⟨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.

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.

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 or Guinea Pig	0.2 mL/animal at each of 10 or 6 sites	B (IC)
	x	x	x	x	x	Extract of *Sample* in *1 in 20 Solution of Alcohol in Sodium Chloride Injection*	Mouse	50 mL/kg	A (IP)
	x	x	x	x	x		Rabbit or Guinea Pig	0.2 mL/animal at each of 10 or 6 sites	B (IC)
		x		x	x	Extract of *Sample* in *Polyethylene Glycol 400*	Mouse	10 g/kg	A (IP)
				x	x		Rabbit or Guinea Pig	0.2 mL/animal at each of 10 or 6 sites	B (IC)
		x	x	x	x	Extract of *Sample* in *Vegetable Oil*	Mouse	50 mL/kg	A (IP)
			x	x	x		Rabbit or Guinea Pig	0.2 mL/animal at each of 10 or 6 sites	B (IC)
			x		x	Implant strips of *Sample*	Rabbit	4 strips/animal	C
			x		x	Implant *Sample*	Rat	2 *Samples*/animal	C

[a] Tests required for each class are indicated by "x" in appropriate columns.
[b] Legend: A (IP)—Systemic Injection Test (intraperitoneal); B (IC)—Intracutaneous Test (intracutaneous); C—Implantation Test (intramuscular or subcutaneous implantation).

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*.

This classification does not apply to plastics that are intended for use as containers for oral or topical products, or that may be used as an integral part of a drug formulation. *Table 1* does not apply to natural elastomers, which are to be tested in Sodium Chloride Injection and vegetable oils only.

The *Systemic Injection Test* and the *Intracutaneous Test* are designed to determine the systemic and local, respectively, biological responses of animals to plastics and other polymers by the single-dose injection of specific extracts prepared from a

[1] USP High-Density Polyethylene RS.

Sample. The *Implantation Test* is designed to evaluate the reaction of living tissue to the plastic and other polymers by the implantation of the *Sample* itself into animal tissue. The proper preparation and placement of the specimens under aseptic conditions are important in the conduct of the *Implantation Test*.

These tests are designed for application to plastics and other polymers in the condition in which they are used. If the material is to be exposed to any cleansing or sterilization process prior to its end-use, then the tests are to be conducted on a *Sample* prepared from a specimen preconditioned by the same processing.

Factors such as material composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a material for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of a material for its intended use.

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

Extracting Media—

SODIUM CHLORIDE INJECTION (see monograph). Use Sodium Chloride Injection containing 0.9% of NaCl.

1 IN 20 SOLUTION OF ALCOHOL IN SODIUM CHLORIDE INJECTION.

POLYETHYLENE GLYCOL 400 (see monograph).

VEGETABLE OIL—Use freshly refined Sesame Oil (see monograph) or Cottonseed Oil (see monograph) or other suitable vegetable oils.

DRUG PRODUCT VEHICLE (where applicable).

WATER FOR INJECTION (see monograph).

[NOTE—The Sesame Oil or Cottonseed Oil or other suitable vegetable oil meets the following additional requirements. Obtain, if possible, freshly refined oil. Use three properly prepared animals, and inject the oil intracutaneously in a dose of 0.2 mL into each of 10 sites per animal, and observe the animals at 24, 48, and 72 h following injection. Rate the observations at each site on the numerical scale indicated in *Table 2*. For the 3 rabbits or guinea pigs (30 or 18 injection sites), at any observation time, the average response for erythema is not greater than 0.5 and for edema is not greater than 1.0, and no site shows a tissue reaction larger than 10 mm in overall diameter. The residue of oil at the injection site should not be misinterpreted as edema. Edematous tissue blanches when gentle pressure is applied.]

Table 2. Evaluation of Skin Reactions[a]

Erythema and Eschar Formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to slight eschar formation (injuries in depth)	4
Edema Formation[b]	**Score**
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

[a] Draize JH, Woodward G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther* 1944;82:377–390.

[b] Excludes noninflammatory (mechanical) edema from the blank or extraction fluid.

Apparatus—The apparatus for the tests includes the following.

AUTOCLAVE—Use an autoclave capable of maintaining a temperature of 121 ± 2.0°, 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, but not below, 20° immediately following the heating cycle.

OVEN—Use an oven, preferably a forced-circulation model, that will maintain operating temperatures of 50° or 70° within ±2°.

EXTRACTION CONTAINERS—Use only containers, such as ampuls or screw-cap culture test tubes, of Type I glass. If used, culture test tubes are closed with screw caps having suitable elastomeric liners. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 0.05–0.075 mm in thickness. A suitable disk may be fabricated from a polytef resin.

Preparation of Apparatus—Cleanse all glassware thoroughly with chromic acid cleansing mixture, or if necessary, with hot nitric acid, followed by prolonged rinsing with water. Clean cutting utensils by an appropriate method (e.g., successive cleaning with acetone and methylene chloride) prior to use in subdividing a specimen. Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with water.

Render containers and equipment used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process. [NOTE—If ethylene oxide is used as the sterilizing agent, allow adequate time for complete degassing.]

Procedure—

PREPARATION OF SAMPLE—Both the *Systemic Injection Test* and the *Intracutaneous Test* may be performed using the same extract, if desired, or separate extracts may be made for each test. Select and subdivide into portions a *Sample* of the size indicated in *Table 3*. Remove particulate matter, such as lint and free particles, by treating each subdivided *Sample* or *Negative Control* as follows. Place the *Sample* into a clean, glass-stoppered, 100-mL graduated cylinder of Type I glass, and add about 70 mL of *Water for Injection*. Agitate for about 30 s, and drain off the water. Repeat this step, and dry those pieces prepared for the extraction with *Vegetable Oil* in an oven at a temperature not exceeding 50°. [NOTE—Do not clean the *Sample* with a dry or wet cloth or by rinsing or washing with an organic solvent, surfactant, etc.]

Table 3. Surface Area of Specimen To Be Used[a]

Form of Material	Thickness	Amount of Sample for each 20 mL of Extracting Medium	Subdivided into
Film or sheet	<0.5 mm	Equivalent of 120 cm^2 total surface area (both sides combined)	Strips of about 5 × 0.3 cm
	0.5–1 mm	Equivalent of 60 cm^2 total surface area (both sides combined)	
Tubing	<0.5 mm (wall)	Length (in cm) = 120 cm^2/(sum of ID and OD circumferences)	Sections of about 5 × 0.3 cm
	0.5–1 mm (wall)	Length (in cm) = 60 cm^2/(sum of ID and OD circumferences)	
Slabs, tubing, and molded items	>1 mm	Equivalent of 60 cm^2 total surface area (all exposed surfaces combined)	Pieces up to about 5 × 0.3 cm
Elastomers	>1 mm	Equivalent of 25 cm^2 total surface area (all exposed surfaces combined)	Do not subdivide[b]

[a] When surface area cannot be determined due to the configuration of the specimen, use 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 mL of extracting fluid.
[b] Molded elastomeric closures are tested intact.

PREPARATION OF EXTRACTS—Place a properly prepared *Sample* to be tested in an extraction container, and add 20 mL of the appropriate extracting medium. Repeat these directions for each extracting medium required for testing. Also, prepare one 20-mL blank of each medium for parallel injections and comparisons. Extract by heating in an autoclave at 121° for 60 min, in an oven at 70° for 24 h, or at 50° for 72 h. Allow adequate time for the liquid within the container to reach the extraction temperature.

[NOTE—The extraction conditions should not in any instance cause physical changes such as fusion or melting of the *Sample* pieces, which result in a decrease in the available surface area. A slight adherence of the pieces can be tolerated. Always add the cleaned pieces individually to the extracting medium. If culture tubes are used for autoclave extractions with *Vegetable Oil*, seal screw caps adequately with pressure-sensitive tape.]

Cool to about room temperature but not below 20°, shake vigorously for several minutes, and decant each extract immediately, using aseptic precautions, into a dry, sterile vessel. Store the extracts at a temperature of 20°–30°, and do not use for tests after 24 h. Of importance are the contact of the extracting medium with the available surface area of the plastic and the time and temperature during extraction, the proper cooling, agitation, and decanting process, and the aseptic handling and storage of the extracts following extraction.

SYSTEMIC INJECTION TEST

This test is designed to evaluate systemic responses to the extracts of materials under test following injection into mice. Alternate routes of injection may be used with justification.

Test Animals—Use healthy, not previously used albino mice weighing 17–23 g. For each test group use only mice of the same source. Allow water and food, commonly used for laboratory animals and of known composition, ad libitum.

Procedure— [NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.] Inject each of the five mice in a test group with the *Sample* or the *Blank* as outlined in *Table 4*, except to dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding *Blank*, with 4.1 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 200 mg of polyethylene glycol per mL.

Table 4. Injection Procedure—Systemic Injection Test

Extract or Blank	Dose per kg	Route[a]
Sodium Chloride Injection	50 mL	IV
1 in 20 solution of Alcohol in Sodium Chloride Injection	50 mL	IV
Polyethylene Glycol 400	10 g	IP
Drug product vehicle (where applicable)	50 mL	IV
	50 mL	IP

Table 4. Injection Procedure—Systemic Injection Test *(continued)*

Extract or Blank	Dose per kg	Route[a]
Vegetable Oil	50 mL	IP

[a] IV = intravenous (aqueous sample and blank); IP = intraperitoneal (oleaginous sample and blank).

Observe the animals immediately after injection, again 4 h after injection, and then at least at 24, 48, and 72 h. If during the observation period none of the animals treated with the extract of the *Sample* shows a significantly greater biological reactivity than the animals treated with the *Blank*, the *Sample* meets the requirements of this test. If two or more mice die, or if abnormal behavior such as convulsions or prostration occurs in two or more mice, or if a body weight loss greater than 2 g occurs in three or more mice, the *Sample* does not meet the requirements of the test. If any animals treated with the *Sample* show only slight signs of biological reactivity, and not more than one animal shows gross symptoms of biological reactivity or dies, repeat the test using groups of 10 mice. On the repeat test, all 10 animals treated with the *Sample* show no significant biological reactivity above the *Blank* animals during the observation period.

INTRACUTANEOUS TEST

This test is designed to evaluate local responses to the extracts of materials under test following intracutaneous injection into rabbits or guinea pigs.

Test Animals—Select healthy, rabbits or guinea pigs with fur that can be clipped closely and skin that is free from mechanical irritation or trauma. In handling the animals, avoid touching the injection sites during observation periods, except to discriminate between edema and an oil residue.

Procedure— [NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.] On the day of the test, closely clip the fur on the animal's back on both sides of the spinal column over a sufficiently large test area. Avoid mechanical irritation and trauma. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with diluted alcohol, and dry the skin prior to injection. More than one extract from a given material can be used per rabbit or guinea pig, if it is determined that the test results will not be affected. For each *Sample* use two animals and inject each intracutaneously, using one side of the animal for the *Sample* and the other side for the *Blank*, as outlined in *Table 5*. [NOTE—Dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding *Blank*, with 7.4 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 120 mg of polyethylene glycol per mL.]

Table 5. Intracutaneous Test

Extract or Blank	Number of Sites (per animal)	Dose (µL per site)
Sample	5	200
Blank	5	200

Examine injection sites for evidence of any tissue reaction such as erythema, edema, and necrosis. Swab the skin lightly, if necessary, with diluted alcohol to facilitate reading of injection sites. Observe all animals at 24, 48, and 72 h after injection. Rate the observations on a numerical scale for the extract of the *Sample* and for the *Blank*, using *Table 2*. Reclip the fur as necessary during the observation period. The average erythema and edema scores for *Sample* and *Blank* sites are determined at every scoring interval (24, 48, and 72 h) for each rabbit or guinea pig. After the 72-hour scoring, all erythema scores plus edema scores are totalled separately for each *Sample* and *Blank*. Divide each of the totals by 12 (2 animals × 3 scoring periods × 2 scoring categories) to determine the overall mean score for each *Sample* versus each corresponding *Blank*. The requirements of the test are met if the difference between the *Sample* and the *Blank* mean score is 1.0 or less. If at any observation period the average reaction to the *Sample* is questionably greater than the average reaction to the *Blank*, repeat the test using three additional rabbits or guinea pigs. The requirements of the test are met if the difference between the *Sample* and the *Blank* mean score is 1.0 or less.

IMPLANTATION TEST

The implantation test is designed for the evaluation of plastic materials and other polymeric materials in direct contact with living tissue. Of importance are the proper preparation of the implant strips and their proper implantation under aseptic conditions. The intramuscular implantation test requires healthy adult New Zealand rabbits. The test specimens are placed into needles as the delivery system for implantation. Although most materials lend themselves readily to this method, there are a number of materials that are unsuitable for intramuscular implantation. For materials with physical characteristics unsuitable for routine intramuscular implantation, the subcutaneous rat implantation model is a viable alternative.

Intramuscular Implantation in Rabbits

Prepare for implantation 8 strips of the *Sample* and 4 strips of USP High-Density Polyethylene RS. Each strip should measure not less than 10 × 1 mm. The edges of the strips should be as smooth as possible to avoid additional mechanical trauma upon

implantation. Strips of the specified minimum size are implanted by means of a hypodermic needle (15–19 gauge) with intravenous point and a sterile trocar. Use either presterilized needles into which the sterile plastic strips are aseptically inserted, or insert each clean strip into a needle, the cannula and hub of which are protected with an appropriate cover, and then subjected to the appropriate sterilization procedure. [NOTE—Allow for proper degassing if agents such as ethylene oxide are used.]

Test Animals—Select healthy, adult rabbits weighing not less than 2.5 kg, and with paravertebral muscles that are sufficiently large in size to allow for implantation of the test strips. Do not use any muscular tissue other than the paravertebral site. The animals must be anesthetized with a commonly used anesthetic agent to a degree deep enough to prevent muscular movements, such as twitching. See the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

Procedure—Perform the test in a clean area. On the day of the test or up to 20 h before testing, clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Swab the skin lightly with diluted alcohol, and dry the skin prior to injection.

Implant four strips of the *Sample* into the paravertebral muscle on one side of the spine of each of two rabbits, 2.5–5 cm from the midline and parallel to the spinal column, and about 2.5 cm apart from each other. In a similar fashion implant two strips of USP High-Density Polyethylene RS in the opposite muscle of each animal. Insert a sterile stylet into the needle to hold the implant strip in the tissue while withdrawing the needle. If excessive bleeding is observed after implantation of a strip, place a duplicate strip at another site.

Keep the animals for a period of not less than 120 h, and sacrifice them at the end of the observation period by administering an overdose of an anesthetic agent or other suitable agents. Allow sufficient time to elapse for the tissue to be cut without bleeding. Examine macroscopically the area of the tissue surrounding the center portion of each implant strip. Use a magnifying lens and auxiliary light source. Observe the *Sample* and *Control* implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations. Measure encapsulation, if present, by recording the width of the capsule (from the periphery of the space occupied by the implant *Control* or *Sample* to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to *Table 6*.

Table 6. Evaluation of Encapsulation in the Implantation Test

Capsule Width	Score
None	0
Up to 0.5 mm	1
0.6–1.0 mm	2
1.1–2.0 mm	3
Greater than 2.0 mm	4

Calculate the differences between average scores for the *Sample* and *Control* sites. The requirements of the test are met if the difference does not exceed 1.0, or if the difference between the *Sample* and *Control* mean scores for more than one of the four implant sites does not exceed 1 for any implanted animal.

Subcutaneous Implantation in Rats

Prepare for implantation 10 sample specimens and 10 control specimens. The size and shape of the control specimens shall be as similar to that of the test specimens as practically possible. For example, specimens made of sheeting material shall be 10–12 mm in diameter and from 0.3–1 mm in thickness. The edges of the specimens should be as smooth as possible to avoid additional mechanical trauma upon implantation.

Test Animals—Select healthy albino rats weighing 225–350 g at the time of implantation.

Procedure—Perform the test in a clean area. Anesthetize (see AAALAC guidelines) the animal until a surgical plane is achieved. Clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Clean the clipped area with povidone–iodine solution. Using aseptic technique, make two midline incisions (approximately 1.0 cm long) through the skin at the cranial and caudal regions on the dorsal surface. Using blunt dissection, separate the fascia connecting skin to muscle to form a pocket underneath the skin lateral to each side of the incision (base of pocket approximately 20 mm from the line of implant). Insert a sterile sample into each pocket, and close the incision with wound clips or sutures. Implant two test samples and two control samples in each of five rats. Keep the animals for a period of at least seven days, and sacrifice them at the end of the observation period by CO_2 induced hypoxia or administering an overdose of an anesthetic agent. Allow sufficient time to elapse for the tissue to be cut without bleeding. Cut the skin (dorsal surface) longitudinally and lay back. Carefully examine macroscopically the area of the tissue surrounding the implant. Cut the sample in half and remove for close examination of the tissue in direct contact with the sample. Use a magnifying lens and auxiliary light source, if appropriate. Observe the *Sample* and *Control* implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations. Measure encapsulation, if present, by recording the width of the capsule (from the periphery of the space occupied by the implant *Control* or *Sample* to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to *Table 6*. Calculate the differences between average scores for the *Sample* and *Control* sites. The requirements of the test are met if the difference does not exceed 1.0.

SAFETY TESTS—BIOLOGICALS

The safety test set forth here is intended to detect in an article any unexpected, unacceptable biological reactivity. This in vivo test is provided for the safety assessment of biotechnology-derived products.

Safety Test

Select five healthy mice not previously used for testing, weighing 17–23 g, unless otherwise directed in the individual monograph or elsewhere in this chapter, and maintained on an adequate balanced diet. Prepare a test solution as directed in the individual monograph. Unless otherwise directed in the individual monograph or elsewhere in this chapter, inject a dose of 0.5 mL of the test solution into each of the mice, using a 26-gauge needle of suitable length, or of the length specified below as applicable. Observe the animals over the 48 h following the injection. If, at the end of 48 h, all of the animals survive and not more than one of the animals shows outward symptoms of a reaction not normally expected of the level of toxicity related to the article, the requirements of this test are met. If one or more animals die or if more than one of the animals shows signs of abnormal or untoward toxicity of the article under test, repeat the test using at least another 10 mice similar to those used in the initial test, but weighing 20 ± 1 g. In either case, if all of the animals survive for 48 h and show no symptoms of a reaction indicative of an abnormal or undue level of toxicity of the article, the requirements of the test are met. Body weights of mice before and at the end of the test should be obtained to detect any untoward effects. Animals that show signs of toxicity should be grossly necropsied and subjected to histopathology if necessary.

For biologics, perform the test according to the procedures prescribed in the *Code of Federal Regulations,* Section 610.11.