

# L-NTK-301

## Nitrate Test Kit - Standard Range

### MICROPLATE DETERMINATION FOR 96 NITRATE TESTS

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. All reaction steps occur in the microplate. The concentration of nitrate in the unknown sample is determined by measuring absorbance with a microplate reader using a 540 nm filter (or a filter as close to 540 nm as possible), and then comparing the absorbance to a standard curve generated from 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 0.5 to 10 ppm nitrate-N using a 96-well microplate. The nitrate concentration can also be expressed as µM nitrate, where the range is 36 to 710 µM nitrate. Nitrite can also be determined by omitting NaR and NADH from the test (see **Notes on the Tests**, page 4).

**IMPORTANT - Keep contents refrigerated until day of use. See box for expiration date.**

#### Supplied in NECi Nitrate Test Kit

- Assay Buffer in liquid form - (1) 15 ml amber bottle.
- 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.
- Quench Agent - 1 tube in amber bag.
- Microplate - (1) 96-well microplate with flat bottom wells.
- (2) multichannel pipetter basins.
- Microtubes - 6 tubes for preparing working nitrate standards.

#### Supplied by User

- 25 ml graduated cylinder.
- Pipettors - variable pipettors (0.5µl to 100µl) and (1) multi-pipetter with 8/12 well manifold.
- Several test tubes.
- Vortex-type mixer.
- Microplate mixer.
- Microplate reader capable of reading at 540 nm.
- Timer (0 to 20 minutes) - a clock or stop watch is adequate.
- Deionized or distilled water (di-water)
- 2.5 ml of concentrated hydrochloric acid (HCl).
- Small beaker with ice.

### NECi The Nitrate Elimination Company, Inc.

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2 NITRATE TEST KIT - STANDARD RANGE (Catalog No. M-NTK-301)

#### A. Reagent Preparation

Prepare on day of use. Discard any unused NADH and NaR solutions at end of day. 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.

- Remove tube of NaR from amber bag and add 1.0 ml Assay Buffer. Mix for 3 seconds with a vortex-type mixer. Allow to stand at room temperature for 20 minutes, with 3 sec of vortex-mixing at 10 and 20 minutes. Keep on ice until use.
- Prepare 3 N HCl by adding 2.5 ml concentrated HCl to 7.5 ml di-water. Mix.
- Add 10 ml 3 N HCl to Color Reagent No. 1 bottle. Mix by inverting several times.
- Add 10 ml di-water to Color Reagent No. 2 bottle. Mix by inverting several times.
- Remove tube of NADH from amber bag and tap tube to settle contents. Add 0.6 ml di-water and replace cap. Mix for 3 sec with a vortex-type mixer. Keep on ice until use.
- Add di-water to the 5 ml line of the clear tube labeled Quench Agent. Mix thoroughly.
- In the multichannel basin (included in kit), add 9 ml Assay Buffer, 1 ml NaR solution (prepared in Step 2), and 0.5 ml NADH solution (prepared in Step 6). Mix thoroughly.

#### B. Standard Preparation

Transfer 1 ml of 100 ppm Nitrate-N standard (provided in kit) to a test tube. Dilute with 9 ml di-water to make a 10 ppm Nitrate-N standard. Use Nitrate standard and the 6 microtubes (provided in kit) to prepare nitrate standards as shown in table below.

Volume 10 ppm Nitrate-N Standard	Volume di-water	Working ppm Nitrate-N Standards	Working ppm Nitrate Standards	Working µM Nitrate Standards
1000 µl	0 µl	10 ppm	44 ppm	712 µM
750 µl	250 µl	7.5 ppm	33 ppm	534 µM
500 µl	500 µl	5.0 ppm	22 ppm	356 µM
250 µl	750 µl	2.5 ppm	11 ppm	178 µM
125 µl	875 µl	1.0 ppm	4 ppm	71 µM
50 µl	950 µl	0.5 ppm	2 ppm	36 µM

#### C. Procedure

The following procedure uses 2 replicates of each standard, unknown sample and reagent blank. Using the Microplate Sample Template, assign and record a set of 2 wells for each Working Nitrate Standard, Sample and the Reagent Blanks.

- Pipet 10 µl di-water into 2 wells for Reagent Blanks.
- Pipet 10 µl of Working Nitrate Standards and Samples into designated wells.
- Pipet 90 µl NaR-Assay Buffer-NADH solution (prepared in Step 8 of Reagent Preparation) to each well. Shake on a plate mixer for ~20 minutes @ 800 rpm.
- Transfer Quench Agent solution to multichannel basin. Pipet 30 µl of the solution to each well. Shake on a plate mixer for ~10 minutes at 800 rpm.

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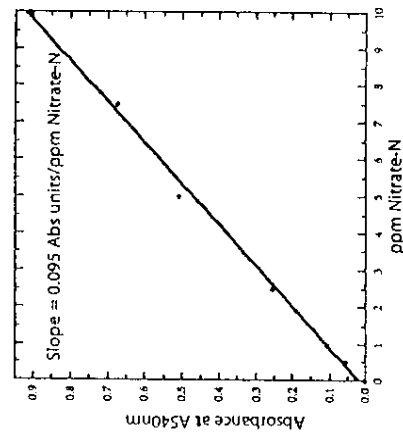
### C. Procedure (cont.)

- 5 If using a multi-pipetter, rinse the 2 multichannel pipetter basins with di-water. Transfer Color Reagent No. 1 to one basin and Color Reagent No. 2 solution to the other basin.
- 6 Add 50  $\mu$ l Color Reagent No. 1 and 50  $\mu$ l Color Reagent No. 2 solution to each well. Shake on a plate mixer for ~10 minutes @ 800 rpm.
- 7 Zero the plate reader with a Reagent Blank well using a 540 nm filter (or a filter within 20 nm of 540 nm).
- 8 Read absorbance of all wells. Transfer results to your computer system for analysis and printing.

### D. Calculations

- 1 To correct for any background absorbance due to the reagents, subtract the mean absorbance of the reagent blanks from the mean absorbance of each nitrate standard and unknown sample:  
Corrected mean sample A-540 nm = (mean A-540 nm for samples) - (mean A-540 nm for reagent blanks)
- 2 Generate a standard curve for the nitrate standards (see example below). Using linear graph paper or computer plotting program such as Sigma Plot<sup>®</sup> or spreadsheet such as Excel<sup>®</sup>, 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 sample.

- 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.



Typical nitrate standard curve generated with NECI Nitrate Test Kit.

Note: 1 ppm Nitrate-N = 4.4  $\mu$ M Nitrate = 71  $\mu$ M nitrate.

### NOTES ON THE TEST

#### Unknown Samples with High Nitrate

This NECI Nitrate Test Kit is capable of determining nitrate levels of up to 10 ppm Nitrate-N (44 ppm Nitrate or 710  $\mu$ M Nitrate). If an unknown sample is found to have more than 10 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 10  $\mu$ l of the diluted sample. After finding the amount of nitrate in 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.

#### Testing for Nitrite?

Nitrite can be determined by replacing the solution in Step 3 of the Procedure with 90  $\mu$ l of di-water and eliminating Step 4.

#### Notes on the Reagents

- Assay Buffer - 25 mM K<sub>2</sub>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 - ~1.1 mM NADH
- Nitrate Reductase (NaR) - approx. 0.5 unit of NaR per tube
- Nitrate Standard - 1 vial of 100 ppm Nitrate-N
- Quench Agent - mild, non-toxic oxidizing chemical to remove excess NADH.

#### Caution

Use care when handling hydrochloric acid (HCl). Gloves are recommended.

#### Waste Disposal

Follow all local guidelines and regulations. If no local guidelines apply to your situation, wash waste down a sink using large amounts of running water.

#### Questions?

Call us at 1-888-NITRATE (1-888-648-7283), or e-mail questions to [tech@nitrate.com](mailto:tech@nitrate.com). Go To NECI Web — [www.nitrate.com](http://www.nitrate.com) — for answers to frequently asked questions.



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