

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.
 - Prepare **3 N HCl** by adding **15 ml concentrated HCl** to **45 ml di-water**. Mix.
 - Add **60 ml 3 N HCl** to **Color Reagent No. 1** bottle. Mix by inverting several times.
 - Add **60 ml di-water** to **Color Reagent No. 2** bottle. Mix by inverting several times.
- To analyze 25 samples and Nitrate Standards:**
- Remove 1 tube of **NADH** from amber bag and add **1.5 ml di-water** and replace cap. Mix with a vortex-type mixer. Keep on ice during use.
 - Remove 1 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 vortex-mixing at 10 and 20 minutes. After 20 minutes, keep on ice.
 - For the 3 remaining sets of 25 Nitrate tests, repeat steps 5 and 6 with unused tubes of **NADH** and **NaR**.

CAUTION

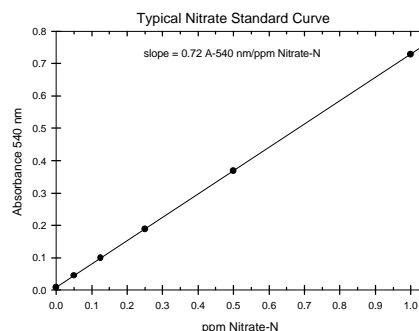
Use care when handling samples containing HCl (gloves are recommended).

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**. Using 15 ml tube (provided in kit) **dilute 1 ml 10 ppm nitrate standard with 9.0 ml di-water** to make **1.0 ppm Nitrate-N Standard** (label & date tube). **Make Working Nitrate Standards** as shown below. Working Nitrate Standards provide enough for 4 sets of nitrate tests and may be stored at 4°C for up to 6 months after mixing (label & date containers). Warm to room temperature to use.

Working Nitrate Standards

Volume of 1.0 ppm Nitrate-N Standard	Volume di-water	Resulting Standard (ppm Nitrate-N)	Resulting Standard (ppm Nitrate)	Resulting Standard (µM)
5.0 ml	0 ml	1.0	4.4	71.2
2.5 ml	2.50 ml	0.5	2.2	35.6
1.25 ml	3.75 ml	0.25	1.1	17.8
625 µl	4.38 ml	0.125	0.55	8.9
250 µl	4.75 ml	0.05	0.22	3.6
0 µl	5.00 ml	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.

- Pipette **500 µl di-water** into one test tube for use as reagent blank.
- Pipette **500 µl** of the **samples** and **standards** into required number of test tubes.
- Add **500 µl Assay Buffer** to each tube.
- Add **50 µl NADH solution** to each tube. Mix thoroughly with vortex-type mixer.
- Add **20 µl NaR solution** to each tube. Mix thoroughly with vortex.
- 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.)
- Add **500 µl Color Reagent No. 1** to each tube. Mix thoroughly with vortex.
- Add **500 µl Color Reagent No. 2** to each tube. Mix thoroughly with vortex.
- Let tubes stand at room temperature for ~10 minutes. To ensure homogeneous samples, briefly mix the tubes with a vortex-type mixer.
- 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

- 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 sample A-540 nm = (mean A-540 nm for sample) - (mean A-540 nm for reagent blank)
- 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.
- 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-PO₄, 0.025 mM EDTA; pH 7.5.
- Color Reagent No. 1** - 1% Sulfanilamide in 3N 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.