Product Manual

OxiSelect™ Intracellular Nitric Oxide (NO) Assay Kit (Fluorometric)

Catalog Number

STA- 800	96 assays
STA- 800- 5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) work together to damage cells and have been implicated in the pathogenesis of several disease states. RNS are a family of molecules derived from nitric oxide (NO) and superoxide anion (O_2^-), produced via nitric oxide synthase (NOS) and NADPH oxidase, respectively (Figure 1). Nitric oxide is an established mediator in vascular diseases, diabetes, renal ischemia, atherosclerosis, inflammatory diseases, and cancer. However, because of its extremely short half life, studies of NO and its physiological role have been challenging.

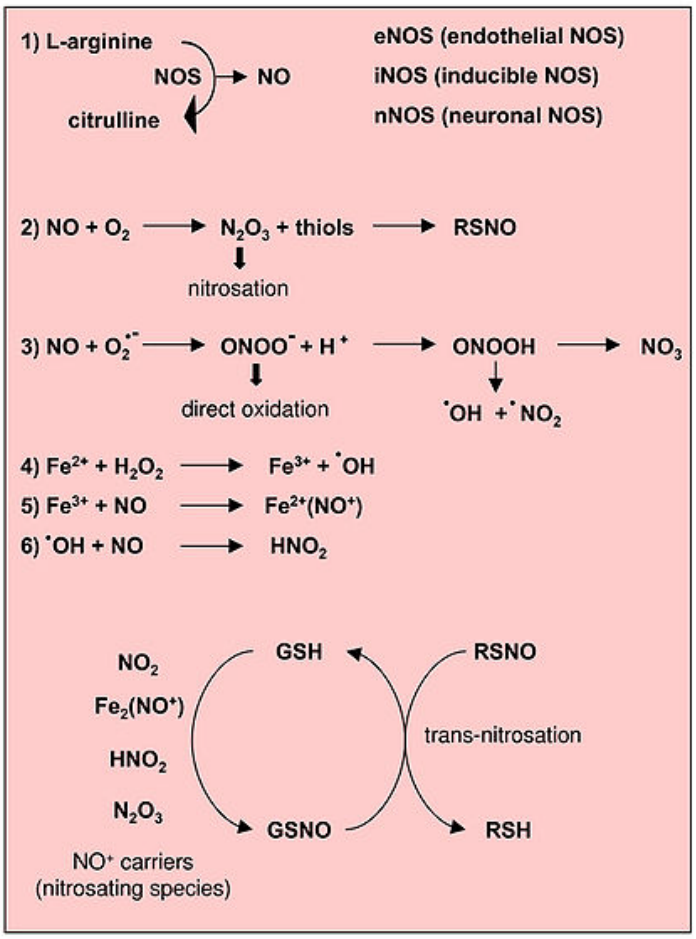
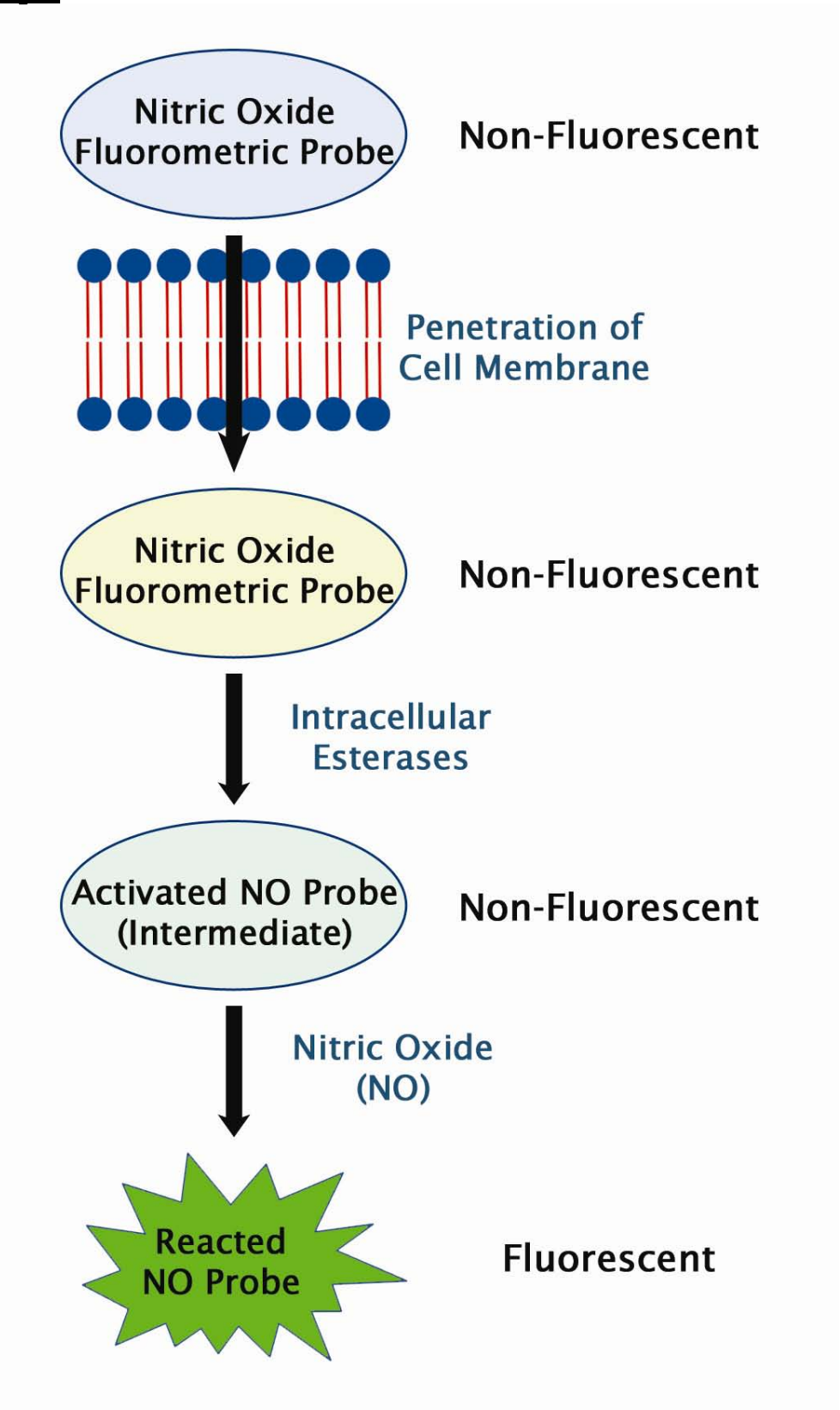


Figure 1: Reactions leading to generation of NO and RNS. [1]

The OxiSelect™ Intracellular Nitric Oxide Assay Kit is a cell-based assay for rapid detection of intracellular NO or NOS activity in cultured cells. In brief, the cell-permeant NO probe passively diffuses into cells and is deacetylated by cellular esterases to a non-fluorescent intermediate. When intracellular nitric oxide encounters this intermediate, it rapidly oxidizes to a highly fluorescent, triazolo-fluorescein analog. The fluorescence intensity is proportional to the NO levels within the cell cytosol (detection limit of ~3 nM). The kit's provided Nitric Oxide Fluorometric Probe is NO specific, has excellent photostablity and pH stability, and is detected with standard fluorescein filters. The probe is suitable for flow cytometry, fluorescent microscopy, and fluorescent microplate detection. Each kit provides sufficient reagents to perform up to 96 assays, including blanks, standards, and unknown samples.

Assay Principle



Related Products

1. STA-310: OxiSelect™ Protein Carbonyl ELISA Kit
2. STA-320: OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)
3. STA-330: OxiSelect™ TBARS Assay Kit (MDA Quantitation)
4. STA-342: OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence)
5. STA-343: OxiSelect™ Hydrogen Peroxide Assay Kit
6. STA-344: OxiSelect™ Hydrogen Peroxide/Peroxidase Assay Kit
7. STA-347: OxiSelect™ In Vitro ROS/RNS Assay Kit (Green Fluorescence)
8. STA-832: OxiSelect™ MDA Adduct Competitive ELISA Kit
9. STA-838: OxiSelect™ HNE Adduct Competitive ELISA Kit

Kit Components

1. NOS Inhibitor, L-NNA (Part No. 280001): One vial containing 100 mg of L-NNA (CAS Number 2149-70-4, MW 219.20). L-NNA is a competitive inhibitor of nitric oxide synthase (NOS).
2. 5X Cell Lysis Buffer (Part No. 280002): Two 1.5 mL vials.
3. 500X Nitric Oxide Fluorometric Probe (Part No. 280003): One 20 µL amber vial.

Materials Not Supplied

1. Sterile DPBS containing magnesium and calcium
2. Cell culture medium
3. 37ºC incubator
4. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
5. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
6. 96-well tissue culture plate

   Note: A tissue culture treated, 96-well fluorescence microtiter plate is required for kinetic/time course experiments.
7. 96-well fluorescence microtiter plate
8. Fluorescent microplate reader capable of reading 485 nm (excitation) and 530 nm (emission)
9. (Optional) L-Arginine

   Note: Found in most culture media
10. (Optional) β-NADPH, LPS, IFN-γ, NOS Inducers, NOS Inhibitors

Storage

Store the entire kit at -20ºC. Avoid multiple freeze/thaws by aliquoting. The Nitric Oxide Fluorometric Probe is light sensitive and should be maintained in amber tubes.

Preparation of Reagents

- NOS Inhibitor, L-NNA: Weigh out the NOS Inhibitor and dissolve at 0.22 mg/mL, or ~1 mM, in water (L-NNA is difficult to dissolve at concentrations above 0.5 mg/mL). Stir to homogeneity, then sterile filter. Further dilute to the desired final concentration in media. Prepare only enough for immediate applications. Do not store diluted solutions.
- 5X Cell Lysis Buffer: Thaw the 5X Cell Lysis Buffer concentrate at room temperature. Immediately before use, dilute to 1X with deionized water. Stir to homogeneity.
- 500X Nitric Oxide Fluorometric Probe: Thaw the NO Fluorometric Probe at room temperature during assay preparation. Immediately before use, dilute the NO Fluorometric Probe to 1X concentration as follows:
    - If detection will be done in a fluorescence microplate reader, dilute 1:500 with either DPBS or culture media, preferably without phenol red or BSA, which may reduce fluorescence. Sensitivity of cells to serum-free environments should be considered when choosing the diluent. Briefly vortex to homogeneity. Do not store diluted solutions.
    - If detection will be done by fluorescence microscopy, dilute 1:500 with either DPBS or serum-free media only. Media containing serum will quench the probe's intracellular fluorescence and should not be used. Briefly vortex to homogeneity. Do not store diluted solutions.

  Note: For longer term storage, the 500X NO Fluorometric Probe should be aliquoted and frozen at -20ºC to avoid multiple freeze/thaws.

Assay Protocol

I. Cell Seeding

1. Harvest and resuspend cells in culture medium at 2-5 x 10^5 cells/mL.
2. Seed 200 µL in each well of either a clear or black, 96-well tissue culture plate. If performing kinetic/time course experiments, a black 96-well tissue culture plate is required.

   Note: Overnight induction of NOS activity (e.g. β-NADPH, LPS, IFN-γ) may be performed immediately after cell plating.
3. Incubate overnight at 37ºC, 5% CO_2 (cells should be > 80% confluent).

II. Induction of NOS Activity

1. Prepare and mix all reagents thoroughly before use.
2. Perform induction of NOS activity to desired wells. Induced, non-induced, media only, and blank wells (without NO Probe) should be assayed in triplicate. NOS inhibitors may also be added.
3. Proceed to Section III, IV or V for the desired detection method.

III. Kinetic/Time Course Experiments in Fluorescence Plate Reader (Black Plate Required)

1. Aspirate/remove media from the wells and add 100 µL of 1X Nitric Oxide Fluorometric Probe (see Preparation of Reagents section above) to each tested well. NOS inducers/inhibitors may also be added.

   Note: Media-only wells (without cells) should be included and subtracted as background.
2. Cover the plate wells to protect the reaction from light.
3. Incubate at 37ºC for the desired time period(s) (typically 30-120 minutes).
4. Read the fluorescence with a fluorometric plate reader at 480 nm/530 nm.

IV. End Point Experiments in Fluorescence Microtiter Plate Reader (Black or Clear Plate)

1. Aspirate/remove media from the wells and add 100 µL of 1X Nitric Oxide Fluorometric Probe (see Preparation of Reagents section above) to each tested well. NOS inducers/inhibitors may also be added.

   Note: Media-only wells (without cells) should be included and subtracted as background.
2. Cover the plate wells to protect the reaction from light.
3. Incubate at 37ºC for 2 hours.
4. Add 100 µL of 1X Cell Lysis Buffer (see Preparation of Reagents section above) to each tested well. Mix well and incubate for 5 minutes.
5. For measurement:
   - If already in a black 96-well tissue culture plate, wells can be directly read in a fluorometric plate reader at 480 nm/530 nm.
   - If in a clear 96-well tissue culture plate, transfer 150 µL of the mixture to a 96-well plate suitable for fluorescence measurement. Read the fluorescence with a fluorometric plate reader at 480 nm/530 nm.

V. Fluorescence Microscopy Detection

1. Aspirate/remove media from the wells and discard. Gently wash the wells 2 times with 250 µL DPBS (containing magnesium and calcium). Remove the last wash and discard.
2. Add 100 µL of 1X Nitric Oxide Fluorometric Probe (see Preparation of Reagents section above) to each tested well.
3. Cover the plate wells to protect the reaction from light.
4. Incubate at 37ºC for 30-120 minutes.
5. View cells with a fluorescence microscope using FITC filter set.

   Note: To reduce the background fluorescence during imaging, aspirate the media/NO Fluorometric Probe and replace with fresh DPBS.

Example of Results

The following figures demonstrate typical Nitric Oxide Assay results. Fluorescence measurement was performed on SpectraMax Gemini XS Fluorometer (Molecular Devices) with a 485/538 nm filter set and 530 nm cutoff. One should use the data below for reference only. This data should not be used to interpret actual results.

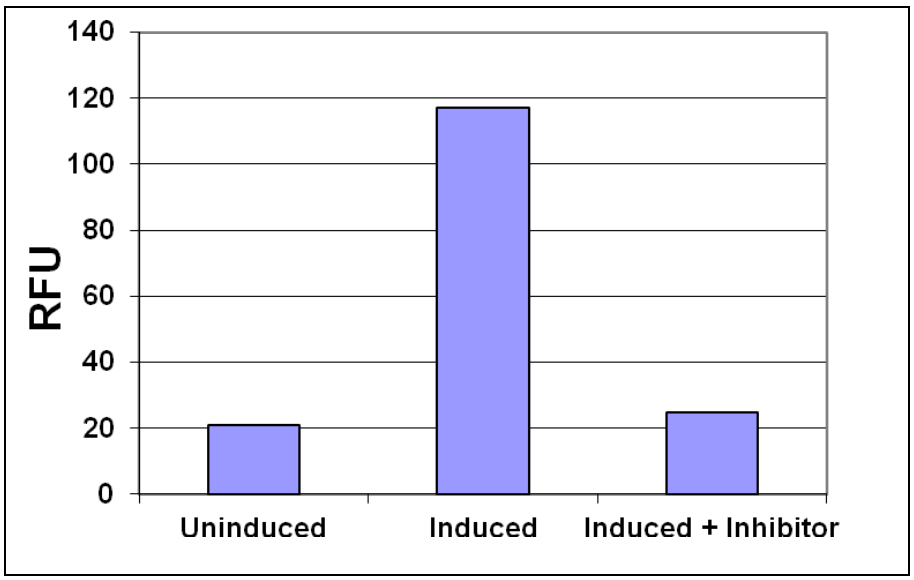


Figure 2. NOS Induction of RAW 264.7 Cells. RAW 264.7 cells were seeded at 100,000 cells/well in a 96-well plate. Induction was performed for 20 hrs with 50 ng/mL LPS and 10 ng/mL IFN-γ. Wells were either uninduced (left), induced (middle), or induced and followed with 2 hr incubation of 50µM L-NNA (right). Microtiter plate detection was performed as described in the Assay Protocol (end point after 2 hours).

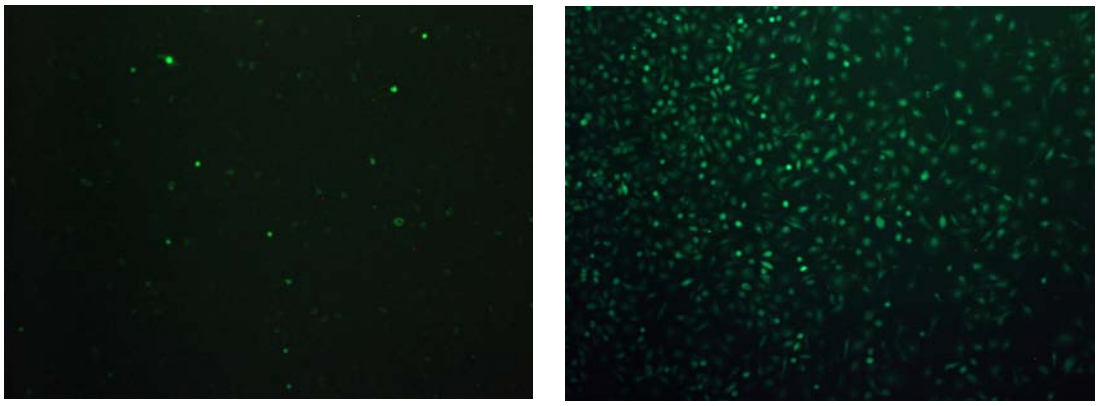


Figure 3. NOS Induction of RAW 264.7 Cells. RAW 264.7 cells were seeded at 100,000 cells/well in a 96-well plate. Wells were either uninduced (left) or induced with 50 ng/mL LPS and 10 ng/mL IFN-γ (right) for 20 hours at 37ºC. Fluorescence microscopy detection was performed as described in the Assay Protocol.

References

1. Novo E, and Parola M. (2008) *Fibrogenesis Tissue Repair*, **1**:5.
2. Ignarro, LJ. (2002) *J. Physiol. Pharmacol*. **53**:503-514.
3. Hickok, JR and Thomas, DD. (2010) *Curr. Pharm. Des.* **16**:381-391.
4. Thomas, DD et al. (2008) *Free Radic. Biol. Med.* **45**:18-31.

Recent Product Citations

1. Paesano, L. et al. (2016). Markers for toxicity to HepG2 exposed to cadmium sulphide quantum dots; damage to mitochondria. *Toxicol*. **374**:18-28.
2. Hung, C. Y. et al. (2016). Card9-and MyD88-mediated IFN-γ and nitric oxide productions are essential for resistance to subcutaneous coccidioides infection. *Infect Immun.* doi:10.1128/IAI.01066-15.
3. Nasrallah, R. et al. (2015). Endoglin potentiates nitric oxide synthesis to enhance definitive hematopoiesis. *Biology Open.* **4**:819-829.
4. Syed, D. N. et al. (2014). Involvement of ER stress and activation of apoptotic pathways in fisetin induced cytotoxicity in human melanoma. *Arch Biochem Biophys.* **563**:108-117.

Warranty

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