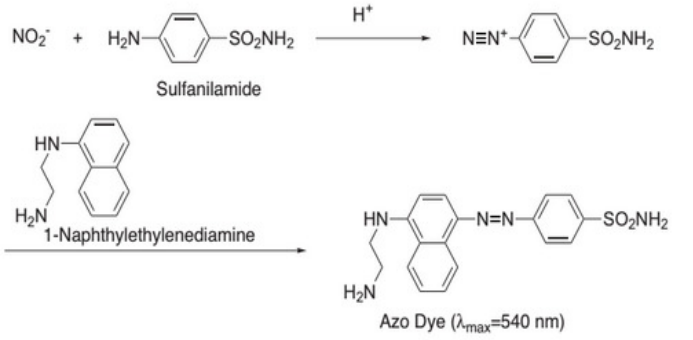
23479 Nitrite/Nitrate Assay Kit, colorimetric

Used for detection of nitric oxide metabolites

Product Description

Nitric oxide (NO), a gaseous paramagnetic radical, is a very important and versatile messenger in biological systems. NO is synthesized from L-arginine by NO synthase (NOS). It has been identified as an endothelial derived relaxation factor (EDRF) and antiplatelet substance. It serves as a neurotransmitter derived from a neutrophile and a cytotoxic substance from an activated macrophage. Although NO's molecular action in the biological system is very versatile, the most important role of NO is the activation of guanylate cyclase.

The Nitrite/Nitrate Assay Kit is a sensitive assay for determining levels of NO metabolites (ranging from 10–100 µM). Total NO metabolite (Figure 2) concentration is determined using the Griess assay. In the process NO_3^- is converted to NO_2^- by the enzyme Nitrate Reductase. The Griess assay's mechanism is summarized as the azo coupling between diazonium species, which are produced from sulfanilamide with NO_2, and naphthylethylenediamine, resulting in a colorimetric (540 or 570 nm) product proportional to the NO metabolite present (Figure 1).



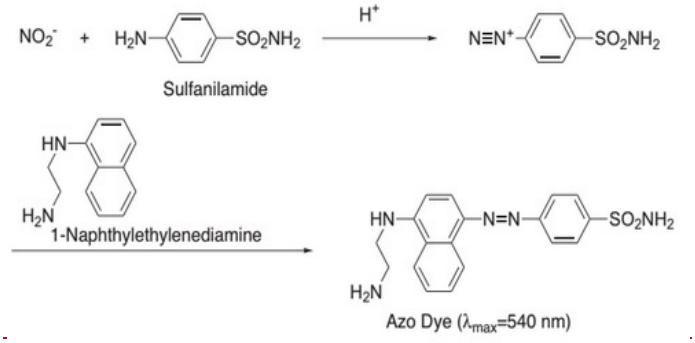


Figure 1 Coloring reaction scheme of NO_2^- detection

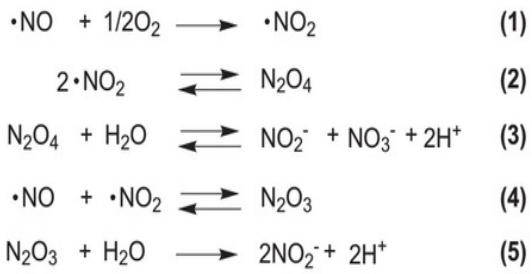


Figure 2 NO reaction and its metabolites

Components
The kit is sufficient for 100 assays in 96 well plates.

- $NaNO_2$ Standard Solution (100 µM) 1 bottle
- $NaNO_3$ Standard Solution (100 µM) 1 bottle
- Buffer Solution (20 mM, pH 7.6) 1 bottle
- Nitrate Reductase 1 vial
- Enzyme Co-factors 1 vial
- Griess Reagent A 1 bottle
- Griess Reagent B 1 bottle

Reagents and Equipment Required but Not Provided
- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
This kit is shipped at ambient temperature. Storage at 2–8°C is recommended.

Preparation Instructions
$NaNO_2$ Standard Solution, $NaNO_3$ Standard Solution, Buffer Solution, Griess Reagent A and Griess Reagent B – Allow each to come to room temperature before use. NO_2 and NO_3 Standard solutions should be used up within 2 months once the vial is opened.

Nitrate Reductase, Enzyme Cofactors – Reconstitute each in 1.2 mL of Buffer Solution prior to use. Since the vials are under pressure, add the Buffer Solution into the vial with a syringe in order to avoid the dispersal of the powder. Please **do not open the rubber septum at the beginning**. Mix well by pipetting. Both solutions should be kept in ice bath during your experiment. Store reconstituted solutions at 2–8 °C. Use within two weeks after reconstitution.

Do not mix Nitrate Reductase with Enzyme Co-factors prior to use.

Procedure
All samples and standards should be run in duplicate.

Standards for Colorimetric Detection of NO_2^-
Add 0, 20, 40, and 80 µL of the 100 µM (0.1 nmole/µL) $NaNO_2$ Standard Solution into a 96 well plate generating 0 (blank), 2, 4, and 8 nmole/well standards. Add Buffer Solution to each well to bring the volume to 100 µL.

Standards for Colorimetric Detection of NO_3^- + NO_2^-
Add 0, 20, 40, and 80 µL of the 100 µM (0.1 nmole/µL) $NaNO_3$ Standard Solution into a 96 well plate generating 0 (blank), 2, 4, and 8 nmole/well standards. Add Buffer Solution to each well to bring the volume to 80 µL.

Sample Preparation
Centrifuge serum and plasma in an Amicon® Ultra-4 Centrifugal Filter Unit with Ultracel®-10 membrane (7000 * g, 4 °C, 20 minutes) to remove hemoglobin and proteins.

Centrifuge cell or tissue culture media at 1000 * g for 15 minutes to remove insoluble material. Collect the supernatant.

Notes: Cell culture medium that contains NO_3^- as a component (such as RPMI-1640) is not a suitable medium to detect [NO_3^- + NO_2^-]. However, NO_2^- in the culture medium can be determined using this kit.

For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Add 1–80 µL of samples into four wells of a 96 well plate. Two wells will be used for the NO_2^- detection and two wells will be used for the [NO_3^- + NO_2^-] detection step (duplicate samples). Add Buffer Solution to bring samples to final volumes of 100 µL for NO_2^- and 80 µL for [NO_3^- + NO_2^-] detection, respectively.

Assay Reaction

1. Add 10 µL of the Nitrate Reductase solution and 10 µL of the Enzyme Co-factors solution to the sample and standard wells for [NO_3^- + NO_2^-] Detection.

2. Mix well using a horizontal shaker or by pipetting and incubate the plate at 25 °C for two hours.
3. Add 50 µL of Griess Reagent A to all wells. Mix well using a horizontal shaker or by pipetting and incubate the plate at 25 °C for five minutes.
4. Add 50 µL of Griess Reagent B to all wells. Mix well using a horizontal shaker or by pipetting and incubate the plate at 25 °C for ten minutes.
5. Measure the absorbance at 540 nm (A540) in a microplate reader.

Results

Calculations

The background for the NO_2^- determination is the value obtained for the 0 nmole/well (blank) $NaNO_2$ Standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate $NaNO_2$ Standards to plot a standard curve.

Follow the same procedure to plot a standard curve for NO_3^- + NO_2^-. Use the blank value for $NaNO_3$ Standard.

Note: New standard curves must be set up each time the assay is run.

Sample NO_2^- Determination: Subtract the NO_2^- Blank value from the NO_2^- sample reading to obtain the corrected measurement. Using the corrected measurement, determine the amount of NO_2^- present in the sample from the standard curve.

Sample NO_3^- + NO_2^- Determination: Subtract the NO_3^- + NO_2^- Blank value from the NO_3^- + NO_2^- sample reading to obtain the corrected measurement. Using the corrected measurement, determine the amount of NO_3^- + NO_2^- present in the sample from the standard curve.

Concentration of Nitrite

$$ANO_2^-/VNO_2^- = CNO_2^-$$

where:
ANO_2^- = Amount of Nitrite in sample well (nmole) from Nitrite standard curve
VNO_2^- = Sample volume (µL) added into the well
CNO_2^- = Concentration of Nitrite in sample

Nitrite molecular weight: 46.01 g/mole

Concentration of Nitrate plus Nitrite

$$A[NO_3^- + NO_2^-]/V[NO_3^- + NO_2^-] = C[NO_3^- + NO_2^-]$$

where:
$A[NO_3^- + NO_2^-]$ = Amount of Nitrate plus Nitrite in sample well (nmole) from standard curve
$V[NO_3^- + NO_2^-]$ = Sample volume (µL) added into the well
$C[NO_3^- + NO_2^-]$ = Concentration of Nitrate plus Nitrite in sample

Concentration of Nitrate

$$CNO_3^- = C[NO_3^- + NO_2^-] - CNO_2^-$$

Nitrate molecular weight: 62.01 g/mole

Sample Calculation

Amount of Nitrite (S_a) = 4.84 nmole (calculated from standard curve)

Sample volume (S_v) = 50 µL

Concentration of Nitrite in sample:

$$4.84 \text{ nmole}/50 \text{ µL} = 0.0968 \text{ nmole/µL}$$

$$0.0968 \text{ nmole/µL} \times 46.01 \text{ ng/nmole} = 4.45 \text{ ng/µL}$$

References

Mária Kovács et al., Effect of Sodium Nitrite on Ischaemia and Reperfusion-Induced Arrhythmias in Anaesthetized Dogs: Is Protein S-Nitrosylation Involved? PLOS ONE, **10(4)**: e0122243. doi:10.1371/journal.pone.0122243. 2015

Attila Kiss et al., The role of nitric oxide, superoxide and peroxynitrite in the anti-arrhythmic effects of preconditioning and peroxynitrite infusion in anaesthetized dogs. Br J Pharmacol. 160(5): 1263-1272. 2010