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.

Designation: E2525 – 22

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 (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.*

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*—ipopolysaccharide

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

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[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.

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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 Ca^{2+}/MG^{2+}-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 % 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 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.