Product Information

Nitric Oxide Fluorometric Assay Kit

I. Kit Contents:

Components	K2099-200 200 assays	Cap Color	Part Number
Assay Buffer	30 ml	WM	K2099-C-1
Enzyme Cofactor	Lyophilized	Blue	K2099-C-2
Enhancer	Lyophilized	Purple	K2099-C-3
Nitrate Reductase	Lyophilized	Green	K2099-C-4
Nitrate Standard	Lyophilized	Yellow	K2099-C-5
Nitrite Standard	Lyophilized	Orange	B1838
DAN Probe	1 ml	Amber Red	K2099-C-6
Sodium Hydroxide	1 ml	Clear	K2099-C-7
Microtiter plate	2 plates	---	K2099-C-8
Plate Cover	2 covers	---	K2099-C-9

II. Introduction:

Nitric oxide is essential in neurotransmission, vascular regulation, immune response and apoptosis. Because Nitric oxide is rapidly converted into nitrite and nitrate, the NO production can be quantitatively determined by the total concentration of nitrite and nitrate. This kit is a simple and accurate two-step measurement of total nitrate/nitrite concentration. Nitrate reductase converts nitrate into nitrite first, and then nitrite reacts with the fluorescent probe DAN (2, 3 diaminonaphthalene). NaOH enhances the fluorescent yield. The total nitric oxide production is proportional to the fluorescent intensity. The kit has been tested with culture media, plasma, and tissue homogenates.

III. Reconstitution of Reagents:

1. Assay Buffer: The assay buffer is ready to use as supplied. Store at 4℃.

2. Enzyme Cofactors: Reconstitute with 110 µl of dH_2O to make 10 mM stock solution. Aliquot and store at -20℃. Freeze/thaw should be limited to 1 time. Dilute appropriate portion 10X to make 1 mM working solution. Keep on ice while in use. Working solution can be stored at 4℃ for 6-8 hrs.

3. Enhancer: Reconstitute with 1.2 ml of Assay buffer. Keep on ice during use. Store at -20℃.

4. Nitrate Reductase: Reconstitute with 1.2 ml of Assay Buffer. Aliquot desired amount and store at –20℃. Keep on ice during use. Freeze/thaw should be limited to 1 time.

5. Nitrate/Nitrite Standards: Reconstitute with 1.0 ml of Assay Buffer, vortex to generate
10 mM standard each. Store at 4℃ when not in use (do not freeze!). The reconstituted standards are stable for 4 months when stored at 4℃.

6. Fluorometric DAN Probe and NaOH: Ready to use. Store at 4℃

IV. Measurement of Nitrate + Nitrite:

1. Prepare standards: Add 5 µl of the reconstituted 10 mM nitrate/nitrite standards to 995 µl assay buffer, vortex to generate 50 µM working Standard Solution. Add 0, 4, 8, 12, 16, 20 µl of the working Standard to 6 consecutive wells to generate 0, 200, 400, 600, 800, 1000 pmol/well standard. Bring the volume to 75 µl with Assay Buffer.

Note: DAN Probe reacts with nitrite, not nitrate. For routine total nitrite/nitrate assay, you may prepare a nitrate standard curve only. However, if you need to measure nitrite and nitrate concentrations separately, you may prepare a nitrite standard curve in the absence of Nitrate Reductase in the standard curve and assay samples. Nitrate = Total – Nitrite.

2. Prepare Samples: Samples containing high protein concentration may need to be filtered through a 10 kDa MW cut-off filter prior to assay. Add 0-75 µl of sample to the wells and adjust the volume to 75 µl with Assay Buffer. Notes: Typical urine nitrite and nitrate levels are in the 0.2 - 2 mM and 1 - 20 µM range respectively. Typical normal serum levels are 0 - 20 µM and 0 - 2 µM respectively with various disease states elevating these levels significantly. Plasma samples or tissue homogenates should be assayed with no more than 10 µl of undiluted sample. Phenol red and serum in cell culture media may decrease the reading, and thus a standard curve should be made in the same media.

3. Add 5 µl of the Enzyme Cofactor working solution to all wells.

4. Add 5 µl of the Nitrate Reductase to nitrate assay wells (unknowns and standards), add 5 µl of buffer in place of Nitrate Reductase (unknowns and standards) when you determine nitrite separately.

5. Cover the plate with the plate cover and incubate at room temperature for 1 - 4 hrs. 1 hour = ~ 90 % conversion of Nitrate to Nitrite, 2 hours = ~ 95 % conversion, 4 hours = ~ 99 % conversion.

6. Add 5 µl of Enhancer to each well. Incubate 30 minutes to quench interfering compounds.

7. Add 5 µl of DAN Reagent to each well. Incubate for 10 minutes at room temperature.

8. Add 5 µl of NaOH to each well. Incubate for 10 minutes at room temperature.

9. Read the plate in a fluorometer using Ex. = 360 nm and Em. = 450 nm.

V. Calculations

1. Plot standard curve: Plot fluorescence vs. picomoles nitrate.

2. Determine sample nitrate and nitrite concentrations:

C = [nitrate + nitrite] (µM) =*(fluorescence - y intercept)/slope/ sample volume (µl) x dilution*

OR

C = Sa/Sv,

where Sa is the amount of samples as read from Standard Curve (in pmol),

Sv is the volume of sample added to the well (in µl), multiplied by the dilution* factor.

Dilution is the sample dilution done prior to addition of the sample to the well.

Frequently Asked Questions

1. Can we use this kit to measure NO that have been generated during culture period and have been released in the culture medium (RPMI 1640)? I know that NO is not stable and is rapidly converted to nitrite/nitrate!

Yes, this kit can be used with RPMI 1640 culture media for measuring NO. Although the NO 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 your final reading will be a measure of the total NO released into the media.

2. Is the Nitrate Reductase provided in this kit NADH dependent or NADPH dependent. What is the sensitivity of this assay kit?

The Nitrate Reductase is NAD(P)H dependent. The detection limit of this assay is 0.03 µM.

3. Can this kit be used with samples like bacteria, plants, drosophila, yeast etc?

We have optimized the kit with mammalian samples. However, theoretically these kits should work with samples from multiple species/sources. Since the optimal conditions depend on the sample type, the protocol has to be be adapted to fit the samples for efficient results. Please refer to this kits citations to see what kind of samples have been used with this kit other than mammalian samples.

4. Can we use frozen samples with this assay?

Fresh samples are always preferred over frozen samples. However, frozen samples can also be used, provided, they were frozen right after isolation, were not freeze thawed multiple time (for which we recommend aliquoting the samples before freezing) and have been frozen for relatively short periods.

5. Can we use a different wavelength than recommended for the final analysis?

It is always recommended to use the exact recommended wavelength for the most efficient results. However, most plate readers have flexibility in their band width of detection in increments of +/- 10 nm. Depending on this flexibility range, you can deviate from the recommended wavelengths within limits.

6. What is the exact volume of sample required for this assay?

There is no specific volume we can recommend for the amount any sample to be used since it is completely sample concentration and quality based. You have to do a pilot expt with multiple sample volumes to determine the optimal volume which gives a reading within the linear range of the standard curve. Please refer to the citations for this product to see what other clients have used with similar sample types.

7. What is the shelf life of this kit?

This kit is good for 12 months from the date of shipment in the unopened form when stored at the appropriate temperature and appropriate conditions. After opening and reconstitution, some of the components in this kit are good for 2 months at -20℃. Please refer to the datasheet for storage information and shelf life of each of the components.

8. Why are my standard curve values lower than those shown on the datasheet?

There are multiple factors which influence the signals like the incubation times, room temperature, handling etc. In general, to increase the value of the standards, you can increase the incubation time. As long as the standard curve is linear, it should be fine to use, since all of your samples will also be measured under the same conditions on this curve.

9. How do I normalize my samples against protein concentration?

You can use a protein quantitation assay on the supernatants you get from cell/tissue lysates or with any other liquid sample in the assay buffer.

10. Can we use an alternate buffer for sample preparation (cell lysis, sample dilutions etc)?

Our assay buffers are optimized for the reactions they are designed for. They not only contain some detergents for efficient lysis of your cells/tissue, but also contain some proprietary components required for the further reactions. Therefore, we highly recommend using the buffers provided in the kit for the best results.

11. Should I make a standard curve for every expt I do, or is one curve/kit enough?

Yes, It is recommended to do the standards every time you do the expt. There is always a chance that something was done differently that day and we do not want any conditions to differ between standards and samples.

For research use only! Not to be used in humans.

Our promise

If the product does not perform as described on this datasheet, we will offer a refund or replacement. For more details, please visit http://www.apexbt.com/ or contact our technical team.

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