



Designation: E2525 – 08

Standard Test Method for Evaluation of the Effect of Nanoparticulate Materials on the Formation of Mouse Granulocyte-Macrophage Colonies¹

This standard is issued under the fixed designation E2525; 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 test method provides a protocol for quantitative analysis of the effect of nanoparticulate materials in physiologic solution on granulocyte-macrophage colony-forming units.

1.2 This test method employs murine bone marrow hematopoietic stem cells which proliferate and differentiate to form discrete cell clusters or colonies which are counted.

1.3 This test method is part of the in vitro preclinical characterization cascade for nanoparticulate materials for systemic administration in medical applications.

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

F1903 Practice for Testing For Biological Responses to Particles In Vitro

2.2 *ANSI Standard*:

ANSI/ AAMI ST72 Bacterial Endotoxins—Test Methodologies, Routine Monitoring, and Alternatives to Batch Testing³

3. Terminology

3.1 *Abbreviations*:

3.1.1 *BM*—bone marrow

3.1.2 *CFU-GM*—colony forming unit of granulocyte and macrophage

¹ This test method is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.03 on Environment, Health, and Safety.

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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 Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, <http://www.ansi.org>.

3.1.3 *Cisplatin*—positive control

3.1.4 *DMSO*—dimethyl sulfoxide

3.1.5 *DPBS*—Dulbecco's phosphate buffered saline

3.1.6 *FBS*—fetal bovine serum

3.1.7 *IMDM*—Iscove's media

3.1.8 *LPS*—lipopolysaccharide

3.1.9 *Physiologic Solution*—isotonic, pH 7.2 \pm 0.2

4. Summary of Test Method

4.1 The effect of nanoparticulate materials on the formation of granulocyte and macrophage colonies is assessed. Bone marrow cells are obtained from mice and cultured in stimulatory media. The number of colony forming units following contact with nanoparticles is counted and compared to baseline and positive control. This determines if the nanoparticulate material in physiologic solution is stimulatory or inhibitory to bone marrow stem cells. Aseptic procedures are necessary.

5. Significance and Use

5.1 Stem cells of hematopoietic origin are pluripotential and may be particularly sensitive to the effects of stimulation by nanoparticulate materials.

5.2 The effect of particles on macrophage responses has an extensive history and can be assessed by Practice F1903. The test method described here will assess the effect on stem cells which can be progenitor cells to the macrophage line.

6. Reagents and Materials

6.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.⁴ Other grades may be used,

⁴ *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

6.2 Reagents and Supplies:

6.2.1 MethoCult medium, StemCell Technologies Inc cat.# 03534.

6.2.2 Fetal Bovine Serum prescreened for hematopoietic stem cells, StemCell Technologies Inc cat.# 06200.

6.2.3 IMDM with 2 % FBS, StemCell Technologies Inc cat.# 07700.

6.2.4 Sterile distilled water.

6.2.5 Cisplatin, (positive control) Sigma cat# P4394.

6.2.6 Sterile $\text{Ca}^{2+}/\text{MG}^{2+}$ -free DPBS, (negative control) Sigma cat.# D8537.

NOTE 1—The source of the reagents is shown for information purposes only to aid laboratories initiating this procedure. Equivalent reagents from other suppliers may be used.

6.3 Equipment—Aseptic procedures are necessary and care should be used in acquiring sterile equipment as needed.

6.3.1 Pipettes covering the range of 0.05 to 10 mL.

6.3.2 35-mm culture dishes prescreened to support stem cell growth and differentiation, StemCell Technologies Inc cat.# 27100.

6.3.3 Blunt-end 16-gauge needles, StemCell Technologies Inc cat.# 03534.

6.3.4 100-mm Petri dishes.

6.3.5 Plastic beakers.

6.3.6 Polypropylene tubes 50 and 15-mL.

6.3.7 Centrifuge.

6.3.8 Refrigerator, 2 to 8°C.

6.3.9 Freezer, -20°C.

6.3.10 Cell culture incubator with 5 % CO_2 and 95 % humidity.

6.3.11 CO_2 euthanasia box, or appropriate equipment approved by institution.

6.3.12 Scissors for tissue dissection.

6.3.13 Forceps.

6.3.14 Biohazard safety cabinet approved for level II handling of biological material.

6.3.15 Inverted microscope.

6.3.16 Vortex.

6.3.17 Hemocytometer.

6.4 Animals—Mice of the strain C56BL/6, males or females 8 to 12 weeks old, are used. Use of the pooled cells derived from at least two (2) animals is highly recommended.

7. Procedure

7.1 Aseptic Precautions are required.

7.2 Reagent and Control Preparation:

7.2.1 *MethoCult Medium*—The MethoCult medium is supplied in 100-mL size batches. It is recommended by manufacturer that the medium be thawed at room temperature or in a refrigerator overnight, vortexed to mix the ingredients, then left at a room temperature for approximately 5 min to allow air bubbles to dissipate. If using another source of media, follow the instructions indicated by the supplier. Use a 16-gauge blunt-end needle to dispense 3-mL aliquots of the MethoCult

medium into sterile 15-mL tubes. Store the aliquoted medium at a nominal temperature of -20°C. Before the test thaw the required number of tubes at room temperature for approximately 20 min and keep on ice prior to use. Repeated freezing and thawing should be avoided.

7.2.2 *50 mM Cisplatin (Positive Control)*—Cisplatin is supplied in a lyophilized form. Reconstitute the lyophilized powder by adding the appropriate amount of DMSO to make a stock solution with nominal concentration of 50 mM. Prepare small aliquots and store at a nominal temperature of -80 °C. Prior to use in the assay, thaw an aliquot of the stock solution at room temperature and dilute in IMDM supplemented with 2 % FBS to bring the Cisplatin concentration to 2 mM. One hundred fifty (150) μL of this intermediate solution is then added to 3 mL of culture medium. Final concentration of Cisplatin in the positive control sample is 100 μM .

7.3 Preparation of Study Samples:

7.3.1 This assay requires 1200 μL of nanoparticles, 150 μL samples in duplicate for each of 4 concentrations. The nanoparticles subjected to the biological test environment should have been characterized as appropriate to allow adequate data interpretation and to help provide information to predict biological responses. For example, lot-to-lot variations in particle size and surface characteristics of the particles could result in different assay results. For this assay, the particles shall be provided in physiologic solution (isotonic with pH 7.2 ± 0.2) and this solution shall be defined. The preparation shall be sterile and the level of LPS provided or determined by the testing laboratory. ANSI/AAMI ST72 may be helpful. The number of particles/mL and mg/mL shall be indicated.

7.3.2 The test sample shall be used at the highest concentration possible and at three serial one to five (1:5) dilutions. The highest possible concentration is that at which the particles appear evenly dispersed in the liquid. If the concentration for its intended use is known, this may serve as the highest concentration to be tested and at least three dilutions made.

7.4 Isolation and Counting of Bone Marrow Cells:

7.4.1 Position the euthanized mouse on its back and rinse fur thoroughly with 70 % alcohol.

7.4.2 Cut a slit in the fur just below the rib cage without cutting the peritoneal membrane.

7.4.3 Firmly grasp skin and peel back to expose hind limbs.

7.4.4 Using sterile sharp dissecting scissors cut the knee joint in the center. Cut through ligaments and excess tissue.

7.4.5 Grasp femur with forceps and cut femur near hip joint.

7.4.6 Free tibia by cutting near the ankle joint.

7.4.7 Trim the ends of the long bones to expose the interior marrow shaft. Put bones in a sterile Petri dish or in sterile culture medium and place on ice. Bones should be collected from multiple animals.

7.4.8 Using a 3-cc syringe with 21 or 22-gauge needle, draw up to 1 to 3 mL of cold Iscove's MDM supplemented with 2 % FBS.

7.4.9 Insert bevel of needle into marrow shaft and flush marrow into 15-mL tube. Repeat this procedure for all bones. The same medium can be used to isolate marrow from 1 to 3 animals. The medullary canal should appear white once all the marrow has been expelled.