Product Manual

OxiSelect™ *In Vitro* Nitric Oxide (Nitrite / Nitrate) Assay Kit (Fluorometric)

Catalog Number

STA-801	100 assays
STA-801-5	5 x 100 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Creating Solutions for Life Science Research

Introduction

Reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) work together to damage cells and have been implicated in the pathogenesis of several disease states. RNI 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. 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, direct quantitation of NO production remains challenging and unsuitable for most detection systems. Therefore, the common method for measurement of total NO has become the sum of its final oxidized products, nitrite (NO_2^-) and nitrate (NO_3^-) (Figure 1).

Cell Biolabs' OxiSelect™ Nitric Oxide (Nitrite/Nitrate) Assay Kit is a simple, fluorometric assay that quantitatively measures NO in various samples by NO_2^-/NO_3^- determination. First, the nitrate (NO_3^-) in the sample is converted to nitrite (NO_2^-) by nitrate reductase enzyme (Figure 2). Next, total nitrite is detected with the fluorescent probe DAN (2,3-diaminonaphthalene). Each kit provides sufficient reagents to perform up to 100 assays using a 96-well microtiter plate format, including blanks, standards and unknown samples. The kit is suitable for serum, plasma, urine, saliva, lysates, and media (see Preparation of Samples) with detection sensitivity limit of ~ 500 nM (in 50 µL sample volume).

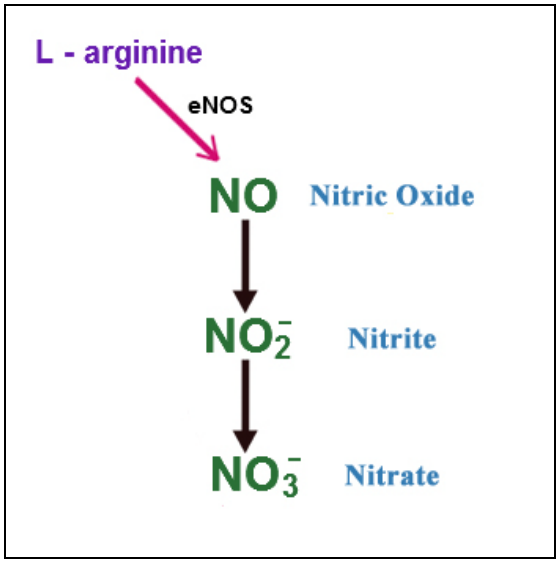

Figure 1: Oxidation of L-arginine to nitrate.

Assay Principle

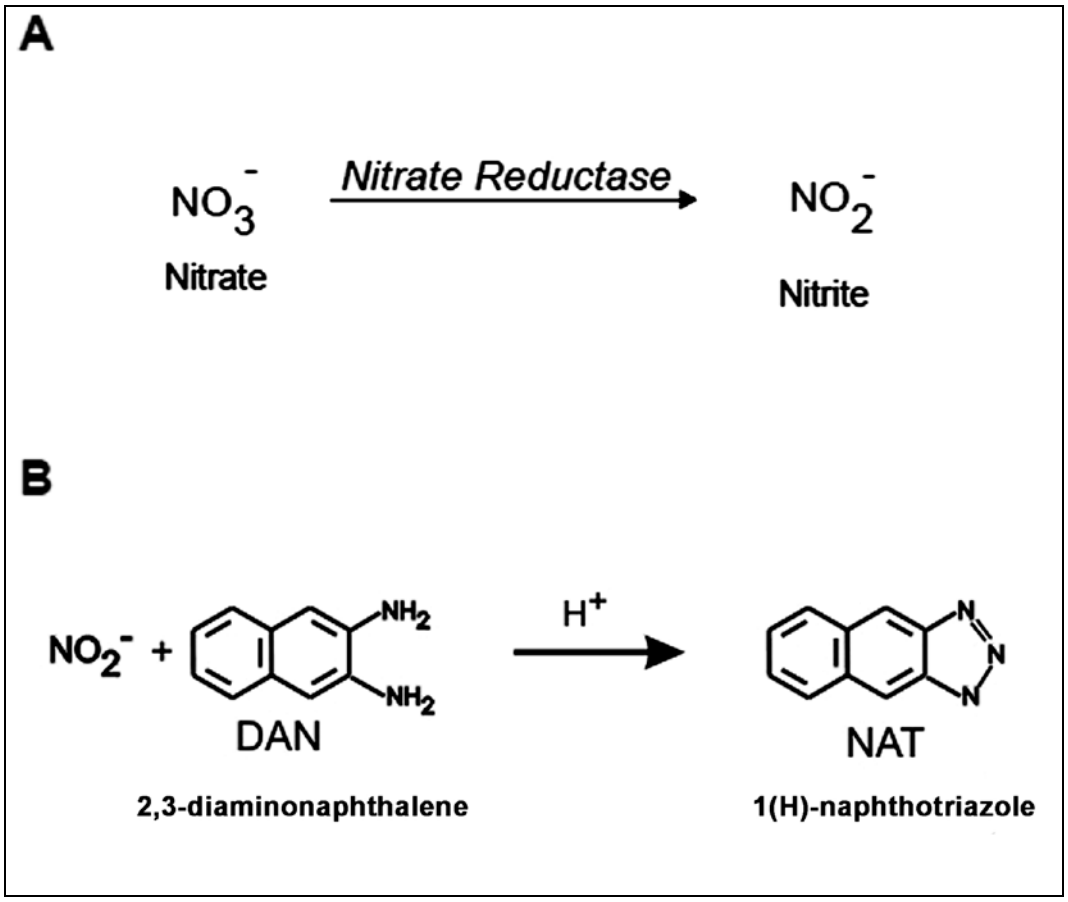

Figure 2: Conversion of nitrate to nitrite by nitrate reductase (A), followed by the reaction with the fluorescent probe DAN (2,3-diaminonaphthalene) (B).

Related Products

1. STA-342: OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence)
2. STA-343: OxiSelect™ Hydrogen Peroxide Assay Kit
3. STA-344: OxiSelect™ Hydrogen Peroxide/Peroxidase Assay Kit
4. STA-347: OxiSelect™ In Vitro ROS/RNS Assay Kit (Green Fluorescence)
5. STA-800: OxiSelect™ Intracellular Nitric Oxide Assay Kit (Fluorometric)
6. STA-802: OxiSelect™ In Vitro Nitric Oxide Assay Kit (Colorimetric)

Kit Components

1. Nitrite Standard (Part No. 280201): One 500 µL vial of 14 mM sodium nitrite.
2. Nitrate Standard (Part No. 280202): One 500 µL vial of 14 mM sodium nitrate.

3. Nitrite Assay Buffer (Part No. 280101): Three 1.4 mL vials.
4. Total Nitrate/Nitrite Assay Buffer (Part No. 280102): Three 1.4 mL vials.
5. Enzyme Cofactor (Part No. 280204): One 20 µL amber vial.
6. 100X DAN Fluorometric Probe (Part No. 280103): One 25 µL amber vial.
7. 2X Fluorometric Probe Diluent (Part No. 280104): One 1.5 mL vial.
8. Stop Solution (Part No. 280105): One 1.1 mL vial.

Materials Not Supplied

1. Deionized or distilled water
2. PBS for sample dilution
3. 10 kDa MWCO ultrafilter (for high protein content samples)
4. Standard 96-well fluorescence black microtiter plate
5. Microtiter plate shaker
6. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
7. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
8. Multichannel micropipette reservoir
9. Fluorescence microplate reader capable of reading excitation in the 360-365 nm range and emission in the 430-450 nm range.

Storage

Store the entire kit at -80ºC. Avoid multiple freeze/thaws by aliquoting. The Enzyme Cofactor and 100X DAN Fluorometric Probe are light sensitive and should be maintained in amber tubes.

Preparation of Reagents

- Nitrite and Nitrate Standards should be thawed/maintained at 4ºC during assay preparation. Both are stable for 2 weeks at 4ºC. For longer term storage, each should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.
- Nitrite Assay Buffer and Total Nitrate/Nitrite Assay Buffer should be thawed/maintained at 4ºC during assay preparation. Both are stable for 1 week at 4ºC. Any unused material should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.

  Note: This component is provided in multiple tubes to minimize multiple freeze/thaws.
- Enzyme Cofactor should be thawed/maintained at 4ºC during assay preparation. The stock solution is stable for 1 week at 4ºC. Immediately before use, dilute the Enzyme Cofactor 1:100 with deionized water and mix well. Do not store diluted solutions. Any unused stock material should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.
- 2X Fluorometric Probe Diluent should be thawed/maintained at room temperature during assay preparation. The stock solution is stable for 1 week at room temperature. Dilute the 2X

Fluorometric Probe Diluent with deionized water (1:1) and mix well. Any unused stock material should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.

- 100X DAN Fluorometric Probe should be thawed/maintained at room temperature during assay preparation. The stock solution is stable for 1 day at room temperature. Immediately before use, dilute the 100X DAN Fluorometric Probe 1:100 with 1X Fluorometric Probe Diluent (see above) and mix well. Do not store diluted solutions. Any unused stock material should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.
- Stop Solution should be thawed/maintained at room temperature during assay preparation. The stock solution is stable for 1 week at room temperature. Any unused stock material should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.

Preparation of Nitrite or Nitrate Standards

- Thaw the nitrite or nitrate standards at 4ºC and mix well. Freshly prepare a dilution series of standard in the concentration range of 0 µM – 140 µM by diluting the standard stock solution (provided at 14 mM) in deionized water (Table 1) or desired sample buffer.

Standard Tubes	14 mM Nitrite or Nitrate Standard (µL)	DI Water or Desired Buffer (µL)	Nitrite or Nitrate Standard (µM)
1	10	990	140
2	500 of Tube #1	500	70
3	500 of Tube #2	500	35
4	500 of Tube #3	500	17.5
5	500 of Tube #4	500	8.75
6	500 of Tube #5	500	4.38
7	500 of Tube #6	500	2.19
8	500 of Tube #7	500	1.09
9	500 of Tube #8	500	0.547
10	0	500	0

Table 1. Preparation of Nitrite or Nitrate Standards

Preparation of Samples

- Plasma: Collect plasma with an anticoagulant such as heparin, citrate or EDTA and mix by inversion. Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C.

  Note: Citrate or EDTA plasma is recommended. Heparin is known to interfere with the assay when samples contain > 1 IU/mL; however, ultrafiltering will remove average MW heparin (15 kDa and larger). If low MW heparin is used, a 3 kDa MWCO ultrafilter should be used.

- Serum: Collect serum with no anticoagulant. Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C.

- Urine: Samples must be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Urine must be diluted before assaying (typically 1:10 or greater in PBS). Use immediately or store at -80°C.
- Saliva: Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C.
- Culture Media: Media known to contain high levels of nitrite/nitrate (e.g. RPMI) should be avoided. Remove particulates/debris in samples by centrifugation or filtration. To account for any background caused by media components, nitrite/nitrate standards should be prepared in the same media. Samples containing phenol red do not interfere with the assay.
- Cell and Tissue Lysates: Remove particulates/debris in samples by centrifugation or filtration. Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C.

Potential Interference from Sample Components

To accurately determine the degree of interference, nitrate and nitrite standard curves should be prepared in water vs. sample buffer. Dilution of the buffer, and ultimately samples, may be required to completely eliminate interference. However, even with some interference, accurate quantitation can be achieved by running standards in the same buffer as samples, although kit sensitivity may be compromised.

Assay Protocol

Note: This kit may be used for measurement of nitrite only, or for total nitrite plus nitrate through nitrate reduction. If desired, nitrate only levels in samples may be calculated by subtracting the nitrite only level from the total nitrite plus nitrate.

I. Measurement of Nitrite only

Note: Each nitrite standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add 50 µL of the nitrite standards, samples, or blanks to the 96-well fluorescence microtiter plate.
2. **Maintain the Nitrite Assay Buffer and Cofactor at 4ºC.** According to Table 2 (below), prepare the desired volume of Reaction Mixture (based on the number of tests) in the following sequence:
   a. In a tube, add the appropriate volume of Nitrite Assay Buffer.
   b. Next, add the corresponding volume of diluted, Enzyme Cofactor Solution (see Preparation of Reagents).

      Note: Enzyme Cofactor is diluted 1:100 in deionized water immediately before use.

   c. Mix well and immediately use.

# of Tests in 96-well Plate (50 µL/test)	Nitrite Assay Buffer (mL)	Diluted Cofactor Solution (mL)	Final Volume of Reaction Mixture (mL)
100	4	1	5
50	2	0.5	2.5
25	1	0.25	1.25

Table 2. Preparation of Reaction Mixture

3. Transfer 50 µL of the above Reaction Mixture to each well (already containing 50 µL of nitrite standard or sample).
4. Cover the plate wells to protect the reaction from light.
5. Incubate at room temperature for 1 hour on an orbital shaker.
6. Add 20 µL of diluted, DAN Fluorometric Probe to each well (see Preparation of Reagents).

   Note: DAN Fluorometric Probe is diluted 1:100 in 1X Fluorometric Probe Diluent immediately before use.
7. Incubate the plate at room temperature for 10 minutes on an orbital shaker, protected from light.
8. Immediately add 10 µL of Stop Solution to each well.
9. Incubate the plate at room temperature for 10 minutes on an orbital shaker, protected from light.
10. Read the plate with a fluorescence microplate reader equipped for excitation in the 360-365 nm range and for emission in the 430-450 nm range.
11. Calculate the concentration of nitrite within samples by comparing the sample absorbance to the standard curve. Negative controls (without nitrite) should be subtracted.

II. Measurement of Total Nitrate/Nitrite via Nitrate Reduction

Note: Each nitrate standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add 50 µL of the nitrate standards, samples, or blanks to the 96-well fluorescence microtiter plate.
2. **Maintain the Total Nitrate/Nitrite Assay Buffer and Cofactor at 4ºC.** According to Table 3 (below), prepare the desired volume of Reaction Mixture (based on the # of tests) in the following sequence:
   a. In a tube, add the appropriate volume of Total Nitrate/Nitrite Assay Buffer.
   b. Next, add the corresponding volume of diluted, Enzyme Cofactor Solution (see Preparation of Reagents).

      Note: Enzyme Cofactor is diluted 1:100 in deionized water immediately before use.
   c. Mix well and immediately use.

# of Tests in 96-well Plate (50 µL/test)	Total Nitrate/Nitrite Assay Buffer (mL)	Diluted Cofactor Solution (mL)	Final Volume of Reaction Mixture (mL)
100	4	1	5
50	2	0.5	2.5
25	1	0.25	1.25

Table 3. Preparation of Reaction Mixture

3. Transfer 50 µL of the above Reaction Mixture to each well (already containing 50 µL of nitrate standard or sample).
4. Cover the plate wells to protect the reaction from light.
5. Incubate at room temperature for 1 hour on an orbital shaker.
6. Add 20 µL of diluted, DAN Fluorometric Probe to each well (see Preparation of Reagents).

   Note: DAN Fluorometric Probe is diluted 1:100 in 1X Fluorometric Probe Diluent immediately before use.
7. Incubate the plate at room temperature for 10 minutes on an orbital shaker, protected from light.
8. Immediately add 10 µL of Stop Solution to each well.
9. Incubate the plate at room temperature for 10 minutes on an orbital shaker, protected from light.
10. Read the plate with a fluorescence microplate reader equipped for excitation in the 360-365 nm range and for emission in the 430-450 nm range.
11. Calculate the concentration of nitrate within samples by comparing the sample absorbance to the standard curve. Negative controls (without nitrate) should be subtracted.

Example of Results

The following figures demonstrate typical Nitric Oxide Assay Kit results. One should use the data below for reference only. This data should not be used to interpret actual results.

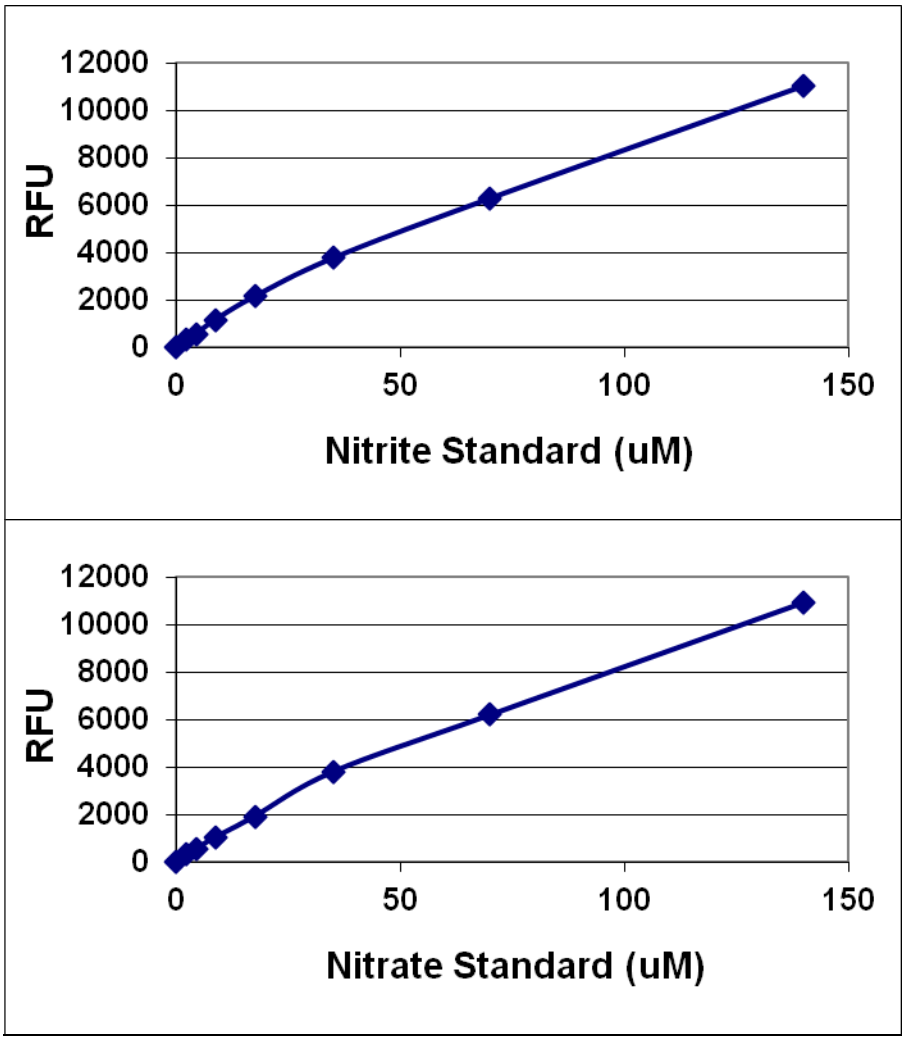

Figure 3: Nitrite and Nitrate Standard Curves. Nitrite (top) and nitrate (bottom) standard curves were performed according to the Assay Protocol.

References

1. Moncada, S. (1992) *Acta Physiol. Scand.* **145**, 201-227.
2. Nathan, C. (1992) *FASEB J.* **6**, 3051-3064.
3. Schreck, R. and Baeuerle, P.A. (1991) *Trends Cell Biol.* **1**, 39-42.
4. Ignarro, L.J. (1990) *Pharmacol. Toxicol.* **67**, 1-7.
5. Green, L.C., et al. (1982) *Anal. Biochem.* **126**, 131-138.

Warranty

These products are warranted to perform as described in their labeling and in Cell Biolabs literature when used in accordance with their instructions. THERE ARE NO WARRANTIES THAT EXTEND BEYOND THIS EXPRESSED

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