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Standard Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity[1]

This standard is issued under the fixed designation F895; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

This standard has been approved for use by agencies of the Department of Defense.

1. Scope

1.1 This test method is appropriate for materials in a variety of shapes and for materials that are not necessarily sterile. This test method would be appropriate in situations in which the amount of material is limited. For example, small devices or powders could be placed on the agar and the presence of a zone of inhibition of cell growth could be examined.

1.1.1 This test method is not appropriate for leachables that do not diffuse through agar or agarose.

1.1.2 While the agar layer can act as a cushion to protect the cells from the specimen, there may be materials that are sufficiently heavy to compress the agar and prevent diffusion or to cause mechanical damage to the cells. This test method would not be appropriate for these materials.

1.2 The L-929 cell line was chosen because it has a significant history of use in assays of this type. This is not intended to imply that its use is preferred, only that the L-929 is an established cell line, well characterized and readily available, that has demonstrated reproducible results in several laboratories.

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1.3 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

2.2 *ATCC Document:*

American Type Culture Collection, (ATCC) Catalogue of Strains II[3]

USP Negative Control Plastic Reference Standard[4]

3. Summary of Test Method

3.1 Cell cultures are grown to a monolayer in culture dishes. The medium is aspirated and replaced with an agar-containing medium that is allowed to solidify. Test control articles are placed on the agar surface to evaluate the cytotoxic properties of a given material or device. Toxic components in the test article can diffuse into the culture medium, forming a concentration gradient and adversely affecting cells at varying distances from the test article. This method is well suited for low-density materials (film, paper, and so forth), powders, liquids, and high-density materials that could physically damage the cells if placed in direct contact with the cell monolayer.

4. Significance and Use

4.1 This test method is useful for assessing the cytotoxic potential of new materials and formulations and as part of a quality control program for established medical devices and components.

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[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Fourth edition, 1983, is available from American Type Culture Collection, 12031 Parklawn Dr., Rockville, MD 10892. Library of Congress No. 76-640122.

[4] *U.S. Pharmacopeia*, current edition, Rockville, MD.

4.2 This test method assumes that assessment of cytotoxicity provides useful information to aid in predicting the potential clinical applications in humans. Cell culture methods have shown good correlation with animal assays and are frequently more sensitive to cytotoxic agents.

4.3 This cell culture test method is suitable for incorporation into specifications and standards for materials to be used in the construction of medical devices that are to be implanted into the human body or placed in contact with tissue fluids or blood on a long-term basis.

4.4 Some biomaterials with a history of safe clinical use in medical devices are cytotoxic. This test method does not imply that all biomaterials must pass this assay to be considered safe for clinical use (Practice F748).

5. Apparatus

5.1 The following apparatus shall be used:

5.2 *Incubator*, which maintains the cultures at 37 ± 2°C, 5 ± 1 % CO_2, and greater than 90 % relative humidity.

5.3 *Water Bath*, capable of maintaining a temperature of 37 ± 2°C and 45 ± 2°C.

5.4 *Microscope*, with inverted phase contrast optics and magnifications of 40, 100, and 200×.

5.5 *Clinical Centrifuge*, capable of attaining 1000 xg.

5.6 *Sterile, Disposable 150-cm^2 Tissue Culture Flasks*.

5.7 *Sterile, Tissue Culture Dishes*, 35 mm in diameter and 10 mm deep.

NOTE 1—Plastic dishes are recommended because they provide a flat surface that promotes the formation of a uniform monolayer of cells.

5.8 *Sterile, Disposable, Centrifuge Tubes*.

5.9 *Sterile Pipettes*, 1, 5, and 10 mL.

5.10 *Filter Disks*, 10 mm in diameter for evaluation of liquids.

NOTE 2—Millipore AP2501000 filter disks have been found satisfactory for use in cytotoxicity evaluations because they elicit no cytopathic effect. Other filter disks that do not elicit a cytopathic effect may also be used.

NOTE 3—A laminar flow work area capable of filtering out 99.99 % of all particles greater than 0.3 μm in diameter, or a Class 100 clean room may be necessary to prevent contamination of cultures.

6. Reagents

6.1 The following reagents shall be used:

6.1.1 *For Cell Culture Maintenance* , 1× Media. Minimum Essential Medium (MEM) is prepared by mixing 90-mL Eagle's MEM (with Earle's salts, without L-glutamine), adjusting the solution to pH of 7.15, ~~add 5-~~ and adding 5 to ~~10-mL~~10 mL of fetal bovine serum, and 1-mL 100× nonessential amino acids (L-glutamine).

6.1.1.1 Opened containers of prepared MEM may be stored at a temperature of 2 to 8°C for periods of not more than two weeks. Glutamine is omitted from this formulation to maximize the shelf life. Immediately before use, 1 mL of L-glutamine solution (see 6.1.3) is added to each 100 mL of MEM.

6.1.1.2 Antibiotics, such as penicillin G10 000 I.U./mL, and streptomycin 10 000 I.U./mL, may be added to the medium to reduce the incidence of bacterial contamination. Use 1 mL of antibiotic per 100-mL media. Care shall be taken to ensure that the antibiotics do not have an adverse effect on the viability of the cell cultures.

6.1.2 *For Agar Media Overlay*, to prepare 2× Media (100-mL final volume). Twice concentrated (2×) MEM is prepared by mixing 20 mL of 10× Eagle's MEM (with Earle's Salts without L-glutamine), 0.22-g sodium bicarbonate (buffer) and sterile distilled water to bring to 70 % volume (70 mL). Adjust the pH to 7.15. Add 20-mL fetal bovine serum and 2-mL 100× nonessential amino acid (L-glutamine). Bring to final volume (100 mL) with sterile distilled water. Filter sterilize the 2× media. Mix with equal amounts of sterilized 3 % agar nobel to give the final concentration of the media as 1×.

6.1.3 *L-Glutamine Solution* (*Lyophilized*), 29.2 mg/mL. Rehydrate with sterile distilled water. (Store frozen.)

6.1.4 *Hanks' Balanced Salt Solution*, calcium- and magnesium-free (store at room temperature).

6.1.5 *Trypsin*, 0.1 % solution in Hanks' balanced salt solution or calcium- and magnesium-free, phosphate-buffered saline (store frozen).

6.1.6 *Water*, sterile, deionized, or distilled water should be used.

6.1.7 *Noble Agar*, 3 %.

6.1.8 *Neutral Red Stain*, 0.01 % by weight in phosphate-buffered saline.

6.2 All reagents shall be tissue-culture grade or equivalent.

6.3 Reagents shall be reconstituted in accordance with the manufacturer's directions, using aseptic technique.

7. Cell Culture

7.1 Cell cultures used in this assay shall be the ATCC, CCL I NCTC clone 929 strain (clone of Strain L, mouse connective tissue) designated L-929. Other suitable validated cell lines may be considered.

8. Control Materials

8.1 Prepare negative control specimens in accordance with Section 10 from a material that consistently elicits negligible cellular response in this assay (for example, USP Negative Control Plastic Reference Standard).

8.2 Prepare positive control specimens in accordance with Section 10 from a material that consistently elicits a moderate and reproducible degree of cytotoxicity (for example, an aqueous solution of phenol (0.45 ± 0.05 % by volume), or other material producing a known cytotoxic response, for example, latex rubber).

8.2.1 Use an aqueous solution of phenol to give a diffuse reaction of cellular degeneration and sloughing; a latex rubber will give a zone of toxicity.

8.2.2 Take care when preparing aqueous solutions of phenol to ensure the homogeneity of the solution since phase separations may occur.

8.2.3 Latex rubber is a widely used control material that has demonstrated reproducible results in several laboratories.

9. General Technique

9.1 Use aseptic technique throughout this assay to minimize microbial contamination.

NOTE 4—Mouth pipetting should not be used to transfer cells, medium, or reagents.

9.2 Warm all solutions and materials to a temperature of 37 ± 2°C before being placed in contact with cells.

9.3 Wash all glass vessels thoroughly with a cleaning solution and rinse thoroughly with copious amounts of deionized water.

9.4 Clean all work surfaces with a disinfectant solution before use.

9.5 Record the culture history of the cells.

9.6 Stock cultures should be periodically screened for mycoplasma contamination.

10. Specimen Preparation

10.1 Sterilize all specimens by a method appropriate to the end use of the device.

10.2 Where a device is sufficiently small (see 10.3 and 10.4) to fit into the culture dish leaving an adequate margin of cells for evaluation, use the entire device as a specimen.

10.3 Cut large solid materials and devices in cross section to obtain a flat surface having an area of 100 to 250 mm^2 to be placed in direct contact with the agar surface.

10.4 Prepare specimens of rod or tubing or of rod- or tube-shaped devices as follows:

10.4.1 Where the diameter is less than 6.4 mm, cut 5 to 15 mm in length.

10.4.2 Where the diameter is 6.4 to 15 mm, cut 2 to 8 mm in length.

10.4.3 Where the diameter exceeds 15 mm, prepare cross sections as described in 10.3.

10.5 Obtain specimens from larger medical items from locations with relatively large cross sections to expose interior material.

10.6 If a device is constructed of two or more materials that are intended to contact body fluids or tissues, either cut the test specimen from the materials' interface or test separate specimens of each material or both.

10.7 Prepare specimens for evaluating the cytotoxicity of liquids or extracts by saturating a sterile filter disk and allowing the excess liquid to drain off while maintaining asepsis. Use the saturated filter disk as a test specimen.

NOTE 5—When ethylene oxide or other chemical sterilants are used, adequate aeration time should be determined to permit dissipation of residues which may adversely affect the results recorded in this assay.

NOTE 6—In general, specimens should be cleaned to remove any residues from specimen preparation and sterilized after they have been cut to size. If the specimen is very hard (for example, ceramics), care should be taken to remove the residues that may be left on the freshly cut surface by the cutting tool. When evaluating the cytotoxic potential of medical materials or devices that are contained in the final sterile package, resterilization, further processing, or delay between the time of opening the package and starting the test must be avoided. With small items, the entire content of the sterile package may be used as the test specimen. When the size of the sterile packaged item is too large, an appropriate, representative, small-sized specimen must be obtained. The application of this assay to items in the final sterile package is limited to items that are small or can be cut and reshaped using aseptic technique.

10.8 Absorbant materials tested in this method ~~must~~shall be prewetted with culture medium to prevent loss of water from the agar and subsequent cellular damage.

11. Cell Culture Maintenance

11.1 Use the following procedures to maintain the cells by serial subculture:

11.2 Aspirate the medium from a 150-cm^2 cell culture flask containing a near-confluent monolayer.

11.3 Rinse the cells with a sufficient volume (for example, 5 to 10 mL) of Hanks' balanced salt solution to remove residual serum ~~(for example, 5 to 10 mL).~~ .

11.4 Aspirate the rinse solution.

11.5 Add a sufficient volume of trypsin solution (0.1 %) to the flask to cover the cell monolayer (approximately 5 mL).

11.6 Incubate for 5 to 10 min to suspend the cells.

11.7 Transfer the cell suspension to a centrifuge tube.

11.8 Centrifuge at 1300 xg for 6 min.

11.9 Discard the supernatant.

11.10 Resuspend the cells in 10 ± 0.1 mL of fresh medium and mix the suspension thoroughly.

11.11 Distribute the cell suspension equally among each of two to eight 150-cm^2 tissue culture flasks.