



NCL Method ITA-7

Detection of Nitric Oxide Production by the Macrophage Cell Line RAW264.7

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

This document describes a protocol for quantitative determination of nitrite (NO_2^-), a stable oxidative end-product of the antimicrobial effector molecule nitric oxide in cell culture medium [1, 2]. The protocol is used to evaluate the capability of nanomaterials to induce nitric oxide production by macrophages. Nitric oxide secreted by macrophages has a half-life of seconds; it interacts with a number of different molecular targets, resulting in cytotoxicity. In the presence of oxygen and water, nitric oxide interacts with itself to generate other reactive nitrogen oxide intermediates and ultimately decomposes to form nitrite (NO_2^-) and nitrate (NO_3^-).

2. Principles

In this assay, nitrite is measured in tissue culture medium using the Greiss reagent. This measurement provides a surrogate marker and quantitative indicator of nitric oxide production. The murine macrophage cell line RAW 264.7 is used as the model in this assay. The upper and the lower limit of quantification are 250 μM and 1.95 μM of nitrate, respectively.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

1. RAW 264.7 murine macrophages
2. Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)
3. Lipopolysaccharide (LPS) O55:B5 or equivalent (Sigma, L6529)
4. Fetal bovine serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)
5. RPMI-1640 **without phenol red** (Invitrogen, 11835-055)
6. Hanks balanced salt solution (HBSS) (Invitrogen, 24020-117)
7. Pen/Strep solution (Invitrogen, 15140-148)
8. β -mercaptoethanol (Sigma, M7522)
9. Trypan Blue solution (Invitrogen, 15250-061)
10. Naphthylethylenediamine dihydrochloride (Sigma, N9125)
11. Sulfanilamide (Sigma, S9251)

12. Phosphoric acid (Aldrich, 22,248-8)
13. Sodium Nitrite (NaNO₂) Standard, 0.1 M stock solution (Sigma, 35271)

3.2 Materials

1. Pipettes covering range from 0.05 to 10 mL
2. Multichannel pipettor
3. Flat bottom 96-well plates
4. 24-well plates
5. Polypropylene tubes, 50 and 15 mL
6. Reagent reservoirs

3.4 Equipment

1. Refrigerator, 2-8°C
2. Freezer, -20°C
3. Cell culture incubator, 5% CO₂ and 95% humidity
4. Biohazard safety cabinet approved for level II handling of biological material
5. Inverted microscope
6. Vortex
7. Hemocytometer
8. Plate reader, 550 nm

4. Reagent and Control Preparation

4.1 Heat-Inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8°C and allow to equilibrate to room temperature. Incubate 30 minutes at 56°C in a water bath mixing every five minutes. Single use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C indefinitely.

4.2 Complete RPMI-1640 Medium

The complete RPMI medium should contain the following reagents: 10% FBS (heat inactivated); 2 mM L-glutamine; 50 µM β-mercaptoethanol, 100 U/mL penicillin; and 100 µg/mL streptomycin sulfate. Store at 2-8°C, protected from light for no longer than one month. Before use, warm in a 37°C water bath.

4.3 Lipopolysaccharide 1 mg/mL (LPS, Stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of LPS and vortex to mix. Store daily use aliquots at a nominal temperature of -20°C. Avoid repeated freezing/thawing.

4.4 Positive control

Dilute stock LPS solution in cell culture medium to a final concentration of 100 ng/mL. Store at room temperature. Discard unused portion after experiment.

4.5 Negative Control

Use PBS as the negative control. Process this control the same way as the test samples. For example, if test nanoparticle samples were diluted 1:10 in complete culture medium, dilute PBS 1:10 in complete culture medium and use this as the negative control.

4.6 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

4.7 Greiss Reagent

- A. Dissolve sulfanilamide in 2.5% phosphoric acid (H_3PO_4) to a final concentration of 1% (w/v), e.g., dissolve 1 g of sulfanilamide in 100 mL of 2.5% H_3PO_4 .
- B. Dissolve naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4 to a final concentration of 0.1% (w/v), e.g., dissolve 100 mg of naphthylethylenediamine dihydrochloride in 100 mL of 2.5% H_3PO_4 .

Store both solutions in glass bottles at 4°C. Discard if discoloration occurs or solutions are not clear. Equal volumes of reagents A and B will be combined just prior to use to form the Griess reagent. This solution should be used immediately after preparation and any remaining should be discarded.

4.8 NaNO₂ Calibration Standards

Example calibration standards are shown in Table 1 below. Volumes can be adjusted based on need. The stock is reagent 3.1.13.

Table 1. Preparation of Calibration Standards

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

4.9 NaNO₂ Quality Controls

Example quality controls are shown in Table 2 below. Volumes can be adjusted based on need. The stock is reagent 3.1.13.

Table 2. Preparation of Quality Controls

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

5. Preparation of Study Samples

This assay requires 2.5 mL of nanoparticle solution dissolved/resuspended in complete culture medium, at a concentration 1X the highest final test concentration. Test concentrations are based on the calculated plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called “theoretical plasma concentration”. Considerations for estimating theoretical plasma concentration have been reviewed elsewhere [3] and are summarized in Box 1 below.

This assay evaluates four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two serial 1:5 dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 2 mg/mL is prepared. This sample is then diluted 10 fold (0.2 mg/mL), followed by two 1:5 serial dilutions (0.04 and 0.008 mg/mL). Use 500 µL of each sample dilution per well. Each nanoparticle concentration is plated three times.

Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration

In this example, we are assuming a known efficacious mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$\text{human dose} = \frac{\text{mouse dose}}{12.3} = \frac{123 \text{ mg/kg}}{12.3} = 10 \text{ mg/kg} \quad (\text{see reference [5]})$$

The blood volume of a human is approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg} \times 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

6. Cell Preparation

Raw 264.7 is a murine macrophage cell line. Grow cells in complete medium. Dislodge cells using trypsin-EDTA solution and resuspend in complete medium. A subcultivation ratio of 1:3 to 1:6 is recommended. Replace or add medium every 2 to 3 days.

7. Experimental Procedure

1. Adjust cell concentration to 1×10^5 cells/mL using complete RPMI medium.
2. Plate 1000 μ L of cell suspension per well in a 24 well plate. Prepare triplicate wells for each sample and duplicate wells for each control. Always leave 1 cell-free well per nanoparticle concentration per plate. These wells will be used to assay potential nanoparticle interference with the assay. This is the “Culture Plate”.
3. Incubate the “Culture Plate” 24 hr in a humidified 37°C, 5% CO₂ incubator.
4. Remove culture medium and add 500 μ L of study samples, controls, or medium blank to appropriate wells. Position samples on the plate such that study samples are bracketed by controls and blank medium.
5. Incubate the “Culture Plate” 48 ± 1 hr in a humidified 37°C, 5% CO₂ incubator.
6. To a fresh 96 well plate, add 50 μ L 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 duplicate wells for each sample and control. This is the “NO⁻ Test Plate”.

Note: Removal of nanoparticles from culture medium may be required prior to this step if nanoparticles interfere with the assay, e.g., if particles react with either or both components of the Greiss reagent, or have absorbance at or close to 550 nm. If particle removal is not feasible, results obtained for “particles only” control may be subtracted from that obtained for particle test-sample to correct for particle background interference.

7. In a separate tube combine equal volumes of reagent A and reagent B; this is the Greiss reagent.
8. Add 100 μ L of the Greiss reagent to each well of the “NO⁻ Test Plate”.
9. Place the plate on a shaker for 2-3 minutes, allowing all ingredients to mix.
10. Measure absorbance at 550 nm.

8. Calculations

8.1 Percent Coefficient of Variation (%CV)

The % CV is used to control precision and calculated for each control or test sample according to the following formula:

$$\frac{\text{Standard Deviation}}{\text{Mean}} \times 100 \%$$

8.2 Percent Difference From Theoretical (PDFT)

PDFT is used to control accuracy of the assay calibration standards and quality controls, and is calculated according to the following formula:

$$\frac{(\text{Calculated NaNO}_2 \text{ Concentration} - \text{Theoretical NaNO}_2 \text{ Concentration})}{\text{Theoretical NaNO}_2 \text{ Concentration}} \times 100 \%$$

9. Acceptance Criteria

1. The %CV for each control and test sample should be within 30%.
2. If the positive control fails to meet acceptance criterion described in 9.1, the assay should be repeated.
3. Within the acceptable assay, if two of three replicates of unknown sample fail to meet acceptance criterion described in 9.1, this unknown sample should be re-analyzed.
4. If two duplicates of the same study sample demonstrate results >30% different, this sample should be re-analyzed.
5. The %CV and PDFT of the calibration standards and quality controls should be within 20%. At least five calibrators should be available. Four of six 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 re-loaded onto a new plate.

10. References

1. Current Protocols in Immunology. Edited by: John E. Coligan (NIAID, NIH); Barbara Bierer (Brigham & Women's Hospital); David H. Margulies (NIAID, NIH); Ethan M. Shevach (NIAID, NIH); Warren Strober (NIAID, NIH); Richard Coico (Weill Medical College of Cornell University); John Wiley & Sons, Inc., 2005.

2. Standard practice for evaluation of immune responses in biocompatibility testing using ELISA tests, lymphocytes proliferation, and cell migration. ASTM F1906-98.
3. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. *J Control Release*. 2013;172(2):456-66.

11. Abbreviations

Cal	calibration standards
CV	coefficient of variation
FBS	fetal bovine serum
HBSS	Hank's buffered saline solution
LPS	lipopolysaccharide
NC	negative control
PBS	phosphate buffered saline
PDFT	percent different from theoretical
PC	positive control
QC	quality control
VC	vehicle control
w/v	weight to volume ratio

12. Appendix

Example Culture Plate Map

	1	2	3	4	5	6
A	Untreated cells	NC	PC	VC	TS 1a 2.0 mg/mL	TS 1a 0.2 mg/mL
B	TS 1a 0.04 mg/mL	TS 1a 0.008 mg/mL	TS 1b 2.0 mg/mL	TS 1b 0.2 mg/mL	TS 1b 0.04 mg/mL	TS 1b 0.008 mg/mL
C	Untreated cells	NC	PC	VC		
D	TS 1 2.0 mg/mL (No cells)	TS 1 0.2 mg/mL (No cells)	TS 1 0.04 mg/mL (No cells)	TS 1 0.008 mg/mL (No cells)		

Example NO⁻ Test Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Diluent	QC 1	QC 2	QC 3	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
B	Diluent	QC 1	QC 2	QC 3	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
C	NC	PC	TS 1a 1.0 mg/mL	TS 1a 0.2 mg/mL	TS 1a 0.04 mg/mL	TS 1a 0.008 mg/mL	TS 1b 1.0 mg/mL	TS 1b 0.2 mg/mL	TS 1b 0.04 mg/mL	TS 1b 0.008 mg/mL	NC	PC
D	NC	PC	TS 1a 1.0 mg/mL	TS 1a 0.2 mg/mL	TS 1a 0.04 mg/mL	TS 1a 0.008 mg/mL	TS 1b 1.0 mg/mL	TS 1b 0.2 mg/mL	TS 1b 0.04 mg/mL	TS 1b 0.008 mg/mL	NC	PC
E	VC	VC	Untreated cells	Untreated cells								
F	VC	VC	Untreated cells	Untreated cells								
G	TS 1 1.0 mg/mL (No cells)	TS 1 0.2 mg/mL (No cells)	TS 1 0.04 mg/mL (No cells)	TS 1 0.008 mg/mL (No cells)					Diluent	QC 1	QC 2	QC 3
H	TS 1 1.0 mg/mL (No cells)	TS 1 0.2 mg/mL (No cells)	TS 1 0.04 mg/mL (No cells)	TS 1 0.008 mg/mL (No cells)					Diluent	QC 1	QC 2	QC 3