Designation: E3351 – 22

# Standard Test Method for Detection of Nitric Oxide Production *In Vitro*[1]

This standard is issued under the fixed designation E3351; 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 delivers a protocol for a quantitative measure of nitrite ($NO_2^-$), a stable end-product of nitric oxide (NO), in cell culture medium due to exposure to nanomaterial(s).

1.2 NO has a critical role in several pathological conditions in addition to its role in many physiological processes.

1.3 This test method uses murine macrophage cell line RAW 264.7 as an *in vitro* model.

1.4 The nitrite is measured in the cell culture medium by a colorimetric analysis using Griess reagent as shown in Fig. 1.

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 *Definitions:*

3.1.1 *Cal*—calibration standards

3.1.2 $C_{max}$—maximum serum concentration

3.1.3 *CV*—coefficient of variation

3.1.4 *DEA NONOate*—diethylamine NONOate/AM

3.1.5 *DMSO*—dimethyl sulfoxide

3.1.6 *FBS*—fetal bovine serum

3.1.7 *Int.*—intermediate

3.1.8 *LPS*—lipopolysaccharide

3.1.9 *PBS*—phosphate buffered saline

3.1.10 *PDFT*—percent difference from theoretical

3.1.11 *RPMI*—Roswell Park Memorial Institute

3.1.12 *QC*—quality control

3.1.13 *SD*—standard deviation

3.1.14 *w/v*—weight to volume ratio

## 4. Summary of Test Method

4.1 This test method is used to assess the capability of nanomaterials to induce nitric oxide production by macrophages *in vitro* (see Fig. 1).

4.2 The NO molecule has a short half-life and reacts quickly with free oxygen, oxygen radicals, redox metals and even with oxygenated hemoglobin to generate other reactive nitrogen intermediates which decomposes to form nitrite ($NO_2^-$) and nitrate ($NO_3^-$) (**1**).[3] NO molecule can react with oxygenated hemoglobin to produce nitrate ($NO_3^-$) (**1, 2**).

4.3 This test method describes a protocol for assessing and measuring nitrite as a replacement marker and quantitative indicator of NO production.

4.4 In this test method, nitrite is measured in cell culture medium using the Griess reagent.

4.5 The upper limit of nitrite quantification is 250 µM and the lower limit of quantification is 1.95 µM.

[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.

[3] The boldface numbers in parentheses refer to the list of references at the end of the standard.

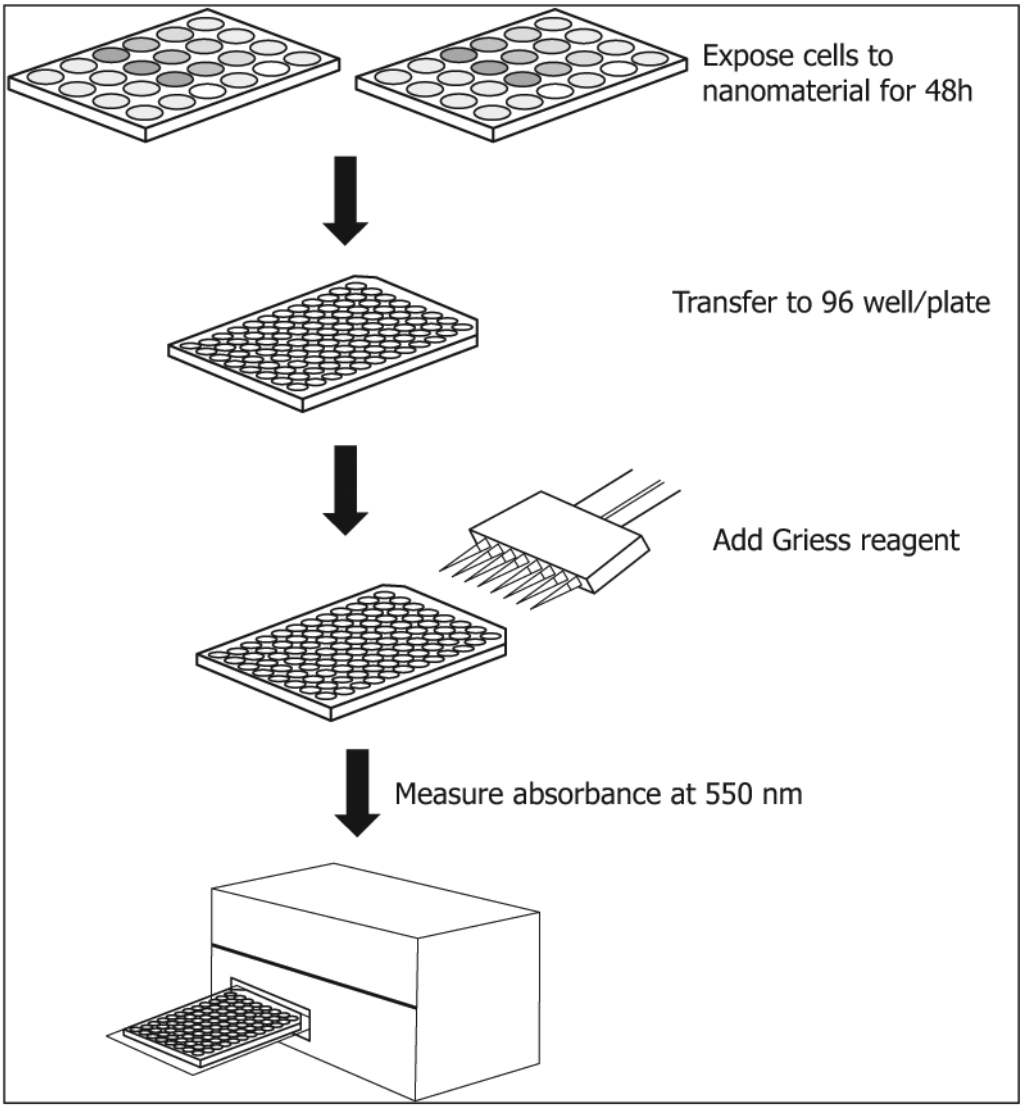


**FIG. 1 Summary of Nitric Oxide Production Assay**

## 5. Significance and Use

5.1 This test method is designed to evaluate nanomaterial capacity to induce nitric oxide production by macrophages.

5.2 Activated macrophages generate large quantities of NO. NO generated from activated macrophages is a cytostatic/cytotoxic agent (**3-6**).

5.3 The production of NO in excessive amounts leads to the generation of peroxynitrite by its spontaneous reaction with superoxide. Peroxynitrite causes tissue injury through its capability to damage lipids, proteins, and DNA (**2**).

5.4 NO is a proinflammatory mediator and it is an important marker for activation of inflammation (**5, 6**).

5.5 Testing the capacity of a nanomaterial to induce NO production *in vitro* helps in predicting the nanomaterial's biocompatibility through anticipating and understanding the potential problems that might be encountered during its *in vivo* administration.

## 6. Materials

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

6.2 Flat bottom 96-well plates.

6.3 24-well plates.

6.4 Polypropylene tubes, 5 mL, and 15 mL.

## 7. Cell Line

7.1 Murine macrophage cell line RAW 264.7 (ATCC (trademarked) TIB-71 (trademarked)).

## 8. Reagents

8.1 β-mercaptoethanol.

8.2 Diethylamine NONOate/AM (DEA NONOate).

8.3 Dimethyl sulfoxide (DMSO).

8.4 Fetal bovine serum (FBS).