

ab65327

Nitric Oxide Assay Kit (Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of nitric oxide in various samples.

This product is for research use only and is not intended for diagnostic use.

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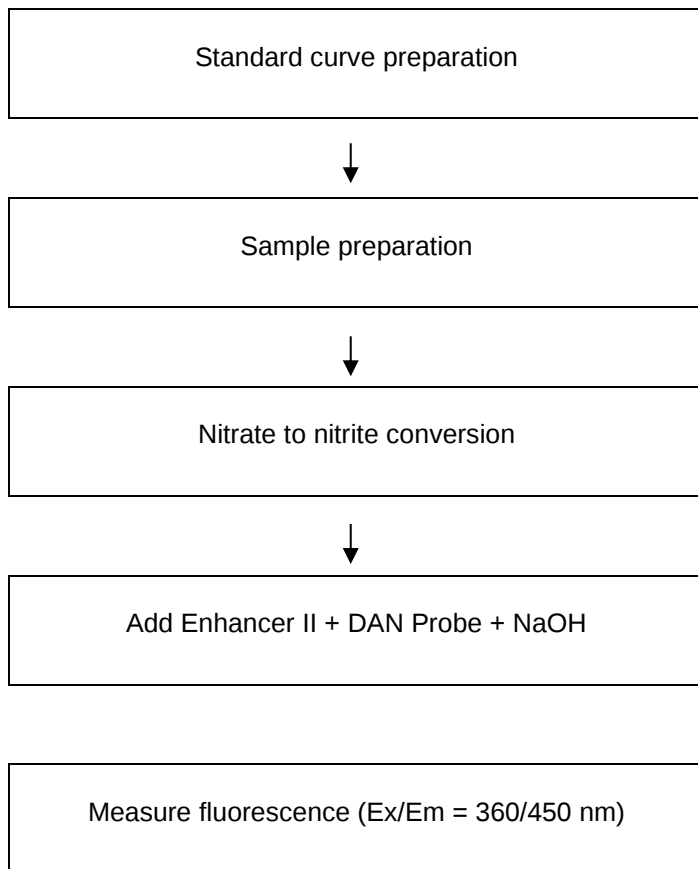
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1. BACKGROUND

Nitric Oxide Assay Kit (fluorometric) (ab65327) provides an accurate and convenient measurement of total nitrate/nitrite concentration in a simple two-step process. In the first step nitrate is converted to nitrite by nitrate reductase. In the second step, nitrite reacts with the fluorescent probe DAN (2,3 diaminonaphthalene). NaOH enhances the fluorescent yield. The fluorescent intensity is proportional to the total nitric oxide production. The kit has been tested with culture media, plasma, and tissue homogenates.

Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. Since NO is rapidly converted to nitrite (NO_2^-) and nitrate (NO_3^-), the total concentration of nitrite and nitrate is used as a quantitative measure of NO production.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

All components in this kit are shipped on blue ice and are suitable for storage at -20°C, unless reconstituted. Upon receipt, immediately store kit at -20°C in the dark. Individual components may be stored at alternative temperatures as shown in in section 5. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Nitric Oxide Assay Buffer	40 mL	-20°C	4°C
Cofactor I	1 vial	-20°C	-20°C
Enhancer II	1 vial	-20°C	-20°C
Nitrate Reductase II	1 vial	-20°C or -80°C	-20°C
Nitrate Standard	1 vial	-20°C	4°C
Nitrite Standard	1 vial	-20°C	4°C
DAN Probe	1 mL	-20°C	4°C
Sodium Hydroxide	1 mL	-20°C	4°C
96-Well White Plate	2 x 1 Unit	-20°C	RT
Microplate Sealing Film	2 x 1 Unit	-20°C	RT

PLEASE NOTE: Cofactor I was previously labelled as NOS Cofactor I and Enzyme Co-factor (Lyophilized). The composition has not changed.

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH₂O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent microplate reader - equipped with filter for Ex/Em = 360/450 nm
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)

If performing deproteinization step, additional reagents are required:

- 10 kD Spin columns (ab93349) – for fluid samples

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Nitric Oxide Assay Buffer:**

Ready to use as supplied. Store at 4°C.

9.2 **Cofactor I:**

Reconstitute with 110 µL of dH₂O to make 10 mM stock solution. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Freeze/thaw should be limited to 1 time.

NOTE: When ready to use, dilute appropriate portion 10X to make 1 mM working solution. Keep on ice during use. Working solution can be stored at 4°C for 6-8 hours.

9.3 **Enhancer II:**

Reconstitute with 1.2 mL of Nitric Oxide Assay Buffer. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice during use.

9.4 **Nitrate Reductase II:**

Reconstitute with 1.2 mL of Nitric Oxide Assay Buffer. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Freeze/thaw should be limited to 1 time. Keep on ice during use.

9.5 **Nitrate and Nitrite Standards:**

Reconstitute with 1.0 mL of Nitric Oxide Assay Buffer. Vortex to generate 10 mM of each standard. Store at +4°C when not in use (**DO NOT FREEZE**). The reconstituted standards are stable for 4 months when stored at +4°C.

9.6 **Fluorometric DAN Probe:**

Ready to use as supplied. Store at 4°C.

9.7 **NaOH:**

Ready to use as supplied. Store at 4°C.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1 For total nitrite/nitrate assay:

10.1.1 Prepare 50 μM Nitrate standard by add 5 μL of the 10 mM Nitrate Standard into 995 μL of Nitric Oxide Assay Buffer.

10.1.2 Using 50 μM Nitrate standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (μL)	Nitric Oxide Assay Buffer (μL)	Final volume standard in well (μL)	End [Nitrate] in well
1	0	225	75	0 pmol/well
2	12	213	75	200 pmol/well
3	24	201	75	400 pmol/well
4	36	189	75	600 pmol/well
5	48	177	75	800 pmol/well
6	60	165	75	1,000 pmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 75 μL).

10.2 For separate nitrate and nitrite measurements:

10.2.1 Prepare 50 μM Nitrite standard by add 5 μL of the 10 mM Nitrite standard into 995 μL of Nitric Oxide Assay Buffer.

10.2.2 Using 50 μM Nitrite standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (μL)	Nitric Oxide Assay Buffer (μL)	Final volume standard in well (μL)	End [Nitrite] in well
1	0	225	75	0 pmol/well
2	12	213	75	200 pmol/well
3	24	201	75	400 pmol/well
4	36	189	75	600 pmol/well
5	48	177	75	800 pmol/well
6	60	165	75	1,000 pmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 75 μL).

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C . When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples:

- 11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 2×10^6 cells).
- 11.1.2 Wash cells with cold PBS.
- 11.1.3 Resuspend cells in 500 μL (or ~ 4 volumes) of Nitric Oxide Assay Buffer on ice.
- 11.1.4 Homogenize cells quickly by pipetting up and down a few times.
- 11.1.5 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
- 11.1.6 Collect supernatant and transfer to a clean tube.
- 11.1.7 Keep on ice.

11.2 Tissue samples:

- 11.2.1 Harvest the amount of tissue necessary for each assays (initial recommendation = 20 mg).
- 11.2.2 Wash in cold PBS.

- 11.2.3 Resuspend tissue in 500 – 1,000 μL (or ~4-6 volumes) of Nitric Oxide Assay Buffer.
- 11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 50 passes.
- 11.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
- 11.2.6 Collect supernatant and transfer to a clean tube.
- 11.2.7 Keep on ice.

Tissue homogenates should be assayed with no more than 10 μL of undiluted sample.

11.3 Plasma, serum and urine:

Plasma samples.

Typical urine nitrite and nitrate levels are in the 0.2 – 2 mM and 1 – 20 μM range respectively.

Typical normal serum levels for nitrate and nitrite are 0 – 20 μM and 0 – 2 μM respectively with various disease states elevating these levels significantly.

Plasma, serum and urine samples generally contain high amount of proteins. Use 10 kDa Spin column (ab93349) to deproteinize biological fluids.

NOTE: We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.
- DAN probe reacts with nitrite, not nitrate.
- For total nitrite/nitrate measurement, prepare a nitrate standard curve only.
- For separate nitrite and nitrate concentration measurement, prepare a nitrite standard curve (i.e. absence of nitrate reductase in standard curve and assay samples).

$$\text{Nitrate} = \text{Total} - \text{Nitrite}$$

12.1 Set up Reaction wells for Nitrate and Nitrite:

- Background wells = 75 μ L Nitric Oxide Assay Buffer.
- Standard wells = 75 μ L standard dilutions.
- Total Nitrate/Nitrite Sample wells = 0 – 75 μ L samples (adjust volume to 75 μ L/well with Nitric Oxide Assay Buffer). (No more than 10 μ L of undiluted plasma or tissue homogenate).
- Nitrite Sample wells = 0 – 75 μ L samples (adjust volume to 75 μ L/well with Nitric Oxide Assay Buffer). (No more than 10 μ L of undiluted plasma or tissue homogenate).

12.2 Add 5 μ L of the Cofactor I solution to all wells.

12.3 Set up of Nitrate and Nitrite measurement:

- Depending on whether nitrate or nitrite is being measured:
- **For nitrate assay:** add 5 μ L of the Nitrate Reductase II to samples and standards. OR
- **For nitrite assay:** add 5 μ L of the Nitric Oxide Assay Buffer to samples and standards.

12.4 Incubate at room temperature for 1 – 4 hours.

NOTE: 1 hour = ~90% conversion of nitrate to nitrite.

2 hours = ~95% conversion of nitrate to nitrite.

4 hours = ~99% conversion of nitrate to nitrite.

- 12.5 Add 5 μ L of Enhancer II to each well.
- 12.6 Incubate at room temperature for 30 minutes (to quench interfering compounds).
- 12.7 Add 5 μ L of DAN Probe to each well.
- 12.8 Incubate at room temperature for 10 minutes.
- 12.9 Add 5 μ L NaOH to each well.
- 12.10 Incubate at room temperature for 10 minutes.
- 12.11 Measure output on a microplate reader.
 - Fluorometric assay: measure Ex/Em = 360/450 nm.
 -

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of nitric oxide.

13.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5 Extrapolate sample readings from the standard curve plotted using the following equation:

$$Sa = \left(\frac{\text{Corrected absorbance} - (y - \text{intercept})}{\text{Slope}} \right)$$

13.6 Concentration of nitrate and nitrite in the test samples is calculated as:

$$\text{Nitrite + nitrate concentration} = \left(\frac{Sa}{Sv} \right) * D$$

Where:

Sa = Amount of nitrate + nitrite in the sample well (conc).

Sv = Sample volume added into the reaction well (μL).

D = Sample dilution factor.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

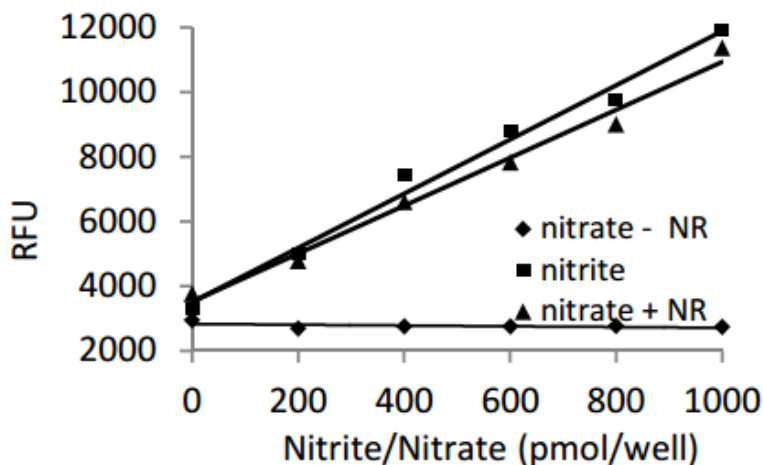


Figure 1: Typical nitrite/nitrate standard calibration curve using fluorometric reading. Assay performed in presence and absence of nitrate reductase, and read after 1 hour conversion of nitrate to nitrite.

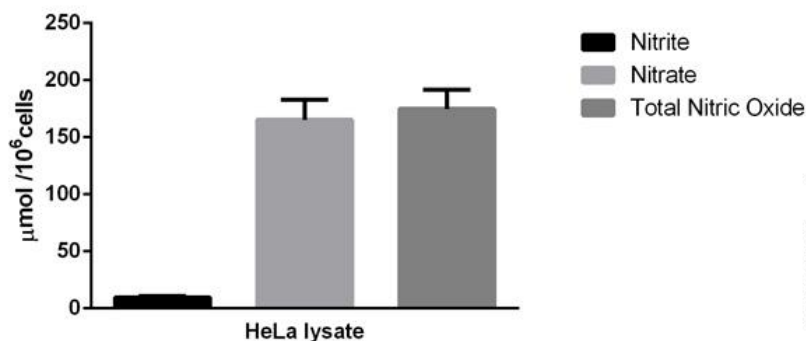
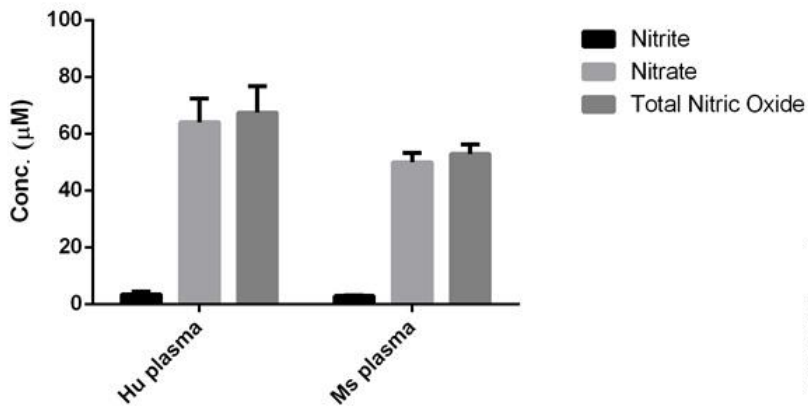


Figure 2: Nitric oxide measured in cell lysates showing quantity (micromol) per 10⁶ cells tested



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Figure 3: Nitric oxide measured in biologicals showing concentration (micromolar).

15. QUICK ASSAY PROCEDURE

NOTE: *This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare standards, DAN probe and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve(s).
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (75 µL), samples (up to 75 µL, with assay buffer) and background (75 µL).
- Add 5 µL of the Cofactor I solution to all wells.
- Add 5 µL of the Nitrate Reductase II to nitrate assay wells (samples and standards) OR
- Add 5 µL of the Nitric Oxide Assay Buffer to nitrite assay wells (samples and standards).
- Incubate at RT for 1 – 4 hrs.
- Add 5 µL of Enhancer II to each well.
- Incubate at RT for 30 min (to quench interfering compounds).
- Add 5µL of DAN Probe to each well.
- Incubate at RT for 10 mins.
- Add 5 µL NaOH to each well.
- Incubate at RT for 10 mins.
- Measure output on a microplate reader, Ex/Em = 360/450 nm.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: white wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

RESOURCES

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQ

What anticoagulant works best for plasma prep for this assay?

You can use either heparin or EDTA for plasma preparation for this assay.

Can we use this kit to measure nitric oxide that have been generated during culture period and have been released in the culture medium (RPMI 1640)? I know that nitric oxide is not stable and is rapidly converted to nitrite/nitrate!

Yes, this kit could be used with RPMI 1640 culture media for measuring nitric oxide. Although the nitric oxide is rapidly converted to nitrite and nitrate, this kit will allow the nitrate to also get converted to nitrite and the total nitrite will then react with the probe. So the final reading will be a measure of the total nitric oxide released into the media.

Note that phenol red in cell culture media may decrease the reading, and therefore we recommend preparing a standard curve in the same culture media.

Is the nitrate reductase provided in this kit NADH dependent or NADPH dependent?

The nitrate reductase is NAD(P)H dependent.

What is the sensitivity of this assay kit?

The detection limit of this assay is 0.03 μM .

18. INTERFERENCES

19. NOTES

Technical Support

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For all technical or commercial enquiries please go to:

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