Designation: E3238 – 20

Standard Test Method for Quantitative Measurement of the Chemoattractant Capacity of a Nanoparticulate Material *in vitro*[1]

This standard is issued under the fixed designation E3238; 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 rapid and quantitative measurement of the chemoattractant capacity of a nanoparticulate material (nanoparticles and their aggregates and agglomerates).

1.2 Immune cells recruitment (by chemotaxis) plays a central role in the immune system function especially in the inflammatory process.

1.3 This test method uses an *in vitro* model. In this model, peripheral blood human acute promyelocytic leukemia cells HL-60 are separated from control chemoattractant or test nanoparticulate material by a 3-µm pore size filter; the cell migration through the filter is monitored and quantified using the fluorescent dye calcein AM (Figs. 1 and 2).

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

1.6 *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]

E2490 Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS)

E2834 Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA)

F1877 Practice for Characterization of Particles

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

3. Terminology

3.1 *Acronyms:*

3.1.1 *BSA*—bovine serum albumin

3.1.2 *calcein AM*—calcein acetoxymethyl ester

3.1.3 C_{max}—maximum serum concentration

3.1.4 *CV*—coefficient of variation

3.1.5 *FBS*—fetal bovine serum

3.1.6 *FU*—fluorescence units

3.1.7 *g*—relative centrifugal force

3.1.8 *NC*—negative control

3.1.9 *PBS*—phosphate buffered saline

3.1.10 *PC*—positive control

3.1.11 *PK*—pharmacokinetic

3.1.12 *RBC*—reagent background control

3.1.13 *RPMI*—Roswell Park Memorial Institute

3.1.14 *SD*—standard deviation

3.1.15 *SM*—starvation medium

3.1.16 *TS*—test sample (of nanoparticulate material dissolved in starvation medium)

3.1.17 *U*—units

4. Summary of Test Method

4.1 This test method describes a protocol for assessing and measuring the chemoattractant capacity of nanoparticulate material.

4.2 The migration of cells through a filter towards the nanoparticulate material is monitored and quantified after staining with calcein AM dye.

[1] This test method is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.08 on Nano-Enabled Medical Products.

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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.

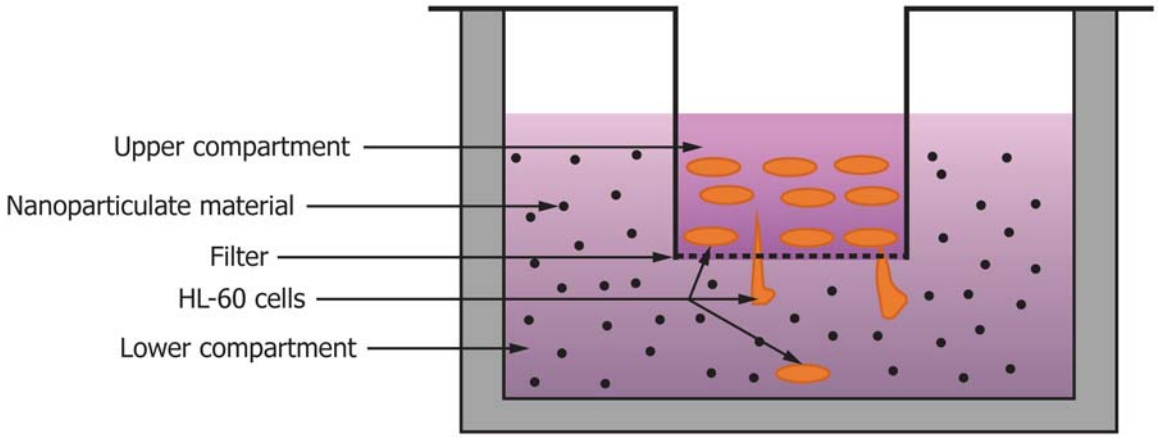
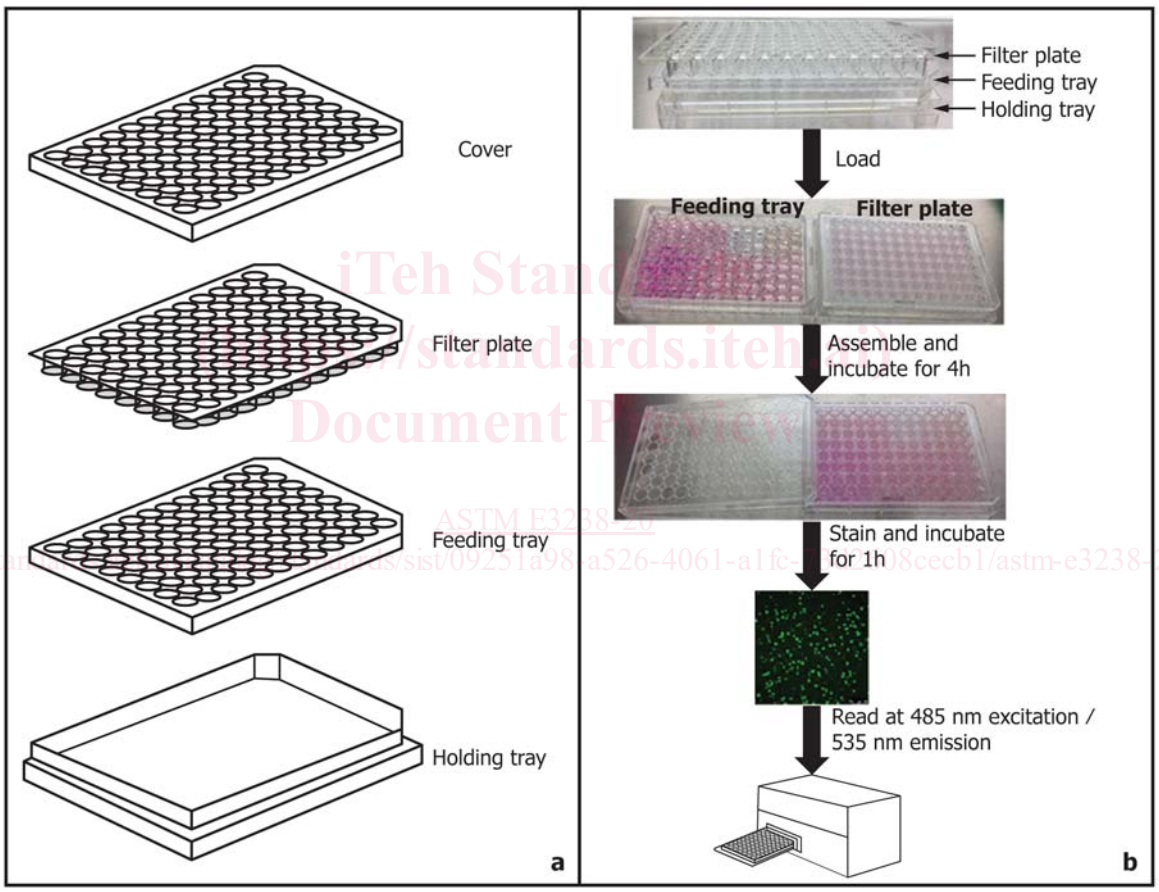


FIG. 1 Chemotaxis Chamber (Boyden Chamber)



a (left)—Parts of the chemotaxis assay assembly.
b (right)—Procedure for testing the chemoattractant capacity of a nanoparticulate material.

FIG. 2 Chemotaxis Assay

4.3 Calcein AM, a non-fluorescent hydrophobic compound, is transported through the cell membrane into live cells. Intracellular esterases remove the acetomethoxy group of calcein AM producing calcein, a hydrophilic green fluorescent compound. Calcein is retained by the live cells.

5. Significance and Use

5.1 This test method will assess whether the test nanoparticulate material has chemoattractant activity.

5.2 This test method will provide a rapid and quantitative measure of the ability of nanoparticulate material to recruit immune cells.

5.3 Recruitment of immune cells by chemotaxis plays an important part in all phases of both humoral and cell-mediated immune responses.

5.4 Testing the capacity of a nanoparticulate material to recruit immune cells *in vitro* helps in predicting the influence of such material on the immune cell response.

6. Materials

6.1 *Pipettes* covering the range of 0.05 to 10 mL.

6.2 *96-well filter plate,* 3.0-µm pore size polycarbonate membrane, clear, sterile.

6.3 *96-well feeding tray,* clear, sterile (culture tray).

6.4 *Holding tray.*

6.5 *Cover* for holding tray.

6.6 *96-microwell clear bottom plates* suitable for fluorescent-based assays.

6.7 *Polypropylene tubes,* 5 and 15 mL.

6.8 *Cell culture flasks.*

7. Cell Line

7.1 Peripheral blood human acute promyelocytic leukemia cells HL-60 (ATCC CCL-240[3]).

8. Reagents

8.1 *Phosphate buffered saline (PBS),* pH 7.4.

8.2 *Bovine serum albumin (BSA),* endotoxin-free and suitable for cell culture.

8.3 *Fetal bovine serum (FBS).*

8.4 *Commercial RPMI-1640 medium* (basal medium).

8.5 *L-glutamine,* 4 mM.

8.6 *Sodium bicarbonate.*

8.7 *β-mercaptoethanol,* 50 µM.

8.8 *Calcein AM,* 1 mM.

8.9 *Trypan blue solution.*

8.10 *Penicillin.*

8.11 *Streptomycin sulfate.*

9. Apparatus

9.1 *Centrifuge.*

9.2 *Water bath* set at 37°C.

9.3 *Refrigerator,* 2–8°C.

9.4 *Freezer,* –20°C.

9.5 *Cell culture incubator* with 5 % carbon dioxide (CO_2) and 95 % humidity.

9.6 *Biohazard safety cabinet* approved for Level II handling of biological material.

9.7 *Inverted microscope.*

9.8 *Vortex mixer.*

9.9 *Hemocytometer.*

9.10 *Fluorescence plate reader.*

10. Preparation of Media, Calcein AM, Test Samples and Controls

10.1 Three solutions of each nanoparticulate material test concentration (referred to as test samples) and three solutions of each control (SM, PC, and NC) shall be independently prepared.

10.2 *Preparation of Complete RPMI-1640 Medium*—The complete RPMI-1640 medium is a solution of RPMI-1640 medium (basal medium) supplemented with 20 % FBS (heat-inactivated), 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 50 µM β-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The medium should be stored at 2–8°C protected from light for no longer than 1 month. Before use, warm in a water bath.

10.3 *Preparation of Starvation Medium (SM)*—The SM is a solution of RPMI-1640 medium (basal medium) supplemented with 0.2 % BSA, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 50 µM β-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The medium is stored at 2–8°C protected from light for no longer than 1 month. Before use, warm in a water bath. Prepare three solutions of SM (see 10.1).

10.4 *Postive Control (PC)*—On the day of the experiment, prepare a solution of RPMI-1640 medium supplemented with 20 % heat-inactivated FBS and 0.2 % BSA. Repeat twice to obtain three independently prepared solutions (see 10.1).

10.5 *Negative Control (NC)*—Use PBS as a NC.

10.6 *Calcein AM Working Solution*—Dilute the calcein AM 1-mM stock solution in prewarmed (37°C) PBS to a final concentration of 4 µM. The working dilution shall be prepared immediately prior to use.

10.7 *Specification of Nanoparticulate Material Test Samples*—The selected nanoparticulate material must be fully dispersed in SM. This assay requires a minimum of 2.3 mL of nanoparticulate material dissolved/resuspended in SM. (This volume is calculated from the number and concentration of test samples prepared as specified below, including replicates.) The following factors shall be considered in choosing the nanoparticulate material concentration:

(1) Dispersibility, stability, and homogeneity of the nanoparticulate material in a biocompatible buffer (that is, SM),

(2) Maintaining the pH in the physiological range, and

(3) Stability of nanoparticulate material during testing.

Before testing, the nanoparticulate material shall be characterized (for example, size, size distribution, and charge) under physiological conditions in accordance with with standard methods such as those recommended in Guides E2490 and E2834 for nanomaterials and Practices F1877 and F1903 for

[3] A trademark of ATCC in Manassas, VA.