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# Biological evaluation of medical devices —

## Part 23: Tests for irritation

*Évaluation biologique des dispositifs médicaux —*

*Partie 23: Essais d'irritation*



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# Foreword

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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

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

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

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

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

# Introduction

This document assesses possible contact hazards from medical devices, which can produce irritation.

Some materials that are included in medical devices have been tested, and their skin or mucosal irritation potential has been demonstrated. Other materials and their chemical components have not been tested and can 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.

The irritation potential of a medical device or its components can be predicted either by an *in vivo* animal irritation test or by an *in vitro* irritation test if qualified for use with medical devices.

ISO 10993-2 describes animal welfare aspects for performing animal studies for the biological evaluation of medical devices thereby also emphasizing the 3R's for replacement, reduction, and refinement of animal studies. This document describes tests to determine the irritancy of medical devices, materials or their extracts either by *in vitro* tests or *in vivo* tests. *In vitro* tests have preference over *in vivo* tests when appropriately validated and providing equally relevant information to that obtained from *in vivo* tests (see ISO 10993-1 and ISO 10993-2).

Traditionally, tests in small animals have been performed prior to testing on humans to help predict human responses. More recently, *in vitro* tests as well as human tests have been added as adjuncts or alternatives. For skin irritation testing of neat chemicals *in vitro* tests were developed using reconstructed human epidermis (RhE) models[31]. The method was adapted for detection of irritant chemicals in medical device extracts. The results of a large round robin study that tested two types of RhE models showed that these models can also be used to detect the presence of irritant chemicals extracted from polymeric materials [polyvinylchloride (PVC) and silicone] commonly used in the manufacture of medical devices[6]. This method was found to be equally sensitive in the detection of low concentrations of some strong irritant compounds when compared to the human patch testing and intracutaneous rabbit test[14]. Therefore, a stepwise approach for irritant testing can start with the *in vitro* RhE model.

The developed and validated RhE models are appropriate to predict skin tissue irritation response. It is recommended to explore the use of other alternative *in vitro* models to assess the irritation potential for mucosal or eye epithelial applications.

It is intended that, for regulatory submission, these studies be conducted using GLP or ISO/IEC 17025 as applicable to the respective country and comply with regulations related to animal welfare. Statistical analysis of data is recommended and can be used whenever appropriate.

This document 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 document are important tools for the development of safe products, provided that they are executed and interpreted by trained personnel.

This document is based on numerous standards and guidelines, including OECD Test Guidelines (TG), U.S. Pharmacopoeia[40] and the European Pharmacopoeia[39]. It is intended to be the basic document for the selection and conduct of tests enabling evaluation of irritation responses relevant to the safety of medical materials and devices.

Instructions are given in normative Annex A for the preparation of materials specifically in relation to the above tests. In normative Annex D several special *in vivo* irritation tests are described for application of medical devices in areas other than skin. In addition, normative Annex E provides information for conducting human skin irritation testing.



# Biological evaluation of medical devices —

# Part 23:
# Tests for irritation

## 1 Scope

This document specifies the procedure for the assessment of medical devices and their constituent materials with regard to their potential to produce irritation. The tests are designed to predict and classify the irritation potential of medical devices, materials or their extracts according to ISO 10993-1 and ISO 10993-2.

This document includes:

— pre-test considerations for irritation, including *in silico* and *in vitro* methods for dermal exposure;

— details of *in vitro* and *in vivo* irritation test procedures;

— key factors for the interpretation of the results.

## 2 Normative references

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

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

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

ISO 10993-9, *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 medical device materials within a risk management process*

ISO 14155, *Clinical investigation of medical devices for human subjects — Good clinical practice*

OECD 404, *Acute Dermal Irritation/Corrosion*

OECD 439, *In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*

## 3 Terms and definitions

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

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

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

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

**3.1**
**blank**
solution prepared in the same way as the sample measuring solution but so that it does not contain the analyte to be determined

[SOURCE: ISO 10136-1:1993, 3.8, modified — the term has been changed from "blank test solution" to "blank".]

**3.2**
**dose**
**dosage**
amount of *test sample* (3.14) administered (e.g. mass, volume) expressed per unit of body weight or surface area

Note 1 to entry: The terms are often used interchangeably (more commonly dosage).

**3.3**
**erythema**
reddening of the skin or mucous membrane

**3.4**
**eschar**
scab or discoloured slough of skin

**3.5**
**extract**
liquid or suspension that results from exposing a test or control material to an extraction *vehicle* (3.16) under controlled conditions

**3.6**
**irritant**
agent that produces *irritation* (3.7)

**3.7**
**irritation**
localized non-specific inflammatory response to single, repeated or continuous application of a substance/material

Note 1 to entry: Skin irritation is a reversible reaction and is mainly characterized by local *erythema* (3.3) (redness) and swelling [*oedema* (3.9)] of the skin.

**3.8**
**necrosis**
cell death as a direct result of irreversible changes caused by injury or disease

Note 1 to entry: Tissue repair will occur either resulting in complete functional restoration or resulting in scar formation.

**3.9**
**negative control**
well-characterized material or substance that, when evaluated by a specific test method, demonstrates the suitability of the procedure to yield a reproducible, appropriately negative, non-reactive or minimal response in the test system

Note 1 to entry: In practice, negative controls (NC) include *blanks* (3.1), *vehicles* (3.16)/solvents and reference materials.

**3.10**
**oedema**
swelling due to abnormal infiltration of fluid into the tissue

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

**3.12**
**skin corrosion**
production of irreversible damage to the skin, manifested as visible *necrosis* (3.8) through the epidermis and into the dermis, following application of a *test sample* (3.14)

EXAMPLE The action of a compound, chemical or a test sample resulting in ulceration of skin (see 3.15).

**3.13**
**test material**
material, device, device portion or component thereof that is sampled for biological or chemical testing

**3.14**
**test sample**
material, device, device portion, component, *extract* (3.5) or portion thereof that is subjected to biological or chemical testing or evaluation

**3.15**
**ulceration**
open sore representing loss of superficial tissue

**3.16**
**vehicle**
liquid used to moisten, dilute, suspend, *extract* (3.5) or dissolve the test substance/material

**3.17**
**vehicle control**
extraction *vehicle* (3.16) not containing the *test material* (3.13), retained in a vessel identical to that which holds the test material and subjected to identical conditions to which the test material is subjected during its extraction

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

## 4 General principles — Step-wise approach

The available methods for testing irritation were developed specifically to detect skin and mucous membrane irritation potential. Other types of adverse effects, such as sensitization, are generally not predicted by these tests. Historically irritation testing was done on rabbits. 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 is indicated as described in 7.2.

Preference for *in vitro* tests instead of *in vivo* tests in accordance with ISO 10993-2, shall be considered, with replacement of the latter as new *in vitro* tests are scientifically validated and qualified for use with medical devices and become reasonably and practicably available. The results of a large round robin study that tested two types of RhE models showed that these models can also be used to detect the presence of irritant chemicals extracted from polymeric materials [polyvinylchloride (PVC) and silicone] commonly used in the manufacture of medical devices[6]. This method was found equally sensitive to detect low concentrations of some strong irritant compounds when compared to the human patch testing and intracutaneous rabbit test[14]. Therefore, the *in vitro* irritation test shall be performed before animal testing or human patch test is considered.

NOTE It can be relevant to provide detailed information of the applicability of the RhE model for the specific medical device being tested.

This document describes a stepwise approach, which shall include one or more of the following:

a) chemical characterization, supplemented where needed with chemical testing of samples in accordance with the general principles specified in ISO 10993-9, ISO 10993-13, ISO 10993-14, ISO 10993-15 and ISO 10993-18;

b) literature review, as indicated in ISO 10993-1, including an evaluation of chemical and physical properties, and information on the irritation potential of any product constituent as well as structurally-related chemicals and materials;

NOTE *In silico* methods (structure activity relationship, QSAR, read across) can indicate potential irritant activity.

c) *in vitro* alternative test using validated RhE per the methods in 6.2 to 6.12;

NOTE For special irritation tests relevant for medical devices intended to be applied to a specific area (Annex D), i.e. mucosal or eye epithelia, the RhE models are not adapted and it is recommended to explore the use of other *in vitro* models with relevant cells or tissues if qualified for use with medical devices.

d) *in vivo* animal tests;

NOTE *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).

e) clinical studies according to ISO 14155 and ethics principles governing human clinical research, shall not be performed before the irritancy potential of a device has been established through one or more of the evaluations described in a) to d).

# 5 Pre-test considerations

## 5.1 General

It is important to emphasize that pre-test considerations can result in the conclusion that testing for irritation is not necessary. For example, if the pH of the test sample is ≤2,0 or ≥11,5 the material shall be considered an irritant and no further irritant testing is required according to OECD 404.

The requirements specified in ISO 10993-1:2018, Clause 5 on the categorization of medical devices and the following apply.

Non-sterile samples shall be investigated *in vivo* 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 should 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 can be used as processing aids, for example, lubricants or mould-release agents. In addition to the chemical components of the starting material and manufacturing process aids, adhesive/solvent residues from assembly, sterilant residues or reaction products resulting from the sterilization process can be present in a finished product. Whether these components pose a health hazard/risk depends on the leaching or degradation characteristics of the finished products. These components shall be taken into account for their potential irritation activity. The following types of materials are often used in medical devices and could introduce risks for irritation.

### 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 ceramics, metals and alloys in terms of composition. A number of reaction products, impurities, and additives can be present and the completeness of polymerization can vary.

### 5.2.4 Biologically derived materials

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

## 5.3 Information on chemical composition

### 5.3.1 General

A description of the medical device chemical constituents shall be established according to ISO 10993-18. As described in ISO 10993-1, the extent of physical and/or chemical characterization required depends on what is known about the material formulation and on the nature and duration of body contact with the medical device. At a minimum, the characterization shall address the constituent chemicals of the medical device and possible residual process aids or additives used in its manufacture. The rigour necessary in the characterization of the chemical constituents is principally determined by the nature, degree, frequency and duration of the exposure and the hazards identified for the medical device or material. 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 (e.g. 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 search is recommended to establish the likely nature of the starting material(s) and any additives, so as to assist in the selection of the most appropriate methods of analysis for the material concerned.

The chemical characterization of a medical device shall be conducted 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 or it can be specified by the user, or both. 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 *In vitro* irritation tests

## 6.1 General

The *in vitro* method with RhE models for testing irritation was developed specifically to detect skin irritation potential for neat chemicals[3][12](see OECD 439). The method was adapted and validated with two RhE models for detection of irritant chemicals in medical device extracts[5][6][12][13][17][18][19]. This method was found equally sensitive to detect low concentrations of some strong irritants in extracts from polymeric medical materials (PVC and silicone) when compared to the human patch testing and intracutaneous rabbit test[20]. Hence, the RhE test as described in this document can replace the *in vivo* rabbit test for irritation by skin exposure and by intracutaneous (intradermal) administration.

NOTE It can be relevant to provide detailed information of the applicability of the RhE model for the specific medical device being tested.

## 6.2 *In vitro* reconstructed human epidermis model

### 6.2.1 Test system — Reconstructed human epidermis model

The RhE model shall consist of normal human-derived epidermal keratinocytes, which have been cultured to form a multi-layered highly differentiated model of the human epidermis. It shall consist of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo*. Normal human keratinocytes obtained from healthy volunteer donors shall be cultured for a number of days on a membrane or filter at an air-liquid interface to form the three-dimensional epidermal model comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum. The model system shall allow for both polar (e.g. saline) and non-polar (e.g. sesame oil) extracts to be directly added to the apical surface of RhE constructs.

Materials not suitable for extraction (e.g. liquids, gels, pastes, and particulates) might be suitable for the test system. If used, validation data should be provided to demonstrate the ability of the assay to detect irritant activity of these forms of materials prior to testing.

### 6.2.2 Principle of the method

Endpoints: cell viability determination is based on cellular reduction of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) and subsequent conversion to a purple formazan salt that is quantitatively measured after extraction from the tissues[9][16]. The cell viability in treated tissues is expressed as a percentage of the negative control. The percent reduction in viability is used to predict the irritation potential.

NOTE 1 Reduced tissue survival can be accompanied by IL-1α release[13][17][19]. Tissue culture media from the exposure can be collected and kept frozen at ≤−20 °C for possible analysis of cytokines.

Brief procedure: studies performed with polymeric biomaterials specifically manufactured to contain irritant chemicals at low concentrations indicated that a prolonged exposure is needed compared to the OECD 439 protocol for neat chemicals. An incubation period of no less than 18 h up to 24 h exposure at 37 °C for exposure to potentially low concentrations of irritants in extracts from biomaterials is sufficient for predicting irritation *in vitro* by reduction of tissue viability below 50 %[4][6][13][17][19]. Both



18 h and 24 h exposure showed similar results in both RhE models evaluated in the round robin study using medical device extracts[13][17][19].

Tissues are incubated at 37 °C, 5 % $CO_2$ in a humidified incubator following the addition of the test and control extracts.

Exposure to the test sample extract is terminated by rinsing with Dulbecco's phosphate buffered saline (DPBS), or PBS without $Ca^{2+}$ and $Mg^{2+}$. After washing, the tissues are manually dried. The viability is assessed by incubating the tissues for 3 h with MTT solution in a 24-well plate (1 mg/ml; 300 µl per well). The formazan crystals are extracted using an appropriate amount (depending on the RhE model used) of isopropanol for at least 2 h at room temperature. Two or three aliquots (depending on the instructions of the supplier) per tissue of extracted formazan is then added to 96-well plates (200 µl/well) and quantified spectrophotometrically at 570 nm.

For direct inoculation assays, a solution with a 1 % volume fraction of sodium dodecyl sulfate (SDS, see 6.4.4) in saline solution of NaCl 0,9 % can be used as positive controls (PCs) and DPBS or PBS without $Ca^{2+}$ and $Mg^{2+}$ treated epidermis are used as the negative control, respectively. For extracted assays, a verified irritant infused control extracted in sesame oil and in saline solutions of NaCl 0,9 % can be used as positive controls.

NOTE 2 Aliquots of culture media collected after 18 h or 24 h exposure can be stored frozen (at a minimum of −20 °C) for potential cytokine (IL-1α) measurements as a complementary endpoint to cell viability. IL-1α measurement determines the inflammation component to the assessment of skin irritation in addition to the cell damage component determined indirectly by the MTT test for cell viability.

Vehicle controls shall include saline (NaCl 0,9 %) solution and sesame oil that have undergone the ISO 10993-12 medical device extraction procedure. For each treated tissue the viability is expressed as a percent relative to negative DPBS or PBS treated control tissues (mean).

Known limitations of the method: The method is not applicable to gases and aerosols. It is also not considered applicable to evaluate irritation by direct contact of solid materials as close contact over the whole test surface cannot be guaranteed.

Known cases of test-compounds requiring specific controls: some chemicals can directly reduce the MTT reagent (e.g. electrophiles, test articles with high pH), while other chemicals can directly colour the tissue or the cells. Such test sample properties can only interfere if sufficient amounts of the chemical are still present on the tissue at the end of the exposure period. In these cases, a special procedure allowing the quantification of the "true" MTT reduction should be applied. A protocol for the determination of possible interactions with MTT is provided in References [20] and [21]. The use of specific and adapted controls enables the calculation of true tissue viability after subtracting the unspecific optical densities (OD) due to direct chemical MTT reduction or chemical residual colour extracted from the tissues, or both.

### 6.2.3 Prediction model

This prediction model is based on the prediction model of the OECD 439 and data further generated during the optimization of the medical device protocol[4][6][14][17][19].

If cell viability after the exposure is ≤50 %: the test sample is classified as an irritant (I).

If cell viability after the exposure is >50 %: the test sample is classified as a non-irritant (NI).

The cell viability test shall be conducted with both polar (e.g. saline) and non-polar (e.g. sesame oil) test extracts. If at least one of the extracts shows a positive effect (viability ≤50 %) the test sample of the medical device is considered to have irritant potential. The device or the device component tested shall then be considered to induce irritant activity. *In vivo* testing might be considered to further evaluate the categorization of the irritant activity when necessary. If the result is non-irritant (viability >50 %) with the two solvents, the device or the device component shall be considered as non-irritant.

## 6.3 Materials

### 6.3.1 Reconstructed human epidermis models — Product description

The epidermal cells are taken from healthy volunteer donors negative to anti-HIV 1 and 2, and to hepatitis C antibodies, and to hepatitis B antigens. Nevertheless, normal handling procedures for biological materials should be followed.

For test samples based on medical device extracts the application of two RhE models described and recognized in the OECD 439 was evaluated in a large international round robin study[6]. In this study the EpiDerm™ tissues EPI-200 model[1)] and the SkinEthic™ RHE model[2)] were used. Specific protocols of these models are available as supplementary material[6][20][21].

Both models have been validated by EURL ECVAM for determining skin irritation of chemicals and are included in OECD 439 and EU Guideline B.46[41]. These models were validated with neat industrial chemicals for the purpose of classification and labelling. The other described and recognized models included in OECD 439, can be used for medical device skin irritation if validated for testing medical device extracts for skin and tissue irritation. The validation of a new RhE based method similar to a reference method is called catch-up validation[3][37].

The validation for a new RhE model listed in the OECD 439 for *in vitro* skin irritation must demonstrate equal performances in terms of predictive capabilities and within- and between-laboratory variability to those of the initial round robin study[6]. The inter-laboratory study (minimum three laboratories) must be carried out blind with three runs (three production batches of the RhE model)[37] on a set of irritant and non-irritant materials equivalent to that of the original round robin study.

### 6.3.2 Preparation of medical device extracts

The preparation of medical device and/or biomaterial extracts shall be performed in accordance with ISO 10993-12.

— Polar extracts shall be prepared in 0,9 % saline solution (900 mg in 100 ml ultrapure or deionized water).

— Non-polar extracts shall be prepared in sesame oil from *sesamum indicum* (examples of quality that are acceptable: super refined and pharmaceutical grade).

NOTE 1 In the round robin study[6] 0,9 % saline solution and sesame oil were demonstrated to be suitable extractants for irritants present in PVC or silicone polymers, or both, to be applied in the *in vitro* RhE irritation assay. Therefore, these vehicles are recommended as extract solutions.

If other vehicles are used for extraction, validation data shall be provided to confirm that a change in the extraction vehicle will not impact the ability of the test system to differentiate between negative, weak, moderate, and strong irritants.

The extraction time and temperature should be justified based on ISO 10993-12.

NOTE 2 In the round robin study to evaluate the RhE for irritation testing of the medical device, extracts spiked polymers (PVC and silicone) were used and extraction was performed at (37 ± 1) °C for (72 ± 2) h with continuous agitation/shaking.



1) EpiDerm™ is a trademark of a product supplied by MatTek *In Vitro* Life Science Laboratories (Bratislava, Slowakia) and MatTek Corporation, (Ashland, MD, USA). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

2) SkinEthic™ RHE is a trademark of a product supplied by EpiSkin SA (Lyon, France), EpiSkin Brazil (Rio, Brazil) and Shanghai EPISKIN Biotechnology (Shanghai, China). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Materials not suitable for extraction (e.g. liquids, gels, pastes, and particulates) can be suitable for the test system. If used, validation data should be provided to demonstrate the ability of the assay to detect irritant activity of these forms of materials prior to testing.

## 6.4 Methods

### 6.4.1 General

**CAUTION — The procedure described in this document requires the use of hazardous reagents. This document does not claim to cover all related safety issues. It is the responsibility of the user of this document to take appropriate precautionary and occupational health and safety measures and to ensure compliance with regulatory and regulatory requirements.**

MTT poses the following hazards:

— H302: harmful if swallowed;

— H315: causes skin irritation;

— H319: causes serious eye irritation;

— H330: fatal if inhaled;

— H335: may cause respiratory irritation;

— H341: suspected of causing genetic defects <state route of exposure if it is conclusively proven that no other routes of exposure cause the hazard>.

Isopropanol poses the following hazards:

— H225: highly flammable liquid and vapour;

— H319: causes serious eye irritation;

— H336: may cause drowsiness or dizziness.

### 6.4.2 Test procedure

Annex B provides a check list for *in vitro* irritation testing using RhE models.

All procedures performed during the RhE irritation test shall be documented. An example of a method documentation sheet (MDS) is presented in Annex C. A description of the various steps involved in performing the *in vitro* skin irritation test is presented below. The following steps shall be followed, with any deviations justified and accompanied with validation data.

— Prepare device/biomaterial test and control samples as extracts in a polar (saline) and non-polar (sesame oil) solvent starting prior to tissue arrival. The timing of initiation of extraction should be based on the time chosen for extraction in accordance with ISO 10993-12 and the time the tissue is ready for treatment (depending on the time of arrival and necessary pre-incubations of the RhE tissues). The test material/medical device extract should be used within the timeframe allowed in ISO 10993-12 for use of the extracts for biocompatibility testing. Apply during the extraction period continuous agitation/shaking.

— Upon receipt transfer RhE tissues from transport plates medium to medium according to tissue manufacturer directions for use. If required by tissue manufacturer's instructions, pre-incubate tissues by placing the tissues in a suitable sized culture plates with assay medium (see 6.5.1).

— If extracts of positive control materials are not included, spike on the day of the experiment the positive control (SDS) into polar (saline) solvent at the concentrations specified. Lower concentrations of SDS can be used (e.g. 0,25 % to 0,5 %) as positive control after demonstrating suitability and positive outcomes[17][19].

— Apply the negative control, the positive control, the vehicle controls and the test sample in a 100 µl volume on the tissue surface.

— Incubate at (37 ± 1) °C, (5 ± 1) % $CO_2$, in a humidified incubator for (18 ± 1) h or (24 ± 2) h.

NOTE In the round robin study the incubation time was 18 h for EpiDerm™ tissues EPI-200 model and 24 h for the SkinEthic™ RHE model. Both models show similar outcomes at either 18 h or 24 h exposure[13][19][17].

— Stop exposure by rinsing the RhE tissues with DPBS or PBS without $Ca^{2+}$ and $Mg^{2+}$ and drying RhE tissues according to tissue manufacturer's instructions.

— Transfer tissues to MTT solution (optional: medium sampling for interleukin mediators release measurements). Store samples in a freezer at ≤–20 °C.

— Incubate tissues for 3 h ± 5 min in MTT [(37 ± 1) °C, (5 ± 1) % $CO_2$, in a humidified incubator].

— Transfer and immerse in formazan extraction medium isopropanol.

— Formazan extraction: for at least 2 h at room temperature or overnight (sealed, at room temperature).

— Shake and homogenize.

— Transfer formazan solution in 96-well plate.

— Read OD using a plate spectrophotometer.

### 6.4.3 Media and end point solutions

#### 6.4.3.1 MTT solution

MTT solution is light sensitive. Protect it from light using silver paper or appropriate material.

Dissolve MTT powder to a final concentration of 5 mg/ml in PBS. Proceed to 0,22 µm filtration. Prepare ready-to-use solutions (e.g. in 1 ml aliquots) in sterile dark 1,5 ml microtubes. Store reagents in a freezer at –20 °C. Storage time: 1 year at –20 °C.

Alternatively, an MTT preparation made available by the supplier of the RhE tissues can be used.

Dilute the MTT stock solution with pre-warmed medium to a final use concentration of 1 mg/ml. Protect from light until use (do not exceed 2 h stocking before use).

#### 6.4.3.2 Isopropanol solution

Use undiluted 2-propanol (CAS N° 67-63-0).

### 6.4.4 Test sample and control preparation

Record the main information about the control and test extracts, including codes or numbers, physical consistency, volumes or weight, expiration date and stocking conditions. Record the preparation and method details. An example for record preparation is presented in Annex C.

Specific procedure for the negative control (DPBS or PBS): the negative control is sterile DPBS or PBS without $Ca^{2+}$ and $Mg^{2+}$. Sterile ready-to-use DPBS or PBS shall be used. A volume of 3 × 100 µl shall be added to three independent tissues.

Specific procedure for the positive control (1 % v/v SDS): 500 µl of 20 % SDS in water shall be mixed with 9,5 ml of the particular extraction vehicle (saline) and thoroughly vortexed. From this preparation, a volume of 100 µl is removed using an appropriate pipette and added to the tissues. Lower concentrations of SDS may be used (e.g. 0,25 % to 0,5 %) as positive control after demonstrating suitability and positive outcomes[17][19].



The positive control in polar extraction vehicle shall be prepared freshly before each use on the day of the experiment.

Twenty percent SDS solution from a commercial supplier shall be used (e.g. Fluka/Sigma, Cat # 05030[3]).

Specific procedure for the vehicles: the vehicle controls (0,9 % of saline and sesame oil) shall be placed into the extraction vessel (e.g. amber glass vial) and be subjected to the identical extraction procedure, e.g. (72 ± 2) h at (37 ± 1) °C as the test materials. If samples are to be directly applied to the tissue, this step can be omitted.

Positive control materials are scarce. The Y-4 polymer[22] as provided by the Food and Drug Safety Center's Hatano Research Institute (Japan) or other proven controls, if validated, provided by suitable suppliers can be used as positive control in the *in vitro* RhE models. A silicone-SDS can be used as a positive control for saline extraction, if validated.

## 6.5 Considerations for test performance

### 6.5.1 Receipt of the reconstructed human epidermis tissues

Kit details and assay procedures shall be documented (e.g. in the MDS).

### 6.5.2 Preparation and pre-incubation

— If required by the tissue manufacturer's instructions, pre-incubate the tissue by placing it in suitable sized culture plates with medium according to the tissue manufacturer's instructions for use.

— Fill an appropriate number of wells with an adapted quantity of fresh medium according to the manufacturer's instructions for use.

— Under sterile conditions, open the cover of plates containing the RhE tissues. Under a sterile airflow, remove the cover and carefully (using sterile forceps) take out each insert containing the epidermal tissue. Remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on the sterile filter paper or gauze, and place the tissues in the sterile prepared wells with fresh medium.

— Check visually that no agarose is remaining and transfer the tissue on fresh medium by inclining the insert to avoid underneath bubble formation.

NOTE The 24-well plate used for shipping can be kept/stored sealed at room temperature to look for signs of possible contamination at the end of the week.

— Check the quality of the RhE tissues and record tissue alterations.

— Transfer inserts with the tissues to the wells of previously pre-filled plates according to the instructions for use of the manufacturer. Pre-incubate (37 ± 1) °C, (5 ± 1) % $CO_2$, in a humidified incubator) the tissues according to tissue producer directions for use.

## 6.6 Application of the test sample and rinsing

### 6.6.1 General

Although the conduct of the *in vitro* assay for irritation testing follows the same principles for all models, one should be aware that minor differences between specific models can be present when performing the assays.

If an SOP is available for a specific RhE model, the SOP of the manufacturer should be followed taking into consideration specific aspects (e.g. exposure time) for medical device extract testing as presented in this document. Before using a RhE model referenced in the OECD 439 for the testing of either medical

3) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

device extracts or medical devices, or both, the suitability of the particular model shall be demonstrated. These commercial or optimized instructions should, when used with medical device systems, result in outcomes that are equivalent to the already qualified methods included in this document.

### 6.6.2 Preparation

— Pre-warm the maintenance culture medium at room temperature.

— Remove the pre-incubation plates from the incubator approximately 5 min before exposure to the extracts.

— Before test extract exposure:

  — prepare suitable sized culture plates for the incubation of the RhE tissues with the test samples and controls;

  — label all plate lids with the test material codes; three tissues/wells per test sample and controls;

  — refresh medium below the inserts (add an appropriate amount of medium) or transfer inserts to a new plate with fresh medium.

### 6.6.3 Test extract and controls exposure

— Apply (100 ± 2) µl (i.e. ~200 µl/cm$^2$) of the undiluted test samples (i.e. medical device extracts), VCs, negative control or PC on the surface of three single tissues each. Use the pipette tip to spread the test sample gently on the epidermis topical surface. The application order is important since it has to be the same for washing.

NOTE The model surface is hydrophobic and it is important to check that the 100 µl are well distributed over the whole surface of the epidermis. Sometimes, with polar solvents, because of surface tension mechanisms, the droplet is distributed only to the periphery of the epidermis. In this case, use either a pipette tip to spread the sample or forceps to tap the insert into the bottom of the plate until the entire surface of the epidermis is covered. In addition, for the oily extracts a bulb headed glass Pasteur pipette can be used to spread the test sample in order to ensure a correct contact with the entire epidermis.

— Keep plates with exposed tissues in the laminar flow hood, until the last tissue is exposed.

— After dosing the last tissue, transfer all plates to the humidified incubator [(37 ± 1) °C, (5 ± 1) % $CO_2$] for the necessary exposure time.

NOTE In the round robin study the incubation time was (18 ± 1) h for EpiDerm™ tissues EPI-200 model and (24 ± 2) h for the SkinEthic™ RHE model.

— After the exposures, thoroughly rinse the tissues with sterile DPBS or PBS, filling and emptying the tissue insert a number of times to remove any residual test sample material. Rinsing of 15 times to 25 times is recommended. Follow specific instructions when provided by the manufacturer of the RhE model. The rinsing should not be too gentle otherwise the test sample will not be completely removed.

— After rinsing the insert, remove residual DPBS or PBS by inverting and gently shaking the insert. Drain the insert by inverting on sterile absorbent paper.

— Dry the insert surface (e.g. use a double-sided swab).

— Transfer the blotted tissue inserts into suitable containers or wells of a tissue culture plate pre-filled with fresh assay medium (0,3 ml/well) until all tissues are rinsed. Proceed to MTT incubation.

In case that traces of the test sample are still present on the surface, try to remove them with a sterile wetted cotton swab. Record this procedure. Tissue surface can be evaluated visually under a dissecting stereoscope.

NOTE Before evaluating the viability of the RhE, the culture medium below the insert can be collected for additional cytokine determination. Reduced tissue survival was accompanied by IL1α release[13][17][19].

## 6.7 MTT test for determination of RhE tissue viability after the exposure period

### 6.7.1 MTT incubation and Isopropanol extraction

RhE tissue viability is assessed by MTT reduction measurement. The MTT assay is performed immediately after the exposures are finished. Residues of test samples are removed by rinsing of the tissues.

The preparation of the MTT-solution and pre-filling of 24-well plate (300 µl/well) shall be performed before starting with the washing procedure.

— Prepare MTT medium and prepare 24-well culture plates with assay medium. Pipette 300 µl of MTT medium (concentration 1 mg/ml) in each well of the required number of 24-well plates.

— Remove inserts from the temporary storage plates, dry the bottom of each insert by pressing it against sterile absorbent paper or with a sterile cotton tipped swab, and transfer them into the 24-well plates, pre-filled with 300 µl of MTT (1 mg/ml). Place the plates in the incubator [(37 ± 1) °C, (5 ± 1) % $CO_2$, in a humidified incubator], record the start time of MTT incubation (e.g. in the MDS) and incubate for 3 h ± 5 min.

— After MTT incubation is complete, blot any residual MTT media off of the tissue inserts and transfer the tissues to suitable containers or wells of tissue culture plates prefilled with an appropriate amount of isopropanol according to the instructions of the RhE supplier.

— Seal the containers or plates (e.g. with Parafilm®)[4] or place into a sealable plastic bag to inhibit extractant evaporation. Record start time of extraction (e.g. in the MDS) and extract formazan for at least 2 h at room temperature with gentle shaking on a plate shaker at the rate of approximately 120 rpm. As an alternative, overnight extraction (18 h to 24 h) is also possible. Seal plates as described above and extract at room temperature or in the refrigerator in the dark, without shaking. Before analysing the extracts, shake for at least 15 min on plate shaker.

— After incubation, pierce tissue and membrane with a tip or injection needle 20 gauge in order to obtain the whole extraction solution in the corresponding well. Obtain solution with solubilized formazan for measurement of absorbance. Before transferring the extract from the containers or plate wells into 96-well plates, pipette up and down at least 3 times until the extractant solution is homogenous.

### 6.7.2 Absorbance measurements

— Transfer per tissue 2 or 3 × 200 µl aliquots (= 2 or 3 wells per tissue) from each test well into a 96-well flat bottom microtiter plate (labelled appropriately) depending on the instructions of the RhE supplier. A single 96-well plate is to be used per measurement.

NOTE Be careful to avoid isopropanol evaporation in 96-well plates. VC1 and VC2 in the plate maps in Annex C are always 0,9 % saline and sesame oil vehicle controls. In the *in vitro* RhE assay a control isopropanol background is used for OD determinations in RhE assay.

4) Parafilm® is a registered trademark of a product supplied by Pechiney Plastic Packaging, 8770 W Bryn Mawr Ave, Chicago, IL 60631. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

— Read the OD in a 96-well plate spectrophotometer using wavelength filter according to the manufacturer's instructions and isopropanol solution as background control. Usually a filter of 570 nm or 630 nm is used.

NOTE Readings at 570 nm are performed without a reference filter, since the "classical" reference filter often used in the MTT test (630 nm) is still within the absorption curve of formazan. Since filters have a tolerance, their use can lead to reduction of the dynamic range of the OD signal.

## 6.8 Test acceptance criteria

Negative control and vehicle control acceptance criteria: the NC and VC data in general meet the acceptance criteria if the mean OD value of the three tissues is ≥0,7 and below 3,0 at 570 nm. These values are the minimum and maximum OD values for various RhE test models and they vary depending on instructions of the RhE model supplier (see OECD 439). The OD values shall meet the acceptance criteria specific for each model as specified in OECD 439. The value is considered valid if the standard deviation of the mean viability of the three exposed tissues after incubation with the NC or VC is ≤20 %. The mean tissue viability of the VC shall be within 80 % to 120 % of the NC.

Positive control acceptance criteria: the PC data meet the acceptance criteria if the mean viability, expressed as % of the NC, is <40 % and the standard deviation of the viability of the replicate tissues is ≤20 %.

NOTE If Y-4 is applied as positive control material, a cell viability of <50 % could be used instead of <40 % in accord with the original article of round robin test for medical device extracts[6].

Test acceptance criteria: all test sample data from an assay are considered as valid if the negative, vehicle and the positive controls data fulfil the above criteria requirements.

Test sample data acceptance criteria: the value is considered valid if the standard deviation of the mean viability of the replicate tissues is ≤20 %. If this requirement is not met the test shall be repeated.

## 6.9 Data calculation steps

### 6.9.1 General

The following calculation steps are applicable to the majority of test-samples characterized as follows: no interaction with the MTT reagent (see 6.2.2), non-coloured, with a low ability to stain the tissues and measured non-specific colour value ≤5 % relative to the NC.

### 6.9.2 Isopropanol background control for OD in RhE assay

Calculate the mean OD from the six replicates of isopropanol solution for each plate.

Calculate the standard deviation between each tissue OD.

### 6.9.3 Negative DPBS or PBS treated controls

Calculate the isopropanol background corrected value by subtracting the mean OD of isopropanol background to the OD of the negative DPBS or PBS treated controls.

Calculate the mean OD per tissue (three replicates).

The mean OD for all tissues corresponds to 100 % viability.

Calculate standard deviation between each tissue OD and viability.

### 6.9.4 Positive control

— Calculate the isopropanol background corrected value by subtracting the mean OD of isopropanol background to the OD of the positive control.



— Calculate the mean OD per tissue (three replicates).

— Calculate the viability per tissue by dividing the mean OD per tissue of PC by the mean OD per tissue of NC and multiplying the result by 100.

— Calculate the mean viability for all tissues.

— Calculate standard deviation between each tissue OD and viability.

#### 6.9.5 Tested extract and VC samples (TTs)

— Calculate the isopropanol background corrected value by subtracting the mean OD of isopropanol background to the OD of the tested extract.

— Calculate the mean OD per tissue (three replicates).

— Calculate the viability per tissue by dividing the mean OD per tissue of TT by the mean OD per tissue of NC and multiplying the result by 100.

— Calculate the mean viability for all tissues.

— Calculate standard deviation between each tissue OD and viability.

The viability percent for each treated epidermis, are calculated relative to the mean of negative controls. Coefficient of variances (CVs) shall also be calculated. The mean relative viability of three tissues is used for classification according to the prediction model.

### 6.10 Data interpretation — Prediction model

The irritant potential of test samples is predicted by the mean tissue viability of tissues exposed to the test sample. The mean viability is calculated on three individual tissues. The irritation potential is predicted if the mean relative viability is equal to or below 50 % of the negative control.

For medical device or medical device component extracted with both polar (e.g. saline) and non-polar (e.g. sesame oil) solvents, if at least one of the extracts shows a positive effect on the tissues (viability ≤50 % and SD <20 %) the device or the device component tested shall be considered to have irritant activity (see Table 1). If the result is non-irritant (viability >50 %) with the two solvents, the device or the device component shall be considered a non-irritant.

**Table 1 — Classification of test sample**

| Criteria for *in vitro* interpretation | Classification |
|---|---|
| Mean tissue viability is ≤50 % in at least one extraction vehicle | Irritant (I) |
| Mean tissue viability is >50 % in the two extraction vehicles | Non-irritant (NI) |

NOTE For neat chemicals, the *in vitro* RhE model for testing of irritant activity detects irritants that are classified according to UN GHS in Category 2 (irritant) and Category 1 (skin corrosion) as indicated in OECD 439 and OECD 431[31]. In addition, it can identify UN GHS No Category "low hazard" non-irritant chemicals when the viability of the tissues is above 50 %. In relation to Table 3 (*in vivo* irritation responses) the RhE models identify response categories as "Negligible" and "Severe".

### 6.11 Method documentation sheet

An MDS is presented as example in Annex C for data management allowing compliant quality control (i.e. correct set up, calibration, function of the equipment and quality of preparations). For each epidermis batch and experiment, make hardcopies of the necessary MDS, fill along the experiments the date, fill in the requested information in the tables, and sign the MDS.

### 6.12 Test report

The test report shall include at least the following details:

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

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

— a description of the RhE model used;

— a copy of the certificate of quality control of batch of tissues used (to be provided by RhE model manufacturer);

— a description of the assay protocol including method of sample administration and exposure time;

— a record of observations on the tissues;

— all individual raw data (e.g. OD for each well) related to viability determination of the tissues;

— an assessment of the results and classification of the test samples/devices;

— the international standard used (including its year of publication);

— any deviations from the procedure;

— any unusual features observed;

— the date of the test.

## 7 *In vivo* irritation tests

### 7.1 General

Irritation testing of either medical devices, components of medical devices or biomaterials, or all, can be performed with either the devices, device components, biomaterials themselves or extracts thereof, or all.

Factors affecting the results of *in vivo* irritation studies include the following:

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

— the dose of the test material;

— the method of application of the test material;

— the degree of occlusion;

— the application site;

— the duration and number of exposures;

— 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 either repeated or prolonged exposure, or both. The study shall be designed to exaggerate the anticipated contact (either time or concentration, or both) in the clinical situation. This shall be borne in mind during interpretation of the result.



If the pH of the test sample is ≤2,0 or ≥11,5 the material shall be considered an irritant and no further testing is required (see OECD 404). 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.

## 7.2 Animal irritation test by skin exposure

### 7.2.1 Principle

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

The rabbit (*Oryctolagus cuniculus*) is the preferred animal model suitable for this test.

### 7.2.2 Test materials

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 produced positive results using the test method within the previous six months.

NOTE A suitable positive control is SDS, also known as sodium lauryl sulfate (SLS).

### 7.2.3 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. As it is not expected that skin irritation will be different for male and female animals, a single sex can be used. If female animals are used, they shall be nulliparous and non-pregnant. 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 2) 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.

### 7.2.4 Test procedure

#### 7.2.4.1 Preparation of animals

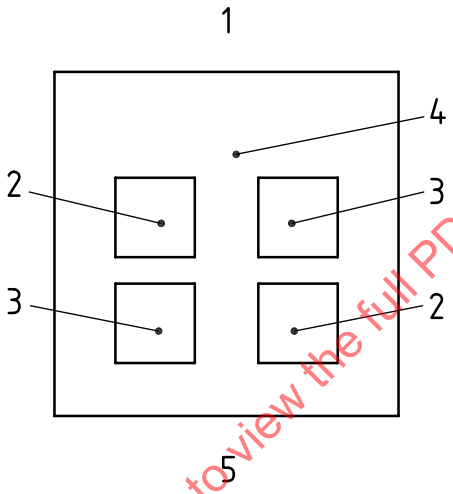
The condition of the skin is a critical factor. Use only animals with healthy intact skin. Fur is generally clipped within 24 h to 4 h of testing from the backs of the animals. A sufficient distance on both sides of the spine should be clipped for application and observation of all test sites (approximately 10 cm × 15 cm). Fur can be re-clipped either to facilitate observation or to accommodate repeated exposures, or both. Depilatories can be used by trained technicians, if the process has been validated at the testing facility and that depilatories will not elicit an irritation response. If repeated exposures are required, follow the procedures in 7.2.4.2.1, 7.2.4.2.2 or 7.2.4.2.3. Repeated exposure testing can be repeated for a maximum of 21 d.

#### 7.2.4.2 Application of test sample

##### 7.2.4.2.1 Application of powder or liquid sample

Apply 500 mg or 500 µl 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). Two observation sites shall be treated with each test and control samples (see Figure 1).

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 using a permanent marker that is considered not to be a skin irritant. 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**



##### 7.2.4.2.2 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 500 µl per patch. Apply one patch on each side of the animal as shown in Figure 2. 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 using a permanent marker that is considered not to be a skin irritant. Remove residual test material by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent and careful drying.

##### 7.2.4.2.3 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 using a permanent marker that is considered not to be a skin irritant. Remove residual test material by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent and careful drying.

### 7.2.5 Observation of animals

#### 7.2.5.1 General

The 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 2, 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.

#### 7.2.5.2 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 the 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. Animals shall be observed at least once a day during this extended observation period.

#### 7.2.5.3 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). The repeated exposure shall be limited by the clinical use of the device.

Repeated exposure after a slight reaction in a single exposure test shall be justified.

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 over a period of time not exceeding 14 d. Animals shall be observed at least once a day during this extended observation period.

### 7.2.6 Evaluation of results

The reactions at the various time points after administration shall be scored according to Table 2.

**Table 2 — 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 |
| Other adverse changes at the skin sites shall be recorded and reported. | |

**Table 2** *(continued)*

| Reaction | Irritation score |
|---|---|
| **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. | |

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

Use only the observations at (24 ± 2) h, (48 ± 2) h and (72 ± 2) h 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 3 and the appropriate response category is recorded for the report.

NOTE The categories of irritation are based on the data comparing 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 3 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 3. In case different extracts have been tested, the one giving the highest PII determines the response category.

**Table 3 — 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 |

#### 7.2.7 Test report

The test report shall include:

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

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

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

— a description of the test animal(s);

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

— how the sites were marked, and the readings performed;

— records of the observations;

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

— evaluation of the results;

— the international standard used (including its year of publication);

— any deviations from the procedure;

— any unusual features observed;

— the date of the test.

### 7.3 Animal irritation test by intracutaneous (intradermal) administration

#### 7.3.1 Introduction

For medical devices that are contacting breached or compromised surface, externally communicating or 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.

#### 7.3.2 Exclusion from test

Any material known or in preliminary tests shown to be a skin, eye or mucosal tissue irritant or material with a pH ≤2,0 or ≥11,5 shall not be tested intradermally (see OECD 404). 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.

#### 7.3.3 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. No positive reference material is available for this test, however some chemicals (SDS, heptanoic acid, lactic acid, nonanoic acid) will give positive responses. Low concentrations inducing a positive irritation score (>1, see 7.3.7) shall be used avoiding induction of skin necrosis. The use of a positive control to confirm sensitivity is only warranted when the testing laboratory has not produced positive results using the test method within the previous six months.

Certain medical devices like dermal fillers can be dosed neat. As the dermal filler will remain, this should not be confused with oedema.

### 7.3.4 Animals and husbandry

Healthy young adult albino rabbits of either sex from a single strain, weighing not less than 2 kg, shall be used. As it is not expected that skin irritation will be different for male and female animals a single sex can be used. If female animals are used, they shall be nulliparous and non-pregnant. 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 two for either erythema or oedema (see Table 4), 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.

### 7.3.5 Test procedure

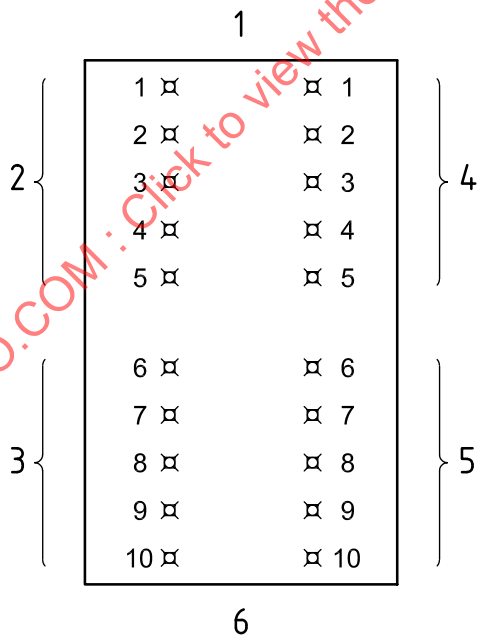
Within a 4 h to 18 h period before testing, closely clip the fur on the backs of the animals, allowing a sufficient distance on both sides of the spine for injection of the extracts.

Inject intracutaneously 200 µl 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 200 µl of the polar or non-polar solvent control on five sites of the contralateral side of each rabbit (e.g. 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 | 4 | 200 µl injections of polar solvent control |
| 2 | 200 µl injections of polar extract | 5 | 200 µl injections of non-polar solvent control |
| 3 | 200 µl injections of non-polar extract | 6 | caudal end |

**Figure 2 — Arrangement of injection sites**

### 7.3.6 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.

Score the tissue reaction for erythema and oedema according to the system given in Table 4 for each injection site and at each time interval observed and record the results.

NOTE 1 Intradermal injection of oil frequently elicits an inflammatory response.

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

NOTE 2 Histological or non-invasive techniques can be used to assist in the evaluation if they are available.

**Table 4 — Scoring system for intracutaneous (intradermal) 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. | |



### 7.3.7 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. Should results be inconsistent between animals or controls not perform as anticipated making interpretation of the overall results questionable, the study can be repeated using three additional rabbits.

A final test score of 1,0 or less would be similar to the classification as non-irritant in the *in vitro* RhE model.

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

### 7.3.8 Test report

The test report shall include:

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

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

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

— a description of the test animals;

— the method of injection;

— how the site readings were performed;

— a record of the observations;

— an assessment of the results;

— the international standard used (including its year of publication);

— any deviations from the procedure;

— any unusual features observed;

— the date of the test.

# 8 Human skin irritation test

## 8.1 Introduction

Potential for irritation risk assessments may also be assessed using data from human skin patch tests.

Human studies can serve several purposes:

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

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

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

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

Clinical tests shall be performed in accordance with ISO 14155. Additional specific requirements for clinical tests are described in Annex E.

NOTE Annex F gives further information on irritation tests.

## 8.2 Initial considerations

Adequate information on the toxicity profile of the material or medical device 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:

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

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

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

— potential corrosivity for human skin can be predicted either on the basis of structure/activity relationships or physicochemical properties such as strong acid or alkaline reserve, or both;



— they present a risk of skin or respiratory tract sensitization;

— they present any acute toxicity hazard under test conditions;

— they present any genotoxic, reproductive or carcinogenic hazard.

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

# Annex A
(normative)

# Preparation of materials for irritation testing

## A.1 General

The conduct of irritation tests and interpretation of their data 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. This annex provides general information on the preparation of test samples for both *in vitro* and *in vivo* irritation testing. The applicability and use of the various types of test samples is described in the relevant clauses in this document.

## 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 for *in vivo* test 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 a non-irritant. Absorbent gauze may be used as a substitute if a more suitable control cannot be identified.

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

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

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

### A.2.2 Liquid test materials

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

## A.3 Extracts of test materials

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

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

## 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 document.

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

# Annex B
(informative)

# Test method check list for *in vitro* irritation testing using reconstructed human epidermis models

— Prepare medical device test and control samples as extracts in a polar (saline) and non-polar (sesame oil) solvent starting prior to tissue arrival (usually 48 h to 72 h before arrival of the RhE tissues depending on the necessary pre-incubations of the RhE tissues). The extraction time and temperature should be justified based on ISO 10993-12.

NOTE In the round robin study to evaluate the RhE for irritation testing of medical device extracts spiked polymers (PVC and silicone) were used and extraction was performed at (37 ± 1) °C for (72 ± 2) h with continuous agitation/shaking.

— Receipt: transfer epidermis from transport plates medium to maintenance medium.

— Incubate for 2 h to 24 h at (37 ± 1) °C and (5 ± 1) % $CO_2$, in a humidified incubator according to the manufacturer's instructions.

— If extracts of positive control materials are not included, spike on the day of the experiment the positive control (SDS) into polar (saline) solvent at the concentrations specified. Lower concentrations of SDS can be used (e.g. 0,25 % to 0,5 %) as positive control after demonstrating suitability and positive outcomes[17][19].

— Apply negative control, positive control, vehicle controls and test samples (extracts from the tested materials) in a 100 µl volume on the tissue surface.

— Incubate at (37 ± 1) °C, (5 ± 1) % $CO_2$, in a humidified incubator overnight (e.g. 18 h to 24 h depending on the manufacturer).

— Stop treatment by DPBS or PBS rinsing.

NOTE Before evaluating the viability of the RhE the culture medium below the insert can be collected for additional cytokine determination.

— Prepare MTT solution and MTT incubation plates.

— Transfer tissues to MTT solution.

— Incubate tissues for 3 h ± 5 min, in MTT at (37 ± 1) °C, (5 ± 1) % $CO_2$, in a humidified incubator.

— Transfer and immerse in formazan extraction medium (isopropanol).

— Formazan extraction: for at least 2 h at room temperature or overnight and sealed, at room temperature.

— Shake and homogenize.

— Transfer extracted solution in 96-well plate.

— Read OD using a plate spectrophotometer.

The check list above is also given in Table B.1.

**Table B.1 — Procedure for *in vitro* irritation testing using reconstructed human epidermis model**

| Time | Procedure |
|---|---|
| Minus 48 h to 72 h | Prepare both polar and non-polar extract (37 ± 1) °C. |
| | Receipt of tissues. |
| | Pre-incubate tissues according to manufacturer's instructions (e.g. 2 h to 24 h). |
| Start of test<br>T = 0 h | Apply NC, PCs, VCs, and test samples in a 100 µl volume on the tissue surface. |
| Incubation<br>T = 18 h or,<br>T = 24 h | Incubate at (37 ± 1) °C and (5 ± 1) $CO_2$, in a humidified incubator overnight.<br>(e.g. 18 h - 24 h depending on the manufacturer). |
| End of incubation next day | Stop treatment by DPBS or PBS rinsing. |
| Measurement viability | Prepare MTT solution. |
| | Transfer tissue into MTT solution. |
| MTT incubation<br>T = 3 h | Incubate tissues for 3 h ± 5 min at (37 ± 1) °C and (5 ± 1) % $CO_2$, in a humidified incubator. |
| Formazan detection | Prepare MTT extraction solution with isopropanol. |
| Formazan extraction<br>T = 2 h | Incubate tissues for at least 2 h at room temperature or overnight in isopropanol. |
| | Shake and homogenize after formazan extraction |
| | Transfer extracted formazan solution in 96-well plate |
| Reading | Read OD using spectrophotometer |

# Annex C
(informative)

# Example of method documentation sheet for reconstructed human epidermis models

Assay number: .....

Date: .....

Corresponding XLS data file name: .....

PERFORMED BY: ..... SIGNATURES: .....

**TIME PROTOCOL (see Table C.1)**

Receipt of RhE tissues (date, day, hour): .....ID:

Manufacturer/supplier: .....

**Table C.1 — Time protocol**

| Procedure | Date (dd-mm-yy) | Set 1 | | Set 2 | |
|---|---|---|---|---|---|
| | | start | stop | start | stop |
| **Pre-incubation** (2 h to 24 h) | | | | | |
| **Exposure** (18 ± 1) h (24 ± 2) h | | | | | |
| **Washing** | | | | | |
| **MTT test** 3 h ± 5 min | | | | | |
| **Extraction** minimum 2 h | | | | | |
| **Measurement** | | | | | |

**EQUIPMENT VERIFICATION**

Equipment verification should be included in the quality system of the laboratory performing the assay. Test parameters such as incubator, refrigerator, and water bath conditions should be documented. Quality aspects of equipment and labware (e.g. pipettes) should be recorded.

**ASSAY PERFORMANCE**

Assay performance is given in Table C.2.

**Table C.2 — Assay components**

| | |
|---|---|
| Reconstructed epidermis (RhE) supplier Lot no.: | Production date: |
| Assay medium Lot no.: | Expiration date: |
| MTT concentrate Lot no.: | Expiration date: |
| MTT diluent<br>Lot no.: | Production date: |
| Isopropanol (MTT extractant) Lot no.: | Expiration date: |
| (D)PBS<br>Lot no: | Expiration date: |
| Other remarks: | |

ID/date:

Visual quality control of the skin is given in Table C.3.

**Table C.3 — Visual quality control of the skin**

| APPEARANCE | KIT 1 | KIT 2 |
|---|---|---|
| MACRO: | | |
| No of excluded tissues with:<br>— edge defects<br>— air bubbles<br>— extensive moisture on the surface | | |
| Use scores: 1-very good, 2-good, 3-acceptable, 4-not acceptable. | | |

Specific observations:

**SOLUTIONS AND VEHICLES**

Spiked chemical positive control.

SDS 1 % (v/v) solution in saline, prepared from 20 % solution of SDS: on the day of the experiment, prepare fresh solutions of 1 % SDS in saline and sesame oil by mixing 100 µl of 20 % SDS with 1,9 ml of vehicle. Vortex thoroughly after preparation and shortly before application to the tissue surface. Expiration is on the same day.

— 20 % SDS stock solution reference, batch N°: .....

— Saline (0,9 % NaCl solution), batch N°: .....

— Preparation date: .....

**Saline (0,9 % NaCl) solution preparation:**

— NaCl reference, batch N°: .....

— Type of sterilisation: .....

— Preparation date: .....

— Expiration date: .....

**(D)PBS solution preparation**:

— (D)PBS concentrate reference, batch N°: .....

— pH adjustment (to 7,0): .....

— Type of sterilisation: .....

— Preparation date: .....

— Expiration date: .....

**Sesame oil:**

— Reference, batch N°: .....

— Expiration date: .....

**REMARKS TO SINGLE TISSUES**

If any abnormalities during the assay are observed, if tissues have to be substituted, or if any technical problems need to deal with, make a note of it in Table C.4.

Record the tissue number, the sample code and the observation or remark.

**Table C.4 — Remarks to single tissues**

| Sample code | Tissue No. | Remark |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |

ID/date:

**SPECTROPHOTOMETRICAL MEASUREMENT**

**PLATE CONFIGURATION FOR READING**

Record the positions of samples on 96-well plate. Strictly adhere to the fixed plate design as indicated in the instructions of the manufacturer. Examples of a plate layout as used in the medical device round robin study for irritation testing[6] are presented in Tables C.5 and C.6.

Switch on the reader 10 min before reading or as specified in the equipment manual.

**Table C.5 — EpiDerm RhE model**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | **bckg** | **bckg** | **bckg** | **bckg** | **bckg** | **bckg** | | | | | | | |
| B | **NC** | **PC** | **VC1** | **VC2** | **TA1** | **TA2** | **TA3** | **TA4** | **TA5** | **TA6** | **TA7** | **TA8** | Tissue1 |
| C | **NC** | **PC** | **VC1** | **VC2** | **TA1** | **TA2** | **TA3** | **TA4** | **TA5** | **TA6** | **TA7** | **TA8** | |
| D | **NC** | **PC** | **VC1** | **VC2** | **TA1** | **TA2** | **TA3** | **TA4** | **TA5** | **TA6** | **TA7** | **TA8** | Tissue2 |
| E | **NC** | **PC** | **VC1** | **VC2** | **TA1** | **TA2** | **TA3** | **TA4** | **TA5** | **TA6** | **TA7** | **TA8** | |
| F | **NC** | **PC** | **VC1** | **VC2** | **TA1** | **TA2** | **TA3** | **TA4** | **TA5** | **TA6** | **TA7** | **TA8** | Tissue3 |
| G | **NC** | **PC** | **VC1** | **VC2** | **TA1** | **TA2** | **TA3** | **TA4** | **TA5** | **TA6** | **TA7** | **TA7** | |
| H | | | | | | | | | | | | | |

**bckg**, isopropanol solution for background measurement; NC, negative control [(D)PBS; PC, positive control (SDS saline (PC)]; VC, vehicle control background [e.g. saline (VC1) and sesame oil (VC2)]; TA1 … TA7, test samples 1–7. For the EpiDerm RhE model the OD is determined *in duplo* (2x 200 µl) according to the manufacturer.

**Table C.6 — SkinEthic RHE model**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | **bckg** | **bckg** | **bckg** | **bckg** | **bckg** | **bckg** | empty | empty | empty | empty | empty | empty |
| B | NC | NC | NC | NC | NC | NC | NC | NC | NC | empty | empty | empty |
| C | NaCl | NaCl | NaCl | NaCl | NaCl | NaCl | NaCl | NaCl | NaCl | empty | empty | empty |
| D | SO | SO | SO | SO | SO | SO | SO | SO | SO | empty | empty | empty |
| E | PC | PC | PC | PC | PC | PC | PC | PC | PC | empty | empty | empty |
| F | NaCl 1 | NaCl 1 | NaCl 1 | NaCl 1 | NaCl 1 | NaCl 1 | NaCl 1 | NaCl 1 | NaCl 1 | empty | empty | empty |
| G | SO 1 | SO 1 | SO 1 | SO 1 | SO 1 | SO 1 | SO 1 | SO 1 | SO 1 | empty | empty | empty |
| H | NaCl 2 | NaCl 2 | NaCl 2 | NaCl 2 | NaCl 2 | NaCl 2 | NaCl 2 | NaCl 2 | NaCl 2 | empty | empty | empty |
| | Tissue 1 | | | Tissue 2 | | | Tissue 3 | | | | | |

**bckg**, isopropanol solution for background measurement; NC, negative control; ((D)PBS vehicle control; SO, sesame oil vehicle control; PCNACL, positive sample control in NaCl extraction vehicle; PCSO, positive sample control in sesame oil extraction vehicle. NaCL 1 and SO 1 isopropanol samples of 200 µl in triplicate for each tissue incubation for test sample 1. Additional test samples can be measured in additional 96-well plates. For the SkinEthic RHE model, the OD is determined *in triplo* (3 × 200 µl) according to the manufacturer.

**Check the wave length**

Tick correct (✓) filter setting in Table C.7.

**Table C.7 — Photometer setting**

| | |
|---|---|
| Wavelength: 570 (550 nm to 570 nm) | |

ID/Date:

**ARCHIVING**

Raw data saved in/as:

Spreadsheet saved in/as:

MDS saved in/as:

**CHARACTERIZATION OF TEST SAMPLES (see Table C.8)**

Laboratory: ..... Study N°: ..... Assay N°: .....

**Table C.8 — Test samples preparation and incubation**

| Test material name or code | Amount of sample used | Volume of extract added | Incubator | Time in: | Time out: | Pre- and post-extraction appearance |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

ID/Date:

# Annex D
(normative)

# Special irritation tests

## D.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.

## D.2 Ocular irritation test

### D.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 come into contact with the eye or eyelid.

OECD 405 on acute eye irritation/corrosion[29] describes a stepwise *in vivo* testing strategy for the determination of the eye irritation/corrosion properties of chemicals. Using this strategy, as well as a weight-of-evidence analysis (where all available information on the eye irritation potential is considered prior to proceeding to *in vivo* testing) is important to avoid the unnecessary use of animals. Reducing substance testing resulting in severe animal responses promotes both animal welfare and sound science. Although TG 405 is an *in vivo* test method it also supports the 3 Rs principle in reduction and refinement of animal testing. This test has not been validated for medical device extracts.

### D.2.2 *In vitro* alternative test for eye irritation

Several *in vitro* alternative tests have been evaluated for eye irritation. However, they have not yet been validated for use in medical device testing. No single *in vitro* test is likely to be able to replace the *in vivo* eye irritation test covering all *in vivo* endpoints. However, strategic combinations of several alternative test methods within a (tiered) testing strategy can be able to replace the Draize eye test. A possible conceptual framework for such a (tiered) testing strategy was developed for chemicals within an EURL ECVAM workshop[23]. The framework is based on alternative eye irritation methods that vary in their capacity to detect either severe irritant substances (EU R41; GHS 'Category 1') or substances considered non-irritant (EU 'Non-Classified'; GHS 'No Category'). According to this framework, the entire range of irritancy can be resolved by arranging tests in a tiered strategy that may be operated from either end: to detect first severe irritants and resolve absence of irritancy ("top-down approach") or to proceed inversely, starting with the identification of non-irritants first ("bottom-up approach"). Mild irritancy will be resolved in a last tier in both approaches.

A number of OECD Test Guidelines based on *in vitro* methods related to the endpoint "Eye Irritation" already exist: OECD 437:2020, OECD 438:2017, OECD 460:2017, OECD 491:2020 and OECD 492:2017. These guidelines for determination of irritant activity of chemicals are based on validated (by ICCVAM, EURL ECVAM, JaCVAM or others) *in vitro* tests. These guidelines describe the bovine corneal opacity and permeability test method (see OECD 437), the isolated chicken eye test method (see OECD 438), the fluorescein leakage test method using cells growing on a semi-permeable insert (see OECD 460), and short time exposure in vitro test method (see OECD 491) composed of a cytotoxicity-based *in vitro* assay that is performed on a confluent monolayer of statens seruminstitut rabbit cornea (SIRC) cells, and a test using human reconstructed cornea-like epithelium the so called reconstructed human cornea-like epithelium (RhCE) test (see OECD 492). None of these OECD tests has been validated for medical devices.

### D.2.3 Principle

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

### D.2.4 Exclusion from test

Either materials or final products, or both, 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 ≤2,0 or ≥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.

### D.2.5 Test material

If the test material is a liquid, instil 100 µl, 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 100 µl 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 100 µl as for liquids.

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

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

— 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 100 µl 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.

### D.2.6 Animals and husbandry

Healthy young adult albino rabbits of either sex from a single strain, weighing not less than 2 kg, shall be used. As it is not expected that eye irritation will be different for male and female animals a single sex may be used. If female animals are used they shall be nulliparous and non-pregnant.

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 D.1) in one animal obviates the need for additional testing. With materials that would be anticipated to not cause significant ocular irritation, e.g. contact lens solutions, well characterized, non-cytotoxic materials, or others, using scientific judgment, three rabbits can be tested initially without the requirement for a one rabbit screen.

When no response (see Table D.1) 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.

### D.2.7 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 % British Pharmacopoeia (BP)[38] or an equivalent 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 D.2.5 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 or blank control when an extract is tested. Water or saline solution may be used as blank in case of samples tested neat.

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.

### D.2.8 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 can 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 gives guidelines for contact lens testing that requires 21 d exposure for 8 h per day. This is an exception to the guidelines[1].

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

**Table D.1 — System for grading ocular lesions**

| Reaction | Numerical grading |
|---|---|
| **a) Cornea** | |
| **1) 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] |
| **2) 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 |
| **b) Iris** | |

[a] Positive result.

**Table D.1** *(continued)*

| Reaction | Numerical grading |
|---|---|
| **1) 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] |
| **c) Conjunctivae** | |
| **1) 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] |
| **2) 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] |
| **3) 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 D.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 D.1, i.e.

— absence of a light reflex (iris 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.

### D.2.9 Evaluation of results

#### D.2.9.1 General

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

#### D.2.9.2 Acute exposure

If the treated eye in more than one animal shows a positive result (see Table D.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 (see Table D.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.

#### D.2.9.3 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 (see Table D.1) at any stage of the observation.

### D.2.10 Test report

The test report shall include:

— the international standard used (including its year of publication);

— a description of the test samples;

— the intended use/application of the test samples;

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

— a description of the test animals;

— the method of instillation;

— how the ocular readings were performed;

— a record of the observations;

— an assessment of the results;

— any deviations from the procedure;

— any unusual features observed;

— the date of the test.

## D.3 Oral mucosa irritation test

### D.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. Similar to the *in vitro* human reconstructed epidermal

models also mucosal *in vitro* models are now available. This includes the use of human reconstructed mucosal structures[10]. However, they have not yet been qualified for use in medical device testing.

### D.3.2 Principle

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

### D.3.3 Exclusion from test

Any material shown to be a skin or eye irritant or any material having a pH ≤2,0 or ≥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.

### D.3.4 Test material

Prepare test materials in accordance with Annex A.

### D.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 a suitable collar with a width of 3 mm to 4 mm to each animal, 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 7 d 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.

### D.3.6 Test procedure

From each animal 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 procedural control. If necessary, negative control animals may be tested in parallel also receiving the procedural control.

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. Other treatment schedules (e.g. based on clinical use) shall be justified and documented.

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

### D.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 D.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 D.2 — Macroscopic grading system for mucosal 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. | |

### D.3.8 Assessment of results

#### D.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 D.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.

#### D.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 D.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.

For the microscopic evaluation in the control cheek pouch a total score greater than nine 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 side or control group average from the test group average to obtain the irritation index (see Table D.4).

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

### D.3.9 Test report

The test report shall include:

— a description of the test samples;

— the intended use/application of the test samples;

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

— a description of the test animals;

— the method of application;

— how the site readings were performed;

— a record of the observations;

— the histological evaluation;

— assessment of the results;

— the international standard used (including its year of publication);

— any deviations from the procedure;

— any unusual features observed;

— the date of the test.

**Table D.3 — Grading system for microscopic examination for mucosal 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 |

**Table D.3** *(continued)*

| Reaction | Numerical grading |
|---|---|
| Minimal | 1 |
| Mild | 2 |
| Moderate | 3 |
| Marked | 4 |

**Table D.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.<br>The microscopic examination grading system given in Table D.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. | |

## D.4 Penile irritation test

### D.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.

### D.4.2 Principle

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

### D.4.3 Exclusion from test

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

### D.4.4 Test sample

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

### D.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 D.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.

### D.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 200 µl) 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 ± 0,1) h after the last application.

For acute exposure, repeat the above procedure every hour (±0,1) h for 4 h. Other treatment schedules (e.g. based on clinical use) shall be justified and documented.

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

### D.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 D.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.

### D.4.8 Assessment of results

#### D.4.8.1 Macroscopic evaluation

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

The grades (see Table D.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.

#### D.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 D.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).

For the microscopic evaluation a total score greater than nine 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 D.4).

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

### D.4.9 Test report

The test report shall include:

— the international standard used (including its year of publication);

— a description of the test sample;

— the intended use/application of the test samples;

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

— a description of the test animals;

— the method of application;

— how the site readings were performed;

— a record of the observations;

— the histological evaluation;

— assessment of the results;

— any deviations from the procedure;

— any unusual features observed;

— the date of the test.

## D.5 Rectal irritation test

### D.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.

### D.5.2 Principle

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

### D.5.3 Exclusion from test

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

### D.5.4 Test material

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

### D.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 either rectal discharge, either swelling or other evidence of lower bowel infection, or both, irritation or injury prior to each treatment, or all.

### D.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. Other treatment schedules (e.g. based on clinical use) shall be justified and documented.

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

### D.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 either excessive discharge, swelling or that are found difficult to dose, or all shall be humanely sacrificed and the tissues examined (see D.5.8.1 and D.5.8.2).

### D.5.8 Evaluation of results

#### D.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.

#### D.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 D.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).

For the microscopic evaluation a total score greater than nine in a control animal can 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 D.4).

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

### D.5.9 Test report

The test report shall include:

— the international standard used (including its year of publication);

— a description of the test samples;

— the intended use/application of the test samples;

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

— a description of the test animals;

— the method of application;

— how the site readings were performed;

— a record of the observations;

— the histological evaluation;

— assessment of the results;

— any deviations from the procedure;

— any unusual features observed;

— the date of the test.