



มาตรฐานผลิตภัณฑ์อุตสาหกรรม

THAI INDUSTRIAL STANDARD

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ISO 10993-5 : 1999

การประเมินทางชีวภาพของเครื่องมือแพทย์

เล่ม 5 : การทดสอบภายนอกร่างกายเพื่อประเมินความเป็นพิษต่อเซลล์

**BIOLOGICAL EVALUATION OF MEDICAL DEVICES -
PART 5 : TESTS FOR *IN VITRO* CYTOTOXICITY**

สำนักงานมาตรฐานผลิตภัณฑ์อุตสาหกรรม

กระทรวงอุตสาหกรรม

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สำนักงานมาตรฐานผลิตภัณฑ์อุตสาหกรรม

การทดสอบความเป็นพิษต่อเซลล์ที่ทำภายนอกร่างกายเป็นสิ่งที่ต้องดำเนินการในการประเมินความปลอดภัยของเครื่องมือแพทย์และวัสดุที่ใช้ทำ เพื่อให้มีการผลิตเครื่องมือแพทย์ที่มีคุณภาพและมีความปลอดภัยต่อผู้ใช้งาน จึงกำหนดมาตรฐานผลิตภัณฑ์อุตสาหกรรม การประเมินทางชีวภาพของเครื่องมือแพทย์ เล่ม 5 : การทดสอบภายนอกร่างกายเพื่อประเมินความเป็นพิษต่อเซลล์ขึ้น

มาตรฐานผลิตภัณฑ์อุตสาหกรรมนี้กำหนดขึ้นโดยรับ ISO 10993-5 : 1999 Biological evaluation of medical devices-Part 5 : Tests for *in vitro* cytotoxicity มาใช้ในระดับเหมือนกันทุกประการ (identical) โดยใช้ ISO ฉบับภาษาอังกฤษเป็นหลัก

มาตรฐานผลิตภัณฑ์อุตสาหกรรมนี้กำหนดขึ้นเพื่อให้ทันกับความต้องการของผู้ใช้ และจักได้แปลเป็นภาษาไทย ในโอกาสอันควร หากมีข้อสงสัยโปรดติดต่อสอบถามที่สำนักงานมาตรฐานผลิตภัณฑ์อุตสาหกรรม

คณะกรรมการมาตรฐานผลิตภัณฑ์อุตสาหกรรมได้พิจารณามาตรฐานนี้แล้ว เห็นสมควรเสนอรัฐมนตรีประกาศตาม มาตรา 15 แห่งพระราชบัญญัติมาตรฐานผลิตภัณฑ์อุตสาหกรรม พ.ศ. 2511



ประกาศกระทรวงอุตสาหกรรม

ฉบับที่ 3908 (พ.ศ. 2551)

ออกตามความในพระราชบัญญัติมาตรฐานผลิตภัณฑ์อุตสาหกรรม

พ.ศ. 2511

เรื่อง กำหนดมาตรฐานผลิตภัณฑ์อุตสาหกรรม

การประเมินทางชีวภาพของเครื่องมือแพทย์

เล่ม 5 : การทดสอบภายนอกร่างกายเพื่อประเมินความเป็นพิษต่อเซลล์

อาศัยอำนาจตามความในมาตรา 15 แห่งพระราชบัญญัติมาตรฐานผลิตภัณฑ์อุตสาหกรรม พ.ศ.2511 รัฐมนตรีว่าการกระทรวงอุตสาหกรรมออกประกาศกำหนดมาตรฐานผลิตภัณฑ์อุตสาหกรรม การประเมินทางชีวภาพของเครื่องมือแพทย์ เล่ม 5 : การทดสอบภายนอกร่างกายเพื่อประเมินความเป็นพิษต่อเซลล์ มาตรฐานเลขที่ มอก. 2395 เล่ม 5-2551 ไว้ ดังมีรายละเอียดต่อท้ายประกาศนี้

ประกาศ ณ วันที่ 16 ตุลาคม พ.ศ. 2551

พลตำรวจเอก ประชา พรหมนอก

รัฐมนตรีว่าการกระทรวงอุตสาหกรรม

มาตรฐานผลิตภัณฑ์อุตสาหกรรม

การประเมินทางชีวภาพของเครื่องมือแพทย์

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มาตรฐานผลิตภัณฑ์อุตสาหกรรมนี้กำหนดวิธีทดสอบภายนอกร่างกายเพื่อประเมินความเป็นพิษต่อเซลล์ โดยกำหนดการเตรียมตัวอย่างทั้งที่เป็นสารที่สกัดได้จากเครื่องมือแพทย์และที่เป็นตัวอย่างวัสดุทดสอบจากเครื่องมือแพทย์ สายพันธุ์และชนิดของเซลล์ที่ใช้ทดสอบ อาหารสำหรับเพาะเลี้ยงเซลล์ การเตรียมเซลล์ เพาะเลี้ยงไว้ใช้ วิธีทดสอบซึ่งมี 3 วิธี คือ การทดสอบสารที่สกัดได้จากเครื่องมือแพทย์ การทดสอบแบบสัมผัสโดยตรงกับวัสดุทดสอบ และการทดสอบแบบสัมผัสทางอ้อมกับวัสดุทดสอบ นอกจากนี้ยังกำหนดการแปลผล ความเป็นพิษต่อเซลล์ การรายงานผล และการประเมินผล

รายละเอียดให้เป็นไปตาม ISO 10993-5 : 1999

Introduction

Due to the general applicability of *in vitro* cytotoxicity tests and their widespread use in evaluating a large range of medical devices and materials, it is the purpose of this part of ISO 10993, rather than to specify a single test, to define a scheme for testing which requires decisions to be made in a series of steps. This should lead to the selection of the most appropriate test.

Three categories of test are listed: extract test, direct-contact test, indirect-contact test.

The choice of one or more of these categories depends upon the nature of the sample to be evaluated, the potential site of use and the nature of the use.

This choice then determines the details of the preparation of the samples to be tested, the preparation of the cultured cells, and the way in which the cells are exposed to the samples or their extracts.

At the end of the exposure time, the evaluation of the presence and extent of the cytotoxic effect is undertaken. It is the intention of this part of ISO 10993 to leave open the choice of type of evaluation. Such a strategy makes available a battery of tests, which reflects the approach of many groups which advocate *in vitro* biological tests.

The numerous methods used and end-points measured in cytotoxicity determination can be grouped into categories of evaluation type:

- a) assessments of cell damage by morphological means;
- b) measurements of cell damage;
- c) measurements of cell growth;
- d) measurements of specific aspects of cellular metabolism.

There are, therefore, several alternative means of producing results in each of these four categories. The investigator should be aware of the categories of test and into which a particular technique fits, in order that comparisons may be made with other results on similar medical devices or materials, and in order that interlaboratory tests may be conducted.

Biological evaluation of medical devices —

Part 5: Tests for *in vitro* cytotoxicity

1 Scope

This part of ISO 10993 describes test methods to assess the *in vitro* cytotoxicity of medical devices.

These methods specify the incubation of cultured cells either directly or through diffusion

- a) with extracts of a device, and/or
- b) in contact with a device.

These methods are designed to determine the biological response of mammalian cells *in vitro* using appropriate biological parameters.

2 Normative references

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

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

ISO 10993-12:1996, *Biological evaluation of medical devices — Part 12: Sample preparation and reference materials*.

3 Terms and definitions

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

3.1

negative control material

material which, when tested in accordance with this part of ISO 10993, does not produce a cytotoxic response

NOTE The purpose of the negative control is to demonstrate background response. For example, high-density polyethylene¹⁾ for synthetic polymers, and aluminium oxide ceramic rods for dental material, have been used as negative controls.

1) High-density polyethylene can be obtained from the U.S. Pharmacopeia (Rockville, Maryland, USA) and Food and Drug Safety Center, Hatano Research Institute (Ochiai 729-5, Hadanoshi, Kanagawa 257 - Japan). This information is given for the convenience of the user of this part of ISO 10993 and does not constitute an endorsement by ISO of these products.

3.2

positive control material

material which, when tested in accordance with this part of ISO 10993, provides a reproducible cytotoxic response

NOTE The purpose of the positive control is to demonstrate appropriate test system response. For example, an organo-tin stabilized poly(vinylchloride)²⁾ has been used as a positive control for solid materials and extracts. Dilutions of phenol, for example, have been used as a positive control for extracts.

3.3

reagent control

extraction vehicle without test material subjected to extraction conditions and test procedures

NOTE For the purposes of this part of ISO 10993, this definition replaces that given in 3.1 of ISO 10993-12:1996.

3.4

culture vessels

vessels appropriate for cell culture, including glass Petri dishes, plastic culture flasks or plastic multiwells and microtitre plates

NOTE These vessels may be used interchangeably in these methods provided that they meet the requirements of tissue culture grade and are suitable for use with mammalian cells.

3.5

subconfluency

approximately 80 % confluency, i.e. the end of the logarithmic phase of growth

4 Sample preparation

4.1 General

The test shall be performed on either

- a) an extract of the material; and/or
- b) the material itself.

Sample preparation shall be in accordance with ISO 10993-12.

4.2 Preparation of liquid extracts of material

4.2.1 Principles of extraction

Extraction conditions should attempt to simulate or exaggerate the conditions of clinical use so as to determine the potential toxicological hazard, without causing significant changes in the test material such as fusion, melting or alteration of the chemical structure.

NOTE The concentration of any endogenous or extraneous substances in the extract, and hence the amount of exposure to the test cells, depends on the interfacial area, extraction volume, pH, chemical solubility, diffusion rate, osmolarity, agitation, temperature, time and other factors.

2) Organo-tin poly(vinyl chloride) positive control material is available from SIMS Portex Ltd, Hythe, Kent, CT21 6JL, UK, (product No 499-300-000). The ZDEC and ZDBC polyurethanes are available from Food and Drug Safety Center, Hatano Research Institute, (Ochiai 729-5, Hadanoshi, Kanagawa 257, Japan). This information is given for the convenience of the user of this part of ISO 10993 and does not constitute an endorsement by ISO of the product.

4.2.2 Extraction vehicle

For mammalian cell assays, one or more of the following solvents shall be used. The choice of the extraction vehicle(s) shall be justified:

- a) culture medium with serum;
- b) culture medium without serum;
- c) physiological saline solution;
- d) other suitable solvent.

NOTE The choice of solvent should reflect the aim of the extraction and consideration shall be given to the use of both a polar and a nonpolar solvent. Suitable solvents include purified water, vegetable oil and dimethyl sulfoxide (DMSO). DMSO is known to be cytotoxic in selected assay systems at greater than 0,5 % (volume fraction) concentrations.

4.2.3 Extraction conditions

4.2.3.1 The extraction shall be performed in sterile, chemically inert closed containers using aseptic techniques, in general accordance with ISO 10993-12.

4.2.3.2 Recommended extraction conditions are:

- a) not less than 24 h at $(37 \pm 2) ^\circ\text{C}$;
- b) (72 ± 2) h at $(50 \pm 2) ^\circ\text{C}$;
- c) (24 ± 2) h at $(70 \pm 2) ^\circ\text{C}$;
- d) $(1 \pm 0,2)$ h at $(121 \pm 2) ^\circ\text{C}$.

The recommended conditions may be applied according to the device characteristics and specific conditions for use.

Extraction procedures using culture medium with serum can only be used under the conditions specified in 4.2.3.2 a).

4.2.3.3 If the extract is filtered, centrifuged or processed by other methods prior to being applied to the cells, this shall be included in the final report (see clause 9). Any pH adjustment of the extract shall be reported. Manipulation of the extract, such as by pH adjustment, could influence the result.

4.3 Preparation of material for direct-contact tests

4.3.1 Materials that have various shapes, sizes or physical states (i.e. liquid or solid) may be tested without modification in the cytotoxicity assays.

The preferred sample of a solid specimen should have at least one flat surface.

Adjustments shall be made for other shapes and physical states.

4.3.2 Sterility of the test specimen shall be taken into account.

4.3.2.1 Test materials from sterilized devices shall be handled aseptically throughout the test procedure.

4.3.2.2 Test materials from devices which are normally supplied non-sterile but are sterilized before use shall be sterilized by the method recommended by the manufacturer and handled aseptically throughout the test procedure.

The effect of sterilization methods or agents on the device should be considered in defining the preparation of the test material prior to use in the test system.

4.3.2.3 Test materials from devices not required to be sterile in use shall be used as supplied and handled aseptically throughout the test procedure.

4.3.3 Liquids shall be tested by either

- a) direct deposition; or
- b) deposition onto a biologically inert absorbent matrix.

NOTE Filter discs have been found to be suitable.

4.3.4 If appropriate, materials classed as superabsorbent shall be soaked with culture medium prior to testing to prevent sorption of the culture medium in the testing vessel.

5 Cell lines

5.1 Established cell lines are preferred and where used shall be obtained from recognized repositories³⁾.

5.2 Where specific sensitivity is required, primary cell cultures, cell lines and organo-typic cultures obtained directly from living tissues shall only be used if reproducibility and accuracy of the response can be demonstrated.

5.3 If a stock culture of a cell line is stored, storage shall be at -80°C or below in the corresponding culture medium but containing a cryoprotectant, e.g. dimethylsulfoxide or glycerol. Long-term storage (several months up to many years) is only possible at -130°C or below.

5.4 Only cells free from mycoplasma shall be used for the test. Before use, stock cultures should be tested for the absence of mycoplasma.

6 Culture medium

6.1 The culture medium shall be sterile.

6.2 The culture medium with or without serum shall meet the growth requirements of the selected cell line.

NOTE Antibiotics may be included in the media provided that they do not adversely affect the assays.

The stability of the culture medium varies with the composition and storage conditions. Media containing serum and glutamine shall be stored at 2°C to 8°C for no more than one week. Glutamine-containing media without serum shall be stored at 2°C to 8°C for no more than two weeks.

6.3 The culture medium shall be maintained at a pH of between 7.2 and 7.4.

7 Preparation of cell stock culture

7.1 Using the chosen cell line and culture medium, prepare sufficient cells to complete the test. If the cells are to be grown from cultures taken from storage, remove the cryoprotectant, if present. Subculture the cells at least once before use.

3) For example, recommended cell lines are American Type Culture Collection CCL 1 (NCTC clone 929), CCL 163 (Balb/3T3 clone A31), CCL 171 (MRC-5) and CCL 75 (WI-38), CCL 81 (Vero) and CCL 10 [(BHK-21 (C-13)] and V-79 379A. This information is given for the convenience of the user of this part of ISO 10993 and does not constitute an endorsement by ISO of the products named. Other cell lines may be used if they can be shown to lead to the same or more relevant results.

7.2 Remove and resuspend the cells by enzymatic and/or mechanical disaggregation, using a method appropriate for the cell line.

8 Test procedures

8.1 Number of replicates

A minimum of three replicates shall be used for test samples and controls.

8.2 Test on extracts

8.2.1 This test allows both qualitative and quantitative assessment of cytotoxicity.

8.2.2 Pipette an aliquot of the continuously stirred cell suspension into each of a sufficient number of vessels for exposure to the extracts. Distribute the cells evenly over the surface of each vessel by gentle rotation.

8.2.3 Incubate the cultures at $(37 \pm 2) ^\circ\text{C}$ in air with or without 5 % (volume fraction) carbon dioxide as appropriate for the buffer system chosen for the culture medium.

The test should be performed on a subconfluent monolayer or on freshly suspended cells.

In the colony-forming assay, only an appropriate low cell density shall be used.

8.2.4 Verify the subconfluency and the morphology of the cultures with a microscope before starting the test.

8.2.5 Perform the test on

- a) the original extract; and
- b) a dilution series of the extract, using the culture medium as diluent.

If monolayers are used for the test, remove and discard the culture medium from the cultures and add an aliquot of the extract or dilution thereof into each of the vessels.

If suspended cells are used for the test, add the extract or dilution thereof into each of the replicate vessels immediately after preparation of the cell suspension.

8.2.6 When a non-physiological extract is used, e.g. water, the extract shall be tested at the highest physiologically compatible concentration after dilution in culture medium.

NOTE Concentrated culture medium, e.g. 2×, 5×, is recommended for use in diluting aqueous extracts.

8.2.7 Add known aliquots of the reagent blank and the negative and positive controls into additional replicate vessels.

NOTE A fresh culture medium control may also be tested, if appropriate.

8.2.8 Incubate the vessels, using the same conditions as described in 8.2.3, for an appropriate interval corresponding to the selected specific assay.

8.2.9 After an incubation period of at least 24 h, determine the cytotoxic effects in accordance with 8.5.

8.3 Test by direct contact

8.3.1 This test allows both qualitative and quantitative assessment of cytotoxicity.

8.3.2 Pipette a known aliquot of the continuously stirred cell suspension into each of a sufficient number of vessels for direct exposure to the test sample. Distribute the cells evenly over the surface of each vessel by gentle horizontal rotation.

8.3.3 Incubate the culture at $(37 \pm 2) ^\circ\text{C}$ in air with or without 5 % (volume fraction) carbon dioxide as appropriate for the buffer system chosen for the culture medium until the cultures have grown to subconfluency.

8.3.4 Verify the subconfluency and the morphology of the cultures with a microscope before starting the test.

8.3.5 Remove and discard the culture medium. Then add fresh culture medium to each vessel.

8.3.6 Carefully place individual specimens of the test sample on the cell layer in the centre of each replicate vessel. Ensure that the specimen covers approximately one-tenth of the cell layer surface.

Exercise care to prevent unnecessary movement of the specimens, as this could cause physical trauma to the cells, for example patches of dislodged cells.

NOTE When appropriate, the specimen can be placed in the culture vessel prior to the addition of the cells.

8.3.7 Prepare replicate vessels for both the negative-control and positive-control materials.

8.3.8 Incubate the vessels, under the same conditions as in 8.3.3, for an appropriate interval (a minimum of 24 h) corresponding to the selected specific assay.

8.3.9 Discard the supernatant culture medium and determine the cytotoxic effects in accordance with 8.5.

8.4 Test by indirect contact

8.4.1 Agar diffusion test

8.4.1.1 This test allows a qualitative assessment of cytotoxicity. This assay is not appropriate for leachables that cannot diffuse through the agar layer, or that should react with agar.

8.4.1.2 Pipette a known aliquot of the continuously stirred cell suspension into each of a sufficient number of replicate vessels for the test. Distribute the cells evenly over the surface of each vessel by gentle horizontal rotation.

8.4.1.3 Incubate the cultures at $(37 \pm 2) ^\circ\text{C}$ in air with or without 5 % (volume fraction) carbon dioxide as appropriate for the buffer system chosen for the culture medium until the cultures have grown to approximate confluence at the end of the logarithmic phase of the growth curve.

8.4.1.4 Verify the subconfluency and the morphology of the cultures with a microscope before starting the test.

8.4.1.5 Remove and discard the culture medium from the vessel. Then mix fresh culture medium containing serum with melted agar to obtain a final mass concentration of agar of 0.5 % to 2 %, and pipette an appropriate volume into each vessel. Use only agar that is suitable for the growth of mammalian cells in culture. The agar culture medium mixture should be in a liquid state and at a temperature that is compatible with mammalian cells.

NOTE Agar is available in various molecular weight ranges and purities.

8.4.1.6 Carefully place replicate specimens of the test sample on the solidified agar layer in each vessel. Ensure that the specimen covers approximately one-tenth of the cell layer surface.

Presoak any absorbent material with the culture medium before placing it on the agar to prevent dehydration of the agar.

8.4.1.7 Prepare replicate vessels with both negative-control and positive-control specimens.

8.4.1.8 Incubate the vessels, using the same conditions as described in 8.4.1.3, for between 24 h and 72 h.

8.4.1.9 Examine the cells to determine cytotoxic effects before and after carefully removing the specimens from the agar.

Use of a vital stain, e.g. neutral red, may aid in the detection of cytotoxicity. The vital stain may be added before or after the incubation with the specimen. If the stain is added before the incubation, protect the cultures from light to prevent cell damage elicited by photoactivation of the stain.

8.4.2 Filter diffusion test

8.4.2.1 This test allows a qualitative assessment of cytotoxicity.

8.4.2.2 Place a surfactant-free filter of pore size $0.45\ \mu\text{m}$ into each vessel, and add a known aliquot of the continuously stirred cell suspension into each of a sufficient number of replicate vessels for the test. Distribute the cells evenly over the surface of each filter by gentle rotation.

8.4.2.3 Incubate the cultures at $(37 \pm 2)^\circ\text{C}$ in air with or without 5 % (volume fraction) carbon dioxide as appropriate for the buffer system chosen for the culture medium until the cultures have grown to approximate confluence at the end of the logarithmic phase of the growth curve.

8.4.2.4 Verify the subconfluency and the morphology of the cultures with a microscope before starting the test.

8.4.2.5 Remove and discard the culture medium from the vessels. Then transfer the filters, cell-side down, on to a layer of solidified agar (see 8.4.1.6).

8.4.2.6 Carefully place the replicate specimens of the test sample on the acellular (top) side of the filter. Retain liquid extracts and freshly mixed compounds in non-reactive rings placed on the filter.

8.4.2.7 Prepare replicate filters with both the negative-control and positive-control specimens.

8.4.2.8 Incubate the vessels, using the same conditions described in 8.4.2.3, for $2\ \text{h} \pm 10\ \text{min}$.

8.4.2.9 Carefully remove the specimens from the filter and carefully separate the filter from the agar surface.

8.4.2.10 Determine the cytotoxic effects using an appropriate stain procedure.

8.5 Determination of cytotoxicity

8.5.1 Determine cytotoxic effects by either qualitative or quantitative means.

- a) **Qualitative evaluation:** examine the cells microscopically, using cytochemical stain if desired. Assess changes in, for example, general morphology, vacuolization, detachment, cell lysis and membrane integrity. The change from normal morphology shall be recorded in the test report descriptively or numerically. A useful way to grade test materials is presented below.

Cytotoxicity scale	Interpretation
0	Noncytotoxic
1	Mildly cytotoxic
2	Moderately cytotoxic
3	Severely cytotoxic

The method of evaluation and the results of the evaluation shall be included in the test report.

- b) **Quantitative evaluation:** measure the parameter cell death, inhibition of cell growth, cell proliferation or colony formation. The number of cells, amount of protein, release of enzymes, release of vital dye, reduction of vital dye or other measurable parameter may be quantified by objective means. The objective measure and response shall be recorded in the test report.

NOTE For particular methods of determining cytotoxicity, a zero time or baseline cell culture control may be necessary.

8.5.2 Ensure that care is taken in the choice of evaluation methods, as the test results may be invalid if the test specimen releases substances that interfere with the test system or measurement.

NOTE Materials that may release formaldehyde can only be reliably tested when cell viability is evaluated.

8.5.3 If there are evident differences in the test result for replicate culture vessels, then the test is either inappropriate or invalid.

8.5.4 If the negative, positive and any other controls (reference, medium, blank, reagent, etc.) do not have the expected response in the test system, then repeat the assay(s).

9 Test report

The test report shall include the following details:

- a) description of the sample;
- b) cell line and justification of the choice;
- c) culture medium;
- d) assay method and rationale;
- e) extraction procedure (if appropriate) and if possible the nature and concentration of the leached substance(s);
- f) negative, positive and other controls;
- g) cell response and other observations; and
- h) any other relevant data necessary for the assessment of results.

10 Assessment of results

The overall assessment of the results shall be carried out by a person capable of making informed decisions based on the test data. If the results are inconsistent among replicates or invalid, the tests shall be repeated.