American National Standard

ANSI/AAMI BE78:2002

Biological evaluation of medical devices— Part 10: Tests for irritation and delayed-type hypersensitivity

AAMI Association for the Advancement of Medical Instrumentation

The Objectives and Uses of AAMI Standards and Recommended Practices

It is most important that the objectives and potential uses of an AAMI product standard or recommended practice are clearly understood. The objectives of AAMI's technical development program derive from AAMI's overall mission: the advancement of medical instrumentation. Essential to such advancement are (1) a continued increase in the safe and effective application of current technologies to patient care, and (2) the encouragement of new technologies. It is AAMI's view that standards and recommended practices can contribute significantly to the advancement of medical instrumentation, provided that they are drafted with attention to these objectives and provided that arbitrary and restrictive uses are avoided.

A voluntary *standard* for a *medical device* recommends to the manufacturer the information that should be provided with or on the product, basic safety and performance criteria that should be considered in qualifying the device for clinical use, and the measurement techniques that can be used to determine whether the device conforms with the safety and performance criteria and/or to compare the performance characteristics of different products. Some standards emphasize the information that should be provided with the device, including performance characteristics, instructions for use, warnings and precautions, and other data considered important in ensuring the safe and effective use of the device in the clinical environment. Recommending the disclosure of performance characteristics often necessitates the development of specialized test methods to facilitate uniformity in reporting; reaching consensus on these tests can represent a considerable part of committee work. When a drafting committee determines that clinical concerns warrant the establishment of *minimum* safety and performance criteria, referee tests must be provided and the reasons for establishing the criteria must be documented in the rationale.

A *recommended practice* provides guidelines for the use, care, and/or processing of a medical device or system. A recommended practice does not address device performance *per se*, but rather procedures and practices that will help ensure that a device is used safely and effectively and that its performance will be maintained.

Although a device standard is primarily directed to the manufacturer, it may also be of value to the potential purchaser or user of the device as a fume of reference for device evaluation. Similarly, even though a recommended practice is usually oriented towards health care professionals, it may be useful to the manufacturer in better understanding the environment in which a medical device will be used. Also, some recommended practices, while not addressing device performance criteria, provide guidelines to industrial personnel on such subjects as sterilization processing, methods of collecting data to establish safety and efficacy, human engineering, and other processing or evaluation techniques; such guidelines may be useful to health care professionals in understanding industrial practices.

In determining whether an AAMI standard or recommended practice is relevant to the specific needs of a potential user of the document, several important concepts must be recognized:

All AAMI standards and recommended practices are *voluntary* (unless, of course, they are adopted by government regulatory or procurement authorities). The application of a standard or recommended practice is solely within the discretion and professional judgment of the user of the document.

Each AAMI standard or recommended practice reflects the collective expertise of a committee of health care professionals and industrial representatives, whose work has been reviewed nationally (and sometimes internationally). As such, the consensus recommendations embodied in a standard or recommended practice are intended to respond to clinical needs and, ultimately, to help ensure patient safety. A standard or recommended practice is limited, however, in the sense that it responds generally to perceived risks and conditions that may not always be relevant to specific situations. A standard or recommended practice is an important *reference* in responsible decision-making, but it should never *replace* responsible decisionmaking.

Despite periodic review and revision (at least once every five years), a standard or recommended practice is necessarily a static document applied to a dynamic technology. Therefore, a standards user must carefully review the reasons why the document was initially developed and the specific rationale for each of its provisions. This review will reveal whether the document remains relevant to the specific needs of the user.

Particular care should be taken in applying a product standard to existing devices and equipment, and in applying a recommended practice to current procedures and practices. While observed or potential risks with existing equipment typically form the basis for the safety and performance criteria defined in a standard, professional judgment must be used in applying these criteria to existing equipment. No single source of information will serve to identify a particular product as "unsafe". A voluntary standard can be used as one resource, but the ultimate decision as to product safety and efficacy must take into account the specifics of its utilization and, of course, cost-benefit considerations. Similarly, a recommended practice should be analyzed in the context of the specific needs and resources of the individual institution or firm. Again, the rationale accompanying each AAMI standard and recommended practice is an excellent guide to the reasoning and data underlying its provision.

In summary, a standard or recommended practice is truly useful only when it is used in conjunction with other sources of information and policy guidance and in the context of professional experience and judgment.

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American National Standard

ANSI/AAMI BE78:2002
(Revision of ANSI/AAMI/ISO 10993-10:1995)

Biological evaluation of medical devices—Part 10: Tests for irritation and delayed-type hypersensitivity

Approved 1 October 2002 by
Association for the Advancement of Medical Instrumentation

Approved 6 November 2002 by
American National Standards Institute, Inc.

Abstract: This standard describes the procedure for the assessment of medical devices and their constituent materials with regard to their potential to produce irritation and delayed-type hypersensitivity.

Keywords: biological evaluation, hypersensitivity, irritation, medical devices

AAMI Standard

This Association for the Advancement of Medical Instrumentation (AAMI) standard implies a consensus of those substantially concerned with its scope and provisions. The existence of an AAMI standard does not in any respect preclude anyone, whether they have approved the standard or not, from manufacturing, marketing, purchasing, or using products, processes, or procedures not conforming to the standard. AAMI standards are subject to periodic review, and users are cautioned to obtain the latest editions.

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Glossary of equivalent standards

International Standards adopted in the United States may include normative references to other International Standards. For each International Standard that has been adopted by AAMI (and ANSI), the table below gives the corresponding U.S. designation and level of equivalency to the International Standard.

NOTE—Documents are sorted by international designation.

Other normatively referenced International Standards may be under consideration for U.S. adoption by AAMI; therefore, this list should not be considered exhaustive.

International designation	U.S. designation	Equivalency
IEC 60601-1-2:2001	ANSI/AAMI/IEC 60601-1-2:2001	Identical
IEC 60601-2-21:1994 and Amendment 1:1996	ANSI/AAMI/IEC 60601-2-21 & Amendment 1:2000 (consolidated texts)	Identical
IEC 60601-2-24:1998	ANSI/AAMI ID26:1998	Major technical variations
ISO 5840:1996	ANSI/AAMI/ISO 5840:1996	Identical
ISO 7198:1998	ANSI/AAMI/ISO 7198:1998/2001	Identical
ISO 7199:1996	ANSI/AAMI/ISO 7199:1996/(R)2002	Identical
ISO 10993-1:1997	ANSI/AAMI/ISO 10993-1:1997	Identical
ISO 10993-2:1992	ANSI/AAMI/ISO 10993-2:1993/(R)2001	Identical
ISO 10993-3:1992	ANSI/AAMI/ISO 10993-3:1993	Identical
ISO 10993-4:2002	ANSI/AAMI/ISO 10993-4:2002	Identical
ISO 10993-5:1999	ANSI/AAMI/ISO 10993-5:1999	Identical
ISO 10993-6:1994	ANSI/AAMI/ISO 10993-6:1995/(R)2001	Identical
ISO 10993-7:1995	ANSI/AAMI/ISO 10993-7:1995/(R)2001	Identical
ISO 10993-8:2000	ANSI/AAMI/ISO 10993-8:2000	Identical
ISO 10993-9:1999	ANSI/AAMI/ISO 10993-9:1999	Identical
ISO 10993-10:2002	ANSI/AAMI BE78:2002	Minor technical variations
ISO 10993-11:1993	ANSI/AAMI 10993-11:1993	Minor technical variations
ISO 10993-12:1996	ANSI/AAMI/ISO/CEN 10993-12:1996	Identical
ISO 10993-13:1998	ANSI/AAMI/ISO 10993-13:1999	Identical
ISO 10993-14:2001	ANSI/AAMI/ISO 10993-14:2001	Identical
ISO 10993-15:2000	ANSI/AAMI/ISO 10993-15:2000	Identical
ISO 10993-16:1997	ANSI/AAMI/ISO 10993-16:1997	Identical
ISO 10993-17:2002	ANSI/AAMI/ISO 10993-17:2002	Identical
ISO 11134:1994	ANSI/AAMI/ISO 11134:1993	Identical
ISO 11135:1994	ANSI/AAMI/ISO 11135:1994	Identical
ISO 11137:1995 and Amdt 1:2001	ANSI/AAMI/ISO 11137:1994 and A1:2002	Identical

International designation	U.S. designation	Equivalency
ISO 11138-1:1994	ANSI/AAMI ST59:1999	Major technical variations
ISO 11138-2:1994	ANSI/AAMI ST21:1999	Major technical variations
ISO 11138-3:1995	ANSI/AAMI ST19:1999	Major technical variations
ISO TS 11139:2001	ANSI/AAMI/ISO 11139:2002	Identical
ISO 11140-1:1995 and Technical Corrigendum 1:1998	ANSI/AAMI ST60:1996	Major technical variations
ISO 11607:2002	ANSI/AAMI/ISO 11607:2000	Identical
ISO 11737-1:1995	ANSI/AAMI/ISO 11737-1:1995	Identical
ISO 11737-2:1998	ANSI/AAMI/ISO 11737-2:1998	Identical
ISO TR 13409:1996	AAMI/ISO TIR 13409:1996	Identical
ISO 13485:1996	ANSI/AAMI/ISO 13485:1996	Identical
ISO 13488:1996	ANSI/AAMI/ISO 13488:1996	Identical
ISO 14155:1996	ANSI/AAMI/ISO 14155:1996	Identical
ISO 14160:1998	ANSI/AAMI/ISO 14160:1998	Identical
ISO 14161: 2000	ANSI/AAMI/ISO 14161:2000	Identical
ISO 14937:2000	ANSI/AAMI/ISO 14937:2000	Identical
ISO 14969:1999	ANSI/AAMI/ISO 14969:1999	Identical
ISO 14971:2000	ANSI/AAMI/ISO 14971:2000	Identical
ISO 15223:2000	ANSI/AAMI/ISO 15223:2000	Identical
ISO 15223/A1:2002	ANSI/AAMI/ISO 15223:2000/A1:2001	Identical
ISO 15225:2000	ANSI/AAMI/ISO 15225:2000	Identical
ISO 15674:2001	ANSI/AAMI/ISO 15674:2001	Identical
ISO 15675:2001	ANSI/AAMI/ISO 15675:2001	Identical
ISO TS 15843:2000	ANSI/AAMI/ISO TIR15843:2000	Identical
ISO TR 15844:1998	AAMI/ISO TIR15844:1998	Identical
ISO TR 16142:1999	ANSI/AAMI/ISO TIR16142:2000	Identical

Committee representation

Association for the Advancement of Medical Instrumentation

Biological Evaluation Committee

The adoption of ISO 10993-10:2002 (with a minor national deviation) as an American National Standard was initiated by the AAMI Biological Evaluation Committee, which also functions as a U.S. Technical Advisory Group to the relevant work in the International Organization for Standardization (ISO). U.S. representatives from the AAMI Irritation and Sensitization Working Group (U.S. Sub-TAG for ISO/TC 194/WG 8), cochaired by Paul Upman of NAmSA and Katharine Merritt of the U.S. Food and Drug Administration/Center for Devices and Radiological Health, played an active part in developing the ISO standard.

At the time this document was published, the **AAMI Biological Evaluation Committee** had the following members:

Cochairs:	Donald F. Gibbons, PhD
	Donald E. Marlowe
Members:	James M. Anderson, MD, PhD, Case Western Reserve University
	Sumner A. Barenberg, PhD, Bernard Technologies
	Eric R. Claussen, PhD, Becton Dickinson
	Roger Dabbah, PhD, U.S. Pharmacopeial Convention, Inc.
	Donald F. Gibbons, PhD, 3M
	Lawrence H. Hecker, PhD, Abbott Laboratories
	Donald E. Marlowe, U.S. Food and Drug Administration/Center for Devices and Radiological Health
	Edward Mueller, Annapolis, MD
	Barry F. Page, Garner, NC
	Melvin E. Stratmeyer, PhD, U.S. Food and Drug Administration/Center for Devices and Radiological Health/OST
	Paul Upman, NAmSA
Alternates:	Raju G. Kammula, DVM, PhD, U.S. Food and Drug Administration/Center for Devices and Radiological Health/ODE
	Sharon Northup, PhD, U.S. Pharmacopeial Convention, Inc.

At the time this document was published, the **AAMI Irritation and Sensitization Working Group** had the following members:

Cochairs:	Katharine Merritt, PhD
	Paul J. Upman, PhD
Members:	William C. Bradbury, PhD, Viromed Biosafety Laboratories
	Lee Ellis, Boston Scientific Corp.
	Mitchell B. Friedman, PhD, Abbott Laboratories
	Gloria Frost, PhD, Allegiance Healthcare Corporation
	Anita Kore, DVM, PhD, 3M Healthcare
	Howard Maibach, MD, University of California, San Francisco
	Katharine Merritt, PhD, U.S. Food and Drug Administration/Center for Devices and Radiological Health
	Anita Sawyer, Becton Dickinson
	Jeff Sturm, St. Jude Medical Inc.
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	Joel Gorski, PhD, NAmSA
	Lawrence H. Hecker, PhD, Abbott Laboratories
	Peter Pendergrass, Boston Scientific Corporation

NOTE—Participation by federal agency representatives in the development of this standard does not constitute endorsement by the federal government or any of its agencies.

Background of ANSI/AAMI adoption of ISO 10993-10:2002 and rationale for minor technical deviation

As indicated in the foreword to the main body of this document (page ix), the International Organization for Standardization (ISO) is a worldwide federation of national standards bodies. The United States is one of the ISO members that took an active role in the development of this standard.

International Standard ISO 10993-10 was developed by Technical Committee ISO/TC 194, *Biological evaluation of medical devices*, to provide guidance on the assessment of medical devices and their constituent materials with regard to their potential to produce irritation and delayed-type hypersensitivity.

U.S. participation in this ISO TC is organized through the U.S. Technical Advisory Group for ISO/TC 194, administered by the Association for the Advancement of Medical Instrumentation on behalf of the American National Standards Institute. The U.S. made a considerable contribution to this International Standard.

AAMI encourages its committees to harmonize their work with International Standards in the area of biological evaluation of medical devices as much as possible in order to help reduce unnecessary repetition of testing.

Upon review of ISO 10993-10, the AAMI Biological Evaluation Committee and AAMI Irritation and Sensitization Working Group decided to adopt ISO 10993-10:2002 with a minor technical deviation as a revision of ANSI/AAMI/ISO 10993-10:1995. The rationale for the deviation was to restore the text to its original text as voted on by the U.S. committee. A substantive change was introduced to ISO 10993-10:2002 that should not have been, and ANSI/AAMI BE78:2002 restores the original text (see below).

One major change to the previous edition was made. Annex B describes the very important intracutaneous reactivity test. Historically, this was in the normative section and it is now informative.

AAMI (and ANSI) have adopted other ISO standards. See the Glossary of equivalent standards for a list of ISO standards adopted by AAMI, which gives the corresponding U.S. designation and level of equivalency with the ISO standard.

The concepts incorporated in this standard should not be considered inflexible or static. This standard, like any other, must be reviewed and updated periodically to assimilate progressive technological developments. To remain relevant, it must be modified as technological advances are made and new data comes to light.

Suggestions for improving this standard are invited. Comments and suggested revisions should be sent to Standards Department, AAMI, 1110 N. Glebe Road, Suite 220, Arlington, VA 22201-4795.

NOTE—Beginning with the ISO foreword on page ix, this American National Standard is identical to ISO 10993-10:2002 with the exception of the national deviation to subclause 7.5.4.3.1 (see below).

ANSI/AAMI deviation from ISO 10993-10:2002

Subclause 7.5.4.3.1 Induction phase

The third sentence has been changed from “Repeat this procedure three times a week for three weeks” to “Repeat this procedure **one to** three times a week for three weeks.”

Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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

Attention is drawn to the possibility that some of the elements of this part of ISO 10993 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

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

This second edition cancels and replaces the first edition (ISO 10993-10:1995), which has been technically revised.

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

— *Part 1: Evaluation and testing*

— *Part 2: Animal welfare requirements*

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

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

— *Part 5: Tests for in vitro cytotoxicity*

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

— *Part 7: Ethylene oxide sterilization residuals*

— *Part 8: Selection and qualification of reference materials for biological tests*

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

— *Part 10: Tests for irritation and delayed-type hypersensitivity*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

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

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

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

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

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

— *Part 18: Chemical characterization of materials*

Future parts will deal with other relevant aspects of biological testing.

This part of ISO 10993 is a harmonization of numerous standards and guidelines, including BS 5736, OECD Guidelines, U.S. Pharmacopoeia, and the European Pharmacopoeia. It is intended to be the basic document for the selection and conduct of tests enabling evaluation of irritation and dermal sensitization responses relevant to safety of medical materials and devices.

Annex A forms a normative part of this part of ISO 10993. Annexes B and C are for information only.

Introduction

This part of ISO 10993 assesses possible contact hazards from chemicals released from medical devices that may produce skin and mucosal irritation, eye irritation, and delayed contact hypersensitivity.

Some materials that are included in medical devices have been tested, and their skin or mucosal irritation or sensitization potential has been documented. Other materials and their chemical components have not been tested and may induce adverse effects when in contact with biological tissues. The manufacturer is thus obliged to evaluate each device for potential adverse effects prior to marketing.

Traditionally, small animal tests are performed prior to testing on humans to help predict human response. More recently, *in vitro* tests and human tests have been added as alternatives. Despite progress and considerable effort in this direction, a review of findings suggests that currently no satisfactory *in vitro* test has been devised to eliminate the requirement for *in vivo* testing. Where appropriate, the preliminary use of *in vitro* methods is encouraged for screening purposes prior to animal testing. In order to reduce the number of animals used, this part of ISO 10993 presents a step-wise approach, with review and analysis of test results at each stage. An animal test is usually required prior to human testing.

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

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

Biological evaluation of medical devices—Part 10: Tests for irritation and delayed-type hypersensitivity

1 Scope

This part of ISO 10993 describes the procedure for the assessment of medical devices and their constituent materials with regard to their potential to produce irritation and delayed-type hypersensitivity.

This part of ISO 10993 includes

a) pretest considerations,

b) details of the test procedures, and

c) key factors for the interpretation of the results.

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

Supplementary tests which are required specifically for devices used intradermally in the ocular, oral, rectal, penile, and vaginal areas are given in annex B.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 10993. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 10993 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 10993-1:1997, *Biological evaluation of medical devices—Part 1: Evaluation and testing*.

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

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

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

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

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

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

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

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

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

3 Terms and definitions

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

3.1 allergen (sensitizer): Substance/material which is capable of inducing specific hypersensitivity such that, on subsequent exposure to the same substance/material characteristic, allergic effects are produced.

3.2 blank liquid: Solvent portion treated in the same manner as the identical solvent used for the preparation of test samples but without test material, and which is intended for the determination of a background response of the solvent.

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

3.4 corrosion: Slow destruction of the texture or material of a tissue.

NOTE—The action of a strong irritant.

3.5 delayed-type hypersensitization: Induction of specific T-cell mediated immunological memory for an allergen to which an individual is exposed, resulting in a delayed-type hypersensitivity reaction after secondary contact with the allergen.

3.6 dose: Quantity to be administered to the test system at one time.

3.7 edema: Swelling due to abnormal infiltration of fluid into the tissues.

3.8 erythema: Reddening of the skin or mucous membrane.

3.9 eschar: Scab or discolored slough of skin.

3.10 induction: Process that leads to the *de novo* generation of an altered state of immunological reactivity in an individual to a specific material.

3.11 irritant: Agent that produces irritation.

3.12 irritation: Localized non-specific inflammatory response to single, repeated, or continuous application of a substance/material.

3.13 necrosis: Death of one or more cells, or portion of tissue or organ, resulting in irreversible damage.

3.14 negative control: Material or substance which, when tested by the procedure described, demonstrates the suitability of the procedure to yield a reproducible, appropriate negative, nonreactive, or background response in the test system.

3.15 positive control: Material or substance which, when tested by the procedure described, demonstrates the suitability of the procedure to yield a reproducible, appropriate positive, or reactive response in the test system.

3.16 solvent: Material or substance used to moisten, dilute, suspend, extract, or dissolve the test substance material. Examples: Chemical, vehicle, medium, etc.

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

3.18 test sample: Extract or portion of the test material that is subjected to biological or chemical testing.

3.19 ulceration: Open sore representing loss of superficial tissue.

4 General principles—Step-wise approach

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

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

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

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

c) consideration of *in vitro* tests in preference to *in vivo* tests, and replacement of the latter as new *in vitro* methods become available and validated; at the present time, there are no validated *in vitro* tests (other than simple screens) to detect irritants or sensitizers;

d) *in vivo* animal tests;

NOTE—Acute *in vivo* animal studies are undertaken to test for materials not already classified as severe irritants or strong sensitizers by step a) or b). Materials that do not demonstrate an acute dermal irritation at single exposure may then be further evaluated following repeated exposure.

A test of a positive-control substance for skin sensitization [7] shall be run at least every six months by the testing laboratory to validate the test system and demonstrate a positive response.

e) non-invasive human tests/clinical trials.

If the material has been demonstrated not to be an irritant, a sensitizer, or toxic in animals, studies on skin irritation may then be considered in humans.

5 Pretest considerations

5.1 General

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

The requirements given in clause 5 of ISO 10993-1:1997 and the subclauses below apply.

5.2 Types of material

5.2.1 Initial considerations

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

5.2.2 Ceramics, metals, and alloys

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

5.2.3 Polymers

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

5.2.4 Biologically derived materials

These materials are inherently complex in their composition. They often also contain process residues, e.g., cross-linkers and anti-microbial agents. Biological materials may not be consistent from sample to sample.

The methods in this part of ISO 10993 have not been designed for testing of biologically derived materials and may therefore be less adequate. For example, the tests in this part of ISO 10993 do not consider cross-species sensitization.

5.3 Information on chemical composition

5.3.1 General

Full qualitative data on the chemical constituents of the material shall be established. Where relevant to biological safety, quantitative data shall also be obtained. If quantitative data is not obtained, the rationale shall be documented and justified.

5.3.2 Existing data sources

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

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

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

In the absence of any data on composition, a literature study to establish the likely nature of the starting material and any additives is recommended to assist in the selection of the most appropriate methods of analysis for the material concerned.

NOTE—The composition of ceramics, metals, and alloys may be in accordance with ISO or American Society of Testing Materials (ASTM) standards and/or may be specified by the user. However, in order to obtain full qualitative and quantitative details on composition, it may 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.

5.4 Material characterization

When details of composition are unavailable, or only qualitative information is available, or new or unknown substances may be expected to develop during the manufacturing process, it may be necessary to undertake analysis of a material.

Analytical methods appropriate for the material under investigation shall be used. All analytical techniques shall be justified, validated, and reported and, if not already known, the pH of the material (chemical solutions) shall be measured prior to any *in vivo* or *in vitro* testing when possible. Chemical analysis (qualitative as well as quantitative) of extracts may give useful information. In this context, it should also be emphasized that chemical analysis of the extract may give results that make testing for irritation and sensitization unnecessary, as information on irritation and sensitization potential of the compounds present in the extract solution may already be available.

6 Irritation tests

6.1 *In vitro* irritation tests

Two *in vitro* methods, the rat skin Transcutaneous Electrical Resistance (TER) test and the EPISKIN test, have been internationally validated as alternative tests to assess the skin corrosivity of chemicals. However, no validated methods to assess skin irritancy yet exist.

National and international organizations continue work to develop and validate *in vitro* tests for skin irritancy in parallel with the search for alternative methods; others have been developing methods to quantify the responses of animals and humans in order to better define endpoints using non-invasive techniques. See C.1.

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

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

Factors affecting the results of irritation studies include:

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

b) the dose of the test material;

c) the method of application of the test material;

d) the degree of occlusion;

e) the application site;

f) the duration and number of exposures; and

g) the techniques used in evaluating the test.

Additional background information is provided in annex C.

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

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

If the pH of the test sample is less than or equal to 2 or equal to or greater than 11.5, the material shall be declared an irritant and no further testing is required. However, experimental evidence suggests that acidity and alkalinity of the test material are not the only factors to be considered in relation to the capacity of a material to produce severe injury. The concentration of the test material, its period of contact, and many other physical and chemical properties are also important.

NOTE—For products intended to be used extensively on normal and compromised skin, no substantial risk is normally accepted; however, many products, in spite of a potential to irritate, are fully acceptable because of their inherent benefit or intended biological activity.

6.3 Animal skin irritation test

6.3.1 Principle

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

The rabbit is the preferred test animal.

6.3.2 Test material

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

In order to demonstrate the sensitivity of the assay, it is advisable to include, in addition to the negative control, a positive control on each animal. As there are two test sites and two control sites on each animal, a maximum of two test materials may be applied together with the control materials, provided that the same vehicle is used.

6.3.3 Animals and husbandry

Healthy young adult albino rabbits of either sex from a single strain, weighing not less than 2 kg, shall be used.

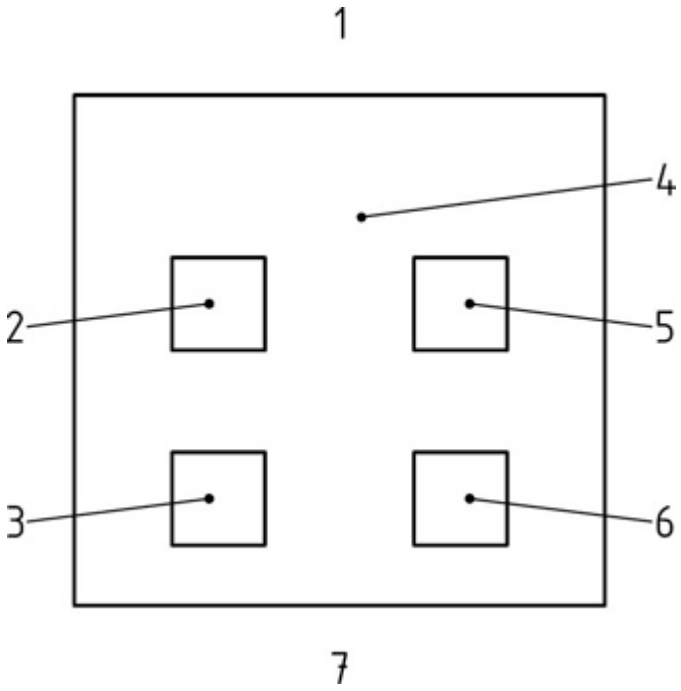
The animals shall be acclimatized and cared for as specified in ISO 10993-2.

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 edema; see Table 1) is observed, a minimum of two further animals shall be used. If no response is expected, initial testing may be conducted using three animals. If the response in the test using the minimum of three animals is equivocal, further testing shall be considered.

6.3.4 Test procedure

6.3.4.1 Preparation of animals

The condition of the skin is a critical factor. Use only animals with healthy intact skin. Fur is generally clipped within 24 h to 4 h of testing on the backs of the animals a sufficient distance on both sides of the spine for application and observation of all test sites (approximately 10 cm $\times$ 15 cm). Fur may be re-clipped to facilitate observation and/or to accommodate repeated exposures. Depilatories may be used by trained technicians, if the process has been validated at the testing facility. If repeated exposure is required, follow the procedures in 6.3.4.2, 6.3.4.3, or 6.3.4.4, repeated for a maximum of 21 days.



Key

1 Cranial end
2 Test site
3 Control site
4 Clipped dorsal region
5 Control site
6 Test site
7 Caudal end

Figure 1—Location of skin application sites

6.3.4.2 Application of powder or liquid sample

Apply 0.5 g or 0.5 mL of the test material directly to each test skin site as shown in Figure 1. For solid and hydrophobic materials, there is no need for moistening. If the material is a powder, it should be slightly moistened with water or other suitable solvent before application (see annex A).

Cover the application sites with a 2.5 cm × 2.5 cm non-occlusive dressing (such as an absorbent gauze patch) and then wrap the application site with a bandage (semi-occlusive or occlusive) for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites with permanent ink. Remove residual test material by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent and careful drying.

6.3.4.3 Application of extracts and extract vehicle

Apply the appropriate extract(s) to the 2.5 cm × 2.5 cm absorbent gauze patches. Use a volume of extract sufficient to saturate the gauze; generally 0.5 mL per patch. Apply one patch on each side of the animal as shown in Figure 1. Apply a control patch of gauze moistened with the extract vehicle as indicated in Figure 1.

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

6.3.4.4 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 by the test material shall be taken into account.

Cover the application sites with 2.5 cm × 2.5 cm non-occlusive dressings (such as a gauze patch) and then wrap the application sites with a bandage (semi-occlusive or occlusive) for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites with permanent ink. Remove residual test material by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent and careful drying.

6.3.5 Observation of animals

6.3.5.1 General

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

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

6.3.5.2 Single-exposure tests

For single-exposure tests, record the appearance of each application site at 1 h, 24 h, 48 h, and 72 h following removal of the patches. Extended observation may be necessary if there are persistent lesions, in order to evaluate the reversibility or irreversibility of the lesions. This need not exceed 14 days.

6.3.5.3 Repeated-exposure tests

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

For repeated exposure tests, record the appearances of the application site at 1 h after removal of the patches and immediately prior to the next application. The number of exposures may vary.

After the last exposure, note the appearance of each application site at 1 h, 24 h, 48 h, and 72 h following removal of the patches. Extended observation may be necessary if there are persistent lesions, in order to evaluate the reversibility or irreversibility of the lesions. This need not exceed 14 days.

Table 1—Scoring system for skin reaction

Reaction	**Primary 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
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Well-defined edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond exposure area)	4
Total possible score for irritation	8

Other adverse changes at the skin sites shall be recorded and reported.

6.3.6 Evaluation of results

For single exposure tests, determine the Primary Irritation Index (PII) as follows.

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

For each animal, add together the Primary Irritation Scores for the test material for both erythema and edema at each time point and divide the sum by the total number of observations. (One observation in this context includes both erythema and edema at each test site.) When blank liquid or negative control is used, calculate the Primary Irritation Score for the controls and subtract that score from the score for the test material to obtain the Primary Irritation Score. Add the scores for each animal and divide the total by the number of animals. This value is the Primary Irritation Index.

For repeated exposure, determine the Cumulative Irritation Index as follows.

For each animal, add together the Primary Irritation Scores for both erythema and edema at each time specified. Divide this total by the total figure of observations to obtain the Irritation Score per animal.

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 to the categories of Irritation Response defined in Table 2 and the appropriate Response category is recorded for the report.

NOTE—The categories of Cumulative Irritation Index are based on the data relating the Primary Irritation Index (PII) for chemicals in rabbits to the primary irritation response in humans for a number of chemicals that have been tested in both species.

For any response, record the maximum Primary Irritation Score from Table 1 for each animal, the time of onset of the response, and the time to maximum response.

The Primary or Cumulative Irritation Index is characterized by number (score) and description (Response category) in Table 2. In case different extracts have been tested, the one giving the highest PII determines the Response category.

Table 2—Irritation Response categories in rabbit

Mean score	Response category
0 to 0.4	Negligible
0.5 to 1.9	Slight
2 to 4.9	Moderate
5 to 8	Severe

6.3.7 Test report

The test report shall include:

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

b) the intended use/application of the test material(s) or device,

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

d) a description of the test animals,

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

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

g) records of the observations,

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

i) evaluation of the results.

6.4 Human skin irritation test

6.4.1 Introduction

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

Human studies can serve several purposes:

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

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

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

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

Clinical tests shall be performed in accordance with ISO 14155-1 and ISO 14155-2.

NOTE—C.1 gives further information on irritation tests.

6.4.2 Initial considerations

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

Materials shall not be tested in humans if:

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

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

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

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

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

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

Further guidance on the selection of human volunteers can be found in 6.4.4.1 and C.1.

6.4.3 Principle

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

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

6.4.4 Description of the method

6.4.4.1 Selection of human volunteers

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

6.4.4.2 Preparation of doses

Liquid test materials are generally used undiluted. When testing solids, moisten the test material with a small amount of water (typically 0.2 mL) or, where necessary, with another suitable vehicle, in order to ensure good contact with

the skin. The structure of the solid shall be considered and the choice of test material preparation shall be justified. When using moistened samples, take care to ensure that each subject receives the same amount of the test material. Use the same amount of water for moistening for each individual in the test and record this amount.

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

6.4.4.3 Procedure

6.4.4.3.1 Number of volunteers

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

6.4.4.3.2 Application of the test material

Apply the test material to intact skin at a suitable site, e.g., the upper outer arm, by means of an occlusive chamber containing a gauze pad. The application site shall be the same in all volunteers and shall be recorded. Generally, the patch shall measure at least 1.8 cm, preferably 2.5 cm, in diameter. The patch shall be held in contact with the skin by means of a suitable non-irritating dressing, including non-irritating tape, for the duration of the exposure period.

The patch shall deliver an adequate dose per unit area: approximately 50 mg to 100 mg test material per square centimeter is considered optimal. When applying liquid test materials, in general 0.2 mL to 0.4 mL is added onto the gauze pad until it is moistened. When testing solid materials, in general 0.2 g of the test material are moistened and added onto the gauze pad. As an alternative method of application for solids, the gauze pad is moistened and the test material covers the entire test site.

6.4.4.3.3 Duration of exposure

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

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

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

6.4.4.3.4 Limited exposure

In addition to the phased increase in duration of application as described in 6.4.4.3.3, if it is suspected that the material might produce severe irritation, a substantially reduced exposure time shall be employed, possibly in a pilot group of volunteers. The progress of the study can then be defined on the basis of the data produced. Subsequent patches are only applied after the 48 h/72 h readings.

6.4.4.3.5 Clinical observation and grading of skin reactions

Treatment sites are examined for signs of irritation and the responses are graded immediately after patch removal and at 1 h to 2 h, 24 h, 48 h, and 72 h after patch removal. If necessary to determine reversibility of the response, the observation period may be extended beyond 72 h. In addition, the condition of the skin before and after the test shall be described thoroughly (e.g., pigmentation and extent of hydration). Skin irritation is graded and recorded according to the grading in Table 3.

Noninvasive bioengineering methods may be applied (see annex C).

Table 3—Human skin irritation test, grading scale

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

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

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

6.4.4.3.6 Rationale for and selection of a concurrent positive control substance

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

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

6.4.5 Data and reporting

6.4.5.1 Data

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

6.4.5.2 Data evaluation/interpretation

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

6.4.5.3 Test report

The test report shall include the following information:

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

b) test material:

— physical nature and, where relevant, physicochemical properties;

— identification data;

c) vehicle:

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

d) volunteers:

— number of volunteers who were treated with the test material;

— age/sex distribution of the volunteers;

e) results:

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

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

— description of all irritant reactions observed;

— description of any other effects in addition to irritation observed;

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

— description or reference of an *in vitro* or *in vivo* animal test, if such is performed before the test in human volunteers, including details of the procedure and results obtained with test and reference materials.

f) discussion of the results.

7 Delayed hypersensitivity tests

7.1 Choice of test

The two most commonly used methods for testing delayed hypersensitivity are the guinea pig maximization test (GPMT) and the closed patch test (Buehler test).

The maximization test is the most sensitive method and is preferred for single chemicals. It has also been reported to be useful for the evaluation of extracts. However, the value of this test method is best documented for single chemicals. Recently, the Murine Local Lymph Node Assay (LLNA) was internationally accepted for testing single chemicals as a stand-alone alternative to the guinea pig assays [83].

NOTE—A rationale and a list of alternative methods is given in annex C.

7.2 Choice of test sample concentrations

7.2.1 General

Current guidelines for testing the sensitizing potential of single chemicals recommend using only one concentration for the test. However, the test result is highly dependent on dose and, if the test is used for the evaluation of an extract, qualitative and quantitative analyses of the extract to be tested are recommended.

7.2.2 Induction

Sensitization rate is highly dependent on the induction dose, which shall be moderately irritating. If the irritating threshold is not reached, then select the highest possible concentration. However, it shall not interfere with the health of the animals. The induction dose is selected based on pilot experiments as described for the individual tests. Undiluted extracts with the usual solvents for parenteral dosing need not be subjected to a pilot study.

7.2.3 Challenge

The challenge concentration is also based on pilot experiments on animals previously not exposed to the test material. A concentration below irritation threshold shall be used. The use of more than one concentration is advised for the challenge procedure, in order to facilitate the evaluation of the results (see C.2).

7.3 Other important factors affecting the outcome of the test

The biochemical and physical characteristics of the test sample may influence the choice of test. Since the maximization test requires intradermal injections, if the test sample cannot be injected intradermally, an alternative method shall be used.

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

Variation among results from different laboratories can have several sources. The following factors in the test procedure are important: ambient test conditions, test site on the animal, method of hair removal (clipping/shaving) or chemical depilation, type of patch design, quantity of test material, quality of occlusion, exposure time, and reading of the animals. Animal responsiveness also varies according to genetic factors and husbandry.

Comparison of the number of test animals having a positive response at challenge with the appropriate controls is essential for indication of a positive test result, though the severity of reactions will aid in the interpretation. Borderline reactions at challenge are best clarified by rechallenge. Histopathology has not been shown to be of help in the evaluation of test results.

In order to ensure reproducibility and sensitivity of the test procedure, tests with well-known contact allergens, e.g., mercaptobenzothiazole, hexyl cinnamic aldehyde, and benzocaine, shall be performed regularly.

7.4 Maximization test for delayed hypersensitivity

7.4.1 Principle

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

7.4.2 Test sample preparation

If the test material is solid or a liquid, the test sample shall be prepared as specified in annex A. The concentration of test sample shall be the highest possible without affecting the ability to interpret the results (see 7.4.4.2).

7.4.3 Animals and husbandry

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

The animals shall be acclimatized and cared for as specified in ISO 10993-2. Preliminary tests should be carried out on one set of animals to determine test concentrations (see 7.4.4.2).

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

For testing extracts, a minimum of ten animals shall be treated with the test sample and a minimum of five animals shall act as a solvent control group. If a preliminary test is needed, it shall be carried out on additional animals.

If testing on ten test and five control animals is completely negative, it is unlikely that testing of a further ten plus five animals will give positive results. However, if any equivocal responses develop, rechallenge (see 7.4.6) shall be carried out. If equivocal responses remain, conduct a new study on a minimum of 20 test and ten control animals.

7.4.4 Test procedure

7.4.4.1 Preparation

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

For intradermal injections, inject 0.1 mL per site.

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

7.4.4.2 Preliminary tests

The preliminary tests are intended to determine the concentration of the test samples to be used in the main test in 7.4.4.3.

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

Consideration shall be given to the following pretreatment of all animals by injection with Freund's complete adjuvant (FCA) in order to evaluate the possible excited skin status during the main test and thus interference with the readings. Topically apply a range of dilutions of the test sample to the flanks of at least three animals. Remove the occlusive dressings and patches after 24 h, and assess the application sites for erythema and edema using the Magnusson and Kligman grading scale given in Table 4.

For the topical induction phase in the main test, select the highest concentration that causes no more than slight erythema but does not otherwise adversely affect the animal.

For the challenge phase in the main test, select the highest concentration that produces no erythema.

Table 4—Magnusson and Kligman scale

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

7.4.4.3 Main test

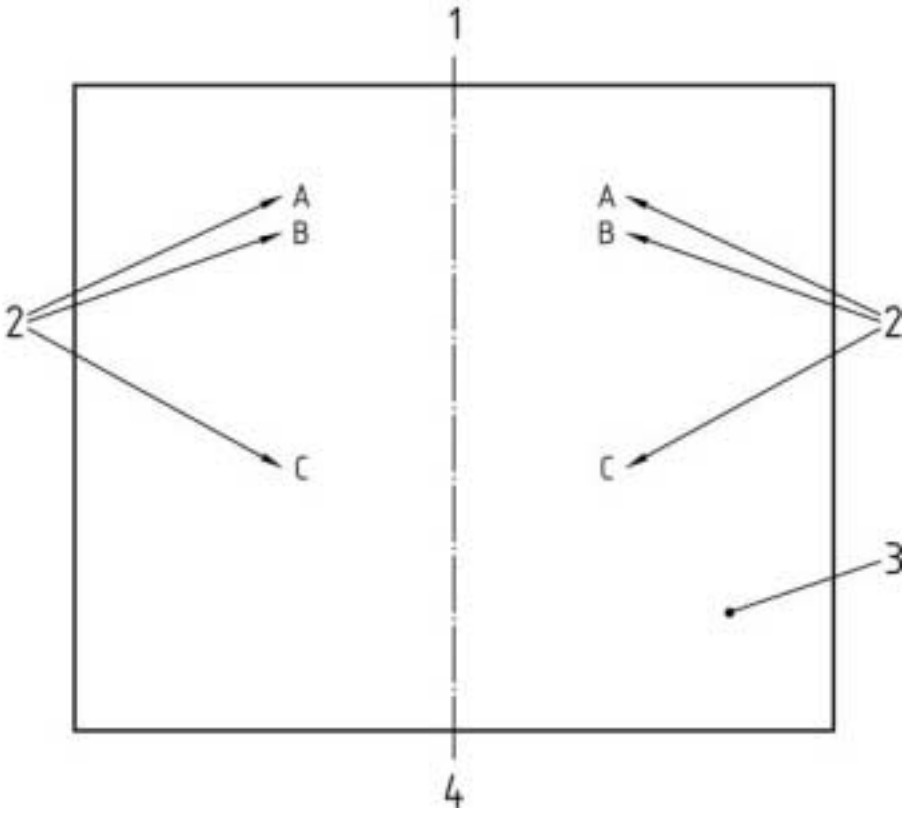
7.4.4.3.1 Intradermal induction phase

Make a pair of 0.1 mL intradermal injections of each of the following, into each animal, at the injection sites (A, B, and C) as shown in Figure 2 in the clipped intrascapular region.

Site A: A 50:50 (volume ratio) stable emulsion of Freund's complete adjuvant mixed with the chosen solvent. Use physiological saline (BP, USP, or equivalent) for water-soluble materials.

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

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



Key

1 Cranial end
2 0.1 mL intradermal injections (see 7.4.4.3.1)
3 Clipped intrascapular region
4 Caudal end

Figure 2—Location of intradermal injection sites

7.4.4.3.2 Topical induction phase

Seven days (± 1 day) after completion of the intradermal induction phase, administer the test sample by topical application to the intrascapular region of each animal, using a patch of area approximately 8 cm^2 (filter paper or absorbent gauze), so as to cover the intradermal injection sites. Use the concentration selected in 7.4.4.3.1 for site B. If the maximum concentration that can be achieved in 7.4.4.3.1 does not produce irritation, pretreat the area with 10 % sodium dodecyl sulfate massaged into the skin 24 h ± 2 h before the patch is applied. Secure the patches with an occlusive dressing. Remove the dressings and patches after 48 h ± 2 h.

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

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

7.4.4.3.3 Challenge phase

At 14 days (± 1 day) after completion of the topical induction phase, challenge all test and control animals with the test sample. Administer the test sample and a vehicle control by topical application to sites that were not treated during the induction stage, such as the upper flank of each animal, using appropriate patches or chambers soaked in the test sample at the concentration selected in 7.4.4.3.1 for site C. Dilutions of this concentration may also be applied to other untreated sites in a similar manner. Secure with an occlusive dressing. Remove the dressings and patches after 24 h ± 2 h.

7.4.5 Observation of animals

Observe the appearance of the challenge skin sites of the test and control animals 24 h and 48 h after removal of the dressings. Use of natural or full-spectrum lighting is highly recommended to visualize the skin reactions. Describe and grade the skin reactions for erythema and edema according to the Magnusson and Kligman grading given in Table 4 for each challenge site and at each time interval. It is highly recommended that reading be done without knowledge of the treatment, in order to minimize bias in the evaluation of the results.

7.4.6 Evaluation of results

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

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

A new FCA treated control group is recommended.

7.4.7 Test report

The test report shall include:

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

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

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

d) a description of the test animals,

e) method of application to the test sites,

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

g) records of the observations,

h) assessment of the results.

7.5 Closed-patch test for delayed hypersensitivity

7.5.1 Principle

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

7.5.2 Test sample preparation

If the material cannot be dosed as is, it shall be prepared as specified in annex A using polar and non-polar extractions. Where shape and size permit, topical devices (e.g., electrode) may be patched as is.

7.5.3 Animals and husbandry

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

The animals shall be acclimatized and cared for as specified in ISO 10993-2. Preliminary tests should be carried out on one set of animals to determine concentrations of test sample (see 7.5.4.2).

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

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

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

7.5.4 Test procedure

7.5.4.1 Preparation

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

For all topical applications, saturate a patch (filter paper or an absorbent gauze) of the appropriate dimensions with the test material or extract and apply the patch to the clipped area under an occlusive dressing for 6 h. The use of restraint on each animal is highly recommended to ensure occlusion of the test sites. If wrapping is used, its adequacy should be evaluated in every experiment.

7.5.4.2 Preliminary tests

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

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

Topically apply four concentrations of the test sample to the flanks of each of at least three animals using appropriate patches. Remove the occlusive dressings and patches after 6 h. Assess the application sites for erythema and edema using the Magnusson and Kligman grading given in Table 4 at 24 h and 48 h after patch removal.

Select:

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

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

7.5.4.3 Main test

7.5.4.3.1 Induction phase

Administer the test sample by topical application to the clipped left upper back region of each animal using appropriate patches soaked in the test material at the concentration selected in 7.5.4.2(a). Remove the restrainer and occlusive dressings and patches after 6 h. Repeat this procedure one to three times a week for three weeks. Treat the control animals similarly, using the blank liquid alone.

7.5.4.3.2 Challenge phase

Fourteen days (± 1 day) after the last induction application, challenge all test and control animals with the test sample. Administer the test sample by a single topical application to a clipped untested area of each animal using appropriate patches soaked in the test sample at the concentration selected in 7.5.4.2 b). Remove the restrainer and occlusive dressings and patches after 6 h.

7.5.5 Observation of animals

At 24 h ± 2 h after the primary challenge or rechallenge exposure, either:

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

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

Thoroughly wash the depilated area with warm water and dry the animals with a towel before returning them to their cages. A minimum of 2 h after removal of hair, grade the test sites according to Table 4. Repeat the grading 48 h ± 2 h after removal of the challenge patch. Use of natural or full-spectrum lighting is highly recommended to visualize the skin reactions. It is highly recommended that reading be done without knowledge of the treatment, in order to minimize bias in the evaluation of the results.

7.5.6 Evaluation of results

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

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

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

A naive control group is recommended.

7.5.7 Test report

The test report shall include:

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

b) the intended use/application of the test material(s) or device,

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

d) a description of the test animals,

e) method of application to the test sites,

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

g) records of the observations, and

h) assessment of the results, including statistical methods.

8 Key factors in interpretation of test results

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

Evidence of delayed contact hypersensitivity by any method does not necessarily exclude the test material or device from use, because the amount of the test material in the test procedure may be exaggerated compared to actual conditions of use. An adverse finding using any of the validated procedures indicates the need for further analysis that would allow risk assessment of intended human exposure.

Predictive test results generated by the procedures described in the standard cannot stand alone. A negative test result does not always exclude the possibility that a product may cause allergic skin reactions. Both positive and negative test results in any of the assays should be scrutinized by rigorous follow-up in order to minimize the likelihood of false positive or false negative results. The results should be validated by comparison with other sources of information, such as:

a) industry and consumer complaint data,

b) experience with devices containing similar components,

c) diagnostic test results in dermatologic clinics, and

d) retrospective epidemiologic data.

Annex A
(normative)

Preparation of materials for irritation/sensitization testing

A.1 General

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

A.2 Materials for direct-contact exposure

A.2.1 Solid test materials

Solid materials, which have appropriate physical states (e.g., sheets, films), shall be tested without modification. Prepare samples 2.5 cm × 2.5 cm of a thickness that approximates normal use but is not greater than 0.5 cm. Prepare suitable negative control samples in the same way. The negative control shall physically resemble the test material closely and should be non-irritant. Absorbent gauze may be used as a substitute if a more suitable control cannot be identified.

The solid may be pulverized, care being taken to ensure that 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 physicochemical properties of the ceramic may be altered by reducing the ceramic to a powder, with potentially marked effects on biological activity.

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

NOTE—Surface area and/or particle size are important factors in biological responses such as phagocytosis, which plays an important role in inflammatory and immune responses.

A.2.2 Liquid test materials

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

A.3 Extracts of test materials

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

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

A.4 Solvents

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

A.5 Sterile test methods

If the final product is supplied in a sterile condition, then the test material shall be sterilized using the same process prior to testing. Products sterilized by ethylene oxide present a technical difficulty in that ethylene oxide and its reaction products can produce a biological response in the tests described in this part of ISO 10993.

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

Annex B
(informative)

Additional irritation tests

B.1 General

The following special evaluation tests should be considered as additional to the basic tests but not as replacements for them. If used, a rationale shall be provided for the choice of test method. They are only relevant for medical devices intended to be applied to these specific areas.

B.2 Intracutaneous (intradermal) reactivity test

B.2.1 Principle

An assessment is made of the potential of the material under test to produce irritation following intradermal injection of extracts of the material.

B.2.2 Exclusion from test

Any material shown to be a skin, eye, or mucosal tissue irritant or material with a pH of ≤ 2 or ≥ 11.5 shall not be tested intradermally.

B.2.3 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 may be applied together with the appropriate negative controls or blank liquids.

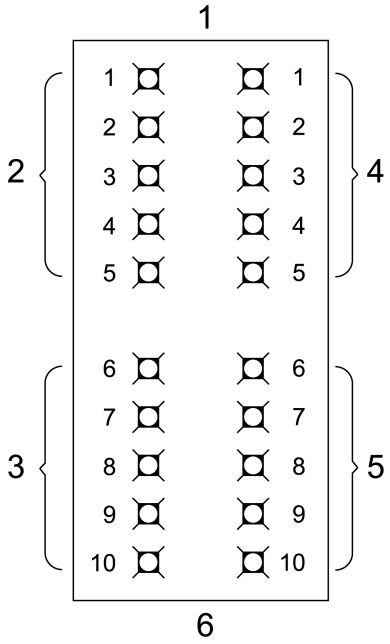
B.2.4 Animals and husbandry

Healthy young adult albino rabbits of either sex from a single strain, weighing not less than 2 kg, shall be used. The animals shall be acclimatized and cared for as specified in ISO 10993-2. A minimum of two animals shall be used initially to evaluate the test material. If the response in the initial test is equivocal or not clear, additional testing shall be considered.

B.2.5 Test procedure

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

Inject intracutaneously 0.2 mL of the extract obtained with polar solvent at five sites on one side of each rabbit (see Figure B.1). Use the smallest needle appropriate to the viscosity of the test material for the intradermal injections.



Key

1 Cranial end
2 0.2 mL injections of polar extract
3 0.2 mL injections of polar solvent control
4 0.2 mL injections of non-polar extract
5 0.2 mL injections of non-polar solvent control
6 Caudal end

Figure B.1—Arrangement of injection sites

Similarly, inject 0.2 mL of the polar solvent control at five posterior sites on the same side of each rabbit (see Figure B.1).

Repeat the above procedures for the extract obtained with the non-polar solvent and the non-polar solvent control on the other side of each rabbit (see Figure B.1).

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

B.2.6 Observation of animals

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

Grade the tissue reaction for erythema and edema according to the system given in Table B.1 for each injection site and at each time interval observed, and record the results.

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

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

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

B.2.7 Evaluation of results

After the 72 h grading, all erythema grades plus edema grades are totaled separately for each test sample and vehicle blank. Divide each of the totals by 12 (2 animals $\times$ 3 grading periods $\times$ 2 grading categories) to determine the overall mean score for each test sample versus each corresponding vehicle blank. The requirements of the test are met if the difference between the test sample mean score and the vehicle blank mean score is 1.0 or less. If at any observation period the average reaction to the test sample is questionably greater than the average reaction to the

vehicle blank, repeat the test using three additional rabbits. The requirements of the test are met if the difference between the test sample and the vehicle blank mean score is 1.0 or less.

Table B.1—Grading system for intracutaneous (intradermal) reactions

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Well-defined edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond exposure area)	4
Total possible score for irritation	8

Other adverse changes at the injection sites shall be recorded and reported.

B.2.8 Test report

The test report shall include:

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

b) the intended use/application of the test material(s) or device,

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

d) a description of the test animals,

e) method of injection,

f) how the site readings were performed,

g) a record of the observations, and

h) assessment of the results.

B.3 Ocular irritation test

B.3.1 General

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

NOTE—*In vitro* test systems are under development which, when validated, may be used in place of this *in vivo* ocular irritation test.

B.3.2 Principle

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

B.3.3 Exclusion from test

Materials and/or final products which have demonstrated definite corrosion or severe irritation in a dermal study shall not be further tested for eye irritation. Any material shown to be a skin irritant or those with a pH of ≤ 2 or ≥ 11.5 should not be tested but should be labeled a potential eye irritant.

B.3.4 Test material

If the test material is a liquid, instill 0.1 mL undiluted into the lower conjunctival sac of one eye.

If the test material is a solid or granular product, grind to a fine dust. When gently compacted, instill that amount which occupies a volume of 0.1 mL and does not weigh more than 100 mg into the lower conjunctival sac of one eye.

NOTE—Some products may not be amenable to 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 instill 0.1 mL as for liquids.

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

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

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

If the test material is such that it can only be applied as an extract, prepare extracts as described in annex A. Instill a 0.1 mL aliquot of the extract into the lower conjunctival sac of one eye.

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

B.3.5 Animals and husbandry

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

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

One animal shall initially be used to evaluate the test material. If no response is expected, initial testing may be conducted using three animals.

A well-defined positive response (see Table B.2) in the one animal obviates the need for additional testing.

Unless a well-defined response is observed for solid or liquid materials, a minimum of two further animals shall be used. For extracts, a minimum of two further animals per extract shall be used.

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

B.3.6 Test procedure

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

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

Instill the test sample as specified in B.3.4 in one eye.

Following instillation, hold the eyelids together for approximately 1 s.

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

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

B.3.7 Observation of animals

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

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

NOTE—U.S. FDA guidelines for contact lens testing require 21 days' exposure for 8 h per day. This is an exception to the guidelines.

Grade and record any reactions observed in accordance with the scale for grading ocular lesions given in Table B.2.

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

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

Grade and record any reactions observed in accordance with Table B.2.

Withdraw an animal immediately from the study and humanely sacrifice it, if at any time it shows:

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

b) blood-stained or purulent discharge; or

c) significant corneal ulceration.

Withdraw from the study any animal showing maximum effects on the grading system in Table B.2, i.e.,

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

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

and sacrifice it humanely.

Table B.2—System for grading ocular lesions

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

[a] Positive result.

B.3.8 Evaluation of results

Differences between the test and control eyes shall be characterized and explained in the terms of the grading system given in Table B.2.

a) Acute exposure

If the treated eye in more than one animal shows a positive result (footnoted grades in Table B.2) 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 positive reaction or the reactions are equivocal, treat further animals.

When further animals have been treated, the test material is considered to be an eye irritant if more than half of the eyes treated in the test group exhibit a positive result (footnoted grades in Table B.2) at any stage of the observation.

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

b) Repeated exposure

The test material is considered an eye irritant if more than half of the animals in the test group exhibit a positive result (footnoted grades in Table B.2) at any stage of the observation.

B.3.9 Test report

The test report shall include:

a) a description of the test samples,

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

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

d) a description of the test animals,

e) method of instillation,

f) how the ocular readings were performed,

g) a record of the observations, and

h) assessment of the results.

B.4 Oral mucosa irritation test

B.4.1 General

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

B.4.2 Principle

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

B.4.3 Exclusion from test

Any material shown to be a skin or eye irritant or material having a pH of ≤ 2 or ≥ 11.5 shall not be tested and shall be labeled as a potential oral tissue irritant.

B.4.4 Test material

Prepare test materials in accordance with annex A .

B.4.5 Animals and husbandry

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

In addition to the above, when appropriate, fit to each animal a suitable collar of width 3 mm to 4 mm, placed around the neck so that it permits normal feeding and respiration but prevents the animal from removing the cotton-wool

pellet. Weigh each animal daily for seven days during the test period. Examine any animal showing a loss of body mass during this period and adjust its collar, if necessary. If the animal continues to lose mass, exclude it from the test.

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

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

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

B.4.6 Test procedure

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

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

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

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

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

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

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

For acute exposure, repeat the above procedure every hour for 4 h.

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

B.4.7 Observation of animals

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

Describe the appearance of the cheek pouches for each animal and grade the pouch surface reactions for erythema according to the system given in Table B.3 for each animal at each time interval. Record the results for the test report.

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

Table B.3—Grading system for oral and penile reactions

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4

Other adverse changes of the tissues should be recorded and reported.

B.4.8 Assessment of results

B.4.8.1 Macroscopic evaluation

Compare the untreated 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 scores (Table B.3) 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 may assist in the histological evaluation.

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

B.4.8.2 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 presented in Table B.4.

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

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

Subtract the control group average from the test group average to obtain the Irritation Index (see Table B.5).

For repeated-exposure tests, Table B.4 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.4.9 Test report

The test report shall include:

a) a description of the test samples,

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

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

d) a description of the test animals,

e) method of application,

f) how the site readings were performed,

g) a record of the observations,

h) histological evaluation, and

i) assessment of the results.

Table B.4—Grading system for microscopic examination for oral, penile, rectal, and vaginal tissue reaction

Reaction	Numerical grading
1. Epithelium	
Normal, intact	0
Cell degeneration or flatting	1
Metaplasia	2
Focal erosion	3
Generalized erosion	4
2. 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
3. Vascular congestion	
Absent	0
Minimal	1
Mild	2
Moderate	3
Marked, with disruption of vessels	4
4. Edema	
Absent	0
Minimal	1
Mild	2
Moderate	3
Marked	4

Table B.5—Irritation Index

Average grade	Description of response
0	None
1 to 4	Minimal
5 to 8	Mild
9 to 11	Moderate
12 to 16	Severe

Other adverse changes of the tissues should be recorded and included in the assessment of the response.

The microscopic examination grading system in Table B.4 applies for all tests listed. The “Irritation Index” was developed for use with the vaginal irritation model but may be used for the other tests.

B.5 Penile irritation test

B.5.1 General

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

B.5.2 Principle

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

B.5.3 Exclusion from test

Any material shown to be a skin or eye irritant or material having a pH of ≤ 2 or ≥ 11.5 shall not be tested and shall be labeled as a potential penile irritant.

B.5.4 Test sample

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

B.5.5 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 classification system given in Table B.3 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 be used initially to evaluate the test material, and three animals as the control group.

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

B.5.6 Test procedure

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

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

When the penis is protruded, apply enough (approximately 0.2 mL) of the test sample to be sure that the penis is coated.

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

Alternatively, the animal may be secured in an appropriately designed restrainer for 1 h after the last application.

For acute exposure, repeat the above procedure every hour for 4 h.

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

B.5.7 Observation of animals

For acute exposure, note the appearance of the penis 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 h, 24 h, and 48 h after the last application.

For prolonged repeated exposure, note the appearance of the penis at 1 h after the initial application and immediately prior to the next application.

Grade the skin surface reactions for erythema according to the system given in Table B.3 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 score for one observation is four.

B.5.8 Assessment of results

B.5.8.1 Macroscopic evaluation

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

The grades (Table B.3) for each observation are added and divided by the number of observations to determine the average grade per animal.

NOTE 1—These observations may 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 into an appropriate fixative prior to processing for histological examination.

B.5.8.2 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 presented in Table B.4.

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

Repeat for the control group(s).

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

Subtract the control group average from the test group average to obtain the Irritation Index (see Table B.5).

For prolonged repeated-exposure tests, Table B.4 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.5.9 Test report

The test report shall include:

a) a description of the test sample,

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

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

d) a description of the test animals,

e) method of application,

f) how the site readings were performed,

g) a record of the observations,

h) histological evaluation, and

i) assessment of the results.

B.6 Rectal irritation test

B.6.1 General

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

B.6.2 Principle

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

B.6.3 Exclusion from test

Any material shown to be a skin or eye irritant or those having a pH of ≤ 2 or ≥ 11.5 shall not be tested and shall be labeled as a potential rectal irritant.

B.6.4 Test material

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

B.6.5 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 be used initially to evaluate the test material, and three animals used as the control group.

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

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

B.6.6 Test procedure

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

Secure the animal by placing it in a restraining device which permits access to the 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 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 h intervals every day for five consecutive days.

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

B.6.7 Observation of animals

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

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

B.6.8 Evaluation of results

B.6.8.1 Macroscopic evaluation

At 24 h after the last dose, humanely sacrifice 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 may assist in the histological evaluation.

B.6.8.2 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 presented in Table B.4.

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

Repeat for the control group(s).

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

Subtract the control group average from the test group average to obtain the Irritation Index (see Table B.5).

For prolonged repeated-exposure tests, Table B.4 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.6.9 Test report

The test report shall include:

a) a description of the test samples,

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

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

d) a description of the test animals,

e) method of application,

f) how the site readings were performed,

g) a record of the observations,

h) histological evaluation, and

i) assessment of the results.

B.7 Vaginal irritation test

B.7.1 General

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

B.7.2 Principle

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

B.7.3 Exclusion from test

Any material shown to be a skin or eye irritant or material having a pH of ≤ 2 or ≥ 11.5 shall not be tested and shall be labeled as a potential vaginal irritant.

B.7.4 Test material

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

B.7.5 Animals and husbandry

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

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

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

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

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

B.7.6 Test procedure

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

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

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

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

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

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

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

B.7.7 Observation of animals

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

Animals exhibiting excessive discharge, erythema, and/or edema, and found difficult to dose, shall be humanely sacrificed and the tissues examined (see B.7.8.1).

B.7.8 Evaluation of results

B.7.8.1 Macroscopic evaluation

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

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

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

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

NOTE—These observations may assist in the histological evaluation.

B.7.8.2 Histological evaluation

The irritant effects on vaginal tissue shall be evaluated by a pathologist. The pathologist may grade each tissue according to the system presented in Table B.4.

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

Repeat for the control group(s).

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

Subtract the control group average from the test group average to obtain the Irritation Index (see Table B.5).

For prolonged repeated-exposure tests, Table B.4 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

B.7.9 Test report

The test report shall include:

a) a description of the test samples,

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

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

d) a description of the test animals,

e) method of application,

f) how the site readings were performed,

g) a record of the observations,

h) histological evaluation, and

i) assessment of the results.

Annex C
(informative)

Background information

C.1 Background information on irritation tests

Dermal irritation testing in small animals is performed to help identify materials which may be potential human skin and/or mucosal tissue irritants. A primary irritant is a material which produces inflammatory changes in the skin as a result of a direct damaging effect characterized by the presence of inflammation, or, in the case of severe irritant, vesiculation and/or necrosis.

The rabbit is the preferred test animal, as evidenced by the large amount of dermal irritation information on this animal in the Registry of Toxic Effects of Chemical Materials (RTECS). Out of over 2000 RTECS entries, 85 % report test results with the rabbit, 7.5 % with human, 4 % with the mouse, and 3 % with the guinea pig. As a result, rabbits have been used to generate the vast majority of the available data in the open literature. Abrasion of the test site is not necessary, as evidence indicates similar responses between abraded and non-abraded sites.

Skin irritation tests may give varying results due to variation in a number of test-related factors such as host, test dose, patch size, degree of occlusion, length of exposure, vehicle, time for reading, and quality of reading. Therefore, in human skin irritation tests, it is important to include well-known positive and negative control materials in order to compare the test results with the control materials, making the results relative. As a positive irritant control, sodium dodecyl sulfate (SDS) of purity > 99 % is the preferred choice, since it is the most widely used control irritant in clinical investigations [2], [4], [31]. It is also easily and widely available and free from other adverse effects. Nonanoic acid, which has a mode of action different from SDS, may also be used as a positive control [18], [19].

SDS exposure calibrates the panel of human volunteers and acts as a reference point. SDS is classified as a skin irritant according to EU criteria (88/379/EEC Council Directive of 7 June 1988). It is not clear, however, whether SDS is at, or close to, the threshold level of response at which chemicals should be regarded as skin irritants. Thus, rather than using the neat material, it is more appropriate to take as a reference point the minimum level of SDS regarded by at least one regional group (the EU) as a significant acute irritant to skin, which is a 20 % (mass concentration) aqueous preparation [31].

The use of laboratory animals for skin irritation testing is decreasing due to the development of *in vitro* models and more frequent use of human volunteers [10], [14]. Bioengineering or non-invasive, objective measuring methods are utilized to quantify the irritant response and thereby decrease the dependency on the more subjective visual reading scales [12], [16], [17]. However, decades of experience have been obtained with the Draize dermal irritation test on albino rabbits. This method is in reference [8]. The test material is introduced under gauze patches to intact sites on the clipped dorsum. Applications are made on three rabbits. The patches are secured by adhesive tape and the entire trunk of the animal is wrapped in a semi-occlusive or occlusive dressing for 4 h. After 4 h, the patches are removed, the test sites cleaned, and 1 h later any resulting reaction graded for erythema and edema. The reactions are also graded at 24 h, 48 h, and 72 h.

The rabbit eye irritation test has been developed to predict ocular irritancy in man [27]. Draize [26] published a grading system to assist in the evaluation of ocular irritation. Illustrated guides have been published as aids in assessing ocular lesions.

Alternative *in vitro* methods for investigating effects of eye irritation are being developed but are not yet validated and internationally approved [21].

Extensive human data on skin irritation comes from the Research Institute for Fragrance Materials Monographs on essential oils and other aromatics published in *Food and Cosmetic Toxicology*. An OECD Guideline Draft on Acute Dermal Irritation study in human volunteers gives additional background information. The chemicals group of the OECD Guideline program has not yet reached consensus on the need to develop an OECD Guideline for local skin effects in human volunteers.

C.2 Background information on sensitization tests for delayed hypersensitivity

Sensitization in man occurs after single or multiple epicutaneous exposures, and is initiated and elicited by components of the immune system. Most importantly, the hapten (chemical) must be substantive to skin and be able to penetrate. It then reacts with skin proteins to form immunogenic complexes. Langerhans cells at the epidermal/dermal border present the antigen to specific lymphocytes which are then activated to initiate the immune

responses. A small percentage of these lymphocytes are long-lived memory cells, and these serve as the primary activators during the challenge phase. Thus, subsequent re-exposures can result in adverse reactions that are mediated by lymphokines released by the activated lymphocytes and other inflammatory cells that are attracted to the area of the lesion.

In 1895, Jadassohn employed the patch test to disclose contact allergy to mercury in a clinical patient. This innovative approach provided the scientific basis for subsequent tests aimed at diagnosis and prediction of contact allergy in man and animals. The development of prospective/predictive tests for evaluating the sensitizing potential of chemicals followed the pioneer work of Landsteiner and Chase [49], who firmly substantiated the use of the guinea pig for studying delayed hypersensitivity.

Magnusson and Kligman [50] explored many of the variables of guinea pig testing and presented a procedure, the guinea pig maximization test (GPMT), based on intradermal injections (with and without Freund's complete adjuvant, FCA), followed by topical application of the test material to the same area. The original procedure requires pretreatment of the test site if the test material is non-irritant. By definition, it reputedly detects weak sensitizers, because "weak" included a zero incidence of positive reactors. It is a sensitive test and has been extensively used. The use of Freund's complete adjuvant increases the sensitivity of the test method and may, in some cases, overestimate the sensitizing potential of the compound in question.

In 1965, Buehler [41] advocated the use of the closed patch to provide occlusion as a method to optimize exposure and mimic the procedures used in human (Human Repeat Insult Patch Test: HRIPT). It was suggested that the occlusive patch procedure was sensitive and would accurately predict moderate to severe sensitizers, thus avoiding exposure of human subjects to the prospect of adverse reaction during HRIPTs. The data presented demonstrated the superiority of occlusion over intradermal injections and open-type topical application. Stimulation of the immune system by adjuvants was not used. This method is established as a technique that is sufficiently sensitive to detect most weak sensitizers and has been shown to be sufficiently flexible to be used in the Risk Assessment Process. However, the closed-patch test (Buehler test) is less sensitive compared to the GPMT [46].

These two tests, the closed-patch test in the United States and the GPMT in Europe, have been the most frequently used for safety assessment. They are also the preferred test methods in current OECD and EU test guidelines. The result from guinea pig sensitization assays depends on many animal-related and technical factors explaining the interlaboratory variation in test results, e.g., animal strain, sex, age, ambient test conditions, test site on the animal, method of hair removal (clipping/shaving or chemical depilation), type of patch design, quantity of test material, quality of occlusion, exposure time, and reading of the tissue response. Numerous other tests have been employed and investigated, and all of these have their proponents. There are currently several procedures that have been recognized as acceptable for regulatory purposes, provided the procedure is properly documented and validated by the investigator. In all cases, the procedures should be performed in accordance with to the original references. A list of other tests is provided in Table C.1.

A recent ECETOC monograph gives an update on skin sensitization testing [44].

Table C.1—Alternative delayed-contact sensitization tests

1	Freund's complete adjuvant test
2	Split adjuvant test
3	Open epicutaneous test
4	Mauer optimization test
5	Foot-pad test in guinea pig
6	Cumulative contact enhancement test
7	Scratched skin (adjuvant and patch) test
8	Mouse ear swelling test
9	Local lymph node assays

The last assay in Table C.1, the murine local lymph node assay (LLNA), deserves attention. The local lymph node assay (LLNA) has been accepted by the Organization for Economic Cooperation and Development (OECD) as a stand-alone alternative to the current guinea pig tests, and as an improvement for animal welfare [83].

The scientific basis for the test is measurement of the incorporation of 3H-methyl thymidine into lymphocytes in draining lymph nodes of mice topically exposed to the test article as a measurement of sensitization. It does not include a challenge phase. The endpoint of interest is a stimulation index giving the ratio of thymidine incorporation in lymph nodes from dosed animals compared to the incorporation in lymph nodes from control animals. The test is positive when the stimulation index exceeds 3 (SI >3). An intra- and inter-laboratory evaluation of the LLNA has demonstrated reproducible dose-response relationship within and between laboratories [59], [60], [65], [72], [76], [78], [82]. However, difficulties in differentiating between irritating and allergenic substances with the LLNA have been reported [62], [72], [79]. Thus, the LLNA may give false positive results with irritants and may overestimate the allergenicity of substances with both irritating and allergenic properties [59]. However, the LLNA has advantages compared to the guinea pig assays because of shorter test duration, a more objective end point, and less test substance required, and it omits the Freund's complete adjuvant injections. Improvements of the test procedure by use of analysis of cell activation markers and flow cytometry are possible [68], [69]. Whether they practically can be implemented in standard LLNA protocols for routine toxicology is not determined. On the other hand, the LLNA allows a more limited choice of test vehicles; most studies have used a mixture of acetone and olive oil. A recent study shows the variability of the results using different vehicles [77]. Further, it is not possible with the LLNA to study the challenge phase or cross-reactivity patterns because the animals are sacrificed after induction treatment before the lymph nodes are harvested.

The popliteal lymph node assay (PLNA) using subcutaneous administration in the footpad [63], [66], [81] is an alternative lymph node assay. In the latter assay, in addition to direct measurement of lymph node activation, reporter antigens may be used for further clarification of the immunomodulation caused by the chemical under investigation [58].

The risk assessment process should not rely on a single model or approach, but should be thoughtfully conducted to provide maximum assurance of safety to the consumer. Generally, this entails both animal and human experimental models. There should be flexibility in the choice of models and approaches as long as the rationale is documented and/or validated.

Negative tests in guinea pigs, when they are properly conducted, can generally be definitive if the test concentration has a sufficient safety factor over use conditions. However, one should avoid classifying test materials solely on the basis of incidence and/or severity, without due consideration of eventual product usage.

The risk, i.e., incidence and severity, of the allergic reaction to the product is determined mainly with the following four factors: the sensitizing potency of the chemical allergen, its amount in the product, bioavailability, and the exposure conditions. The relative sensitizing potencies of chemicals can be defined in terms of the minimum induction concentration required to induce a given level of sensitization: the lower this concentration, the more potent the sensitizer [80], [40]. It was shown that the significant incidence of allergic contact dermatitis was found in users when the residue level of the allergen in the product exceeded its minimum induction concentration obtained by GPMT [51].

On the other hand, predictive testing of mixtures and products is much less validated and may be performed following testing of product ingredients. Accordingly, test design and result interpretation is subject to uncertainty, but several examples document this possibility. In animal experiments with acetone extracts from a sweater that had caused contact dermatitis in human, allergens (phosgene chlorophenylhydrazones) were demonstrated [48]. In another case, animal experiments with acetone/chloroform extracts from rubber boots that had caused contact dermatitis in man, mercaptobenzothiazole and dibenzothiazyldisulfide were eventually found to be the causative allergens [47]. The importance of using an appropriate organic solvent was clearly demonstrated. The extracts made with organic solvent induced hypersensitivity in the guinea pigs, while the saline extracts failed to do so.

The Japanese Guidelines of Basic Biological Tests of Medical Materials and Devices (1995) adopts the sample preparation procedure with organic solvent followed by evaporation of the solvent to obtain the residue, and the risk assessment procedure by comparing the percent residue yield from the material with the minimum percent dilution of the residue (mixture) that still induced delayed hypersensitivity in animals.

In vitro methods for skin sensitization testing are currently not available for routine use [13].

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