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Notice
Assessment of medical devise No. 36
March 19, 2003

To: Department of Regulatory Affairs,
Medical and Sanitary Affairs Office in Each Prefecture

Evaluation and Licensing Division,
Pharmaceutical and Food Safety Bureau,
Ministry of Health, Labor and Welfare

Re: Reference document regarding a basic idea on biological safety tests

A basic idea on biological safety tests required for application for approval to manufacture (import) medical devices was notified by notification No. 0213001 by Ministry of Health, Labour and Welfare dated on February 13, 2003 (hereinafter referred to as "Notification"). Regarding tests to be conducted based on this Notification, specific test methods with good sensitivity and those easy to conduct have been recently organized by our research team on "Research on International Harmonization regarding Efficacy and Safety Evaluation Methods of Medical Devices" based on their experience and knowledge. The research report is enclosed as an attachment. Please read it carefully and keep parties involved under your jurisdiction informed.

Please know that test methods given in this report are examples and tests conducted in other test methods are also acceptable as long as they are based on the same basic idea and the adequacy can be explained.

The copy of this notification will be sent to the Chairman of Japan Association for the Advancement of Medical Equipment, Chairman of the Japan Federation of Medical Devices Associations, Chair of Medical Devices and Diagnostics Committee, American Chamber of Commerce and Chairman of Medical Diagnostics Committee, European Business Community in Japan.

Research on International Harmonization regarding Efficacy and Safety Evaluation Methods of Medical Devices

Report

Test Methods for Biological Safety Evaluation of Medical Devices

Research on International Harmonization regarding Efficacy and Safety Evaluation Methods of Medical Devices

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Test Methods for Biological Safety Evaluation of Medical Devices

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Test Methods for Biological Safety Evaluation of Medical Devices

Introduction

Regarding biological safety evaluation of medical devices, “Guidelines for Biological Safety Tests Required for Applications for Approval to Manufacture (Import) Medical Devices” was issued on June 27, 1995; however, revision of the Guideline has been required due to rapid improvement of scientific technology. Based on this background, various issues on biological safety evaluation of medical devices including basic issues as well as roles of guidelines were investigated in 2003 in a research program of Ministry of Health, Labour and Welfare “Research on International Harmonization regarding Efficacy and Safety Evaluation Methods of Medical Devices”. Additionally, a “Committee on Preparation of Revised Guidelines for Basic Biological Tests of Medical Devices and Materials” was established within this research group in February 2002 to discuss basic ideas on biological safety evaluation and “A Basic Idea on Biological Safety Evaluation of Medical Devices” was organized. This was issued as a Notification on February 13, 2003, following a predefined procedure.

The base of this “Basic Idea” is to conduct biological safety tests in accordance with an international standard, ISO 10993 “Biological Evaluation of Medical Devices” series. A lot of test methods are described in each part of ISO 10993 series but there is no description on which method is to be selected. Tests should be selected considering (1) principle of the test, (2) usage method and objective of the medical device and (3) characteristic of the material. For convenience, this report gives specific examples of test methods with good sensitivity and those easy to conduct in premarket safety evaluation of medical devices. However, we think it important to keep investigating because better methods may be developed at any time with rapid improvement of science. We do not force test methods in this report because there may be a case in which methods described in this report are not suitable for usage method or objective or material of the device. When conducting biological safety evaluation of a medical device, it is required to select the most adequate test method for the medical device and conduct the test adequately in accordance with “A Basic Idea on Biological Safety Evaluation”, referring to each section of this report. As false-positive or false-negative results may be derived from the test sample and extraction or preparation methods of the test solutions, it is necessary to always consider characteristics of the test method such as sensitivity and accuracy and be prepared to explain the adequacy for the selection of the test method. It is useful to conduct a comparison test in a case there is a reference material that can be used as a control.

Section 1 Cytotoxicity Test

1. Scope of application

This test is to evaluate cytotoxicity of a medical device or material *in vitro*.

The extraction method [colony and subconfluent methods (such as MEM Elution method)], indirect contact method (agar overlay and filter diffusion methods) and direct contact method are included in ISO 10993-5 Biological evaluation of medical devices Part 5: Tests for in vitro cytotoxicity. In this section, we introduce the colony method by extraction and direct contact methods, methods with high sensitivity based on accumulated data in the past, referring to ISO 10993-5 Biological evaluation of medical devices Part 5: Tests for in vitro cytotoxicity, so that appropriate test methods are selected and the results are effectively used in a safety evaluation.

Other test methods adopted in ISO 10993-5 can be selected as long as sensitivity and reversibility of the method are adequately confirmed taking into account the tissues that have contact with the medical device to be evaluated (Refer to 5.2). However, when a test method other than the colony method is selected for cytotoxicity evaluation of contact lenses or other intraocular lenses, it is necessary to present test results to indicate that the test method applied has equivalent or better sensitivity than the colony method using positive control materials.

2. Standard quoted

3. Colony method by extraction method

3.1 Objective

The objective of this test is to confirm cytotoxicity of extractant extracted from a test sample (final product or material) (hereinafter referred to as “test solution”) using cells.

3.2 Test summary

A test sample will be extracted using serum-containing culture media, and transferred to cell cultures, and colony-forming ability will be evaluated after incubation.

3.3 Preparation of test solution

3.3.1 Test sample

1) Appropriately sterilize a test sample, considering its biochemical or physicochemical characteristics. This procedure can be omitted for

sterilized products.

2) In case ethylene oxide is used for sterilization, aerate thoroughly before initiation of the test in order to avoid residue of ethylene oxide. Sterilization by immersion in ethanol is not allowed.

3) In case that a test sample is a liquid or solution, use it after diluting with an appropriate vehicle or culture medium. When necessary, clarify the effect of a vehicle by testing after diluting the vehicle with a culture medium to an adequate concentration.

3.3.2 Control materials

1) Negative control materials

Negative control materials are materials that meet the defined standard value when tested in accordance with a procedure described in 4.4.3, and the followings are available.

For the extraction method: high density polyethylene sheet (qualified, refer to 5.5 for a vendor)

For the direct contact method: Wako plastic sheet for tissue culture, resistant to toluene, (Wako Pure Chemical Industries, Ltd., catalogue No. 160-08893 or 164-08891)

2) Positive control materials and substance

There are two kinds of positive control materials; positive control material A, which shows moderate cytotoxicity, and positive control B, which shows weak cytotoxicity when tested in accordance with a procedure described in 4.4.3, and the followings are available (refer to 5.5 for a vendor). Use qualified materials.

Positive control material A: polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC)

Positive control material B: polyurethane film containing 0.25% zinc dibutyldithiocarbamate (ZDBC)

Positive control substance is a substance used to clarify sensitivity and accuracy of cells. The following is available.

Positive control substance: ZDBC (for example, Wako Pure Chemical Industries, Ltd., 1st grade)

3.3.3 Preparation of test solution

1) Cut a 1 g piece of a test sample into as small pieces as possible (approximately 2 x 15 mm). A sample piece not being cut into smaller pieces will be also tested for the test sample of which the surface is

specially coated.

2) Transfer a test sample cut into pieces to a sterilized glass container or plastic tubewith a screw caps, add 10 mL of culture medium (refer to 3.4.3, 4)) per 1 g test sample and loosely put a screw cap.
3) Confirm that pH of culture medium being between 7.2 and 7.4, and put the container or tube in a carbon dioxide incubator set at 37 ºC and incubate for total 24 hours under static condition.
4) Remove the test solution from a container. This solution will be regarded as 100% test solution.
5) Dilute the 100% test solution less than 3-fold using serial dilution with culture medium. This solution will be used as a test solution.
6) The test sample may be prepared with constant surface areas. In that case, make the virtual total surface areas of test and control samples taking into account the thickness as close as 6 cm^2/mL.
7) In a case an extraction condition not included in this test method is applied, thoroughly consider the condition in which the test material is used and select an appropriate extraction condition for safety evaluation on cytotoxicity. It is necessary to confirm that the extraction condition meet the conditions specified in 4.4.3.

3.4 Test method

3.4.1 Cell strain

Use one of the following cell strains. In a case that other cell strain or primary cell is used, detection sensitivity with the cell should be judged from positive and negative control materials and it should be confirmed that the cell strain possesses a certain level of detection sensitivity and accuracy (refer to 4.4.3).

(1) CCL1 (NCTC clone 929) (hereinafter referred to as “L929 cell”)
(2) CCL163 (Balb/3T3 clone A31) (hereinafter referred to as “Balb/3T3 cell”)
(3) JCR B 0603 (V79) (hereinafter referred to as “V79 cell”)

Cell to be used in the test should be checked for a negative mycoplasma infection and possession of good colony-forming ability because colony-forming ability differs depending on cell strains and incubation conditions.

3.4.2 Apparatus and equipment

(1) Carbon dioxide incubator (temperature 37±2 ºC, concentration of

carbon dioxide 5±1%)

(2) Phase-inverted microscope

(3) Stereoscopic microscope

(4) Low-speed centrifuge

(5) Flasks for tissue culture

(6) Petri dishes and plates for tissue culture

(7) Sterilized centrifuge tubes

(8) Sterilized test tubes

(9) Hemocytometer

(10) Clean bench

3.4.3 Reagents and a culture medium

1) Use regents suitable for cell culture.

2) Use an aseptic culture medium.

3) Prepare and store a culture medium and reagents in accordance with methods designated by vendors.

4) Use (1) MEM10 culture medium when using Balb/3T3 or L929 cell, use (2) M05 culture medium in of the test solution using V79 cell and use (1) MEM10 culture medium in a test using direct contact method. In a case that other culture medium is used or a component of other culture medium is added, detection sensitivity of toxicity in the test should be judged from positive control substance and materials and it should be confirmed that the culture medium or a component of the culture medium possesses a certain level of detection sensitivity and accuracy (refer to 4.4.3).

(1) MEM10 culture medium

Add L-glutamine (0.292 g/L), sodium hydrogen carbonate (2.2 g/L) and fetal bovine serum (10%v/v) to Eagle's Minimum Essential Medium (MEM) which includes Earle's balanced salt solution. Antibiotics may be added at concentrations that do not interfere with cells.

(2) M05 culture medium

Add MEM nonessential amino acids, sodium pyruvate (0.11 g/L), L-glutamine (0.292 g/L), sodium hydrogen carbonate (2.2 g/L) and fetal bovine serum (5%v/v) to Eagle's Minimum Essential Medium (MEM) which includes Earle's balanced salt solution. Antibiotics may be added at concentrations that do not interfere with cells.

5) Use phosphate buffers solution which does not include calcium or

magnesium [PBS (-)] as balanced salt solution.

6) Prepare trypsin solution by adding 0 to 0.02% EDTA and 0.05 to 0.25% trypsin (crystal) to balanced salt solution described in 5).
7) Use methanol or 10% formaline solution as a fixative.
8) Dilute Giemsa staining solution (commercial product) approximately 20-fold with phosphate buffer solution (M/15, pH 6.4) immediately before use.
9) Use distilled water with higher purity that is deionized and further diluted.

3.4.4 General cautions on operation

1) Conduct all procedures under aseptic conditions to avoid contamination with microbe. Do not use your mouth to pipette when transferring cells, culture medium and test solution.
2) Heat solutions and test samples up to approximately 37 ºC before contacting cells.
3) Wash all glass containers well with distilled water after cleaning with washing agent for tissue culture.
4) Clean up locations to be used in the test before starting procedures.
5) Record history of cells.
6) Check stored cells periodically for the presence and absence of mycoplasma infection.

3.4.5 Subculture of cells

1) Grow cells in a monolayer in a flask and remove culture medium when nearly confluent.
2) Wash cells once with balanced salt solution (refer to 3.4.3).
3) Discard balanced salt solution in a flask.
4) Add trypsin solution (refer to 3.4.3) (1 to 2 mL for 25 cm^2 flask).
5) Incubate the flask in a carbon dioxide incubator for 1 to 2 minutes at 37 ºC.
6) Add serum-containing culture medium and inactivate trypsin.
7) Transfer cell suspension to a centrifuge tube.
8) Centrifuge the tube for 2 minutes at 1000 rpm.
9) Discard the supernatant.
10) Add fresh culture medium (2 mL) to cells and prepare a uniform cell suspension. Implant the cell suspension in a new flask at most appropriate concentration or subculture ratio with the cell strain.
11) Add fresh culture medium (approximately 10 mL for 25 cm^2 flask).
12) Make culture medium changes and subcultures with appropriate intervals with the cell strain to be used.

13) Store a cell strain in culture media containing 5 to 10% dimethyl sulfoxide or glycerin as a freeze protectant and serum. A cell strain can be stored at -80 ºC or lower for a short term (approximately for a year) but should be stored in liquid nitrogen for a longer term.

3.4.6 Test operation

1) Treat subcultured cells with trypsin (refer to 3.4.5), prepare isolated cells and suspend them in culture media (refer to 3.4.3).
2) Seed cells so that 100 to 200 colonies will appear on a 60 mm petri dish (4 to 8 mL of culture medium), 50 to 100 colonies will appear on a 35 mm petri dish (1 to 3 mL of culture medium) and 40 to 50 colonies will appear on a 12 or 24-well plate (0.5 to 2 mL of culture medium).
3) Incubate petri dishes (or plates) in a carbon dioxide incubator under static condition for 4 to 24 hours at 37 ºC so that cells will adhere to the bottom of a petri dish.
4) Discard culture medium and add 100% test solution or test solutions at various concentrations prepared in accordance with a procedure described in 10.1 (4 to 8 mL to a 60 mm petri dish, 1 to 3 mL to a 35 mm petri dish, 1 to 2 mL to a 12-well plate and 0.5 to 1 mL to a 24-well plate).
5) Add test solutions of negative and positive control materials in the same manner as above.
6) Use 3 to 8 wells or 3 to 8 petri dishes for a test solution each at the same concentration.
7) Put petri dishes (or plates) in a carbon dioxide incubator immediately after adding test solutions and incubate under static condition.
8) Incubating period depends on a cell culture to be used. Incubate for 9 to 11 days with Balb/3T3 cell, 7 to 9 days with L929 cell and 6 to 7 days with V79 cell.
9) Discard culture medium after completion of incubation. Wash petri dishes (or plates) with balanced salt solution and add methanol or 10% formaline for fixation.
10) Add Giemsa staining solution after fixation (refer to 3.4.3) in order to stain colonies.
11) Discard staining solution after confirming that colonies are well stained, and count colonies in each petri dish (or well).
12) A colony counter can be used for a prompt judgment; however, in that case reliability of results obtained by the equipment such as accuracy should be validated.

13) Fixation or staining methods or microplate readers etc., that are different from those used in this test method can be used. In such cases, evaluation results of negative control material and positive control materials A and B should meet the conditions described in 4.4.3.

3.4.7 Observation

1) Count stained colonies in each petri dish (or well). Observe colonies with a stereoscopic microscope and colonies consisting with 50 cells or more will be counted.
2) Take petri dishes (or wells) incubated only with fresh culture media as a control group. Obtain colony-forming ability (number of colonies formed / number of cells seeded) from the number of cells seeded and the number of colonies actually formed in the control group. Take the mean number of colonies in the control group as 100%, indicate number of colonies formed by 100% test solution and test solutions at various concentrations (refer to 3.3.3) in percentage.
3) Plot the test results on a graph with its vertical axis indicating colony-forming ratios (take the mean number of colonies in the control group as 100%) and horizontal axis indicating concentrations of test solutions (log). Obtain the concentration (%) of test solution at which the number of colonies formed in the control group is inhibited to 50% and take it as IC50 (%).
4) It is also possible to calculate IC50 obtained by a theoretical formula of statistics using a computer.
5) Use the IC 50 value (%) as an indicator for a degree of cytotoxicity caused by the test solution of the test sample.

3.4.8 Evaluation

Cytotoxicity of a test sample can be fairly evaluated in tests meeting the following conditions.

1) Good colony-forming ability is confirmed in the control group.
2) The number of colonies formed in each petri dish (or well) with 100% test solution of the negative control material is similar to that in the control group.
3) In case a vehicle is used, the number of colonies formed in each petri dish (or well) with vehicle at the concentration used in the test is similar to that in the control group.
4) Investigate the degree of cytotoxicity (IC50) of the positive control substance (ZDBC) as necessary and use the value as a reference for

evaluation of detection sensitivity and accuracy of test system (refer to 5.4).

5) When test solutions of positive control materials A and B are extracted and tested in the same manner as a test sample, dose-response relationship is noted between concentration of the test solution and degree of colony-forming inhibition. Additionally, obtained IC50s of positive control materials A and B meet the following criteria (refer to 5.4).

   IC50 of positive control material A: less than 7%

   IC50 of positive control material B: less than 80%

3.5 Test report

The test report should include following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample
   (Example: name, manufacturer, serial number, materials, etc. of the medical devise)
4) Control materials used (negative and positive control materials and positive control substance)
5) Application method of the test sample in the test (sterilization procedure included, if applicable)
   (Example: weight and area of the sample, cutting procedure, sterilization procedure, etc.)
6) Preparation of test solution
7) Established cell strain used
8) Culture medium used (kind and amount of antibiotics used)
9) Colony-forming ability (number of colonies formed / number of cells seeded) in the control group with cells and culture medium used
10) Concentration of positive control substance (ZDBC) at which the number of colonies formed in the control group is inhibited by 50% [IC50 (μg/mL)]
11) Result of cytotoxicity test in test solution: table of individual data and calculated values (mean value and standard deviation) of test samples and negative and positive control materials, graph in which data is plotted and IC50s
12) Evaluation and discussion of results
13) Reference

4. Colony method using direct contact method

4.1 Objective

The objective of this test is to confirm cytotoxicity by directly contacting a test sample (final product or material) with seeded cells.

4.2 Test summary

A test sample will be adhered to the bottom of a petri dish, cells seeded on top, and colony-forming ability will be evaluated after incubation.

4.3 Test sample

1) Appropriately sterilize a test sample in accordance with the procedure described in 3.3.
2) Cut the test sample in a size that fit in one of a 60 mm or 35 mm petri dish or a 12- or 24-well plate. Prepare at least 4 circular or semicircular samples and weigh and measure the surface area.
3) Cut the negative control material and positive control material B in the same manner as the test sample.
4) Sterilize the test sample in accordance with an appropriate method for the usage objective of the test sample.
5) Closely contact the test sample, negative control material or positive control material B to a petri dish (or well).

4.4 Test method

4.4.1 Test operation

1) Use V79 cell and MEM10 culture medium (refer to 3.4.3).
2) Add 100 to 200 cells to a 60 mm petri dish (4 to 8 mL of culture medium), 50 to 100 cells to a 35 mm petri dish (2 to 3 mL of culture medium), 40 to 50 cells to a 12-well plate (1 to 2 mL of culture medium) and 40 to 50 cells to a 24-well plate (0.5 to 1.0 mL of culture medium).
3) Incubate petri dishes (or plates) in a carbon dioxide incubator under static condition for 6 to 7 days at 37 ºC.
4) Discard culture medium after completion of incubation. Wash petri dishes (or plates) with balanced salt solution and add a fixative appropriate for the test sample.
5) Add Giemsa staining solution after fixation in accordance with the procedure described in 4.3 in order to stain colonies.

6) Discard staining solution after confirming that colonies are well stained, and count colonies in each petri dish (or well).
7) Fixation or staining methods different from those used in this test method can be used unless the methods are officially approved in academic conferences and appropriate for cytotoxicity test for safety evaluation.

4.4.2 Observation

1) Take the number of colonies formed after directly seeding cells on petri dishes (or wells) as the control group and take the mean number of colonies in the control group as 100%.
2) Count number of colonies formed after directly seeding cells on test samples and obtain the ratio to the number of colonies in the control group (%).
3) Obtain colony-forming ratios (%) of negative control material and positive control material B.

4.4.3 Evaluation

1) Cytotoxicity of a test sample can be fairly evaluated in tests with direct contact method meeting the following conditions.
   Colony-forming ratio of negative control material: 80% or above
   Colony-forming ratio of positive control material B: 10% or below
2) Investigate a degree of cytotoxicity (IC50) of the positive control substance (ZDBC) as necessary and use the value as a reference for evaluation of detection sensitivity and accuracy of test system.
3) In case that colony-forming ratio being <30% after directly seeding cells on test samples and IC50 of the test solution being >100%, a test sample will be extracted for 72 hours and re-tested in accordance with a procedure described in 4.4.1. It is not necessary to repeat test procedure with the test solution obtained by 72-hour extraction if the cause of low colony-forming ratio can be specified.

4.5 Test report

The test report should include at least following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample (final product or material)
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Control material used (negative and positive control materials and positive

control substance)

5) Application method of the test sample in the test (sterilization procedure included if applicable)
   (Example: weight and area of the sample, cutting procedure, sterilization procedure, etc.)
6) Preparation of test solution
7) Established cell strain used
8) Culture media used (kind and amount of antibiotics used)
9) Colony-forming ability (number of colonies formed / number of cells seeded) in the control group with cells and culture media used
10) Concentration of positive control substance (ZDBC) at which the number of colonies formed in the control group is inhibited by 50% [IC50 (μg/mL)]
11) Result of cytotoxicity test in direct contact method: colony-forming ratios and micrograms (those that can identify a whole plate and individual colonies) in test samples and negative and positive control materials
12) Evaluation and discussion of results
13) Reference

5. Reference information

5.1 Position of cytotoxicity test

Cytotoxicity test is required for evaluation of products in all categories and play very important role in the biological toxicity evaluation of products.

The objective of this test system is to clarify the toxicity test results in animals in more simple experiment system, in cells. Primary or established cell strains have been used mainly as a measure to demonstrate emergence mechanism of toxicity. However, it is important to know that cell strains usually used in cytotoxicity tests have different sensitivity from cells that constitute human body and organs, and therefore results of tests may not necessarily correlate with in vivo adverse effects.

However, tests using cell cultures are expected as alternative methods to animal experiment in tests such as mucosal irritation test as these tests have advantages in aspects of sensitivity, economy and efficiency over in vivo tests. It is important to adopt new test methods to obtain data with high accuracy that are based on scientific justifications without sticking to conventional methods also in the field of biomaterials.

5.2 Characteristics of various cytotoxicity test methods

For cytotoxicity test of a medical material, there are methods to use the test solution of the material and methods to contact a material to cells indirectly or directly. There are two ways for a direct contact method, to put a material on cells or to seed cells on top of a material.

In the method putting a material on cells, there is a possibility of injury to cells by physical weight of a material. On the other hand, in the method seeding cells on top of a material, it is difficult to evaluate cytotoxicity in case the cells hardly adhere to the material to be tested. Even though each method has a defect as such, these test methods are exellent in the detection of toxicity unstable compounds such as peroxides because eluted component from the material immediately reacts will cells, and are generally considered to have high detection sensitivity of cytotoxicity.

In the test method by indirect contact of a material to cells, there are methods such as agar overlay method and its improved version, Millipore filter overlay method. In these methods, there exist agar or filter between cells and a material. Fat-soluble compounds are hardly diffuse and therefore the detection sensitivity turns out to be low, and the detection sensitivity of a filter method is comparable with or lower than the agar method or below that. Scoring is used for a judgment and still semi-quantitative. This method is effective for materials that polymerize in situ (example: composite resin); however, not appropriate for devices that have direct contact with eye mucosa because detection sensitivity of cytotoxicity is low and occasionally the result does not turn out to be positive even though the material shows irritation to eye mucosa.

The method to use the test solution extracted using the culture medium and is most popular one. The test solution extracted from the positive control B for 24 hours using non-serum containing culture medium at 6 cm^2/mL and 37 ºC is evaluated in accordance with the USP XXIV <87> Biological reactivity tests in vitro (hereinafter referred to as Elution Test), the result stands the test with score 2. When the test solution of the same material in 5 to 10% serum-containing culture medium is tested, the result does not stands the test with score 4. When the test solution of the positive control materials A and B extracted with distilled water at 6 cm^2/mL and 37 ºC for 24 hours are evaluated in accordance with the Elution Test, results of both test solutions show score 0 and cytotoxicity of materials can not be detected. When both positive control materials A and B were extracted with distilled water for 72 hours at 50 ºC, 24 hours at 70 ºC and 1 hour at 121 ºC and evaluated in accordance with the Elution Test, cytotoxicity could not be detected

in both positive control materials. Facts that substances such as oligomer and additives are hardly elute in distilled water or non serum-containing culture medium, and that some compounds are decomposed at high temperature are considered to be causes that cytotoxicity could not be detected. Therefore, solutions extracted with 5 to 10% serum-containing culture media will be used as the test solution for cytotoxicity tests. Detection sensitivity and accuracy depends on the density of cells and judgment methods used in the test. Guidelines in other countries adopt cells at subconfluent growth stage and a quantitative method to evaluate the uptake of the pigment (neutral red) by the living cells bymeasuring the absorbance spectrophotometrically and a semi-quantitative method to categorize a degree of cytotoxicity into scores 3 to 5 using gross observation of morphological changes of cells are used for indication of a degree of cytotoxicity. Based on the site of body in which the material would be used and the contact period, these methods can be adopted as long as the rationale for of the application of the method is established.

In this report, we adopted a colony method which has high detection sensitivity and in which results can be judged quantitatively without special counting apparatus, as the objective of the test is to evaluate the safety of medical devices.

5.3 Positive control material

Positive control materials and a positive control substance are adopted as indicators to judge appropriateness and detection sensitivity of the experiment system. Positive control material A, which shows moderate cytotoxicity and positive control material B, which shows weak cytotoxicity are adopted. The objective to use two kinds of positive control materials is (1) to obtain relative degree of cytotoxicity of the test sample by comparing with these positive control materials even when test method and cells used in the test are different and experimental room are changed during the test and (2) to estimate degree of tissue irritancy by relative degree of cytotoxicity.

5.4 IC50s of positive control materials and positive control substance

IC50 ranges of positive control materials and positive control substance when L929 cell, Balb/3T3 cell and V79 cell are used are shown below.

IC50 of a positive control substance (ZDBC) (μg/mL)

	L929 cell	Balb/3T3 cell	V79 cell

ZDBC	2.5 - 5.5	0.2 - 0.4	1.0 - 4.0*

IC50 of positive control materials A and B (%)

	L929 cell	Balb/3T3 cell	V79 cell
Positive control material A	2 - 5	2 - 6	1 - 3*
Positive control material B	50 - 60	15 - 25	50 - 60*

* IC50s of a positive control substance (ZDBC) and positive control materials A and B in V79 cells in MEM10 culture medium show weaker cytotoxicity than that in M05 culture medium (example: IC50 of ZDBC (μg/mL) 4 to 8 μg/mL, IC50 (%) of positive control material A 3 to 8% and IC50 (%) of positive control material B > 100%).

5.5 Vendor where negative and positive control materials were obtained

Hatano Research Institute, Food and Drug Safety Center
Reference Material Division
TEL: 0463-82-4751, FAX: 0463-82-9627
e-mail: RM.Office@fdc.or.jp

5.6 Correlation between the degree of cytotoxicity and tissue irritancy

The relationship between IC50 (%), which shows the degree of cytotoxicity and a degree of irritancy in various vital tissues is shown in Figure 1. IC50 values were obtained in a colony method using Balb 3T3 cell with the test solutions of control materials that contain ZDEC at various concentrations prepared in accordance with the method described in this report. Control materials were coated on contact lenses and tested with rabbit eyes, and reference materials were tested in implantation test in rabbits and patch test on healthy human skin, and the relationship between IC50 (%) and the degree of in vivo irritancy was elucidated. As a result, eye mucosa has the highest sensitivity among three and materials with IC50 (%) 35% or below caused eye irritancy. Inflammatory response was noted with materials with IC50 (%) 5% or below in muscular tissue. No dermal irritancy was noted in healthy skin even with materials with strong cytotoxicity with IC50 (%) of 0.1%. As shown above, differences in sensitivity among tissues can be revealed by using control materials.

Comparison between positive control materials	Intensity of cytotoxicity and expected biological response
Over 100%	No cytotoxicity or very weak cytotoxicity (see footnote)
Weaker than positive control material B	Weak cytotoxicity indicated Possibly raise irritancy against eye mucosa

Between positive control materials A and B	Moderate cytotoxicity indicated Inflammatory reaction may occur in mucosal tissue
Stronger than positive control material A	Strong cytotoxicity indicated. Inflammatory reaction may occur in muscular tissue

Irritancy against eye mucosa with Draize score below 4 rarely occurs. For example, when a contact lens material is extracted for 24 hours and evaluated in accordance with a colony method, IC50 was 100%; however, when it was extracted for 72 hours, IC50 was 89%. Mean Draize score of this material in lens wear test in rabbits was 3.2.

6. Reference

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Section 2 Sensitization Test

1. Scope of Application

This test is conducted to evaluate the potential of a medical device or its raw material to induce a delayed-type hypersensitivity reaction (sensitization). In this study, appropriate test and extraction methods were selected referring to "ISO 10993-10 Biological evaluation of medical devices Part 10: Tests for irritation and delayed-type hypersensitivity" and "Part 12: Sample preparation and reference materials. Maximization Test using an adjuvant and Adjuvant and Patch Test (also referred to as Scratched Skin Method)" are thus described as test methods with high detection power from the accumulated data to allow utilizing these tests effectively in safety evaluation.

The aim of this test is not to detect immediate-type hypersensitivity (antigenicity).

2. Cited References

2.1 ISO 10993-10 (1995) Biological evaluation of medical devices Part 10: Tests for irritation and sensitization

2.2 ISO 10993-10(2002) Biological evaluation of medical devices Part 10: Tests for irritation and delayed-type hypersensitivity

2.3 ISO 10993-12 (2002) Biological evaluation of medical devices Part 10: Sample preparation and reference materials

3. Selection of Test Sample and Test Methods

3.1 Principle

Maximization Test is the most sensitive method. When an extract of a test sample (final product or raw materials) can be dissolved or evenly dispersed in an appropriate solvent that can be used for intradermal administration (passes through an injection needle without causing flocking), a Maximization Test by intradermal administration is desirable, in principle. When the extract cannot be dissolved or dispersed in an appropriate solvent that can be used for intradermal administration (does not pass through an injection needle due to flocking), the Adjuvant and Patch Test should be conducted.

When the solvent used to dissolve or disperse the extract of a test sample exhibits potent systemic toxicity or local irritability, a test method must be selected considering its toxicity.

The biochemical and physicochemical properties of a test sample affect selection of a test method. Outline of the relationship between a test sample and selection of a test is shown as a flow chart in Figure 1. The details are shown below.

3.2 Preparation of Test Sample and Test Solution

3.2.1 Metals or Ceramics

Known findings shall be utilized for metals and ceramics (See 6.3). When the metal ion constituting a test sample has already been confirmed to have a sensitization potential by the Maximization Test, Adjuvant and Patch Test, etc., no further test is needed.

When the material contains metal element species with no sufficient data concerning the potential of sensitization, the sensitization potency shall be evaluated for an ion solution of the metal concerned by the ordinary Maximization Test or Adjuvant and Patch Test (See 6.2.3).

3.2.2 Substances Soluble in Water or Alcohol

For substances soluble in water or alcohol, a sensitization test shall be conducted by the Maximization Test after dissolving in water or an appropriate alcohol.

3.2.3 Low Molecular Weight Organic Compounds

For low molecular weight organic compounds, the sensitization test shall be conducted by the Maximization Test after dissolving or evenly dispersing in an appropriate solvent that does not affect the judgment of test results. Vegetable oils, dimethylsulfoxide (DMSO), and distilled water are suitably used as solvents. When the compound is insoluble in these solvents but soluble in acetone, however, it is also preferable to dissolve the compound in acetone and then evaporate acetone while mixing the resultant solution with a vegetable oil or DMSO.

3.2.4 Polymer Resins

For polymer resins, the sensitization test shall be conducted by the Maximization Test or Adjuvant and Patch Test using, as a test solution, a solution of the extract obtained using a solvent with the highest extraction efficiency (See 6.4). Regarding an extactant and preparation of a test solution, attention should be paid on the matters described blow. In addition, for medical devices intended for single use and transient contact (within 24 hours), when the test results of a solvent other than organic solvents are available, the risk may be assessed using these results

As an extractant, a solvent with the highest extraction efficiency should be selected from those described in ISO 10993-10 (1995) Annex B.2.10.

Methanol or acetone is generally employed as an organic solvent (See 6.5). When (1) the test sample is dissolved or deformed or degenerated at such a degree that its original form is lost in a solvent, or (2) when extraction with methanol or acetone does not provide a

sufficient amount of the extract, a mixture of cyclohexane and 2-propanol (1:1) can be employed as an alternative extractant, since organic chlorine-based solvents such as chloroform place large burden upon environment.

Extraction should be conducted by adding a solvent to the chopped test sample, at a volume 10 times as great as its weight, and stirring or agitating at a room temperature. Extraction should be continued for 24 hours or more.

The following 2 methods may be used for preparation of a test solution from an extract with an organic solvent (See 6.6).

Method 1

The solvent is removed from the extraction solution by evaporation using a rotary evaporator at a temperature as low as possible to obtain a residue, and the residue is dissolved or evenly dispersed in vegetable oil, dimethylsulfoxide (DMS), or distilled water to prepare the test solution. The test solution thus obtained is used for the sensitization test. When the residue is insoluble in these solvents but soluble in acetone, however, it is also preferable to dissolve the compound in acetone and then evaporate acetone while mixing the resultant solution with vegetable oil or DMSO. Concentration for local application is a critical factor in affecting the success of sensitization and the concentration of the test article is desirable to be as high as possible within the range not adversely affecting the results for hazard detection. Therefore, the concentration of the extract is generally on the level of 10%, and the basis for setting the concentration used in the test should be explained.

Method 2

The extract is concentrated or dried using a Kuderna-Danish concentrator (graduated) to prepare a solution containing the test sample at a concentration of 1 g/mL or the extractant is evaporated from the extract and then the appropriate solvent is added to make 1 mL. The solution thus prepared is used as the test solution for the sensitization test (See 6.8).

For both Methods 1 and 2, it is required to obtain the extraction efficiency, which is determined by direct measurement of the dried extract or in accordance to 6.5 of Part 3 Gene Toxicity Test.

4. Maximization Test

4.1 Test Method

4.1.1 Test Animals and Number of Animals

Healthy young white guinea pigs (generally 1-3 moths old) weighing about 400 g are used

(See 6.10). Both male and female animals can be employed; however, nonpregnant nulliparous animals should be used for females.

The number of animals should be at least 5 or 10 both for the test and control groups. In Japan, it is allowed to use at least 5 animals per group, if appropriate results are obtained. In foreign countries, however, at least 10 animals must be employed per group. When the potential of sensitization cannot be evaluated, measures such as rechallenge or increase in the number of animals are required. Animals should be allocated to groups in a random manner.

4.1.2 Sensitization

The following preparations shall be intradermally injected at a dose of 0.1 mL at 6 symmetrical sites as shown in Figure 2 on the clipped skin area (about 2 x 4 cm) of the upper blade bone of the guinea pig (See 6.11)

(a) Water in oil (W/O) emulsion of distilled water and Freund's complete adjuvant (FCA) at 1:1 (E-FCA)

(b) Test solution or control solution

(c) Emulsified mixture of the test solution (at a concentration 2 times that of the test solution in (b)) and FCA at 1:1

The positive control substance is required for comparison of the sensitivity of test animals and the potential of sensitization. The following substances are employed: p-phenylenediamine (CAS No.106-50-3), 1-chloro-2,4-dinitrobenzene (CAS No.97-00-7), potassium dichromate (CAS No.7778-50-9), neomycin sulfate (CAS No.1405-10-3), and nickel sulfate (CAS No.7786-81-4). Other sensitizing substances known from literature can be employed.

At week 1 after intradermal injection, sodium lauryl sulfate (10% in vaseline) is applied on the intradermal injection sites (clipped skin area on the upper blade bone). When the sample is irritable, this procedure can be omitted.

On the following day, sodium lauryl sulfate (10% in Vaseline) is wiped off, and 0.2 mL of the test solution (b) is closely patched at the same sites for 48 hours.

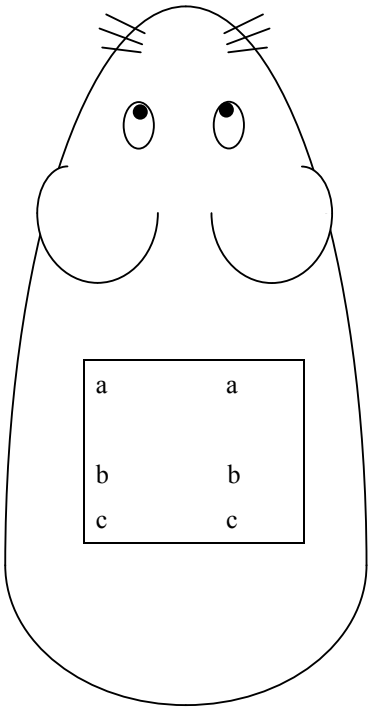

Figure 2 Sensitization sites for intradermal injection and patch

a, b and c show intradermal injection sites, and ▭ shows patched regions (2 cm × 4 cm).

4.1.3 Challenge

At week 2 after close patch, the sample solution prepared by dissolving or mixing the sample in an appropriate solvent or serially diluting it is applied on the clipped back or flank area. The highest concentration at which no irritability was shown in the preliminary test and concentrations obtained by serial dilution from the highest concentration are used. Each 0.1 mL of these solutions is applied on the skin of each guinea pig.

Application is conducted by close patch or open application. When a raw material chemical substance or metal material is tested and it is water soluble, an aqueous solution may be employed (See 6.12).

Since vegetable oils (olive oil, cottonseed oil, sesame oil, etc.) may exhibit irritability or sensitization potential, assessment should be made fully considering reactions etc., found in the negative control group.

4.1.4 Assessment of Skin Reactions

For close patch, the patch is removed at 24 hours later, and skin reactions are scored according to the general assessment criteria at 24 and 48 hours later and described as shown below. The general assessment criteria include Draize's criteria (See 6.13).

For open application, skin reactions are scored at 24, 48, and 72 hours later.

The concentration providing an average score of about 1.0 allows estimation of the approximate lowest sensitization concentration (See 6.14).

Table 1 Score of Skin Reactions by Draize's criteria

Erythema and escar formation		
No erythema	.....	0
Very slight erythema (barely perceptible)	.....	1
Well defined erythema	.....	2
Moderate to severe erythema	.....	3
Severe erythema to mild escar formation (injuries in depth)	.....	4
		[Highest score 4]
Oedema formation	.....	
No oedema	.....	0
Very slight oedema (barely perceptible)	.....	1
Slight oedema (edges are well defined by definite raising	.....	2
Moderate oedema (raised approximately 1 mm)	.....	3
Severe oedema (Raised 1 mm or more and extending beyond area of exposure)	.....	4
		[Highest score 4]
		[Highest summed score for erythema/escar and oedema 8]

4.2 Study Report

The study report should include at least the following items:

1) Test facility and test manager

2) Test period

3) Factors to identify the test sample (medical device or its raw materials)

(Examples: Name of medical device, name of manufacturer, manufacturing number, names of raw materials)

4) Control Substance (positive control substance) used

(Examples: Name of control substance, supplier, manufacturing number, etc.)

5) Method of preparation of the test solution

6) Species and strain, number, age in weeks, and sex of test animals

7) Test method

8) Individual body weight at the start and completion of the experiment

9) Results for skin reactions for individual animals and summary table

10) Evaluation of results and discussion

11) References

Summary tables for the results of scoring should be prepared so that challenge concentrations, positive rates, average scores are easily understood as exemplified in the following tables.

(Example of summary table for test results)

Example of Summary Table for Method 1 (Extraction rate: 0.5%)

Sensitization concentration	Challenge concentration (%)	Observation timing (hr)*1	Evaluation	
			Positive rate*2	Average score*3
5%	5	24	100	3.1
		48	100	4.0
	0.5	24	80	1.5
		48	90	2.0
	0.05	24	20	0.2
		48	20	0.2
	0.005	24	0	0
		48	0	0
	0	24	0	0
		48	0	0

*1 Observation timings are 24 and 48 hours later.

*2 (Number of positive animals/total number of animals in the group) × 100

*3 Total reaction score by Draize criteria, etc. for the group/total number of animals

Example of Summary Table for Method 2(Extraction rate: 0.15%)

Sensitization concentration	Dilution rate of test solution for challenge	Observation timing (hr)*1	Evaluation	
			Positive rate *2	Average score *3
1 (0.15%)	1	24	100	3.6
		48	100	3.2
	1/2	24	100	2.2
		48	100	2.0
	1/4	24	100	1.2
		48	100	1.0
	1/8	24	100	1.0
		48	0	0
	1/16	24	0	0
		48	0	0

*1 Observation timings are 24 and 48 hours later.

*2 (Number of positive animals/total number of animals in the group) x 100

*3 Total reaction score by Draize criteria, etc. for the group/total number of animals

5. Adjuvant and Patch Test

5.1 Test Method

5.1.1 Sensitization

1) E-FAC shown in 4.1.1(a) is injected intradermally at a dose of 0.1 mL on 4 corners of the clipped skin area (about 1 x 4 cm) of the upper blade bone of the guinea pig.
2) #-shaped injury is made using an injection needle on the E-FCA injection sites. About 0.1 ml of a sample is closely patched for 24 hours.
   When a test solution in a volatile organic solvent is used, open application is allowed.
3) Procedure in (2) is repeated 3 times, once daily.
4) At week 1 after the start of sensitization, sodium lauryl sulfate (10% in vaseline) is applied on the intradermal injection sites (clipped skin area on the upper blade bone).
5) On the following day, sodium lauryl sulfate (10% in Vaseline) is wiped off, and 0.2 mL of the test solution (b) is closely patched at the same sites for 48 hours. 5.1.2 Challenge

Challenge is conducted at week 2 after application as shown in 4.1.2.

5.1.3 Evaluation

After challenge, evaluation is made in according to 4.1.3.

5.2 Study Report

A study report is prepared in accordance to 4.2.

6. Reference Information

6.1 Background

The purpose of the sensitization test on new medical devices and medical materials is to predict, at the preclinical stage, the risk of contact hypersensitivity of chemical substances released from them in humans. When the potential of sensitization is observed in appropriate tests, it is necessary to reduce the risk of contact hypersensitivity in humans by evaluating the potency of the sensitizing substance in advance and taking adequate measures.

Factors affecting development of contact hypersensitivity include (1) sensitization potency of a chemical substance, (2) its concentration in a product and dissolution property, (3) usage

form of a product (use frequency and contact duration, etc.), and (4) predisposition of the user. Among these, (1) and (2) can be detected prior to application to humans by the sensitization test in animals and chemical analysis, respectively.

Here, the skin sensitization test design using an adjuvant in guinea pigs that can determine the MR1 inducing concentration is introduced. The use of this method allows predicting not only the presence of sensitization potential but also the lowest sensitization concentration based on the MR1 inducing concentration upon positive results. Thus, a standard for controlling exposure amount in humans can be obtained. The content of the sensitizing substance in the test sample is estimated from the amount of the extract, allowing prediction of the risk in humans.

Here, the following points are amended for the "Guidelines for Basic Biological Tests for Medical Devices and Medical Materials" (Yakuki No. 99 dated June 27, 1995, hereinafter referred to as the previous guideline," and it is considered helpful for appropriate hazard detection of sensitization and risk assessment.

1) A flow chart for the selection of tests is shown.
2) A material or a product soluble in water or alcohols is dissolved and subjected to testing.
3) Two organic solvents were used for extraction conventionally, but it is decide that one solvent with the highest extraction efficiency is to be employed.
4) For Adjuvant and Patch Test, a test using a crushed product is deleted, and a test method using the extracted test solution is described.
5) Two methods using organic solvent extraction are introduced.

6.2 Test Method

Skin sensitization test can be systematically classified into 2 methods. One is an adjuvant method using Freund complete adjuvant (FCA), an immunostimulant, in combination to promote hypersensitivity induction, and another is a non-adjuvant method not using an adjuvant. The adjuvant method is conducted to enhance sensitivity to sensitization in animals so that substances with low sensitization potential can be detected and thus to detect the potential risk in humans with high sensitivity. Sato et al., compared adjuvant methods (Maximization Test and Adjuvant and Patch Test) and non-adjuvant methods (Buehler Test and Open Epicutaneous Test). They reported that the adjuvant methods were more sensitive than the non-adjuvant tests and that when the sensitization concentration was fixed and a challenge concentration is varied and the degree of skin reaction was scored, positive sensitization reaction could be detected depending on a challenge concentration (See Reference 2). There are also many reports stating that test methods using FCA are more

sensitive. Among these, many reports state that the Maximization Test is the best (See References 3-8). In addition, "ISO 10993-10 Biological evaluation of medical devices Part 10: Tests for irritation and sensitization" also recommends Maximization Tests as the most sensitive method.

Although many researchers recognize that the Maximization Test is excellent, the method has defects as described below. Since the sample is injected intradermally to induce sensitization, it is difficult to directly apply crystalline powder or metal materials insoluble in water or FCA and polymer resins. In addition, since preparation of water in oil (W/O) emulsion of the sample is required, sensitization concentrations of the sample are limited. Adjuvant and Patch Test is a modified method of the Maximization Test, in which all intradermal injections of the sensitizing substance are replaced by subcutaneous application, and it is said to have high detection sensitivity (See Reference 2). However, “a comparison using the same eluate from the material gave lower score in the Adjuvant and Patch Test than in the Maximization Test.”

Based on the above, the Maximization Test is the first choice as the test method, and the Adjuvant and Patch Test is recommended when the Maximization Test cannot be applied even with some modification.

6.3 Sensitization Test of Inorganic Materials

Sensitization with inorganic materials such as metal materials and ceramics in human relies on the metal ion incorporated in the body through corrosion or abrasion of or elution from the material. Sensitization with Ni, Cr, Cu, Hg, and Co in humans is well known, but sensitization may rarely occur with Fe, Al, Ti, Zn, or Pt. The sensitization potency thus differs depending on metal ions. Another factor to determine the sensitization potential of a material (See Reference 9) is elution of the metal ion from the material (including that caused by abrasion and corrosion) (See Reference 10). However, it is currently difficult to predict *in vivo* long-term fate of the material or simulate elution, corrosion, or abrasion. A sensitization test following extraction with a physiological saline solution or buffer is meaningless, since the circumstance is far from simulation of the *in vivo* reaction. This can be easily understood from the fact that although no positive result is obtained by testing Ni-Cr alloys with such a procedure, a large number of sensitized cases in humans are reported.

On the above premise, it is considered appropriate to conduct a sensitization test of inorganic materials using a metal-iron aqueous solution at a concentration of 10-25% (or the highest soluble concentration or the maximum non-irritable concentration) with a pH as close to neutral as possible.

6.4 Reasons for Selecting Organic Solvent Extraction for Polymer Devices

The products that caused allergic contact dermatitis in humans and the residual amounts of causal substances in these products are shown in the table below (See Reference 1). Supposing that the extraction efficiency is 100% when these products are tested in accordance to ISO 10993-10 (1995) B.2.10, (a) and (b) and extracted with 10 mL physiological saline solution or a vegetable oil per 1 g of a material, the concentrations of PCPHs extracted from a pullover in a solvent are 0.6 to 0.8 ppm and the concentration of emulsion of the PCPHs (FAC : test sample = 1:1) upon intradermal injection in the sensitization test (Maximum Test) is 0.3 to 0.4. When similar calculation is made on other compounds, the sensitization concentrations were equal to or lower than the lowest sensitization concentration obtained in Reference 1 for all compounds other than IPPD. This suggests that when tested in accordance to ISO 10993-10 (1995) B.2.10, (a) and (b), since the concentration of the sensitization substance in the test solution is equal to or lower than the lowest sensitization concentration and thus sensitization cannot be established in an animal experiment, it is highly probable that the substance is judged to be negative. Therefore, in order to detect a trace amount of an unknown sensitizing substance in the test sample by an animal experiment, it is effective to extract with an organic solvent that has high extraction efficiency and can be concentrated and use the concentrate for the experiment. Attention should be paid, on the other hand, on that sensitivity may not be detected after extraction with an organic solvent, since extraction with an organic solvent is accompanied by an operation to remove the organic solvent and thus volatile components such as monomers may be lost during the concentration process.

A comparison of the residual amounts in the product of an accident case with the lowest sensitization concentrations, the residual amounts exceeded the lowest sensitization concentrations for all substances except Naphthol-AS. This means that sensitization potential can be detected even after the amount of the extract with an organic solvent is recovered to the original amount of the sample. In other words, when 1 g of the sample is extracted with 10 mL of an organic solvent and the extract is concentrated to 1 mL, supposing the extraction efficiency is 100%, the concentration of the sensitizing substance in the test solution is equal to its concentration in the product. This method allows the conduct of the Maximization Test with a small amount of the test sample, regardless of extraction efficiency (amount of an extract).

Unlike these cases (products of frequent and long-term contact), medical devices for single and transient contact (within 24 hours) are likely to have low sensitization risk, and risk management is easy even when delayed type hypersensitivity is induced. When sensitization tests using a physiological saline solution and cottonseed oil have already been conducted and the result is negative, it is considered that the product has no strong sensitization

potential. Sensitivity of the medical device concerned is thus evaluable.

Causal substance	Residual amount in a product (ppm)	Accident product	Amount of a compound in emulsified solution (ppm)	Lowest sensitization concentration (ppm)
1) PCPHs	6 - 8	Pullover	0.3 - 0.4	1
2) DPTU	100-200	Tape	5-10	20
3) MBT	678	Rubber boots	33.9	500
4) IPPD	5, 000-6, 000	Rubber pad	250-300	10
5) MBTS	285	Rubber boots	14.3	100
6) Naphthol-AS	4, 600	Night cloth	230	10, 000

6.5 Selection of Extractant for Test Sample

This report recommends methanol or acetone as an extractant. The initial draft of the previous guideline described that 3 extractants should be selected from water, methanol, acetone, chloroform, and hexane, because it is empirically known that the optimum solvent depends on the combination of the sensitizing substance and the material containing the substance. However, since it makes the procedure complicated and obscure, methanol is also useful for extraction of water-soluble sensitizing substances, and it is sufficient to use acetone for extraction of most apolar sensitizing substances, the previous guideline recommended the 2 solvents. Subsequent accumulation of various cases has clarified that the sensitization potency of the material can be evaluated by extraction with one appropriate solvent. It is thus described herein that one solvent with the highest extraction efficiency (including mixtures) should be used.

6.6 Matters to Be Considered in Selection of Preparation Method for Test Solution from Organic-solvent Extract

Methods 1 and 2 are available for preparation of a test solution from an organic-solvent extract for polymers and elastomers. It is necessary to understand the characteristics of the respective methods in risk assessment and hazard detection and then select a preparation method.

Method 1 is a more desirable test method, and especially excellent when hazard must be detected, such as when new raw materials with no biological safety information for implant medical devices are tested, since the concentration of the extract in the test solution can be adjusted and hazard upon exposure to a high concentration can be detected. However, the test

cannot be conducted unless a certain amount of an extract is obtained.

Method 2 can be conducted regardless of the amount of the extract, and it is useful when a sufficient amount of the extract cannot be obtained by Method 1 or for risk assessment for small-sized devices. When no sensitization potential is detected by this method, it is considered that there is at least no large risk in clinical practice. However, hazard is not always detected, and when weak sensitization potential is observed, the sensitization potency cannot be evaluated so that risk assessment is. In this case, some modifications such as an increase in concentration rate of an extract from the test sample (for example, the volume of the test solution is adjusted to 0.1 mL/g test sample) may allow quantitative risk assessment. Especially, large-size frequently-used devices may sometimes require such modifications.

6.7 Solvent for Preparation of Test Solution

Solvents for preparation of the test solution should be selected to solubilize the extract and enhance permeability.

It is known for the sensitization test that detection sensitivity is higher when the test sample is dissolved before administration. Water, vegetable oils (such as olive oil, cottonseed oil, sesame oil), DMSO, and acetone are generally employed. For DMSO and acetone, reduction in sensitivity is expected due to the occurrence of necrosis by intradermal injection. However, when the effect is limited to a very local area and produces no systemic toxicity, sensitivity is frequently elevated when a substance is dissolved and then administered.

6.8 Preparation of Test Solution

Many accident cases occur when the content Y (weight/weight %) of the sensitizing substance in product is larger than the lowest sensitization concentration LD (weight/volume %) obtained in an animal experiment. In other words, assuming that 100% of the sensitizing substance in the product can be extracted, the result of an animal experiment would be positive for a product with a risk of sensitization when the content Y (weight/weight %) of the sensitizing substance in product is equal to or higher than the lowest sensitization concentration LD (weight/volume %). That is to say, no positive result is obtained, unless the final test solution volume (for example, V mL) is equal to or larger than the amount of collected sample (for example, W g). The sample is usually extracted with 10 volumes of the solvent. Thus, when the extract is used as it is, the above conditions are not satisfied. In such a case, the extract must be concentrated 10 fold. The method to prepare a test solution from A g of a material in accordance to Method 2 is exemplified below. The extract obtained from A g of the material is weighed and an extraction efficiency is recorded. The sample is extracted with 10 mL of a solvent several times, and the extracts are combined. The combined extracts are concentrated to the final volume of A mL using a Kuderna-Danish concentrator to prepare a test solution, which is then used for sensitization. At the administration site (C), the extract from 2 x A g of the material is concentrated to the final volume of A mL, since the final concentration must be twice that of the test solution.

6.9 Amount of Test Sample Required for Test

When the test is conducted using conventional extract obtained by Method 1, even 5 kg of a sample may be required in rare cases. There is a critic that the situation may sometimes deviate from the actual practice. From accumulated cases, it is judged that the risk can be evaluated also by Method 2. In the present amendment, this test is thus also adopted. A test

by Method 2 can be conducted on each 10 animals in test and control groups using an extract obtained from 7 g of the test sample, in calculation. A specific test method considering losses, etc., during operation processes is shown in Figure 3.

6.10 Test Animals

Animals with high sensitivity are selected as test animals, in principle.

Guinea pigs are employed for the Maximization Test and Adjuvant and Patch Test. Guinea pigs are selected mainly for the following reasons: They exhibit excellent sensitivity in sensitization reaction; erythema and oedema formation can be visually observed; it is known that reactions to various chemicals are similar to those in humans; and rich background data has been accumulated. Body weight of an animal is an important factor. It is desirable to use healthy young white guinea pigs (generally 1-3 months old) weighing 400 mg at the start of experiment, since handling of very small animals is difficult and reactivity reduces in very large animals (600 g or more). Both male and female animals can be used, and nonpregnant nulliparous animals should be used for females.

6.11 Sensitization Induction

A volume of intradermal administration at each site is 0.05 mL in the Magnusson and Kligman's Maximization Test, while 0.1 mL is adopted in the OECE guideline, and ISO, BS, and drug toxicity test guidelines. The important point for deciding the intradermal dose is that the sensitizing substance to be detected is sufficiently administered and detected correctly, rather than that an ulcer is formed at the administration site. It is desirable to determine an intradermal dose referring the matters described in 2.2.2.

An administration concentration is an important factor for establishment of sensitization. Two-fold concentrated solution of test solution (b) must be prepared to obtain the final concentration the same as that of test solution (b) at administration site (c). When it is difficult to prepare this solution, test solution (b) may inevitably be employed. When a solvent for test solution (c) is vegetable oil or DMSO and it is impossible to emulsify using a mixture with FCA (1:1), an appropriate amount of distilled water or physiological saline solution may be added for emulsification.

Since ethanol may induce contact hypersensitivity, it is better to avoid using ethanol in the step of sensitization induction (See References 11-12).

An important point in the sensitization test is to contact with a sufficient amount of a test article during the period of sensitization induction. An excessively small amount does not provide information about allergic property of the substance. When the dose for intradermal administration is very small due to flocking or local irritability, sufficient induction treatment

cannot always be conducted. Thus treatment to compensate the insufficiency shall be added. For example, treatment similar to the Adjuvant and Patch Test is given at the intradermal administration site.

It is important for challenge to set an appropriate negative control group and compare the results of skin reactions, since it is known that a threshold against stimulation falls in the method using an adjuvant.

6.12 Application Method for Test Article

Since it is desirable to apply a completely dissolved sample to promote induction of challenge reaction, a water-base system must be used in the case where the sample is soluble only in water-base system. However, aqueous solution has low affinity to the skin, and thus it is desirable to dissolve the sample in the solvent with higher affinity to the skin, such as vaseline. For example, it is known that, in challenge reaction with metal salt that is generally considered to be water soluble, challenge reaction is more easily detectable with a metal salt solution containing ethanol than with an aqueous solution of metal salt due to a higher permeation to the skin (References 13, 14). It is necessary to understand that solubility of the test article and skin permeability of the test solution is different phenomena and it is necessary to select an appropriate solvent. Since vaseline is rarely contaminated with an irritable or sensitizing substance, it is necessary to try various vaselines and select one that does not induce a skin reaction.

Challenge is conducted by close patch of the sample as in the patch test in humans. Considering that contact hypersensitivity actually induced on human skin is frequently by open contact, close patch is not considered necessary at the level of challenge. In addition, it is necessary to fix the body of the guinea pig by winding an elastic bandage for close patch. Thus, if a small lint cloth with a diameter of 0.8 cm is used, the cloth can be applied on the back at utmost 4 points. On the other hand, open application advantageously allows challenge application at 6 sites or more on the back symmetrical from the midline. In close patch, skin reactions may difficult to be judged due to the effect of bandage, etc. As solvents for open application, volatile solvents with no primary irritation are preferable for prevention of enlargement of applied site. Ethanol and acetone can be mentioned as solvents satisfying theses conditions.

It is preferable to apply test solutions of various concentrations to one sensitized animal at challenge. Since a largest dilution rate to allow a positive reaction can be judged at one time, a lot of information can be obtained together and sensitization condition can be correctly grasped. It is premise that a certain level of sensitized condition of the animal continues, but it is difficult to ensure it. Thus, it is better to conduct challenge simultaneously as far as

possible.

6.13 Scoring Criteria for Skin Reactions

When guinea pigs are employed, since erythema due to vasodilation is easily distinguishable from oedema due to promoted vascular permeability, criteria for assessment of skin reactions often rely on the combination of the severity of erythema and oedema formation. This report exemplifies Draize's criteria.

6.14 Evaluation of Sensitization Potency

This report recommends that the sensitization potency is comprehensively evaluated by determining 3 parameters: (1) mean score for skin reactions, (2) lowest sensitization concentration, and (3) an MR1 inducing concentration.

The mean score for skin reactions is obtained by scoring the severity of skin reactions (erythema and oedema) and dividing a total score by the number of animals used and represents the severity of skin inflammation (See Reference 2).

The lowest sensitization concentration represents the lowest concentration at which sensitization is observed. Although it can be obtained experimentally, the study scale becomes enormous and thus its conduct is unpractical. It has been shown that the lowest sensitization concentration is almost the same level as the MR1 inducing concentration (the lowest inducing concentration for the mean score for skin reactions of about 1.0) in the highest sensitization concentration group (See Reference 1). Thus, it is possible to estimate an approximate lowest sensitization concentration from the MR1 inducing concentration. Multiple solutions diluted with an appropriate common ratio are applied on the back of the guinea pig for challenge, and skin reactions at the application sites are observed at 48 hours later to obtain an MR1 inducing concentration at which a positive reaction is observed. When dose relationship is observed between the inducing concentration and mean score for skin reactions, the approximate lowest sensitization concentration can be predicted from the MR1 inducing concentration. Attention should be paid that the lowest sensitization concentration cannot be predicted depending on physicochemical properties (skin permeability, binding property with epidermal protein) of a chemical substance.

The bases for setting "the mean score for skin reactions of about 1.0" for the MR1 inducing concentration are as follows: (1) At the mean score for skin reactions of 1.0, positive reaction is observed in almost all animals; (2) Mean scores for skin reactions are almost the same as the positive rate up to the positive rate (sensitization rate) of 60%, but at a positive rate exceeding 60%, the mean score for skin reactions exceeds the positive rate (sensitization rate), and thus the mean score for skin reactions represents sensitization

potency more accurately (See Reference 1).

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Section 3 Genotoxicity Study

1. Scope

The objective of this study is to evaluate the genotoxicity of medical devices or raw materials.

In ISO 10993-3 "Biological evaluation of medical devices Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity", tests to investigate the effects on DNA damage are also included in addition to gene mutation and chromosomal aberration tests. Here however, reverse mutation test with bacteria and chromosomal aberration test with cells in culture or mouse lymphoma TK test will basically be performed without essential requirement of tests at DNA levels based on the consistency with the "Guidelines for Toxicity Studies of Drugs" in Japan and with the "Guidelines for Toxicity Studies" in the Law concerning the Examination and Regulation of Manufacture etc. of Chemical Substances. However, in case of a positive test result or depending on the duration and condition of use of the medical device or raw material, testing other systems should be considered (see the section 6.2).

2. Reference Standards

2.1 Guidelines for Toxicity Studies of Drugs

2.2 Guidelines for Toxicity Studies in the Law concerning the Examination and Regulation of Manufacture etc. of Chemical Substances

2.3 OECD Guidelines: 471, 473 (476)

2.4 ISO 10993-3 "Biological evaluation of medical devices Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity"

3. Application of the study

3.1 If the safety of the raw material chemical substances and inactive ingredients (excipients) etc. contained in the test sample (final product or raw material) concerning genotoxicity has been confirmed and it is unlikely that any unknown substance is produced by interactions of the contained raw material chemical substances etc., it is not necessary to perform the study with the final product or raw material. However, the validity must be demonstrated.

3.2 If no data is available in the literature, the following tests should be performed according to one of the guidelines shown in the Reference Standards (see the section

6.2).

1) Reverse mutation test with bacteria
2) Chromosomal aberration test with cells (or mouse lymphoma TK test) (see the section 6.3)

4. Preparation of the test fluid

The test sample (final product or raw material) will be classified into the followings according to the data whether extracts can be obtained or not considering its characteristics such as material, physical properties and solubility, and the test fluid applied for the test will be prepared.

① Test sample which is insoluble in water and from which extracts can be obtained with organic solvent

② Test sample which is insoluble in water and from which extracts cannot be obtained even with organic solvent

③ Test sample which is soluble or suspendable in water

4.1 In case of organic material

4.1.1 In case of test sample which is soluble or suspendable in water

In case of test sample which is soluble or suspendable in water, it will be dissolved or suspended in a solvent (water or culture fluid) to perform the test.

4.1.2 In case of test sample which is neither soluble nor suspendable in water

In case of test sample which is neither soluble nor suspendable in water, it will be checked for the extraction ratio with methanol and acetone (see the sections 6.4 and 6.5).

If extracts can be obtained with methanol or acetone (see the section 6.6), solvent with the higher extraction ratio will be employed. Chopped test sample will be added with the solvent at a volume of 10 times its weight and then extracted while stirring at room temperature for 24 hr (see the section 5.6). The solvent will be evaporated by an evaporator to obtain the extracts at an amount required for the test. The amount of extracts obtained from the sample will be recorded. Each test will be performed using the obtained extracts.

If extracts cannot be obtained either with methanol or acetone (see the section 6.7), DMSO extracts will be used for reverse mutation test and culture medium extracts for chromosomal aberration test or mouse lymphoma TK test according to the following methods 1) and 2).

1) In case of reverse mutation test

   The test sample will be chopped and each 0.2 g will be added with 1 mL of DMSO (or the test sample of each 6 cm^2 will be added with 1 mL of the solvent) and then extracted while shaking at 37 °C for 48 hr, and the extraction fluid of 100 μL per plate at the maximum will be added to perform the reverse mutation test.

2) In case of chromosomal aberration test (or mouse lymphoma TK test)

   The test sample will be chopped and each 0.2 g will be added with 1 mL of the culture medium employed in the test (including serum) (or the test sample of each 6 cm^2 will be added with 1 mL of the culture medium) and then extracted at 37 °C for 48 hr, and the extraction fluid will be used to perform the test.

4.2 In case of inorganic material

In case of inorganic material such as metal or ceramics, what should be investigated is the genotoxicity due to metal ions in most cases. On the premise of this matter, genotoxicity study of inorganic material will be performed as follows.

1) If it has been demonstrated in the literature or previous studies that the genotoxicity of the ions of metal elements that constitute the material is negative, it is not necessary to perform the study.
2) If the genotoxicity data concerning the constituting metal elements is insufficient, the study will be performed with the solution of the representative metal ion.

5. Study report

The study report must include the following items at least.

1) Testing organization (facility) and study director
2) Study period
3) Factors specifying the test sample (final product or raw material)
   (e.g.: name of medical device, name of manufacturer, serial number, name of raw material, etc.)
4) Factors specifying the control fluid if any
   (e.g.: name of the control fluid, supplier, serial number, etc.)
5) Preparation method for the test fluid
   (e.g.: extraction method with solvent and extraction ratio, sterilization method, etc.)
6) Testing methods
7) Results
   If necessary, tables, figures and photographs should be attached.
8) Evaluation of the results and discussion
9) References

6. Reference information

6.1 Background

Genotoxicity test is a test to detect genotoxic substances that cause gene mutation and chromosomal aberration at levels of cells and individuals deriving from DNA damage developed in a single cell. Effects of genotoxic substances differ in the expression of disorders depending on whether the damage is developed in the somatic cells or in the reproductive cells in the living body. If DNA damage has been developed in the somatic cells of various tissues, it may cause cancer. Therefore, genotoxicity test plays a role of a short-term screening test for carcinogenic substances. On the other hand, when DNA damage has been developed in the reproductive cells in the living body such as eggs and sperm, although most of the cells with damage undergo the selection during the process of development of reproductive cells and embryos, there is a risk that the gene mutation and chromosomal aberration may be transferred to the next generation. Besides, in case pregnant mother has received the exposure and DNA damage has been developed in the fetal somatic cells, a risk of newborn with malformation and physical disturbance also arises.

As described above, since genotoxic substances act on DNA to develop cancer or to exert genetic effects on the next generation, it is desirable that medical devices do not affect the living body resulting in no genotoxicity in either long-term or short-term use conditions.

6.2 Selection of the testing method

In the present report, it is described that it is desirable to perform 2 tests, i.e., reverse mutation test with bacteria (*Salmonella typhimurium* and *Escherichia coli*) and chromosomal aberration test with mammalian cells in culture (or mouse lymphoma TK test) according to the Guidelines for Toxicity Studies of Drugs, Guidelines for Toxicity Studies in the Law concerning the Examination and Regulation of Manufacture etc. of Chemical Substances, OECD Guidelines, etc. These tests can detect the major events of genotoxicity, i.e., induction of mutation and chromosomal aberration. In case of a positive test result or depending on the duration and condition of use of the medical device, testing other systems such as those described below should also be considered. In ISO 10993-3 "Biological evaluation of medical devices – Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity", tests to investigate the effects on DNA damage are regarded as essential in addition to gene mutation and chromosomal aberration tests. However in the present report, tests at DNA levels are not regarded as essential based on the consistency with the "Guidelines for Toxicity Studies of Drugs" in Japan and with the "Guidelines for Toxicity Studies" in the Law concerning the Examination and Regulation of Manufacture etc. of Chemical Substances.

1) System to detect DNA damage
   * Unscheduled DNA synthesis (UDS) test with mammalian hepatocytes (OECD Guidelines 486)
   * Single cell gel (SCG) electrophoresis test (Comet test)
2) System to detect chromosomal aberration
   * *In-vitro* micronucleus test with cultured cells
   * Micronucleus test in mammalian animals (OECD Guidelines 474)
   * Spermatogonial chromosomal aberration test in mammalian animals (OECD Guidelines 483)
3) System to detect malignant alteration (canceration) of cells
   * Transformation test with BALB 3T3 cells

6.3 Outline of chromosomal aberration test

Chromosomal aberration test will be performed according to the following flow in all the Guidelines for Toxicity Studies of Drugs, Guidelines for Toxicity Studies in the Law concerning the Examination and Regulation of Manufacture etc. of Chemical Substances and OECD Guidelines. It takes time especially in the analysis of chromosomal aberration since a lot of cells are analyzed.

① As the test cells, Chinese hamster-derived CHL/IU cells and CHO cells, and human lymphocytes are often used.

② First, in order to determine the maximum concentration to apply in this test, cell growth inhibition test is performed in the presence and absence of S9 by the metabolic activation method with various treatment times to investigate the growth inhibition concentration of the test substance in each condition.

③ A positive control group and a negative control group are prepared for each treatment group consisting of 3 concentrations per series.

④ In the short-time treatment condition, 2 series consisting of presence and absence of S9 mix are prepared, and after treatment for 3 to 6 hr, culture is conducted for another 21 to

18 hr. If a negative result is obtained in the short-time treatment, additional test with continuous treatment for at least 1.5 times the normal cell cycle is performed.

6.4 Extraction solvent

As an extraction solvent to obtain extracts from the material, 2 solvents, i.e., methanol to extract mainly water-soluble substances and acetone to extract fat-soluble substances are mentioned. This is a combination to obtain a lot of substances as far as possible. In the extraction of test sample shown in ISO 10993-3 "Biological evaluation of medical devices – Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity", use of 2 solvent systems consisting of physiological solvent and organic solvent is recommended.

6.5 Extraction ratio

If no data concerning how much methanol and acetone extracts can be obtained is available, the extraction ratio should be determined by either method. The operation of the test differs depending on whether the extraction ratio is at least 0.5% (or 1%) or below 0.5% (or 1%). Accordingly, the protocol should be prepared based on the data of the extraction ratio. There are several methods to investigate the extraction ratio, and it can be determined according to the following procedures by way of example (ISO 10340, Optical and optical instruments – Contact lenses – Method for determining the extractable substances from contact lenses, 1995).

1) Desiccate the test sample at 60 ± 5 °C until it reach a constant weight, in a vacuum desiccator if possible. Return it to room temperature in a desiccator.
2) Confirm that the test sample weighs at least 200 mg as a sample for extraction.
3) Weigh the desiccated test sample accurately to ± 0.1 mg and record the weighed value (m1).
4) Put the test sample in a cylinder filter for extraction and put boiling stones in Soxhlet flask if necessary, and then add an appropriate solvent up to approximately 70% of the flask capacity. Attach the cylinder filter to Soxhlet extractor, assemble the flask and cooler and then set up them on a mantle heater.
5) Extract the test sample for at least 4 hr.
6) Pick up the test sample from the cylinder filter after cooling down. Desiccate the test sample until it reaches a constant weight according to the procedure 1).
7) Weigh the desiccated test sample to ± 0.1 mg and record the weighed value (m2).
8) Calculate the value (m1 – m2) / m1 × 100 as the extraction ratio.

6.6 Criteria for "a case extracts can be obtained"

Taking the medical device weight of 0.5 g as a standard, the limit values to obtain extracts are set as follows.

"A case extracts can be obtained" is generally a case where the amount of extracts obtained from the test sample is at least 0.5% of the weight of the test sample (in case the medical device weighs at least 0.5 g) or at least 1% of the weight of the test sample (in case the medical device weighs less than 0.5 g).

The limit values for extraction ratio, i.e., 0.5% (in case the medical device weighs at least 0.5 g) and 1% (in case the medical device weighs less than 0.5 g) have been established based on the amount of the test sample to obtain necessary amount of extracts for the test.

6.7 Criteria for "a case extracts cannot be obtained"

"A case extracts cannot be obtained" is generally a case where the amount of extracts obtained from the test sample is less than 0.5% of the weight of the test sample (in case the medical device weighs at least 0.5 g) or less than 1% of the weight of the test sample (in case the medical device weighs less than 0.5 g). In case the material is dissolved in the solvent or deformed beyond recognition, it is judged that extracts cannot be obtained.

If the test sample is expensive, it may sometimes be difficult to obtain necessary amount of extracts for the test in practice. In such a case, although the operation is performed according to the section 3.1.3, the judgment is made according to the same criteria. Instead of operation according to the section 3.1.3, it is also acceptable to conduct tests with the raw material chemical substances (monomers and inactive ingredients (excipients)) contained in the raw material in combination with quantitation of the amount of eluted raw material chemical substances from the test sample followed by evaluation.

6.8 Amount of extracts

The amount of extraction residues required for a genotoxicity study using extracts is, as a rough indication although it depends on the protocol, at least 1 g for reverse mutation test and approximately 2 g for chromosomal aberration test. Extraction is conducted by adding 10-volume of solvent to the material followed by stirring for a basic extraction time of 24 hr. Extraction may be conducted for a longer time depending on the circumstances.

6.9 Evaluation

If a positive result has been obtained in the genotoxicity study, the safety should be evaluated considering all the various conditions such as intensity and concentration-dependency of toxicity, type of solvent used for extraction and extraction ratio, and contact site of the medical device and contact period. Based on the importance of genotoxicity, additional conduct of other genotoxicity studies including *in-vivo* studies will contribute to the risk assessment concerning the safety in humans.

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Section 4 Implantation Test

1. Scope of application

This test is to grossly or histologically evaluate local effects of a medical device or material to be implanted in vivo by animal experiments. This test will be conducted in order to estimate troubles caused by a material, such as a tissue response induced by the material and its intensity. Therefore, it is necessary to conduct a test separately regarding troubles caused by the design and function of the device (refer to 5.2.1).

Examples of subcutaneous, intramusclar and intraosseous implantation methods are shown in ISO 10993-6: Biological evaluation of medical devices Part 6: Tests for local effects after implantation, and it is indicated in this section to adjust the duration of implantation in accordance with the duration of application of a medical device. For medical devices to be implanted subcutaneously or intraosseously, or materials of medical device to be implanted over a month, studies should be selected in accordance with the implant site and contact duration referring to ISO 10993-6.

2. Standard quoted

2.1 ISO 10993-6 (1994): Biological evaluation of medical devices Part 6: Tests for local effects after implantation

2.2 USP25: BIOLOGICAL REACTIVITY TESTS, IN VIVO Implantation Test

3. Short-term intramuscular implantation test

3.1 Objective

The objective of this test is to evaluate a tissue response induced by an implant material and the intensity by observing tissue response appeared in surrounding tissues of an implanted subject at the early stage after implantation of a test sample (medical device or material) in soft tissues.

Exclusion by absorption/phagocytosis, organization and loculation are general types of biological reaction against a foreign substance. Test samples to be tested in this test are mainly solid samples, and it is expected that materials easy to be absorbed *in vivo* will be phagocytized by macrophages and material difficult to be absorbed will be loculated by fibers. Both are series of biological responses that initiate with infiltration of inflammatory cells and followed by processes of granulation, locutation, etc., and it is considered that these responses will be modofied (such as retardation, acceleration or enhancement) by the kind of implanted material. This test method is to

histologically investigate the effect of a material and to evaluate tissue toxicity of the material. Non-solid sample such as paste, should be evaluated in accordance with ISO 10993-6 3.2.2.

3.2 Test material

3.2.1 Test animal

Use more 4 or more male rabbits weighing 2.5 kg or more per observation period.

Use healthy animal with no medical history and with matured paravertebral muscle, an implant site of a test sample. Do not use animals previously used in other studies and possibly sensitized with any chemical substances.

Effective numbers of animals for the final evaluation is 2 or more for gross observation and 2 or more for histological examination per observation period. Clip the hair from dorsal regions before implant of a test material.

3.2.2 Preparation of test sample

3.2.2.1 Size of test sample

It is desirable to prepare columnar test sample strips with length 10 to 12 mm and width 1.0 to 1.5 mm so that the samples can be implanted by an injection needle with the gauge around 15. However, a test sample with its size different from a standard described above can be used with an rationale in case it is impossible to prepare a test sample in the standard size described above because of the material, shape or size of the product, or in case the size of the final product is narrower or thinner than the standard size described above and a tissue response far different from that may appear in the clinical use is expected if the product is tested after re-shaped in the standard size. Special attention should be paid in case the size or shape of a test sample is greatly different from that of a control sample because the difference may affect the type and range of the tissue response.

An appropriate implant method should be developed when implantation by an injection needle and a stylet is difficult because of the size or shape of the test sample. For example, samples like a string,

such as a loop of an intraocular lens, should be implanted with an injection needle with a smaller gauge, and large samples should be implanted by a surgical operation. In such cases, it should be considered in the evaluation that tissue response differs from that in a negative control sample.

3.2.2.2 Shape of test sample

Columnar shape with its both ends rounded is an ideal shape.

It is desirable to make the surface of a test sample smooth because an acute angle on the surface may physically injure tissues and the "size of inflammation regions", which will be explained later, tends to be narrower at the region of the sharp angles. Shapes of test samples to be used in the same test should be made as similar as possible because it is known that the shape of an implanted test sample affects the intensity of the surrounding tissue response. A test sample can be tested in an appropriate shape that does not interfere with the test procedure when the final product cannot be prepared in the standard size. However, the final evaluation is assumed to be very difficult in a case that shapes of test and control samples are very different, therefore, a careful evaluation considering the difference in types of tissue response and kinds and numbers of infiltrated cells based on the shape of the test sample is required (reference 5, 6).

3.2.2.3 Sterilization method

A non-sterilized test sample should be sterilized after preparation in accordance with an appropriate sterilization method considering the characteristic of the test sample before use (refer to 4.3). Prepare a negative control sample in the same manner as a test sample. When necessary, prepare a positive control sample in the same manner as a test sample.

3.2.2.4 Control materials

Use high-density polyethylene or an equivalent product as a negative control material. An appropriate control material (for example SUS316) can be used when available in such a case that a test sample is a metal.

Use polyurethane containing zinc diethyldithiocarbamate (ZDEC) as a

positive control material. Use a positive control sample when necessary in order to confirm the positive response (refer to 4.4).

3.3 Test method

3.3.1 Implantation method

Insert samples separately in a sterilized injection needle with gauge 15. Animals should be anesthetized because implantation procedure may become difficult by agitation of animals or cramp of muscles at implantation site and accordingly surrounding tissues may be unnecessarily injured. Implant 4 strips of test samples in the unilateral side of the paravertebral muscle approximately 3.5 cm away from spinal column with approximately 2.5 cm interval using a stylet. Implant 2 strips of a negative control sample in the other side of the paravertebral muscle in the same manner as the test sample. Maximum 4 strips of a sample can be implanted on the unilateral side of the paravertebral muscle. There is extra space for 2 more strips on the side in which a negative control sample is implanted, therefore, it can be used to implant a positive control sample or for a re-implantation due to excessive bleeding, etc.. The number of strips to be implanted can be increased at the test planning.

Try to keep the direction, angle and depth of samples consistent at implantation not only because it will make it easier to find the location of samples at explantation, but also because these may affect measurement of "size of inflammation". Keep an injection needle parallel to the spinal column and introduce it into the muscle with a constant angle to the skin surface. Depth can be kept consistent by marking needles at the same points from tips. Marking the location in which a needle is introduced will make explantation and excision easier.

It is impossible to implant samples without causing bleeding or tissue injury; however, histological examination will be difficult because of excessive inflammatory response around samples in case excessive bleeding is noted or surrounding tissues are

injured more than necessary. In such case, it is better to re-implant samples in different sites.

When a test sample is a soft material, there is a possibility that the sample bends in a muscle and the measurement of "width of inflammation" become difficult. It is important not to squeeze samples into muscle after introducing a needle into a muscle but to draw a needle out carefully. It is difficult to implant a test sample extremely thin and soft, like a suture, under constant conditions by method using an injection needle and a stylet. As a counter-measure thereto, the implantation method using a suture needle for surgery can be adopted instead; however, there is a high risk of causing nonspecific tissue injuries by the suture needle, especially like bleeding and necrosis of muscle, and by this a fair evaluation of the test sample may be interfered. Therefore, implantation to other sites such as subcutaneous tissues should be considered.

3.3.2 Implant duration

Test samples will be implanted basically for 1 or 4 weeks. Other implant duration may be set in accordance with the planned clinical duration or duration recommended in ISO 10993-6: Biological evaluation of medical devices Part 6: Tests for local effects after implantation, as long as there is an adequate justification.

3.3.3 Animal care

During the in-life portion of the test, animals will be observed individually and abnormal findings will be recorded. Animals that died before scheduled date of sacrifice will be necropsied immediately for confirmation of the cause of death. When the cause of death is considered to be related to the implanted test sample, the animal will be included in the evaluation. When the cause of death is considered to be something other than the implanted sample, the animal will be excluded from the evaluation. The test will be regarded to be effective when numbers of animals for the final evaluation is 2 or more for gross observation and 2 or more for histological examination. When the effective number of animals cannot be obtained, the test should be re-conducted to make up the shortage.

3.3.4 Sacrifice of animals and collection of samples

Allocate animals so that 2 or more animals can be obtained for each of gross observation and histological examination per observation period. The animals will be anesthetized and euthanized by exsanguination and muscular tissues will be excised with explanted samples after 1 or 4 weeks of implantation.

Samples will be explanted with paravertebral muscles. Tissue injury at explantation should be limited to minimal in order not to interfere with a histological examination.

3.3.5 Gross observation

Surrounding tissues and test samples will be observed grossly or by using a magnifying glass and the following items will be recorded.

1) Hemorrhage, loculation and discoloration in surrounding tissues
   Describe its degree, area and thickness when abnormalities noted.
2) Discoloration or degeneration of the sample
   Describe its color, cracks and hardness when abnormalities noted.
3) All the other abnormalities noted
   Using the following evaluation criteria stated in the United States Pharmacopoeia (USP25) is one of the judgment methods that can be adopted.

Evaluation criteria of coating in an implantation test

Coating thickness	Score
No coating	0
Up to 0.5mm	1
0.6 to 1.0mm	2
1.1 to 2.0mm	3
Over 2.0mm	4

3.3.6 Histological examination

3.3.6.1 Preparation of histological specimens

Muscular tissues removed for a histological examination will be placed in a fixative immediately for a preparation of histopathological specimens. Generally, tissue samples will be fixed in 10% buffered formalin and trimmed after completion of fixation so that a cross-section of a sample can be observed. Samples will

be paraffin-embedded, sectioned by a microtome, hematoxylin-eosin stained and histologically examined. Other fixation, embedding or staining methods can be used when necessary to the extent that may not adversary affect the final evaluation.

Fixed tissues will be trimmed by several millimeters so that a cross-section of a sample can be observed on the histological specimen. When trimming tissues, it will make it easier to confirm the embedded direction of a test sample on the tissue specimen and to select the site to measure "the width of inflammation regions" if you make the shape of the trimmed face rectangular in which the embedded direction becomes the major axis.

In case a test sample is soluble in organic solvents, a test sample will not interrupt a procedure of sectioning as it dissolves during preparation of specimens. In case a test sample is soft and can be sectioned by a microtome, sectioning a test sample with surrounding tissues will make less injury to surrounding tissues. In case a test sample is hard, its forced sectioning may injure the surrounding tissues, therefore it is necessary to remove a test sample before sectioning. It is important to prepare specimens in a good condition as possible considering the characteristic of a test sample.

In case a test sample is hard and cannot be sectioned by a microtome, it is better to remove it before sectioning. However, attention should be paid when removing a test sample so that surrounding tissues will not be injured. Surrounding coatings may be also removed if a test sample is roughly removed and it will make a histological examination impossible. As for a test sample , the details of *in vivo* degradation status can be found by preparing specimens without removing a test sample. A test sample such as a ceramic cannot be removed because it will be fixed by fiber components; however, it cannot be sectioned by a microtome as it is too hard. In such a case, polished specimens using resin-embedding should be prepared.

3.3.6.2 Measurement of "width of inflammation"

Tissues surrounding a test sample show a slight inflammatory change even

with a negative control sample. Measurement of "width of inflammation" enables a quantitative evaluation of toxicity of a test sample against surrounding tissues. The measurement of the width of inflammation can be omitted when impossible with an adequate rationale. For example, the width of inflammation cannot be measured with a test sample degradable *in vivo* because the shape of the test sample will not be maintained due to phagocytosis, and with a porous test sample because of invasion of fibers or fibroblasts.

The "width of inflammation" will be obtained by calculating the mean "width of inflammation" after measuring the width of inflammation between the test sample and surrounding tissues vertically from the major axis of a test sample microscopically using a micrometer at several points. An image analysis system can be used.

Width of inflammation will normally be noted with uniform width around a test sample when the effect of a test sample against surrounding tissues is uniform. However, if there exist fibrous connective tissues that extend vertically against a test sample in muscular fibers around the inflammatory region, the inflammatory region tends to expand in the direction of the fibrous connective tissues. Also, the inflammatory region tends to extend in the direction of muscle contraction and show spindle shape. Select appropriate measuring points based on the shape of the test sample considering these characteristics so that an actual mean width of inflammation can be obtained.

3.3.6.3 Histopathological observation

Observe surrounding tissues of a test sample by a light microscope, and record kinds and degrees of appearance of inflammatory cells and other abnormal findings noted (refer to 4.5). For example, components of coatings and its condition, hyperplasia of fibroblasts, infiltration of pseudo-eosinophil (neutrophils), lymphocytes, plasmacytes, macrophages and giant cells, necrosis and fatty infiltration will be observed and evaluated. Infiltration of inflammatory cells and inflammatory response tend to expand in the direction of fibrous connective tissues in

muscular fibers and tend to extend in the direction of muscle contraction and show spindle shape. Record findings considering these characteristics.

3.4 Evaluation

After gross observation and histological examination, the result will be judged positive when tissue response against a test sample in all animals is significantly stronger than that against a negative control sample (refer to 4.6) in either 1 or 4-week observation period.

A gross observation reveals spreading of response as a whole and a histological examination reveals the kind of cells that cause the response noted in gross observation. Weak tissue response may be perceived only by histological examination and focal response may be perceived only by gross observation. Therefore, results from gross observation must be considered in the evaluation of results from histological examination.

There is a rare case that an individual animal has very high sensitivity (response such as cell infiltration is observed even with a negative control sample) and this makes evaluation difficult. In such a case, the animal should be excluded from evaluation and supplimented by the other animal. However, data from the animal excluded from the evaluation should be included in the report.

Kinds and degrees of appearance of inflammatory cells noted in surrounding tissues of a test sample and the "width of inflammation" both are important indices of histological examination. It is desirable that a pathologist integrally evaluates tissue findings as they can be important factors for judgment in case significant differences are noted in a type of tissue response when compared with a negative control sample.

3.5 Test report

The test report should include at least following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample (final product or material)
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Control materials used (Example: name of the control material, vendor, date of acquisition, serial number and etc.)
5) Preparation methods of test and control samples
   (Example: cutting, extraction, sterilization, size and etc.)
6) Test methods (implantation and explantation methods of samples, animal care

conditions and preparation method of histopathological specimens)

7) Test results

Results from gross observation of test samples and surrounding tissues of test samples
Results from histological examination of surrounding tissues of test samples
Measurement results of width of inflammation
Photographs of representative gross and histological findings

8) Evaluation and discussion of results

9) Reference

4. Reference information

4.1 Background

Several histological examination methods have been tested so far regarding the evaluation of a medical device or material by intramuscular implantation in laboratory animals. J.E Turner *et al.* tried to examine degrees of various tissue response finings noted after implantation of test samples and judge a degree of toxicity of a test sample by integral evaluation of those results (reference 1). M. Therin *et al.* try to judge biocompatibility of the test sample from cell density and the condition of distribution after computer image analysis of information on various cell components that appear around a test sample after implantation of the test sample (reference 2). Either evaluation method requires complicated procedures and data on various indices are obtained; however, it seem that most of those indices do not necessarily reflect the intensity of tissue toxicity of the test sample and it is considered important to select appropriate indices for evaluation from among those.

A. Nakamura *et al.* conducted a 7-day intramuscular implantation test in rabbits and investigated the correlation between various tissue response indices and *in vitro* cytotoxicity test results and contents of toxic compounds in samples (references 3 and 4). As a result, indices that showed good correlation with cytotoxicity results were "width of inflammation around a test sample", "a degree of residue of degenerated/necrotic muscular fibrous cells in inflammatory region" and "a degree of infiltration of inflammatory cells in surrounding muscular fibers", and they were all findings that reflect the intensity of inflammatory response after sample implantation. Objective scores were provided to each index of tissue response and correlation between the total score and results from *in vitro* cytotoxicity test was examined, however not good correlation was obtained. Form results above, indices reflecting a degree of inflammatory response and suggesting a correlation with results from cytotoxicity test were considered to be effective for the evaluation of tissue toxicity. Among these

indices, “width of inflammation around the test sample” was adopted as an index for tissue toxicity evaluation in a short-term intramuscular implantation test as the index indicates a degree of inflammatory response quantitatively and has advantages in aspects of simplicity, objectivity and correlation. However, “width of inflammation around the test sample” is an index to perceive inflammatory response caused by a sample implantation quantitatively, and integrated judgment considering tissue findings that suggest qualitative information on inflammatory response, such as existence of macrophages and kinds and amount of other inflammatory infiltrating cells, are required for the final evaluation.

4.2 Selection of test method

ISO 10993-6 (1994): Biological evaluation of medical devices Part 6: Tests for local effects after implantation includes methods for an implantation test. Following the ISO standard is basically enough when testing materials of implantable medical devices. However, it is better to use not only histological evaluation but also quantitative indices such as width of inflammation in intramuscular implantation studies because the width of inflammation have good correlation between cytotoxicity, as reported by Nakamura *et al*.

A short-term intramuscular implantation test described in this report is almost equivalent to the method to evaluate a short-term local effect in intramuscular implantation test in ISO 10993-6. According to ISO, an appropriate site for implantation should be selected based on the implant material, and subcutaneous, intramusclar and intraosseous implants are given as examples, and it is also indicated in this section to change the duration of implant in accordance with the duration of application of a medical device. We also support this way of thinking in Japan and there is no problem to change the test method in accordance with the location and duration of application of the medical device.

4.3 Sterilization method

Attention should be paid for degeneration or deformation of test samples in case test samples are sterilized by heat, by autoclaving, dry-heat sterilization or boiling water sterilization. In case ethylene oxide gas is used for sterilization, attention should be paid to avoid residue of gas. Alcohol immersion is not an appropriate sterilization method in this test because in a lot of cases a compound in a test sample elutes in alcohol and actual toxicity cannot be detected (reference 7).

Additionally, there is a sterilization method using irradiation of □- or ultraviolet ray; however, attention should be paid because irradiation may cause degradation or deterioration of samples. Including methods described in other sections, all

sterilization methods have advantages and disadvantages. Regardless of a sterilization method selected, changes that may cause tissue response different from that of the final product applied for human body should not happen with test and control samples by degradation or deterioration of the material or residue or absorption of gas or chemical compound by the sterilization selected for the test. An appropriate sterilization method should be selected by thoroughly considering a characteristic of the material and sterilization method used for the clinical application.

4.4 Positive material

Polyurethane sheets containing zinc diethyldithiocarbamate (ZDEC), one of causative substances of toxicity of natural rubber products, at various concentrations were prepared and the positive material sample was selected from those in accordance with the content of ZDEC and results from a test on correlation between "width of inflammation" in an intramuscular implantation test in rabbits and *in vitro* cytotoxicity test (reference 8). Negative and positive control samples are available from Hatano Research Institute, Food and Drug Safety Center.

4.5 Changes in histology based on implantation duration

When tissue response was chronologically examined using a positive control sample, the peak with maximum "width of inflammation" in most of cases corresponds to peak of inflammatory cell infiltration such as pseudo-eosinophils (neutrophils), and it seem that the width become narrower as tissue response accelerates to granulation and to formation of fibrous coating by scarring. It is considered that the peak of inflammatory cell infiltration differs in accordance with absolute amount of toxic substance contained in samples, rate of elution and degree of toxicity. Therefore, the measurement site is conventionally described as "width of inflammation", but with a substance with weak tissue toxicity 1 week after implantation is considered to be a stage where infiltration of cells (mainly macrophages and fibroblasts) shifts to granulation, and 4 weeks after implantation is considered to be a stage where fibrous coating is formed and actually thickness of fibrous coating will be mainly measured 4 weeks after implantation.

4.6 Tissue response

"Significantly strong tissue response" does not only mean a judgment using a statistical method, but a case in which a broader "width of inflammation" was noted when compared to that with a control sample, or strong inflammatory or tissue toxicity changes such as degenerated/necrotic changes are noted also can be considered as "significantly strong tissue response". Regarding the "width of inflammation", it is

expected to be difficult to distinguish a slight difference between a test and control sample, and therefore it is considered that using statistical method is a counter-measure to bring objectivity to judgment. In evaluation of histology, it is important to consider time courses as inflammation is not a static reaction but it is a dynamic change in which kinds of circulatory disorder and infiltrated cells and intensity of reaction may change with passage of time. Two observation periods, 1 and 4 weeks are set in a short-term intramuscular implantation test in order to determine effects of a test sample on biological response from a broader perspective. Therefore, information from either observation period should not be emphasized but the tissue response should be evaluated integrally based on information from both observation periods.

5. References

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2) M. Therin, P. Christel, and A. Meunier, J. Biomed. Mater. Res., 28, 1267-1276 (1994).
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4) Y. Ikarashi, K. Toyoda, N. Ohsawa, T. Uchima, T. Tsuchiya, M. -A. Kaniwa, M. Sato, M. Takahashi, and A. Nakamura, J. Biomed. Mater. Res., 26, 339-356 (1992).
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6) B.F. Matlaga, L.P. Yasenchak, T.N. Salthouse, J. Biomed. Mater. Res., 10, 391-397 (1976).
7) T. Bouet, K. Toyoda, Y. Ikarashi, T. Uchima, A. Nakamura, T. Tsuchiya, M. Takahashi, and R. Eloy, J. Biomed. Mater. Res., 25, 1507-1521 (1991).
8) T. Tsuchiya, Y. Ikarashi, H. Hata, K. Toyoda, M. Takahashi, T. Uchima, N. Tanaka, T. Sasaki, and A. Nakamura, J. Applied Biomat., 4, 153-156 (1993).

Section 5 Irritation Test

1. Scope of application

This test is to evaluate tissue toxicity and irritancy of extracts of a medical device or material. Standard methods for skin irritation, intracutaneous response and eye irritation studies are described in this report. Select an appropriate irritation test based on the expected clinical application site of the medical device. Oral mucosa irritation or vaginal mucosa irritation test can be selected as they are also included in ISO 10993-10. It is not necessary to conduct an irritation test in a case there are test results from studies conducted in accordance with test standards described in section 2 "Standard quoted".

2. Standard quoted

2.1 ISO10993-10 Biological evaluation of medical devices Part 10－Tests for irritation and sensitization

2.2 ASTM Standard F749-87 Standard Practice for Evaluating Material Extracts by Intracutaneous Injection in the Rabbit

2.3 USP25 Biological Reactivity Tests, InVivo.－Intracutaneous Test

2.4 ASTM Standard F719-81 Standard Practice for Testing Biomaterials in Rabbits for Primary Skin Irritation.

3. Intracutaneous response test

3.1 Objective

The objective of this test is to confirm tissue toxicity or inducibility of inflammation of extracts (hereinafter referred to as "test solution") extracted from a test sample (final product or material) after intracutaneous administration.

3.2 Test summary

Test solutions extracted from test samples using physiological saline or vegetable oil will be administered intracutaneously to dorsal regions of 3 rabbits to evaluate tissue toxicity and inducibility of inflammation by observation of administration sites until 72 hours of administration.

3.3 Preparation of test solution

3.3.1 Extraction vehicle

Use physiological saline (Japanese Pharmacopoeia or equivalent products) and vegetable oil (cotton seed oil or sesame oil, Japanese Pharmacopoeia or equivalent products) for extraction.

3.3.2 Ratio of vehicle and test sample

Basically follow standards shown in Attachment 1.1.

3.3.3 Extract condition

Select the highest temperature condition with which the test sample can tolerate among conditions shown in Attachment 1.2.

The highest temperature condition with which the test sample can tolerate should meet the followings.

(1) Extraction temperature is lower than the melting point of a test sample.
(2) A test sample does not degrade at extraction temperature.
(3) Eluted substance does not evaporate or degrade at the extraction temperature.

3.3.4 Operation method

Cool down the test solution immediately to room temperature (not more than 20 ºC) and shake well. Transfer the solution to a sterilized dry container under aseptic condition and store it at 20 to 30 ºC. Use the solution within 24 hours.

3.3.5 Preparation of control solution

Treat a vehicle (without test sample) under the same condition as test solution preparation and use it as a control solution.

3.4 Test method

3.4.1 Test animal

Use healthy rabbits in good nutritional state (refer to 6.2). Body weight and age will not be specified. Animals should be maintained under specific conditions with a specific food for 1 week or longer before initiation of test.

Clip (or shave) the hair from a dorsal region by the day before administration to make administration and observation easier

(refer to 6.3).

3.4.2 Dose level

Basically administer 0.2 mL test solution at 1 administration site.

3.4.3 Administration route and period

Administer intracutaneously to dorsal regions once.

3.4.4 Administration site

Administer test and control solutions prepared by 2 kinds of vehicles (five injections for each solution, total 20 injections) on both sides of the spinal column (10 injections on each side) (refer to figure 1 for example).

3.4.5 Observation

Observe and record conditions of injection sites of all animals before and approximately 24, 48 and 72 hours after administration in accordance with Table 1 (Scale for scoring skin response according to Draize).

3.4.6 Evaluation

Evaluate tissue toxicity and inducibility of inflammation from observation results (refer to 6.4).

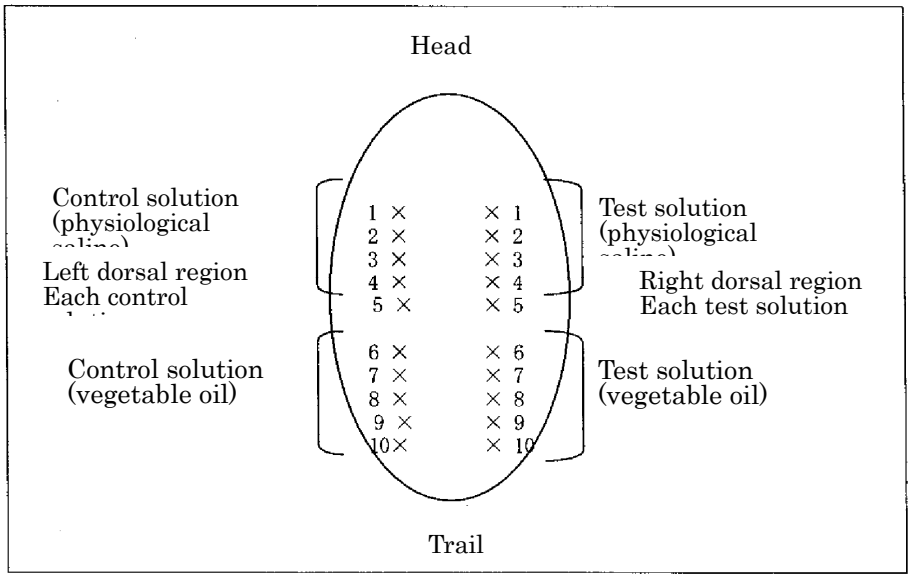

Figure 1 Administration site (example)

Table 1　Scale for scoring skin response according to Draize

Formation of erythema and crust	
No erythema	0
Very slight erythema (barely notable)	1
Clear erythema	2
Moderate to severe erythema	3
Sever erythema to slight crust formation (up to injury at the deep portion of skin)	4
Formation of edema	
No edema	0
Very slight edema (barely notable)	1
Slight edema (Clear margin identified by definite swelling)	2
Moderate edema (Approximately 1 mm swelling)	3
Severe edema (Swelling 1 mm or larger and spreading of edema over exposed area)	4

3.5　Test report

The test report should include at least following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Factors to identify the control sample when used (Example: name, vendor, serial number, etc.)
5) Preparation method of test solution
6) Species, strain, number, age and sex of animals
7) Test method
8) Test results
   Table:　Individual animal weights at initiation and completion of test
   Skin response results of individual animals (score points)
   Photograph: Conditions of administration sites
   (only representatives allowed).
9) Evaluation and discussion of results
10) Reference

4. Skin irritation test

4.1 Objective

The objective of this test is to confirm whether substances that possess skin irritancy exist in extracts (hereinafter referred to as “test solution”) extracted from a test sample (final product or material).

4.2 Test summary

Test solutions extracted from test samples using physiological saline or vegetable oil will be applied on dorsal regions either abraded or not abraded (6 rabbits for each vehicle) to evaluate irritancy.

4.3 Preparation of test solution

Follow the procedure described in 3.3.

4.4 Test method

4.4.1 Test animal

Use healthy rabbits in good nutritional state (refer to 6.2). Body weight and age will not be specified. Animals should be maintained under specific conditions with a specific food for 1 week or longer before initiation of test.

Clip (or shave) the hair from a dorsal region by the day before administration to make administration and observation easier (refer to 6.3).

4.4.2 Dose level

Basically administer 0.5 mL test solution at 1 administration site.

4.4.3 Administration route and period

Apply on dorsal regions once.

4.4.4 Administration site

Divide a dorsal region into 4 regions, top and bottom and right and left portions (refer to figure 2 for example). Prepare abrasion with a shape of # (approximately 2.5cm x 2.5cm) on a stratum corneum of epidermis (be careful not to damage corium) in 2 of 4 regions by using a sterilized surgical knife with vertical angle to epidermis. Top 2 regions will not be abraded. Apply the

test solution (0.5 mL each) on one abraded area and one non-abraded area and cover each area with a sterilized gauze pad (2.5cm x 2.5cm, 4 gauze pads in one package) and a tape. Then fix the dorsal area by covering with a polyethylene film. Follow the same procedure for control solutions.

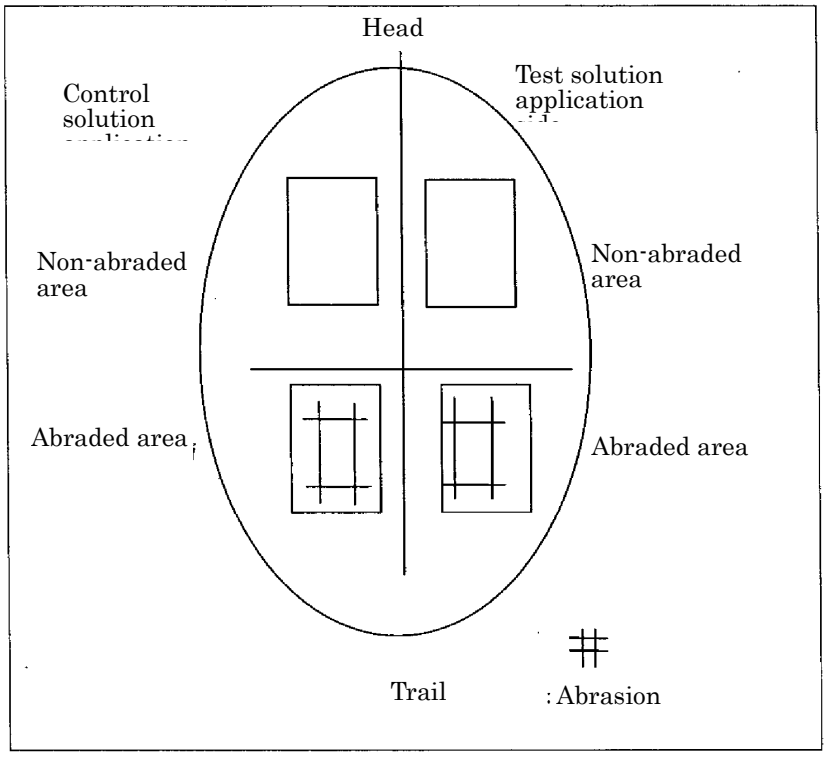

Figure 2 Skin irritation test (example) rabbit dorsal region

4.4.5 Observation

Observe the condition of skin immediately before administration. Remove gauze pads 24 hours after administration and wipe the application area carefully. Observe and record conditions of skin 1, 24 and 48 hours after removal of gauze pads in accordance with Table 1 (Scale for scoring skin response according to Draize, refer to 6.5).

4.4.6 Evaluation

Evaluate tissue toxicity and irritancy from observation results (refer to 6.4).

Abraded area may easily become infected and reddening and edema similar to those noted in a primary irritation may be caused.

In a case that infection is suspected, re-conduct the test with different animals and conduct an aseptic test of a test sample.

4.5 Test report

The test report should include following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Factors to identify the control sample when used (Example: name, vendor, serial number and etc.)
5) Preparation method of test solution
6) Species, strain, number, age and sex of animals
7) Test method
8) Test results
   Table: Individual animal weights at initiation and completion of test
   Skin response results of individual animals (score points)
   Photograph: Conditions of administration sites
   (only representatives allowed).
9) Evaluation and discussion of results
10) Reference

5. Eye irritation test

5.1 Objective

The objective of this test is to confirm effects of a test sample (medical device or material, excluding intraocular implant materials) which may have contact with ocular tissues by instillation of the test sample (final product or material) in rabbit eyes.

Measure pH of test solutions before initiation of the test and do not initiate the test in case pH of test solution indicates strong acidity or strong alkaline ($pH \leq 2$ or ≥ 15).

5.2 Equipment, apparatus and reagent (or equivalent products)

1) Slit lamp (x 6)
2) Autoclave
3) Incubator

4) pH meter
5) Hermetic heat-resistant hard glass container
6) Physiological saline (Japanese Pharmacopoeia)
7) Vegetable oil (olive oil (Japanese Pharmacopoeia), sesame oil (Japanese Pharmacopoeia), etc.)
8) Water for injection (Japanese Pharmacopoeia)
9) Fluorescein sodium solution (approximately 2%, Japanese Pharmacopoeia) or test paper

5.3 Preparation of test solution

Follow the procedure described in 3.3.

5.4 Test method

5.4.1 Test animal

1) Use healthy rabbits that were not previously used in studies using eyes.
2) Identify animals in an appropriate method, house them in breeding cages with appropriate size and breed them under specific conditions with a specific food for 1 week or longer before initiation of thetest.
3) Observe anterior portions of eyes before initiation of the test for absence of abnormalities such as conjunctivae hyperemia or corneal opacity (refer to Attachments 1 and 2). Additionally, observe corneas after instillation of fluorescein sodium solution or using a test paper for absence of stained area (refer to 6.6). Use rabbits that showed no abnormalities in examinations above.

   As it will make corneal observation easier, nictitating membranes may be incised as appropriate. When incising, it should be completed at least 2 weeks before initiation of test.
4) All animals should be maintained under appropriate conditions and provided with rabbit food and water with an appropriate quality during acclimation and test periods.

5.4.2 Test method

1) Select 6 rabbits in accordance with 5.4.1, weigh, and record body weights before initiation of the test.
2) Open a lower eyelid of the one side of eyes, instill 0.1 mL of the test solution extracted with physiological saline into the conjunctival sac, and then close eyes for 30 seconds.

3) Instill a control solution prepared with physiological saline into the other side of eyes in the same manner as the test solution.
4) Repeat procedures 2) and 3) to 3 rabbits.
5) Instill a test solution extracted with vegetable oil to the one side of eyes of the rest of 3 rabbits and a control solution prepared with vegetable oil into the other side of eyes.
6) Observe both eyes using a slit lamp 1, 24, 48 and 72 hours after instillation, evaluate in accordance with Draize or McDonald-Shadduck's standard and record scores.
7) Take photographs of anterior portions of eyes when total evaluation score based on Draize's standard is 6 points or higher.
8) Weigh animals and record body weights of animals after completion of the test.

5.5 Test report

The test report should include following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Factors to identify the control sample when used (Example: name, vendor, serial number and etc.)
5) Preparation method of test solution
6) Species, strain, number, age and sex of animals
7) Test method
8) Test results
   Table: Individual animal weights at initiation and completion of test
   Skin response results of individual animals (score points)
   Photograph: Conditions of anterior portions of eyes
   (when evaluation score is 6 points or higher).
9) Evaluation and discussion of results
10) Reference
   It is desirable to attach all data from individual animals as a table like Table 2.

6. Reference information

6.1 Amendments

The major amended points from the previous Guideline are as follows.

1) Only names and numbers of the standard were referred in the previous Guideline with skin irritation and intracutaneous response studies; however, the details of procedures were added.
2) The eye irritation test was included in an irritation test.
3) Consistency was assured for overall composition of the Guideline and contents of reports.

6.2 Animal species

Japanese white or New Zealand white rabbits are commonly used in studies.

6.3 Clipping hair

A hair-removing cream may be used when it is confirmed that the cream does not possess irritancy.

6.4 Judgment method

Judgment methods described in the quoted standards may be used in order to judge that test results (tissue toxicity and irritancy) are within the acceptable range. For example, it is indicated in ISO 10993-10 to obtain the primary irritation index (IIP) as a mean score for judgment.

6.5 Contact duration of test solution

This report includes the method to evaluate the primary irritation on skin. Therefore, the contact duration of a test solution was set at 24 hours in accordance with the standard; however, it is possible to change the duration based on the actual contact duration of the medical device (refer to ISO 01993-10). In case the medical device is used repeatedly in the clinical practice, it is necessary to evaluate the effect of the test sample after repeated administration.

6.6 Instillation of fluorescein sodium solution

It is desirable not to instill fluorescein sodium solution (Japanese

Pharmacopoeia) directly into eyes for staining. It is difficult to identify stained tissues when 1 to 2 % fluorescein sodium solution (Japanese Pharmacopoeia) is directly instilled into eyes of rabbits that can secrete only a small amount of lacrimal fluid and the fluorescent turn out to be too strong. When staining rabbit eyes with fluorescein sodium solution (Japanese Pharmacopoeia), first instill physiological saline and then administer fluorescein sodium solution with a glass bar while pulling an upper or lower eyelid. Close both eyelids gently with fingers in order to stain corneas, etc. In case staining of corneas by direct instillation of fluorescein sodium solution (Japanese Pharmacopoeia) is desirable, it is recommended to dilute 2% fluorescein sodium solution 5 to 10-fold with physiological saline. In this case do not store the diluted fluorescein sodium solution (Japanese Pharmacopoeia) for a long period, as it loses the antiseptic property.

In observations using fluorescein sodium solution or a test paper, scratches or scars likely to be caused by a hair gotten in the eye may be stained and observed frequently. In such cases the animals may be used in the test, but findings should be recorded.

7. Reference

1) T.O. McDonald, J.A. Shadduck, Dermatoxicology and Pharmacology, p.139, John Wiley & Sons, New York, 1977.

2)Francis N. Marzulli, H.L. Maibach edited, Dermatoxicology, 4th ed., Eye irritation (Robert B. Hackett, T.O. McDonald), pp749-815, hemisphere Publishing, 1991

Table 2

		Eyes instilled with a test solution (right eye)			Eyes instilled with a control solution (left eye)		
Animal No.		2481	2482	2483	2481	2482	2483
Body weight at initiation of the test (kg)		2.96	3.31	2.99			
Body weight at completion of the test (kg)		3.05	3.34	3.02			
Before instillation	Corneal opacity	0 × 0	0 × 0	0 × 0	0 × 0	0 × 0	0 × 0
	Corneal neovascularity	0	0	0	0	0	0
	Corneal staining	0	0	0	0	0	0
	Anterior chamber	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctival hyperemia	0	0	0	0	0	0
	Chemosis	0	0	0	0	0	0
	Discharge	0	0	0	0	0	0
1 hour after instillation	Corneal opacity	0 × 0	0 × 0	0 × 0	0 × 0	0 × 0	0 × 0
	Corneal neovascularity	0	0	0	0	0	0
	Corneal staining	0	0	0	0	0	0
	Anterior chamber	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctival hyperemia	0	0	0	0	0	0
	Chemosis	0	0	0	0	0	0
	Discharge	0	0	0	0	0	0
24 hour after instillation	Corneal opacity	0 × 0	0 × 0	0 × 0	0 × 0	0 × 0	0 × 0
	Corneal neovascularity	0	0	0	0	0	0
	Corneal staining	0	0	0	0	0	0
	Anterior chamber	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctival hyperemia	0	0	0	0	0	0
	Chemosis	0	0	0	0	0	0
	Discharge	0	0	0	0	0	0

Attachment 1) Draize Scale for Scoring Ocular Lesions

I. Cornea

A. Opacity: degree of turbidity (area which is most turbid is taken for reading)

No opacity	0
Scattered or diffuse opacity in which iris is clearly visible	1
Details of iris slightly obscured	2
No details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4

B Area of cornea involved

Normal	0
$0 < A < 1/4$	1
$1/4 \leq A < 1/2$	2
$1/2 \leq A < 3/4$	3
$3/4 \leq A$	4

Score = A x B x 5 (Maximum score: 80)

II. Iris (A)

Normal	0
Enhanced plica formation, congestion, swelling, circumcorneal congestion (any one or all of these or any combination thereof), still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage; extensive destruction (any one or all of these)	2

Score = A x 5 (Maximum score: 10)

III. Conjunctivae

A. Redness (Palpebral and bulbar conjunctivae, excluding cornea and iris)

Normal	0
Enhanced congestion	1
Extensive, deeper crimson red, individual vessels not easily discernible	2
Beefy red in all region	3

B. Chemosis

Normal	0
Enhanced swelling (includes nictating membrane)	1

Swelling with partial eversion of the eyelids	2
Approximately half closed eyelids with swelling	3
Approximately half to completely closed eyelids with swelling	4

C. Discharge

No discharge	0
Amount of discharge is more than normal (does not include small amount observed in inner canthus of normal animals)	1
Discharge moisturizing the eyelids and eyelashess	2
Discharge moisturizing the eyelids and considerable area around the eye	3

Score = (A + B + C) x 2(Maximum score: 20)

Judgment criteria

Total score	Evaluation
0~5	No irritancy
6~15	Slight irritancy
16~30	Irritancy
31~60	Moderate irritancy
61~80	Moderate to severe irritancy
81~110	Severe irritancy

Attachment 2) Scale for Scoring Ocular Lesions - Slit Lamp (McDonald-Shadduck)

Cornea

0 = Normal, the epithelial or endothelial surface looks light-gray with slit lamp and the parenchyma marble gray

1 = Lose clarity slightly, Anterior 1/2 portion of the parenchyma injured, substructure slightly cloudy, but clearly visible with scattering light

2 = Lose clarity moderately, Cloudiness extends to the endothelium, the parenchyma becomesevenly white, substructure clearly visible with scattering light

3 = The parenchyma overall injured but the endothelium visible, substructure slightly visible with scattering light

4 = The parenchyma overall injured and the endothelium not visible, substructure invisible with scattering light

Corneal opacity

0 = Normal cornea with no opacity

1 = 1 to 25% cloudiness of the parenchyma

2 = 26 to 50% cloudiness of the parenchyma

3 = 51 to 75% cloudiness of the parenchyma

4 = 76 to 100% cloudiness of the parenchyma

Corneal angiogenesis

0 = No angiogenesis

1 = Angiogenesis exists but vessels not invading within circumcorneal region and invaded area limited

2 = Vessels invading into cornea from various directions 2 mm or over

Corneal staining

0 = No fluorescein staining

1 = Slight fluorescein staining in limited area, easy to observe substructure with scattering light

2 = Moderate fluorescein stain in a limited area, details of substructure not clear with scattering light

3 = Marked fluorescein staining, substructure is barely visible, but difficult to observe

4 = Marked fluorescein stain, impossible to observe substructure with scattering light

Anterior chamber

0 = No diffusion of light noted in the anterior chamber

1 = Tyndall phenomenon slightly noted, light within an anterior chamber is

weaker than the transmitted light through a crystalline lens

2 = Tyndall phenomenon cleary noted, light within an anterior chamber is equivalent to the transmitted light through a crystalline lens

3 = Tyndall phenomenon cleary noted, light within an anterior chamber is stronger than the light transmitted through a crystalline lens

Iris

0 = Iris with no congestion, occasionally mydriasis noted at the direction of 12:00 to 01:00 and 06:00 to 07:00, 1 to 3 mm diameter slight congestion noted at the margin.

2 = Secondary vessels slightly congested but tertiary vessels not congested

3 = Moderate congestion of secondary and tertiary vessels with slight swelling of the iris parenchyma

4 = Marked congestion of secondary and tertiary vessels with marked swelling of the iris parenchyma

Conjunctival congestion

0 = Normal

1 = Eyelids with congestion around limbus limited to a portion in the direction of 04:00 to 07:00 and 11:00 to 01:00.

, reddening of conjunctiva

2 = Reddening of eyelids and conjunctiva with approximately 75% congestion around limbus.

3 = Dark-red eyelids accompanied by clear conjestion around limbus and petechial hemorrhage of congestion, congestion of bulbar conjunctiva

Chamosis

0 = Normal

1 = Swelling with no palpebral valgus

2 = Swelling with partial valgus of upper eyelid

3 = Swelling with partial valgus of lower and upper eyelids in the same level

4 = Swelling with partial valgus of lower eyelid and marked valgus of upper eyelid

Discharge

0 = Normal

1 = Amount of discharge is more than normal in the eye but none on eyelids and eyelashes

2 = Abundant amount of discharge attaching to the eyelids and eyelashes

3 = Moisturizing the eyelids and eyelashes and flows from the eyelids

Section 6 Systemic Toxicity Test

1. Scope of application

This test is conducted to evaluate the systemic toxicity of a medical device or material.

2. Standard quoted

2.1 ISO10993-11 (1993) Biological evaluation of medical devices. – Test for systemic toxicity

2.2 ASTM Standard F750-87 Standard Practice for Evaluating Material Extracts by Systemic Injection in the mouse

2.3 USP25 Biological Reactivity Tests, In-vivo.-Systemic Injection Test

2.4 BS5736-3 (1981) Method of test for systemic toxicity; assessment of acute toxicity of extracts from medical devices.

2.5 The Chemical Substances Control Law, a partial amendment to "Test Methods Concerning a New Chemical Substance" (formulation of concerning screening toxicity test method), December 5, 1986

3. Acute toxicity test

3.1 Objective

The objective of this test is to confirm whether substances that possess acute toxicity exist in extracts (hereinafter referred to as "test solution") extracted from a test sample (final product or material).

3.2 Test summary

The test method described in this report is basically prepared in accordance with the standard quoted 2.2. Test solutions extracted from test samples using physiological saline or vegetable oil will be administered intravenously (physiological saline test solutions) or intraperitoneally (vegetable oil test solutions) to 5 mice in each group. Animals will be observed until 72 hours after administration (refer to 5.1) and existence of acute toxicity will be evaluated by comparison with the control solution group.

3.3 Preparation of test solution

3.3.1 Extraction vehicle

Use physiological saline (Japanese Pharmacopoeia or equivalent products) and vegetable oil (cotton seed oil or sesame oil, Japanese Pharmacopoeia or equivalent products) for extraction.

3.3.2 Ratio of vehicle and test sample

Basically follow standards shown in Attachment 1.1.

3.3.3 Extract condition

Select the highest temperature condition with which the test sample can tolerate among conditions shown in Attachment 1.2.

The highest temperature condition with which the test sample can tolerate should meet the followings.

(1) Extraction temperature is lower than the melting point of a test sample.
(2) A test sample does not degrade at extraction temperature.
(3) Eluted substance does not evaporate or degrade at extraction temperature.

3.3.4 Operation method

Cool down extracts immediately to room temperature (not below 20 °C) and shake. Transfer the solution to a sterilized dry container under aseptic condition and store at 20 to 30 °C. Use the solution within 24 hours.

3.3.5 Preparation of control solution

Treat a vehicle without test sample under the same condition as test solution preparation and use it as a control solution.

3.4 Test method

3.4.1 Test animal

Use healthy albino mice in good nutritional state weighing 17 to 23 g. Animals should be raised under specific conditions with a specific food for approximately 1 week before initiation of the test and select animals that showed no body weight decrease. Use male animals. However, the sex of animals should be determined based on the use of the test sample and

the chemical substance of the material which constitutes the test sample. For example, female animals should be used in the test of a medical device which will be only applied to females.

3.4.2 Dose level

Basically administer 50 mL test solution per 1 kg body weight (refer to 5.2).

3.4.3 Administration route

Administer a test solution and control solution extracted in physiological saline intravenously and a test solution and control solution extracted in vegetable oil intraperitoneally.

3.4.4 Observation and measurement parameter

Clinical signs observation:	Observe all animals immediately after and 4, 24, 48 and 72 hours after administration. Record clinical signs basically in accordance with Table 1. Immediately necropsy animals that found dead.
Body weight measurement:	Weigh all animals immediately before and 24, 48 and 72 hours after administration (refer to 5.3).
Gross pathology:	Grossly observe administration sites, hearts, lungs, gastrointestinal tracts, livers, spleens, kidneys and reproductive organs of all animals at completion of an observation period.

Table 1. Classification of clinical signs

Normal, no symptoms	No adverse effects noted.
Slight (slight reaction)	Slight decline in motor function, dyspnea, and peritoneal irritation are noted.
Moderate (moderate reaction)	Peritoneal irritation, dyspnea, decline in motor function, palpebral ptosis and diarrhea are clearly noted.
Marked (marked reaction)	Collapse, cyanosis, tremor or sever peritoneal irritation, diarrhea, palpebral ptosis and dyspnea are noted.

Dead	Dead

3.4.5 Judgment method

The test solution will be judged to have no acute toxicity when no animal in the test solution group reveal any strong toxic symptom (clinical signs, body weight changes and results from gross pathology) when compared with animals in the control solution group.

The test solution will be judged to have acute toxicity when 2 or more animals in the test solution group are found dead or reveal marked toxic symptoms (Symptoms in “Marked” class in a Table 1).

Re-conduct the test with 10 animals in each of the test and control solution groups when any animal in the test solution group reveal slight abnormality (Symptoms in “Slight” class in a Table 1) when compared with animals in the control solution group, or 1 animal in the test solution group reveal marked symptom (Symptoms in “Marked” class in a Table 1) or is found dead. Re-conduct the test (10 animals in each of the test and control solution groups) when all animals in the test solution group reveal significant decreases in body weights even no abnormal clinical signs are noted (for example, statistically significant body weight decrease are noted when compared with animals in the control solution group). As a result of re-conducted test, the test solution will be judged to have no acute toxicity when no marked toxic symptoms are noted in any animal in the test solution group when compared with animals in the control solution group.

3.5 Test report

The test report should include following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample (medical device or material)
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Preparation method of test solution
5) Test method
6) Test results
   Table: Clinical signs, mortality (when necessary), body weights and

results from gross pathology

Photograph: Gross pathology (when findings considered to be toxicologically problem are found)

7) Evaluation and discussion of results
8) Reference

4. Sub-acute toxicity test (Sub-chronic toxicity test)

4.1 Objective

The objective of this test is to confirm whether substances that possess sub-acute (sub-chronic) toxicity exist in extracts (hereinafter referred to as "test solution") extracted from a test sample (final product or material). The test method described in this report is basically prepared in accordance with the standard quoted 2.5 and only an example of a sub-acute toxicity test. Therefore, examination (observation) parameters to detect a systemic toxicity should be carefully investigated at preparation of the test protocol based on a kind of test sample and an expected kind of a medical device.

4.2 Test summary

Test solutions extracted from test samples using physiological saline will be administered repeatedly to 5 male and 5 female rats for 28 days (90 days for sub-chronic toxicity test) by intravenous administration and toxicity will be compared between animals in the control group.

4.3 Preparation of test solution

Use physiological saline (Japanese Pharmacopoeia or equivalent products) as a vehicle and follow a procedure described in 3.3 for other extraction conditions.

4.4 Test method

4.4.1 Test animal

Use male and female rats, 5 to 6-week old with body weight within a range ± 20% of the mean body weight.

4.4.2 Dose level

Basically administer 20 mL test solution per 1 kg body weight (refer to 5.5).

4.4.3 Administration route and period

Administer a test solution intravenously. Administration period is 28 days in a sub-acute toxicity test (refer to 5.6).

4.4.4 Observation and measurement parameter

Clinical signs observation:	Observe at least once daily.
Body weight measurement:	Weigh at least once weekly.
Food consumption:	Weigh at least once weekly.
Hematological examination:	Measure leukocyte count, erythrocyte count, hemoglobin, hematocrit value, blood platelet count and differential leukocyte. Conduct blood coagulation test to determine parameters such as prothrombin time and activated partial thromboplastin time.
Serum biochemistry:	Measure alkaline phosphatase (ALP), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), lactate dehydrogenase (LDH), γ-glutamyltranspeptidase (γ-GTP), total protein, albumin, total cholesterol, triglyceride, phospholipid, glucose, blood urea nitrogen, creatinine, inorganic phosphorus, calcium, sodium, potassium, chlorine and albumin/globulin ratio.
Gross pathology:	Grossly observe all animals and store the following organs in accordance with a routine method. Brain*, pituitaries, eye balls, thyroids (including parathyroids), hearts, lungs*, livers*, kidneys*, spleens*, adrenals*, stomach, urinary bladder, bone marrows (femurs), testes*, epididymides, vesicular glands, prostates or ovaries*, uteri and gross lesions, and other organs and tissues decided to be target organs from gross observation results. Organs with * marks will be weighed.

Histopathological examination: Hearts, livers, spleens, kidneys, adrenals and other gross lesions, and other organs and tissues decided to be target organs from gross observation results will be examined histopathologically.

4.5 Test report

The test report should include at least following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample (final product or raw material)
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Preparation method of test solution
5) Test method
6) Test results
   Table: Clinical signs, mortality (when necessary), mean body weights, results from blood tests and results from pathological examinaiton
   Photograph: Gross pathology (when findings considered to be toxicologically problem are found)
   Histopathology (when findings considered to be toxicologically problem are found)
7) Evaluation and discussion of results
8) Reference

5. Reference information

5.1 Observation period in an acute toxicity test

Standard observation period in an acute toxicity test is set as 72 hours after administration. However, observation period can be extended based on the characteristic of a test sample and animal conditions during the test. BS5736-3 (1981) set the maximum observation period of 14 days, and so use this as an indication.

5.2 Dose level in an acute toxicity test

The dose level adopted in ASTM Standard F750-87 and USP 25, those possess solid accomplishments, was set as a standard. It is preferable to set as high dose level as possible for the objective to detect toxicity; however, the dose level is one of test conditions that should be considered thoroughly from the aspect of effects on animals by temporal increase in amount of circulating blood and hemodilution and animal welfare[2]. When setting a dose level different from the guidelines given above, the justification for the selection of the dose level should be included in the test protocol and final report.

5.3 Body weights of test animals

Body weights of test animals can be used as a parameter to investigate systemic toxicity of the test sample. USP25 states the standard that the test sample will be judged to be inappropriate (systemic toxicity noted) when body weight decrease 2 g or above is noted in 3 or more animals among 5.

5.4 Usage of an implantation test

Methods of sub-acute toxicity test by administration of extracts are described in "4. Sub-acute toxicity test (Sub-chronic toxicity test)"; however, results from an implantation test can be used as results of sub-acute toxicity test (sub-chronic toxicity test) when the test sample is implantable in an appropriate animal species (animals other than rats can be used) and as long as evaluation parameters described in this report are appropriately evaluated.

5.5 Dose level in a sub-acute toxicity test

The dose level in rats was set at 20 mL/kg in "4. Sub-acute toxicity test (Sub-chronic toxicity test)" in accordance with a reference[1]. It is important to select an adequate dose level considering the expected clinical use of the medical device, and to give a justification of the selection.

5.6 Administration and observation period

Set administration and observation periods considering the duration of expected clinical use of the medical device, and give a justification. ISO10993-11 states that sub-acute toxicity is a toxicity noted 14 to 28 days after administration of test

sample, and sub-chronic toxicity is a toxicity noted generally 90 days after administration of the test sample (however not exceeding 10% of duration of life).

6. Reference

1) Derelanko、 M. J. & Hollinger、 M. A. : CRC Handbook of Toxicology. CRC Press、 New York、 p78

2) Diehl、 K-H、 Hull、 R.、 Morton、 D.、 Pfister、 R.、 Rabemampianina、 Y.、 Smith、 D.、 Vidal、 J-M and van de Vorstenbosch C. : A Good Practice Guide to the Administration of Substances and Removal of Blood、 Including Routes and Volumes. J. Appl. Toxicol. 2001 21、 15–23

Section 7 Pyrogen Test

1. Scope of application

This test consists of 2 methods, rabbit pyrogen test and endotoxin test. The objective of rabbit pyrogen test is to investigate the existence of a pyrogen substance (endotoxic or nonendotoxic pyrogen substance) in a medical device or material. For a medical device consists of a naturally derived material, such as collagen, gelatin or alginate, it is preferable to conduct both studies described above in order to determine the amount of endotoxin as there is a possibility of endotoxic contamination of the material.

Pyrogen test is included in Part 11: Systemic toxicity in ISO10993 Biological evaluation of medical device, and the United States Pharmacopoeia (USP24) recommends both pyrogen and endotoxin tests. It is considered that test methods described in this report have the equivalent sensitivity to those of tests described above. Therefore, it is not necessary to re-conduct this test in the case there are results from the test conducted in accordance with ISO10993-11 or USP24.

2. Standard quoted

2.1 The Japanese Pharmacopoeia, fourteenth edition, Pyrogen Test / General Tests

2.2 The Japanese Pharmacopoeia, fourteenth edition, Endotoxin Test / General Tests

2.3 JIS K8008-1992 4.3.1-4.3.3

2.4 USP 24 Biological Reactivity Tests, In-vivo

2.5 ISO10993-11: Biological evaluation of medical devices Part 11: Systemic toxicity

3. Pyrogen test

3.1 Objective

The objective of this test is to confirm whether endotoxic or nonendotoxic pyrogen substance derived from the material exists in extracts (hereinafter referred to as “test solution”) extracted from a test sample (final product or raw material) (refer to 5.1).

3.2 Test summary

A test solution extracted from a test sample using physiological saline (Japanese Pharmacopoeia) will be administered intravenously to 3 rabbits in accordance with the pyrogen test method described in the Japanese Pharmacopoeia. The existence of a pyrogen substance will be evaluated by monitoring rectal temperature for 3 hours after administration and by comparing that with the temperature immediately before injection.

3.3 Preparation of test solution

3.3.1 Extraction vehicle

Use physiological saline (Japanese Pharmacopoeia) for extraction.

3.3.2 Ratio of vehicle and test sample

Basically follow standards shown in Attachment 1, 1.1.

3.3.3 Extract condition

Select the most appropriate condition from temperature and time conditions shown in Attachment 1, 1.2 (refer to 5.2).

3.3.4 Operation method

Cool down extracts immediately to room temperature (not more than 20 °C) and shake well. Transfer the solution to a sterilized dry container under aseptic condition and store at 20 to 30 °C. Conduct the pyrogen test within 24 hours. It is preferable to sonicate the test solution immediately before use in the test (refer to 5.3).

3.4 Pyrogen substance test method (refer to 5.4)

Conduct the test in accordance with the pyrogen test method described in the Japanese Pharmacopoeia, fourteenth edition, using a test solution prepared in accordance with the method described in 3.3 (refer to 5.5).

3.4.1 Test animal (refer to 5.6)

Use healthy rabbits in good nutritional state weighing 1.5 kg or above, and those did not show body weight decrease after being raised with a specific food for 1 week or longer before initiation of test.

When using rabbits previously used in other test, take a wash out period as long as possible. Do not use animals used in a previous test in which a

positive pyrogen result was confirmed. Body temperature of animals will be measured for 5 to 6 hours after fixing animals with restraints at least once between 1 to 3 days before initiation of the test in order to get animals accustomed to the fixation and body temperature measurement procedures. Animals will be housed individually in cages. Try not to make animals get exited during this period, and pay special attention when handling animals on the day of the test.

Maintain room temperature at 20 to 27 °C for 48 hours before initiation of the test and during the test and try to maintain consistent temperature and humidity.

3.4.2 Equipment and apparatus (refer to 5.7)

Thermometer: Use a rectal thermometer or other kind of thermometer with an equivalent sensitivity. Time period necessary for rectal temperature measurement should be decided before initiation of the test.

Sterilization of apparatus: Remove pyrogen substance from glass apparatus, containers, syringes and injection needles by heating for 30 minutes or more at 250 °C. Endotoxin-free disposable products may be used.

3.4.3 Dose level (refer to 5.8)

Basically administer 10 mL test solution per 1 kg body weight.

3.4.4 Operation (refer to 5.9)

Conduct the test in the room with the same temperature and humidity as in the room in which animals were raised before initiation of the test.

Fix animals with an appropriate restraint.

Measure rectal temperature by intrarectally inserting a thermometer at a constant depth within a range of 60 to 90 mm for an appropriate time period and read the temperature.

Do not provide food to animals from several hours before the initiation of the first temperature measurement until the completion of the final temperature measurement on the same day.

Measure body temperature 3 times with 1-hour interval before injection of the test solution, and use the third temperature as a control body temperature when the second and third body temperatures are almost equivalent. Exclude the animal from the test when the second and third body temperatures are not equivalent, or equivalent but over 39.8 °C.

Heat the test solution at 37 °C and administer it intravenously into auricle

veins of animals within 15 minutes after the third body temperature measurement.

Measure body temperature 3 times with 1-hour interval after injection.

A difference from the highest body temperature will be regarded as a body temperature increase.

Body temperature may be measured automatically by a thermistor body temperature measurement system and a personal computer.

3.4.5 Judgment (refer to 5.10)

Use 3 animals for the first test. The result will be judged to be pyrogen positive when 2 or 3 animals shows body temperature increase of 0.6 °C or above after injection.

Conduct an additional test when there is only 1 animal that show body temperature increase of 0.6 °C or above, or the total temperature increase of 3 animals exceeds 1.4 °C.

Use 5 animals for the second test. The result will be judged to be pyrogen positive when 2 or more animals show body temperature increase of 0.6 °C or above after injection.

When test solution prepared under conditions described in Attachment 1, 1.2 (1) or (3) is judged to be positive, existence of endotoxin should be confirmed by conducting the test using endotoxin-specific limulus (LAL) reagent (example, JIS K8008 4.3.3) with the same test solution used in the pyrogen test and the test solution prepared under conditions described in 1.2 (5). The origin of pyrogen substance should be discussed by integrating these results.

Refer to references shown in "6. Reference" regarding the endotoxin test by endotoxin-specific limulus (LAL) reagent (refer to 5.11).

3.5 Test report

The test report should include following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample (medical device or raw material)
   (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Preparation method of test solution
5) Test method

6) Test results
   Table: Body temperature of each animal
7) Evaluation and discussion of results
8) Reference

4. Endotoxin test (refer to 5.11)

Conduct the endotoxin test by endotoxin-specific limulus (LAL) reagent (The Japanese Pharmacopoeia, fourteenth edition, Endotoxin Test or JIS K8008 4.3.3) after selecting an appropriate condition from Attachment 1 1.2 (1) – (5) based on the characteristics of the material or usage objective, and when possible, after extracting test solution by successive shaking or sonication, because a possibility of contamination by endotoxin derived from the material cannot be denied for a naturally derived medical material (example, chitin, chitosan, natural gum, pectin, alginate, collagen or gelatin) (refer to 5.12).

5. Reference information

5.1 Test objective

The primary objective of this test is to determine the existence of pyrogen substance in the test sample, not for quality assurance purpose. In GMP, it is definitely necessary to check endotoxic contamination of the material at receipt or during the manufacturing process. In this case, the test method should be defined in GMP or specifications/standards of individual products. The following 2 points are important.

The possibility of exothermic reaction by an added chemical substance cannot be denied with a so-called synthetic polymer, and it is necessary to investigate the existence of nonendotoxic pyrogen substance in a material in a test using rabbits.

With a naturally derived material, such as collagen, gelatin or alginate, it is necessary to determine the amount of endotoxin at the designing phase because endotoxic contamination during its manufacturing process cannot be avoided and it is not easy to remove endotoxin. This test was schemed based on this recognition.

This test is to detect pyrogenicity of a substance extracted from a test sample. There may be a case that pyrogenic activity cannot be detected when a substance is extracted under conditions described in Attachment 1, 1.2 (1) – (3) even though a low concentration of endotoxin exists in a test sample. It is considered that this phenomenon has been caused not by deactivation of endotoxin by heat, but by

decreased activity of endotoxin by hydrolysis associated with recovery losses by absorption/attachment of endotoxin to a material (surface) or pH changes of extracts.

This report is not targeted to endotoxin tests as a quality control test of a final product. Contamination by endotoxin derived from gram-negative bacteria (extremely strong pyrogen substance) or by pyrogen substances derived from gram-positive bacteria and fungi (molds and yeasts) by bioburdens during manufacturing process should be tested in accordance with GMP of medical devices.

5.2 Extraction temperature

In conventional pyrogen substance tests of test samples, test solution has been prepared under high temperature and long-time conditions as described in Attachment 1.2 "Extraction temperature/time" (1) – (3). In these tests, a pyrogen substance noted in a test solution has been judged to be "endotoxin" based on the rationale that endotoxin is a lipopolysaccharide which possesses extremely strong heat tolerance. However, as suggested in a reference 1) and other reports, endotoxin solution may lose its activity after heat processing and it is suggested that this phenomenon depends on three factors, the concentration of endotoxin, the temperature of heat-processing and time period of heat-processing. Especially, low concentration of endotoxim has high possibility to lose its activity under conditions as 1.2 (1) – 1.2 (3). Endotoxin is easily hydrolyzed under mild acid or alkaline condition (decreased solubility by the release of lipid A, detachment of glycoside binding type phosphoric acid or fatty-acid residue that are directly related to decreased activity). Therefore, decomposition of endotoxin may be caused after changes in pH of the test solution by an effect of a chemical substance released from an active group on the surface of a material or material itself. Additionally, nonspecific absorption of endotoxin on the surface of a material and recovery losses by ionic bond cannot be neglected when endotoxin concentration is low. Decreased activity reported in the reference 1) and other reports is considered to be related to these factors.

5.3 Extract condition

Contact of extract vehicle and sample surface, the time period and temperature, cooling, shaking (example: sonication), aseptic handling and storage are important factors of extraction. Solubility may be good when extraction is conducted at

high temperature; however, when temperature at storage gets lower, insoluble substance may be produced as a result of decreased solubility. It is necessary to transfer the test solution to an endotoxin-free container under aseptic condition after cooling it not more than 20 °C. Collect the test solution by decantation. When insoluble substances are noted by gross observation, centrifuge the solution to remove them. It is preferable not to use membrane filters for sterilization to remove insoluble substance (because endotoxin may be absorbed by the membrane filter). It is prescribed to try to conduct procedures (extraction and storage) under aseptic condition as much as possible and conduct a pyrogen test within 24 hours of extraction. It is recommended to sonicate the test solution before administering it to rabbits in order to resolve endotoxin attached to a container wall and to prepare uniform micelle.

The justification for centrifugation and the condition should be specified in the test final report when insoluble substance noted in the test solution is removed by centrifugation after extraction or before injection. When a membrane filter is unavoidably used, the justification and the name of membrane filter used should be indicated as well.

5.4 Pyrogen test method

The pyrogen test method described in this report is in accordance with the method described in the Japanese Pharmacopoeia. The pyrogen test method has been hardly changed except for elimination of upper limit of the rabbit body weight since it was included in the general tests in the Japanese Pharmacopoeia, sixth edition. There are some points (to be described later) currently not appropriate in details because equipment and apparatus has been improved since then, therefore, methods in the current USP or European Pharmacopoeia (EP) can be referred instead.

The rabbit pyrogen test was developed with its primary objective to detect endotoxin, considering the response relationship between rabbit and human being. Most of studies on temperature regulation mechanism in homeotherms are conducted in accordance with procedures in this test method using rabbits. Regarding temperature regulation, there are a lot of things still unexplained; however, it is considered that hypothalamus, vertebra and mucosa are related and body temperature is regulated by action of nerve transmitter substances such as central monoamine (noradrenaline and serotonin) and acetylcholine at temperature regulation neural network in hypothalamus. It is considered that heat is

generated by endotoxin as a result of disturbance of metabolic balance of nerve transmitter substances caused by the action of prostaglandin, which is considered to be a final mediator, against hypothalamus, accompanying synthesis and release of several kinds of cytokines, endogenous pyrogen substances (interleukin-1, tumor necrosis factor, interferon, etc.). There are several known neurotropic substances (example, LSD, morphine) other than endotoxin that cause temperature increase by disturbing temperature regulation mechanism.

5.5 Reported case of heat generation by a chemical substance

There are not so many reported case of heat generation caused by a chemical substance related to a medical device; however, Shimohira *et al.* (reference 2) reported that N-phenyl-β-naphthylamine and aldol-α-naphthylamine, substances that had been used as rubber anti-oxidants, showed pyrogenicity against rabbits and the peak temperature increase was noted 1 to 2 hours after injection. Additionally, they reported that N-phenyl-β-naphthylamine had been actually detected from rubber for gastric catheter. Recently, these naphtylamines are not used because carcinogenicity is suspected with these substances.

There are other chemical substances that cause temperature increase as follows (reference 3). 4, 6-dinitro-o-cresol, which is used as an antiparasitic agent and dinitrophenol, which is used as an intermediate for cobalt sulfide staining cause temperature increase because these substances promote heat generation *in vivo* by stimulating oxidative metabolism after decreasing high-energy phosphorylate substance by uncoupling of oxidative phosphorylation. O-nitrophenol, m-nitrophenol and p-nitrophenol are used as intermediates for organic synthesis, fungicides and insecticides, and these are known to cause high body temperature experimentally. Body temperature increase was noted in dog studies with a picric acid, which is used in a bactericide or during process of stain manufacture.

5.6 Test animal

It is stated that healthy rabbits weighing 1.5 kg or above should be used, and upper limit of the body weight was eliminated. Mature rabbits should be used because juvenile rabbits with age 4 to 5 weeks old have low sensitivity against endotoxin and reaction may vary widely among individual animals. It is better to raise animals individually in cages from the beginning in order to prevent contagious disease and because rabbits make noise when caged together. Both male and female animals may be used in a test; however, it is better to use either

sex of animals in the test in order to avoid emotional stimulation.

The change of temperature in animal and test room is limited within ± 3 °C in the Pharmacopoeias of each country. It is preferable that an animal room and a test room are separated by a door and both rooms are controlled under the same temperature and humidity conditions. Regarding the handling of rabbits while body temperature measurement, conventional unfixed cloth rapping method (test technician softly press rabbits by his/her knees and arms) is currently not frequently used, and neck fixation is widely used. As animals are fixed on their necks for several hours, it is better to use a restraint which does not fix backs and legs of animals in order to reduce their stress. Sometimes rabbits agitate while being fixed against noise and other stimulations, and reveal hypothermia as a result of being frightened. Once rabbits get into this state, almost all animals will never recover to the normal status and die within several days. Therefore, it is important to get animals accustomed to the fixation posture during the raising period.

Regarding the frequency of use of rabbits in the test, the Japanese Pharmacopoeia does not specify the wash out period of animals once used in the other test. USP stated that at least 48-hour wash out period should be set when the same rabbit is repeatedly used in the other test. EP states that 3-day wash out period should be set. It may be better to take at least 2-day wash out period.

Animals once judged to have positive pyrogen result should not be re-used. This is because animals once administered with endotoxin may obtain endotoxin tolerance and show weaker reaction or no reaction after the second endotoxin administration. USP states that animals can be re- used after 2-week wash out period and EP states that animals can be re-used after 3-week wash out period.

5.7 Thermometer

Mercurial, thermocouple and electric-resistance thermometers are used. However, recently, automatic measurement by a thermistor body temperature measurement system (accuracy of ± 0.1 °C) and a personal computer are adopted in majority of test facilities. For a measurement of rectal temperature, the Japanese Pharmacopoeia states that a thermometer should be inserted intrarectally within a range of 60 to 90 mm and this is almost consistent to the standards in USP and EP. This range was determined based on the necessity to check the depth of themometer insertion as electrical serial measurement method pervades because some thermocouple and electric-resistance thermometers show a

temperature at one point and some show an algebraic mean of temperature detected at a certain area.

Heat-resistance glass apparatuses, containers and syringes and injection needles should be sterilized by dry-heat sterilization at 250 °C for at least 30 minutes before use in order to deactivate endotoxic pyrogenicity derived from gram-negative bacteria. A strong heat-processing condition is required to deactivate endotoxin (chemically heat-resistant lipopolysaccharide), the component of outer membrane of gram-negative bacteria, since these apparatuses are contaminated by gram-negative bacteria that exist in the air.

Because limulus reaction by endotoxin has several hundred times stronger sensitivity than that in the pyrogen substance test, sterilization at 250 °C for 30 minutes is not enough for dry-heat sterilization of glass apparatuses and containers to be used for preparation of test solution to be used in the endotoxin test, and it is safer to sterilize at 250 °C for at least 60 minutes. Regarding syringes and injection needles, there are disposable products available on market from which a pyrogen substance cannot be detected (pyrogen-free), so these can be used instead.

Physiological saline is used for extraction and dilution. The Japanese Pharmacopoeia physiological saline is assured to be pyrogen-free. The negative control test should be conducted in order to confirm that apparatuses and containers used are pyrogen-free by treating with physiological saline used in the test.

5.8 Dose level

Generally the dose level is set at 10 mL test solution per 1 kg body weight, and administer from the auricle vein within 15 minutes of control body temperature measurement. EP states dose level range of 0.5 to 10 mL/kg, and it is considered unnecessary to heat up test solution when the amount is small. It is necessary not to contact with test solution when drawing it into syringes in order to avoid endotoxin contamination of the test solution. It is considered unnecessary to conduct test solution drawing procedure under aseptic conditions because the effect of dropping bacteria in the test room can be neglected when the procedure is completed immediately.

5.9 Operation method

It is stated to measure body temperature 3 times with 1-hour interval before injection of the test solution, and use the third temperature as a control body

temperature when the second and third body temperatures are almost equivalent. This means that at least 3 hours are required to determine the control body temperature. USP states that the control body temperature should be determined by 30 minutes before injection of the test solution (EP requires 90 minutes).

Control body temperature range was conventionally (The Japanese Pharmacopoeia, seventh edition) stated as 38.9 to 39.8 °C. This range was amended to "39.8 °C or below" in the Japanese Pharmacopoeia eighth edition, because only approximately 30% of rabbits show body temperature within the conventional range when they are fixed using a restraint however it was observed that sensitivity of rabbits against a pyrogen substance may not be affected unless body temperature markedly deviate from the lower limit (38.9 °C). USP stated the same body temperature condition as the Japanese Pharmacopoeia. EP requires to exclude animals with body temperature 38.0 °C or below or 39.8 °C or above.

It is stated to measure body temperature 3 times within 1-hour period after injection. Measurement interval was amended to 30-minute period in the USP XXIII revised in 1995, and this is almost consistent to the method described in the EP, which requires body temperature measurement with constant intervals within 30 minutes. Currently, thermometers that enable electrical serial measurement are used in most of test facilities and temperature can be recorded with 2 to 3-minute intervals. Therefore, the method to detect the highest temperature during 3-hour observation period can be adopted, referring to the method described in the EP. For example, it is known that the peak temperature increase is noted approximately 1.5 hours after endotoxin administration, and then when a large amount of endotoxin is administered, the second peak is noted again 3 to 3.5 hours after administration. As described above, it is meaningful to record body temperature with as close intervals as possible during 3-hour observation period. Therefore, it is recommended to judge pyrogenicity by paying attention to body temperature changes within 3 hours of test solution administration even in a case that 1-hour body temperature measurement interval is adopted in accordance with this report.

5.10 Judgment

Since judgment in this test is based on a slight changes in body temperature of rabbits, the animals with high sensitivity, very careful judgment procedure is set and when a body temperature increase at a certain degree is noted, the test is

re-conducted to make the final judgment. This test method is the one included in the current Japanese Pharmacopoeia, fourteenth edition.

5.11 Endotoxin test method

Regarding endotoxin test methods, JIS K8008 4.3.3, which is described in this report and "Endotoxin Test" included in the general test methods in the Japanese Pharmacopoeia, fourteenth edition can be referred. Additionally, technical information journal, the Japanese Pharmacopoeia TI (reference 4) can be referred for measurement procedures, test examples and instructions.

Endotoxin test method is *in vitro* test method to detect or determine the quantity of endotoxin based on a reaction of endotoxin derived from gram-negative bacteria to induce gelation after activating blood cell extracting component, LAL (Limulus Amebocyte Lysate) of limuloids (Limulus polyphemus or Tachypleus tridentatus). There are 3 test methods, gelation method, in which gelation is set as an indicator, turbidimetric method, in which the turbidity of gel is set as an indicator, and colorimetric method, in which the coloring by hydrolysis of coloring synthetic substrate is set as an indicator. Endotoxin test method has been widely used in fields of pharmaceutical, clinical and medical device as an alternative method for a pyrogen substance test method (for endotoxin) because it has high reaction specificity against endotoxin and several hundred times higher sensitivity when compared with rabbit pyrogen test.

This test method can be adopted in cases that endotoxin test is set as a specification of a final product to check bacterial contamination during manufacturing process.

5.12 Endotoxin-specific LAL reagent

It is stated that β-glucan, component of fungus cell walls, and cellulosic substances (extracts from artificial kindneys such as cuprophan membrane) do not possess pyrogenic activity; however, it is understood that they strongly react against LAL. Endotoxin-specific LAL reagents that do not react to β-glucan are available on market. It is known that β-glucan has possibility to enhance bioactivity of endotoxin[9].

6. Reference

1) Y. Ogawa, T. Murai, K. Kawasaki: Endotoxin Test Method of Medical

Device – relationship between limulus test and pyrogen test – Biocontrol Science, 19, 561-566 (1991)

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3) Toxicity Test Course: Industrial Chemical Substance, Environmental Chemical Substance, edited by M. Wada, p.129-151, Chijin Shokan, Co., Ltd. (1993)
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8) M. Doi *et al.*: Development and Application of Endotoxin-specific Limulus Test using G-factor Inhibitory Activity by excessive carboxymethylated curdian, Japanese Journal of Bacteriology 45, 903-911 (1990)
9) Y. Adachi, M. Okazaki, N. Ohno, and T. Yadomae. “Enhancement of cytokine production by macrophages stimulated with (1-3)-beta-D-glucan, grifolan (GRN), isolated from Grifola frondosa.” Biol. Pharm. Bull., 17, 1554-1560 (1994)

Section 8 Hemocompatibility Test

1. Scope of application

This test is conducted to evaluate hemocompatibility of a medical device or material that have contact with blood.

2. Standard quoted

2.1 ISO 10993-4 (1992): Biological evaluation of medical devices. Part 4: Selection of tests for interactions with blood

2.2 ISO/DIS 10993-4 (2000): Biological evaluation of medical devices. Part 4: Selection of tests for interactions with blood

2.3 ASTM Standards: F756-93 Practice for Assessment of Hemolytic Properties of Materials

3. Selection of test items

It is preferable to evaluate items described in Table 1 or 2 as hemocompatibility test (refer to 6.1). These tests can be included in a series of function tests of the medical device.

Standard test method of hemolytic toxicity test is described after section 4 in this report.

Table 1. Evaluation item for hemocompatibility test (1) Evaluation items for medical devices (or the raw material) that connect external and internal body and that have contact with circulating blood

Evaluation category	Method (example)	Note
Thrombosis	Light microscopic observation Platelets, leukocyte count, hemagglutination, erythrocyte count, adhesion and its condition of fibrins	Scanning microscope can be used instead.
Coagulation	PTT measurement	
Platelets	Platelet count	
Hematology	leukocyte count and differential leukocyte measurement	Refer to the following section for the

	plasma hemoglobin concentration measurement or conduct hemolytic toxicity test	standardized hemolytic test method in this report.
Complement system	Complement product measurement (C3a or C5a)	

Table 2. Evaluation item for hemocompatibility test (2) Evaluation items for implantable medical devices (or the material) that have contact with circulating blood

Evaluation category	Method (example)	Note
Thrombosis	Measurement of occlusion rate and decrease in blood flow by thrombosis in implantation test Observation results of the medical device after implantation test (appearance and adhesion of thrombosis under microscopic observation) Observation of organs after implantation test (observe the effect of thrombosis formed by the medical device)	Scanning microscope can be used instead.
Coagulation	PTT, PT, TT measurement Plasma fibrinogen concentration measurement FDP assay	
Platelets	Blood platelet count Blood platelet coagulation	
Hematology	Leukocyte count and differential leukocyte measurement Plasma hemoglobin concentration measurement or conduct hemolytic toxicity test	Refer to the following section for the standardized hemolytic test method in this report.
Complement system	Complement product measurement (C3a or C5a)	

4. Hemolytic toxicity test

4.1 Preparation of test solution

Regarding the amount ratio of test sample (medical device or material) and extract vehicle (physiological saline), follow standards shown in Attachment 1.1. When cutting a test sample into pieces, be careful for contamination during the procedure. In the extraction, select the highest temperature condition with which the test sample can tolerate among conditions shown in Attachment 1.2. and prepare a test solution. Extract 3 different test samples with the same lot number separately once and obtain test solutions E1, E2 and E3.

The highest temperature condition with which the test sample can tolerate should meet the followings.

(1) Extraction temperature is lower than the melting point of a test sample.

(2) A test sample does not degrade at extraction temperature.

(3) Eluted substance does not evaporate or degrade at extraction temperature.

When degradation of the test sample within the specified temperature range is temperature-dependent, the test should be conducted at different extraction temperatures in order to analogize the biological reaction under actual usage conditions.

4.2 Preparation of defibrinated blood

Prepare defibrinated blood from healthy rabbit blood and use for test after confirming the followings.

Add 0.2 mL of prepared blood to 10 mL of physiological saline and centrifuge at 750 x g for 5 minutes. Measure the absorbance of the supernatant at 576 nm and confirm that there is no hemolysis (0.01 or below) (refer to 6.2).

Blood treated with anti-coagulant agents may be used; however it should be recorded on the test report. Some kinds of test sample (example: ceramics) may deactivate anti-coagulant agents, so be careful for the usage.

4.3 Preparation of control solution

4.3.1 Negative control (non-hemolytic) solution

Use physiological saline instead of the test solution, and follow the procedure described in 4.4.

4.3.2 Positive control (completely-hemolytic) solution

Add 0.2 mL of prepared blood to 10 mL of distilled water (refer to 6.3) and the completely-hemolytic solution will be used as a positive control solution.

4.4 Test operation

Mix the test solution and defibrinated blood in a ratio of 10:0.2 and mix by inversion once. Incubate the solution for 1, 2 or 4 hours at 37 ± 1 °C, centrifuge at 750 x g for 5 minutes and remove the supernatant (refer to 6.4).

Determine the absorption spectrum of the supernatant and calculate the hemolysis ratio using method I when the absorption waveform indicates oxygenated hemoglobin and using method II when absorption of methemoglobins is noted. Conduct this procedure once for each test solution (E1, E2 and E3), calculate the mean value and regard it as the hemolysis ratio at the time point (refer to 6.5, 6.6 and 6.7).

[Method I]

Determine absorbance of obtained supernatant at 540 nm or 576 nm, the maximum absorptions of oxygenated hemoglobin. Determine absorbance of negative and positive control solutions separately and obtain the hemolysis ratio in accordance with the following formula.

$$\text{Hemolysis ratio} = \frac{\text{(absorbance of the test solution) – (absorbance of the negative control solution)}}{\text{(absorbance of the positive control solution) - (absorbance of the negative control solution)}} \times 100$$

[Method II]

Determine the total hemoglobin in the supernatant by cyanmethemoglobin method and obtain the hemolysis.

1) Drabkin reagent; K_3 [$Fe(CN)_6$] 200 mg/L, KCN 50 mg/L, $NaHCO_3$ 1.0 g/L (refer to 6.8)

2) Operation; Add 4.5 mL of Drabkin reagent in test tubes, and add 0.5 mL of either test solution supernatant, positive control solution or negative control solution. Leave at room temperature for 20 minutes after mixing and determine the absorbance of generated cyanmethemoglobin at 540 nm. Obtain the hemolysis ratio in accordance with the following formula.

$$\text{Hemolysis ratio} = \frac{\text{(absorbance of the test solution) – (absorbance of the negative control solution)}}{\text{(absorbance of the positive control solution) - (absorbance of the negative control solution)}} \times 100$$

4.5 Evaluation

Obtain hemolysis ratios after 1, 2 and 4 hours of incubation. Degrees of hemolysis can be categorized using Table 3. It is preferable to discuss the risk considering the kind of the medical device with which the material is used and the contact period of the medical device with blood (refer to 6.9).

Table 3 Judgment table (based on ASTM F756-93)

Hemolysis ratio	Grade
Hemolysis ratio ≤ 2	No hemolysis
2 < hemolysis ratio ≤ 10	Slight hemolysis
10 < hemolysis ratio ≤ 20	Moderate hemolysis
20 < hemolysis ratio ≤ 40	Strong hemolysis
40 < hemolysis ratio	Extremely strong hemolysis

5. Test report

The test report should include following items.

1) Test facility and Test Director
2) Period of the test
3) Factors to identify the test sample (medical device or raw material) (Example: name, manufacturer, serial number, materials and etc. of the medical devise)
4) Preparation method of test solution
5) Test method
6) Test results (hemolysis ratio)
7) Evaluation and discussion of results
8) Reference

6. Reference information

6.1 Selection of test items

Evaluation items shown in Tables 1 and 2 are set in order to investigate various reactions that may be caused when a medical device or raw material contact with blood and these were set in accordance with 5 evaluation categories (thrombosis, coagulation, platelets, haematology, complement system*) described in ISO 10993-4 (1992) and ISO/DIS 10993-4 (2000). It is preferable to select evaluation items, methods and conditions (*in vitro*, *ex vivo*, *in vivo*) considering

conditions of clinical usage (conditions such as contact duration with blood and surface area) of the medical device. Medical devices that have indirect contact with circulating blood is not included in this test report; however, evaluation items of these medical devices should be selected considering the assumed usage conditions, such as selecting hemolytic toxicity test using the test solution to evaluate hemolysis.

*note This report adopted the category "complement system" in accordamce with ISO/DIS 10993-4 (2000) even though ISO 10993-4 (1992) expresses the category as "immunology".

6.2 Preparation of defibrinated blood

Blood used for preparation of defibrinated blood can be drawn from carotid artery, auricle vein, auricle artery or heart. There is almost no possibility of hemolysis up to 6 hours of incubation. Therefore, it is not necessary to draw blood from carotid artery in a case that the amount of required blood is small.

Blood can tolerate the test when absorbance of supernatant obtained after being mixed in physiological saline (supernatant obtained by procedures described in 4.4 except for incubation) is 0.01 or below at 576 nm.

6.3 Distilled water

The primary objective is to completely hemolyze erythrocytes therefore we do not designate distillation as the only method of water purification.

6.4 Incubation time

Although the longest incubation duration of 4 hours is enough, we set 3 incubation durations, 1, 2 and 4 hours because usually reaction occurs chronologically and understanding the process may help the final evaluation, and this brings higher reliability to data when comparing with data obtained at only one time point. There are various patterns for hemolysis, which is noted at early stage, which is noted in accordance with time progress and which is noted at later stage for the first time. These patters should be included in the evaluation and therefore it is important to understand chronological changes of hemolysis.

When only small amount of test solution is obtained, incubation time can be set only at 4 hours.

6.5 Methemoglobins

Methemoglobins include those other than oxygenated hemoglobin, such as methemoglobin and carboxyhemoglobin.

6.6 Calculation of hemolysis ratio

Regarding the selection of method I or II, it is preferable to consistently use the test method II, using cyanmethemoglobin method. However, in order to avoid unnecessary use of cyanides, it was decided to use test method I in the case no absorbance of methemoglobin is noted.

6.7 Waveform and absorption peak of oxygenated hemoglobin

In order to confirm the waveform of oxygenated hemoglobin, it is good to compare waveforms at 500 to 700 nm wavelength between the positive control solution using spectrophotometer. Waveforms should be observed carefully referring to reference 2 or 3 because there are some cases that a part of oxygenated hemoglobin changes to methemoglobin and clear oxygenated hemoglobin peak at 540 nm and 576 nm is mixed with methemoglobin peak. Attention should be also paid to pH of solutions because it may affect waveform of methemoglobin.

Oxygenerated hemoglobin show peak absorption at around 540 nm and 576 nm, and it is preferable to determine at 576 nm, which show sharper peak than at 540 nm.

6.8 Expiration date of Drabkin reagent

Drabkin reagent prepared should be transferred to an amber bottle and stored in a refrigerator. Expiration data is approximately within 1 month after preparation.

6.9 Evaluation

Whether the obtained hemolysis rate is acceptable or not should be judged considering individual conditions such as *in vivo* contact duration, contact frequency and surface area of the medical device. Table 3 is a judgment table quoted from ASTM F756-93, which states almost equivalent test conditions such as incubation time to the conditions in this test. It has been reported that a normal polymer medical device showed hemolysis rate below 0.5%. On the contrary, there was a case in which clear hemolysis was noted in accordance with

ion concentration increase when a certain kind of glass ceramics was extracted at high temperature. In this case hemolysis rate was dependent on extraction temperature and no hemolysis was noted at 37 °C. The relationship to an extraction temperature may be one of evaluation factors for a certain raw material as shown above.

7. Reference

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Attachment

Extraction vehicle/sample ratio and extraction temperature and time in preparation of the test solution from a medical device or material

1.1 Extraction vehicle/sample ratio

Vehicle/sample ratio should be selected in accordance with the feature or thickness of the test sample.

Feature	Thickness and material	The amount of test sample against 1 mL of extract vehicle (acceptable range ± 10%)
Film or sheet	≤ 0.5 mm	6 cm^2 (total surface area (both sides))
	> 0.5 mm	3 cm^2 (total surface area (both sides))
Tubular material	Wall thickness < 0.5 mm	6 cm^2 (total area of both inside and outside)
	Wall thickness 0.5 to 1 mm	3 cm^2 (total area of both inside and outside)
Slab, tubular material, molded material	> 1 mm	3 cm^2 (total surface area)
Material in which the measurement of surface area is difficult	Elastomer	0.1 g
	Plastics or other polymers	0.2 g

1.1 Extraction temperature and time

(1) 121 ± 2 °C	1 ± 0.2 hours	autoclave
(2) 70 ± 2 °C	24 ± 2 hours	incubator
(3) 50 ± 2 °C	72 ± 2 hours	incubator
(4) 37 ± 1 °C	72 ± 2 hours	incubator
(5) Room temperature	72 ± 2 hour	