material(s) or device;

c) a detailed description of the method employed in preparing the test samples;

d) a description of the test animals;

e) the method of injection;

f) how the site readings were performed;

g) a record of the observations;

h) an assessment of the results.

6.5 Human skin irritation test

6.5.1 Introduction

At present, the prediction of human cutaneous irritation for the purpose of hazard identification relies primarily on the use of experimental animals (see Annex F). There are, however, problems in extrapolating from animals to humans. For chemicals to which human exposure is high (e.g. cosmetics and detergents), risk assessments are performed using human skin patch tests.

Human studies can serve several purposes:

a) direct identification of human hazard by testing chemicals in humans rather than in laboratory animals;

b) provision of risk assessment of certain chemicals to which human exposure is high;

c) facilitation of extrapolation to humans of data obtained previously from laboratory animal studies.

This part of ISO 10993 allows skin irritation data to be obtained directly from humans for purposes of hazard identification. Its aim is to determine whether a material presents a significant skin irritation hazard following acute exposure.

Clinical tests shall be performed in accordance with ISO 14155-1 and ISO 14155-2. Additional specific requirements for clinical tests are described in Annex C.

NOTE F.1 gives further information on irritation tests.

6.5.2 Initial considerations

Adequate information on the toxicity profile of the material and (where relevant) its constituent chemicals, including percutaneous absorption data, shall be available to indicate that the study does not present any significant health risk.

Materials shall not be tested on humans if:

a) they have been shown to be irritant in a predictive assay, either *in vitro* or *in vivo*;

NOTE In certain situations it might be necessary to perform additional testing of irritant samples/extracts of products in humans in order to further characterize the potential human risk.

b) they have been shown to be corrosive in a predictive assay, either *in vitro* or *in vivo*;

c) potential corrosivity for human skin can be predicted on the basis of structure/activity relationships and/or physicochemical properties such as strong acid or alkaline reserve;

d) they present a risk of skin or respiratory tract sensitization;

e) they present any acute toxicity hazard under test conditions;

f) they present any genotoxic, reproductive or carcinogenic hazard.

Further requirements and guidance on the selection of human volunteers can be found in Annex C and Annex F.

7 Skin sensitization tests

7.1 Choice of test methods

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. So far, the two most commonly used methods for testing for skin sensitization are the Guinea Pig Maximization Test (GPMT) and the closed-patch test (Buehler test). Of these the maximization test is the most sensitive method. See Reference [51]. The closed-patch test is suitable for topical products.

The murine Local Lymph Node Assay (LLNA) was internationally accepted for testing single chemicals as a stand-alone alternative to the guinea pig assays, and is now the preferred assay for chemicals. See References [69] [88] [91]. In some instances guinea pig assays can be necessary for the evaluation of the sensitizing potential of certain test samples. Such might be true in the case of false negatives, false positives, certain metals and high molecular weight substances, which do not penetrate the skin. One should be aware that irritant activity can also result in positive lymph node responses.

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

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

7.2 Murine Local Lymph Node Assay (LLNA)

7.2.1 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 The Bibliography contains representative LLNA publications. Laboratories conducting this assay are encouraged to review these and other relevant publications available. In addition, *in vitro* alternatives for the LLNA are currently being developed. For up-to-date information for these developments, references to the ECVAM, ICCVAM and JaCVAM websites are provided in the Bibliography. See References [114] [115] [116]).

7.2.2 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. 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 an agent like carboxy methyl cellulose or hydroxyethyl cellulose (0,5 % w/v). For water soluble chemicals, dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF) are preferred above the surfactant Pluronic® L92. See Reference [89]. Alternatively, other extract vehicles can be used, as mentioned. See Reference [88]. The effect of additions to the extract media and/or changes in vehicle composition shall be validated and documented. This might be done by experiments using weak to moderate sensitizers as commonly used as positive control. In addition, spiking of the test sample with a positive control might be performed in order to demonstrate that the prepared extract is still able to detect the presence of potential sensitizers. The methods of extraction are described in ISO 10993-12.

For each administration, a separate extract shall be prepared.

NOTE For polymers, information on a specific method for preparation of extracts is given in Annex E.

7.2.3 Animals and husbandry

Healthy female mice of the CBA/Ca or CBA/J strain shall be used, unless another strain is validated. See References [88] [96]. Several mouse strains have been reported as acceptable (DBA/2, B6C3F1, BALB/c). See Reference [90]. The mice shall be non-pregnant and eight to twelve weeks of age; the mice in each study shall be matched in age (within a one-week age range). Male mice might be used if shown to be equivalent to the female sensitivity level.

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.

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

7.2.4 Test procedure

For chemicals, the LLNA is generally performed in a dose-response manner. For medical devices, samples to be tested may 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, when investigating highly toxic extracts (see ISO 10993-5) to perform the LLNA in a dose-response manner and to dilute the extract.

In order to ensure reproducibility and sensitivity of the test procedure, tests with well-known weak to moderate contact allergens, e.g. mercaptobenzothiazole, hexyl cinnamic aldehyde and benzocaine, shall be included in each assay. The examples mentioned might not be suitable for each vehicle used for sample preparation (i.e. water based vehicle); in such cases, another positive control might be selected. This shall be justified and documented. When the assay is performed frequently, positive controls do not have to be included in each assay, however they shall be included at least once every six months.

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.

NOTE 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 [88] states that periodic testing (i.e. at intervals $\leqslant$ 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 could 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 could be questioned. In order to demonstrate that the prior negative test substance results are acceptable, a laboratory could be expected to repeat all negative tests, which would require additional expenses and increased animal use.

7.2.5 Treatment groups

When the LLNA is performed, the data of a minimum of four 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, e.g. 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 might 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 might be included to demonstrate the sensitivity of the assay. See Reference [88].

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 might interfere with interpreting results. See References [73] [82] [84].

7.2.6 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 might be used.

At (72 $\pm$ 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 20 µCi (740 KBq) 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 2 µCi (74 KBq), and for fluorodeoxyuridine inject 0,25 ml containing 10^{-5} mol/l into the tail vein. See Reference [88].

Other alternative procedures not requiring radiolabelling are available and may be used when justified [e.g. bromodeoxyuridide BrdU, adenosine triphosphate (ATP) determination (DA method)].

NOTE 1 For more information, see References [88] [91] [100] [114].

Euthanize the mice (5 ± 0,75) h after the administration of the labelling solution. 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. Single cell preparations are prepared by gently pressing the lymph nodes through a 200 µm stainless steel wire mesh or nylon mesh. Data from each individual animal is preferred as it provides the variability between each animal in a group. 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 Reference [70] [90] [91].

NOTE 2 Alternatively, labelling and determination of cellular proliferation can be performed *ex vivo*. See References [92] [93].

7.2.7 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 (only for individual sampling method) of the cpm or dpm for each group of mice. See Reference [88]. Subtract the background value from each result.

Determine the Stimulation Index (SI) by dividing the test cpm or dpm by the blank cpm or dpm. An SI of three or more ($\geqslant$ 3,0) shall be considered positive for designating a test sample as a sensitizer. See Reference [64].

Positive control samples shall produce an SI of $\geqslant$ 3,0.

For a valid study, the positive control shall be conducted either concurrently or within the previous six months. See Reference [88].

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

d) a description of the test animals;

e) method of application to the ears;

f) description of method for determining cellular proliferation;

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

h) assessment of the results, including positive control.

7.3 Guinea pig assays for the detection of skin sensitization

7.3.1 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.

7.3.2 Choice of test sample concentrations

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

NOTE Information on a specific method for preparation of extracts from polymeric test materials is given in Annex E.

7.3.3 Induction

Sensitization rate is highly dependent on 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. Undiluted extracts with the usual solvents for parenteral dosing need not be subjected to a preliminary test.

7.3.4 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 (see F.2).

7.4 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. The extract solutions shall be prepared under aseptic conditions. Non-sterile solutions should not be used for intradermal applications.

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, propyleneglycol 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.

Assays with positive controls shall be performed regularly in order to ensure reproducibility and sensitivity of the test procedure. Positive controls should preferably be weak to moderate contact allergens, e.g. mercaptobenzothiazole, hexyl cinnamic aldehyde and benzocaine. Positive controls shall be performed at least once every six months. See Reference [6].

NOTE In order to get a positive response, dilutions of moderate to strong 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.

7.5 Guinea pig maximization test (GPMT)

7.5.1 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.

7.5.2 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 7.5.4.2).

NOTE Polymer information on a specific method for preparation of extracts is presented in Annex E.

7.5.3 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 7.5.4.2).

If the test material is powder or liquid, a minimum of ten 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.

For testing extracts, a minimum of ten animals shall be treated with the test sample, 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 ten test and five control animals is completely negative, it is unlikely that testing of a further ten plus five animals will give positive results. However, if any equivocal responses develop, rechallenge (see 7.5.6) shall be carried out. If equivocal responses remain, conduct a new study on a minimum of 20 test and ten control animals.

7.5.4 Test procedure

7.5.4.1 Preparation

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

For intradermal injections, inject 0,1 ml per site.

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

NOTE 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.

7.5.4.2 Preliminary tests

The preliminary tests are intended to determine the concentration of the test sample to be used in the main test in 7.5.4.3.

Undiluted extracts using the usual solvents need not be subjected to preliminary testing.

Topically apply a range of dilutions of the test sample to the flanks of at least three 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 4.

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. It should be recognised that for extracts of medical devices, the irritating threshold may 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.

Table 4 — 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) in order to evaluate the possibility of hyperreactive skin status during the main test and thus interference with the readings.

7.5.4.3 Main test

7.5.4.3.1 Intradermal induction phase

Make a pair of 0,1 ml intradermal injections of each of the following, into each animal, at the injection sites (A, B and C), as shown in Figure 3, 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) for water-soluble materials.

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

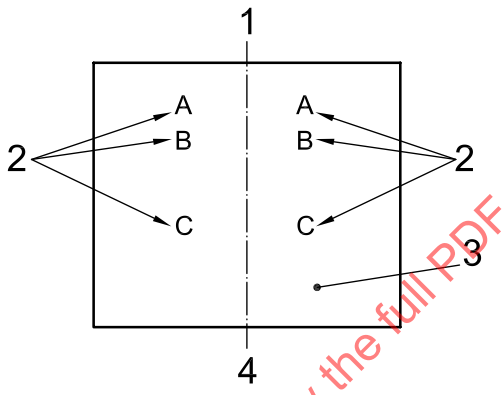
Site C: The test sample at the concentration used at site B, emulsified in a 50:50 volume ratio stable emulsion of Freund's complete adjuvant and the solvent (50 %); inject the control animals with an emulsion of the blank liquid with adjuvant.

7.5.4.3.2 Topical induction phase

At (7 ± 1) d after completion of 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 (Figure 3). Use the concentration selected in 7.5.4.3.1 for site B. If the maximum concentration that can be achieved in 7.5.4.3.1 does not produce irritation, pretreat the area with 10 % sodium dodecyl sulfate massaged into the skin (24 ± 2) h before the patch is applied. 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 7.5.4.3.1)
3 clipped intrascapular region
4 caudal end

Figure 3 — Location of intradermal injection sites

7.5.4.3.3 Challenge phase

At (14 ± 1) d after completion of the topical induction phase, challenge all test and control animals with the test sample. Administer the test sample and a blank by topical application to 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 7.5.4.3.1 for site C. Dilutions of this concentration might also be applied to other untreated sites in a similar manner. Secure with an occlusive dressing. Remove the dressings and patches after (24 ± 2) h.

7.5.5 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 given in Table 4 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.

7.5.6 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 might 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 naive side on the animal.

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

d) a description of the test animals;

e) the method of application to the test sites;

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

g) records of the observations;

h) assessment of the results.

7.6 Closed-patch test (Buehler test)

7.6.1 Principle

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

7.6.2 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 7.6.4.2). Where shape and size permit, topical devices (e.g. electrodes) might be used as they are.

7.6.3 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 7.5.4.2).

For testing powders or liquids, a minimum of ten 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.

For testing extracts, a minimum of ten 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 ten test and five control animals is completely negative, it is unlikely that testing of a further ten plus five animals will give positive results. However, if any equivocal responses develop, rechallenge (see 7.5.6) shall be carried out. If equivocal responses remain, conduct a new study in a minimum of 20 tests and ten control animals.

7.6.4 Test procedure

7.6.4.1 Preparation

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

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 might be used to ensure occlusion of the test sites. If wrapping is used, its adequacy should be evaluated in every experiment.

NOTE 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.

7.6.4.2 Preliminary tests

The preliminary tests are intended to determine the concentrations of the test sample to be used in the main test described in 7.6.4.3.

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

Topically apply four concentrations of the test sample to the flanks of each of at least three 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 4 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.

7.6.4.3 Main test

7.6.4.3.1 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 7.6.4.2 a). Remove the restrainer of any occlusive dressings and patches after (6 ± 0,5) h. Perform this procedure on three days a week for three weeks. Treat the control animals similarly, using the blank liquid alone.

7.6.4.3.2 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 7.6.4.2 b). Remove the restrainer and occlusive dressings and patches after (6 ± 0,5) h.

7.6.5 Observation of animals

At (24 ± 2) h after 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. A minimum of 2 h after removal of hair, grade the test sites using the scale given in Table 4. 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.

7.6.6 Evaluation of results

The Magnusson and Kligman grading scale given in Table 4 is applied.

Grades of 1 or greater in the test group generally indicate sensitization, provided grades of less than 1 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.

7.6.7 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 material(s) or device;

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

d) a description of the test animals;

e) the method of application to the test sites;

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

g) records of the observations;

h) assessment of the results, including statistical methods.

8 Key factors in interpretation of test results

The tests included in this part of ISO 10993 are important tools for development of safe products, provided that they are executed and interpreted by trained personnel.

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

Predictive test results generated by the procedures described in this part of ISO 10993 cannot stand alone. A negative test result does not always exclude the possibility that a product might cause allergic skin reactions. Both positive and negative test results in any of the assays should be scrutinized by rigorous follow-up in order to minimize the likelihood of false positive or false negative results. The results should be validated by comparison 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 irritation/sensitization testing

A.1 General

The conduct of the tests and interpretation of the data from irritation/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 and of 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-irritant. Absorbent gauze may be used as a substitute if a more suitable control cannot be identified.

The solid might 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 Surface area and/or particle size 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 might be tested by preparing extracts from the solid. If extracts are tested, they shall be prepared as described 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.

A.4 Solvents

If the test material has to be extracted, diluted, suspended or moistened, a suitable non-irritant 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 part of ISO 10993.

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.

Annex B
(normative)

Special irritation tests

B.1 General

The following special irritation tests exist. These tests are relevant for medical devices intended to be applied to specific areas. If used, a rationale shall be provided for the choice of the test method.

B.2 Ocular irritation test

B.2.1 General

The ocular irritation test should only be considered if safety data cannot be obtained by other means, and only for materials that will come into contact with the eye or eyelid.

Recently, four *in vitro* alternative test systems were evaluated by ICCVAM, two of which were sufficiently developed to replace *in vivo* animal testing for identifying severe irritants and corrosives. These assays are the Bovine Corneal Opacity and Permeability (BCOP) test method, and the Isolated Chicken Eye (ICE) test method. See Reference [107]. For weak irritants, an *in vivo* assay can still be necessary.

B.2.2 Principle

An assessment is made of the potential of the material under test to produce ocular irritation.

B.2.3 Exclusion from test

Materials and/or final products that have demonstrated definite corrosion or severe irritation in a dermal study shall no longer be tested for eye irritation. Any material shown to be a skin irritant or those with a pH $\leqslant$ 2,0 or $\geqslant$ 11,5 should not be tested but should be labelled as a potential eye irritant.

In exceptional cases where further risk characterization/assessment is needed, it might be necessary to test materials that are minimally irritant. These cases shall be justified and documented.

B.2.4 Test material

If the test material is a liquid, instil 0,1 ml, undiluted, into the lower conjunctival sac of one eye.

If the test material is a solid or granular product, grind it to a fine dust. When gently compacted, instil that amount which occupies a volume of 0,1 ml and does not weigh more than 100 mg into the lower conjunctival sac of one eye.

NOTE Some products are not suitable for testing directly in the eye. Mechanical damage can result in making the test useless.

If the test material is contained in a pump spray, expel and instil 0,1 ml as for liquids.

If the test material is contained in an aerosol container, examine by either

a) spraying a single burst of 1 s duration at a distance of 10 cm directed at the open eye or

b) expelling the aerosol into a cool container and treating as for a liquid.

If the test material is such that it can only be applied as an extract, prepare extracts as described in Annex A. Instil a 0,1 ml aliquot of the extract into the lower conjunctival sac of one eye.

Under conditions identical with those used above, prepare a blank liquid, using both the polar and the non-polar solvent, in the absence of the test material.

B.2.5 Animals and husbandry

Healthy young adult albino rabbits of either sex from a single strain, weighing 2 kg to 3 kg, shall be used.

The animals shall be acclimatized and cared for as specified in ISO 10993-2.

One animal shall initially be used to evaluate the test material. A well-defined positive response (see Table B.1) in one animal obviates the need for additional testing.

When no response is observed for solid or liquid materials, a minimum of two further animals shall be used. For extracts, a minimum of two further animals per extract shall be used.

If the response in the test using the minimum of three animals is equivocal or not clear, additional testing shall be considered.

B.2.6 Test procedure

No longer than 24 h before commencement of the test, visually examine both eyes of each rabbit for evidence of ocular abnormality. If either eye shows any abnormality, the rabbit shall be replaced.

When the eyes are examined, sodium fluorescein 2 % BP (British Pharmacopoeia) may be used in order to visualize any corneal damage. The use of an ophthalmoscope, hand slit-lamp or other suitable device is recommended.

Instil the test sample as specified in B.2.4 in one eye.

Following instillation hold the eyelids together for approximately 1 s.

The contralateral eye of each animal serves as control and should be treated with blank liquid when an extract is tested.

If repeated exposure to the material is anticipated and the test material has not demonstrated a significant response in the acute test, a repeat-exposure study may be conducted. Repeated exposure shall only be carried out after completion of the acute exposure test [after at least (72 ± 2) h]. The duration of the exposure should bear resemblance to the length of use of the test material/device in the clinical situation.

B.2.7 Observation of animals

For animals receiving a single instillation of test material, examine both eyes of each animal approximately (1 ± 0,1) h, (24 ± 2) h, (48 ± 2) h and (72 ± 2) h after instillation.

Extended observation may be necessary if there are persistent lesions, in order to determine the progress of the lesions or their reversal; this need not exceed 21 d. Extended observation cannot be justified for animals with severe lesions.

NOTE ISO 9394[25] gives guidelines for contact lens testing that requires 21 d exposure for 8 h per day. This is an exception to the guidelines.

Grade and record any reactions observed in accordance with the scale for grading ocular lesions given in Table B.1.

Table B.1 — System for grading ocular lesions

Reaction	Numerical grading
1. Cornea	
Degree of opacity (most dense area)	
No opacity	0
Scattered or diffuse areas, details of iris clearly visible	1[a]
Easily discernible translucent areas, details of iris slightly obscured	2[a]
Opalescent areas, no details of iris visible, size of pupil barely discernible	3[a]
Opaque, detail of iris not visible	4[a]
Area of cornea involved	
One-quarter (or less), not zero	0
Greater than one-quarter, but less than half	1
Greater than half, but less than three-quarters	2
Greater than three-quarters, up to whole area	3
2. Iris	
Normal	0
Folds above normal, congestion swelling, circumcorneal injection (any or all or combination of these), iris still reacting to light (sluggish reaction is positive)	1[a]
No reaction to light, haemorrhage, gross destruction (any or all of these)	2[a]
3. Conjunctivae	
Redness (refers to palpebral and bulbar conjunctiva excluding cornea and iris)	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2[a]
Diffuse beefy red	3[a]
Chemosis	
No swelling	0
Any swelling above normal (include nictitating membrane)	1
Obvious swelling with partial eversion of lids	2[a]
Swelling with lids about half-closed	3[a]
Swelling with lids about half-closed to completely closed	4[a]
Discharge	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of lids and hairs, and considerable area around the eye	3
[a] Positive result.	

For animals receiving multiple instillations of test material, examine both eyes of each animal immediately before and approximately (1 ± 0,1) h after each instillation.

If there is evidence of irritation after the last treatment, the observations may be extended. Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation, in order to determine the progress of the lesions and their reversibility.

Grade and record any reactions observed in accordance with Table B.1.

Immediately withdraw an animal from the study and humanely euthanize it if, at any time, it shows:

— very severe ocular damage (e.g. sloughing and ulceration of conjunctival membrane, corneal perforation, blood or pus in the anterior chamber);

— blood-stained or purulent discharge;

— significant corneal ulceration.

Withdraw from the study any animal showing maximum effects on the grading system given in Table B.1, i.e.

— absence of a light reflex (iridial response grade 2) or corneal opacity (grade 4) without evidence of recovery within (24 ± 2) h or

— maximum conjunctival inflammation (chemosis grade 4 together with redness grade 3) without evidence of recovery within (48 ± 2) h

and sacrifice it humanely.

B.2.8 Evaluation of results

Differences between the test and control eyes shall be characterized and explained in terms of the grading system given in Table B.1.

Acute exposure

If the treated eye in more than one animal shows a positive result (footnoted grades given in Table B.1) at any of the observations, then the material is considered an eye irritant and further testing is not required.

If only one of three treated eyes shows a mild or moderate reaction or the reactions are equivocal, treat further animals.

When further animals have been treated, the test material is considered to be an eye irritant if more than half of the eyes treated in the test group exhibit a positive result (footnoted grades given in Table B.1) at any stage of the observation.

A severe reaction in only one animal is considered sufficient to label the material as an eye irritant.

Repeated exposure

The test material is considered an eye irritant if more than half of the animals in the test group exhibit a positive result (footnoted grades given in Table B.1) at any stage of the observation.

B.2.9 Test report

The test report shall include:

a) a description of the test samples;

b) the intended use/application of the test samples;

c) a detailed description of the method employed in preparing the test samples;

d) a description of the test animals;

e) the method of instillation;

f) how the ocular readings were performed;

g) a record of the observations;

h) assessment of the results.

B.3 Oral mucosa irritation test

B.3.1 General

The oral irritation test shall only be considered for materials with intended contact with oral tissue and if safety data cannot be obtained by other means.

B.3.2 Principle

An assessment is made of the potential of the material under test to produce irritation of the oral tissue.

B.3.3 Exclusion from test

Any material shown to be a skin or eye irritant or material having a pH $\leqslant$ 2,0 or $\geqslant$ 11,5 shall not be tested and shall be labelled as a potential oral tissue irritant.

In exceptional cases where further risk characterization/assessment is needed, it might be necessary to test materials that are either an irritant or have a pH outside the range mentioned above. These cases shall be justified and documented.

B.3.4 Test material

Prepare test materials in accordance with Annex A.

B.3.5 Animals and husbandry

Healthy young adult Syrian hamsters of either sex from a single outbred strain shall be used. The animals shall be acclimatized and cared for as specified in ISO 10993-2.

In addition to the above, when appropriate, fit to each animal a suitable collar of width 3 mm to 4 mm, placed around the neck so that it permits normal feeding and respiration but prevents the animal from removing the cotton-wool pellet. Weigh each animal daily for seven days during the test period. Examine any animal showing a loss of body mass during this period and adjust its collar, if necessary. If the animal continues to lose mass, exclude it from the test.

A minimum of three animals shall initially be used to evaluate the test material.

NOTE The use of additional animals treated with a negative control material or blank liquid can be appropriate.

If the response in the initial test is equivocal or not clear, additional testing shall be considered.

B.3.6 Test procedure

Remove the collar from each animal and evert the cheek pouches. Wash the pouches with physiological saline solution, and examine for any abnormality.

For solid test materials, place a sample (no larger than 5 mm diameter) directly into the cheek pouch.

For liquid test materials or extract samples, soak a cotton-wool pellet in the sample, record the volume absorbed, and place a pellet in one pouch of each animal. Alternatively, an appropriate volume of a sample may be flushed into the cheek pouch.

No sample is placed in the other cheek pouch, which serves as a control. Appropriate control animals shall be tested in parallel.

When required, replace the collar and return the animal to its cage.

The duration of exposure shall be that expected for actual use of the material, but no shorter than 5 min.

Following the exposure, remove the collar and cotton-wool pellet and wash the pouch with physiological saline solution, taking care not to contaminate the other pouch.

For acute exposure, repeat the above procedure every hour (±0,1 h) for 4 h.

For repeated-exposure tests, base the number of applications, their duration and their interval on the exposure time anticipated in the clinical situation.

B.3.7 Observation of animals

Examine the pouches macroscopically following removal of the pellets and, if repeated applications are required, immediately prior to the next dosing.

Describe the appearance of the cheek pouches for each animal and grade the pouch surface reactions for erythema according to the system given in Table B.2 for each animal at each time interval. Record the results for the test report.

At (24 ± 2) h after the final treatment, examine the cheek pouches macroscopically, and humanely sacrifice the hamsters and remove tissue samples from representative areas of the pouches. Place in an appropriate fixative prior to processing for histological examination.

Table B.2 — Grading system for oral and penile reactions

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Other adverse changes of the tissues should be recorded and reported.	

B.3.8 Assessment of results

B.3.8.1 Macroscopic evaluation

Compare the treated cheek pouch with the cheek pouch on the contralateral side and, if a control group is included, with the pouches of animals in the control group.

The grades (see Table B.2) for each observation are added and the sum is divided by the number of observations to determine the average grade per animal.

NOTE 1 These observations can assist in the histological evaluation.

NOTE 2 The initial observations made prior to the first application of the test material are not included in the grade average.

B.3.8.2 Histological evaluation

The irritant effects on oral tissue shall be evaluated microscopically by a pathologist. The pathologist may grade each tissue according to the system given in Table B.3.

The grades for microscopic evaluation for all the animals in the test group are added and the sum is divided by the number of observations to obtain a test group average. Repeat for the control group(s). The maximum score is 16.

A total score greater than nine for the microscopic evaluation in the control cheek pouch can indicate underlying pathology or, in a control animal, it can indicate trauma at dosing. Either situation can require a retest if other test or control animals exhibit equivalent high scores.

Subtract the control group average from the test group average to obtain the irritation index (see Table B.4).

For repeated-exposure tests, Table B.3 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.3.9 Test report

The test report shall include:

a) a description of the test samples;

b) the intended use/application of the test samples;

c) a detailed description of the method employed in preparing the test samples;

d) a description of the test animals;

e) the method of application;

f) how the site readings were performed;

g) a record of the observations;

h) the histological evaluation;

i) assessment of the results.

Table B.3 — Grading system for microscopic examination for oral, penile, rectal and vaginal tissue reaction

Reaction	Numerical grading
Epithelium	
Normal, intact	0
Cell degeneration or flatting	1
Metaplasia	2
Focal erosion	3
Generalized erosion	4
Leucocyte infiltration (per high power field)	
Absent	0
Minimal (less than 25)	1
Mild (26 to 50)	2
Moderate (51 to 100)	3
Marked (greater than 100)	4
Vascular congestion	
Absent	0
Minimal	1
Mild	2
Moderate	3
Marked, with disruption of vessels	4
Oedema	
Absent	0
Minimal	1
Mild	2
Moderate	3
Marked	4

Table B.4 — Irritation index

Average grade	Description of response
0	None
1 to 4	Minimal
5 to 8	Mild
9 to 11	Moderate
12 to 16	Severe
Other adverse changes of the tissues should be recorded and included in the assessment of the response. The microscopic examination grading system given in Table B.3 applies for all tests listed. The “irritation index” was developed for use with the vaginal irritation model but may be used for the other tests.	

B.4 Penile irritation test

B.4.1 General

The penile irritation test shall only be considered for materials intended for contact with penile tissue and if safety data cannot be obtained by other means.

B.4.2 Principle

An assessment is made of the potential of the material under test to produce irritation of the penile tissue.

B.4.3 Exclusion from test

Any material shown to be a skin or eye irritant or materials with a pH $\leqslant$ 2,0 or $\geqslant$ 11,5 shall not be tested and shall be labelled as a potential penile irritant.

B.4.4 Test sample

If the test sample is a solid or a liquid, it shall be prepared as specified in Annex A.

B.4.5 Animals and husbandry

Male albino rabbits or guinea pigs shall be used. They shall be healthy young adults, weighing not less than 2 kg for rabbits and 300 g to 500 g for guinea pigs.

The animals shall be acclimatized and cared for as specified in ISO 10993-2.

The length of the penis which can be exposed shall be at least 1 cm.

Due to individual pigment variation, animals shall be observed and graded for erythema prior to the first test application. The system given in Table B.2 shall be used for grading any erythema. Animals showing severe discoloration or having an erythema grade of 2 or greater shall not be used.

A minimum of three animals shall initially be used to evaluate the test material, and three animals as the control group.

If the response in the initial test is equivocal or not clear, additional testing shall be considered.

B.4.6 Test procedure

Place the animal in a supine position with the limbs secured by an assistant.

With the index and middle finger, gently press the genital area to protrude the penis.

When the penis is protruded, apply enough (approximately 0,2 ml) of the test sample to be sure that the penis is coated.

Allow the penis to retract into the sheath. Take measures to prohibit the animal from licking the test site and confounding the primary irritation by secondary factors (e.g. Elizabethan collar).

Alternatively, the animal may be secured in an appropriately designed restrainer for (1 $\pm$ 0,1) h after the last application.

For acute exposure, repeat the above procedure every hour ($\pm$0,1) h for 4 h.

For prolonged repeated-exposure tests, base the number of applications, their duration and their interval on the exposure time anticipated in the clinical situation.

B.4.7 Observation of animals

For acute exposure, note the appearance of the penis (1 ± 0,1) h after the initial application (e.g. immediately prior to the next application) and subsequent treatments. Note and record the appearance of the penis at (1 ± 0,1) h, (24 ± 2) h and (48 ± 2) h after the last application.

For prolonged repeated-exposure tests, note the appearance of the penis at (1 ± 0,1) h after the initial application and immediately prior to the next application.

Grade the skin surface reactions for erythema according to the system given in Table B.2 for each animal at each time interval and record the results for the test report.

If any animal exhibits redness prior to the first test application, the grade given prior to the first application of the test sample is subtracted from the grades for erythema at the timed observations to determine the erythema grade due to the test sample. The highest possible grade for one observation is four.

B.4.8 Assessment of results

B.4.8.1 Macroscopic evaluation

Compare the treated penis and sheath with the penis of the control animals.

The grades (see Table B.2) for each observation are added and divided by the number of observations to determine the average grade per animal.

NOTE 1 These observations can assist in the histological evaluation.

NOTE 2 The initial observations made prior to the first application of the test material are not included in the grade average.

Immediately after the 48 h observation, humanely sacrifice the animals. Dissect free the distal penis and sheath and place in an appropriate fixative prior to processing for histological examination.

B.4.8.2 Histological evaluation

The irritant effects on the penile skin shall be evaluated by a pathologist. The pathologist may grade each tissue according to the system given in Table B.3.

The grades for microscopic evaluation for all the animals in the test group are added and the sum is divided by the number of observations to obtain a test group average. The maximum score is 16.

Repeat for the control group(s).

A total score greater than nine for the microscopic evaluation in a control animal can indicate trauma at dosing. A retest can be required if other test or control animals exhibit equivalent high grades.

Subtract the control group average from the test group average to obtain the irritation index (see Table B.4).

For prolonged repeated-exposure tests, Table B.3 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.4.9 Test report

The test report shall include:

a) a description of the test sample;

b) the intended use/application of the test samples;

c) a detailed description of the method employed in preparing the test samples;

d) a description of the test animals;

e) the method of application;

f) how the site readings were performed;

g) a record of the observations;

h) the histological evaluation;

i) assessment of the results.

B.5 Rectal irritation test

B.5.1 General

The rectal irritation test shall only be considered for materials intended for contact with rectal tissue and if safety data cannot be obtained by other means.

B.5.2 Principle

An assessment is made of the potential of the material under test to produce irritation of the rectal tissue.

B.5.3 Exclusion from test

Any material shown to be a skin or eye irritant or those with a pH $\leqslant$ 2,0 or $\geqslant$ 11,5 shall not be tested and shall be labelled as a potential rectal irritant.

B.5.4 Test material

If the test material is a solid or a liquid, it shall be prepared as specified in Annex A.

B.5.5 Animals and husbandry

Healthy young adult albino rabbits of either sex from a single strain, weighing not less than 2 kg, shall be used. If other species are used, the choice shall be justified.

The animals shall be acclimatized and cared for as specified in ISO 10993-2.

A minimum of three animals shall initially be used to evaluate the test material, and three animals used as the control group.

If the response in the initial test is equivocal or not clear, additional testing shall be considered.

The animals shall be checked for rectal discharge, swelling and/or other evidence of lower bowel infection, irritation and/or injury prior to each treatment.

B.5.6 Test procedure

Attach a short (6 cm) soft catheter or blunt-tipped cannula to a syringe with a capacity to deliver more than 1 ml, and fill the syringe and catheter such that 1 ml of the test sample will be dosed. Prepare a separate syringe with attached catheter for each animal.

Secure the animal by placing it in a restraining device which permits access to the perineum, or by an assistant carefully restraining the animal and securing the back legs in such a way to expose the perineum.

Just prior to insertion, moisten the catheter with either the control sample or with a suitable lubricant.

Grasp and raise the animal's tail to expose the perineum. Gently insert the moistened catheter deep into the rectum and deposit the entire 1 ml dose from the syringe. Withdraw the catheter and discard it appropriately.

Due to differences in the capacity of the rectum of individual animals, some of the test sample may be discharged during or immediately after it is deposited. Gently remove any of the expelled material with a soft tissue.

Repeat the above procedure at (24 ± 2) h intervals every day for five consecutive days.

For prolonged repeated-exposure tests, base the number of applications, their duration and their interval on the exposure time anticipated in the clinical situation.

B.5.7 Observation of animals

At (24 ± 2) h after the initial application and immediately prior to each treatment, note and record the appearance of the perineum for signs of discharge, erythema and irritation.

Animals exhibiting excessive discharge, swelling and/or that are found difficult to dose shall be humanely sacrificed and the tissues examined (see B.5.8.1 and B.5.8.2).

B.5.8 Evaluation of results

B.5.8.1 Macroscopic evaluation

At (24 ± 2) h after the last dose, humanely kill the animals. Dissect free the entire lower bowel, open longitudinally and examine for signs of irritation, injury to the epithelial layer of tissue and necrosis.

Place the rectum and distal portion of the large bowel in an appropriate fixative prior to processing for histological examination.

Compare the rectal tissues of the test rabbits with the rectal tissue of the control rabbits.

Record and describe the macroscopic appearance of the rectal tissue for each animal, noting differences between the test and control sites.

NOTE These observations can assist in the histological evaluation.

B.5.8.2 Histological evaluation

The irritant effects on the rectal tissue shall be evaluated by a pathologist. The pathologist may grade each tissue according to the system given in Table B.3.

Add the grades for microscopic evaluation for all the animals in the test group and divide the sum by the number of observations to obtain a test group average. The maximum score is 16.

Repeat for the control group(s).

A total score greater than nine for the microscopic evaluation in a control animal may indicate trauma at dosing. A retest may be required if other test or control animals exhibit equivalent high scores.

Subtract the control group average from the test group average to obtain the irritation index (see Table B.4).

For prolonged repeated-exposure tests, Table B.3 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.5.9 Test report

The test report shall include:

a) a description of the test samples;

b) the intended use/application of the test samples;

c) a detailed description of the method employed in preparing the test samples;

d) a description of the test animals;

e) the method of application;

f) how the site readings were performed;

g) a record of the observations;

h) the histological evaluation;

i) assessment of the results.

B.6 Vaginal irritation test

B.6.1 General

The vaginal irritation test shall only be considered for materials intended for contact with vaginal tissue and if safety data cannot be obtained by other means.

B.6.2 Principle

An assessment is made of the potential of the material under test to produce irritation of the vaginal tissue.

B.6.3 Exclusion from test

Any material shown to be a skin or eye irritant or materials with a pH $\leqslant$ 2,0 or $\geqslant$ 11,5 shall not be tested and shall be labelled as a potential vaginal irritant.

B.6.4 Test material

If the test material is either a solid or a liquid, it shall be prepared as specified in Annex A.

B.6.5 Animals and husbandry

Healthy young adult female albino rabbits from a single strain weighing not less than 2 kg shall be used. If other species are used, the choice shall be justified.

The animals shall be acclimatized and cared for as specified in ISO 10993-2.

A minimum of three animals shall initially be used to evaluate the test material, and three animals as the control group.

If the response in the initial test is equivocal or not clear, additional testing shall be considered.

The animals shall be checked for vaginal discharge, swelling and/or other evidence of vaginal infection, irritation and/or injury prior to each treatment. A check shall also be made on the stage in oestrus cycle to ensure a false positive reaction is not given based on physiological changes in the vagina.

B.6.6 Test procedure

Attach a short (6 cm) soft catheter or blunt-tipped cannula to a syringe with a capacity to deliver more than 1 ml, and fill the syringe and catheter such that 1 ml of the test sample will be dosed. Prepare a separate syringe with attached catheter for each animal.

Secure the animal by placing it in a restraining device which permits access to the vagina or by an assistant carefully restraining the animal and securing the back legs in such a way to expose the vagina.

Moisten the catheter in either the control sample or a suitable lubricant.

Grasp and raise the animal's tail to expose the vaginal opening. Gently insert the moistened catheter deep into the vagina and deposit the entire 1 ml dose from the syringe. Withdraw the catheter and discard it appropriately.

Due to differences in the capacity of the vagina of individual animals, some of the test sample may be discharged during or immediately after it is deposited. Gently remove any of the expelled material with a soft tissue.

Repeat the above procedure at (24 ± 2) h intervals every day for a minimum of five consecutive days.

For prolonged repeated-exposure tests, base the number of applications, their duration and their interval on the exposure time anticipated in the clinical situation.

B.6.7 Observation of animals

At (24 ± 2) h after the initial application and immediately prior to each treatment, note and record the appearance of the vaginal opening and perineum for signs of discharge, erythema and oedema.

Animals exhibiting excessive discharge, erythema and/or oedema, and that are found difficult to dose shall be humanely sacrificed and the tissues examined (see B.6.8.1 and B.6.8.2).

B.6.8 Evaluation of results

B.6.8.1 Macroscopic evaluation

At (24 ± 2) h after the last dose, humanely kill the animals. Dissect free the entire vagina, open longitudinally and examine for signs of irritation, injury to the epithelial layer of tissue and necrosis.

Place the vagina in an appropriate fixative prior to processing for histological examination. Three sections, to include the cervical, central and caudal portions of each vagina, shall be taken.

Compare the vaginas of animals treated with the test material with the vaginas of the control animals.

Record and describe the macroscopic appearance of the vaginal tissue for each animal, noting differences between the test and control groups.

NOTE These observations can assist in the histological evaluation.

B.6.8.2 Histological evaluation

The irritant effects on vaginal tissue shall be evaluated by a pathologist. The pathologist may grade each tissue according to the system given in Table B.3.

The grades for microscopic evaluation for all the animals in the test group are added and the sum is divided by the number of observations to obtain a test group average. The maximum score is 16.

Repeat for the control group(s).

A total score greater than nine for the microscopic evaluation in a control animal may indicate trauma at dosing and may require a retest if other test or control animals exhibit similar high scores.

Subtract the control group average from the test group average to obtain the irritation index (see Table B.4).

For prolonged repeated-exposure tests, Table B.3 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.6.9 Test report

The test report shall include:

a) a description of the test samples;

b) the intended use/application of the test samples;

c) a detailed description of the method employed in preparing the test samples;

d) a description of the test animals;

e) the method of application;

f) how the site readings were performed;

g) a record of the observations;

h) the histological evaluation;

i) assessment of the results.

Annex C
(normative)

Human skin irritation test

C.1 General

The following requirements apply in addition to ISO 14155-1 and ISO 14155-2 to the extent to which they are more specific.

C.2 Principle

A single dose of the material to be tested is applied under occlusion to the skin of human volunteers. Irritation is kept to a minimum by applying the test material for short periods. Longer exposure periods can also be appropriate under certain circumstances.

The principal means of evaluation is by determining the proportion of the human volunteers who develop skin irritation relative to a reaction to a concurrent positive control material.

C.3 Description of the method

C.3.1 Selection of human volunteers

This part of ISO 10993 is designed for use with healthy human volunteers. The selected human volunteers shall be at least 18 years of age, not pregnant and not breast-feeding. In addition, human volunteers with a known sensitivity to the test material or showing any signs of dermatitis shall be excluded from the test. The selection of volunteers shall be supervised by a dermatologist or other qualified person.

C.3.2 Preparation of doses

Liquid test materials are generally used undiluted. When testing solids, moisten the test material with a small amount of water (typically 0,2 ml) or where necessary with another suitable vehicle in order to ensure good contact with the skin. The structure of the solid shall be taken into consideration and the choice of test material preparation shall be justified. When using moistened samples, take care to ensure that each subject receives the same amount of the test material. Use the same amount of water for moistening for each individual in the test and record this amount.

When vehicles are used, the influence of the vehicle on irritation of the skin by the test material shall be taken into account. If a vehicle other than water is used as the wetting agent for solid compounds, consider the application of a blank liquid (blank) patch on each subject.

C.3.3 Procedure

C.3.3.1 Number of volunteers

At least 30 volunteers shall complete the test, with no less than one-third of either sex.

C.3.3.2 Application of the test material

Apply the test material to intact skin at a suitable site, e.g. the upper outer arm, by means of an occlusive chamber containing a gauze pad. The application site shall be the same in all volunteers and shall be recorded. Generally, the patch shall measure at least 1,8 cm, preferably 2,5 cm in diameter. The patch shall be held in contact with the skin by means of a suitable non-irritating dressing, including non-irritating tape, for the duration of the exposure period.

The patch shall deliver an adequate dose per unit area: approximately 50 mg to 100 mg test material per square centimetre is considered optimal. When applying liquid test materials, in general 0,2 ml to 0,4 ml is added to the gauze pad until it is moistened. When testing solid materials, in general 0,2 g of the test material are moistened and added to the gauze pad. As an alternative method of application for solids, the gauze pad is moistened and the test material covers the entire test site.

C.3.3.3 Duration of exposure

To avoid unacceptably strong reactions, a cautious approach to testing shall be adopted. A sequential patch procedure permits the development of a positive, but not severe, irritant response. The patches are applied progressively starting with durations of 15 min and 30 min, and up to 1 h, 2 h, 3 h and 4 h. The 15 min and/or 30 min exposure periods may be omitted if there are sufficient indications that excessive reactions will not occur following the 1 h exposure. Progression to longer exposures, including 24 h closed-patch exposure at a new skin site, will depend upon the absence of skin irritation (evaluated up to at least 48 h) arising from the shorter exposures, in order to ensure that any delayed irritant reaction is adequately assessed.

Application of the material for a longer exposure period is always made to a previously untreated site.

At the end of the exposure period, residual test material shall be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

C.3.3.4 Limited exposure

In addition to the phased increase in duration of application as described in C.3.3.3, if it is suspected that the material might produce severe irritation, a substantially reduced exposure time shall be employed, possibly in a pilot group of volunteers. The progress of the study can then be defined on the basis of the data produced. Subsequent patches are only applied after the (48 ± 2) h and (72 ± 2) h readings.

C.3.3.5 Clinical observation and grading of skin reactions

Treatment sites are examined for signs of irritation and the responses are scored immediately after patch removal and at (1 ± 0,5) h to (2 ± 1) h, (24 ± 2) h, (48 ± 2) h and (72 ± 2) h after patch removal. If necessary to determine reversibility of the response, the observation period may be extended beyond 72 h. In addition, the condition of the skin before and after the test shall be thoroughly described (e.g. pigmentation and extent of hydration). Skin irritation is graded and recorded according to the grading given in Table C.1.

Non-invasive bioengineering methods may be applied (see Annex E).

Table C.1 — Human skin irritation test, grading scale

Description of response	Grading
No reaction	0
Weakly positive reaction (usually characterized by mild erythema and/or dryness across most of the treatment site)	1
Moderately positive reaction (usually distinct erythema or dryness, possibly spreading beyond the treatment site)	2
Strongly positive reaction (strong and often spreading erythema with oedema and/or eschar formation)	3

For volunteers who have a grading of 1 or greater following an exposure of less than 4 h, it is assumed that they will present a stronger reaction if exposed to the material for 4 h. Once a grading of 1 or greater has been obtained, there is no need to subject the reacting volunteer to further treatment with the material. Further observations can be required for proper volunteer care. In addition to the observation of irritation, any other effects shall be recorded and fully described. For example, volunteers shall be trained to make comments related to the patch applications (e.g. sensory effects), and assessors shall be trained to note immediate responses (e.g. urticaria) when the patches are removed. Such observations do not necessarily indicate an irritant effect, but they shall be included in the test report if noted. If significant, they shall be considered in the management of the study to ensure proper volunteer care.

The critical data obtained are the number of volunteers who had, or would be expected to have, skin irritation after an exposure up to 4 h. The time required for an individual to develop a response (if any) does not form part of the results to be evaluated; it relates only to ensuring proper care of the volunteers.

C.3.3.6 Rationale for and selection of a concurrent positive control substance

As humans show variation in their responses to irritants, a positive control shall be included to determine the suitability of a test panel to detect irritant effects of the test compound. Preferably, 20 % sodium dodecyl sulfate (SDS) shall be used as positive control since its irritant effects are well characterized (see F.1). Other controls may be used if justified.

A routine positive control can be included as a benchmark. Skin irritation is not an absolute phenomenon. All materials can give rise to skin irritation; it is simply a matter of dose and the nature and extent of exposure. Thus, skin irritation tests in humans are almost always comparative and shall be related to known chemical irritancy.

C.4 Data and reporting

C.4.1 Data

Data, including results with positive and negative control materials, shall be summarized in tabular form, showing for each individual the irritation grading at (24 ± 2) h, (48 ± 2) h and (72 ± 2) h after patch removal and any other effects observed.

C.4.2 Data evaluation/interpretation

The aim of this test is to determine whether a material presents a significant skin irritation potential hazard following acute exposure. Thus if the material produces a frequency of skin irritation in the test subjects which is similar to, or greater than, the positive control, it shall be regarded as a significant skin irritant. On the other hand, if it produces a frequency of skin irritation in the test subjects which is substantially and significantly less than the positive control, then it shall not be regarded as a significant skin irritant. It is important that interim data generated in the context of volunteer care are not confused with the endpoint data, i.e. the proportion of the subjects that exhibit an irritant reaction. It is also important not to confuse individual variation in the susceptibility to skin irritation with the issue of the general skin irritation potential of the test material.

C.4.3 Test report

For the test report, the requirements of ISO 14155-1 and ISO 14155-2 shall be followed. For specific reporting on the human skin irritation test, the following information shall also be included:

a) ethical considerations and confirmation of consent from the volunteers;

b) test material:

   1) physical nature and, where relevant, physicochemical properties;

   2) identification data;

c) vehicle:

1) identification of and justification for the choice of vehicle used to moisten a solid test material;

d) volunteers:

1) number of volunteers who were treated with the test material;

2) age/sex distribution of the volunteers;

e) results:

1) response rate at 0 h, (1 ± 0,1) h to (2 ± 0,2) h, (24 ± 2) h, (48 ± 2) h and (72 ± 2) h and at any other times scored;

2) tabulation of irritation reaction data for each individual for each observation time period (with summarised frequency of irritant reaction rate at e.g. (24 ± 2) h, (48 ± 2) h and (72 ± 2) h after patch removal);

3) description of all irritant reactions observed;

4) description of any other effects in addition to irritation observed;

5) statistical treatment of the results (comparison with positive control, e.g. using Fisher's exact test);

6) description or reference of an *in vitro* or *in vivo* animal test, if one is performed before the test in human volunteers, including details of the procedure, and results obtained with test and reference materials;

f) discussion of the results.

Annex D
(informative)

In vitro tests for skin irritation

D.1 Background information

Recently, various studies have been published on the evaluation and validation of *in vitro* assays for the determination of irritating activity of chemicals as an alternative for the rabbit skin irritation test. See References [101] [102] [103] [104]. In 2007, the ECVAM Scientific Advisory Committee (ESAC) evaluated the validation process of an *in vitro* human skin model for the determination of skin irritation of chemicals. See Reference [101]. After this evaluation, ESAC endorsed the following.

"After a review of scientific reports and peer reviewed publications on the following range of *in vitro* tests, which had been subjected to a full validation study:

1. EpiDerm™ (with MTT reduction and IL-1α release)

2. EPISKIN™ (with MTT reduction and IL-1α release)

the EPISKIN™ method showed evidence of being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction, and for being used as a replacement (based on the performance of the assay as specified in the annex) for the Draize Skin Irritation Test (OECD TG 404 & Method B.4 of Annex V to the Directive 67/548/EEC) for the purposes of distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances. At the present time, the IL-1α endpoint should be regarded as a useful adjunct to the MTT assay, as it has the potential to increase the sensitivity of the test, without reducing its specificity. This endpoint could be used to confirm negatives obtained with the MTT endpoint.

At this time, due to its high specificity, the EpiDerm™ model reliably identifies skin irritants, but negative results may require further testing (e.g. according to the tiered strategy, as described in Reference [7]). Improvement of the EpiDerm™ protocol should be made to increase the level of sensitivity."

Both EPISKIN™ and EpiDerm™ models reliably identify skin irritating chemicals, while only the EPISKIN™ model can be used to demonstrate non-irritating (no-label) properties of chemicals. In the EPISKIN™ model, the determination of IL-1α production may be used for further confirmation of negative responses. A negative response in the EpiDerm™ model needs confirmation in the rabbit skin irritation test.

In addition to the commercially available skin models (see also D.2.2), "open source" models are also available; these are based on the same principle of using human cells in a skin equivalent matrix. See Reference [109].

It should be noted that the *in vitro* test for skin irritation has so far been validated only for neat chemicals and not for medical device extracts. In order to apply these assays for the testing of irritation potential of medical devices, further validation for this specific area is essential. Certain aspects of the testing of medical devices, such as extraction techniques and possible low concentrations of chemicals in these extracts, can result in adaptations of the testing protocol, such as changing extraction techniques or incubation times.

D.2 Principle of the *in vitro* skin irritation tests

D.2.1 General

The principle of the *in vitro* skin model irritation assay is based on the premise that irritant chemicals are able to penetrate the stratum corneum by diffusion and are cytotoxic to the cells in the underlying layers. Moreover, if the cytotoxic effect is absent or weak, a quantifiable number of inflammatory mediators are released by the epidermis and may be used in a tiered approach to increase the sensitivity of the test.

The test material is applied topically to a three-dimensional human epidermal model, comprised of at least a reconstructed epidermis with several epidermal cell layers and a functional stratum corneum.

Irritant materials are identified by their ability to decrease cell viability below defined threshold levels (e.g. 50 %). As an additional measure of skin irritation, release of inflammatory mediators (e.g. Interleukin 1α) can be determined.

In the validation studies, carefully selected chemicals representing a wide spectrum of chemical classes were included for the validation of the *in vitro* human skin model test system for skin irritation. See References [102] [103] [104]. The method is expected to be generally applicable across chemical classes, except for gases and aerosols.

D.2.2 General model characteristics

Human skin models can be obtained commercially (e.g. EpiDerm™, EPISKIN™, Vitrolife-Skin, TESTSKIN, Labcyte EPI-MODEL) or be developed or constructed in the testing laboratory. Any new model should be validated and compared with existing models. Human keratinocytes should be used to construct the epithelium. Multiple layers of viable epithelial cells (basal layer, stratum spinosum, stratum granulosum) should be present under a functional stratum corneum. Stratum corneum should be multilayered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic marker chemicals, e.g. sodium dodecyl sulphate (SDS) or Triton X-100. This property may be estimated by the determination of IC_{50} or ET_{50} after application of an established cytotoxic marker chemical. The containment properties of the model should prevent the passage of material around the stratum corneum to the viable tissue, which would lead to poor modelling of the exposure to skin.

For general characterization of a new skin or epidermal model, histological evaluation (H&E staining), identification of the keratins (immune histochemistry) and lipid profiling [high performance thin layer chromatography (HPTLC)] should be preformed. See Reference [110].

The skin model should be free of contamination by bacteria, mycoplasma or fungi.

D.2.3 Functional model requirements

D.2.3.1 General

The functional model conditions are described in Reference [105]. The following criteria are applicable to the use of the *in vitro* skin irritation test.

D.2.3.2 Viability

The magnitude of viability is usually quantified by using MTT or other metabolically converted vital dyes. In these cases the optical density (OD) of the extracted (solubilized) dye from the negative control tissue should be at least 20 fold greater than the OD of the extraction solvent alone. The OD of the negative control tissues should preferably be above 0,8. It should be documented that the negative control tissue is stable in culture for the duration of the test. This can be done by performing the viability assay at various time points during the test period. The measurements should provide similar viability for each time point.

D.2.3.3 Barrier function

The stratum corneum (SC) and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic marker chemicals, e.g. SDS or Triton X-100. This property can be estimated either by determination of the concentration at which a marker chemical reduces the viability of the tissues by 50 % (IC_{50}) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50 % (ET_{50}) upon application of the marker chemical at a specified, fixed concentration. The ET_{50} of a sufficiently functional SC should be above 2 h.

D.2.3.4 Morphology

An ongoing histological examination of the reconstructed skin/epidermis should be performed, showing human skin/epidermis-like structure (including functional SC). This information can be provided by the manufacturer of the skin construct.

D.2.3.5 Reproducibility

The results of the method using a specific model should demonstrate reproducibility over time. The model shall be capable of demonstrating correct prediction of reference chemicals over an extended time period (see Table D.1).

Table D.1 — Examples of QC batch release criteria

	Lower acceptance limit	Mean of acceptance range	Upper acceptance limit
EPISKIN™ (18 h SLS)	IC_{50} = 1,0 mg/ml	IC_{50} = 2,32 mg/ml	IC_{50} = 3,0 mg/ml
EpiDerm™ (1 % Triton X100)	ET_{50} = 4,8 h	ET_{50} = 6,7 h	ET_{50} = 8,7 h

D.2.3.6 Quality controls (QC) of the model

Each batch of the epidermal model used shall meet defined production release criteria, among which those for viability and for barrier function are the most relevant. An acceptability range (upper and lower limit) for the IC_{50} or the ET_{50} shall be established by the skin model supplier (or investigator when using an in-house model). Only results produced with qualified tissues can be accepted for reliable prediction of irritation effects. As an example, the acceptability ranges for EPISKIN™ and EpiDerm™ are given in D.3.

D.3 Test material

The test material may be composed of solids, liquids, semisolids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Solids should be ground to a powder before application; no other prior treatment of the sample is required. For medical devices or biomaterial extracts, both in polar and non-polar solvents could be used.

A negative reference control and a positive reference control should be tested concurrently with the test substances to demonstrate that viability (negative reference control), barrier function and resulting tissue sensitivity (positive reference control) of the tissues are within a defined historical acceptance range. For a listing of positive control substances, see Reference [105].

A non-irritating negative control (NC) (e.g. PBS, water or blank) shall be tested concurrently with the test substance. The negative control tissues should be stable in culture and provide similar viability measurements throughout the test chemical exposure and post-incubation periods. A minimum viability (e.g. expressed as absolute OD of the vital dye) shall be established as a test acceptance criterion. In general, such a negative control should have an OD above 0,8.

An appropriate positive control (PC) should be used in the assay (e.g. 5 % SDS) to avoid complete "knock-out" of the model. The range of responses to the PC shall be developed and based on data obtained in a sufficient number of independent experiments. In each assay, the positive control shall be correctly classified as irritant, be within the established range of responses, and the standard deviation of the three tissue replicates shall be below a defined maximum. If these criteria are not fulfilled, the assay is declared not valid and should be repeated. Typical ranges for two skin models used in the ECVAM skin irritation validation study (EPISKIN™ and EpiDerm™) are given in Table D.2.

Table D.2 — Example for range of model responses to a positive control (5 % SDS)

Skin model	Viability	Range	SD
EPISKIN™	< 40 %	1,5 to 32,2 (1,3 to 41,6)[a]	⩽ 18 %
EpiDerm™	< 20 %	3,7 to 13,8 (4,7 to 13,6)[a]	⩽ 18 %
[a] 95 % confidence interval.			

D.4 Test procedures

The tissue constructs are cultured in tissue culture medium according to protocols as provided by the manufacturer. If the tissues have been transported, care should be taken to apply the recommended procedures for conditioning before the use of the tissues in the laboratory.

At least three tissue replicates of sufficient size (at least 10 mm diameter, 0,63 cm^2) should be incubated with the test samples and controls. The test samples and controls should be incubated with the skin constructs for at least (15 ± 5) min.

A sufficient amount of test substance should be applied to uniformly cover the skin surface. A minimum of 25 µl/cm^2 or 25 mg/cm^2 should be used. Solid substances should be moistened with deionized or distilled water after application to ensure good contact with the skin. If appropriate, solids should be ground to a powder before application.

After (15 ± 5) min of incubation, the test samples are removed by thorough washing and rinsing with an appropriate buffer or 0,9 % NaCl. The washing and rinsing procedure should be adequate to remove all test materials. The tissues are further incubated in fresh medium for (42 ± 2) h as a post exposure recovery period which allows for recovery from weakly irritant effects. After (42 ± 2) h, cellular survival is determined.

NOTE It is important to realize that extracts of medical devices can contain low concentrations of irritating chemicals. It can therefore be necessary to adapt the exposure period.

Only quantitative methods can be used to measure cell viability. Furthermore, the measure of viability should be compatible with use in a three-dimensional tissue construct. Non-specific dye binding should not interfere with the viability measurement. Protein binding dyes and those that do not undergo metabolic conversion (e.g. neutral red) are therefore not appropriate.

The most frequently used assay is MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1)] reduction, which has been shown to give accurate and reproducible results. The skin sample is placed in MTT solution of appropriate concentration (e.g. 0,3 mg/ml to 1 mg/ml) for 3 h. The precipitated blue formazan product is then extracted using a solvent (isopropanol), and the concentration of formazan is measured by determining the OD at a wavelength between 540 nm and 595 nm.

NOTE 1 Chemical interaction of the test material with the vital dye can mimic that of cellular metabolism, leading to a false estimate of viability. This can occur when a test material is not completely removed from the skin by rinsing. If the test material acts directly on the vital dye, additional controls should be used to detect and correct for test substance interference with the viability measurement.

NOTE 2 Other variants of the use of tetrazolium salts for the detection of cellular metabolism as a measurement for cell viability are available, such as XTT, MTS and WST-1.

In addition to cellular survival in the skin constructs, Interleukin 1α can be determined as a complementary endpoint, especially in the EPISKIN™ model. For epidermis tissues showing a cell viability > 50 %, the amount of IL-1α released into the tissue culture medium at the end of the post incubation period [after (42 ± 2) h post-incubation] is measured in the medium (immediately or frozen) using a commercially available ELISA kit, see Reference [107]. The amount of IL-1α should be expressed in international units.

D.5 Results and interpretation

The optical density (OD) values obtained with each test sample can be used to calculate the percentage of viability compared with the negative control, which is set at 100 %. The cut-off value of percentage cell viability distinguishing irritant from non-irritant test materials and the statistical procedure(s) used to evaluate the results and identify irritant materials should be clearly defined and documented, and proven to be appropriate.

The test substance is considered to be irritant to skin if in the EPISKIN™ or EpiDerm™ model the tissue viability after exposure and post incubation is ⩽ 50 %.

Although IL-1α might be useful to acquire additional information on the irritant potential of chemicals, only results from the MTT assay are currently used for considering a test sample an irritant. Further investigations are on-going to improve the reproducibility of the IL-1α assay to allow combination of two endpoints for reliable prediction of irritancy.

In the EPISKIN™ model for tissues showing a cell viability > 50 %, the amount of IL-1α released into the tissue culture medium at the end of the post incubation period [after (42 ± 2) h post-incubation] is measured in the medium (immediately or frozen).

The test substance is considered to be an irritant to skin if the viability after (15 ± 5) min of exposure and (42 ± 2) h of post incubation is more than 50 %, and the amount of IL-1α release is more than 9 IU/ml.

The test substance is considered to be non-irritant to skin if the viability after (15 ± 5) min of exposure and (42 ± 2) h of post incubation is more than 50 %, and the amount of IL-1α release is ⩽ 9 IU/ml.

In the EpiDerm™ model a negative response needs confirmation in the rabbit skin irritation test.

D.6 Test report

The test report shall include the following information:

a) justification of the skin model and protocol used;

b) description of test samples and control samples including chemical name(s) such as IUPAC or CAS name and CAS number, if known;

c) purity and composition of the substance or preparation [in percentage(s) by weight];

d) physical-chemical properties such as physical state, volatility, pH, stability, water solubility relevant to the conduct of the study;

e) treatment of the test/control substances prior to testing, if applicable (e.g. warming, grinding);

f) stability, if known;

g) cell system used;

h) calibration information for the equipment used for the measuring device used for measuring cell viability (e.g. spectrophotometer);

i) complete supporting information for the specific skin model used including its validity;

j) details of the test procedure used;

k) test doses used;

l) description of any modifications of the test procedure;

m) reference to historical data of the model;

n) description of evaluation criteria used;

o) assessment of results, including tabulation of data from individual test samples and validity of assay controls;

p) description of other effects observed;

q) discussion of the results;

r) conclusions.