Instruction Manual

Nitrate/Nitrite Colorimetric Assay Kit

BioDynamics Laboratory Inc.
1. Principle of Assay

Discovery of nitric oxide (NO) as a mediator of endothelium-dependent vascular relaxation (Furchgott and Zawadzki, 1980), led to the discovery of NO synthetase, responsible for the generation of NO from amino acid L-arginine (Palmer et al., 1987, 1988). NO generated by the action of NO synthetase has been found to associate with many important physiological and biological functions such as signal transduction and self-defense. NO acts in a paracrine fashion and undergoes a series of reactions with several molecules present in biological fluids and is eventually metabolized to NO₂ (nitrite) and NO₃ (nitrate). Thus, the best index of total NO production is the sum of both nitrite and nitrate.

For the quantification of total NO produced, a two-step assay method is commonly used. The first step is the conversion of nitrate to nitrite by the use of NADH or NADPH-dependent nitrate reductase. Subsequently, the converted nitrite can be quantified by the addition of Griess Reagents, which converts nitrite into a purple colored azo compound. Accurate concentration of nitrite can be determined by photometric measurement of the colored azo compound. In this two-step assay method, NADPH is commonly used as an essential cofactor for nitrate reduction. However, an excess amount of NADPH interferes with the subsequent Griess Reagents reaction. In order to remove this interference activity, the two-step assay method has been improved by the addition of one more step (LDH assay method). This additional step includes a lactate dehydrogenase (LDH) reaction after the reductase reaction in order to degrade the excess NADPH. The commonly used two step assay has low sensitivity while the LDH assay method is tedious.

We provide a simple and sensitive assay kit for nitrate determination based on the commonly used two-step assay method with no need to use lactate dehydrogenase. The sensitivity of the assay is equivalent to that of LDH assay method, but more sensitive than that of a commonly used two-step assay method. This new system involves the addition of two cofactors for the nitrate reductase reaction. With the help of an additional cofactor, the reductase reaction to convert nitrate to nitrite is accelerated and simultaneously, excess NADPH is degraded to NADP. Thus, the reductase reaction can be completed within 30 minutes and colorimetric determination can directly be measured by the addition of Griess Reagents without the treatment of lactate dehydrogenase. This kit is fast and simple and can be applied to the assay of nitrite and nitrate determination in urine, plasma, serum, saliva and tissue culture medium.
2. Precautions
1. Read these instructions carefully before beginning the assay.
2. This kit is for research use only, not for human or diagnostic use.
3. We take great care to ensure the quality as well as the reliability of our products. However, it is possible that in some cases, unusual results may be obtained due to high levels of interfering factors.

3. Contents of the kit
<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Assay buffer</td>
<td>1 bottle</td>
</tr>
<tr>
<td>B. Nitrate Reductase</td>
<td>2 vials</td>
</tr>
<tr>
<td>C. Co-factors</td>
<td>2 vials</td>
</tr>
<tr>
<td>D. Nitrate Standard</td>
<td>1 vial</td>
</tr>
<tr>
<td>E. Nitrite Standard</td>
<td>1 vial</td>
</tr>
<tr>
<td>F. Griess Reagent-A</td>
<td>1 bottle</td>
</tr>
<tr>
<td>G. Griess Reagent B</td>
<td>1 bottle</td>
</tr>
<tr>
<td>H. Microtiter Plate</td>
<td>1 bottle</td>
</tr>
<tr>
<td>I. Plate cover</td>
<td>2 covers</td>
</tr>
</tbody>
</table>

In case any of the items listed above are damaged or missing, please feel free to contact our customer service department or local distributor. We cannot accept returns without prior authorization.

4. Storage and stability
Unopened kit should be stored at 0-4°C. The expiration date for unopened kits is indicated on the outside of the box. Once the kit is opened and each component reconstituted, store at -20°C except for assay buffer, Griess Reagent-A and -B, Nitrate Standard and Nitrite Standard. The storage conditions for each component after reconstitution is described below.

5. Other Supplies Required
A. Adjustable pipettor(s).
B. Plate reader with a 540nm filter for samples and 620nm filter for reference.
6. Reconstitution of Components

Reconstitution has to be carried out on ice since the components, especially nitrate reductase and co-factors are labile. Prior to reconstitution, the water bottle and the assay buffer should be pre-cooled on ice. Follow the directions carefully to ensure that the proper volumes of water or assay buffer are used to reconstitute the each component.

A. Nitrate Reductase

Two vials are provided in lyophilized form. Each vial contains enough for 50 samples. Reconstitute the contents of the vial with 0.6ml of assay buffer. Keep on ice during use. Store at -20°C when not in use. Freezing and thawing of the reconstituted enzyme solution should be limited to one time.

B. Co-factors

Two vials are provided in lyophilized form. Each vial contains enough for 50 samples. Reconstitute the contents of the vial with 1.2ml of HPLC-grade water. Keep on ice during use. Store at -20°C when not in use. Freezing and thawing of the solution should be limited to one time.

C. Nitrate standard

This vial contains a solution of 200μM NaNO₃. Do not add water or assay buffer to this vial. The nitrate standard should be stored at 4°C (DO NOT FREEZE).

D. Nitrite standard

This vial contains a solution of 200μM NaNO₂. Do not add water or assay buffer to this vial. The nitrite standard should be stored at 4°C (DO NOT FREEZE).

E. Griess Reagents

Griess reagents are composed of Griess Reagent-A and Griess Reagent-B. Do not add water or assay buffer to these bottles.

F. Buffer (Assay Buffer)

Dilute the 10x Buffer 10times with HPLC-grade water to create the assay buffer. This should be used for dilution of samples and preparation of standard curves for Nitrate and Nitrite.

7. Sample Preparation

This kit may be used for the samples including urine, plasma, saliva and culture
media.

A. Urine sample
Urine may be used directly after proper dilution with assay buffer, but in some cases, precipitation will form after the addition of Griess Reagents. In this case, the sample should be filtered through a 10,000 Dalton micropore filter prior to assay.

B. Plasma, Serum and Saliva
Citrate, EDTA or heparin may be used as an anticoagulant for the collection of plasma. All samples require at least a 2-fold dilution with the assay buffer followed by centrifugation to remove particulate matter. After above treatments, samples must be ultrafiltered through a 10,000 Dalton micropore filter prior to assay.

C. Culture Media
The use of a media containing high levels of intrinsic nitrate/nitrite must be avoided for tissue/cell culture, since the kit measures minute changes of nitrate/nitrite produced from cultured cells. Cellular nitrite/nitrate production can be quantified by subtracting the level of nitrite/nitrate present in the media from the nitrite/nitrate level present during cell growth. The effect of components in media on color development can be assessed by making nitrate or nitrite standard curves in the presence of a fixed volume of the culture media. RPMI culture medium is not recommended for this assay. Precipitation may form by addition of Griess Reagents and interfere in the measurement of color. The sample must be filtered through a 10,000 Dalton micropore filter prior to assay.
8. Determination of Nitrate
Prior to starting the nitrate and nitrite determination, make sure that all of the reagents needed are prepared. Keep the reagents cool on ice.

A. Preparation of nitrate standard curve
The standard curve for nitrate is prepared by the addition of reagents to the plate wells in the following way:

<table>
<thead>
<tr>
<th>Well</th>
<th>Nitrate Standard (µl)</th>
<th>Assay Buffer (µl)</th>
<th>Final Nitrate Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>5</td>
<td>65</td>
<td>5</td>
</tr>
<tr>
<td>C1</td>
<td>10</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>D1</td>
<td>15</td>
<td>55</td>
<td>15</td>
</tr>
<tr>
<td>E1</td>
<td>20</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>F1</td>
<td>25</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>G1</td>
<td>30</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>H1</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
</tbody>
</table>

*The Concentration is indicated in the final 200µl assay volume after addition of the Griess Reagents.

B. Nitrate assay procedure
(1) Add 70µl of Assay Buffer to the blank wells.
(2) Add up to 70µl of sample or sample dilution to the wells. The final volume must be adjusted to 70µl using the assay buffer.

Note: The pH of the sample should be approximately neutral since the enzyme would be inactivated at high and low pH values. Sample volumes for plasma, serum or tissue homogenates are recommended to be less than 40µl.

(3) Add 20µl of the reconstituted co-factors solution to each well.
(4) Add 10µl of the reconstituted nitrate reductase solution to each well.
(5) Put the plate on a plate mixer and mix well.
(6) Cover the plate with a plate cover.
(7) Incubate the plate for 30 minutes at room temperature. Avoid light exposure during incubation. It is not necessary to shake the plate during incubation.
(8) Add 50µl of the Griess Reagent-A to each well (standard and unknowns).
(9) Immediately add 50µl of Griess Reagents-B to each well (standard and unknowns).
(10) Allow the color to develop for 20 minutes at room temperature.
(11) Read the absorbance at 540nm using the plate reader.

**Note:** This assay procedure measure total nitrite by converting nitrate to nitrite, thus the results obtained indicate the sum of endogenous nitrate and nitrite concentration in the sample. To determine the nitrate concentration in the sample, the endogenous nitrite concentration obtained by the nitrite assay procedure must be subtracted from the concentration obtained by the above assay.

9. **Determination of Nitrite**

Prior to starting the nitrate and nitrite determination, make sure that all of the reagents needed are prepared. Keep the reagents cool on ice.

**A. Preparation of Nitrite Standard Curve**

Nitrite concentrations can be measured directly without treatment of nitrate reductase and co-factors. The nitrite standard curve is prepared as following:

<table>
<thead>
<tr>
<th>Well</th>
<th>Nitrite Standard(µl)</th>
<th>Assay Buffer(µl)</th>
<th>Final Nitrite Concentration(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>C1</td>
<td>10</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>D1</td>
<td>15</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>E1</td>
<td>20</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>F1</td>
<td>25</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>G1</td>
<td>30</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>H1</td>
<td>35</td>
<td>65</td>
<td>35</td>
</tr>
</tbody>
</table>

*The concentration is indicated in the final 200µl assay volume after addition of the Griess Reagents.

**B. Measurement of nitrite in samples**

Measurement of unknown nitrite concentrations in samples is done using up to 100µl sample. If the sample volume is less than 100µl for nitrite determination, the volume must be adjusted to 100µl using diluted assay buffer.

1. Add 100µl of diluted assay buffer to the blank wells.
2. Add up to 100µl of sample to the chosen wells. If the sample volume is less than 100µl, add diluted assay buffer up to 100µl.
3. Add 50µl of the Griess Reagents-A to each well (standard and unknown).
(4) Immediately add 50µl of Griess Reagents-B to each well (standard and unknown).
(5) Allow the color to develop for 10 minutes at room temperature.
(6) Read the absorbance at 540nm using the plate reader.

10. Calculations

A. Subtract the blanks
Average the absorbance values of the blank wells and the sample wells, followed by subtraction of the blank value from all the absorbance values of the samples.

B. Plotting the standard curves
Make a plot of absorbance as a function of nitrate or nitrite concentration. The nitrate standard curve is used for determination of total nitrate and nitrite concentration, whereas the nitrite standard curve is used only for the nitrite concentration in the sample. The nitrate and nitrite standard curves are supposed to be indentical, however, in practice a small discrepancy often occurs. The typical standard curves are used as in the following:

\[ \square \] Measure the endogenous nitrite concentration using the nitrite assay procedure and the standard curve of nitrite.

\[ \square \] Measure the nitrate concentration using the nitrate assay procedure and the standard curve of nitrate.

\[ \square \] Determine the nitrate concentration in the sample by subtracting the endogenous nitrite concentration obtained in (\[ \square \]) from the nitrate concentration obtained in (\[ \square \]).

Note: If the sample has been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
11. Typical Standard Curve
These nitrate and nitrite standard curves are provided for demonstration only. Standard curves should be generated for each set of samples assayed.

12. Recovery
There is the possibility that the sample contains interference(s) for nitrate reductase activity and/or Griess Reaction. If concentration of interference(s) is high in the sample, the recovery of nitrate and nitrite should be determined using the nitrite and nitrate provided with the kit. To determine the recovery, the nitrite and nitrate at concentrations similar to those used for the standard curve should be added to the sample and assay buffer, followed by nitrite and nitrate determination by the kit. Typical recovery data of nitrate in plasma and average % recovery data for various samples are shown in the following:

A. Typical recovery data
Human urine sample was collected, centrifuged, and filtered through 10,000 Dalton micropore filter. The amount of nitrate in various volumes of the filtrate (0-5µl) was measured in the presence or absence of exogenous nitrate (final concentration: 10µM) by the nitrate assay procedure.
13. Interference
Oxidative reagents as well as reducing reagents such as ascorbic acid, dithiothreitol and mercaptoethanol etc. will interfere with color development. Higher concentration of phosphate, more than 50mM, will interfere the nitrate reductase activity. There is a possibility that the sample contains an interference(s) for reductase activity or color development, therefore, it is recommended to determine the nitrate or nitrite recovery rate by addition of exogenous nitrate or nitrite in the sample.

14. Linearity and Sensitivity
Linearity of the nitrate standard curve can be obtained at the concentrations between 2.5 to 55µM. The sensitivities of the nitrate and nitrite assays were typically less than 2µM.

15. Troubleshooting
Problem: Erratic Values or dispersion of duplicates.
Cause: a) Poor pipetting technique, or careless mistake; b) Bubble in the well.

Problem: No color development in the nitrate standard curve.
Cause: Careless mistakes such that substrate or enzyme was not added.
Solution: Repeat the experiment.

Problem: The nitrate standard curve is not linear at high concentration of nitrate.
Cause: a) Concentration of nitrate is more than 55\(\mu\)M; b) Enzyme activity is lost due to repeated freeze and thawing; c) Sensitivity of plate reader is not adequate.
Solution: a) Use only the points in the linear portion at lower nitrate concentration to make a standard curve; b) Avoid freeze and thawing of the enzyme; c) Use the enzyme in the kit before expiration.

Problem: Nitrite standard curve is not linear at high absorbance.
Cause: a) Sensitivity of plate reader is not adequate; b) nitrite concentration is high.
Solution: Use the points at lower concentration in the linear portion to make a standard curve.

16 References
