



Designation: F1903 – 98 (Reapproved 2003)

Standard Practice for Testing For Biological Responses to Particles *in vitro*¹

This standard is issued under the fixed designation F1903; 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 the production of wear debris and degradation products from implanted materials that may lead to a cascade of biological responses resulting in damage to adjacent and remote tissues. In order to ascertain the role of particles in stimulating such responses, the nature of the responses, and the consequences of the responses, established protocols are needed. This is an emerging, rapidly developing area and the information gained from standard protocols is necessary to interpret responses and to determine if there is correlation with the *in vivo* responses. Since there are many possible and established ways of determining responses, a single standard protocol is not stated. However, well described protocols are needed to compare results from different investigators using the same materials and to compare biological responses for evaluating (ranking) different materials. For laboratories without established protocols, recommendations are given and indicated with an *.

1.2 Since the purpose of these studies is to predict the response in humans, the use of human cells would provide much information. However in the interest of safety in laboratory protocols, the use of non-human and non-primate cells is described.

1.3 The values stated in SI units are to be regarded as the 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

F619 Practice for Extraction of Medical Plastics

¹ This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical 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.

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

3. Summary of Practice

3.1 Biological responses to particles may be evaluated using specimens from animals being tested according to the Practice **F748** matrix for irritation and sensitivity, or for implantation. Blood, organs, or tissues from the animals may be used.

3.2 Biological responses to particles may be evaluated using materials or extracts according to Practice **F619**. These materials or extracts may be used for *in vivo* tests or for the *in vitro* tests. Particles generated by other methods may also be used.

4. Significance and Use

4.1 This practice is to be used to help assess the biocompatibility of materials used in medical devices. It is designed to test the effect of particles from the materials on macrophages. For safety reasons, the use of non human, non primate cells is recommended in this practice. For laboratories equipped and approved to work with human blood and tissue, the use of these same protocols would be advantageous for development of understanding of the interaction of cells and particles.

4.2 The appropriateness of the methods should be carefully considered by the user since not all materials or applications need be tested by this practice.

4.3 Abbreviations:

4.3.1 **LPS**—lipopolysaccharide (endotoxin).

4.3.2 **LAL**—Limulus Amebocyte Lysate.

4.3.3 **ATCC**—American Type Culture Collection.

4.3.4 **FCS (FBS)**—Fetal Calf Serum.

4.3.5 **NCS**—Newborn Calf Serum.

4.3.6 **PBS**—Phosphate Buffered Saline.

4.3.7 **HANKS**—A balanced salt solution.

4.3.8 **MMPS**—Matrix Metallo Proteases.

4.3.9 **RPMI 1640**—Specific Growth Medium (Roswell Park Memorial Institute).

4.3.10 **HEPES**—A buffering salt.

5. Responses from Cells Grown *In Vitro*

5.1 **Particles**—Define the nature of the particles used:

5.1.1 Source,

5.1.2 Chemistry,

5.1.3 Size (mean and range),

5.1.4 Shape,

5.1.5 Method of sterilization,

5.1.6 If the presence of bacterial lipopolysaccharide (LPS) was determined, specify how this was done and the sensitivity of the method. (LAL testing with a sensitivity of at least 0.06 EU is recommended),

5.1.7 Concentration of particles used as weight or number or surface area/ 10^6 cells, and

5.1.8 Surface charge (if known).

5.2 *Cells*—Define the nature of the cells used:

5.2.1 *Established Cell Line* (if not, go to 5.2.2)—The use of established cell lines provides a known cell type with a reproducible response. Although the correlation with the *in vivo* system may not be known at this time, careful studies with established cell lines could eventually allow determination of correlation between *in vivo* and *in vitro* systems.

5.2.1.1 Source of cell and identifying number or code,

5.2.1.2 Nature of the cell (for example, macrophage), and

5.2.1.3 Special attributes of the cell line (for example, nonphagocytic),

5.2.1.4 *ATCC murine macrophages such as RAW 264.7, J774A, P388D1, or IC-21 are recommended.

5.2.2 *Primary Isolate* (if not, go to 1.2.1):

5.2.2.1 Source of cell including species and location (for example, murine, alveolar),

5.2.2.2 Nature of the cell (for example, macrophage),

5.2.2.3 Mechanism of isolation (for example, lavage), and

5.2.2.4 Specify if stimulant used and if so which one (for example, mineral oil).

5.2.2.5 *Mouse (specify strain, age, and sex used) peritoneal exudate cells are recommended with a mild stimulant such as nutrient broth.

5.3 *Culture Conditions*:

5.3.1 Specify source and type of medium. If not a commercial source, list ingredients and sources of ingredients.

5.3.2 Specify source and type of serum, and whether it was heat inactivated. If the presence of LPS was determined, specify method and sensitivity of the method.

5.3.3 Specify culture conditions (for example, 37°C, humidified, 5 % CO₂ incubator).

5.3.4 Specify time of termination of culture or sampling of culture medium.

5.3.5 If cell counts were determined specify as to when and how. If estimates of cell number/mL specify when and how.

5.3.5.1 *Medium and serum specified by the supplier of the cells are recommended. Generally RPMI 1640 with heat inactivated 10 % newborn or fetal calf serum are recommended. LPS levels are generally provided or available from the distributor. Recommended culture conditions are 37°C, with 5 % CO₂, in a humidified incubator. Cell counts at the time of initial plating and at the termination of the culture are recommended using a hemocytometer with monolayer cells resuspended by trypsin solution (not recommended for macrophages), washing with Ca and Mg free PBS or Hanks, or scraping in 1 mL of Ca and Mg free PBS or Hanks. The addition of trypan blue is helpful. The supernatant of the medium from macrophages exposed to particles for specified time periods is assayed.

5.3.6 *Controls*:

5.3.6.1 Cells not stimulated with particles should be maintained at the same time under the same conditions.

5.3.6.2 Polystyrene particles, spherical, size range 1 to 5 μ m, should be used as a reference control.

5.3.6.3 LPS is a known stimulant and is a good positive control. A concentration between 0.25 and 1 ng/mL of culture medium is sufficient.

5.3.6.4 Culture medium is a recommended diluent for the assays.

5.4 *Products or Response Determined*—One or more of the following:

5.4.1 Cell viability can be determined by any of several methods:

5.4.1.1 From the total cell counts and viable cell count with trypan blue dye exclusion,

5.4.1.2 From flow cytometry with vital stains,

5.4.1.3 From uptake of DNA precursors, and

5.4.1.4 From total DNA or RNA in the culture.

5.4.2 *Soluble Cell Products Elaborated*:

5.4.2.1 Reliable reagents or kits are available to detect products. The products are classified into three classes: growth factors such as TNF α , TGF beta, and PGE₂, interleukins such as IL-1, IL-1 receptor antagonist, IL-1 beta, and IL-6, and reactive oxygen species such as superoxide or nitric oxide (NO) (usually measured as nitrite produced using the Griess reagent). The release of at least two products, which are from different classes, should be determined.

5.4.2.2 The time in culture should be specified since these products peak at different times (for example, TNF α peaks by 24 h, NO peaks around 72 h, the others generally peak at 48 to 72 h). The detection of NO and TNF α are strongly recommended and the assay procedures are described in some detail in this recommended practice.

5.4.3 *Assay for Nitric Oxide*:

5.4.3.1 The production of NO is most conveniently measured by detection of the stable endproduct nitrite. This reflects the action of NO on arginine. The technique is simple, inexpensive and quantitative. The reagent used to quantitate nitrite is the Griess reagent. This is prepared by dissolving 100 mg of Sulfanilamide and 10 mg of N-(1-naphthyl)ethylenediamine in 2.5 % phosphoric acid. Assay of culture medium: 100 μ L of the culture medium is placed in a 96-well microtiter plate and then 100 μ L of the Griess reagent is added. This is incubated at room temperature for 10 min and the optical density determined at 540 nm wavelength. A standard curve is prepared with sodium nitrite in water. It is recommended that two-fold serial dilutions be prepared encompassing the range of 32 to 0.125 nmoles/mL. The standard curve is prepared using 100 μ L of the dilutions of sodium nitrite and 100 μ L of the Griess reagent.

5.4.4 *Assay for TNF α* :

5.4.4.1 Kits for assay of TNF α using immunological assays are available from supply houses including, but not limited to, R&D Systems and Endogen. Instructions are provided with the kits. These are sensitive and reliable.