Dynamic Test Kits for R&D and Quality Control

NITRATES / NITRITES ASSAY KIT (enzymatic / colorimetric method)

Product code : NITRATES-100C
(version : 022023-8)

Assay kit for the detection of nitrates, nitrites – Enzymatic / Colorimetric Method (540nm)

- Accurate, Economical and Safe nitrate analysis.
- Kit for 100 tests manuals.
- Nitrate standards included in the kit.

Nitrate Units	In Europe
Standard range	2-44 ppm nitrates

TEST PRINCIPLE

This Nitrate test kit is based on the enzyme Nitrate Reductase (NaR), catalyzing the reduction of Nitrate to Nitrite using the natural electron donor NADH. The Nitrite reacts with color reagents (dyes) under acidic conditions to produce a visible color. The concentration of Nitrate in the original sample is determined by measuring absorbance at 540nm versus Nitrate concentration in Nitrate Standards. Nitrate can be determined in water samples and extracts of plant tissues, soils and foods. The test is designed to measure Nitrate in the range 2.0 to 44 ppm Nitrates. The Nitrate concentration can also be expressed as µM Nitrate, where range is 36 to 714 µM Nitrate, or ppm Nitrate-N, where range is 0.5 to 10 ppm Nitrate-N. Nitrite can also be determined by omitting NaR and NADH from the test (see Determining Nitrite, page 5).
If testing seawater, salt water or brackish water, follow the blue high-lighted instructions. Chloride is a mild inhibitor of Nitrate Reductase. The color development when analyzing seawater is not as intense as other types of samples. Nitrate Standards prepared in salt water solves the problem.

KIT COMPONENT

- **Buffer** (28 mM KH_2PO_4, 0,025 mM EDTA ; pH 7.5) – 3 tubes of 50 ml.
- **Color reagent #1** (1% Sulfanilamide in 3N HCl) powder format – 1 amber bottle for 60ml.
- **Color reagent #2** (0.02% N-Naphthylethylenediamine in dionised water) powder format – 1 amber bottle for 60ml.
- **NADH** in freeze-dried form (approx. 2 mM NADH per tube) – 4 tubes in a sachet with dessicant.
- **Nitrate Reductase (NaR)** in freeze-dried form (1 unit per tube) – 4 tubes in a sachet with dessicant.
- **Diluant enzyme** – 4 squeeze-bulb plastic ampoules of 1ml.
- **Nitrate standard (440 ppm nitrates)** – in liquid form – 1 tube of 1.5 ml.
- **Microcentrifuge tubes** – 6 tubes for preparing nitrate standards.
- **Salt Water** – one 20 ml tube, green cap, only if you are testing seawater. (N.B.: may contain 0.22 ppm nitrates, giving your blank a slight pink color).

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MATERIALS NOT PROVIDED

- 100 ml graduated cylinder.
- Variable pipettes (10 to 100 µl and 100 to 1000µl).
- Test tube vortex-type mixer or other means to mix contents of tubes.
- Colorimeter or Spectrophotometer capable of reading at 540 nm ± 20 nm, with a glass or plastic cuvette (approx. volume 2 ml).
- (100) 13 x 100 mm test tubes (Clean and Nitrate-free).
- Timer (0 to 20 minutes) – a clock or stop watch is adequate.
- Deionized or distilled water (d-I water; must be "Nitrate-free" to avoid high background).
- 15 ml of concentrated HCI.
- Ice and Ice Bucket.

REAGENT PREPARATION

Step 1 **Assay Buffer** – ready to use from kit. Warm to room temperature for nitrate tests. If desired, the assay buffer may be more quickly warmed in a 30ºC water bath**.**

Step 2 Prepare **3 N HCl** by adding **15 ml concentrated HCl** to **45 ml d-I water.** Mix.

Step 3 Add **60 ml 3 N HCl** to **Color reagent #1** bottle. Mix by shaking well.

Step 4 Add **60 ml d-I water** to **Color reagent #2** bottle. Mix by shaking well.

Step 5 Remove a tube of **NADH** from amber bag, tap tube to settle contents, add 1.5 ml **d-I water** and replace cap. Mix by inversion several times. Keep on ice during use.

Step 6 Remove a **NaR** vial from foil pouch and tap tube to settle contents before opening. Twist off the end of the **Enzyme Diluent Squeeze Bulb** and completely empty the contents into the **NaR** vial. Replace the cap and mix by inversion 3 times. Allow to stand at room temperature for at least 10 minutes, with mixing at 5 and 10 minutes. Then keep on ice during use.

Step 7 For the remaining unused vials of NADH and NaR, repeat steps 5 and 6.

REAGENTS STABILITY

Before opening, the reagents are stable at 2-8°C up to the expiry date mentioned on the kit label.
For an extended stability, NADH and NaR can be stored at -20°C.

Stability of the reconstituted NaR enzyme:
The preparation of NaR reconstituted with diluent is stable 7 days at 2-8°C.
For an extended stability (6-12 months) store it at -20°C. Freeze/thaw cycles can be repeated.

Stability of the reconstituted NADH:
The preparation of NADH is stable 7 days at 2-8°C.
For an extended stability (>30 days), divide into appropriately sized aliquots the NADH preparation to avoid repetitive freeze / thaw cycles and store it at -20°C.

STANDARD PREPARATION

Transfer **1 ml of 440 ppm Nitrates Standard** into a test tube containing **9 ml d-I water** to make a **44 ppm Nitrates Standard.** Use the 6 microtubes (provided in kit) to prepare Nitrate Standards as shown in table below. Cap and mix the tubes by inversion before use. **If you are testing seawater,** use the **Salt Water** provided instead of d-I water to prepare standards.

			Equivalence other units	
Volume to pipet from tube of 44 ppm nitrates (µl)	Volume d-I (µl)	**Concentration (ppm nitrates)**	*Concentration (ppm nitrates-N)*	*Concentration (µM)*
1000	0	**44**	*10*	*712*
750	250	**33**	*7.5*	*534*
500	500	**22**	*5*	*356*
250	750	**11**	*2.5*	*178*
100	900	**4.4**	*1*	*71.2*
50	950	**2.2**	*0.5*	*35.6*

ASSAY PROCEDURE

The following procedure is written for single determinations. For greater accuracy, replicates can be run.

WASTE DISPOSAL
Follow all local guidelines and regulations. If there are no local guidelines, wash the waste down the sink with large amounts of running water.

STEP 1 Pipette **50 µl d-I water** into one test tube for use as "Reagent Blank". If testing seawater, use the **Salt Water** provided instead of d-I water.
STEP 2 Pipette **50 µl** of the **samples** and **standards** into the required number of test tubes.
STEP 3 Add **900 µl Assay Buffer** to each tube.
STEP 4 Add **50 µl NADH solution** to each tube. Mix thoroughly with a vortex- type mixer.
STEP 5 Start reaction by adding **40 µl NaR** solution to each tube. Mix thoroughly with a vortex-type mixer.
STEP 6 Let tubes sit for ~20 minutes at room temperature. (NOTE: Exact timing is not critical but at least 20 minutes are required for complete reduction of nitrate.)
STEP 7 Add **500 µl of solution Color reagent #1** to each tube. Mix thoroughly with a vortex-type mixer.
STEP 8 Add **500 µl of solution Color reagent #2** to each tube. Mix thoroughly with a vortex-type mixer.
STEP 9 Let tubes stand at room temperature for ~10 minutes. To ensure homogeneous samples, briefly mix the tubes with a vortex-type mixer.
STEP 10 Read absorbance at 540 nm ± 20 nm in a colorimeter or spectrophotometer for the samples and Nitrate Standards. To ensure accurate results, read absorbance between 10 and 30 minutes after color reagents are added.
(NOTE: Zero the colorimeter with d-I water in the cuvette; rinse cuvette with d-I water between readings.)

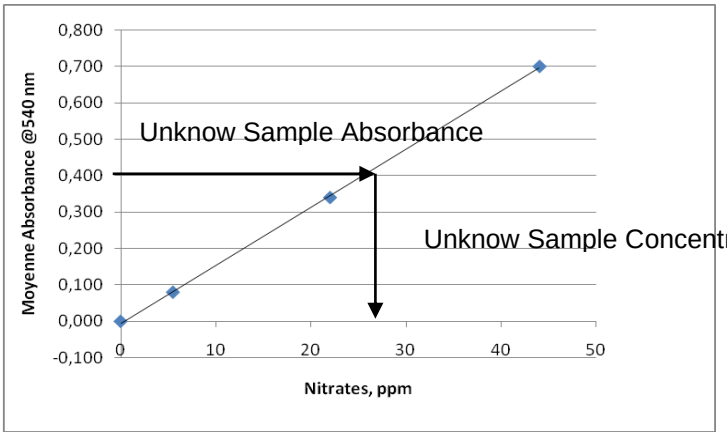
CALCULATIONS

STEP 1 To correct for any background absorbance due to the reagents, subtract the mean absorbance of the reagent blank(s) from the mean absorbance of each nitrate standard and unknown sample:

(Corrected mean absorbances) $_{\text{sample or std}}$ = (mean absorbances) $_{\text{sample or std}}$ – (mean absorbances) $_{\text{reagent blank}}$

STEP 2 Generate a standard curve for the Nitrate Standard (see example below). Using linear graph paper or a computer plotting program or spreadsheet such as Excel®, plot the ppm Nitrate on the x-axis, and the absorbance @ 540 nm for each nitrate standard on the y-axis. If plotting by hand, draw a straight line through the points for the Nitrate Standards. If plotting by computer, the slope of the line can be calculated for determining Nitrate ppm in the unknown samples.

STEP 3 Using the standard curve, determine the ppm Nitrate for the sample: (a) Find the corrected absorbances @ 540 nm for the sample on the y-axis of the standard curve. (b) Follow over along a horizontal line to where the line intersects the standard curve. Trace down the x-axis and read the ppm of Nitrate on the x-axis.



UNKNOW SAMPLES WITH HIGH NITRATE

This kit is capable of determining Nitrate levels of up to 44 ppm Nitrate (equivalent to 11 ppm Nitrate-N or 714 µM Nitrate). If an unknown sample is found to have more than 44 ppm Nitrate, the sample may be diluted with d-I water 1:10 to allow an exact determination. For example, take 100 µl of sample and add 900 µl of deionized water to make a 1:10 dilution and then assay 50 µl of the diluted sample. After finding the Nitrate content of the diluted sample, multiply the Nitrate concentration by 10 to find the Nitrate concentration in the original sample.

NOTE: Keep the sample volume constant by diluting the sample rather than using a smaller volume of sample in the assay.

PREPARATION OF FOOD SAMPLES

Before starting the detection of nitrates, prepare food samples by following appropriate standard sample preparation method.

NITRATE DETERMININATION IN PLANT LEAF EXTRACTS

To determine how much Nitrate is in one gram of leaf tissue, grind the tissue in 10 ml d-I water and measure the total volume of extract after the solids are filtered off. Take 50 µl of extract for the Nitrate assay, conduct the Nitrate Test Kit assay and find the amount of Nitrate present in nmoles. Determine the total amount of Nitrate in the extract [= (total volume of extract) x (nmol Nitrate/50 µl of extract)]. Divide this total amount of Nitrate by the weight of plant tissue to find the amount of Nitrate per unit of tissue (grams in this example). The green or brown color of the leaf extract does not significantly interfere with Nitrate determinations since the plant extract is diluted 20-fold in the assay. The most

quantitative analysis of leaf Nitrate content is obtained when the leaves are boiled for 20 min. After boiling, cool on ice and then filter the sample to recover aqueous extract. Finally, make the volume back up to 10 ml to compensate for water lost during boiling.

Ask LIBIOS for a detailed protocol for extracting either fresh plant leaves or dried leaf material for Nitrate Assays

NITRITE DETERMINATION

Nitrite can be determined by omitting NaR and NADH from the samples. (That is, skipping steps 4, 5 and 6 on page 3). Prepare Nitrate standards as described in the normal Nitrate Assay Procedure with both NADH and NaR added and use the Nitrate Standard Curve for estimating Nitrite content.

AUTO-ANALYZER BIOCHEMISTRY PROTOCOLS

Ask LIBIOS for a detailed protocol.

TECHNICAL ASSISTANCE

For more information, please contact our services:

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