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[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[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[3].

Materials

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

Product	Quantity	Storage
Reagent A	1 vial	4ºC
Reagent B	1 vial	-20ºC
Reagent C	1 vial	4ºC
Reagent D	1 vial	-20ºC
Reagent E	2 vials	4ºC
Reagent F	2 vials	4ºC
Standard	1 vial	4ºC

- **This kit is for R&D use only**

All these chemicals should be handled with care

Assay principle

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).

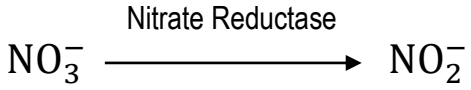

Figure 1. Nitrate reduction

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

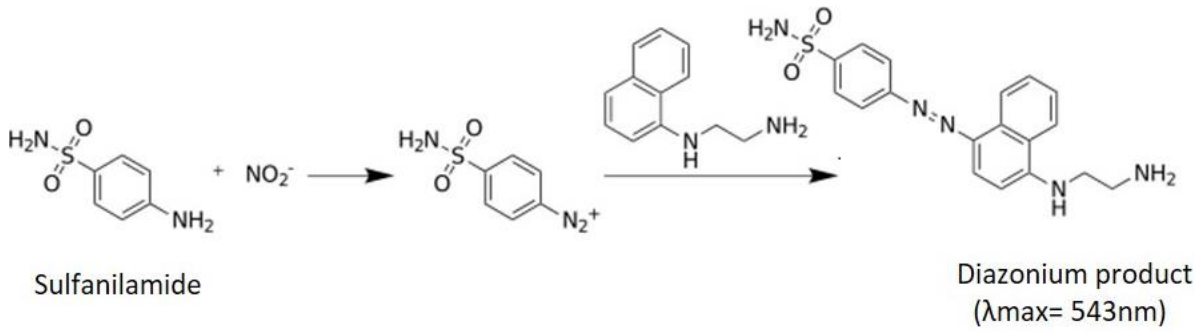

Figure 2. Reaction obtaining diazonium product

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 1. Reagent volumes needed to carry out the standard curve

Sample	Standard (µl)	H_2O ultrapure (µl)	Nitrite (µM)
S1(Blank)	0	1000	0
S2	25	975	25
S3	50	950	50
S4	75	925	75
S5	100	900	100

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.

Plate set up

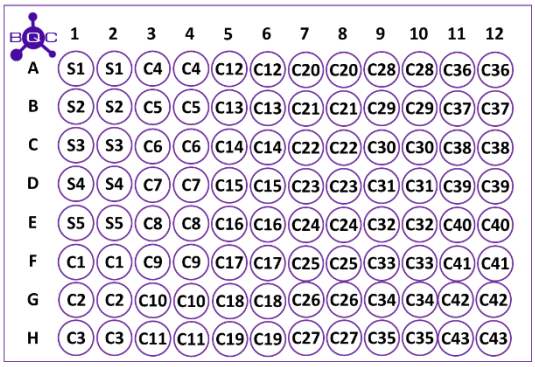

Figure 3. 96-well plate filling format

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).

Nitrite (µM) = (ΔA_{540nm}– intercept)/ slope

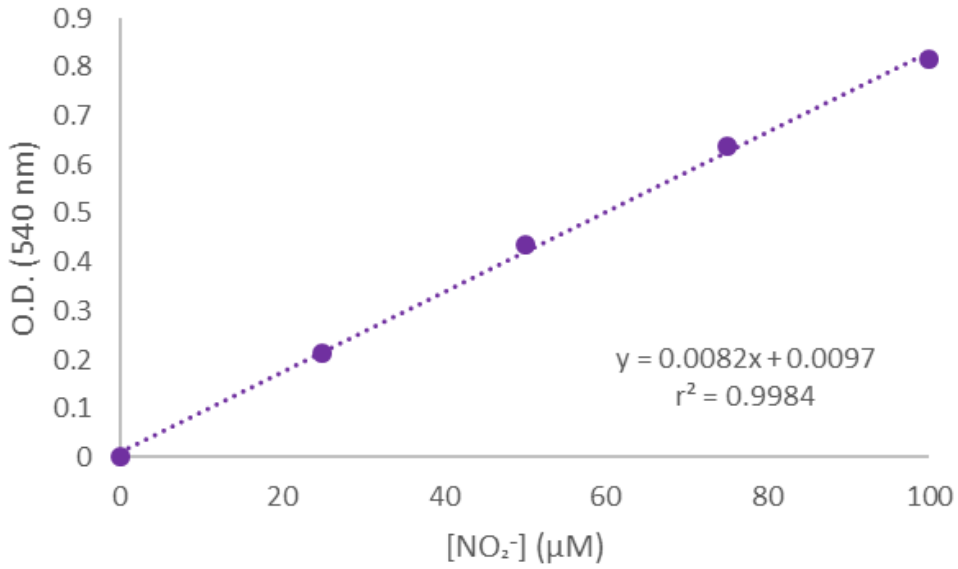

Figure 4. Nitrite standard reference curve using the microplate procedure

References

1. Virág, L., Szabó, É., Gergely, P. & Szabó, C. Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. Toxicol. Lett. 140–141, 113–124 (2003).

2. Drew, B. & Leeuwenburgh, C. Aging and the role of reactive nitrogen species. Ann. N. Y. Acad. Sci. 959, 66–81 (2002).

3. Carmeli, E., Bachar, A., Rom, O. & Aizenbud D. Oxidative stress and nitric oxide in sedentary older adults with intellectual and developmental disabilities. Advs. Exp. Medicine. 18: 21 – 27 (2016).

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