For life science research only.
Not for use in diagnostic procedures.

Nitrite/Nitrate, Colorimetric Test

Version: 07
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Photometric endpoint determination.

Cat. No. 11 746 081 001 1 test combination
approximately 64 assays

Store the product at +2 to +8°C.

1. General Information

1.1. Contents

Vial / bottle	Label	Function / description	Content
1	Nitrite/Nitrate, Colorimetric Test, Potassium phosphate buffer	Contains potassium phosphate buffer, pH 7.5 and stabilizers.	1 bottle, 22 ml
2	Nitrite/Nitrate, Colorimetric Test, NADPH/FAD tablets	Each tablet contains 0.5 mg NADPH, 0.01 mg FAD, and stabilizers.	1 bottle, 7 tablets
3	Nitrite/Nitrate, Colorimetric Test, Nitrate reductase	Lyophilized	2 bottles, 4 U each
4	Nitrite/Nitrate, Colorimetric Test, Color reagent I: Sulfanilamide	Contains stabilizers.	2 bottles, 8 ml each
5	Nitrite/Nitrate, Colorimetric Test, Color reagent II: N-(-1-naphthyl)-ethylenediamine × 2 HCl	Contains stabilizers.	2 bottles, 8 ml each

1.2. Storage and Stability

Storage Conditions (Product)

When stored at +2 to +8°C, the product is stable through the expiry date printed on the label.

Vial / bottle	Label	Storage
1	Potassium phosphate buffer	Store at +2 to +8°C.
2	NADPH/FAD tablets	
3	Nitrate reductase	
4	Color reagent I: Sulfanilamide	
5	Color reagent II: N-(-1-naphthyl)-ethylenediamine × 2 HCl	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Spectrophotometer or spectral line photometer with mercury vapor lamp
- Volumetric or conical flasks, beakers
- Glass or disposable cuvettes
- Centrifuge
- Water bath
- Microwave oven
- Membrane filters
- Spatula
- Parafilm

For preparation of solutions

See section, **Working Solution** *for information on preparing solutions.*

- Double-distilled water
- Sodium nitrite
- Potassium nitrate
- Carrez solutions (standard and concentrated)
- NaOH

1.4. Application

The Nitrite/Nitrate, Colorimetric Test is a semimicro method for the determination of nitrite and/or nitrate in various samples:

- Water and waste water
- Environmental
- Plant material
- Foodstuffs
- Drugs
- Cosmetics
- Biological

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Sample preparation

1. Use clear, colorless, or faintly colored solutions directly or dilute to a concentration of <5.00 mg nitrite or nitrate/l before using in the test.

2. Filter turbid solutions or clarify using Carrez solutions and dilute such that the nitrite or nitrate concentration is <5.00 mg/l.

3. The diluted solution can be used in the test without further treatment.

The concentration of the sample solution should be diluted for a nitrite or nitrate concentration between 0.05 mg/l and 5.00 mg/l.

General Considerations

Guidelines for performing the test

Calibration curves

Calibration curves do not have to be plotted every time a determination is made. It is sufficient to occasionally check the calibration curves and to include a sodium nitrite or potassium nitrate standard solution as a control.

Interference

- Manganese ions in the sample (>5 µg/cuvette) considerably delay the reaction of nitrate.
- Chloride ions only have a distinct effect at concentrations >4.3 mg/cuvette.
- Cyanide ions (>1.6 µg/cuvette) and sulfide ions (>100 µg/cuvette) completely deactivate nitrate reductase.

Checking of reagents and equipment

The following points are important because of the high sensitivity of the nitrite/nitrate colorimetric test.
- The filter papers or membrane filters must be free from nitrite and nitrate. If in question, test the filter material by washing with double-distilled water and using the washings in the test.
- The solutions used to prepare the samples must also be free from nitrite and nitrate.

Clarification with Carrez solutions

After adding Carrez solutions I and II, it is important that the pH is adjusted to 8.0 ± 0.2.

Safety Information

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Working Solution

Preparation of kit working solutions		
Solution	**Preparation/Composition**	**Storage and Stability**
Solution 1	Use the contents of Bottle 1 (Potassium phosphate buffer) undiluted.	Store 1 year at +2 to +8°C. ⚠ ***Bring to +15 to +25°C before use.***
Solution 2 (Reaction mixture)	Using the supplied tweezers, place one Coenzyme-tablet (Bottle 2) in a beaker and dissolve in 3 ml of the solution from Bottle 1. ⓘ *The resulting Solution 2 is sufficient for 12 nitrate determinations.*	Prepare solution immediately before use. ⚠ ***Bring to +15 to +25°C before use.***
Solution 3	Dissolve the contents of one Bottle 3 (Nitrate reductase) in 0.7 ml of double-distilled water.	Store 2 weeks at +2 to +8°C.
Solution 4	Use the contents of one Bottle 4 (Color reagent I) undiluted.	Store 3 months at +2 to +8°C.
Solution 5	Use the contents of one Bottle 5 (Color reagent II) undiluted.	
Preparation of standard solutions		
Solution	**Preparation/Composition**	**Storage and Stability**
Sodium nitrite standard solution	▪ Accurately weigh 75.0 mg (± 0.1 mg) sodium nitrite into a 100 ml volumetric flask. ▪ Dissolve in double-distilled water and dilute up to the mark with the same solvent (stock solution = 500 mg nitrite/l). ▪ Prepare a nitrite calibration curve by diluting the stock solution with double-distilled water to give concentrations in the range of 5.00 to 0.05 mg nitrite/l.	–
Potassium nitrate standard solution	▪ Accurately weigh 81.5 mg (± 0.1 mg) potassium nitrate into a 100 ml volumetric flask. ▪ Dissolve in double-distilled water and dilute up to the mark with the same solvent (stock solution = 500 mg nitrate/l). ▪ Prepare a nitrate calibration curve by diluting the stock solution with double-distilled water to give concentrations in the range of 5.00 to 0.05 mg nitrate/l.	–
Preparation of additional solutions		
Solution	**Preparation/Composition**	**Storage and Stability**
Carrez I	3.60 grams potassium hexacyanoferrate(II) $K_4(Fe[CN]_6)$ × 3 H_2O/100 ml	–
Carrez II	7.20 grams zinc sulfate, $ZnSO_4$ × 7 H_2O/100 ml	–
NaOH solution	1 M sodium hydroxide solution	–
Concentrated Carrez I	15 grams potassium hexacyanoferrate(II) $K_4(Fe[CN]_6)$ × 3 H_2O/100 ml	–
Concentrated Carrez II	30 grams zinc sulfate, $ZnSO_4$ × 7 H_2O/100 ml	–

2.2. Protocols

Pipetting scheme

Prepare the measurement as shown in the table.

If only nitrite is to be determined, follow the pipetting scheme shown only in Columns 1 and 2 (Blank nitrite and Sample nitrite).

Pipette into cuvettes	Blank nitrite [ml]	Sample nitrite [ml]	Blank nitrite + nitrate [ml]	Sample nitrite + nitrate [ml]
Sample	–	0.500	–	0.500
Double-distilled water	0.770	0.270	0.500	–
Reaction mixture 2	–	–	0.250	0.250
Solution 3[1]	–	–	0.020	0.020
Mix[2] and incubate for 30 minutes at +15 to +25°C; read off A_1 and add:				
Color reagent I	0.250	0.250	0.250	0.250
Color reagent II	0.250	0.250	0.250	0.250
Mix and allow to stand in the dark at +15 to +25°C for 10 to 15 minutes; read off A_2				

[1] Before adding the sample solution, rinse the pipette supplied with the test or the tip of the piston pipette with the sample.
[2] For example, using a spatula or by swirling after sealing, for example, with Parafilm.

Determination of the absorbance

Parameter	Value
Wavelength[1]	540 nm (Hg 546 nm)
Glass cuvette[2]	1.00 cm, semimicro
Temperature	+20 – +25°C
Volume	1.27 ml
Measurement	against blank
Sample solution	0.05 – 5.00 mg nitrite or nitrate/l

[1] Carryout the measurement at 540 nm if using a spectrophotometer, or at 546 nm if using a spectral line photometer with a mercury vapor lamp.
[2] Disposable cuvettes may be used instead of glass cuvettes.

Sample protocols

The following protocols are examples of nitrite and nitrate determinations in various samples.

See section, **Working Solution** *for information on preparing solutions.*

Determination in water, waste water, drinking, and mineral water

1. If necessary, dilute clear, colorless water samples to a nitrite or nitrate concentration of <5.00 mg/l and use in the test.

2. Filter turbid water samples before use.

3. Filter samples containing carbon dioxide or swirl for one minute to remove the gas; neutralize if necessary and use in the test.

Determination in fermentation samples and cell culture media

1. Centrifuge the samples if necessary and stop enzymatic processes.
2. Place in a water bath at +80°C for approximately 15 minutes.
3. Centrifuge, and if necessary, dilute the supernatant such that the nitrite or nitrate concentration is <5.00 mg/l, and use in the test.
   – Alternatively, the samples may be deproteinated with concentrated Carrez I and Carrez II solutions.

Determination in juice

1. Accurately weigh approximately 5 grams of well homogenized sample into a 100 ml volumetric flask.
2. Add approximately 20 ml double-distilled water and mix.
3. Add 5 ml of diluted Carrez I solution, mix well, and add 5 ml of Carrez II solution; mix well.
4. Adjust to pH 8.0 with Sodium hydroxide solution.
5. Dilute up to the mark with double-distilled water and mix.
6. Filter and if necessary, centrifuge; use the clear filtrate for the test.

Determination in fruit and vegetables

1. Accurately weigh approximately 3 grams of well-homogenized sample into a 100 ml beaker.
2. Add 60 ml of hot (approximately +60 to +70°C) double-distilled water.
3. Shake and allow to stand for approximately 15 minutes on a water bath at +60 to +70°C.
4. Treat samples that are intensely colored or that have a high starch content with Carrez solutions.
5. Allow the contents of the beaker to cool to +15 to +25°C and transfer quantitatively to a 100 ml volumetric flask.
6. Dilute up to the mark with double-distilled water and mix.
7. Filter and if necessary, centrifuge; discard the first few ml and use the clear filtrate for the test.

Determination in meat and meat products

1. Accurately weigh approximately 5 grams of well-homogenized sample into a 100 ml beaker.
2. Add approximately 50 ml of boiling double-distilled water and mix and boil on a water bath for approximately 15 minutes.
3. Allow to cool to +15 to +25°C and successively add 3 ml each of concentrated Carrez I and Carrez II solutions, mixing well after each addition.
4. Adjust to pH 8 with Sodium hydroxide solution and mix.
5. Transfer the contents of the beaker quantitatively to a 100 ml volumetric flask.
6. Dilute up to the mark with double-distilled water and mix.
7. Filter and if necessary, centrifuge; discard the first few ml and use the clear filtrate for the test.

Determination in dairy products

1. Accurately weigh approximately 2 grams of well-homogenized sample into a 100 ml conical flask.
2. Add approximately 50 ml of boiling double-distilled water and mix and boil on a water bath for approximately 15 minutes.
3. Allow to cool to +15 to +25°C and successively add 3 ml each of concentrated Carrez I and Carrez II solutions, mixing well after each addition.
4. Adjust to pH 8 with Sodium hydroxide solution and mix.
5. Transfer the contents of the conical flask quantitatively to a 100 ml volumetric flask.
6. Dilute up to the mark with double-distilled water and mix.
7. Transfer an aliquot to a centrifuge tube, centrifuge at 3,000 × *g* (6,000 rpm, r = 7 cm) for 15 minutes.
8. Filter the supernatant (folded filter paper or membrane filter); discard the first few ml and use the clear filtrate for the test.

Determination in cheese and processed cheese

1. Accurately weigh approximately 3 grams of well-homogenized sample into a 100 ml volumetric flask.
2. Add approximately 20 ml of double-distilled water and heat in a microwave oven until the cheese is melted, for example, at 1000 W for approximately 10 seconds.
3. Stir with a spatula and microwave again until the mixture boils gently.
4. Add approximately 50 ml of boiling double-distilled water and boil on a water bath for approximately 15 minutes.
5. Allow to cool to +15 to +25°C and successively add 5 ml each of concentrated Carrez I and Carrez II solutions, mixing well after each addition.
6. Adjust to pH 8 with Sodium hydroxide solution and mix.
7. Transfer the contents of the conical flask quantitatively to a 100 ml volumetric flask.
8. Dilute up to the mark with double-distilled water.
9. Transfer an aliquot to a centrifuge tube, centrifuge at 3,000 × *g* (6,000 rpm, r = 7 cm) for 20 minutes.
10. Filter the supernatant through a membrane filter; discard the first few ml and use the clear filtrate for the test.

Determination in baby food

1. Accurately weigh approximately 1.5 grams of well-homogenized sample into a 100 ml conical flask.
2. Add approximately 50 ml of boiling double-distilled water and mix and boil on a water bath for approximately 15 minutes.
3. Allow to cool to +15 to +25°C and successively add 3 ml each of concentrated Carrez I and Carrez II solutions, mixing well after each addition.
4. Adjust to pH 8 with Sodium hydroxide solution and mix.
5. Transfer the contents of the conical flask quantitatively to a 100 ml volumetric flask.
6. Dilute up to the mark with double-distilled water.
7. Transfer an aliquot to a centrifuge tube, centrifuge at 3,000 × *g* (6,000 rpm, r = 7 cm) for 15 minutes.
8. Filter the supernatant through a membrane filter; discard the first few ml and use the clear filtrate for the test.

2.3. Parameters

Detection range

Detection limit

0.02 mg/l for nitrite and nitrate.
A high degree of scatter should not be ignored in this trace range because of the small measurement signal.

Linearity

The method is linear in the range from 0.02 to 5.00 mg nitrite or nitrate/l sample solution.

Specificity

Under the provided conditions, nitrate reductase reacts specifically with nitrate ions.

3. Results

Calculations

Graphical evaluation

The result is calculated from the calibration curves constructed using the standard solutions.

1. Plot the change in absorbance obtained for the sodium nitrite and potassium nitrate standard solutions on the Y-axis against the corresponding nitrite or nitrate concentrations in mg/l on the X-axis.

$$\Delta A_{nitrite} = (A_2\text{-}A_1)_{nitrite} - (A_2\text{-}A_1)_{Blank\ nitrite}$$
$$\Delta A_{nitrite\ +\ nitrate} = (A_2\text{-}A_1)_{nitrite\ +\ nitrate} - (A_2\text{-}A_1)_{Blank\ nitrite\ +\ nitrate}$$
$$\Delta A_{nitrate} = \Delta A_{nitrite\ +\ nitrate} - \Delta A_{nitrite}$$

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2. Determine the concentrations of nitrite and nitrate in the sample from the calibration curves using the change in absorbance measured.
   – If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

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3. When analyzing solid or semisolid samples that must be weighed out, calculate the result with respect to the mass of sample.

$$Content_{nitrite} = \frac{C_{nitrite} \times 1000}{Mass_{Sample}\ in\ g/l\ sample\ solution}\ (mg\ nitrite/kg\ sample)$$

$$Content_{nitrate} = \frac{C_{nitrate} \times 1000}{Mass_{Sample}\ in\ g/l\ sample\ solution}\ (mg\ nitrate/kg\ sample)$$

4. The results are determined as sodium nitrite and potassium nitrate.

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5. The conversion factor from $NaNO_2$ to nitrite (NO_2^-) is 46.006:68.995 = 0.667 and from KNO_3 to nitrate (NO_3^-) 62.005:101.11 = 0.613.

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4. Additional Information on this Product

4.1. Test Principle

Assays of nitrite and nitrate have become increasingly important in recent years for health care and economic reasons. While nitrate is an essential plant nutrient, it constitutes a risk to human health because it plays a part in the formation of methemoglobin and nitrosamines. Negligible amounts of nitrite occur in plants and drinking water. However, under unfavorable conditions, nitrite may enter the food chain via microbial reduction of nitrate thus endangering human health.
The test principle is shown in the following steps:

1. Nitrate is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase:
   Nitrate + NADPH + H^+ → Nitrite + $NADP^+$ + H_2O

2. The nitrite formed reacts with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye:
   Nitrite + sulfanilamide + N-(1-naphthyl)-ethylenediamine → diazo dye

3. The diazo dye is measured on the basis of its absorbance in the visible range at 540 nm (Hg 546 nm).

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

Layout changes.
Editorial changes.
Update to include new safety Information to ensure handling according controlled conditions.

5.3. Trademarks

All product names and trademarks are the property of their respective owners.

5.4. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

5.5. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.6. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.7. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany