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Optics and optical instruments — Contact lenses — Determination of cytotoxicity of contact lens material —

Part 1:
Agar overlay test and growth inhibition test

Optique et instruments d'optique — Lentilles de contact — Détermination de la cytotoxicité des matériaux des lentilles de contact —

Partie 1: Essai de recouvrement par de l'agar-agar et essai d'inhibition de croissance

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Foreword

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

International Standard ISO 9363-1 was prepared by Technical Committee ISO/TC 172, *Optics and optical instruments*, Subcommittee SC 7, *Ophthalmic, endoscopic, metrological instruments and test methods*.

Annex A forms an integral pat of this part of ISO 9363. Annex B is for information only.

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Optics and optical instruments — Contact lenses — Determination of cytotoxicity of contact lens material —

Part 1:
Agar overlay test and growth inhibition test

1 Scope

This part of ISO 9363 specifies two *in vitro* methods for determining the cytotoxicity of contact lens materials:

— the agar overlay test; and

— the growth inhibition test.

The primary purpose of these tests is to reveal the presence of leachable cytotoxic substances in or on contact lenses.

NOTES

1 Attention is drawn to ISO 10993-5.

2 A minimum of one *in vitro* test is recommended for preclinical evaluation of new types of contact lenses. Either one of the following two *in vitro* tests may be used for the *in vitro* requirement.

2 Principle

The proposed tests are designed to ascertain the absence of extractable cytotoxic substances.

The agar overlay test is designed to assess the presence of leachable toxic substances in solid materials. The test sample is placed in contact with the surface of an agar layer which covers a monolayer of cells treated with a vital stain. After 24 h of incubation, the presence of leachable toxic substances is manifested by the loss of dye from the cells within the diffusion zone of the soluble substance(s) leaching from the sample and by lysis of the cells within the zone if the concentrations and toxicity of the diffusing substance(s) are sufficiently high.

The growth inhibition test is designed to ascertain the presence of extractable cytotoxic substances. The growth rate of mammalian cells is significantly decreased in the presence of toxic substances. Usually, the growth rate is determined by comparing the cell number or the protein content of cells at different time intervals. Since there is a linear relationship between cell number and protein concentration under conditions of the assay to be described, the growth rate is determined by protein measurement. Cell counting can be used as an alternative method of assessment.

In the growth inhibition test, medium extracts of contact lenses are added to the culture medium of cells and the protein content of the cell culture after 72 h in the presence of the extract is compared with the protein content in cell cultures without the extract.

In order to ensure high quality work, the cytotoxicity testing of contact lenses should be carried out in experienced laboratories according to GLP guidelines. The overall assessment of the results should be carried out by an expert in the field of toxicology who is informed about the final product and the conditions of its use and has appropriate chemical and biological data concerning it.

3 Agar overlay test

3.1 Apparatus and solutions

3.1.1 Apparatus

Standard tissue culture facilities, including sterilization equipment (autoclave and membrane filtration), laminar airflow hood, 37 °C carbon dioxide-air

incubator, water bath, tissue culture glass and plastics ware.

3.1.2 Culture medium

The culture medium shall be sterile.

NOTE 3 To ensure this, the culture medium may be purchased sterile and ready for use, may be prepared from sterile ingredients using aseptic techniques, or, when one or more ingredients are not available in sterile form, may be sterilized after preparation by membrane filtration.

The complete culture medium shall be Dulbecco's Modified Eagle Medium containing 3,7 g/l sodium hydrogen carbonate, 10 % by volume fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin or any other culture medium which can be demonstrated to give reproducible results five times.

3.1.3 Agar medium

The agar medium shall be comprised of one part double concentration of sterile complete culture medium (all supplements are double concentration as well) plus one part of sterile 3 g/l agarose or equivalent in bidistilled water or equivalent.

Bring melted agarose to approximately 50 °C and medium to 37 °C in a water bath or both to 42 °C and mix aseptically. Use at 42 °C.

3.1.4 Phosphate buffered saline (PBS), calcium- and magnesium-free

The phosphate buffered saline shall consist of 8,0 g of sodium chloride, 0,2 g of potassium chloride, 2,9 g of disodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), 0,2 g of potassium dihydrogen orthophosphate (KH_2PO_4) dissolved in bidistilled water or equivalent (cell culture grade) to give 1 000 ml of solution. Adjust to pH 7,2, sterilize by an appropriate method. Warm up to 37 °C before use.

3.1.5 Vital stain

The vital stain shall be neutral red or an equivalent vital stain. Neutral red vital stain shall be prepared as follows:

Stock solution: 1,0 g/l neutral red in bidistilled water. Adjust to pH 7,2, sterilize by filtration. Protect from strong light.

Vital stain: 1:10 stock solution in sterile PBS, prepare freshly and protect from strong light.

3.1.6 Trypsin solution

A suitable concentration (0,1 g/l to 0,25 g/l) of trypsin in PBS or other agent to dissociate the cell monolayer shall be used for preparation of cell suspensions.

3.2 Test material

The test material shall be representative of the final product. At least two contact lenses of each sample are necessary.

3.3 Controls

3.3.1 Positive control material

The positive control shall be any material which, when tested by the procedure described in 3.5.2, produces a cytotoxic response.

3.3.2 Negative control material

The negative control shall be only material which, when tested by the procedure described in 3.5.2, is known not to produce a cytotoxic response.

3.4 Cells

One of the following cell lines shall be used:

a) American Type Culture Collection CCL 1, NCTC Clone 929 (connective tissue, mouse), clone of Strain L (referred to hereafter as L 929 cells).

b) Any other cell line, provided that when tested in accordance with this part of ISO 9363 a reproducible toxic titre is obtained for the positive control material and no cytotoxicity is observed for the negative control material and fresh cell culture medium.

NOTES

4 The passage number should be recorded.

5 Stock cultures should be tested for the absence of mycoplasma before use. Test for mycoplasma can be performed in accordance with W. C. Russell *et al.* or any other established method. Only cells free from mycoplasma should be used for the test.

3.5 Test procedure and evaluation

3.5.1 Sample preparation

The contact lenses should be applied directly. The agar overlay test does not require sterile samples, although sterility is desirable.

3.5.2 Procedure

Use L 929 cells from an exponentially growing monolayer culture.

Prepare a working stock of cells. Remove the medium, wash twice with PBS, add approximately 3 ml trypsin solution per 75 cm^2 tissue culture flask and incubate until cells become detached (approximately 3 min at 37 °C with 0,25 % trypsin in PBS).

Stop the enzyme reaction by adding 10 ml of complete culture medium, centrifuge for 10 min at 100 *g* and bring to $2,5 \times 10^5$ cells/ml medium. The viability of the cells shall be more than 75 % as determined by trypan blue staining or any other appropriate method.

Plate 10 ml (4,5 ml) of the adjusted cell suspension into 90 mm (60 mm) diameter disposable Petri dishes and incubate for 24 h at 37 °C in humidified air containing 5 % by volume carbon dioxide.

Aspirate the medium after incubation and add 10 ml (4,5 ml) of agar medium at 42 °C to each Petri dish. Allow the agar medium to solidify (approximately 30 min in the incubator).

Dispense 10 ml (4,5 ml) of freshly prepared vital stain solution onto the solidified agar surface. Incubate the Petri dishes for 30 min at 37 °C in the dark, and aspirate the excess stain solution.

Place two test specimens, together with one negative and one positive control, symmetrically on each of two 90 mm Petri dishes. If 60 mm Petri dishes are used, place one sample per plate and use two separate plates for each control.

For rigid lenses it is recommended that a drop of agar be placed on the agar surface prior to placing the lens, to prevent the lens from shifting position on the agar surface.

For hydrophilic lenses it is recommended that three or four equidistant cuts be made around the circumference of the lens to allow the lens to be placed flat on the agar surface. Incubate for a further 24 h at 37 °C in humidified air containing 5 % by volume carbon dioxide.

3.5.3 Test results

Monitor the response of the stained monolayer by the extent of decolorization (if any) under and around the test sample, using an inverted microscope at 100-fold magnification. Use the scoring system given in annex A.

Reject a plate (for 90 mm plate) or the test (for 60 mm plates), if the cell monolayer under or around the negative control has lost colour or if the standard response of the positive control is not observed.

Neutral red is a redox dye which particularly concentrates in the lysosomes of living viable cells. Decolorization of cells is a first sign of cell damage and precedes detachment from the substrate and cell lysis. Depending on the water solubility and concentration of toxic substances of low molecular mass in the specimen, those cells under and around the sample are decolorized (zone-index). Frequently, a decolorization of the cells directly under the sample is observed but without cell lysis. However, a material is judged as "cytotoxic" only if lysis is observed simultaneously. If a material releases highly diffusible cytotoxic compounds in a low concentration, decolorization of the cells without lysis is possible. Therefore, a zone-index of 2 or higher without cell lysis is also considered a significant reaction. In any case, the reaction-index shall be given and the result has to be interpreted.

NOTE 6 Hydrophylic contact lens materials can passively absorb dye, giving a discoloration which would not be due to cytotoxic effects.

3.5.4 General considerations

A positive response index of 1/1 or higher is a definite indication of the presence of a diffusible toxic substance in the sample.

3.5.5 Assessment of results

The overall assessment of the test results shall be carried out by an expert in the field of toxicology familiar with this type of test system. If the toxicologist considers the results to be inconclusive or invalid, the test shall be repeated using new test materials and, if necessary, an alternative cell line and/or culture medium.

4 Growth inhibition test (measured by protein determination)

4.1 Apparatus and solutions

4.1.1 Apparatus

Standard tissue culture facilities, including sterilization equipment (autoclave and membrane filtration), laminar airflow hood, 37 °C carbon dioxide air incubator, water bath, tissue culture glass and plastic ware.

4.1.2 Culture medium

The culture medium shall be sterile.

NOTE 7 To ensure this, the culture medium may be purchased sterile and ready for use, may be prepared from

sterile ingredients using aseptic techniques, or, when one or more ingredients are not available in sterile form, may be sterilized after preparation by membrane filtration.

The complete culture medium shall be Dulbecco's Modified Eagle Medium containing 3,7 g/l sodium hydrogen carbonate, 10 % by volume fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin or any other culture medium which can be demonstrated to give reproducible results five times.

4.1.3 Phosphate buffered saline (PBS), calcium- and magnesium-free

The phosphate buffered saline shall consist of 8,0 g of sodium chloride, 0,2 g of potassium chloride, 2,9 g of disodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), 0,2 g of potassium dihydrogen orthophosphate (KH_2PO_4) dissolved in bidistilled water or equivalent (cell culture grade) to give 1 000 ml of solution. Adjust to pH 7,2, sterilize by an appropriate method and store at 4 °C. Warm up to 37 °C before use.

4.1.4 Trypsin solution

A suitable concentration (0,1 g/l to 0,25 g/l) of trypsin in PBS or appropriate cell disintegration agent shall be used for preparation of cell suspensions.

4.1.5 Lowry solutions

Solution A: 20,0 g sodium carbonate
4,0 g sodium hydroxide
to 1 000 ml distilled water or equivalent.

Solution B: 2,0 g sodium-potassium-tartrate
to 1 000 ml distilled water or equivalent.

Solution C: 2,0 g copper sulfate
to 1 000 ml distilled water or equivalent.

Solution D: Mix solution B and solution C in the ratio 1:1 by volume.

Solution E: 98 parts of solution A
2 parts of solution D
Mix freshly before use.

Solution F: 1 part Folin-Ciocalteus phenol reagent
1 part distilled water or equivalent
Mix freshly before use.
Control each new batch of phenol reagent with a bovine serum albumin standard curve.

4.2 Test materials

6 cm^2 of contact lens surfaces for extraction with 1 ml of culture medium. 6 ml of extract per test are necessary.

NOTES

8 Both polar and non-polar extractions are recommended.

9 Good results have been obtained by the application of an additional solvent extraction, for example 20 cm per 1 ml of dimethylsulfoxide (DMSO).

4.3 Controls

The culture medium is used as the negative control. Material which gives a reproducible cytotoxic response shall be treated as the positive control.

4.4 Cells

Cell lines as specified in 3.4 shall be used.

4.5 Test procedure and evaluation

4.5.1 Preparation of extracts

Test material from pre-sterilized devices shall be handled aseptically throughout the extraction and test procedure. Test material from devices which are normally supplied non-sterile, but are sterilized before use, shall be sterilized before the extraction procedure.

Test materials from devices not required to be sterile in use shall be used as supplied. Materials shall be handled aseptically throughout the extraction and test procedure. If, in the case of non-sterile devices, extracts are filtered sterile prior to application, this shall be recorded in the final report.

Extract 36 cm^2 of contact lens material with 6 ml of complete culture medium for at least 24 h at 37 °C.

When using DMSO as a solvent, extract 20 cm^2 of contact lens material with 1 ml of DMSO for at least 24 h at 37 °C. Add to 100 ml of complete medium.

Subsequent to extraction, dilute the extracts with complete medium to 30 %, 10 % and 3 % by volume.

Likewise prepare the extract of the positive control material but use it only undiluted. For negative control, incubate culture medium without test material under identical conditions.

4.5.2 Preparation of cell cultures

Use L 929 cells from an exponentially growing monolayer culture.

Remove the medium, wash twice with PBS, add 3 ml of trypsin solution per 75 cm^2 tissue culture flask and incubate for approximately 3 min at 37 °C. Support cell disintegration by rocking the flask after incubation.

Stop enzyme reaction by adding 10 ml of complete culture medium, centrifuge for 10 min at 100 *g* and bring to $2,0 \times 10^5$ cells/ml medium. Viability of the cells shall be more than 75 % as determined, for example, by trypan blue dye exclusion.

Use multiwell plates (24 wells with an area for all growth of 2 cm^2 each). Three wells each shall be used for the

— negative control at the beginning of the experiment (C_0)

— negative control (protein content of the cell culture after 72 h of incubation) (C_{72})

— extract of the specimen (100 %) to be tested after 72 h ($T_{100\ \%}$)

— extract of the specimen (30 %) to be tested after 72 h ($T_{30\ \%}$)

— extract of the specimen (10 %) to be tested after 72 h ($T_{10\ \%}$)

— extract of the specimen (3 %) to be tested after 72 h ($T_{3\ \%}$)

— extract of the positive control material (C_p)

Test medium extracts of one sample by adding 1,0 ml of extract (undiluted and dilutions) into 3 wells each (T), 1,0 ml of preincubated medium into each of 3 wells for C_0 and C_{72}, 1,0 ml of positive control extract into 3 wells (C_p) and 0,2 ml of the adjusted cell suspension into each of the wells.

Incubate the multiwell plates at 37 °C in humidified air containing 5 % by volume carbon dioxide. After 1 h aspirate the medium from the wells of the control (C_0) and wash three times with PBS. Assay the control cells for protein content. Continue the incubation of the multiwell plates for 72 h. Then aspirate the supernatants and wash the cell layers three times with PBS at 37 °C.

Perform the protein determination according to Lowry as follows: Add 2,0 ml of Lowry solution E to each well and keep the multiwell plates in the dark for 1 h at room temperature. Then dispense 0,2 ml of Lowry solution F into each well, mix thoroughly and continue incubation in the dark for a further 30 min. Measure the optical density at 660 nm immediately after this incubation time.

NOTES

10 If required, proper dilution should be done one hour after adding Lowry solution E by adding more Lowry solution E before adding Lowry solution F.

11 The optical density is proportional to the protein content of the wells. Cell counting with a hemocytometer can be done as an alternative to protein determination.

4.5.3 Test results

Since there is a linear relationship between cell number and protein concentration under conditions of the assay, the percentage of growth inhibition, % GI, of the cells in the presence of the extract is calculated using the following formula:

$$\%\ GI = 100 - 100 \times \frac{A_{660}(T) - A_{660}(C_0)}{A_{660}(C_{72}) - A_{660}(C_0)}$$

where

$A_{660}(T)$ is the optical density at 660 nm in the presence of the extract or dilution;

$A_{660}(C_0)$ is the optical density at 660 nm of the negative control at the beginning of the experiment;

$A_{660}(C_{72})$ is the optical density at 660 nm in the presence of the negative control after 72 h of incubation;

$A_{660}(C_p)$ is the density at 660 nm in the presence of the positive control material.

The growth inhibition of the positive control material is calculated by substituting (C_p) for (T) in the formula.

4.5.4 General considerations

A sample is reported as cytotoxic if an unequivocal dose/dilution response curve is observed and at least one test dilution shows a cytotoxic response. Under the test conditions described, an undiluted test sample giving a growth inhibition of more than 30 % is considered cytotoxic.

NOTE 12 This is statistically significant.

The test shall be repeated if a standard response of the medium control or the positive control is not observed.

4.5.5 Assessment of results

The overall assessment of the test results shall be carried out by an expert in the field of toxicology. If the expert considers the results to be inconclusive or invalid, the test shall be repeated using new extracts and, if necessary, an alternative cell line and/or culture medium.

Annex A
(normative)

Scoring system

A.1 Zone index (Z)

Zone index, Z	Zone description
0	No detectable zone around or under sample.
1	Zone limited to the area directly under the sample.
2	Zone not greater than 5 mm around the sample.
3	Zone not greater than 10 mm around the sample.
4	Zone greater than 10 mm around the sample but not covering entire plate.
5	Zone covering entire plate.

A.2 Lysis index (L)

The extent of lysis of the cells (if any) within the zone of decolorization is rated as follows:

Lysis index, L	Description of lysis extent
0	No observable lysis.
1	Up to 20 % of cells lysed.
2	Between 20 % and 40 % of the cells lysed.
3	Between 40 % and 60 % of the cells lysed.
4	Between 60 % and 80 % of the cells lysed.
5	More than 80 % of the cells lysed.

A.3 Response index (R)

The response is reported in terms of the "response index" R (= Z/L).

Evaluation	Interpretation	Response index, R (mean of two samples)
0	Non-cytotoxic.	0/0 to 0,5/0,5[1] or 1/0
1	Slightly cytotoxic.	1/1 to 1,5/1,5
2	Moderately cytotoxic.	2/2 to 3/3
3	Strongly cytotoxic.	4/4 to 5/5

1) If a response index 0,5/0,5 is obtained from the average of 2 tests with results 1/1 and 0/0 the test shall be repeated.

Annex B
(informative)

Bibliography

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