



**NECi**

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NECi**

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[www.nitrate.com](http://www.nitrate.com)

**Important!**

Keep contents of kit refrigerated until day of use.

See Kit Box for expiration date.

Catalog No. L-NTK-202

## Lab Nitrate Test Kit – Low Range 25 Colorimetric Determinations (Test Tube)

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 in a colorimeter and comparison to a standard curve of absorbance 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 of 0.05 to 1.0 ppm Nitrate-N in up to 25 samples or standards. The Nitrate concentration can also be expressed as  $\mu\text{M}$  Nitrate, where range is 3.6 to 71.2  $\mu\text{M}$  Nitrate, or ppm Nitrate, where range is 0.2 to 4.4 ppm Nitrate. Nitrite can also be determined by omitting NaR and NADH from the test (see Determining Nitrite, page 4).

### EQUIPMENT AND REAGENTS:

#### Supplied in NECi Nitrate Test Kit

- **Assay Buffer** in liquid form - (1) 50 ml tube.
- **Color Reagent No. 1** in solid form - (1) 15 ml amber bottle.
- **Color Reagent No. 2** in solid form - (1) 15 ml amber bottle.
- **NADH** - 1 tube in amber bag.
- **Nitrate Reductase (NaR)** - 1 tube in amber bag.
- **Nitrate Standard (100 ppm Nitrate-N)** in liquid form - 1 tube.
- **Microcentrifuge tubes** - 6 tubes for preparing Nitrate Standards.

#### Supplied by User

- **25 or 100 ml graduated cylinder.**
- **Variable pipettors** (10 to 100  $\mu\text{l}$  and 100 to 1000  $\mu\text{l}$ ).
- **Test tube vortex-type mixer** or other means to mix contents of tubes.
- **Colorimeter or Spectrophotometer** capable of reading at 540 nm  $\pm$  20 nm, with a glass or plastic cuvette (approx. volume 2 ml).
- **(25) 13x100 mm test tubes** (Clean and Nitrate-free).
- **Timer** (0 to 20 minutes) - a clock or stop watch is adequate.
- **Deionized or distilled water** (di-water; must be "Nitrate-free" to avoid high background).
- **4 ml of concentrated HCl.**
- **Ice and Ice Bucket.**

### Problems? Need More Information? Need to Re-Order?

- Call NECi Toll-Free: 1-888-NITRATE (648-7283)
- FAX: 1-906-296-8003
- Email: tech@nitrate.com
- Visit the NECi Web – <http://www.nitrate.com>

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## Reagent Preparation

Prepare on day of use. Discard any unused NADH and NaR solutions at end of day. Unused Assay Buffer and Color Reagent solutions may be kept in a refrigerator for up to 6 months after mixing (remember to label and date containers).

- 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 30°C water bath.
- 2** Prepare **3 N HCl** by adding **4 ml concentrated HCl** to **12 ml di-water**. Mix.
- 3** Add **15 ml 3 N HCl** to **Color Reagent No. 1** bottle. Mix by inverting several times.
- 4** Add **15 ml di-water** to **Color Reagent No. 2** bottle. Mix by inverting several times.
- 5** Remove tube of **NADH** from amber bag and add **1.5 ml di-water** and replace cap. Mix with a vortex-type mixer for about 5 sec. Keep on ice during use.
- 6** Remove tube of **NaR** from amber bag and add **0.6 ml Assay Buffer**. Mix for 3 sec with a vortex-type mixer. Allow to stand at room temperature for 20 minutes, with 3 sec vortex-mixing at 10 and 20 minutes. After 20 minutes, keep on ice.

### CAUTION

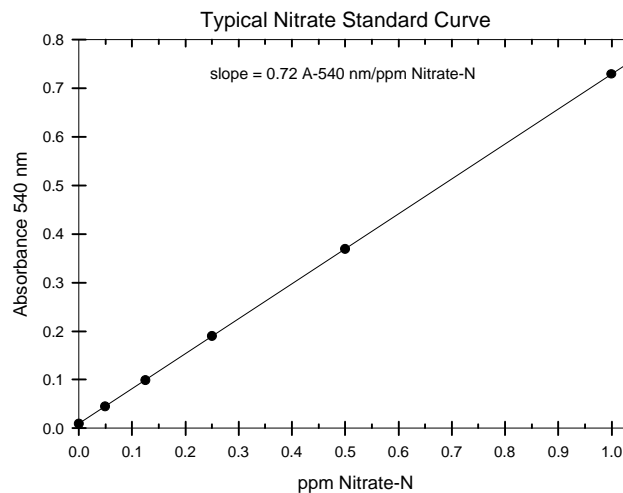
Use care when handling samples containing HCl (gloves are recommended).

## Nitrate Standard Preparation

Transfer **0.5 ml of 100 ppm Nitrate-N Standard** into test tube containing **4.5 ml di-water** to make a **10 ppm Nitrate-N Standard**. In another test tube, **dilute 1 ml of 10 ppm Nitrate-N Standard** with **9.0 ml di-water** to make **1.0 ppm Nitrate-N Standard**. **Make Working Nitrate Standards** as shown below.

### Working Nitrate Standards

<i>Volume of 1.0 ppm Nitrate-N Standard</i>	<i>Volume di-water</i>	<i>Resulting Standard (ppm Nitrate-N)</i>	<i>Resulting Standard (ppm Nitrate)</i>	<i>Resulting Standard (µM)</i>
1000 µl	0 µl	1.0	4.4	71.2
500 µl	500 µl	0.5	2.2	35.6
250 µl	750 µl	0.25	1.1	17.8
125 µl	875 µl	0.125	0.55	8.9
50 µl	950 µl	0.05	0.22	3.6
0 µl	1000 µl	0	0	0



## Nitrate Assay Procedure

### 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.

- 1 Pipette **500 µl di-water** into one test tube for use as reagent blank.
- 2 Pipette **500 µl** of the **samples** and **standards** into required number of test tubes.
- 3 Add **500 µl Assay Buffer** to each tube.
- 4 Add **50 µl NADH solution** to each tube. Mix thoroughly with vortex-type mixer.
- 5 Add **20 µl NaR solution** to each tube. Mix thoroughly with vortex.
- 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.)
- 7 Add **500 µl Color Reagent No. 1** to each tube. Mix thoroughly with vortex.
- 8 Add **500 µl Color Reagent No. 2** to each tube. Mix thoroughly with vortex.
- 9 Let tubes stand at room temperature for ~10 minutes. To ensure homogeneous samples, briefly mix the tubes with a vortex-type mixer.
- 10 Read absorbance at 540 nm ± 20 nm in colorimeter or spectrophotometer for the **reagent blank, samples and nitrate standards**. Rinse cuvette with di-water between readings.  
To ensure accurate results, read absorbance between 10 and 30 minutes after color reagents are added.

**NOTE: Zero the colorimeter with di-water in the cuvette to permit you to check for background color in the reagent blank, which should be less than 0.03.**

## Calculations

- 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:  
$$\text{Corrected mean sample A-540 nm} = (\text{mean A-540 nm for sample}) - (\text{mean A-540 nm for reagent blank})$$
- 2 Generate a standard curve for the Nitrate Standards (see example on previous page). Using linear graph paper or a computer plotting program such as Sigma Plot® or spreadsheet such as Excel®, plot the ppm Nitrate-N on the x-axis, and the A-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-N ppm in the unknown samples.
- 3 Using the standard curve, determine the ppm Nitrate-N for the sample: (a) Find the corrected A-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 to the x-axis and read the ppm of Nitrate-N on the x-axis.

## Notes on the Reagents

- **Assay Buffer** - 25 mM K-PO<sub>4</sub>, 0.025 mM EDTA; pH 7.5.
- **Color Reagent No. 1** - 1% Sulfanilamide in 3 N HCl.
- **Color Reagent No. 2** - 0.02% N-Naphthylethylenediamine in di-water.
- **NADH** - approx. 1 mM NADH.
- **Nitrate Reductase (NaR)** - approx. 0.5 unit of NaR per tube.
- **Nitrate Standard** - 1 vial of 100 ppm nitrate-N.

## Notes on Nitrate Testing

**UNKNOWN SAMPLES WITH HIGH NITRATE:** This NECi Nitrate Test Kit is capable of determining Nitrate levels of up to 1.0 ppm Nitrate-N (71  $\mu$ M Nitrate). If an unknown sample is found to have more than 1.0 ppm Nitrate-N, the sample may be diluted with di-water 1:10 to allow an exact determination. For example, take 100  $\mu$ l of sample and add 900  $\mu$ l of deionized water to make a 1:10 dilution and then assay 500  $\mu$ l 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.

**DETERMINING NITRATE IN MOLAR UNITS:** Use a conversion of 1 ppm Nitrate-N = 71  $\mu$ M Nitrate. The standard curve shown has a slope = 0.01 A-540 nm/ $\mu$ M Nitrate. One ppm Nitrate-N = 1 mg of Nitrogen/liter. Since Nitrogen has a molecular weight of 14 g/mole, then the molar concentration is:

$$(0.001 \text{ g/l}) \div (14 \text{ g/mole}) = 0.000071 \text{ M Nitrogen} = 0.000071 \text{ M Nitrate} = 71 \mu\text{M Nitrate}$$

**DETERMINING NITRATE AS A QUANTITY:** (Using the 500  $\mu$ l Sample Size). In the tube where the 1.0 ppm nitrate-N standard is determined, there is approx. 36 nmol of Nitrate [(71 nmol Nitrate/ml) x (0.5 ml) = 35.5 nmol]. So the example standard curve would have a slope of 0.02 A-540 nm/nmol Nitrate (calculated from slope = 0.7 A-540nm/35.5 nmol Nitrate).

**NITRATE DETERMINATION IN PLANT LEAF EXTRACTS:** To determine how much Nitrate is in one gram of leaf tissue, grind the tissue in 10 ml di-water and measure the total volume of extract after the solids are filtered off. Take 500  $\mu$ l 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/500  $\mu$ l 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 may significantly interfere with Nitrate determinations since the plant extract is diluted only 2-fold in the assay. Using activated charcoal may over come the interference when quantitative analysis of leaf Nitrate content is done by boiling leaves for 20 min. After boiling, cool on ice and then filter the sample to recover the aqueous extract and make up to 10 ml. *Ask NECi for a detailed protocol for extracting either fresh plant leaves or dried leaf material for Nitrate Assays.*

## Determining Nitrite

**INTERESTED IN DETERMINING NITRITE?** 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.

## Notes on Nitrate in Water

The Clean Water and Safe Drinking Water Acts (U.S. EPA 1974) set Maximum Contaminant Level (MCL) for portable water at 10 ppm Nitrate-N (10 mg Nitrate-N per liter). California and European standards are 45 ppm nitrate (45 mg nitrate per liter). If you find drinking water with 7 to 10 ppm Nitrate-N or more, advise users to seek a professional test of their water. Environmental water samples usually contain 1 to 2 ppm Nitrate-N or less.

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To Order NECi Products: Call Toll-Free 1-888-NITRATE (648-7283)

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