ab65328
Nitric Oxide Assay Kit
(Colorimetric)

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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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. 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.
INTRODUCTION

2. **ASSAY SUMMARY**

- Standard curve preparation
  
- Sample preparation*
  
- Add enzyme, cofactor and incubate RT for 60 min
  
- Add enhancer, Griess reagents and develop at RT for 10 min
  
- Measure optical density (OD540 nm)

*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 -20°C 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.**
## MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Buffer</td>
<td>30 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Enzyme Cofactor</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Enhancer (lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Nitrate Reductase (lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Nitrate Standard (lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Nitrite Standard (lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Griess Reagent R1</td>
<td>10 mL</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Griess Reagent R2</td>
<td>10 mL</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Microtiter plate</td>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Plate cover</td>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
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$_2$O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD540 nm
- 96 well plate: clear plates for colorimetric assay
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)

For deproteinization step, additional reagents are required:

- Perchloric acid (PCA) 4M, ice cold
- Potassium Hydroxide (KOH) 2M
- 10 kD Spin Columns (ab93349) – for fluid samples, if not performing PCA precipitation

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:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 Enzyme Cofactor:

Reconstitute in 1.1 mL 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:

Reconstitute in 1.1 mL ddH₂O. Aliquot 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:

Reconstitute in 1.1 mL 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 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 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.

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.

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

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

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 10⁶ cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of ice cold 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 as described in section 11.4.

11.2 **Tissue samples:**

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

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue in 100 µL of ice cold Assay Buffer.
11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 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.

11.2.8 Perform deproteinization step as described in section 11.4.

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

Plasma, serum and urine samples generally contain high amount of proteins, so they should be deproteinized as described in section 11.4.

Alternatively, you can use 10kD Spin column (ab93349) to deproteinize biological fluids.

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 1-20 mM.

11.4 **Deproteinization step:**

Prepare samples as specified in protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.

11.4.1 Add ice cold PCA 4 M to a final concentration of 1 M in the homogenate solution and vortex briefly to mix well. **NOTE:** *high protein concentration samples might need more PCA.*

11.4.2 Incubate on ice for 5 minutes.
11.4.3 Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.

11.4.4 Precipitate excess PCA by adding an equal volume of ice-cold 2 M KOH to supernatant obtained in previous step and vortex briefly. This will neutralize the sample and precipitate excess PCA. After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 µL of sample). Any left over PCA will interfere with the assay.

11.4.5 Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.

Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

**Sample Recovery**

The deproteinized samples will be diluted from the original concentration.

To calculate the dilution factor of your final sample, simply apply the following formula:

\[
\text{% original concentration} = \left( \frac{\text{Initial sample volume}}{\text{Initial sample volume} + \text{vol PCA} + \text{vol KOH}} \right) \times 100
\]

**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.

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).
- Sample Blank wells= 1 – 85 µL samples (adjust volume to 85 µL/well with Assay Buffer).

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Standard (µL)</th>
<th>Sample wells (µL)</th>
<th>Sample Blank wells (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reductase*</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Enzyme cofactor</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>0</td>
<td>0</td>
<td>115</td>
</tr>
</tbody>
</table>

**NOTE**: *If measuring nitrite levels omit 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 to standard and sample wells only.
12.5 Incubate at room temperature for 10 minutes.
12.6 Add 50 µL Griess Reagent R1 to standard and sample wells only.
12.7 Add 50 µL 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:

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

Or

\[
\text{Nitrate and/or nitrite concentration} = \left( \frac{A}{B} \right) \times D
\]

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.

![Typical nitrite standard calibration curve using colorimetric reading.](image)

*Figure 1*. Typical nitrite standard calibration curve using colorimetric reading.
**Figure 2:** Typical nitrate standard calibration curve using colorimetric reading.

**Figure 2:** Nitrite and nitrate measured in biologicals showing concentration (micromolar).
**Figure 3:** 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, enzyme cofactor, 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Standard (µL)</th>
<th>Sample wells (µL)</th>
<th>Sample Blank wells (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reductase</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Enzyme cofactor</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>0</td>
<td>0</td>
<td>115</td>
</tr>
</tbody>
</table>

**NOTE:** *If measuring nitrite levels omit 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 to Standard and Sample wells only.
- Incubate for at RT for 10 mins.
- Add 50 µL Griess Reagent R1 and 50 µL 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**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use PCA precipitation protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions on protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
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.
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