50% to 200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the *Sample Solution* under test is considered to contain interfering factors. Then, repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the *Sample Solution* or diluted *Sample Solution* not to exceed the MVD may be eliminated by suitable validated treatment such as filtration, neutralization, dialysis, or heat treatment. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above, using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

### Test Procedure

Follow the procedure described for *Test for Interfering Factors* under *Preparatory Testing*, immediately above.

### Calculation

Calculate the endotoxin concentration of each of the replicates of *Solution A*, using the standard curve generated by the positive control *Solution C*. The test is considered valid when the following three requirements are met.

1. The results of the control *Solution C* comply with the requirements for validation defined for *Assurance of Criteria for the Standard Curve* under *Preparatory Testing*.
2. The endotoxin recovery, calculated from the concentration found in *Solution B* after subtracting the concentration of endotoxin found in *Solution A*, is within the range of 50% to 200%.
3. The result of the negative control *Solution D* does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

### Interpretation

In photometric assays, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of *Solution A*, after correction for dilution and concentration, is less than the endotoxin limit for the product.

## ⟨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*).* 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 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.

**USP Reference Standards** ⟨11⟩—*USP High-Density Polyethylene RS*. *USP Positive Bioreaction RS*.

**Cell Culture Preparation**—Prepare multiple cultures of L-929 (ATCC cell line CCL 1, NCTC clone 929) mammalian fibroblast cells in serum-supplemented minimum essential medium having a seeding density of about $10^5$ cells per mL. Incubate the cultures at $37 \pm 1°$ in a humidified incubator for not less than 24 hours in a $5 \pm 1\%$ 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 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 hours.

**Apparatus—**

*Autoclave*—Employ an autoclave capable of maintaining a temperature of $121 \pm 2°$, 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° to 70° within $\pm 2°$.

*Incubator*—Use an incubator capable of maintaining a temperature of $37 \pm 1°$ and a humidified atmosphere of $5 \pm 1\%$ 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 to 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 containers and devices used for extraction, transfer, or administration of test material. If ethylene oxide is used as the sterilizing agent, allow not less than 48 hours for complete degassing.

**Procedure—**

*Preparation of Sample for Extracts*—Prepare as directed in the *Procedure* under *Biological Reactivity Tests, In Vivo* ⟨88⟩.

*Preparation of Extracts*—Prepare as directed for *Preparation of Extracts* in *Biological Reactivity Tests, In Vivo* ⟨88⟩ using either Sodium Chloride Injection (0.9% NaCl) or serum-free

* 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.

mammalian cell culture media as *Extraction Solvents.* [NOTE—If extraction is done at 37° for 24 hours 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 not less than 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 under *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 not more than 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, Negative Control Preparation,* and *Positive 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 not less than 24 hours 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 to 4 (see *Table 1*). Measure the responses of the cell cultures to the *Sample Preparation,* the *Negative Control Preparation,* and the *Positive 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 |
| 3 | Moderate | Zone extends 0.5 to 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 not less than 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 under *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 *Negative Control Preparation,* and a *Positive Control Preparation* in each of duplicate cultures. Incubate all cultures for not less than 24 hours at 37 ± 1° in a humidified incubator containing 5 ± 1% of carbon dioxide. Examine each culture around each *Sample, Negative Control,* and *Positive Control Preparation,* either visually or under a microscope, using a suitable stain, if desired.

*Interpretation of Results*—Proceed as directed for *Interpretation of Results* under *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% NaCl) or serum-free mammalian cell culture media as *Extraction Solvents.* If the size of the *Sample* cannot be readily measured, a mass of not less than 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 hours 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 under *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, Negative Control Preparation,* or *Positive 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 hours at 37 ± 1° in a humidified incubator preferably containing 5 ± 1% of carbon dioxide. Examine each culture at 48 hours, under a microscope, using a suitable stain, if desired.

*Interpretation of Results*—Proceed as directed for *Interpretation of Results* under *Agar Diffusion Test* but using *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 | Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present |
| 2 | Mild | Not more than 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells |
| 3 | Moderate | Not more than 70% of the cell layers contain rounded cells or are lysed |
| 4 | Severe | Nearly complete destruction of the cell layers |

# ⟨88⟩ BIOLOGICAL REACTIVITY TESTS, IN VIVO

The following tests are designed to determine the biological response of animals to elastomerics, plastics, and other polymeric material with direct or indirect patient contact, or by the injection of specific extracts prepared from the material under test. It is essential to make available the specific surface area for extraction. 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. Also, it is essential to exercise care in the preparation of the materials to be injected or instilled to prevent contamination with microorganisms and other foreign matter. Three tests are described. The *Systemic Injection Test* and the *Intracutaneous Test* are used for elastomeric materials, especially to elastomeric closures for which the appropriate *Biological Reactivity Tests, In Vitro* ⟨87⟩ have indicated significant biological reactivity. These two tests are used for plastics and other polymers in addition to a third test, the *Implantation Test,* to test the suitability of these materials intended for use in fabricating containers and accessories thereto, for use in parenteral preparations, and for use in medical devices, implants, and other systems.

These three tests are applied to materials or medical devices, if there is a need for classification of plastics and other polymers based on in vivo biological reactivity testing.

For the purpose of this chapter, these definitions apply: the *Sample* is the specimen under test or an extract prepared from such a specimen. A *Blank* consists of the same quantity of the same extracting medium that is used for the extraction of the specimen under test, treated in the same manner as the extracting medium containing the specimen under test. A *Negative Control*[1] is a specimen that gives no reaction under the conditions of the test.

***Change to read:***

## CLASSIFICATION OF PLASTICS

Six Plastic Classes are defined (see *Table 1*). This classification is based on responses to a series of in vivo tests for which extracts, materials, and routes of administration are specified. These tests are directly related to the intended end-use of the plastic articles. The choice of extractants is representative of the vehicles in preparations with which the plastics are likely to be in contact. The *Table 1* classification facilitates communication among suppliers, users, and manufacturers of plastics by summarizing the tests to be performed for containers for injections and medical devices if a need for classification exists.

With the exception of the *Implantation Test*, the procedures are based on the use of extracts that, depending on the heat resistance of the material, are prepared at one of three standard temperatures: 50°, 70°, and 121°. Therefore, the class designation of a plastic must be accompanied by an indication of the temperature of extraction (e.g., IV-121°, which represents a class IV plastic extracted at 121°, or I-50°, which represents a class I plastic extracted at 50°).

Plastics may be classified as USP Plastic Classes I–VI only on the basis of the response criteria prescribed in *Table 1*.

[1] USP High-Density Polyethylene RS.

**Table 1. Classification of Plastics**

| Plastic Classes[a] | | | | | | Tests to be Conducted | | | |
|---|---|---|---|---|---|---|---|---|---|
| I | II | III | IV | V | VI | Test Material | Animal | Dose | Procedure[b] |
| x | x | x | x | x | x | Extract of *Sample* in *Sodium Chloride Injection* | Mouse | 50 mL/kg | A (iv) |
| x | x | x | x | x | x | | Rabbit | 0.2 mL/animal at each of 10 sites | B |
| | x | x | x | x | x | Extract of *Sample* in *1 in 20 Solution of Alcohol in Sodium Chloride Injection* | Mouse | 50 mL/kg | A (iv) |
| | x | x | x | x | x | | Rabbit | 0.2 mL/animal at each of 10 sites | B |
| | | x | | x | x | Extract of *Sample* in *Polyethylene Glycol 400* | Mouse | 10 g/kg | A (ip) |
| | | | | x | x | | Rabbit | 0.2 mL/animal at each of 10 sites | B |
| | | x | x | x | x | Extract of *Sample* in *Vegetable Oil* | Mouse | 50 mL/kg | A (ip) |
| | | | x | x | x | | Rabbit | 0.2 mL/animal at each of 10 sites | B |
| | | | x | | x | Implant strips of *Sample* | Rabbit | 4 strips/animal | C |
| | | | ▲x | | x | Implant *Sample* | Rat | 2 *Samples*/animal | C▲*USP35* |

[a] Tests required for each class are indicated by "x" in appropriate columns.

[b] Legend: A (ip)—Systemic Injection Test (intraperitoneal); A (iv)—Systemic Injection Test (intravenous); B—Intracutaneous Test (intracutaneous); C—Implantation Test (intramuscular ▲or subcutaneous▲*USP35* implantation).