ab65328

Nitric Oxide Assay Kit (Colorimetric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Nitric Oxide in various samples.

View kit datasheet: www.abcam.com/ab65328
(use www.abcam.cn/ab65328 for China, or www.abcam.co.jp/ab65328 for Japan)

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

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Version v10, Last Updated 24 November 2023

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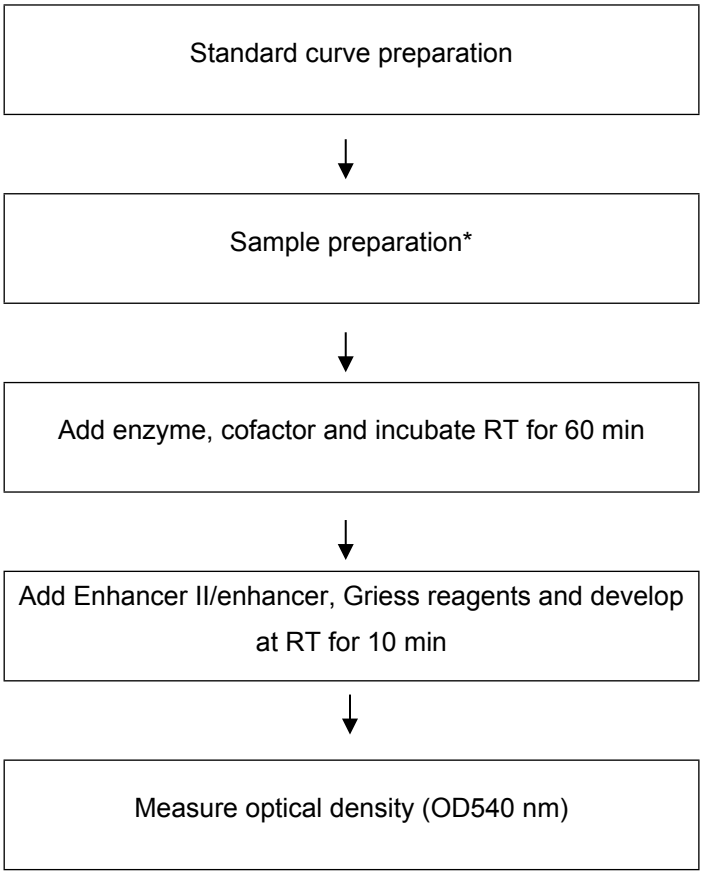
1. BACKGROUND

Nitric Oxide Assay Kit (colorimetric) (ab 65328) provides an accurate, convenient measure of total nitrate/nitrite in a simple two-step process. The first step converts nitrate to nitrite utilizing Nitrate Reductase I/nitrate reductase. The second step uses Griess Reagents to convert nitrite to a deep purple azo compound. The amount of the azochromophore accurately reflects nitric oxide amount in samples. The detection limit of the assay is approximately 1 nmol nitrite/well, or 10 µM.

Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. NO is rapidly oxidized to nitrite and nitrate which are used to quantitate NO production.

2. ASSAY SUMMARY



*Samples might require deproteinization.

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

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

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)
Assay Buffer XVII/Assay Buffer	30 mL	-20°C	-20°C
Enzyme Cofactor	1 vial	-20°C	-20°C
Enhancer II/Enhancer (lyophilized)	1 vial	-20°C	4°C
Nitrate Reductase I/Nitrate Reductase (lyophilized)	1 vial	-20°C	-20°C
Nitrate Standard (lyophilized)	1 vial	-20°C	4°C
Nitrite Standard (lyophilized)	1 vial	-20°C	4°C
Griess Reagent I/Griess Reagent R1	10 mL	-20°C	4°C
Griess Reagent II/Griess Reagent R2	10 mL	-20°C	4°C
96-Well Clear Plate/Microtiter plate	2	N/A	N/A
Plate cover	2	N/A	N/A

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 (ddH_2O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD540 nm
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)

For deproteinization step:

- 10 kD Spin Columns (ab93349)

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 **Assay Buffer XVII/Assay Buffer:**

Ready to use as supplied. Store at -20°C. Keep on ice while in use.

9.2 **Enzyme Cofactor:**

Reconstitute in 1.1 mL Assay Buffer XVII/Assay Buffer. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.3 **Enhancer II/Enhancer:**

Reconstitute in 1.1 mL ddH_2O. Aliquot Enhancer II/enhancer so that you have enough volume to perform the desired number of assays. Store at 4°C. Keep on ice while in use.

9.4 **Nitrate Reductase I/Nitrate Reductase:**

Reconstitute in 1.1 mL Assay Buffer XVII/Assay Buffer. This enzyme dissolve slowly, so gently vortex 2-3 times over 15 minutes. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.5 **Nitrate Standard:**

Reconstitute the Nitrate Standard in 100 µL of Assay Buffer XVII/Assay buffer to generate a 100 mM standard stock solution. Vortex and mix well. Aliquot nitrate standard so that you have enough volume to perform the desired number of assays. Store at 4°C (do not freeze). Use within 4 months of reconstitution. Keep on ice while in use.

9.6 **Nitrite Standard:**

Reconstitute the Nitrite Standard in 100 µL of Assay Buffer XVII/Assay buffer to generate a 100 mM standard stock solution. Vortex and mix well. Aliquot nitrite standard so that you have enough volume to perform the desired number of

assays. Store at 4°C (do not freeze). Use within 4 months of reconstitution. Keep on ice while in use.

9.7 **Griess Reagents R1:**

Ready to use as supplied. Aliquot reagent R1 so that you have enough volume to perform the desired number of assays. Store at 4°C. Keep on ice while in use.

9.8 **Griess Reagents R2:**

Ready to use as supplied. Aliquot reagent R2 so that you have enough volume to perform the desired number of assays. Store at 4°C. Keep on ice while in use.

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.

NOTE: *Reagents react with nitrite, not nitrate. For routine total nitrite/nitrate assay, it is only necessary to* prepare *the nitrate standard curve*. *To measure nitrite and nitrate separately, prepare a nitrite standard curve in absence of Nitrate Reductase I/nitrate reductase.*

10.1 Prepare a 1 mM nitrite or nitrate standard by diluting 5 µL of the appropriate reconstituted 100 mM standard with 495 µL of Assay Buffer XVII/Assay Buffer.

10.2 Using 1mM nitrite or nitrate standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Assay Buffer XVII/Assay Buffer (µL)	Final volume standard in well (µL)	End [nitrite or nitrate] in well
1	0	255	85	0 nmol/well
2	6	249	85	2 nmol/well
3	12	243	85	4 nmol/well
4	18	237	85	6 nmol/well
5	24	231	85	8 nmol/well
6	30	225	85	10 nmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 85 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 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 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of ice cold Assay Buffer XVII/Assay Buffer.

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.

11.1.8 Perform deproteinization step. We recommend using 10 kD spin columns (ab93349).

Tissue samples:

11.1.9 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

11.1.10 Wash tissue in cold PBS.

11.1.11 Resuspend tissue in 100 µL of ice cold Assay Buffer XVII/Assay Buffer.

11.1.12 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.1.13 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.14 Collect supernatant and transfer to a clean tube.

11.1.15 Keep on ice.

11.1.16 Perform deproteinization step. We recommend using 10 kD spin columns (ab93349).

11.2 **Plasma, Serum and Urine and other biological fluids:**

Plasma, serum and urine samples generally contain high amount of proteins, so they should be deproteinized through a10kD Spin column (ab93349).

Serum samples can be tested directly by adding sample to the microplate wells. Typical serum levels are ~20 µM and ~2 µM for nitrate and nitrite respectively (various disease states elevate these levels significantly). However, serum proteins will have a slight (~10%) effect on apparent nitrite levels.

Urine samples have a high nitrate content therefore a 10 fold dilution should be used. Typical urine levels are 0.2-2 mM and 1-20mM.

12. ASSAY PROCEDURE and DETECTION

- **It is recommended to assay all standards, controls and samples in duplicate.**

12.1 **Set up Reaction wells:**

- Nitrite or Nitrate Standard wells = 85 µL standard dilutions.
- Sample wells = 1 – 85 µL samples (adjust volume to 85 µL/well with Assay Buffer XVII/Assay Buffer).
- Sample Blank wells= 1 – 85 µL samples (adjust volume to 85 µL/well with Assay Buffer XVII/Assay Buffer).

12.2 Add the following reagents to the wells as shown below:

Component	Standard (µL)	Sample wells (µL)	Sample Blank wells (µL)
Nitrate Reductase I/Nitrate reductase*	**5**	**5**	**0**
Enzyme cofactor	**5**	**5**	**0**
Assay Buffer XVII/Assay Buffer	**0**	**0**	**115**

***NOTE**: *If measuring nitrite levels omit Nitrate Reductase I/nitrate reductase from standard and sample wells*.

12.3 Cover and incubate at room temperature for 1 hour to convert nitrate to nitrite.

12.4 Add 5 µL Enhancer II/Enhancer to standard and sample wells only.

12.5 Incubate at room temperature for 10 minutes.

12.6 Add 50 µL Griess Reagent I/Griess Reagent R1 to standard and sample wells only.

12.7 Add 50 µL Griess Reagent II/Griess Reagent R2 to standard and sample wells only.

12.8 Measure output on a microplate reader.

- Colorimetric assay: measure OD540 nm.

NOTE: *If OD is read with filters further away from the recommended wavelength of 540 nm e.g. 570 nm, the sensitivity of the assay will be reduced by approximately 35%.*

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 If the sample background control is significant, then subtract the sample background control from sample reading.

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

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of nitrate or nitrite.

13.5 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).

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

$$Nitrate \frac{and}{or} nitrite\ concentration = \left(\frac{Sample\ absorbance - Blank\ absorbance}{Slope\ of\ standard\ curve}\right)/B$$

Or

$$Nitrate \frac{and}{or} nitrite\ concentration = \left(\frac{A}{B}\right) * D$$

Where:

A = Amount of nitrate or nitrite in the sample well (nmol).

B = Sample volume added into the reaction well (µL) nmol/µL or mM nitrate (nitrite).

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.

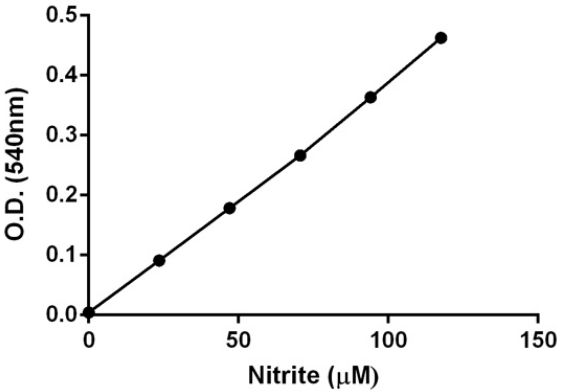


Figure 1. Typical nitrite standard calibration curve using colorimetric reading.

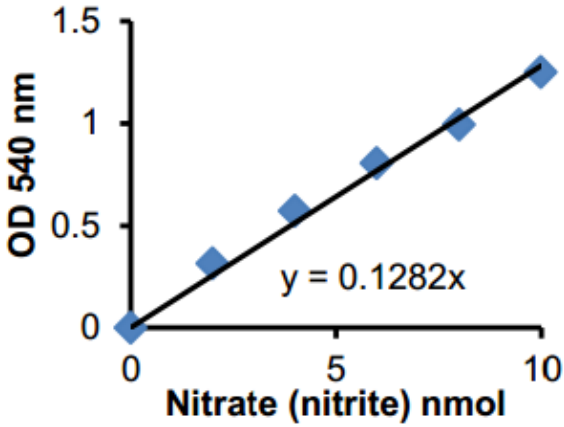


Figure 2: Typical nitrate standard calibration curve using colorimetric reading.

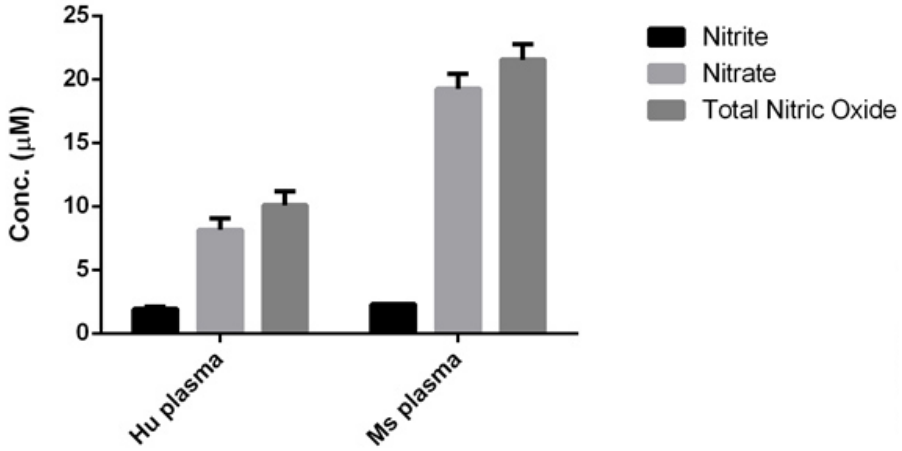


Figure 3: Nitrite and nitrate measured in biologicals showing concentration (micromolar).

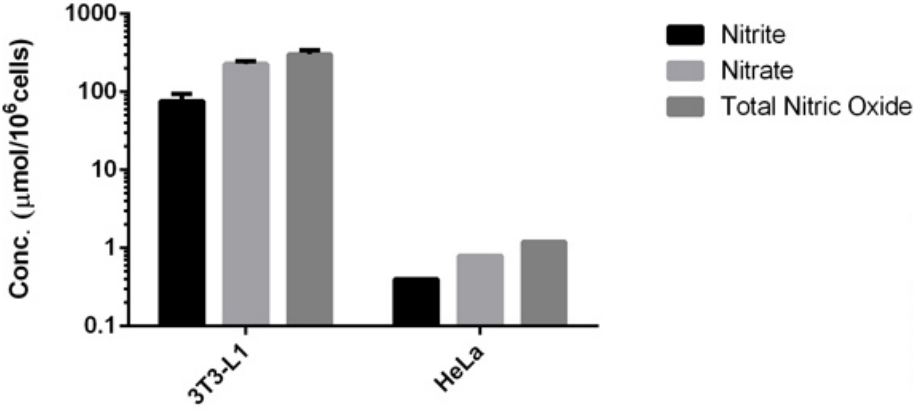


Figure 4: Nitrite and nitrate measured in cell lysates showing quantity (micromol) per 10^6 cells.

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 Nitrate Reductase I/nitrate reductase, enzyme cofactor, Enhancer II/enhancer, Griess Reagents 1 and 2 (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve (total nitrite/nitrate or nitrite only).
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings), including deproteinization step.
- Set up plate for standard (85 µL), samples (85 µL) and sample blank wells (85 µL).
- To standard and sample wells, add the following:

Component	Standard (µL)	Sample wells (µL)	Sample Blank wells (µL)
Nitrate Reductase I/Nitrate reductase	**5**	**5**	**0**
Enzyme cofactor	**5**	**5**	**0**
Assay Buffer XVII/Assay Buffer	**0**	**0**	**115**

***NOTE**: *If measuring nitrite levels omit Nitrate Reductase I/nitrate reductase from standard and sample wells*.*

- Cover and incubate at room temperature for 1 hour to convert nitrate to nitrite.
- Add 5 µL Enhancer II/Enhancer to Standard and Sample wells only.
- Incubate for at RT for 10 mins.

- Add 50 µL Griess Reagent I/Griess Reagent R1 and 50 µL Griess Reagent II/Griess Reagent R2 to standard and sample wells only.
- Incubate plate at RT 10 mins.
- Measure plate at OD540 nm for colorimetric assay.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use 10 kD Spin columns 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

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 preparation for this assay?

You can use either heparin or EDTA for plasma preparation. EDTA should be avoided when assaying for enzyme analytes.

Can this kit be used with MEM tissue culture medium that contains phenol red?

This kit is compatible with MEM with phenol red. Media that contain phenol red as a pH indicator do not interfere with the Griess reaction as the indicator is typically yellow colored under the conditions of the Griess reaction.

What are the main differences between this assay kit and ab65327?

This kit is colorimetric based, whereas ab65327 is fluorometric. The fluorometric assay is therefore ~ 10 times more sensitive than this kit.

18. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- RIPA: contains SDS which can destroy/decrease the activity of the enzyme.
- RPMI: contains nitrate (calcium nitrate).
- High concentrations of NADPH (0.5 – 1 mM) may inhibit Griess color reaction slightly.

19. **NOTES**

For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)