



Designation: E2525 – 22

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

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1. Scope

1.1 This test method provides a protocol for quantitative analysis of the effect of nanoparticulate materials in physiologic solution (isotonic, pH 7.2 ± 0.2) on granulocyte-macrophage colony-forming units (CFU-GM).

1.2 CFU-GM reflects the number of viable bone marrow cells which differentiate into granulocytes and macrophages. A decrease in CFU-GM count is indicative of a test substance's toxicity to bone marrow and is commonly used for the identification of drug products with myelosuppressive properties, a form of immunosuppression.

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

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

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

1.6 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

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. Referenced Documents

2.1 *ASTM Standards*:²

F1903 Practice for Testing for Cellular Responses to Particles *in vitro*

2.2 *ANSI Standard*:³

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

3. Terminology

3.1 *Abbreviations*:

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

3.1.2 *DMSO*—dimethyl sulfoxide

3.1.3 *DPBS*—Dulbecco's phosphate buffered saline

3.1.4 *FBS*—fetal bovine serum

3.1.5 *IMDM*—Iscove's media

3.1.6 *LPS*—lipopolysaccharide

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 visually 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 pluripotent and may be particularly sensitive to the effects of stimulation by nanoparticulate materials.

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>.

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, 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 (see Note 1)*:

6.2.1 MethoCult medium, (optimized for growth and enumeration of granulocyte-macrophage progenitor cells) StemCell Technologies Inc cat.# 03534.

6.2.2 FBS 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, [cis-diammineplatinum(II) dichloride], (positive control) Sigma-Aldrich cat# P4394.

6.2.6 Sterile Ca²⁺/Mg²⁺-free DPBS, (negative control) Sigma-Aldrich 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 (see Note 2)*—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 mL 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 mL and 15 mL.

6.3.7 Centrifuge.

6.3.8 Refrigerator, 2 °C to 8 °C.

6.3.9 Freezer, -20 °C.

6.3.10 Cell culture incubator with 5 % CO₂ and 95 % humidity.

6.3.11 CO₂ 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 weeks to 12 weeks old, are used. Use of the pooled cells derived from at least two (2) animals is highly recommended.

NOTE 2—The source of the equipment, where given, is shown for information purposes only to aid laboratories initiating this procedure. Equivalent equipment from other suppliers may be used.

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 the 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 then keep on ice prior to use. Repeated freezing and thawing should be avoided.

7.2.2 *50 mmol/L 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 mmol/L. 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 mmol/L. 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 µmol/L.

7.3 *Preparation of Study Samples*:

7.3.1 This assay requires 1200 µL of nanoparticles, 150 µL samples in duplicate for each of four (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

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.

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 gauge or 22 gauge needle, draw up to 1 mL 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.

7.4.10 Keep the needle below the surface of the medium and gently draw the cell suspension up and down with 3 cc syringe and 21 gauge needle 3 to 4 times to make a single cell suspension without introducing air bubbles.

7.4.11 Keep cells in medium on ice until use.

7.4.12 Perform a nucleated cell count. Dilute an aliquot sample of the cells with 3 % acetic acid with methylene blue 1:50 (20 μ L cells + 980 μ L 3 % acetic acid methylene blue) or 1:100 (10 μ L cells + 990 μ L 3 % acetic acid methylene blue). Then use hemocytometer to determine the number of viable nucleated cells. An average cell count is expected to be $1-2 \times 10^7$ for femur and $0.6-1 \times 10^7$ for tibia from each mouse.

7.4.12.1 An automatic cell counter that counts nucleated live cells, if available, can also be used in place of hemocytometer.

7.4.13 If cell viability and count are acceptable providing at least 10^6 viable cells, proceed to the next step.

7.5 Experimental Procedure:

7.5.1 Label lids of 35 mm culture dishes at the edge using a permanent fine felt marker. There will be two 35 mm culture dishes for each control and test article.

7.5.2 Thaw the aliquoted tubes of MethoCult medium at room temperature or in a refrigerator overnight.

7.5.3 Vortex tubes to ensure all components are thoroughly mixed.

7.5.4 Dilute bone marrow cells isolated according to the procedure described in Section 6 with Iscove's medium supplemented with 2 % FBS to 4×10^5 cells per mL.

7.5.5 Add 150 μ L of cell suspension and 150 μ L of either Iscove's medium with 2 % FBS (baseline), PBS (negative control), Cisplatin (positive control), or nanoparticles (test sample) to 3 mL of MethoCult medium.

7.5.6 Vortex tubes to ensure all cells and medium components are mixed thoroughly.

7.5.7 Let the tubes stand for 5 min to allow bubbles to dissipate.

7.5.8 Label two 35 mm Petri dishes for each sample: baseline, negative control, positive control, and the test articles. Each sample is thus tested in duplicate.

7.5.9 Attach a 16 gauge blunt-ended needle to a 3 cc syringe, place the needle below the surface of the solution prepared in 7.5.5. Draw up approximately 1 mL, then gently depress the plunger and expel medium back into the tube. Repeat this step until no air space is visible.

7.5.10 With this syringe and needle, draw up the solution and dispense 1.1 mL into each of two 35- mm dishes. Distribute the cell suspension evenly by gently tilting and rotating each dish. Cover the 35 mm dishes and place the two replicates into a 100 mm Petri dish. Place a third 35 mm dish into the 100 mm Petri dish and fill this 35 mm dish with 3 mL of sterile water and do not cover this dish. This will provide a humidity chamber. Place the lid on the 100 mm Petri dish.

7.5.11 Repeat 7.5.10 for all samples and place the cultures in an incubator maintained at 37 °C, 5 % CO₂ and 95 % humidity.

7.5.12 Incubate for 12 days. On the 12th day, remove dishes from incubator, identify and count colonies as described below. Representative values of CFU-GM for C57BL6 mice at 8 weeks to 12 weeks of age range from 48 to 80.

7.6 Count the CFU-GM Colonies:

7.6.1 This count includes the CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), and CFU-granulocyte macrophage (CFU-GM). Count the colonies by visually examining plates and recording the respective numbers for each plate. CFU-GM colonies often contain multiple clusters and appear as dense core surrounded by cells. CFU-M and CFU-G colonies may also be present and included in the counts. The monocytic lineage cells are large cells with an oval to round shape and appear to have a grainy or grey center. The granulocytic lineage cells are round, bright, and are much smaller and more uniform in size than macrophages. It is easy to see individual cells of a CFU-GM colony, especially in the periphery of the colony.

7.6.2 See Fig. 1 for the following colony examples. Colonies seen on Figures A and B are CFU-GM. Colonies on Figures C and D are CFU-M. Figure E demonstrates an example of single CFU-G colony. Few CFU-G colonies growing together are shown on Figure F.

8. Calculation or Interpretation of Results

8.1 Percent CFU inhibition is calculated as follows:

$$\% \text{CFU (inhibition)} = \frac{(\text{baseline CFU}_{\text{GM}}) - (\text{test CFU}_{\text{GM}})}{(\text{baseline CFU}_{\text{GM}})} \times 100 \quad (1)$$

Where CFU_{GM} includes all counted colonies (G, M, and GM) for the baseline, controls, or test (sample) material. In the numerator, (test CFU_{GM}) is the total colony count for a test material, positive control, or negative control.