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⟨87⟩ BIOLOGICAL REACTIVITY TESTS, IN VITRO

The following tests are designed to determine the biological reactivity of mammalian cell cultures following contact with the elastomeric plastics and other polymeric materials with direct or indirect patient contact or of specific extracts prepared from the materials under test. It is essential that the tests be performed on the specified surface area. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Exercise care in the preparation of the materials to prevent contamination with microorganisms and other foreign matter.

Three tests are described (i.e., the *Agar Diffusion Test*, the *Direct Contact Test*, and the *Elution Test*).[1] The decision as to which type of test or the number of tests to be performed to assess the potential biological response of a specific sample or extract depends upon the material, the final product, and its intended use. Other factors that may also affect the suitability of a sample for a specific use are the polymeric composition; processing and cleaning procedures; contacting media; inks; adhesives; absorption, adsorption, and permeability of preservatives; and conditions of storage. Evaluation of such factors should be made by appropriate additional specific tests before determining that a product made from a specific material is suitable for its intended use. Materials that fail the *in vitro* tests are candidates for the *in vivo* tests described in *Biological Reactivity Tests, In Vivo* ⟨88⟩.

PROCEDURES

• Test Control

Positive control: Polyurethane film containing zinc diethyldithiocarbamate (ZDEC)[2] or zinc dibutyldithiocarbamate (ZDBC)

Cell culture preparation: Prepare multiple cultures of L-929 (ATCC cell line CCL 1, NCTC clone 929; alternative cell lines obtained from a standard repository may be used with suitable validation) mammalian fibroblast cells in serum-supplemented minimum essential medium having a seeding density of about 10^5 cells per mL. Incubate the cultures at 37 ± 1° in a humidified incubator for NLT 24 h in a 5 ± 1% carbon dioxide atmosphere until a monolayer, with greater than 80% confluence, is obtained. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers. [Note—The reproducibility of the in vitro biological reactivity tests depends upon obtaining uniform cell culture density.]

Extraction solvents: *Sodium Chloride Injection* [see monograph—use *Sodium Chloride Injection* containing 0.9% of sodium chloride (NaCl)]. Alternatively, serum-free mammalian cell culture media or serum-supplemented mammalian cell culture media may be used. Serum supplementation is used when extraction is done at 37° for 24 h.

• Apparatus

Autoclave: Employ an autoclave capable of maintaining a temperature of 121 ± 2°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20°, but not below 20°, immediately following the heating cycle.

Oven: Use an oven, preferably a mechanical convection model, that will maintain operating temperatures in the range of 50°–70° within ±2°.

Incubator: Use an incubator capable of maintaining a temperature of 37 ± 1° and a humidified atmosphere of 5 ± 1% carbon dioxide in air.

Extraction containers: Use only containers, such as ampuls or screw-cap culture test tubes, or their equivalent, of Type I glass. If used, culture test tubes, or their equivalent, are closed with a screw cap having a suitable elastomeric liner. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 50–75 µm in thickness. A suitable disk can be fabricated from polytef.

Preparation of apparatus: Cleanse all glassware thoroughly with chromic acid cleansing mixture and, if necessary, with hot nitric acid followed by prolonged rinsing with *Sterile Water for Injection*. Sterilize and dry by a suitable process for containers and devices used for extraction, transfer, or administration of test material. If ethylene oxide is used as the sterilizing agent, allow NLT 48 h for complete degassing.

• Procedure

Preparation of sample for extracts: Prepare as directed in the *Procedure* in ⟨88⟩.

Preparation of extracts: Prepare as directed for *Preparation of extracts* in ⟨88⟩ using either *Sodium Chloride Injection* [0.9% sodium chloride (NaCl)] or serum-free mammalian cell culture media as *Extraction solvents*. [Note—If extraction is done at 37° for 24 h in an incubator, use cell culture media supplemented by serum. The extraction conditions should not in any instance cause physical changes, such as fusion or melting of the material pieces, other than a slight adherence.]

• Agar Diffusion Test

This test is designed for elastomeric closures in a variety of shapes. The agar layer acts as a cushion to protect the cells from mechanical damage while allowing the diffusion of leachable chemicals from the polymeric specimens. Extracts of materials that are to

be tested are applied to a piece of filter paper.

Sample preparation: Use extracts prepared as directed, or use portions of the test specimens having flat surfaces NLT 100 mm^2 in surface area.

Positive control preparation: Proceed as directed for *Sample preparation*.

Negative control preparation: Proceed as directed for *Sample preparation*.

Procedure: Using 7 mL of cell suspension prepared as directed in *Cell culture preparation*, prepare the monolayers in plates having a 60-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with serum-supplemented culture medium containing NMT 2% of agar. [Note—The quality of the agar must be adequate to support cell growth. The agar layer must be thin enough to permit diffusion of leached chemicals.] Place the flat surfaces of *Sample preparation*, *Positive control preparation*, and *Negative control preparation* or their extracts in an appropriate extracting medium, in duplicate cultures in contact with the solidified agar surface. Use no more than three specimens per prepared plate. Incubate all cultures for NLT 24 h at 37 ± 1°, preferably in a humidified incubator containing 5 ± 1% of carbon dioxide. Examine each culture around each sample, negative control, and positive control under a microscope, using a suitable stain, if desired.

Interpretation of results: The biological reactivity (cellular degeneration and malformation) is described and rated on a scale of 0–4 (see *Table 1*). Measure the responses of the cell cultures to the *Sample preparation*, the *Positive control preparation*, and the *Negative control preparation*. The cell culture test system is suitable if the observed responses to the *Negative control preparation* is grade 0 (no reactivity) and to the *Positive control preparation* is at least grade 3 (moderate). The sample meets the requirements of the test if the response to the *Sample preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

Table 1. Reactivity Grades for Agar Diffusion Test and Direct Contact Test

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen and less than 0.45 cm beyond specimen
3	Moderate	Zone extends 0.45–1.0 cm beyond specimen
4	Severe	Zone extends greater than 1.0 cm beyond specimen

- **Direct Contact Test**

This test is designed for materials in a variety of shapes. The procedure allows for simultaneous extraction and testing of leachable chemicals from the specimen with a serum-supplemented medium. The procedure is not appropriate for very low- or high-density materials that could cause mechanical damage to the cells.

Sample preparation: Use portions of the test specimen having flat surfaces NLT 100 mm^2 in surface area.

Positive control preparation: Proceed as directed for *Sample preparation*.

Negative control preparation: Proceed as directed for *Sample preparation*.

Procedure: Using 2 mL of cell suspension prepared as directed in *Cell culture preparation*, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the cultures, and replace it with 0.8 mL of fresh culture medium. Place a single *Sample preparation*, a *Positive control preparation*, and a *Negative control preparation* in each of the duplicate cultures. Incubate all cultures for NLT 24 h at 37 ± 1° in a humidified incubator containing 5 ± 1% of carbon dioxide. Examine each culture around each *Sample preparation*, a *Positive control preparation*, and a *Negative control preparation*, under a microscope, using a suitable stain, if desired.

Interpretation of results: Proceed as directed for *Interpretation of results* in *Agar Diffusion Test*. The sample meets the requirements of the test if the response to the *Sample preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

- **Elution Test**

This test is designed for the evaluation of extracts of polymeric materials. The procedure allows for extraction of the specimens at physiological or nonphysiological temperatures for varying time intervals. It is appropriate for high-density materials and for dose-response evaluations.

Sample preparation: Prepare as directed in *Preparation of extracts*, using either *Sodium Chloride Injection* [0.9% sodium chloride (NaCl)] or serum-free mammalian cell culture media as *Extraction solvents*. If the size of the sample cannot be readily measured, a mass of NLT 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per mL of extraction medium may be used. Alternatively, use serum-

supplemented mammalian cell culture media as the extracting medium to simulate more closely physiological conditions. Prepare the extracts by heating for 24 h in an incubator containing 5 ± 1% of carbon dioxide. Maintain the extraction temperature at 37 ± 1°, because higher temperatures may cause denaturation of serum proteins.

Positive control preparation: Proceed as directed for *Sample preparation*.

Negative control preparation: Proceed as directed for *Sample preparation*.

Procedure: Using 2 mL of cell suspension prepared as directed in *Cell culture preparation*, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with extracts of the *Sample preparation*, *Positive control preparation*, or *Negative control preparation*. The serum-supplemented and serum-free cell culture media extracts are tested in duplicate without dilution (100%). The *Sodium Chloride Injection* extract is diluted with serum-supplemented cell culture medium and tested in duplicate at 25% extract concentration. Incubate all cultures for 48 h at 37 ± 1° in a humidified incubator preferably containing 5 ± 1% of carbon dioxide. Examine each culture at 48 h, under a microscope, using a suitable stain, if desired.

Interpretation of results: Proceed as directed for *Interpretation of results* in *Agar Diffusion Test* but use *Table 2*. The sample meets the requirements of the test if the response to the *Sample preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed. For dose-response evaluations, repeat the procedure, using quantitative dilutions of the sample extract.

Table 2. Reactivity Grades for Elution Test

Grade	Reactivity	Conditions of All Cultures
0	None	Discrete intracytoplasmic granules; no cell lysis
1	Slight	Less than or equal to 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present
2	Mild	Greater than 20% to less than or equal to 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells
3	Moderate	Greater than 50% to less than 70% of the cell layers contain rounded cells or are lysed
4	Severe	Nearly complete destruction of the cell layers

ADDITIONAL REQUIREMENTS

- **USP Reference Standards ⟨11⟩**
  USP High-Density Polyethylene RS
  (Negative Control)

[1] Further details are given in the following publications of the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103: Standard test method for agar diffusion cell culture screening for cytotoxicity, ASTM Designation F 895-84; Standard practice for direct contact cell culture evaluation of materials for medical devices, ASTM Designation F 813-83.

[2] ZDEC and ZDBC polyurethanes are available from the Food and Drug Safety Center, Hatano Research Institute, Ochiai 729–5, Hadanoshi, Kanagawa 257, Japan.

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
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