Nitric Oxide (NO)

Colorimetric Assay kit

(96 Tests)

Zellbio GmbH (Germany)

CAT No. ZX-44107-96

www.zellx.de

Sample Types Validated for:

Serum, Plasma, Urine, Saliva, Water, Buffers, and Tissue Culture Medium

!!! Caution: This product is for Research Use Only. Not for *in vitro* Diagnostics !!!

Table of Contents

Please read this insert completely prior to using the product.

ZELLX®
Krantorweg 48c
13503 Berlin
Tel.: +49(0)30 81309085
Fax: +49(0)30 81309086
E-Mail: support@zellx.de
Web: www.zellx.de

Introduction

Background

Nitric oxide (NO) is a free radical and signaling molecule that plays an important role in vasodilation, immune system function, apoptosis and many other physiological and pathological processes. Nitric oxide is generated either via the oxidation of L-arginine by NO synthases (NOSs) or the reduction of dietary Nitrite (NO2) and Nitrate (NO3) by various metal-containing proteins.

Nitric oxide is an unstable molecule that quickly degrades, which makes it challenging for direct detection methods. However, as the NO2 and NO3 are the stable metabolites of NO oxidation, their concentration is conveniently measured via colorimetric methods and reported as the total NO content in biological samples.

Assay principle

The ZellX® Nitric Oxide (NO) assay kit is designed to quantitatively measure the total NO content, i.e. the sum of Nitrite (NO2) and Nitrate (NO3) in a variety of samples. Both NO2 and NO3 standards are provided to generate standard curves for the assay and all samples should be read off the standard curve. The total Nitric Oxide kit has two assay options:

For NO2 Determination: Samples are mixed with Color Reagents A and B, and incubated at room temperature for 5 minutes and read at 540-570 nm to quantify the endogenous NO2 level.

For total NO Determination: As mentioned above, the total NO content is derived from the sum of NO2 and NO3, for which, endogenous NO3 is first reduced to NO2 with Nitrate Reductase and NADH, and then processed similar to the first option. To obtain the NO3 concentration, endogenous NO2 is subtracted from the total NO value.

This kit uses NO2 and NO3 Standard solutions calibrated to the US National Institute for Science and Technology Standard Reference Materials and ISO/IEC standards.

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General information

Materials supplied in the Kit

Component	*Quantity*
Nitrate (NO3) Standard (2000 µM)	100 µL
Nitrite (NO2) Standard (2000 µM)	100 µL
Assay Buffer	30 mL
NADH Concentrate	600 µL
Nitrate Reductase	1 Vial
Reagent A	2.5 mL
Reagent B	2.5 mL
Enzyme Stabilization Buffer	500 µL
Clear Half Area 96 Well Plate	1 plate

Storage instruction

All reagents should be stored at 4° C until the expiration date of the kit. Upon reconstitution, the Nitrate Reductase must be stored at -20°C

Materials required but not supplied

10,000 Molecular Weight Cut Off (MWCO) polysulfone filters (Corning Spin-X UF 500, Cat No. 431478) or similar products

Double distilled water (ddH_2O) free of detectable nitrate or nitrite

Phosphate Buffer Saline (PBS)

Microplate/ELISA reader capable of reading optical absorption at 450-570 nm

Centrifuge, Vortex mixer

Precision pipettes, multichannel/repeater pipette and disposable pipette tips

Disposable 1.5-2 mL microtubes for sample preparation

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Precautions

This kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The Reagents A and B are both acid solutions and should be handled like any laboratory acid.

General remarks

- Equilibrate all kit components to room temperature (RT) 30 minutes before use.
- The instruction must be strictly followed.
- The reading of Microplate/ELISA reader must be set at the appropriate wavelength.
- Pipette tips should not be used more than once to prevent cross contamination.
- Reagents of different batches should not be mixed or used after their expiration dates.
- All samples must be filtered through a 10,000 MWCO spin filter to remove protein.

Assay protocol

Reagent preparation

i. **NADH Solution:** Prepare a 1:2 dilution of NADH Concentrate with Assay Buffer (mix 500 µL of NADH Concentrate with 500 µL of Assay Buffer). Discard any excess NADH Solution.

ii. **Nitrate Reductase Solution:** Allow the ziploc bag to warm completely to RT prior to opening. Add 550 µL of Enzyme Stabilization Buffer to the vial, vortex thoroughly and leave it at RT for 5 min. reconstituted Nitrate Reductase must be used within 2 hours of preparation; for extended periods of time (> 2 hours) store it on ice.
Store any unused reconstituted NR at -20°C.
Prepare a 1:4 dilution of reconstituted NR with Assay Buffer (mix 1 part of reconstituted NR with 3 parts of Assay Buffer).

Sample preparation

After collecting the sample, extraction should be immediately carried out in accordance with related instruction. After extraction, experiment should be conducted immediately, otherwise, keep the sample at -70 or -80˚C. Avoid repeated freeze-thaw cycles.

This assay has been validated for serum, plasma, urine, and saliva, as well as water and buffer samples. Tris, HEPES, and PBS buffers are compatible at pH 7.2, as is EDTA at ≤ 10 mM. Most cell lysates and

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tissue homogenates should also be compatible. Detergents such as Triton X-100, Tween 20 and CHAPS are compatible at concentrations of ≤ 0.1%. Samples containing these detergents should be diluted at least 1:2 with the Assay Buffer. **Samples containing SDS or azide are not compatible with the assay.** Samples containing visible particulate should be centrifuged prior to filtration and using.

All samples must be Equilibrate to RT and filtered through a 10000 MWCO spin filter to remove protein.

Serum, Plasma, saliva, or urine:

- Dilute the sample with Assay Buffer and filter through a 10000 MWCO device following the manufacturer's recommendations.
- Collect the filtered sample and either further dilute with Assay Buffer as appropriate or use directly in the assay.
- For serum and plasma, the recommended dilution is ≥ 1:4. For urine and saliva, the recommended dilution is ≥ 1:8.

Standard preparation

Standard solutions:

- Prepare a 1:10 dilution of Standard with Assay Buffer (mix 40 µL of either Nitrite or Nitrate standard with 360 µL of Assay Buffer), and label as the Standard No.7 (200 µM).
- Apply series of other dilutions as described in the table.
- The Assay Buffer is used as the 0 µM standard.

No.	*Concentration Nitrite or Nitrate*	*Material needed*
Standard No.7	200 µM	40 µL Standard (2mM) + 360 µL Assay Buffer
Standard No.6	100 µM	200 µL Standard No.7 + 200 µL Assay Buffer
Standard No.5	50 µM	200 µL Standard No.6 + 200 µL Assay Buffer
Standard No.4	25 µM	200 µL Standard No.5 + 200 µL Assay Buffer
Standard No.3	12.5 µM	200 µL Standard No.4 + 200 µL Assay Buffer
Standard No.2	6.25 µM	200 µL Standard No.3 + 200 µL Assay Buffer
Standard No.1	3.125 µM	200 µL Standard No.2 + 200 µL Assay Buffer
Standard No.0	0 µM	200 µL Assay Buffer

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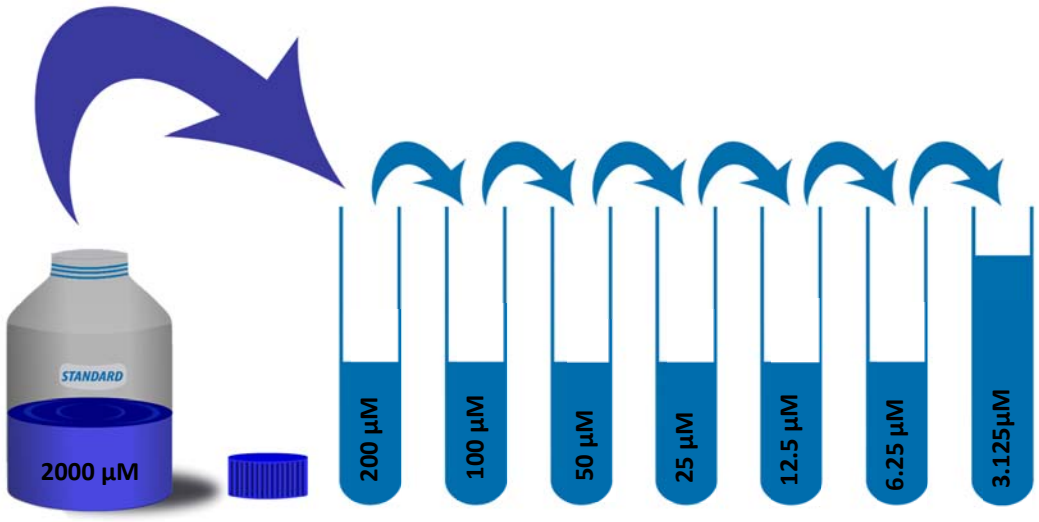

All standard must be used within 2 hours of preparation

Assay Procedure

For Nitrite (NO2) Determination:

1. Pipette 50 µL of either samples or NO2 standards into duplicate wells in the plate.
2. Pipette 50 µL of Assay Buffer into duplicate wells as the Zero standard.
3. Add 25 µL of the Reagent A to each well using a multichannel/repeater pipette.
4. Add 25 µL of the Reagent B to each well using a multichannel/repeater pipette.
5. Incubate at room temperature for 5 minutes.
6. Read the optical density at 540-570 nm.

➢ These data will be used to determine NO2 concentration.

For total Nitric Oxide (NO) Determination:

1. Pipette 50 µL of either samples or Nitrate (NO3) standards into duplicate wells in the plate.
2. Pipette 50 µL of Assay Buffer into duplicate wells as the Zero standard.
3. Add 10 µL of the NADH Solution to each well using a multichannel/repeater pipette.
4. Add 10 µL of the Nitrite Reductase Solution to each well using a multichannel/repeater pipette.
5. Incubate at room temperature for 20 minutes.
6. Add 25 µL of the Reagent A to each well using a multichannel/repeater pipette.
7. Add 25 µL of the Reagent B to each well using a multichannel/repeater pipette.
8. Incubate at room temperature for 5 minutes.

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9. Read the optical density at 540-570 nm.

➢ These data will be used to determine Total NO concentration.

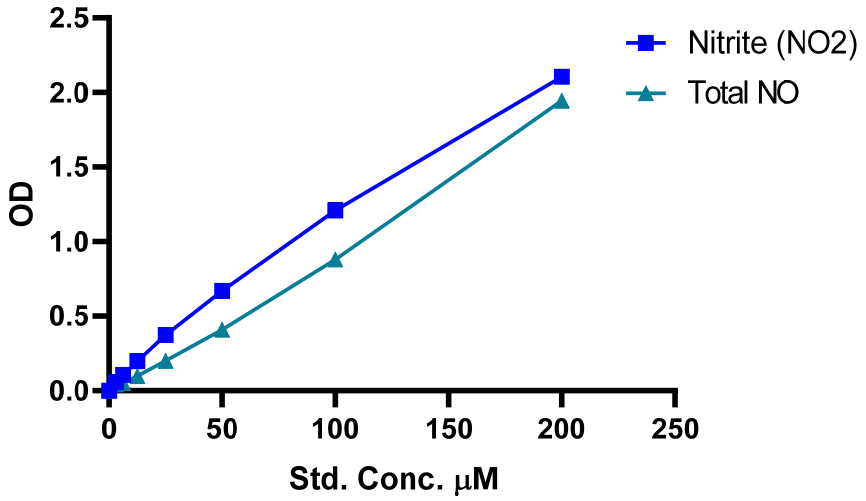
Calculation

- Average the duplicate optical density (OD) readings for each standard and sample.
- Subtract the mean ODs for the zero standard from all OD values

  (for example if the OD value of zero standard, and standard 6 are 0.087, and 1.086 respectively; then the adjusted ODs equal 0 and 0.999 respectively.)
- Create a standard curve by reducing the data using the four parameter logistic curve (4PLC) fitting routine on the plate reader using the adjusted OD values
- The concentrations obtained should be multiplied by the dilution factor to obtain sample values.

Nitrate concentrations are calculated by subtracting Nitrite concentration of each sample from total NO concentration

$$NO3\ Conc. = Total\ NO\ Conc. - NO2\ Conc.$$



A typical standard curve of ZellX® NO Assay kit

Run your own standard curves for calculation of results

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Assay range

The detection limit of ZellX® NO assay was determined as 0.94 µM for Nitrite and 3.0 µM for Total NO.

Sensitivity

The sensitivity of the ZellX® NO assay was determined as 2.63 µM for Nitrite and 1.02 µM for Total NO.

Precision

Intra-Assay Precision (Precision within an assay): 3 samples were tested 20 times in an assay.

Inter-Assay Precision (Precision between assays): 3 samples were tested in duplicate on 20 different assays over multiple days.

Nitrite:

Item	*%CV*
Intra assay	9.1, 1.3, 4.4
Inter assay	3.1, 6.3, 4.0

Total NO:

Item	*%CV*
Intra assay	1.8, 6.8, 4.4
Inter assay	5.7, 7.4, 4.1

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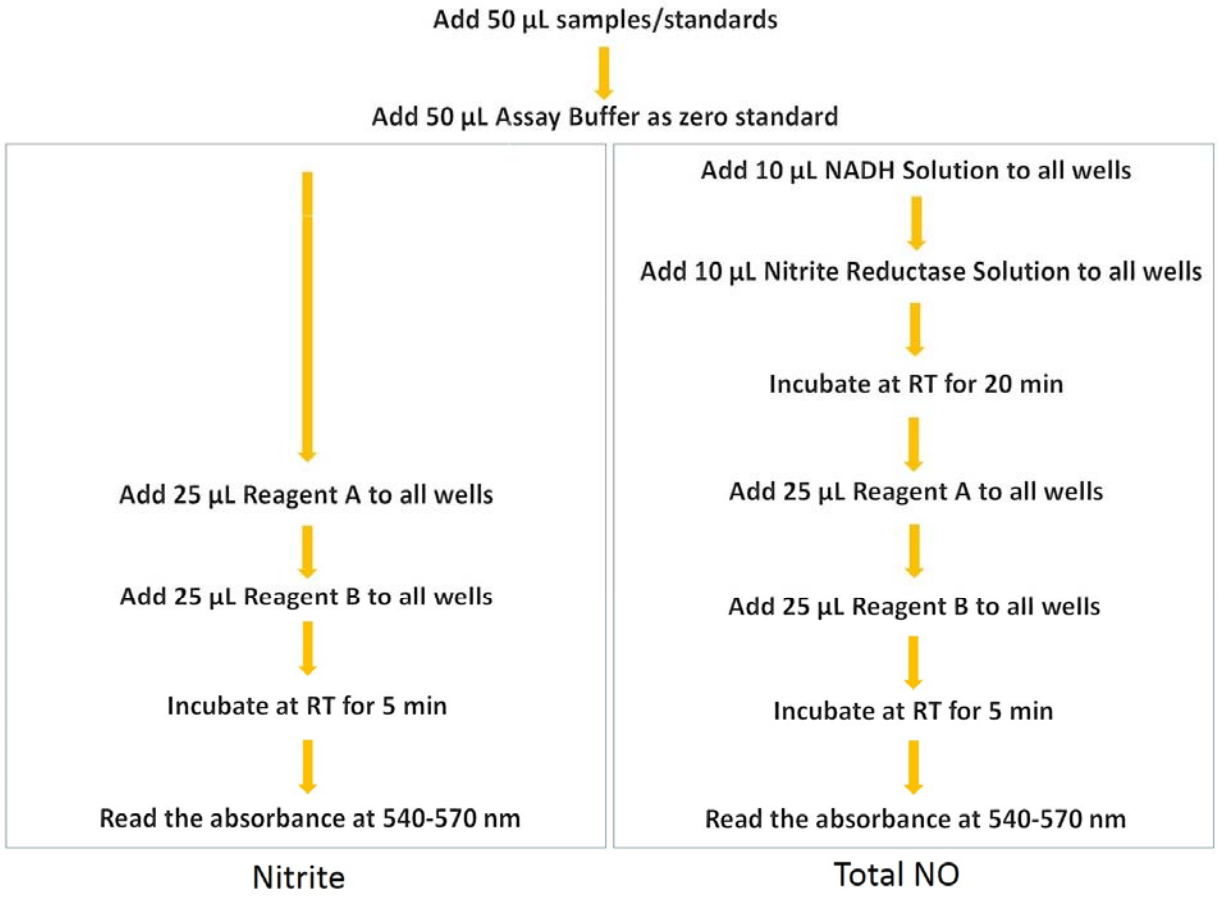
Protocol summary



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