Designation: E2525 – 08 (Reapproved 2013)

Standard Test Method for Evaluation of the Effect of Nanoparticulate Materials on the Formation of Mouse Granulocyte-Macrophage Colonies[1]

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

1.5 *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:*[3]

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

3. Terminology

3.1 *Abbreviations:*

3.1.1 *BM*—bone marrow

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

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*—ipopolysaccharide

3.1.9 *Physiologic Solution*—isotonic, pH 7.2 ± 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

[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.

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:*

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 Ca^{2+}/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 peal 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.

[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.

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.

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 µL cells + 980 µL 3 % acetic acid methylene blue) or 1:100 (10 µL cells + 990 µL 3 % acetic acid methylene blue). Then use either hemocytometer or automatic cell counter (that counts nucleated live cells) 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.

7.4.13 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 µL of cell suspension and 150 µL 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 no 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 % CO_2 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 to 12 weeks of age range from 64 ± 16.

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 containing at least 30 CFU-G, CFU-M or both cell types (CFU-GM). CFU-GM colonies often contain multiple clusters and appear as dense core surrounded by cells. 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 Determine the mean and standard deviation and the Percent Coefficient of Variation for each control or test according to the following formula:

$$\%\text{CV} = \text{SD/Mean} \times 100\,\% \quad (1)$$

8.2 Percent CFU Inhibition is calculated as follows:

$$\%\ \text{CFU} - \text{Inhibition} = \frac{(\text{Baseline CFU} - \text{GM} - \text{Test CFU} - \text{GM})}{\text{Baseline CFU} - \text{GM}} \times 100\,\% \quad (2)$$

9. Report

9.1 %CV for each control and test sample should less than 30 %.

9.1.1 If the positive control or negative control fail to meet acceptance criterion described in 9.1 the assay should be repeated.

9.1.2 If the assay meets the acceptable criteria but a test sample fails to meet acceptance criterion described in 9.1 this test sample should be re-analyzed.

9.1.3 If two duplicates of the same test sample demonstrate results different by more then 20 %, this sample should be reanalyzed.

9.1.4 Determine if the results of the test sample are different from the media control and indicate if the test sample is stimulatory or inhibitory.

9.1.5 Determine if there is a dose response.

10. Precision and Bias

10.1 Precision and bias have not been determined for this test method and will be determined within 5 years of publication of the standard. At this time there is no commercially

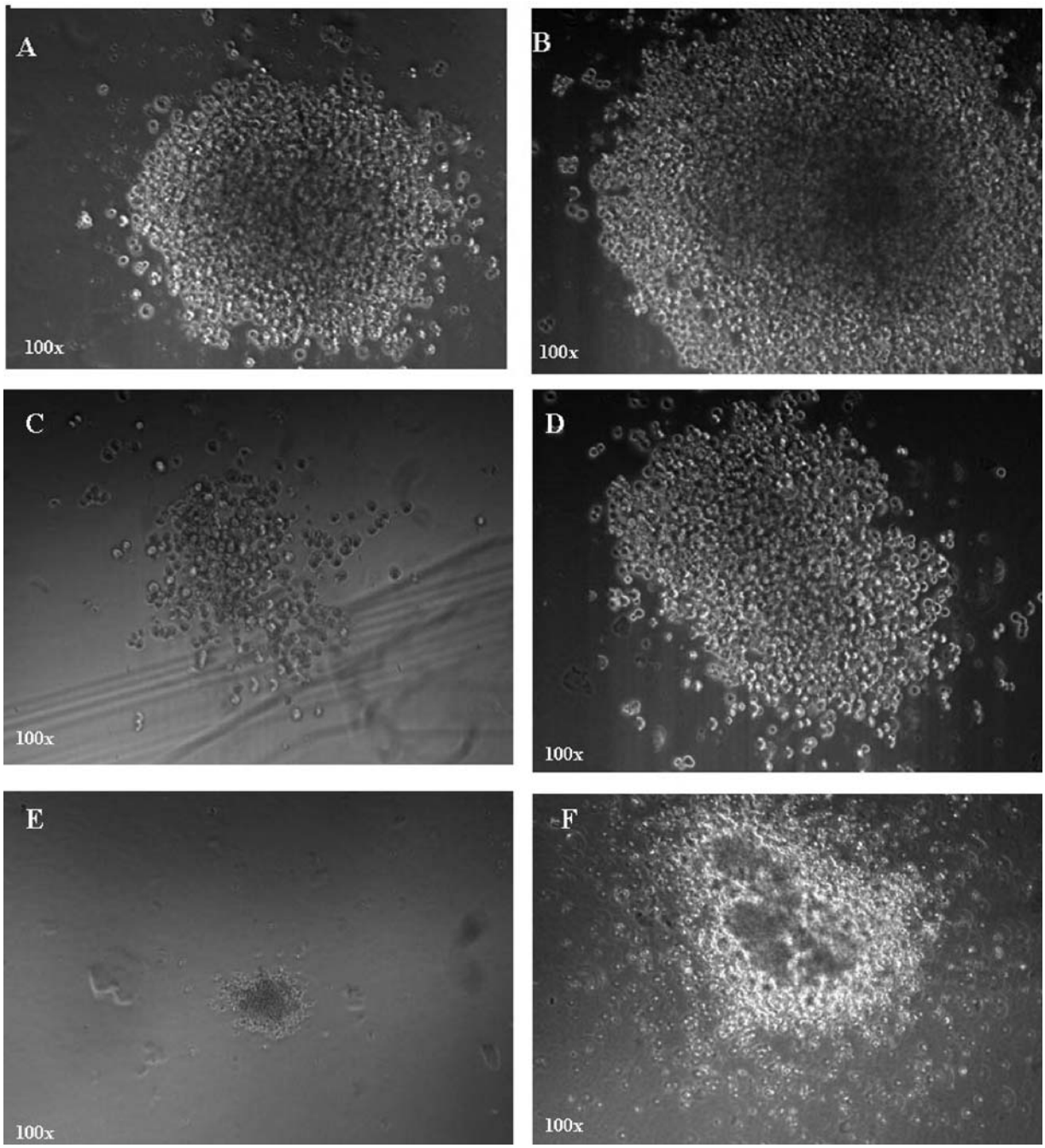

FIG. 1 Example of colonies observed on day 12 of CFU-GM culture (see step 7.5.12)

available test article of sufficient quantity and reproducible quality to conduct intra- or interlaboratory comparisons of precision and bias.

11. Keywords

11.1 bone marrow; CFU-GM; clonogenic assay; granulocyte; macrophage; myelosuppression; nanoparticles; preclinical development; stem cells

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