Nitrite/ Nitrate Determination Kit

KB-03-010

100 tests (96 well plate)
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Introduction

Nitric oxide is an important molecular messenger in the vascular and nervous systems. It has multiple physiological roles, such as vasorelaxation or neuronal signaling, but it also has other complex pathophysiological effects\textsuperscript{1}. It is synthesized by the three isoforms of the nitric oxide synthases (eNOS, nNOS and iNOS) from L-arginine in the endothelial cells, neurons, macrophages, etc. and in biological systems it is decomposed to nitrite and nitrate.

The overproduction of nitric oxide may lead to oxidative and nitrosative stress. It has been demonstrated that they enhance the development of a variety of diseases, as well as the ageing process\textsuperscript{2}.

Regarding nitrosative stress, high levels of iNOS have been found in various inflammatory diseases such as arthritis and obesity, and increased levels of NO have been also associated to other cardiovascular diseases\textsuperscript{3}. 
**Materials**

---

Bioquochem **Nitrite/Nitrate Determination Kit** contains:

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
<tr>
<td>Reagent B</td>
<td>1 vial</td>
<td>-20°C</td>
</tr>
<tr>
<td>Reagent C</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
<tr>
<td>Reagent D</td>
<td>1 vial</td>
<td>-20°C</td>
</tr>
<tr>
<td>Reagent E</td>
<td>2 vials</td>
<td>4°C</td>
</tr>
<tr>
<td>Reagent F</td>
<td>2 vials</td>
<td>4°C</td>
</tr>
<tr>
<td>Standard</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
</tbody>
</table>

- This kit is for R&D use only

![Warning] All these chemicals should be handled with care
Bioquochem Nitrate/Nitrite Determination Kit is recommended for the
determination of nitrite and nitrate, which is a method for the detection of
nitric oxide formation.

The assay described here measures the nitrite and nitrate anions. Firstly,
the nitrate is reduced to nitrite catalyzed by the nitrate reductase with
cofactors and specific compounds to eliminate interferences* (very
important this step to eliminate interference).

\[
\text{Nitrate Reductase} \quad \text{NO}_3^- \rightarrow \text{NO}_2^- \\
\text{Figure 1. Nitrate reduction}
\]

The detection is based on the final product detection (diazonium
compound, \(\lambda_{\text{max}}\) = 540 nm) obtained after nitrite reaction in several steps
with sulfanilamide.

\[
\text{Sulfanilamide} \rightarrow \text{Diazonium product} \quad (\lambda_{\text{max}}= 543\text{nm}) \\
\text{Figure 2. Reaction obtaining diazonium product}
\]

Nitrite only determinations can then be made in a parallel assay where the
samples where not reduced before the colorimetric assay. The nitrate
levels are determined by the subtraction of nitrite levels from the total.
Pre-Assay

Reagent Preparation

- To prepare **Reagent A solution**: Add exactly 1000 µl of ultrapure water to Reagent A and mix thoroughly. This reagent must be freshly prepared.

- To prepare **Reagent C solution**: Add exactly 1000 µl of ultrapure water to Reagent C and mix thoroughly. This reagent must be freshly prepared.

Sample Preparation

- It is recommended to assay the samples in duplicate.

- Plasma samples may be deproteinized before performing the assay.
**Assay Protocol**

**Standard Preparation**

Prepare the calibrate in 1 ml tubes following the Table 1. Use ultrapure water as diluent.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Standard (µl)</th>
<th>H₂O ultrapure (µl)</th>
<th>Nitrite (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1(Blank)</td>
<td>0</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>S2</td>
<td>25</td>
<td>975</td>
<td>25</td>
</tr>
<tr>
<td>S3</td>
<td>50</td>
<td>950</td>
<td>50</td>
</tr>
<tr>
<td>S4</td>
<td>75</td>
<td>925</td>
<td>75</td>
</tr>
<tr>
<td>S5</td>
<td>100</td>
<td>900</td>
<td>100</td>
</tr>
</tbody>
</table>

**Performing the assay**

- The following procedure is for the determination of nitrite + nitrate:
  1. Add 50 µl of the sample or standard in each well (96-well plate).
  2. Add 10 µl of Reagent A and 20 µl of Reagent B. Incubate for 60 minutes.
  3. Add 10 µl of Reagent C and 10 µl of Reagent D. Incubate for 20 minutes.
  4. Add 50 µl of Reagent E in each well. Incubate for 10 minutes protected from light.
  5. Add 50 µl of Reagent F in each well. Incubate for 10 minutes protected from light.
  6. Read the absorbance at 540 nm within 30 minutes.

In order to measure only the nitrite in the sample (not both nitrite and nitrate, “total nitrite”), add ultrapure water instead of Reagents A, B, C and D and continue the assay by adding Reagents E and F as shown in the procedure.
Assay Protocol

Plate set up

Figure 3. 96-well plate filling format

S1-S5 = Standards
C1-C43 = Samples

Attention

- This scheme is just a recommendation of how to perform the assay.
- If the nitrite/nitrate concentration in the samples is not known or it is expected to be beyond the range of the standard curve, it is recommended to assay the samples at several dilutions.
- For optimal results, it is recommended to run the standards and the samples for duplicate, but it is the user’s discretion to do so.
Data analysis

1. Calculate the average absorbance of each sample and control.

2. Determine the concentration in the sample by comparison to the Nitrite Standard reference curve (Figure 4).

\[
\text{Nitrite (µM)} = (\Delta A_{540\text{nm}} - \text{intercept}) / \text{slope}
\]

Figure 4. Nitrite standard reference curve using the microplate procedure
References


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Said refund or replacement is conditioned on buyer giving written notice to Bioquochem within 30 days after arrival of the material at its destination.

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