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.

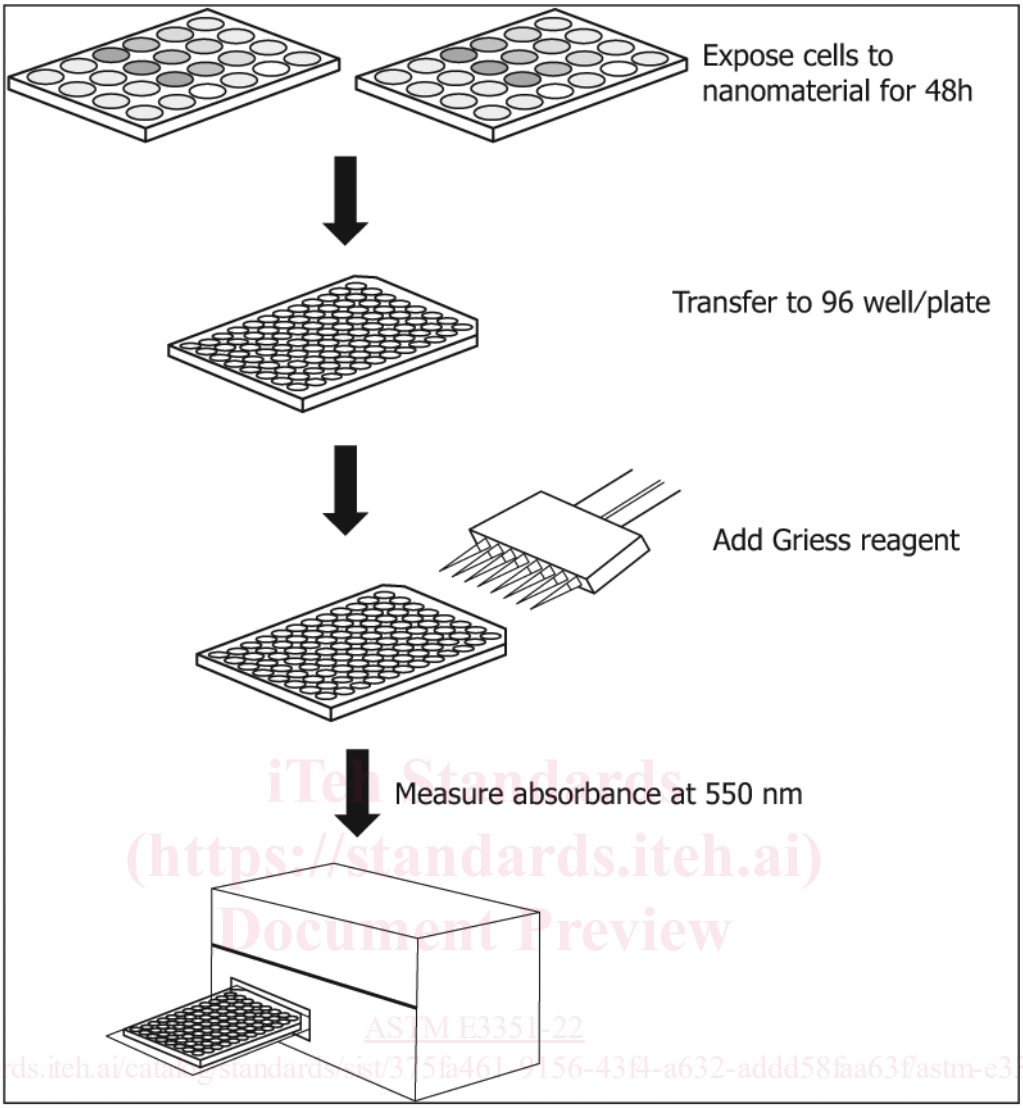

FIG. 1 Summary of Nitric Oxide Production Assay

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).

8.5 Lipopolysaccharides from *Escherichia coli* O55:B5 (LPS).

8.6 Naphthylethylenediamine dihydrochloride.

8.7 Penicillin-Streptomycin.

8.8 Phosphate buffered saline (PBS), pH 7.4.

8.9 Phosphoric acid.

8.10 RPMI-1640 without phenol red.

8.11 Sodium nitrite ($NaNO_2$) standard, 0.1 M stock solution.

8.12 Sulfanilamide.

8.13 Trypan blue solution.

9. Apparatus

9.1 Biohazard safety cabinet approved for level II handling of biological material.

9.2 Cell culture incubator with 5 % CO_2 and 95 % humidity.

9.3 Centrifuge.

9.4 Freezer, –20 °C.

9.5 Hemocytometer or cell counter.

9.6 Inverted light microscope.

9.7 Plate reader.

9.8 Refrigerator, 2 °C to 8 °C.

9.9 Vortex mixer.

9.10 Water bath set at 37 °C.

10. Preparation of Complete Cell Culture Medium, Lipopolysaccharide, Diethylamine NONOate/AM, Griess Reagent, Controls, Nitrite Calibration Standards and Test Samples

10.1 *Preparation of Complete RPMI-1640 Medium*—The complete RPMI-1640 medium should contain 10 % FBS (heat inactivated), 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 U⁄mL penicillin, and 100 µg⁄mL streptomycin sulfate. The medium should be stored at 2 °C to 8 °C protected from light for no longer than 1 month. Before use, warm in a water bath.

10.2 *Lipopolysaccharide 1 mg/mL (LPS, Stock)*—Add 1 mL of sterile PBS or cell culture medium per 1 mg of LPS to the vial and vortex to mix. Aliquot the stock into 100 µL portions and store at –20 °C. Individual aliquots are thawed and used only once.

10.3 *Diethylamine NONOate/AM*—Reconstitute with DMSO, aliquot (20 µl aliquots) and freeze (–20 °C). DMSO stock solution aliquots are stable for 2 months at –20 °C.

10.4 *Positive Control*—Dilute stock LPS solution in cell culture medium to final concentrations of 100 ng⁄mL. Store at room temperature. Discard unused portion after experiment. For DEA NONOate; dilute stock aliquots in cell culture medium to final concentrations of 6.25 µM, 12.5 µM, and 25 µM. Discard unused portion after experiment.

10.5 *Negative Control*—Use PBS as a negative control.

10.6 *Griess Reagent*—Consists of 2 reagents: reagent A and reagent B.

10.6.1 *Reagent A*—Dissolve sulfanilamide in 2.5 % phosphoric acid (H_3PO_4) to a final concentration of 1 % (w/v), for example, dissolve 1 g of sulfanilamide in 100 mL of 2.5 % H_3PO_4.

10.6.2 *Reagent B*—Dissolve naphthylethylenediamine dihydrochloride in 2.5 % H_3PO_4 to a final concentration of 0.1 % (w/v), for example, dissolve 100 mg of naphthylethylenediamine dihydrochloride in 100 mL of 2.5 % H_3PO_4.

10.6.3 Store both solutions in amber glass bottles at 4 °C; discard if discoloration occurs or solutions are not clear.

10.6.4 Combine equal volumes of reagents A and B just prior to use to form the Griess reagent.

10.6.5 Griess should be used immediately after preparation and any remaining reagent should be discarded.

10.7 *Preparation of Sodium Nitrite ($NaNO_2$) Calibration Standards:*

10.7.1 Prepare $NaNO_2$ calibration standards as shown in the example table below. These calibration standards will be used for building the standard curve. The standard curve will be used to calculate the NO concentration of the test samples. Stock is reagent 8.11 (sodium nitrite ($NaNO_2$) standard, 0.1 M stock solution). The stock will be diluted to prepare the intermediate (Int.) stock A, intermediate stock B, and calibration standard (Cal) 1–8.

10.7.2 Volumes of $NaNO_2$ calibration standards can be adjusted based on the need.

Level	Nominal Concentration (µM)	Preparation Procedure
Int. A	10 000	100 µL Stock + 900 µL complete RPMI-1640 medium
Int. B	1000	100 µL Int. A + 900 µL complete RPMI-1640 medium
Cal 1	250	200 µL Int. B + 600 µL complete RPMI-1640 medium
Cal 2	125	400 µL Cal 1 + 400 µL complete RPMI-1640 medium
Cal 3	62.5	400 µL Cal 2 + 400 µL complete RPMI-1640 medium
Cal 4	31.3	400 µL Cal 3 + 400 µL complete RPMI-1640 medium
Cal 5	15.6	400 µL Cal 4 + 400 µL complete RPMI-1640 medium
Cal 6	7.81	400 µL Cal 5 + 400 µL complete RPMI-1640 medium
Cal 7	3.91	400 µL Cal 6 + 400 µL complete RPMI-1640 medium
Cal 8	1.95	400 µL Cal 7 + 400 µL complete RPMI-1640 medium

10.8 *Preparation of Sodium Nitrite ($NaNO_2$) Quality Controls:*

10.8.1 Prepare $NaNO_2$ quality control as shown in the example table below. Stock is reagent 8.11 (sodium nitrite ($NaNO_2$) standard, 0.1 M stock solution). The stock will be diluted to prepare intermediate (Int.) stock A, intermediate stock B, and quality control (QC) 1–3.

10.8.2 Volumes of $NaNO_2$ can be adjusted according to the need.

Level	Nominal Concentration (µM)	Preparation Procedure
Int. A	10 000	100 µL Stock + 900 µL complete RPMI-1640 medium
Int. B	1000	100 µL Int. A + 900 µL complete RPMI-1640 medium
QC 1	100	100 µL Int. B + 900 µL complete RPMI-1640 medium
QC 2	50	400 µL QC1 + 400 µL complete RPMI-1640 medium
QC 3	5	100 µL QC 2 + 900 µL complete RPMI-1640 medium

10.9 *Specification of Nanomaterial Test Samples*—This assay requires a minimum of 3500 µL of nanomaterial (at 1× the highest final test concentration, this volume is calculated from the number and concentration of test samples prepared as

specified below including the replicates) dissolved/resuspended in complete culture medium. The following shall be considered when choosing the nanomaterial concentration: *(1)* Dispersibility, solubility, and homogeneity of nanomaterials in a biocompatible buffer, *(2)* pH shall be maintained in the physiological range, and *(3)* stability of nanomaterials during testing. Before testing, the nanomaterial shall be characterized (for example, size, size distribution, and charge) under physiological conditions and according to standard methods including those recommended in Guides E2490 and E2834 for nanomaterials and Practices F1877 and F1903 for powders. Nanomaterials are tested at four concentrations. For example, in the absence of data from PK studies, if the highest tested concentration is 1.0 mg/mL then the stock nanomaterial concentration should also be 1.0 mg/mL and the three serial 1:5 dilutions will be 0.2 mg/mL, 0.04 mg/mL, and 0.008 mg/mL. Six 0.5 mL samples will be used for each test concentration (four for the assay test samples and two for a cell-free control). However, when the nanomaterial's plasma concentration is known from *in vivo* PK studies, the highest concentration tested *in vitro* is chosen as 10×, 30×, or 100× of that C_{max} (depending on the solubility of the nanomaterial).

10.10 *Preparation of Nanomaterial Test Samples*—Prepare a nanomaterial stock solution in complete cell culture medium at a concentration of 1 mg/ml. Prepare three serial 1:5 dilutions using this stock to obtain nanomaterial test samples of 0.2 mg/mL, 0.04 mg/mL, and 0.008 mg/mL.

10.11 *Cell Maintenance*—Grow RAW 264.7 cells in complete RPMI-1640 medium. For subculturing, dislodge cells from cell culture vessel using a cell scraper; aspirate and add appropriate aliquots of the cell suspension into new culture vessel. A subcultivation ratio of 1:3 to 1:6 is recommended. Replace or add medium every 2 to 3 days.

11. Experimental Procedure

11.1 Adjust cell concentration to 1×10^5 cells/mL using complete RPMI-1640 medium.

11.2 Plate 1000 µL of cell suspension per well in a 24-well plate. Prepare four replicate wells for each sample and control. Leave two cell-free well per nanomaterial per plate. These wells will be used to test nanomaterial interference with the assay. This is the "Culture Plate" (see plate map example in Appendix X1, Fig. X1.1).

11.3 Incubate the "Culture Plate" 24 h in a humidified 37 °C, 5 % CO_2 incubator.

11.4 Remove culture medium and add 500 µL of test samples, controls, or medium blank to appropriate wells (see culture plate map example in Appendix X1, Fig. X1.1).

11.5 Incubate the "Culture Plate" 48 h ± 1 h in a humidified 37 °C, 5 % CO_2 incubator.

11.6 To a fresh 96-well plate, add 50 µL per well of reagent blank [culture medium used to prepare calibration standards and quality controls], calibration standards, quality controls, and medium from each well of the "Culture plate". Load four replicate wells for each sample and control. This is the "NO Test Plate" (see plate map example in Appendix X1, Fig. X1.2).

NOTE 1—Removal of nanomaterials from culture medium may be required prior to this step if nanomaterials can interfere with assay, for example, if nanomaterial reacts with either or both components of the Griess reagent or have absorbance at or close to 550 nm. If nanomaterial removal is not feasible, results obtained for "no cells nanomaterials" control may be subtracted from that obtained for "nanomaterial test sample" to correct for particle background interference. See Annex A1 for an optional pre-test plate procedure that can be used to obtain information about potential nanomaterial interference before performing the full assay.

11.7 In a separate tube, combine equal volumes of reagent A and reagent B at room temperature; this is the Griess reagent (for example, combine 6 ml of reagent A with 6 ml of reagent B).

11.8 Add 100 µL of the Griess reagent to each well of the "NO Test Plate".

11.9 Place the plate on a shaker for 2 min to 3 min, allowing all ingredients to mix.

11.10 Measure absorbance at 550 nm using a 96-well plate reader machine.

12. Calculations

12.1 Note that superimposing the two culture plates maps on the NO plate map in Appendix X1 (Fig. X1.1 and Fig. X1.2) yields four replicate wells for each test sample, positive control, negative control and blank. There are three replicates for each calibration standard and quality control.

12.2 Subtract the mean absorbance values of the blank wells from all the absorbance values of all other wells.

12.3 Mean is a simple average of absorbance values. For example, for Test Sample One TS1:

$$\text{Mean absorbance} = (TS1a + TS1b + TS1c + TS1d) / 4 \quad (1)$$

Where a, b, c, and d represent the four replicates for TS1. Similarly, Mean values shall be calculated for TS2, TS3, TS4, PC, NC, Blank etc.

Where 4 (the number of replicate measurements for each sample concentration) is used to calculate the Mean values.

12.4 A percent coefficient of variation (%CV) shall be calculated for blank, PC, NC, and test samples according to the following formula:

$$\%CV = SD/Mean \times 100\% \quad (2)$$

12.4.1 SD is the square root of the variance; it is the mean of the square differences of absorbance values for each of the four replicates from the mean value. For example, for Test Sample One TS1:

$$SD\ TS = \sqrt{(((TS1a - Mean\ TS1) + (TS1b - Mean\ TS1) + (TS1c - Mean\ TS1) + (TS1d - Mean\ TS1)^2) / 4)} \quad (3)$$

Where a, b, c, and d represent the four replicates for TS1. Similarly, SD values shall be calculated for TS2, TS3, TS4, PC, NC, blank, etc.

Where 4 (the number of replicate measurements for each sample concentration) is used to calculate the SD values.

12.4.2 %CV calculation, for example, for Test Sample One TS1:

$$\%CV\ TS1 = SD\ TS1/Mean\ TS1 \times 100 \quad (4)$$

Similarly, %CV values shall be calculated for TS2, TS3, TS4, PC, NC, etc.

12.5 Percent Difference from Theoretical (PDFT) is used to control accuracy of the assay calibration standards and quality controls, and is calculated according to the following formula:

$$\text{PDFT} = \frac{\text{Calculated } NaNO_2 \text{ concentration} - \text{Theoretical } NaNO_2 \text{ concentration}}{\text{Theoretical } NaNO_2 \text{ concentration}} \times 100 \quad (5)$$

12.6 Generate a standard curve with $NaNO_2$ in concentrations from 1.95 µM to 250 µM by making a plot of absorbance at 550 nm as a function of $NaNO_2$ concentration.

12.7 Calculate nitrite concentration of controls and Test Samples using the standard curve.

13. Acceptance Criteria

13.1 %CV for each control and test sample should be within 20 %.

13.2 If %CV of positive control is more than 20 %, the assay should be repeated.

13.3 If %CV of three out of four replicates of Test Sample is more than 20 %, this test sample should be reanalyzed.

13.4 %CV and PDFT of calibration standards and quality controls should be within 20 %. At least 5 calibrators should be available. Six of nine QC and at least one of each level should be acceptable. If not, a new set of calibration standards and quality controls should be prepared, and test samples reloaded onto a new plate.

14. Report

14.1 Determine the calculated amount of nitrite in µM produced by cells exposed to the test sample from the equation of the generated standard curve.

14.2 The calculated nitrite represents the amount of NO generated upon exposure of the murine macrophage cell line (RAW 264.7) to the test nanomaterials.

15. Precision and Bias

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

16. Keywords

16.1 immune response; macrophages; nanomaterial; nanoparticles; nitric oxide

ANNEX

(Mandatory Information)

A1. PRE-TEST PLATE PROCEDURE

A1.1 *Aim and Significance*—This is an optional procedure that provides information for users who want to evaluate potential test sample interference before performing the NO production assay.

A1.2 *Materials*—See Materials section (Section 6).

A1.3 *Reagents*—See Reagents section (Section 8).

A1.4 *Apparatus*—See Apparatus section (Section 9).

A1.5 *Preparation of Complete Cell Culture Medium, Griess reagent, Nitrite Calibration Standards and Test Samples*:

A1.5.1 Cell culture medium (see 10.1).

A1.5.2 Griess reagent (see 10.6).

A1.5.3 Specification of nanomaterial test samples (see 10.9).

A1.5.4 Preparation of nanomaterial test samples (see 10.10).

A1.5.5 Preparation of sodium nitrite ($NaNO_2$) calibration standards.

A1.5.5.1 Prepare $NaNO_2$ calibration standards as shown in the example table below. These calibration standards will be used for building the standard curve and for evaluating test sample interference. The standard curve will be used to calculate the NO concentration of the test samples. The stock is reagent 8.11 (sodium nitrite ($NaNO_2$) standard, 0.1 M stock solution).The stock reagent will be diluted to prepare intermediate (Int.) stock A, intermediate stock B, and calibration (Cal) standard curve 1–8.

A1.5.5.2 Volumes of $NaNO_2$ calibration standards can be adjusted based on the need.

Level	Nominal Concentration (µM)	Preparation Procedure
Int. A	10,000	100 µL Stock + 900 µL complete RPMI-1640 medium
Int. B	1000	100 µL Int. A + 900 µL complete RPMI-1640 medium
Cal 1	250	600 µL Int. B + 1800 µL complete RPMI-1640 medium
Cal 2	125	1200 µL Cal 1 + 1200 µL complete RPMI-1640 medium
Cal 3	62.5	1200 µL Cal 2 + 1200 µL complete RPMI-1640 medium
Cal 4	31.3	1200 µL Cal 3 + 1200 µL complete RPMI-1640 medium
Cal 5	15.6	1200 µL Cal 4 + 1200 µL complete RPMI-1640 medium
Cal 6	7.81	1200 µL Cal 5 + 1200 µL complete RPMI-1640 medium
Cal 7	3.91	1200 µL Cal 6 + 1200 µL complete RPMI-1640 medium
Cal 8	1.95	1200 µL Cal 7 + 1200 µL complete RPMI-1640 medium

A1.6 *Experimental Procedure*:

A1.6.1 Using a new 96-well plate, add 50 µL per well of calibration standards, cell culture medium and test samples to the appropriate wells as shown in the example Pre-test Plate in Fig. A1.1.

A1.6.2 In a separate tube combine equal volumes of reagent A and reagent B at room temperature; this is the Griess reagent.

A1.6.3 Add 100 µL of the Griess reagent to each well of the "Pre-test Plate".

A1.6.4 Place the plate on a shaker for 2 min to 3 min, allowing all ingredients to mix.

A1.6.5 Measure absorbance at 550 nm.

A1.7 *Calculations*—See Calculations section (Section 12)

A1.8 *Data Analysis and Conclusions*:

A1.8.1 To evaluate test sample ability to adsorb nitrite; compare the calculated mean of each calibration standard to its corresponding calibration standard and test sample for each test sample concentration (for example, compare mean Cal1 absorption value to mean TS1 Cal1).

A1.8.2 To evaluate whether test sample interferes with Griess reagent; compare the caluculated mean of each test sample Griess reagent for each test sample concentration to mean Blank 1 (for example, compare mean TS1 Gr to mean Blank1).

A1.8.3 Information obtained from A1.8.1 and A1.8.2 helps to decide on how to run Detection of Nitric Oxide Production *In Vitro* (for example, If interferences are observed only at the highest TS concentrations, it may be helpful to decrease the maximum concentration tested, or evaluate if centrifugation could reduce the interference). Other possible interferences caused by the unique physicochemical properties of some engineered nanomaterials should be also considered (**7-12**).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal 1	Cal 1	TS1 Cal 1	TS1 Cal 1	TS2 Cal 1	TS2 Cal 1	TS3 Cal 1	TS3 Cal 1	TS4 Cal 1	TS4 Cal 1	TS1 Gr	TS1 Gr
B	Cal 2	Cal 2	TS1 Cal 2	TS1 Cal 2	TS2 Cal 2	TS2 Cal 2	TS3 Cal 2	TS3 Cal 2	TS4 Cal 2	TS4 Cal 2	TS2 Gr	TS2 Gr
C	Cal 3	Cal 3	TS1 Cal 3	TS1 Cal 3	TS2 Cal 3	TS2 Cal 3	TS3 Cal 3	TS3 Cal 3	TS4 Cal 3	TS4 Cal 3	TS3 Gr	TS3 Gr
D	Cal 4	Cal 4	TS1 Cal 4	TS1 Cal 4	TS2 Cal 4	TS2 Cal 4	TS3 Cal 4	TS3 Cal 4	TS4 Cal 4	TS4 Cal 4	TS4 Gr	TS4 Gr
E	Cal 5	Cal 5	TS1 Cal 5	TS1 Cal 5	TS2 Cal 5	TS2 Cal 5	TS3 Cal 5	TS3 Cal 5	TS4 Cal 5	TS4 Cal 5	Blank1	Blank2
F	Cal 6	Cal 6	TS1 Cal 6	TS1 Cal 6	TS2 Cal 6	TS2 Cal 6	TS3 Cal 6	TS3 Cal 6	TS4 Cal 6	TS4 Cal 6	Blank1	Blank2
G	Cal 7	Cal 7	TS1 Cal 7	TS1 Cal 7	TS2 Cal 7	TS2 Cal 7	TS3 Cal 7	TS3 Cal 7	TS4 Cal 7	TS4 Cal 7	Blank1	Blank2
H	Cal 8	Cal 8	TS1 Cal 8	TS1 Cal 8	TS2 Cal 8	TS2 Cal 8	TS3 Cal 8	TS3 Cal 8	TS4 Cal 8	TS4 Cal 8	Blank1	Blank2

NOTE 1—Cal, Calibration Standard; TS Cal, Test sample and Calibration Standard; TS Gr, Test Sample and Griess reagent; Blank1, Griess reagent only; Blank2, Griess reagent and cell culture media.

FIG. A1.1 NO Pre-Test Plate Map

APPENDIX

(Nonmandatory Information)

X1. PLATE MAP TEMPLATES

X1.1 See Fig. X1.1 and Fig. X1.2.