06239 Nitrite/Nitrate Assay Kit

Principle

Nitrite (NO_2^-) and Nitrate (NO_3^-) can be detected with this kit:

- NO_2^- is determined by direct reaction with DAN and fluorescence measurement
- NO_3^- can only be detected after enzymatic conversion to NO_2^- and measurement of total [NO_2^-+ NO_3^-].

Subsequent subtraction of NO_2^- yields the NO_3^- concentration:

$$[NO_3^-] = [NO_2^- + NO_3^-] - [NO_2^-]$$

$$NO_3^- + NADPH + H^+ \xrightarrow{\text{enzyme}} NO_2^- + NADP^+ + H_2O$$

$$NO_2^- + DAN + H^+ \longrightarrow \text{Naphthotriazole (fluorescent)} + 2H_2O$$

One of the enzyme-cofactors (NADPH) quenches the fluorescence, so we recommend to add the cofactors also to the NO_2^- test for better comparison. (If only NO_2^- has to be determined, this is not necessary)

The optimal detection range is 1-10 µM for [NO_2^- + NO_3^-]. Dilute samples with higher concentrations with buffer until the specified concentration-range is reached.

Excitation maximum of naphthotriazole is 360-365 nm, emission maximum 410-425 nm. However, in order to reduce the fluorescence blank of 2,3-Diaminonaphthalene and increase the sensitivity, the use of a 450 nm emission-filter is recommended.

Fluorescence-based enzymatic assays are generally more susceptible to interfering substances than colorimetric tests (see list of interfering substances).

Content

- $NaNO_2$ stock solution ([NO_2^-]=10mM): dilute 1:1000 with buffer solution to create a 10 µM working standard (e.g. 10µl ad 10ml)
- KNO_3 stock solution ([NO_3^-]=10mM): dilute 1:1000 with buffer solution to create a 10 µM working standard (e.g. 10µl ad 10ml)
- Phosphate Buffer Solution (50 mM, pH 7.5)
- Nitrate Reductase (lyophilized): Dissolve in 0,5 ml of water prior to use ⇒ aliquot and store at –20°C
- FAD, solid: Dissolve in 10 ml of Phosphate Buffer Solution prior to use ⇒ aliquot and store in the dark at –20°C
- NADPH, solid: Dissolve in 5 ml of Phosphate Buffer Solution prior to use ⇒ aliquot and store in the dark at –20°C
- Fluorescence Reagent Solution (Diaminonaphthalin, DAN): store in the dark
- NaOH Solution

Storage

Please store kit at 4°C. Enzyme and cofactors should be stored at –20°C after dissolution and aliquotation. After that, they should be used up within 1 month.

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Kit format

According to the lab equipment, the test can be performed in different formats:

		equipment :			
		Thermomixer for microplates	Thermomixer for vials	Fluorescence microplate reader	Fluorescence spectrometer
formats :	directly in microplates (black plate or white plate)	x		x	
	in vials (1.5 ml tubes) + transfer to microplate		x	x	
	in vials + transfer to cuvettes		x		x

Preparation of Nitrite Calibration Curve

1. Add $NaNO_2$ Standard Solution and Buffer Solution to each well as follows

Well	$NaNO_2$ Standard Solution (µl)	Buffer Solution (µl)	Final concentration of NO_2^- (µM)
A	0	80	0
B	20	60	2.5
C	40	40	5.0
D	80	0	10.0

2. Add 7 µl Buffer Solution to each well
3. Add 5 µl FAD
4. Add 8 µl NADPH, but no enzyme
5. Incubate at 25°C for 1 hour (only in order to do it in parallel with the NO_3^- test, otherwise not necessary)
6. Add 10 µl Fluorescence Reagent Solution (DAN)
7. Leave the plate for 15 min at room temperature for complete reaction
8. Add 10 µl NaOH Solution
9. Fill up with water to the desired end-volume for measurement (e.g. up to 200 µl for 96-well plate or up to 1ml for cuvette)
10. Measure the fluorescence intensity with a fluorescence microplate reader or fluorophotometer. (excitation 360 nm, emission 425 or 450 nm)
11. Subtract the blank value of well A from the fluorescence intensity of each other well. Plot the concentration of the $NaNO_2$ solution on the X-axis and the fluorescence intensity on the Y-axis to prepare the NO_2^- calibration curve.

Determination of Nitrite Concentration in Sample Solution

1. Add 80 µl of a sample solution to a well (dilute with buffer if necessary)

2.-10. proceed with steps 2-10 as described in the NO_2^- calibration method above

11. Subtract the blank value of well A from the fluorescence intensity of the sample. Use the NO_2^- calibration curve for the determination of the NO_2^- sample-concentration. Consider the dilution factor, if the sample volume was less than 80ul.

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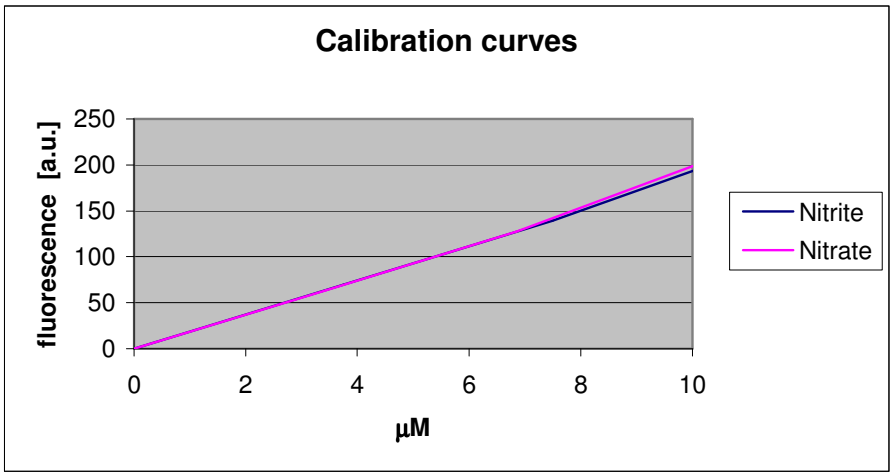

Preparation of Nitrate Calibration Curve

1. Add KNO_3 Standard Solution and Buffer Solution to each well as follows

Well	NaNO3 Standard Solution (µl)	Buffer Solution (µl)	Final concentration of NO_3^- (µM)
E	0	80	0
F	20	60	2.5
G	40	40	5.0
H	80	0	10.0

2. Add 5 µl Buffer Solution to each well
3. Add 5 µl FAD
4. Add 8 µl NADPH
5. Add 2 µl enzyme
6. Incubate at 25°C for 1 hour
7. Add 10 µl Fluorescence Reagent Solution (DAN)
8. Leave the plate for 15 min at room temperature for complete reaction
9. Add 10 µl NaOH Solution
10. Fill up with water to the desired end-volume for measurement (e.g. up to 200 µl for 96-well plate or up to 1ml for cuvette)
11. Measure the fluorescence intensity with a fluorescence microplate reader or fluorophotometer. (excitation 360 nm, emission 425 or 450 nm)
12. Subtract the blank value of well E from the fluorescence intensity of each other well. Plot the concentration of the $NaNO_3$ solution on the X-axis and the fluorescence intensity on the Y-axis to prepare the NO_3^- calibration curve.

Determination of Nitrate Concentration in Sample Solution

Whether or not a sample contains NO_3^-, can only be determined by a 2-step procedure:

First, NO_3^- has to be converted to NO_2^- by the enzyme, and the fluorescence-measurement will yield total $[NO_2^- + NO_3^-]$. Second, subtract the previously measured NO_2^- concentration from the $[NO_2^- + NO_3^-]$ value.

1. Add 80 µl of a sample solution to a well (dilute with buffer, if necessary)

2.-11. proceed with steps 2-11 as described in the NO_3^- calibration method above

12. Subtract the blank value of well E from the fluorescence intensity of the sample. Use the NO_3^- calibration curve for the evaluation, which will give the total $[NO_2^- + NO_3^-]$ concentration in the sample solution. Consider the dilution factor, if the sample volume was less than 80ul.
13. Subtract the previously measured NO_2^- concentration from the $[NO_2^- + NO_3^-]$ concentration

Schematic Overview

80ul sample	Buffer [ul]	FAD [ul]	NADPH [ul]	Enzyme [ul]		DAN [ul]		NaOH [ul]
NO_2^- standards	7	5	8	-		10		10
NO_2^- sample	7	5	8	-	⇒ 1h incubation at 25°C	10	⇒ 15min incubation at RT	10
NO_3^- standards	5	5	8	2		10		10
NO_3^- sample (=NO_2^- + NO_3^-)	5	5	8	2		10		10

$[NO_3^-] = [NO_2^- + NO_3^-] - [NO_2^-]$

Notes

- Keep the enzyme and the cofactors on ice during use
- Do not mix enzyme and cofactors prior to use
- Do not mix the 2 cofactors before use
- Store FAD and NADPH in the dark
- DAN is light sensitive. Keep it in the dark
- Dilute too concentrated samples with buffer
- Samples should be clear
- Samples must not contain NADPH-consuming enzymes
- If the sample volume is less than 80 ul, add buffer to be 80 ul
- The sample solution should not contain fluorescent materials
- Results may be influenced by quenching substances in the sample
- Cell culture media must be diluted and clear
- Use Phenol red free medium
- A cell culture medium, which contains NO_3^- as a component (such as RPMI1640), is not a suitable medium to detect NO_3^-
- When assaying a cell culture medium, use a supernatant (centrifuge the medium at 1000xg for 15 min)
- Not tested for Plasma or serum samples
- If only NO_2^- has to be determined (not NO_3^-), then the cofactors need not to be added
- For enhanced accuracy, we strongly recommend double or triple measurement of the same sample and subsequent average value calculation
- Do not mix components from different kits

Interfering substances

Phenol red, Tissue culture media containing high amounts of nitrate, Fetal bovine serum, BSA, Hemoglobin, DTT, SH-group reagents (e.g. Iodoacetamide), NADPH, Selenum, Azide, Cyanide, Sulfide

References

1. Gilliam MB et al.; Anal.Biochem. 212, 359-365 (1993)
2. Misko TP et al.; Anal. Biochem. 214, 11-16 (1993)