NCL Method ITA-7

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

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov
https://ncl.cancer.gov

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.

Method written by:

Timothy M. Potter

Edward Cedrone

Barry W. Neun

Marina A. Dobrovolskaia*

Nanotechnology Characterization Lab, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, MD 21702

*- address corespondence to: marina@mail.nih.gov

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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 and 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^-). Interestingly, despite expression of inducible nitric oxide synthase (iNOS), an enzyme responsible for the generation of nitric oxide in both human and mouse immune cells, the induction of nitric oxide by immunologically active agonists (e.g., bacterial lipopolysaccharide) and the levels of produced nitric oxide are different between human and mouse immune cells [3]. The response in human immune cells, especially under in vitro conditions is substantially lower than in murine cells. For this reason, a murine macrophage cell line is a better model for in vitro analysis of nitric oxide production than human monocytes and macrophages.

2. Principles

In this assay, nitrite is measured in tissue culture medium using the Griess 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, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

3.1.1 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

3.1.2 LPS-EK UltraPure (*E. coli K12)* or equivalent (Invivogen, tlrl-peklps)

3.1.3 Fetal bovine serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)

3.1.4 RPMI-1640 **without phenol red** (GE Life Sciences, Hyclone, SH30605.01)

3.1.5 Hanks balanced salt solution (HBSS) (Gibco, 24020-117)

3.1.6 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)

3.1.7 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.1.8 β-mercaptoethanol (Sigma-Aldrich, M7522)

3.1.9 Trypan Blue solution (Gibco, 15250-061)

3.1.10 Naphthylethylenediamine dihydrochloride (Sigma-Aldrich, N9125)

3.1.11 Sulfanilamide (Sigma-Aldrich, S9251)

3.1.12 Phosphoric acid, 1N (Sigma-Aldrich, 438081)

3.1.13 Sodium Nitrite ($NaNO_2$) Standard, 1 M stock solution (Fisher, 60-026-33)

3.2 Materials

3.2.1 Pipettes, 0.05 to 10 mL

3.2.2 Multichannel pipettor

3.2.3 Flat bottom 96-well plates

3.2.4 24-well plates

3.2.5 Polypropylene tubes, 50 and 15 mL

3.2.6 Reagent reservoirs

3.3 Cell Lines

3.3.1 RAW 264.7 murine macrophages

3.4 Equipment

3.4.1 Centrifuge

3.4.2 Refrigerator, 2-8ºC

3.4.3 Freezer, -20ºC

3.4.4 Cell culture incubator, 5% CO_2 and 95% humidity

3.4.5 Biohazard safety cabinet approved for level II handling of biological material

3.4.6 Inverted microscope

3.4.7 Vortex

3.4.8 Hemocytometer

3.4.9 Plate shaker

3.4.10 Plate reader capable of operating at 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 2-8ºC, and allow to equilibrate to room temperature. Incubate 30 minutes at 56ºC in a water bath, mixing every 5 minutes. Single use aliquots may be stored at 2-8ºC for up to one month or at a nominal temperature of -20ºC 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

100 μg/mL streptomycin sulfate

Store at 2-8ºC protected from light for no longer than 1 month. Before use, warm in a water bath.

4.3 Lipopolysaccharide 1 mg/mL (LPS, Stock)

K12 LPS is provided as lyophilized powder. Reconstitute the powder by adding 1 mL of water per 1 mg of LPS to the vial and vortex to mix. Stocks with higher concentration (5-10 mg/mL) can also be prepared. Store daily use aliquots at a nominal temperature of -20ºC. Avoid repeated freezing/thawing.

4.4 Positive Control

On the day of experiment thaw a stock aliquot at room temperature, vortex well and dilute this stock LPS solution in cell culture medium to a final concentration of 100 ng/mL. Store at room temperature during the experiment, and discard unused portion after use.

4.5 Negative Control

Use PBS as a negative control. Process this sample the same way you do study samples. For example, if stock nanoparticle test samples are diluted 1:10 in complete culture medium, dilute PBS 1:10 in complete culture medium and use this sample 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 Griess 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 Preparation of $NaNO_2$ Calibration Standards

First dilute the 1N stock (section 3.1.13) 1:10 in distilled water then proceed as in the example shown in the table below. Volumes can be adjusted based on need.

4.9 Preparation of $NaNO_2$ Quality Controls

As in section 4.8 above, start with a 1:10 dilution of stock reagent (section 3.1.13) Example is shown in the table below. Volumes can be adjusted based on need.

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

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 nanoparticles at 1X the highest final test concentration dissolved/resuspended in complete culture medium. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol, this concentration is called the “theoretical plasma concentration”. Considerations for estimating theoretical plasma concentration were reviewed elsewhere [4] and are summarized in Box 1 below.

The assay will evaluate four concentrations: 10X (or when feasible 100X or 30X; or 5X if 10X is not feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 2 mg/mL will be prepared and 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 of these samples per well. Each nanoparticle concentration is plated 3 times.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

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

$$Human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\ mg/kg}{12.3} = 10\ mg/kg$$

Blood volume constitutes 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 blood, which is used as the in vitro test concentration.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

6. Cell Preparation

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

7. Experimental Procedure

7.1 Adjust cell concentration to 1 x 10^5 cells/mL using complete RPMI medium.

7.2 Plate 1000 µL 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 assess potential nanoparticle interference with the assay. This is the "Culture Plate".

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

7.4 Remove culture medium and add 500 µL 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.

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

7.6 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 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 can interfere with assay, e.g. if particles react with either or both components of the Griess 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.

7.7 In a separate tube combine equal volumes of reagent A and reagent B; this is the Griess reagent.

7.8 Add 100 µL of the Griess reagent to each well of the "NO^- Test Plate".

7.9 Mix the well contents using a plate shaker for 2-3 minutes.

7.10 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:

$$\%CV = \frac{standard\ deviation}{mean} * 100\%$$

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:

$$PDFT = \frac{(Calculated\ NaNO_2\ Concentration - Theoretical\ NaNO_2\ Concentration)}{Theoretical\ NaNO_2\ Concentration} * 100\%$$

9. Acceptance Criteria

9.1 The %CV for each control and test sample should be within 30%.

9.2 If the positive control fails to meet acceptance criterion described in 9.1, the assay should be repeated.

9.3 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.

9.4 If two duplicates of the same study sample demonstrate results >30% different, this sample should be reanalyzed.

9.5 The %CV and PDFT of 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

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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
RPMI	Roswell Park Memorial Institute
VC	vehicle control
w/v	weight to volume ratio

12. Appendix

Example Culture Plate Map

	1	2	3	4	5	6
A	Untreated cells	Untreated cells	NC	NC	PC	PC
B	TS 1b 1.0 mg/mL	TS 1b 1.0 mg/mL	TS 1b 1.0 mg/mL	TS 1b 0.2 mg/mL	TS 1b 0.2 mg/mL	TS 1b 0.2 mg/mL
C	TS 1 0.04 mg/mL	TS 1 0.04 mg/mL	TS 1 0.04 mg/mL	TS 1 0.008 mg/mL	TS 1 0.008 mg/mL	TS 1 0.008 mg/mL
D	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)	VC?	VC?

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	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	TS 1c 1.0 mg/mL	TS 1c 0.2 mg/mL	TS 1c 0.04 mg/mL	TS 1c 0.008 mg/mL
D	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	TS 1c 1.0 mg/mL	TS 1c 0.2 mg/mL	TS 1c 0.04 mg/mL	TS 1c 0.008 mg/mL
E	NC	PC	VC	VC	Untreated Cells	Untreated Cells	NC	PC				
F	NC	PC	VC	VC	Untreated Cells	Untreated Cells	NC	PC				
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