List of Titles

American National Standard

ANSI/AAMI/ISO 10993-10:1995

Biological evaluation of medical devices, Part 10: Tests for irritation and sensitization

AAMI Association for the Advancement of Medical Instrumentation

List of Titles

Association for the Advancement of Medical Instrumentation
1110 N. Glebe Rd., Suite 220
Arlington, VA 22201-4795

Copyright and Permissions

10993-10 Tests for irritation and sensitization

American National Standard

ANSI/AAMI/ISO 10993-10—1995

Biological evaluation of medical devices—Part 10: Tests for irritation and sensitization

Approved 24 July 1995 by7
Association for the Advancement of Medical Instrumentation

Approved 11 September 1995 by
American National Standards Institute, Inc.

Abstract:

This standard describes test methods to evaluate the potential of devices and their constituent materials to produce irritation; and to evaluate the potential of devices and their constituent materials to produce sensitization.

Committee representation

Association for the Advancement of Medical Instrumentation

The adoption of ISO 10993-10:1995 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 Adelbert L. Stagg, PhD, of Allergan Pharmaceuticals, Inc., and Katharine Merritt, PhD of Case Western Reserve University, played an active part in developing the ISO standard.

The AAMI **Biological Evaluation Committee** has the following members:

Cochair: Paul Didisheim, MD

Members:
James M. Anderson, MD, PhD, Case Western Reserve University
Sumner A. Barenberg, Bernard Technologies
Arthur J. Coury, PhD, Society for Biomaterials
Roger Dabbah, PhD, U.S. Pharmacopeial Convention, Inc.
Paul Didisheim, MD, National Heart, Lung, and Blood Institute
Robert L. Fuson, MD, Bristol-Myers Squibb
Jean A. Goggins, PhD, Meadox Medicals, Inc.
Sharon Northup, PhD, Baxter Healthcare Corporation
Barry F.J. Page, Consultant, Garner, NC
John W. Stanford, PhD, American Dental Association
Mel Stratmeyer, PhD, FDA Center for Devices and Radiological Health

Alternates:
John G. Miller, DVM, National Institutes of Health
Ed Mueller, FDA Center for Devices and Radiological Health
Harold Stanley, DDS, American Dental Association

The AAMI **Irritation and Sensitization Working Group** has the following members:

Cochairs: Adelbert L. Stagg, PhD
Katharine Merritt, PhD

Members: Daniel W.C. Brown, PhD, FDA Center for Devices and Radiological Health
Edwin Buehler, PhD, Hill Top Biolabs, Inc.
Howard I. Maibach, MD, University of California Medical Center, San Francisco, CA
Katherine Merritt, PhD, Case Western Reserve University
Herbert N. Prince, PhD, Gibraltar Biological Labs
Van M. Seabaugh, Environmental Protection Agency
Adelbert L. Stagg, PhD, Allergan Pharmaceuticals, Inc.
Richard F. Wallin, DVM PhD, North American Science Associates, Inc.
Anne Wolven-Garrett, Consultant, Atlanta, GA

Alternate: Joel Gorski, North American Science Associates, Inc.

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 10993-10:1995

Tests for irritation and sensitization

As indicated in the foreword to the main body of this document (page vi), 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, which is part of the ISO 10993 series of standards, created by ISO Technical Committee 194, Biological evaluation of medical devices, to fill a need for the international harmonization of test methods for various kinds of biological aspects of medical devices.

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 (AAMI). The U.S. TAG for ISO/TC 194 supports the international harmonization of methods used in evaluating the biocompatibility of medical devices in order to help reduce unnecessary repetition of testing.

AAMI and ANSI procedures require that standards be reviewed and, if necessary, revised every five years to reflect technological advances that may have occurred since publication.

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 as new data come to light.

Suggestions for improving this standard are invited. Comments and suggested revisions should be sent to Standards Department, AAMI, 3330 Washington Boulevard, Suite 400, Arlington, VA 22201.

NOTE Beginning with the ISO foreword on page vi, this American National Standard is identical to ISO 10993-10:1995

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.

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.

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

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

— *Part 1: Guidance on selection of tests*

— *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 cytotoxicity: in vitro methods*

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

— *Part 7: Ethylene oxide sterilization residuals*

— *Part 9: Degradation of materials related to biological testing [Technical Report]*

— *Part 10: Tests for irritation and sensitization*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

— *Part 13: Identification and quantification of degradation products from polymers*

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

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

— *Part 16: General guidance on toxicokinetic study design for degradation products and leachables*

— *Part 17: Glutaraldehyde and formaldehyde residues in industrially sterilized medical devices*

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 overall guidance document for the selection and conduct of tests enabling evaluation of irritation and sensitization responses relevant to material and device safety.

Annexes A, B and C form an integral part of this part of ISO 10993. Annexes D, E and F are for information only.

Introduction

This part of ISO 10993 assesses possible contact hazards from device-released chemicals that may produce skin and mucosal irritation, eye irritation, and delayed contact sensitization.

Some materials that are included in these 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 act differently when exposed to biological tissues. It is incumbent upon the manufacturer to evaluate each device for its human toxic potential prior to marketing.

Traditionally, small animal tests are performed prior to human testing to help predict human response. More recently, *in vitro* tests have been added as an alternative. 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 as screening tests prior to animal testing. In order to reduce the number of animals used, these standards use a step-wise approach with review and analysis of test results at each stage.

It is incumbent upon the investigator to conduct these studies using good scientific laboratory practices, complying with regulations related to animal welfare. Since the number of animals is restricted, the data obtained may be insufficient to warrant the application of statistics.

American National Standard ANSI/AAMI/ISO 10993-10--1995

Biological evaluation of medical devices—Part 10: Tests for irritation and sensitization

1 Scope

This Part of ISO 10993 describes test methods:

a) to evaluate the potential of devices and their constituent materials to produce irritation; and

b) to evaluate the potential of devices and their constituent materials to produce sensitization.

These test methods are recommended for most categories of device and mode of body contact given in ISO 10993-1. Of the tests listed, those appropriate to the end use of the device are to be selected. Guidance is also given for the preparation of materials specifically in relation to the above tests.

NOTE 1 Guidance on the conduct of supplementary tests which may be required specifically for use in the oral, rectal, penile and vaginal areas is given in annex D.

2 Normative references

The following standards contain procedures which, through reference in this text, constitute provisions of this part of ISO 10993. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 10993 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 10993-1:1992, *Biological evaluation of medical devices—Part 1: Guidance on selection of tests.*

ISO 10993-12:—1), *Biological evaluation of medical devices—Part 12: Sample preparation and reference materials.*

3 Definitions

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

3.1 (allergic contact) sensitization; delayed contact hypersensitivity: Allergic response involving immunological systems that have been activated by prior exposure.

3.2 irritation: Localized inflammatory response to single, repeated or continuous application of the test substance, without involvement of an immunological mechanism.

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

3.4 erythema: Reddening of the skin or mucous membrane.

3.5 eschar: Scab or discolored slough of skin.

3.6 corrosion: Production of irreversible tissue damage at the site of contact with the skin following the application of a test substance.

3.7 ulceration: Open sore representing loss of superficial tissue.

3.8 necrosis: Death of cells and/or tissues.

3.9 negative control: Substance that closely resembles the test substance in form and, when tested in accordance with this part of ISO 10993, is neither an irritant nor a sensitizer.

3.10 positive control: Substance that, when tested in accordance with this part of ISO 10993, gives a reproducible irritation or sensitization response.

3.11 solvent: Substance (chemical, vehicle, medium, etc.) used to moisten, dilute, suspend, extract or dissolve the test substance.

3.12 reagent control: Solvent used to moisten, dilute, suspend, extract or dissolve the test substance, which is evaluated concurrently with the moistened, diluted, suspended, extracted or dissolved test substance.

4 General principles, step-wise approach

This part of ISO 10993 advocates a step-wise approach which may include any or all of the following:

a) literature review;

b) *in vitro* tests (if available and when validated);

c) *in vivo* tests;

d) non-invasive human tests/clinical trials.

The first stage is a literature review and shall include an evaluation of chemical and physical properties, and information on structurally related chemicals and materials. If not already known, the pH and pKa of the material (liquid, solution or extracts of materials) shall be measured prior to any *in vivo* or *in vitro* testing.

The second stage provides for *in vitro* assessments. These should always be considered in preference to *in vivo* tests and should replace these as new *in vitro* methods become available and validated.

At the third stage acute *in vivo* studies are undertaken to test for materials not already classified as severe irritants or strong sensitizers by stages a) or b). Materials that do not demonstrate an acute potential may be further evaluated following repeated exposure.

At the present time there are no validated *in vitro* tests (other than simple screens) to detect irritants or sensitizers; guidance is therefore provided only in the conduct of *in vivo* tests in species other than humans.

It is not necessary to use positive controls in every *in vivo* test. A positive control should be run periodically to validate the test system and demonstrate a positive response.

If assessment is not possible using the above stages, consideration should be given to non-invasive testing in humans.

5 Irritation tests

5.1 Factors to be considered in design and selection of tests

Factors affecting the results of irritation studies include

a) the patch test unit;

b) the degree of occlusion;

c) application of the test substance;

d) the application site;

e) the duration of exposure; and

f) the techniques used in evaluating the test.

Additional background information is provided in annex E.

While increased flexibility will allow 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 investigator, in consultation with the device manufacturer, should design the study to exaggerate the anticipated contact (time and/or concentration) in the clinical situation. While use of an exaggerated concentration or extract of the material is acceptable, this should be borne in mind during interpretation of the results.

For products intended to be used extensively on normal and abnormal 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.

If the pH of the test material is less than or equal to 2 or equal to or greater than 11.5, the material may 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 substance to produce severe injury. The concentration of the test material, its period of contact, and many other physical and chemical properties are also important.

As dose levels in test procedures can be exaggerated, a positive test does not necessarily exclude the material from use.

5.2 Skin irritation test

5.2.1 Principle

Assessment of the potential of the material under test to produce dermal irritation.

5.2.2 Test material

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

If the test material is to be tested as an extract, it shall be prepared as specified in annex B.

5.2.3 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 annex C.

One animal shall initially be used to evaluate the test material.

A well-defined response 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.

5.2.4 Test procedure

5.2.4.1 Preparation of animals

On the day before the test, closely clip the fur 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 x 15 cm). Use only animals with healthy intact skin.

NOTE 2 Abrasion of the test site is not necessary, as evidence indicates similar responses between abraded and non-abraded sites.

If repeated exposure is required, follow the procedures in 5.2.4.2, 5.2.4.3 or 5.2.4.4, repeated for a maximum of 21 days.

5.2.4.2 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. If the substance is a powder, it should be slightly moistened with water or other suitable solvent before application.

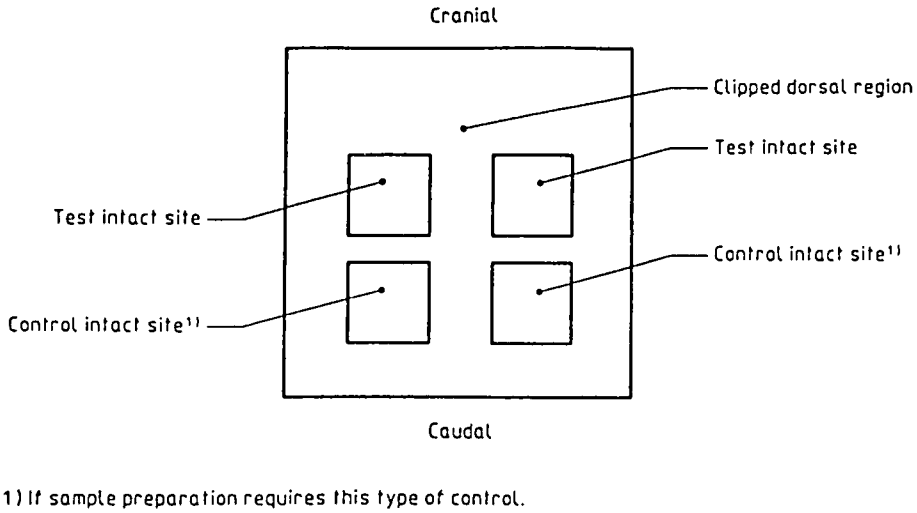

Figure 1—Location of skin application sites

Cover the application sites with a 25 mm x 25 mm non-occlusive dressing (such as a gauze patch) and then wrap the application site with a semi-occlusive bandage for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites. Remove residual test substance by appropriate means, such as washing with lukewarm water or other suitable, non-irritating solvent, and careful drying.

5.2.4.3 Extracts and extractants

Apply the appropriate extract(s) to the 25 mm x 25 mm four-ply gauze patches (0.5 ml per patch), one patch on each side of the animal as shown in figure 1. Apply a control patch of gauze moistened with the extracting medium to the other side.

Cover the application sites with a semi-occlusive bandage for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites. Remove residual test substance by appropriate means,

such as washing with lukewarm water or other suitable, non-irritating solvent, and careful drying.

5.2.4.4 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 substance shall be moistened sufficiently with water or, where necessary, an alternative solvent, to ensure good contact with the skin. When solvents are used, the influence of the solvent on irritation of skin by the test substance shall be taken into account.

Cover the application sites with a 25 mm x 25 mm non-occlusive dressing (such as a gauze patch) and then wrap the application sites with a semi-occlusive bandage for a minimum of 4 h. At the end of the contact time, remove the dressings and mark the positions of the sites. Remove residual test substance by appropriate means, such as washing with lukewarm water or other suitable, non-irritating solvent, and careful drying.

5.2.5 Observation of animals

For acute (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.

For repeated exposure, note the appearances of the application site at 1 h after removal of the patches and immediately prior to the next application. 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.

Describe and grade 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 3 Histological and non-invasive techniques may assist in certain cases.

5.2.6 Evaluation of results

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

For each animal, add together the Primary Irritation Scores for the test material for both erythema and edema at each time specified and divide by the total number of observations (six: two at each time specified). When vehicle controls are used, calculate the Primary Irritation Score for the vehicle controls and subtract that score from the score for the test material to obtain the Primary Irritation Score.

Only use 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.

Table 1—Classification system for skin reaction

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

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 Irritation Scores for both erythema and edema at each time specified. Divide this total by the total number of observations to obtain the Irritation Score per animal.

Add the Irritation Scores of each animal 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 Cumulative Irritation Index defined in table 2 and the appropriate Category is recorded for the report.

NOTE 4 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, determine the Maximum Irritation Response, the time of onset of the response and the time to maximum response.

The Primary or Cumulative Irritation Index is characterized by number and description in table 2.

Table 2—Irritation response categories in the rabbit

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

5.2.7 Presentation of results

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 material or device;

d) the test animals;

e) method of application to the test sites;

f) how the site readings were performed and a record of the observations;

g) assessment of the results.

5.3 Ocular irritation test

5.3.1 Principle

Assessment of the potential of the material under test to produce ocular irritation.

5.3.2 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. Strongly acidic or alkaline substances (pH 2 or 11.5) shall not be tested owing to their predictive corrosive properties. These products shall be considered eye irritants.

5.3.3 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 5 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 B.

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 reagent controls, using both the polar and the non-polar solvent, in the absence of the test material.

5.3.4 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 annex C.

One animal shall initially be used to evaluate the test material.

A well-defined response 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.

5.3.5 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 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 material as specified in 5.3.3.

Following instillation hold the eyelids together for approximately 1 s.

NOTE 6 The contralateral eye of each animal serves as an untreated control.

If repeated exposure of 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. The duration of the exposure should bear resemblance to the length of use of the test material/device in the clinical situation.

5.3.6 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.

Grade and record any reactions observed in accordance with the scale for grading ocular lesions given in table 3.

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 the scale for grading ocular lesions given in table 3.

Withdraw an animal immediately from the study and humanely kill 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 3—absence of a light reflex (iridial response 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 kill it humanely.

5.3.7 Evaluation of results

Differences between the test and control eyes shall be characterized and explained in the terms of the classification system used in table 3.

5.3.7.1 Acute exposure

If the treated eye in more than one animal shows a positive response (asterisked figures in table 3) at any of the observations, then the material is considered an eye irritant and further testing is not required.

If only one of three eyes treated shows a 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 reaction (asterisked figures in table 3) at any stage of the observation.

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

5.3.7.2 Repeated exposure

The test material is considered an eye irritant if more than half of the animals in the test group exhibit a positive reaction (asterisked figures in table 3) at any stage of the observation.

5.3.8 Presentation of results

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 material or device;

d) the test animals;

e) method of instillation;

f) how the ocular readings were performed and a record of the observations;

g) assessment of the results.

Table—3 Classification system for grading ocular lesions

Reaction	Numerical grading	
1. Cornea		
Degree of opacity (most dense area used)		
No opacity	0	
Scattered or diffuse areas, details of iris clearly visible		1*
Easily discernible translucent areas, details of iris slightly obscured	2*	
Opalescent areas, no details of iris visible, size of pupil barely discernible	3*	
Opaque, iris invisible	4*	
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*	
No reaction to light, hemorrhage, gross destruction (any or all of these)	2*	
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*	
Diffuse beefy red	3*	
Chemosis		
No swelling	0	
Any swelling above normal (include nictitating membrane)	1	
Obvious swelling with partial eversion of lids	2*	
Swelling with lids about half-closed	3*	
Swelling with lids about half-closed to completely closed	4*	
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 round the eye	3	

*positive result

5.4 Intracutaneous (intradermal) reactivity test

5.4.1 Principle

Assessment of the potential of the material under test to produce irritation following intradermal injection of extracts.

5.4.2 Exclusion from test

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

5.4.3 Test material

The test materials shall be extracts and shall be prepared according to the procedures specified in annex B.

5.4.4 Animals and husbandry

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

The animals shall be acclimatized and cared for as specified in annex C.

A minimum of three 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.

5.4.5 Test procedure

On the day before the test, 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 the polar solvent at five sites on one side of each rabbit (see figure 2). Use the smallest sized needle, appropriate to the viscosity of the test material, for the intradermal injections.

Similarly, inject 0.2 ml of the polar solvent control at five posterior sites on the same side of each rabbit (see figure 2).

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 2).

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

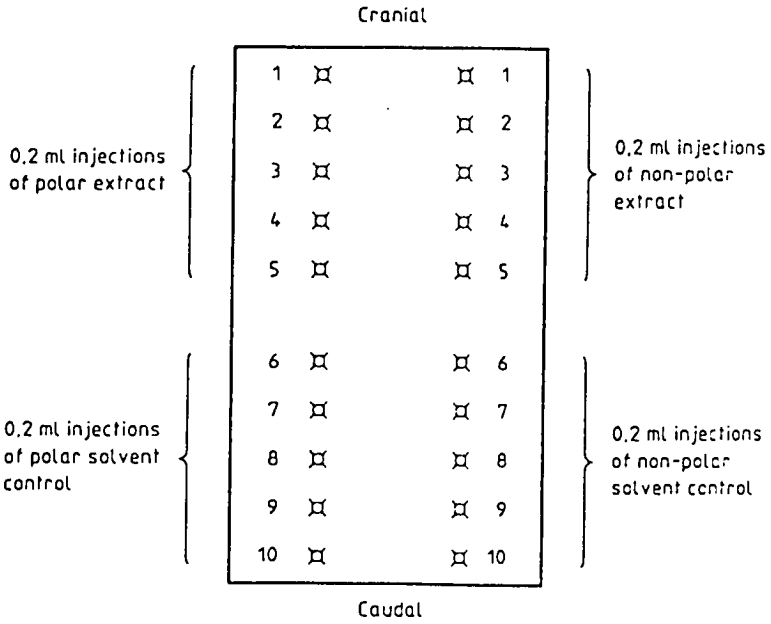

Figure 2 — Arrangement of injection sites

5.4.6 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 classification system given in table 4 for each injection site and at each time interval observed, and record the results.

NOTE 7 Intradermal injection of an oil frequently elicits an inflammatory response.

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

Table 4—Classification 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
NOTE Other adverse changes at the injection sites shall be recorded and reported.	

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

5.4.7 Evaluation of results

Determine the Primary Irritation Index as follows.

For each animal, add together the Primary Irritation Scores for both erythema and edema separately for each test extract at each time specified and divide by the total number of observations. A similar assessment is made of the sites injected with the reagent control. Subtract that score for the reagent control from the score for the test material to obtain the Primary Irritation Score to be used in determining the Primary Irritation Index.

Only use the 24 h, 48 h and 72 h observations for calculations.

Add the Primary Irritation Scores of each animal and divide the total by the number of animals. This value is the Primary Irritation Index (PII).

The Primary Irritation Index is characterized by number and description in table 5.

5.4.8 Presentation of results

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 material or device;

d) the test animals;

e) method of injection;

f) how the site readings were performed and a record of the observations;

g) assessment of the results.

Table 5—Primary irritation response categories in rabbit

Response Category	Mean Score (PII)[1)]
Negligible	0 to 0.4
Slight	0.5 to 1.9
Moderate	2 to 4.9
Severe	5 to 8
1) The Primary Irritation Index (PII) is determined by adding the Primary Irritation Score for each animal and dividing the total score by the number of animals.	

6 Sensitization tests

There are several methods for determining skin sensitization in guinea pigs. The two most commonly used methods are the maximization (Magnusson & Kligman) and closed patch (Buehler) methods. It is only necessary to evaluate by one of these methods. The maximization test (6.2) is regarded as the most sensitive and is the preferred method, particularly with regard to the evaluation of extracts. A list of alternative methods is given in annex E.

6.1 Factors to be considered in design and selection of tests

The biochemical and physical characteristics of the test material may influence the choice of test. The maximization test requires intradermal injections; consequently if the test material cannot be injected intradermally, the closed patch or alternative method shall be used.

A solvent should be selected that optimizes exposure by solubilization and penetration. The concentration of test material should be the highest possible without affecting the ability to interpret the results. The concentration of the test material at the skin surface is an important criterion for topical administration and not the total volume of test material. The latter will be determined by the capacity of the patch system.

Damage to the skin cannot be avoided when Freund's complete adjuvant is injected intradermally.

The times of exposure for all phases of each experiment need to be sufficient for experimental success. They should be varied only to the extent that they achieve this end. All phases (preliminary, induction and challenge) are critical but the scoring intervals are critical only to the extent that they demonstrate the delayed and persistent characteristic of hypersensitivity. Scoring of induction sites is not generally informative and is not critical for interpretation.

The actual position of patch sites on the flank of the guinea pig is not a critical parameter, provided all the challenge sites are conducted on naive skin.

Comparison of test animals at challenge with the appropriate controls is essential for indication of a positive test result, though the severity of lesions will aid in the interpretation.

Borderline reactions at challenge are best clarified by rechallenge. Histopathology is not generally of diagnostic value.

A positive test does not necessarily exclude the test material or device from use because the doses of the test substance in the test procedure may be exaggerated compared to actual conditions of use. A positive test using any of the validated procedures indicates the need for additional data, either in guinea pigs or humans, that

would allow risk assessment of intended human exposure. The relative sensitizing potencies of substances can be defined in terms of the minimum (lowest) induction concentration required to induce a given level of sensitization.[37] This entails, as a minimum, verification with appropriate concentrations and vehicles. Repeating this assay with other techniques and utilization of open challenges (to avoid non-specific effects of tape) are of scientific and practical value. Provocative tests followed by ad libitum use tests with appropriate diagnostic patch testing can effectively determine safe levels of use.

For products to be used extensively on normal and abnormal skin, no substantial risk is acceptable. However, many ingredients, in spite of sensitizing potential, are fully acceptable in products at reasonable concentrations because of their inherent benefit.

6.2 Maximization sensitization test

6.2.1 Principle

Assessment of the potential of the material under test to produce skin sensitization in the guinea pig.

6.2.2 Test material

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

If the test material is to be tested as an extract, it shall be prepared as specified in annex B.

6.2.3 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 annex C.

For testing powders or liquids, a minimum of ten animals shall be treated with the test material and a minimum of five animals acts as a control group. Additional animals shall be used for the preliminary test.

For testing extracts, a minimum of ten animals shall be treated with each extract and a minimum of five animals acts as a control for each solvent. Additional animals shall be used for the preliminary test.

NOTE 8 It may be necessary to double the number of animals in order to confirm weak sensitizers. [34], [35]

6.2.4 Test procedure

6.2.4.1 Preparation

Clip the fur on all treatment sites the day prior to treatment.

For intradermal injections, inject 0.1 ml per site.

For all topical applications, saturate a patch of filter paper of the appropriate dimensions with the test material and apply the patch to the clipped skin surface under an occlusive dressing wound around the torso of the animal.

6.2.4.2 Preliminary tests

NOTE 9 The preliminary tests are intended to determine the concentrations of the test materials to be used in the main test in 6.2.4.3.

Consideration shall be given to the pretreatment of all animals by injection with Freund's complete adjuvant.

Inject a range of concentrations of the test material or extract (in the selected solvent) intradermally into at least two animals.

Select for the intradermal induction phase in the main test the highest concentration that does not cause

extensive destruction of the skin and does not otherwise adversely affect the animals.

Topically apply a range of concentrations of the test material or extract to the flanks of at least three additional animals. Remove the occlusive dressings and patches after 24 h, and assess the application sites for erythema and edema using the grading given in table 6.

Select

a) if possible, for the topical induction phase in the main test, the highest concentration that causes slight erythema but does not otherwise adversely affect the animals;

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

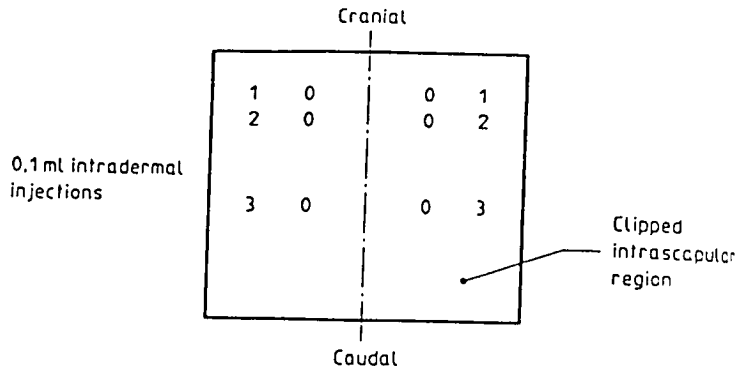

Figure 3—Location of intradermal injection sites

6.2.4.3 Main test

6.2.4.3.1 Intradermal induction phase

Make a pair of 0.1 ml intradermal injections of each of the following, into each animal, at the injection sites (1, 2 and 3) shown in figure 3 in the clipped intrascapular region.

a) A 50:50 (V/V) mixture of Freund's complete adjuvant mixed with the chosen solvent. Water for injection or physiological saline (BP, USP or equivalent) for water-soluble substances. For non-aqueous soluble substances, examples of solvents are given in annex B, B.2.10.

b) The test material or extract at the concentration selected in the preliminary tests: inject the control animals with the solvent alone.

c) The test material or extract at the concentration used in b), emulsified in a 50:50 (V/V) mixture of Freund's complete adjuvant and the solvent; inject the control animals with the solvent mixed/emulsified with adjuvant.

Table 6—Classification system for skin reactions

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema to slight eschar formation	4
Edema formation	
No edema	0
Slight edema	1
Well-defined edema	2
Moderate edema	3
Severe edema	4

NOTES

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

2 For the purposes of standardization,, the grading system has been modified from the original method.

6.2.4.3.2 Topical induction phase

Seven days after completion of intradermal induction phase, administer the test material or extract by topical application to the intrascapular region of each animal, using 20 mm x 40 mm filter paper, so as to cover the intradermal injection sites. Use the concentration selected in 6.2.4.2a). Secure with an occlusive dressing. Remove the dressings and patches after 48 h 2 h.

Treat the control animals similarly, using the solvent alone.

If the maximum concentration that can be achieved in 6.2.4.2a) does not produce irritation, pre-treat the area with 10% sodium lauryl sulphate in petrolatum massaged into the skin 24 h 2 h before the topical induction patch is applied. Treat the control groups similarly.

6.2.4.3.3 Challenge phase

At least 14 days after completion of the topical induction phase, challenge all test and control animals with the test material or extract. Administer the test material or extract by topical application to one flank of each animal using appropriate patches soaked in the test material or extract at the concentration selected in 6.2.4.2b). Secure with an occlusive dressing. Remove the dressings and patches after 24 h 2 h.

NOTE 10 Dilutions of this concentration may also be applied to other untreated sites in a similar manner.

6.2.5 Observation of animals

Observe the appearance of the challenge skin sites of the test and control animals 24 h, 48 h and 72 h after removal of the dressings. Describe and grade the skin reactions for erythema and edema according to the grading given in table 6 for each challenge site and at each time interval.

6.2.6 Evaluation of results

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.

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. If necessary a rechallenge shall be carried out approximately 7 days after the first challenge. The method used shall be as described for the first challenge, using the other flank of the animal.

6.2.7 Presentation of results

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 material or device;

d) the test animals;

e) method of application to the test sites;

f) how the site readings were performed and a record of the observations;

g) assessment of the results.

6.3 Closed patch sensitization test

6.3.1 Principle

Assessment of the potential of the material under test to produce skin sensitization in guinea pigs.

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.

If the test material is to be tested as an extract, it shall be prepared as specified in annex B.

6.3.3 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 annex C.

For testing solids, powders or liquids, a minimum of ten animals shall be treated with the test material and a minimum of five animals acts as a control group. Additional animals shall be used for the preliminary test.

For testing extracts, a minimum of ten animals shall be treated with each extract and a minimum of five animals acts as a control for each solvent. Additional animals shall be used for the preliminary test.

NOTE 11 It may be necessary to double the number of animals in order to confirm weak sensitizers.[35]

6.3.4 Test procedure

6.3.4.1 Preparation

Clip the fur on all treatment sites the day prior to treatment.

For all topical applications, saturate a patch (a woven dressing) 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.

Use either wrapping or restraint of the animal to ensure occlusion of the test sites.

If wrapping is used, its adequacy should be evaluated in every experiment, since wrapping can be stressful.

6.3.4.2 Preliminary tests

NOTE 12 The preliminary tests are intended to determine the concentrations of the test material or extract to be used in the main test described in 6.3.4.3.

Topically apply four concentrations of the test material or extract 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 grading given in table 6, 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.

6.3.4.3 Main test

6.3.4.3.1 General

Use a minimum of ten animals as a test group and a minimum of five animals as a control group. If the response to solvent is not known, there shall be an appropriate solvent control group.

6.3.4.3.2 Induction phase

Administer the test material or extract 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 6.3.4.2a). Remove the occlusive dressings and patches after 6 h.

Repeat this procedure at weekly intervals for three weeks. Additional induction application may be warranted.

Treat the reagent control animals similarly, using the solvent alone.

6.3.4.3.3 Challenge phase

Fourteen days after the last application, challenge all test and control animals with the test material or extract. Administer the test material or extract by a single topical application to a clipped untested area of each animal using appropriate patches soaked in the test material or extract at the concentration selected in 6.3.4.2b). Remove the dressings and patches after 6 h.

6.3.5 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 7. Repeat the grading 48 h 2 h after removal of the challenge patch.

NOTE 13 For reporting purposes the first and second gradings are designated as 24 h and 48 h readings respectively.

Table 7—Classification system for skin reactions

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema to slight eschar formation	4
Edema formation	
No edema	0
Slight edema	1
Well-defined edema	2
Moderate edema	3
Severe edema	4

NOTES

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

2 For the purposes of standardization,, the grading system has been modified from the original method.

6.3.6 Evaluation of results

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.

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. If necessary a rechallenge shall be carried out approximately 7 days 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.

6.3.7 Presentation of results

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 material or device;

d) the test animals;

e) method of application to the test sites;

f) how the site readings were performed and a record of the observations;

g) assessment of the results.

Annex A
(normative)

Preparation of materials for 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 man. One of the parameters critical to these tests is the preparation of the test material.

NOTE 14 A general guideline (ISO 10993-12) is in preparation but specific problems are associated with irritation and sensitization extract preparation.

A.2 Direct contact

A.2.1 Solid materials which have appropriate physical states (e.g. sheets, films) shall be tested without modification.

A.2.2 Powders (e.g. super-absorbents) shall be tested by direct deposition or by making a paste in an appropriate solvent.

A.2.3 Liquids shall be tested by either direct deposition or in diluted solution made with an appropriate solvent.

A reagent control using the same solvent shall be evaluated in parallel with the moistened, diluted or suspended test material.

A.2.4 If the test material is a solid, prepare samples 25 mm x 25 mm of a thickness that approximates to normal use but is not greater than 5 mm. Prepare suitable negative control samples in the same way. The solid may be pulverized, care being taken to ensure no contamination occurs during this process, or moistened sufficiently with water or a suitable non-irritant solvent to ensure good contact with the tissues. In the case of ceramics where pulverization is required, it must be remembered that the physico-chemical properties of the ceramic may be altered by reducing the ceramic to a powder.

The negative control shall physically resemble the test material closely and should be non-irritant. Four-ply gauze may be used as a substitute.

A.2.5 If the test material has to be diluted, suspended or moistened, a suitable non-irritant solvent shall be used. Refer to annex B for a list of appropriate solvents.

A.2.6 If the test material is a solid, it may be tested by preparing extracts from the solid. If extracts are tested, extracts shall be prepared as described in annex B, using polar, non-polar and/or additional solvents when appropriate.

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

A.2.7 If the final product is sold 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. Any adverse biological response shall be evaluated. To enable differentiation to be made between effects produced by the test material and those produced by ethylene oxide residuals when an adverse irritant response is observed, consideration shall be given to evaluation of this response to the device pre- and post-ethylene oxide sterilization.

Annex B
(normative)

Method for extraction of materials for biological tests

B.1 Introduction

To conduct biological evaluations of biomaterials, it is often necessary to obtain extracts with suitable extraction media. °This procedure outlines methods used on a routine basis to obtain such extracts for testing and supplements, but does not supersede methods contained in specific study protocols.

B.2 Apparatus

B.2.1 Autoclave capable of maintaining cycles at 121 °C 2 °C.

B.2.2 Oven or incubator capable of maintaining temperature at 70 °C 2 °C.

B.2.3 Oven or incubator capable of maintaining temperature at 50 °C 2 °C.

B.2.4 Incubator capable of maintaining temperature at 37 °C 2 °C.

B.2.5 Cutting tools as required (e.g. scissors, scalpel, saw).

Clean metal cutting tools with a volatile organic solvent such as alcohol or acetone. Do not use acids to clean metal equipment.

B.2.6 Pipettes, sizes as appropriate: usually 1 ml, 5 ml, 10 ml and 25 ml.

B.2.7 Balance, accurate to at least 0.1 g.

B.2.8 Measuring devices (e.g. millimeter scale, calliper).

B.2.9 Extraction vessels:

a) borosilicate glass tubes with screw caps lined with an inert material (e.g. PTFE);

b) other suitable glass jars or extraction vessels as required for specific materials.

The vessel should not affect the extract obtained from the test material.

B.2.10 Extraction media—at least one of the following shall be used:

a) polar solvent: physiological saline;

b) non-polar solvent: either vegetable oil (e.g. cottonseed oil or sesame oil, EP or USP) oleum neutral (DAC, Fract. Coconut, BP73);

c) suitable extraction media other than those specified in a) and b), such as ethanol/water, ethanol/saline, polyethylene glycol 400, dimethylsulfoxide, propanone (acetone), methanol, chloroform, dilute surfactant, mineral oil.

NOTE 15 Solvent extraction methods may be appropriate for skin sensitization tests.

B.3 Sample preparation

B.3.1 Determination of surface area

Use surface area calculations for samples which can be considered as resembling simple geometric shapes. Porous samples such as gauze, woven articles or spongy materials with simple geometric shapes may be regarded and measured as solid objects.

If the thickness of the material is less than 0.5 mm, use a portion with a total surface area equivalent to 120 cm2.

Extract this in 20 ml of the extraction medium ensuring all exposed surfaces are covered.

NOTE 16 This is a 6:1 surface area to volume extraction ratio.

If the thickness of the material is greater than 0.5 mm, use a portion with a total surface area equivalent to 60 cm2. Extract this in 20 ml of the extraction medium ensuring all exposed surfaces are covered.

NOTE 17 This is a 3:1 surface area to volume extraction ratio.

B.3.2 Mass

For materials with an irregular shape whose surface area cannot easily be determined, use a ratio of 2 g to 4 g of sample to 20 ml of extraction medium. Weigh all materials to the nearest 0.1 g.

B.3.3 Other materials

For materials whose characteristics are such that the volume of medium will not cover the required surface area or mass of homogeneous subdividable sample (e.g. foam, sponge), use the maximum amount of sample that can be covered by the required volume of extraction medium. Indicate the surface area ratio used and weigh the amount of the sample to the nearest 0.1 g.

Super-absorbents present a problem: no standard methods are available.

B.3.4 Non-subdividable materials

For materials that cannot be subdivided without loss of sample character, identity or integrity, and for which the calculated volume of extraction medium will not cover the entire sample (e.g. complex devices, metal objects, interiors of bags), use the minimum amount of medium which will cover the test surfaces. Depending on the type of material, designate either mass (to the nearest 0.1 g) or exposed surface area (to the nearest 1 cm2) extracted, and record the volume of extract to the nearest 1 ml.

B.3.5 Final preparation

B.3.5.1 Examine the material to be extracted for signs of particulate or dust contamination. Conduct rinsing and drying procedures when the material to be extracted does not appear free of surface contaminants or when otherwise required. Rinse the material with either Purified Water or Water for Injection, USP or BP, using a ration of 70 ml of water for each 60 cm2 surface area. Agitate for at least 30 s and then decant. Repeat rinsing if necessary and dry prior to extraction.

NOTE 18 The majority of materials are provided sterile and/or cleanly packaged. The extra manipulations and exposure to the drying temperatures are not usually warranted and in fact may adversely affect the outcome of some studies. Omission of the rinsing procedure is recommended for apparently clean materials as it may permit a more realistic evaluation of the manufacturing process and material.

B.3.5.2 After the correct amount of material has been calculated, subdivide, where practical, into small pieces.

B.3.5.3 When the thickness of the material is greater than 1 mm and subdivision is not practical, calculate the exposed areas of all pieces to determine the amount to be used.

B.4 Extraction procedure

B.4.1 Place the test material into an appropriately sized extraction vessel. Measure the volume of extraction medium required to the nearest 1 ml with a pipette or similar volumetric device and cover the test material in the vessel. Agitate the sample to make sure that the material sections are free and not bound or stuck to each other.

B.4.2 Select the temperature and time of extraction according to the requirement of the material or other requirements. Extraction at 37 °C for at least 72 h is suitable for most devices.

Shorter extraction times at higher temperatures may be considered. If this is done, this approach needs to be

justified. Alternative temperature and time options are as follows:

a) 121 °C for 1 h 0.2 h

b) 70 °C for 24 h 2 h

c) 50 °C for 72 h 2 h

Refer to ISO 10993-12 for a discussion of the appropriate choice of temperature.

B.4.3 Prepare the Reagent Control without the test material in extraction vessels treated in an identical manner (same temperature and time period) as the test extract.

B.4.4 Following extraction, agitate the containers and decant the extracts into clean vessels. Store the extracts at room temperature and use within 24 h of decanting. If an extract is stored longer than 24 h, the reliability of the extract under the conditions of storage should be verified.

B.4.5 An alternative sample extraction procedure using a volatile solvent, followed by evaporation of the solvent and application of the residue to animals, may be undertaken for polymeric materials for sensitization testing.

Annex C

(normative)

Animals and husbandry

The following applies to all tests in this part of ISO 10993.

C.1 Healthy young adult animals of a single strain from a single recognized source shall be used.

C.2 Animals may be housed either in groups or individually. Group housing is strongly recommended.

C.3 The animals shall have access to food and water.

C.4 The animals shall be acclimatized to the laboratory conditions for a minimum of five days before being placed on test.

This time may be longer, depending on the appropriate regulatory requirements.

C.5 All appropriate regulatory requirements governing the care and use of animals shall be followed.

Annex D
(informative)

Additional irritation tests

In contrast to dermal and ocular irritation tests, the oral, rectal, penile and vaginal irritation tests are not well-documented. These tests are special evaluation tests and should be considered as additional to the basic tests but not as replacements. They are only relevant for medical devices intended to be applied to these areas.

The oral irritation test given is less traumatic than some tests in which the test material is secured in the hamster cheek pouch by suturing.

D.1 Oral irritation test

D.1.1 Principle

Assessment of the potential of the material under test to produce irritation of the oral tissue.

D.1.2 Exclusion from test

Any material shown to be a skin or eye irritant or those with a pH of 2 or 11.5 should not be tested and should be labeled as a potential oral tissue irritant.

D.1.3 Test material

Prepare test materials according to the procedures specified in annex A.

Test liquid samples by soaking cotton-wool pellets in the test material or by direct flushing of the cheek pouch with the material.

Test solid samples by placing pellets of the test substance directly into the cheek pouch or by soaking cotton-wool pellets in an extract prepared according to procedures described in annex B.

D.1.4 Animals and husbandry

Syrian hamsters from a single outbred strain should be used. They should be healthy young adults of either sex.

The animals should be acclimatized and cared for as specified in annex C.

In addition to the above, fit to each animal a 3 mm to 4 mm wide suitable collar, placed around the neck so that it permits normal feeding and respiration but prevents the animal from removing the cotton-wool pellet. Weigh each animal daily for 7 days. Examine any animal showing a loss of 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 should be used initially to evaluate the test material.

NOTE 19 Additional animals treated with a negative control substance or control extract may be appropriate.

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

D.1.5 Test procedure

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

For solid materials, place pellets (no larger than 5 mm diameter) of materials directly into the cheek pouch. For liquids or extracts, soak a cotton-wool pellet, recording the volume used, in the test material or extract and place it in one pouch of each animal. Alternatively, an appropriate volume of a liquid may be flushed into the cheek pouch. No sample is placed in the other cheek pouch, which serves as a control. If extracts are tested, appropriate controls should be tested in parallel.

Replace the collar and return the animal to its cage.

The duration of contact should be that expected for actual use of the material, but no shorter than 5 min. Following the exposure, the collar and cotton-wool pellet are removed and the pouch is washed 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, the number of applications, their duration and their interval should be based on the anticipated contact time in the clinical situation.

D.1.6 Observation of animals

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

Describe the appearance of the cheek pouches for each animal and grade the pouch surface reactions for erythema according to the classification system given in table D.1 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 kill the hamsters and remove tissue samples from representative areas of the pouches. Place in an appropriate fixative prior to processing for histological examination.

Table D.1—Classification 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 gradingof erythema	4
NOTE Other adverse changes of the tissues should be recorded and reported.	

D.1.7 Assessment of results

D.1.7.1 Macroscopic evaluation

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

The scores (table D.1) for each observation are added and divided by the number of observations to determine the average score per animal.

NOTES

20 These observations may assist in the histological evaluation.

21 The initial observations made prior to the first application of the test substance are not included in the score average.

D.1.7.2 Histological evaluation

The irritant effects on oral tissue should be evaluated by a pathologist. The pathologist may score each tissue according to the system presented in table D.2.

The scores for microscopic evaluation for all the animals in the test group are added and 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.

For repeated exposure, table D.2 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

D.1.8 Presentation of results

The test report should 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 material or device;

d) the test animals;

e) method of application;

f) how the site readings were performed and a record of the observations;

g) histological evaluation;

h) assessment of the results.

D.2 Penile irritation test

D.2.1 Principle

Assessment of the potential of the material under test to produce irritation of the penile skin.

D.2.2 Exclusion from test

Any material shown to be a skin or eye irritant or those with a pH of 2 or 11.5 should not be tested and should be labeled as a potential penile irritant.

D.2.3 Test material

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

If the test material is to be tested as an extract, it should be prepared as specified in annex B.

D.2.4 Animals and husbandry

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

The animals should be acclimatized and cared for as specified in annex C.

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

Due to individual pigment variation, animals are observed and scored for erythema prior to the first test application. The classification system given in table D.1 should be used for scoring any erythema. Animals showing severe discoloration or having an erythema score of two or greater should not be used.

A minimum of three animals should 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 should be considered.

Table D.2—Microscopic classification system 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. Leucoyte 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
Irritation Index	
Average score	**Adjectival description**
0	None
1 to 4	Minimal
5 to 8	Mild
9 to 11	Moderate
12 to 16	Severe
NOTES	
1 Other adverse changes of the tissues should be recorded and included in the assessment of the response.	

2 The microscopic scoring system in the top portion of the table applies for all tests listed. The “irritation index”was developed for use with the vaginal irritation model buy may be used for other tests.

D.2.5 Test procedure

Place the animal into 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 material to be sure that the penis is coated.

Allow the penis to retract into the sheath and isolate the area by wrapping the body of the animal between the front and rear legs with loose knit dressing (e.g. nylon hose or roll gauze) and secure the dressing to the torso. This is to prohibit the animal from licking the test site and confounding the primary irritation by secondary factors.

Alternatively the animal may be secured in an appropriately designed restrainer for 1 h after the last application of the test material and then returned to its own cage.

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

For prolonged repeated exposure, the number of applications, their duration and their interval should be based on the anticipated contact time in the clinical situation.

D.2.6 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 classification system given in table D.1 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 score given prior to the first application of the test material is subtracted from the scores for erythema at the timed observations to determine the erythema score due to the test material. The highest possible score for one observation is four.

D.2.7 Assessment of results

D.2.7.1 Macroscopic evaluation

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

The scores (table D.1) for each observation are added and divided by the number of observations to determine the average score per animal.

NOTES

22 These observations may assist in the histological evaluation.

23 The initial observations made prior to the first application of the test substance are not included in the score average.

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

D.2.7.2 Histological evaluation

The irritant effects on the penile skin should be evaluated by a pathologist. The pathologist may score each tissue according to the system presented in table D.2.

The scores for microscopic evaluation for all the animals in the test group are added and 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.

For prolonged repeated exposure, table D.2 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

D.2.8 Presentation of results

The test report should 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 material or device;

d) the test animals;

e) method of application;

f) how the site readings were performed and a record of the observations;

g) histological evaluation;

h) assessment of the results.

D.3 Rectal irritation test

D.3.1 Principle

Assessment of the potential of the material under test to produce irritation of the rectal tissue.

D.3.2 Exclusion from test

Any material shown to be a skin or eye irritant or those with a pH of 2 or 11.5 should not be tested and should be labeled as a potential rectal irritant.

D.3.3 Test material

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

If the test material is to be tested as an extract, it should be prepared as specified in annex B.

D.3.4 Animals and husbandry

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

The animals should be acclimatized and cared for as specified in annex C.

A minimum of three animals should 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 should be considered.

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

D.3.5 Test procedure

Attach a short (6 cm) soft catheter or blunt-tipped cannula to a syringe with a capacity to deliver more than 2 ml, and fill the syringe and catheter such that 2 ml of the test solution 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 in either the control solution 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 2 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 material 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, the number of applications, their duration and their interval should be based on the anticipated contact time in the clinical situation.

D.3.6 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, swelling and/or that are found difficult to dose should be humanely killed and the tissues examined (see D.3.7.1).

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

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

D.3.7 Evaluation of results

D.3.7.1 Macroscopic evaluation

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 24 These observations may assist in the histological evaluation.

D.3.7.2 Histological evaluation

The irritant effects on the rectal tissue should be evaluated by a pathologist. The pathologist may score each tissue according to the system presented in table D.2.

Add the scores for microscopic evaluation for all the animals in the test group and divide 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.

For prolonged repeated exposure, table D.2 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

D.3.8 Presentation of results

The test report should 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 material or device;

d) the test animals;

e) method of application;

f) how the site readings were performed and a record of the observations;

g) histological evaluation;

h) assessment of the results.

D.4 Vaginal irritation test

D.4.1 Principle

Assessment of the potential of the material under test to produce irritation of the vaginal tissue.

D.4.2 Exclusion from test

Any material shown to be a skin or eye irritant or those with a pH of 2 or 11.5 should not be tested and should be labeled as a potential vaginal irritant.

D.4.3 Test material

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

If the test material is to be tested as an extract, it should be prepared as specified in annex B.

D.4.4 Animals and husbandry

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

The animals should be acclimatized and cared for as specified in annex C.

A minimum of three animals should 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 should be considered.

The animals should be checked for vaginal discharge, swelling and/or other evidence of vaginal infection,

irritation and/or injury prior to each treatment. A check should 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.

D.4.5 Test procedure

Attach a short (6 cm) soft catheter or blunt-tipped cannula to a syringe with a capacity to deliver more than 2 ml, and fill the syringe and catheter such that 2 ml of the test solution 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 perineum.

Moisten the catheter in either the control solution 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 2 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 material 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, the number of applications, their duration and their interval should be based on the anticipated contact time in the clinical situation.

D.4.6 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 should be humanely killed and the tissues examined (see D.4.7.1).

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

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

D.4.7 Evaluation of results

D.4.7.1 Macroscopic evaluation

Compare the vaginas of animals treated with the test substance 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 25 These observations may assist in the histological evaluations.

D.4.7.2 Histological evaluation

The irritant effects on vaginal tissue should be evaluated by a pathologist. The pathologist may score each tissue according to the system presented in table D.2.

The scores for microscopic evaluation for all the animals in the test group are added and 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.

For prolonged repeated exposure, table D.2 may need to be modified to accommodate additional tissue responses associated with chronic irritation.

D.4.8 Presentation of results

The test report should 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 material or device;

d) the test animals;

e) method of application;

f) how the site readings were performed and a record of the observations;

g) histological evaluation;

h) assessment of the results.

Annex E
(informative)

Background information

E.1 Background information on irritation tests

Dermal irritation testing in small animals is performed to help identify substances which may be potential human skin and/or mucosal tissue irritants. A primary irritant is a substance 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 a severe irritant, vesiculation and/or necrosis.

The Draize dermal irritation test is a patch test on albino rabbits. The test substance 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 dressing for 4 h. After 4 h, the patches are removed, the test sites cleaned, and any resulting reaction graded for erythema and edema. The reactions are also scored at 24 h, 48 h and 72 h.

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 Substances (RTECS). Eighty-five percent of over 2,000 RTECS entries report test results with the rabbit, 7.5% with man, 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.

The majority of human data comes from the Research Institute for Fragrance Materials Monographs on essential oils and other aromatics published in Food and Cosmetic Toxicology. This is also the primary source of mouse skin irritation data.

Mann and Pullinger described the use of rabbits to predict ocular irritancy in man. These authors advocated the use of pigmented rather than non-pigmented (albino) eyes and relied on description of individual animal responses to assess the irritant effects. Friedenwald, et al. reported an albino rabbit model for assessing ocular irritation that provided a scoring system based on the description of individual animal responses. Draize et al.

modified Friedenwalds' procedure and published a grading system to assist in the evaluation of ocular irritation. Illustrated guides have been published as aids in assessing ocular lesions.

There has been concerted effort over the past 20 years to find alternative *in vitro* biological tests to replace acute skin and eye irritation tests. Within the past decade national and international organizations have been established to further the development of alternative test methods. While many test methods have been proposed and evaluated, none of the methods has duplicated the physiological responses of the *in vivo* animal model; consequently, they do not as yet offer a validated alternative test (Bruner, et al.). In parallel with the search for alternative methods, others have been developing methods to quantify the responses of animals to better define endpoints using non-invasive techniques (Nilsson, et al. and Emtestam and Ollmar). After a careful review of the literature, and consultations with experts using these test methods, a decision was made to not include these tests in this part of 10993 until further work has been completed and the methods have been fully validated.

E.2 Background information on sensitization tests

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 become antigenic. 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 raw materials and/or product formulation followed the pioneer work of Landsteiner and Chase, who firmly substantiated the use of the guinea pig for studying delayed hypersensitivity. In 1965, Buehler advocated the use of the closed patch to provide occlusion as a method to exaggerate exposure and to mimic the procedures used in man (Human Repeat Insult Patch Test HRIPT). It was suggested that the occlusive patch procedure was sensitive and would accurately predict moderate to severe sensitizers and avoid exposing human subjects to the prospect of experiencing 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 even weak sensitizers and has been shown to be sufficiently flexible to be used in the Risk Assessment Process.

Magnusson and Kligman explores many of the variables of guinea pig testing and presented a procedure 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. Because of its abnormal route of exposure (injection) and the use of FCA, it bypasses important components of the immune system (Langerhans cells) and can reorder sensitizers and/or produce experimental tolerance.

These two tests have been the most frequently used for safety assessment, the closed patch test in the United States and the FCA test in Europe. They are also the preferred test methods in current OECD and EEC test guidelines. 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 according to the original references. A list of these other tests is provided in table E.1.

Table E.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 assay

The last assay in table E.1, the murine local lymph node assay, deserves attention as it measures lymphocyte proliferation in lymph nodes draining the site of exposure. In practice the test material is repeatedly applied in an organic solvent to both ears of mice followed by intravenous injection of phosphate buffered saline/[3H] methyl thymidine. The draining auricular lymph nodes are later excised, a single cell suspension of lymph node cells is prepared, precipitated with trichloroacetic acid, resuspended and transferred to scintillation liquid. [3H] methyl thymidine incorporation as measured by ß-scintillation counting is then compared relative to vehicle treated controls.

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. Currently the U.S. Environmental Protection Agency lists eight guinea pig procedures that they find acceptable for registration and labeling. Generally, it is best to perform the preliminary screens in guinea pigs with those raw materials that are new or are suspect because of structure activity relationships. The selection of a guinea pig model could depend on background information and the experience of the investigator. 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.

Annex F
(informative)

Bibliography

F.1 General reference for skin and eye irritation and skin sensitization tests

[1] MARZULLI, F.N. and MAIBACH, H.I. (eds.) *Dermatotoxicology,* 3rd ed., Hemisphere Publishing Corporation, 1987.

F.2 Bibliography for skin and eye irritation tests

[2] BRUNER, L.H., KAIN, D.J., ROBERTS, D.A. and PARKER, R.D. Evaluation of Seven *in vitro* Alternatives for Ocular Testing. *Fund. and App. Toxicol*. 17, pp. 136-149, 1991.

[3] *Consumer Product Safety Act.* 15 U.S. Congress 2041, 1972.

[4] DRAIZE, J.H. *Dermal Toxicity*. Association of Food and Drug Officials of the U.S., pp. 46-59, 1955, FDA, Washington, D.C.

[5] DRAIZE, J.H., *Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics*, 1959, Austin, Texas,

Association of Food and Drug Officials of the United States, Texas State Department of Health, Texas.

[6] EMTESTAM, L. and OLLMAR, S. Electrical Impedance Index in Human Skin: Measurements after Occlusion, in 5 Anatomical Regions and in Mild Contact Dermatitis. *Contact Dermatitis*, 28, p. 104, 1993.

[7] EUROPEAN CHEMICAL INDUSTRY ECOLOGY AND TOXICOLOGY CENTRE. *Eye Irritation Testing,* Monograph 11, 1988, Brussels, Belgium.

[8] EUROPEAN CHEMICAL INDUSTRY ECOLOGY AND TOXICOLOGY CENTRE. *Skin Irritation*, Monograph 15, 1990, Brussels, Belgium.

[9] GRANT, M.W. *Toxicology of the Eye*, 3rd ed., Springfield, Ill., 1986.

[10] MACMILLAN, F.S., RAFFT, R.R. and CLOERS, W.B. A Comparison of the Skin Irritation Produced by Cosmetic Ingredients and Formulations in the Rabbit, Guinea Pig, and Beagle Dog to that observed in the Human. *Animal Models in Dermatology*, Maibach, H.I. (ed.), N.Y., Churchill Livingstone, pp. 12-22, 1975.

[11] NATIONAL ACADEMY OF SCIENCES. Principles and Procedures for Evaluating the Toxicity of Household Substances, Washington, D.C. Comparisons of Skin Irritancy. *Toxicol. Appl. Pharmacol*. 31, pp. 481-90, 1977.

[12] NATIONAL ACADEMY OF SCIENCES—NATIONAL RESEARCH COUNCIL. In*: Principles for Evaluating Chemicals in the Environment*. NAS Publication for the Environmental Protection Agency: Eye Irritation, pp. 104-105, 1975.

[13] ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT (OECD), Guidelines for Testing of Chemicals No. 404, *Acute Skin Irritation/Corrosion* and No. 405 *Acute Eye Irritation/Corrosion*. OECD Publications.

[14] *Pesticide Assessment Guidelines*, Subdivision F, Hazard Evaluation Human and Domestic Animals. 81-4 Eye Irritation and 81-5 Derman Irritation. Office of Pesticide Programs, U.S. Environmental Protection Agency.

[15] PRINCE, J.H., DIESEM, C.D., EGLITIS, I. and RUSKELL, G.L*. Anatomy and Histology of the Eye and Orbit in Domestic Animals.* Springfield, Ill., 1980.

[16] STEINBERG, M., AKERS, W.A., WEEKS, M., MCCREESH, A.H. and MAIBACH, H.I. A Comparison of Test Techniques Based on Rabbit and Human Skin Responses to Irritants with Recommendations, Regarding the Evaluation of Mildly or Moderately Irritating Compounds. *Animal Models in Dermatology*. Maibach H.I. (ed.), N.Y., Churchill Livingstone, pp. 1-11, 1975.

[17] WEIL, S.C. and SCALA, R.A. Study of Intra- and Interlaboratory Variability in the Results of Rabbit Eye and Skin Irritation Tests. *Toxicol. Appl. Pharmacol*. 12, pp. 276-360, 1971.

[18] WOVEN, A. and LEVENSTEIN, I. Techniques for Evaluating Dermal Irritation. *J. Soc. Cos. Chem*. 18, pp. 199-203, 1967.

F.3 Bibliography for oral irritation tests

[19] NILSSON, R., FALLAN, J.O., LARSSON, K.S., OLLMAR, S. and SUNDSTROM, F. Electrical Impedance—A New Parameter for Oral Mucosal Irritation Tests. *J. Mater. Science: Materials in Medicine*, 3, p. 278, 1992.

[20] PILICRO, S.J. et al. Biocompatibility Evaluation of Casting Alloys in Hamsters. *J. Prosthet. Dentistry*, 41, pp. 221-223, 1979.

[21] ROY, M. and WHITE, H.I. Establishment of an Improved Technique for Hamster Mucous Membrane Irritation Testing. *J. Dent. Res*., 11, pp. 1365-1375, 1986.

F.4 Bibliography for vaginal irritation tests

[22] CHVAPIL, M., CHVAPIL, T.A., OWEN, J.A., KANTOR, M., ULREICH, J.B. and ESKELSON, C. Reaction of Vaginal Tissue of Rabbits to Inserted Sponges Made of Various Materials. *J. Biomed. Mater. Res*., 13:1, 1979.

[23] DRAIZE, J.H. DERMAL TOXICITY. *Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics,* Association of Food and Drug Officials of the United States, Texas State Department of Health, Texas, 1959.

[24] ECKSTEIN, P., JACKSON, H.C.N., MILLMAN, N. and SABRERO, A.J. Comparisons of Vaginal Tolerance Tests of Spermicidal Preparations in Rabbits and Monkeys. *J. Reprod, and Fert*., 20:85, 1969.

[25] KAMINSKY, M. and WILLIGAN, D.A. pH and the Potential Irritancy of Douche Formulations to the Vaginal Mucosa of the Albino Rabbit and Rat. *Fd. Chem. Toxicol*., 20:193, 1982.

[26] MULLER, P., RAABE, G., HAROLD, J. and JURETZEK, U. Action of chronic peracetic acid (wofasteril) administration on the rabbit oral mucosa, vaginal mucosa and skin. *Exp. Pathology*., 34:223, 1988.

F.5 Bibliography for skin sensitization tests

[27] ANDERSON, K.E. and MAIBACH, H.I. *Contact Allergy Predictive Tests in Guinea Pigs*. S. Karger, New York, 1985.

[28] BUEHLER, E.V. Delayed Contact Hypersensitivity in the Guinea Pig. *Arch. Dermatol*., 91, pp. 171-175, 1965.

[29] EUROPEAN CHEMICAL INDUSTRY ECOLOGY AND TOXICOLOGY CENTRE. *Skin Sensitization Testing*, Monograph 14, 1990, Brussels, Belgium.

[30] KIMBER, I. and BASKETTER, D.A. The Murine Local Lymph Node Assay: A Commentary on Collaborative Studies and New Directions. *Fd. Chem. Toxicol*., 30, pp. 165-169, 1992.

[31] LANDSTEINER, K. and CHASE, M.W. Studies on the Sensitization of Animals with Simple Chemical Compounds. *J. Exp. Med*., 69:767, 1939.

[32] MAGNUSSON, B. and KLIGMAN, A.M. The Identification of Contact Allergens by Animal Assay. The Guinea Pig Maximization Test. *J. Invest. Dermatol*., 52, pp. 268-276, 1969.

[33] NEWMAN, E.A., BUEHLER, E.V. and PARKER, R.D. Delayed Contact Hypersensitivity in the Vagina and Skin of the Guinea Pig. *Immunology*, 63:119, 1983.

[34] NORDISKE SEMINAR-OG ARBEJDS-RAPPORTER. *Potency Evaluation of Contact Allergens—Dose response studies using the Guinea Pig Maximization test,* 1993.

[35] ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT (OECD) Guidelines for the Testing of Chemicals No. 406, *Skin Sensitization*, OECD Publications.

[36] RITZ, H.L. and BUEHLER, E.V. *Current Concepts in Cutaneous Toxicity*, Drill, V. and Lazar, P. (eds.) Academic Press, New York, pp. 25-40.

[37] ROBERTS, D.W. *Contact Dermatitis*, 17, pp. 281-289, 1987.

[38] ROBINSON, M.K., STOTTS, J., DANNEMAN, P.J., NUSAIR, T.L. and BAY, P.H.S. *A Risk Assessment Process for Allergic Contact Sensitization.* Fd. Chem. Toxicol., 27, pp. 479-489, 1989.

[39] ROBINSON, M.K., NUSAIR, T.L., FLETCHER, E.R. and RITZ, H.L. A review of the Buehler Guinea Pig Skin Sensitization Test and its use in a Risk Assessment Process for Human Skin Sensitization. *Toxicology*. 61, pp. 91-107, 1990.