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Nitric Oxide Colorimetric Assay

Test combination for determination of nitrogen monoxide (NO) via nitrate on microtiter plates.

Cat. No. 11 756 281 001

Test combination for 96 wells

[30 determination in triplicates plus standard curve]

Version 5.0

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Store at +2 to +8°C

1. Introduction

In recent years it has become apparent that nitrogen monoxide has a number of biologically important functions. The molecule acts as an inter- and intracellular messenger substance with a broad physiological action spectrum, *e.g.*, from the regulation of blood pressure and neurotransmitter functions to involvement in the immune system.

In mammalian cells, NO is synthesized via the enzyme NO synthase with the basic amino acid L-arginine acting as substrate and molecular oxygen (O_2) as cosubstrate. In biological fluids NO is very rapidly deactivated by oxidation to nitrite (NO_2^-) and nitrate (NO_3^-) by physically dissolved oxygen and water. In this test combination, nitrogen monoxide is determined photometrically via its oxidation products nitrite and nitrate.

2. Principle

Nitrogen monoxide is detected in biological fluids via nitrite. The nitrate present in the sample is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase (NR) (a).

(a) $\text{Nitrate} + \text{NADPH} + H^+ \xrightarrow{NR} \text{nitrite} + \text{NADP}^+ + H_2O$

The nitrite formed reacts with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye (b).

(b) Nitrite + sulfanilamide + N-(1-naphthyl)-ethylenediamine → diazo dye

The diazo dye is measured on the basis of its absorbance in the visible range at 550 nm.

3. Constituents of the test combination

Bottle	Contents	Amount
1	Potassium phosphate buffer, pH 7.5, stabilized	22 ml
2	Coenzyme tablets: each tablet contains 0.5 mg NADPH and 0.01 mg FAD, stabilized	10 tablets
3	2 bottles nitrate reductase, lyophilized	4 U each
4	Color reagent I: sulfanilamide, stabilized	8 ml
5	Color reagent II: N-(1-naphtyl)-ethylene diamine dihydrochloride	8 ml
6	Potassium nitrate standard	80.0 mM
7	Microtiter plate, a frame with 12 modules 8 wells each,	1

4. Preparation of the solutions

1) Use the contents of bottle 1 undiluted.
2) Place three tablets from bottle 2 in a beaker and dissolve in 1 ml of buffer from bottle 1 (use the tweezers provided to remove the tablets from the bottle). The resulting solution is reaction mixture 2 and is sufficient for about 20 incubation assays.
3) Dissolve the contents of one bottle 3 in 0.6 ml of double dist. water to give solution 3.
4) Use the contents of bottle 4 undiluted.
5) Use the contents of bottle 5 undiluted.
6) Dilute the contents of bottle 6 according to 6. “calibration curve“.

5. Stability of solutions

Solution 1 is stable for 1 year at +2 to +8°C, bring to +15 to +25°C before use.
The contents of the bottles 2 and 3 are stable at +2 to +8°C (s. pack label).
Prepare reaction mixture 2 immediately before use and bring to +15 to +25°C before use.
Solution 3 is stable at +2 to +8°C for 2 weeks.
Solution 4 is stable at +2 to +8°C (s. pack label).
Solution 5 is stable at +2 to +8°C (s. pack label).
Solution 6 is stable at +2 to +8°C (s. pack label).
Aliquots of the solution 6 are stable for 3 months at −15 to −25°C

6. Calibration curve

Dilute the potassium nitrate standard from its initial concentration of 80.0 mM nitrate to 80.0 µM nitrate (F = 1 000). Dilute the stock solution thus obtained as follows in order to construct a calibration curve.

Dilution with water	Dilution factor	µM Nitrate
-	F = 1	80.0
1 + 4	F = 5	16.0
1 + 9	F = 10	8.00
1 + 19	F = 20	4.00
1 + 49	F = 50	1.60
1 + 99	F = 100	0.80
1 + 199	F = 200	0.40
1 + 299	F = 300	0.25

7. Sample preparation

7.1 for serum

Place 300 µl serum and 300 µl of potassium phosphate buffer from bottle 1 in an ultrafilter (*e.g.*, 10 000 MWCO from SARTORIUS, *VIVASCIENCE* Cat. No. 13 239-E) and centrifuge at 20°C for 45 min (1250 × g resp. 4,000 rpm, r=7 cm) Collect the ultrafiltrate and use in the test.

Note: The membrane filters used must be free from nitrate. In case of doubt it is recommended to test the filter material by washing with double dist. water or isotonic saline solution and checking the washings with the test.

Furthermore note the technical advices in the pack insert of membrane filters, *e.g.,* how to win serum from full blood.

7.2 for urine

Place 500 µl of the urine specimen and approx. 2 ml double dist. water into a tube and incubate for 15 min at 60°C. After cooling add successively 150 µl each of diluted Carrez-I-solution (3,6 g potassium hexacyanoferrate (II), $K_4[Fe(CN)_6]$ x 3 H_2O/100 ml) and diluted Carrez-II-solution (7,2 g zinc sulfate, $ZnSO_4$ × 7 H_2O/100 ml), mixing well after each addition. Adjust to pH 8.0 with sodium hydroxide solution (1 M) and mix again. Transfer the content of the tube into a 5-ml-measuring-cylinder and fill up to 5,0 ml with double dist. water. Transfer the solution into a centrifuge tube and centrifuge at +15 to +25°C for 5 minutes (5000 × g resp. 8,000 rpm; r = 7 cm). Fill the supernatant into another tube and recentrifuge at +15 to +25°C for 3 minutes at maximum speed (9000 × g resp. 11,000 rpm, r = 7 cm). Use 500 µl of the supernatant for testing.

8. Determination

Wavelengt:	550 nm
Incubation temperature:	+15 to +25°C
Incubation period:	30 min
Incubation volume:	570 µl
Determination volume:	300 µl
Sample solution:	0.30 µM – 80.0 µM nitrate

9. Incubation assay

Pipette into the incubation vessel			
	Blank	Sample	Standard
Sample solution	-	500 µl	-
Standard solution	-	-	500 µl
Redist. water	500 µl	-	-
Reaction mixture 2	50 µl	50 µl	50 µl
mix. After 1 min add			
Solution 3	20 µl	20 µl	20 µl
mix and then incubate for 30 min at +15 to +25°C.			

10. Measurement

The measurements should be carried out in triplicate. For this purpose 3 aliquots of 150 µl each of the incubation assay (pt.9) are applied.

After incubation pipette into the microtiter plate wells			
	Blank	Sample	Standard
Incubation solution	150 µl	150 µl	150 µl
Color reagent I	75 µl	75 µl	75 µl
Color reagent II	75 µl	75 µl	75 µl
mix, incubate for 5 min at +15 to +25°C and read the absorbance.			

11. Evaluation

The result is calculated from the calibration curves using the standard solutions. Plot the change in absorbance obtained for the potassium nitrate standard solutions on the y-axis against the corresponding nitrate concentrations in µM on the x-axis.

$\Delta A_{Sample} = A_{Sample} - A_{Blank}$

$\Delta A_{Standard} = A_{Standard} - A_{Blank}$

Determine the concentration of nitrate in the sample from the calibration curves using the change in absorbance measured for the sample and standard, taking dilution factors into account if necessary.

12. Notes on performing the test

12.1 Checking of reagents and equipment

The following points are important because of the high sensitivity of the nitrite/nitrate colorimetric test.

a.) The membrane filter must be free from nitrite and nitrate.

In case of doubts it is recommended to test the filter material (s. point 7.1).

b.) The solution used to prepare the samples must also be free from nitrite and nitrate.

12.2 Clarification with Carrez solutions

After adding Carrez solutions I and II it is important that the pH is adjusted to 8.0 ± 0,2.

12.3 Calibration curves

The calibration curves do not have to be plotted every time a determination is made. It is sufficient to check the calibration from time to time and to include a potassium nitrate standard solution.

12.4 Determination of nitrite

If nitrite is to be determined in the sample, the incubation need not be performed. The sample can then be measured directly after centrifugation (see section 9, measurement). Prepare a standard solution by weighing 6.95 mg sodium nitrite (*e.g.*, Merck, cat. no. 6549) into a 100 ml volumetric flask, dissolving in double dist. water and making up to the mark with the same solvent (stock solution = 1.00 mM nitrite). Construct a calibration curve for nitrite by diluting the stock solution with double dist. water to give concentrations in the range from 0.40 µM to 100.0 µM nitrite. The conversion factor from $NaNO_2$ to nitrite (NO_2) is 0.667.

12.5 Determination of nitrate and nitrite

If the sample solution contains both nitrite and nitrate, the nitrate content is determined from the difference between measurements with and without incubation.

$\Delta A_{Nitrate} = \Delta A_{with\ incubation} - \Delta A_{without\ incubation}$

12.6 Concentration of the sample solution

The nitrate concentration in the sample solution should be between 0.30 µM and 80.0 µM; the nitrite concentration should be between 0.40 µM and 100.0 µM.

Specificity	Under the given conditions, nitrate reductase reacts specifically with nitrate ions.
Limit of detection	The limit of detection of the method is 0.28 µM for nitrate and 0.32 µM for nitrite
Linearity	The method is linear in the range from 0.28 µM to 80.0 µM nitrate and from 0.32 µM to 108.0 µM nitrite
Accuracy	The intra-test variance is ≤10% and the inter-test variance is up to ≤ 20%.
Duration of the test	About 1.5 h

Changes to Previous Version

- Editorial changes
- Changes in Ordering information

References

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Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page www.roche-applied-science.com.

Product	Pack size	Cat. No.
NADPH	100 mg 500 mg 1 g	10 107 824 001 10 621 692 001 10 621 706 001
Nitrate reductase	20 U	10 981 249 001
Nitrite/Nitrate Colorimetric Test	1 kit	11 746 081 001

* available from Roche Applied Science

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