



Standard Practice for Testing For Biological Responses to Particles *In Vitro*¹

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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 asterisk*.

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 this practice, the use of non-human and non-primate cells is described. If the user should wish to employ cell lines from humans, cell lines are available from ATCC and most of the information and recommendations will still apply.

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.*

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. 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

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.

3.3 The purpose of this practice is to assess the response of cells in direct contact with particles and, therefore, this practice is primarily intended to cover the testing of particles placed into culture with the cells. This practice should be equally appropriate for the testing of the response to nanoparticles placed in culture, if particles of that size are the particles of interest.

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. The use of nonhuman, nonprimate 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.

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.

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/10⁶ cells, and
- 5.1.8 Surface charge (if known).
- 5.1.9 Since some particles may have a density less than that of the culture medium being used, careful consideration should be given to determining appropriate procedures for ensuring that the particles and cells are in contact. Techniques in which cells are grown on a substrate and then the substrate is inverted in culture may be appropriate.

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 μm, 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 TNFα, 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, TNFα 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.

5.4.4.2 There is a bioassay for TNF α using L929 cells which may be more convenient for some laboratories. There are several methods available, and a recommended method is described here.

5.4.4.3 L929 cells (available from ATCC and other sources) are seeded at 10^5 cells/mL of RPMI 1640 with 10 % RBS using 100 μ L per well in a 96 well culture plate leaving one column empty as a blank. These are grown for 24 h at 37°C in 5 % CO₂.

5.4.4.4 Assay media is RPMI 1640 with 10 % FBS, 2 μ g/mL of actinomycin D, and 20 μ M HEPES buffer.

5.4.4.5 Prepare TNF α standard curve (TNF α standards are available from supply houses including R&D Systems) of 0.1, 0.25, 0.5, 1, 2.5, and 5 units/mL. A 96-well plate is dedicated to the standards and the unknowns are done in other 96-well plates and the concentration of TNF α is determined from the standard curve. Each standard is run in eight replicates. The recommendation is that 6 of the eight well columns are used for the standards, one column is used for the blank, and three columns are used for cells and media control. The unknowns to be assayed are diluted at least 1/2 in the assay medium.

5.4.4.6 The media in the plates with the 24 h L929 cell cultures are poured off or pipetted off. Then 100 μ L of the assay

media, the standards in the assay media, or the unknowns in the assay media is added to the appropriate wells. These are incubated for 24 h at 37°C with 5 % CO₂. At the end of the 24-h incubation, pour off the media, shake the remaining media off, invert the plates onto absorbent paper for a few minutes to drain.

5.4.4.7 *Stain*—500 mg crystal violet, 8mL of 40 % formaldehyde, 170 mg NaCl, and 22.3 mL ethanol made to 100 mL with water. Dissolve the crystal violet and filter the solution. Add 100 μ L of the stain to each well. Leave at room temperature for 10 min with occasional gentle shaking. Pour off the stain, wash with distilled water, invert the plates onto absorbent paper for 10 min. There should be no stain dripping off. If there is, wash with water again. Place the plates in the upright position and leave to dry. Add 100 μ L of 33 % acetic acid/well, swirl the plate, read the optical density at 595 to 600 nm.

5.4.4.8 Other products detected should be specified and the method used specified. Suggested products would be other reactive oxygen species such as superoxide, other interleukins (IL-2, IL-4, IL-10), collagenase, MMPS.

5.4.5 Effects of the Culture Media on Other Systems:

5.4.5.1 Bone resorption using bone slices or calvaria cultures, and

5.4.5.2 Effect on chondrocyte cultures.

5.4.6 Methods should be clearly described and results compared to a standard that is readily available to other laboratories for validation.

6. Data Collection and Analysis

6.1 Cell viability should be reported as cell number, or percent survival, or DNA or RNA concentrations, or estimated survival and these results compared to the results with the cell control without particles and the results with the polystyrene reference particles. (PS particles are available from commercial suppliers.)

6.2 Soluble products elaborated should be reported as μ g or ng or units/mL, or μ g or ng or units/cell number, or concentration such as mM. Alternatively, the report of the optical density of the solution will suffice. The results with the particles should be compared to the results with the cell control without particles and the results with the cells and the polystyrene reference particles.

7. Keywords

7.1 biocompatibility; growth factors; interleukins; macrophages; nitric oxide; particles; TNF α

APPENDIXES
(Nonmandatory Information)
X1. RATIONALE

X1.1 The primary purpose of this practice is to describe methodology to determine the biological response to particles using *in vitro* cellular responses.

X1.2 It is well recognized that the biological responses to particles could be different from those to solid materials. The interaction of the particles with phagocytic cells, notably macrophages, is a key to the final biological response.

X1.3 The interaction of macrophages with particles has been an active research area for many years. Many investigators have developed procedures for doing these studies. This document is intended to delineate the information necessary so that the results from these various studies can be interpreted and to describe methodology appropriate for those investigators developing such studies.

X1.4 The interaction of phagocytic cells with particles will lead to the production of various soluble mediators which may influence the progression of the biological response and the immune response. It is unknown at this time which of these responses are favorable and which are unfavorable to the host. Similarly it is unknown whether quantitative evaluation of

these factors is predictive of the biological response. Studies such as the ones described here are needed to determine the importance of these responses in biocompatibility and biocompatibility testing of materials.

X1.5 The level of LAL for device evaluation is commonly 0.5 EU/mL. LAL testing with a sensitivity of 0.06 EU/mL is recommended in this practice for evaluating *in vitro* responses to particles since levels of LPS above 0.06 EU/mL may be stimulatory to macrophages. This will permit distinguishing cellular responses to particles from cellular responses to LPS.

X1.6 Polystyrene (PS) particles were chosen as a reference control for this standard practice so that testing results from different laboratories or from different cell lines or assay systems could be compared and normalized to PS. The anticipated responses of the cells to these particles and production of various cytokines and other growth factors are unknown. They may stimulate some factors and not others. These particles are not device material applicable and serve as a neutral territory, commercially available, well characterized particulate material for normalization of results.

X2. ADDITIONAL REFERENCES

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