Designation: F813 – 07 (Reapproved 2012)

Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices[1]

This standard is issued under the fixed designation F813; 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.

1. Scope

1.1 This practice covers a reference method of direct contact cell culture testing which may be used in evaluating the cytotoxic potential of materials for use in the construction of medical materials and devices.

1.2 This practice may be used either directly to evaluate materials or as a reference against which other cytotoxicity test methods may be compared.

1.3 This is one of a series of reference test methods for the assessment of cytotoxic potential, employing different techniques.

1.4 Assessment of cytotoxicity is one of several tests employed in determining the biological response to a material, as recommended in Practice F748.

1.5 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 a well-characterized, readily available, established cell line that has demonstrated reproducible results in several laboratories.

1.6 Since the test sample is not removed at the time of microscopic evaluation and underlying cells may be affected by the specific gravity of the test sample, this practice is limited to evaluation of cells outside the perimeter of the overlying test sample.

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

1.8 *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]

F619 Practice for Extraction of Medical Plastics

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

F895 Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity

F1027 Practice for Assessment of Tissue and Cell Compatibility of Orofacial Prosthetic Materials and Devices

2.2 *Other Documents:*

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

USP Negative Control Plastic Reference Standard[4]

3. Summary of Practice

3.1 Cell cultures are grown to a confluent monolayer in culture dishes. The growth medium is aspirated and replenished to provide a resting, confluent cell layer. Test and control specimens are placed in direct contact with the cell layer to provide an accelerated assessment of the presence or absence of a cytotoxic effect from a given material or device. See Practice F1027 for definitions.

4. Significance and Use

4.1 This practice is useful for assessing cytotoxic potential both when evaluating new materials or formulations for possible use in medical applications, and as part of a quality control program for established medical materials and medical devices.

4.2 This practice assumes that assessment of cytotoxicity potential provides one method for predicting the potential for cytotoxic or necrotic reactions to medical materials and devices during clinical applications to humans. In general, cell culture testing methods have shown good correlation with animal assays and are frequently more sensitive to toxic moieties.

[1] This practice 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] American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108..

[4] *U.S. Pharmacopeia*, Vol 24, Rand McNally, Taunton, MA, 1994, pp. 1652–1653. Use latest publication to ensure current cumulative revisions are used.

4.3 This cell culture test method is suitable for adoption in specifications and standards for materials for use in the construction of medical devices that are intended to be implanted in the human body or placed in contact with tissue, tissue fluids, or blood on a long-term basis. However, care should be taken when testing materials that are resorbable to be sure the method is applicable.

4.4 Since cells in this direct contact test method are not protected by an overlying agarose layer, they are more susceptible to potential mechanical damage imparted by the overlying test sample. Investigators wishing to evaluate the cytotoxic response of cells underlying the test sample should consider agarose-based methods similar to Test Method F895. Alternatively, depending on sample characteristics, extraction methods such as Practice F619 may also be considered.

5. Apparatus

5.1 The following apparatus shall be used:

5.2 *Incubator,* to maintain a temperature of 37 ± 2°C and 4 to 6 % CO_2 with greater than 90 % relative humidity.

5.3 *Tissue Culture Grade Culture Dishes,* that are sterile and 35 mm in diameter by 10 mm deep.

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

5.4 *Disposable, Sterile, Centrifuge Tubes.*

5.5 *Inverted Optical Microscope,* with magnifications of 40×, 100×, and 200×.

5.6 *Clinical Centrifuge,* capable of attaining 1300xg.

5.7 *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.

5.8 *Water Bath,* capable of maintaining a temperature of 37 ± 2°C.

NOTE 3—A laminar flow work area capable of filtering out 99.99 % of all particles greater than 0.5 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 *Minimum Essential Medium* (MEM), prepared without L-glutamine and augmented by the addition of Earle's salts and 5–10 % fetal bovine serum.

NOTE 4—Glutamine is omitted from this formulation in order to maximize the shelf life of the medium. Immediately before use, 5 mL of L-glutamine solution (see 6.1.2) are added to each 500 mL of MEM.

NOTE 5—Opened containers of MEM may be stored at a temperature of 2 to 8°C for periods of not more than one week.

NOTE 6—Antibiotics, such as penicillin G10,000 I.U./ml and streptomycin 10,000 I.U./ml, may be added to the medium (1 ml of antibiotic per 100 ml of media) to reduce the incidence of bacterial contamination. This may, however, have an adverse effect on the viability of the cell cultures.

6.1.2 *L-glutamine Solution,* 29.2 mg/mL of sterile water.

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

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

6.1.5 *Water,* distilled, deionized, and sterile, with a minimum resistivity of 1 MΩ·cm.

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.

6.4 Reagents shall be stored in accordance with the manufacturer's directions unless otherwise indicated in 6.1.

7. Cell Cultures

7.1 Cell cultures used in this assay should be the ATCC, CCL 1 NCTC clone 929 strain (clone of Strain L, mouse connective tissue) designated L-929. Other suitable validated cell lines may be considered. Cells should be tested periodically for Mycoplasma contamination.

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 predictable, moderate degree of cytotoxicity.

8.2.1 Use aqueous phenol (0.45 ± 0.05 % by volume) as a positive control for a diffuse reaction of cellular degeneration and sloughing. Take care to ensure that the preparation is homogenous.

8.2.2 Latex rubber has been used as a positive polymeric control for a zone of inhibition.

9. General Technique

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

NOTE 7—Mouth pipetting should not be employed to transfer cells, medium, or reagents.

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

10. Preparation of Specimens

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 cell monolayer.

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 in order to expose interior material.

10.6 If a device is constructed of two or more materials, cut either the test specimen from the materials' interface or test separate specimens from each material.

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

NOTE 9—In general, specimens should be cleaned to remove any residues from specimen preparation, and sterilized after they have been cut to size. If the large solid materials are very hard, like ceramics, which require cutting with metal or diamond saws, care should be taken to remove any contamination from the metal blade or from the metal bonding the diamonds to the blade. When evaluating the cytotoxicity 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.

NOTE 10—The size and shape of test specimens may vary considerably, providing they physically fit into the culture dish. This test is intended only to provide a qualitative assessment of cytotoxicity potential with no quantitative measurement of cytotoxicity magnitude. Thus specimen size is not considered important.

11. Preparation of Cell Layer

Prepare confluent cell monolayers as follows:

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

11.2 Rinse the cells with 5 ± 0.5 mL of Hanks' solution.

11.3 Aspirate the rinse solution.

11.4 Add 5 ± 0.5 mL of trypsin solution (0.1 %) to the flask.

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

11.6 Dilute the suspension sufficiently to reduce the effect of the trypsin or:

11.7 Transfer the cell suspension to a centrifuge tube and centrifuge at 1300 g for 6 min and discard the supernatant.

11.8 Dilute or suspend the cells in 10 ± 0.1 mL of fresh medium, and mix the suspension thoroughly.

11.9 Add 2.0 ± 0.1 mL of medium to each culture dish.

11.10 Using a sterile 10-mL serological pipette, add 5 to 7 drops of cell suspension to each dish.

11.11 Incubate until a near-confluent monolayer has formed, as observed by microscopic examination. During handling the cell culture dishes and during transport to and from the incubator, agitation of the medium shall be avoided.

NOTE 11—The formation of a near-confluent monolayer usually requires 3 to 5 days. By counting cells with a hemacytometer (to ensure the concentration of the inoculum) the time required for monolayer formation may be regulated. A cell concentration of 1.3×10^5 cells/mL will give a consistent time of 24 h. It should be recognized, however, that this procedure may increase the risk of bacterial contamination.

11.12 If cell suspension remains unused after step 11.10, a subculture may be prepared by adding 9 mL of fresh medium to each millilitre of each suspension in a cell culture flask with a surface area of approximately 3 cm^2 for each millilitre of diluted cells and incubating it until a near-confluent monolayer has formed, as determined by microscopic examination.

12. Test Procedure

12.1 Perform the direct-contact cytotoxicity assay as follows:

12.2 Microscopically examine the cell cultures and reject any in which the cell monolayer is not of correct confluency or the cells show signs of granulation or sloughing.

12.3 Aspirate the medium from all the acceptable cultures and replace it with 1.5 to 2 mL of fresh medium.

12.4 Place a single test or control specimen in each dish in direct contact with the cell monolayer. Prepare triplicate cultures for each test material and both positive and negative controls. The materials should be added to the culture gently and agitation shall be avoided.

NOTE 12—Low-density materials that tend to float in the medium may be held in contact with the cell monolayers by placing a piece of negative control on top to weigh them down.

12.5 Incubate all cultures for 24 ± 1 h.

12.6 Examine each culture microscopically.

NOTE 13—The use of an histologic stain such as 2 % crystal violet in 20 % ethanol is helpful in assessing the cell cultures.

12.7 Quantitative or semi-quantitative assays such as MTT, LDH, and neutral red may be considered if validated against the evaluation of results described in section 13.

13. Evaluation of Results

13.1 A cell culture shall be deemed to show a cytotoxic effect if microscopic examination (see 12.6) reveals the following:

13.1.1 Malformation, degeneration, sloughing, or lysis of cells extending beyond the perimeter of the specimen of material; or

13.1.2 Moderate to severe reduction of cell layer density.

NOTE 14—A slight reduction of cell layer density is acceptable, provided that there is no evidence of the conditions specified in 13.1.1.

13.2 If, for a given set of specimens, a cytotoxic effect is observed for the negative controls or no cytotoxic effect is elicited by the positive controls, the results for that set of specimens shall be considered invalid.

13.3 If neither specimen of a test material elicits a cytotoxic effect as described in 13.1, the material shall be considered to pass this assay.

13.4 If both specimens of a test material elicit a cytotoxic effect, the material shall be considered to fail this assay.

13.5 If only one specimen of a test material elicits a cytotoxic effect, the assay shall be repeated on an additional four specimens. The material shall be considered to pass this assay only if none of the retest specimens elicits a cytotoxic effect, otherwise, it shall be considered to fail.

13.6 It is suggested that moderate to severe reduction of cell density and/or the presence of a zone of cell malformation, degeneration, sloughing, or lysis extending beyond the specimen perimeter be rated on a grading scle of 0 to 4, where:

0 indicates no detectable effect around the specimen
1 indicates a few affected cells around the specimen
2 indicates a zone less than 0.5 cm beyond the specimen
3 indicates a zone 0.5 to 1 cm beyond the specimen
4 indicates a zone greater than 1 cm beyond the specimen

14. Report

14.1 The report of the assay results shall include at least the following:

14.1.1 Test date.
14.1.2 Cell strain used.
14.1.3 Culture history of cells.
14.1.4 Medium used (noting whether antibiotics were used).
14.1.5 Description of all test and control specimens, including:
14.1.5.1 Material or device name (including ASTM designation number, where appropriate).
14.1.5.2 Lot or batch number.
14.1.5.3 Source of supply.
14.1.5.4 Size and method of preparation.
14.1.5.5 Method of sterilization.
14.1.6 Statement of whether each test material passed or failed the assay.
14.1.7 Any other pertinent observations, including any deviations from the exact method described herein.

15. Precision and Bias

15.1 Quantitative precision and bias have not been determined.

16. Keywords

16.1 biocompatibility; cell culture; cytotoxicity testing; direct contact cytotoxicity

APPENDIX

(Nonmandatory Information)

X1. RATIONALE FOR EVALUATING DIRECT CONTACT CELL CULTURE OF MATERIALS FOR MEDICAL DEVICES[5]

X1.1 Medical devices such as implants and certain other devices intended for intimate contact with organs should not produce adverse biological reactions.

X1.2 Laboratory cell culture methods provide a rapid method[6,7,8] for assessment of cytotoxicity potential of both finished medical devices and materials used in their construction. The direct contact method is but one of several such techniques that are in common use. The cell culture method described in this practice is a modification of methods that have been routinely used for more than 10 years. A positive control which produces a cytopathic effect and a negative control which produces no cytopathic effect are assayed with each test to ensure that the cell cultures have expected cytotoxic responses. Test results are considered valid only when the expected responses for both controls are obtained.

X1.3 This practice was revised in recognition of the fact that the mass of a test article could cause damage to cells. Even when the mass of a sample does not appear to be sufficient to cause damage, any cell damage seen under the test article cannot clearly be said to be due to cytotoxicity only. The scoring system was revised to score only the damage around the test article and not under it.

[5] Committee F04 requires a rationale to accompany all standards. This rationale should be readable to lay consumers as well as technical experts.

[6] Guess, W. L., Autian, J., "Toxicity Evaluation of Lexan, Kyonar, Rilsan Short-Term Studies," *Journal of Oral Therapeutics and Pharmacology*, Vol 3, No. 2, 1966, pp. 116–123.

[7] Wilsnack, R. E., Meyer, F. S., Smith, J. G., "Human Cell Culture Toxicity Testing of Medical Devices and Correlation to Animal Tests," *Biomaterials, Medical Devices, and Artificial Organs* Vol 1, 1973, pp. 543–562.

[8] Wilsnack, R. E., "Quantitative Cell Culture Biocompatibility Testing of Medical Devices and Correlation to Animal Tests," *Biomaterials Medical Devices and Artificial Organs*, Vol 4, 1976, pp. 235–261.