Nitrate Reductor Kit Instructions

Product Information
Catalog Numbers: **Nitrate Reductor Kit #50010**
(includes: Reductor, Nunc Microplate, Nunc Holding Tray with Lid, Cleaning Solution Container, 3 Locking Pins for Reductor, and 3 Replacement Locking Pins)

Intended Use
The ParaTechs Nitrate Reductor is designed to facilitate the analysis of nitrate in a wide variety of sample matrixes, including blood serum, urine, natural water, and extracts of soil and plant tissue. The device consists of 96 individual cadmium pins plated with copper, and is used to reduce nitrate to nitrite simultaneously in all wells of a disposable microplate. After reduction is complete, Griess reagent is added, and the final colorimetric determination is made using a microplate reader.

Analytical Instrumentation Required
Tunable microplate reader (or instrument with a 540 nm filter); ultrasonic cleaner; titer-plate shaker; single and multi-channel pipettes; pH meter; vortex mixer. A centrifuge (e.g. Eppendorf Model 5810 with A-2-DWP rotor) is also essential for some applications when using cluster tubes (Fisher Scientific) to prepare samples for analysis.

IMPORTANT
The ParaTechs Nitrate Reductor functions as an array of Cu-Cd micro-batteries with exactly the required voltage to reduce nitrate to nitrite. However, the Reductor will not perform properly until the copper-plating process, and activation with a high level of nitrate to increase the reactive surface area has been successfully completed. Each new Reductor has been partially pre-conditioned at our laboratory, but it still **must be sonicated for 2 min in the acid cleaning solution and then further activated with a high concentration of nitrate before the calibration check is performed** (see below). After initial conditioning, the reductor will improve somewhat in performance as the copper coating becomes more stable. However, **it is essential that the 60 sec sonication step be performed each time the Reductor is used.** The active reducing sites on the cadmium pins will become occluded with cadmium oxide as the Reductor is allowed to dry after each use.

Handling
The Reductor is reusable for several thousand samples without noticeable loss of efficiency. After many uses (40-60+) the cadmium pins will become eroded at the liquid-air interface and pins may start to break off during the ultrasonic cleaning process. **It is recommended that two Reductors be purchased at each initial sale so that a back-up device will always be available**. The Reductor has been pre-conditioned in our laboratory, however, before its initial use the reductor must be activated by sonicating for 2 min in a solution of copper sulfate in hydrochloric acid (2 mL 2.0% CuSO4/175 mL 1.0 M HCl). This solution may be contained in the provided sealable plastic box that fits inside the ultrasonic cleaner. The activation process serves to increase the reactive surface area of the Reductor pins and to initiate the copper plating process. After sonication, the Reductor is rinsed with DI water, dried with a paper towel, and fitted onto a microplate containing 20 µLs of a 200 µg/ml nitrate-N solution and 200 µLs of the standard pH 8.5 ammonium chloride buffer solution (see below). The assembly is then shaken for one hour. Next, the Reductor is rinsed thoroughly with DI water and placed back in the acid cleaning

solution for 60 sec. Finally the reductor is rinsed, blotted dry, and placed in the provided holding tray (Nunc Omintray) containing pH 8.5 ammonium chloride buffer. The calibration check is then performed.

Calibration Check

Before beginning actual sample analyses, the precision of each new Reductor should be verified. All pins should behave identically during the reduction process. The basic analytical procedure (described below) should be used to analyze 96 aliquots of the same standard solution (i.e. 5.0 µg/mL NO_3-N). All resulting optical densities should agree within pipetting error (i.e. ±3%). **Note: When using a multi-channel pipette for the standard solution addition, the tip sets should be wetted before use and changed at least twice to avoid the introduction of pipetting error.**

Pre-analytical Sample Preparation

Samples with simple matrixes such as natural water or potassium chloride extracts of soil can be analyzed using the Reductor with little regard to sample preparation. It is only necessary to match the matrix of the calibration standards with the sample matrix and to provide a sufficient reduction time. However, when analyzing samples with complex matrices, a close match with water standards is never possible. For example, physiological fluids and some extracts of plant tissue containing low levels of nitrate cannot be diluted sufficiently to negate the influence of matrix components on the reduction process, or on the Griess reaction chemistry after the reduction to nitrite is complete. It is therefore desirable to remove these substances to the extent possible before beginning the analysis. The use of Somogyi's reagent ($NaOH/ZnSO_4$), as described below, may help to alleviate this problem. A more complete discussion of these issues is found in the reference at the end of the instructions.

Analytical Reagents (see reference for specific preparation instructions)

1. Acid Cleaning Solution 2 mL 2% $CuSO_4 \cdot 5H_2O$ in 175 mL of 1M HCl.
2. 1% NH_4Cl buffer pH 8.5.
3. 1% NH_4Cl buffer pH 10.0.
4. Griess Reagent Stock #1. 0.1% N(1-naphthyl) ethylenediamine dihydrochloride (NED) in DI water.
5. Griess Reagent Stock #2. 1.0% Sulfanilamide in 3M HCl.
6. Griess Reagent Stock #3. 1.0% Sulfanilamide in 6M HCl.
7. Somogyi Reagent Solution #1 0.300M NaOH.
8. Somogyi Reagent Solution #2 5.00% w/v solution of $ZnSO_4 \cdot 7H_2O$ in DI water.
9. Certified 1000 mg/L NO_3-N Stock Solution (Fisher Scientific).

Basic Analytical Procedure (the procedure can be watched on YouTube http://www.youtube.com/watch?v=HRpQzCGlum4&feature=g-all-u)

1. The Reductor is rinsed with DI water, placed in the acid cleaning solution, and sonicated for ~60 sec to remove surface oxidation. **This step must be performed each time the Reductor is used.**
2. The Reductor is then rinsed with DI water and blotted dry on a paper towel. Also, any liquid adhering to the underside of the device may wick into the samples during the analysis and must be removed with a Q-tip or folded paper towel. The device is then placed in a holding tray (Nunc Omnitray) containing standard pH 8.5 ammonium chloride buffer solution, being careful to submerge only the pins, while leaving the underside dry.
3. For water samples or KCl extracts of soil, standards are prepared in the sample matrix. A 20 µL aliquot will normally produce a linear calibration curve in a range of 0-5 µg/mL NO_3-N. However, the range can be adjusted as needed by varying the aliquot volume.
4. To begin the analysis, typically 20 µL aliquots of your samples are pipetted into a microplate (Nunc) and 200 µLs pH 8.5 ammonium chloride buffer is added.
5. The microplate is placed on the titer-plate shaker. The Reductor is lifted from the holding tray, blotted on a paper towel, and secured to the microplate with locking pins. The shaker speed setting is adjusted so that no spillage will occur (i.e., 6.5). The quantitative reduction of nitrate to nitrite is then accomplished by shaking for ~60 min.

6. While the samples are shaking, Griess reagent may be prepared by mixing equal volumes of stock solutions #1 and #2.
7. After the shaking interval, the Reductor is lifted from the microplate and rinsed thoroughly with DI water. If the Reductor is to be reused immediately, the cleaning and reactivation procedure can be repeated. Otherwise, the Reductor should be allowed to dry on a paper towel.
8. Sixty µL of the mixed Griess reagent is then added to the microplate containing the samples and calibration standards, and placed on the titer-plate shaker for 5 min to complete the color development.
9. Finally, the analysis is completed by determining the optical densities of the samples and standards on the microplate reader at 542 nm.

Sample Matrix Modification using Somogyi's Reagent

For samples with complex matrixes such as blood serum, urine or highly colored plant tissue extracts, Somogyi's reagent ($NaOH/ZnSO_4$) can be used to eliminate many interfering substances in the sample matrix by co-precipitation with $Zn(OH)_2$. Further modifications in the basic procedure such as increasing the pH of the buffer solution used during the reduction process to pH 10.0, and increasing the sulfanilamide concentration in the mixed Griess reagent will also aid in reducing interferences. See the reference for a discussion of a procedural validation protocol using the Standard Addition Method.

1. If a centrifuge with the proper rotor is available (see above), it is efficient to use 1.2 mL cluster tubes to perform the pre-analytical matrix modification. First, an appropriate number of empty tubes are arranged in a cluster tube rack; then 300 µL Somogyi Reagent Solution #1 (0.300 M NaOH) is added to each of these tubes. Next, 150 µL sample is added, and mixed with the NaOH by drawing liquid back into the pipette tip and expelling it at least twice. Then 300 µL Somogyi Reagent Solution #2 (5.00% w/v solution of $ZnSO_4 \cdot 7H_2O$ in DI water) is added using a single channel pipette, and each cluster tube is immediately vortexed. This establishes a dilution factor of five.
2. The filled cluster tube rack is placed in a refrigerator or ice bath for 10 min to allow the co-precipitation reaction to occur. The cluster tube rack assembly is then centrifuged at 3700 rpm (maximum speed) for 20 min. The resulting supernatant should be clear with no loss of nitrate.
3. Calibration standards are prepared in DI water in the appropriate range. For blood serum, the typical operating range is 0-2 µg/mL NO_3-N (i.e., 0-143 µM). Fifty µL aliquots of the treated serum samples and calibration standards are pipetted into a Nunc microplate and 170 µL pH 10 ammonium chloride buffer is added.
4. Griess reagent, to be used with the pH 10 ammonium chloride buffer system, is prepared by adding 5 mL aliquots of stock solutions #1 and #3 to a pre-weighed 200 mg portion of sulfanilamide. Sonification may be employed to dissolve the solid.
5. The reduction process and final colorimetric analysis is conducted as previously described using a 60 min reduction period.

Special Instructions for Nitrate Analysis of Tobacco Plant Tissue

The tobacco plant is known to contain (perhaps) hundreds of soluble chemical compounds that could potentially react with nitrite at near neutral pH. This presents a problem common to all nitrate analysis techniques using cadmium reduction. The ParaTechs cadmium reductor reduces nitrate (NO_3) to nitrite (NO_2) during the analytical process, and this NO_2 may then react with the soluble chemicals found in tobacco to form compounds which do not produce a color response, and thus NO_2 would not be measured. This problem can be minimized by raising the pH of the ammonium chloride (NH_4Cl) buffer from 8.5 to 10.0. At the higher pH, the nitrite is not quite as reactive, so most of the NO_2 is stable while the reductor is in place. However, as soon as the Greiss reagent is added, the pH drops to less than 0.1 and the NO_2 will then react with virtually anything.
The Greiss reaction takes place in two stages. In the first stage NO_2 reacts with sulfanilamide, and this intermediate product then reacts with 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NE) reagent to form the color response. When analyzing tobacco tissue (and other plant tissue) we suggest adding extra sulfanilamide to the mixed color reagent in excess of what is normally recommended. This increases the statistical probability that the NO_2 ions will encounter the sulfanilamide before reaction with some other compound occurs. Typically, we weigh 200 mg of extra sulfanilamide into 10 mL of the mixed Greiss reagent. This results in

a nearly saturated solution, and has been found to increase the calculated nitrate levels significantly. Note that the sulfanilamide stock solution to be used is made up in 6 N HCl instead of 3 N HCL. The extra acid is needed to neutralize the extra ammonia in the pH 10 buffer.

Additional Information

The Nitrate Reductor kit does not include an ultrasonic cleaner. We have found that the "Gemoro 1.2 Quart Ultrasonic Cleaner" brand is the most efficient instrument to use for cleaning the reductor between uses and is compatible with the acid-cleaning solution container that is supplied.

Reference

Crutchfield, J.D. & Grove, J.H. (2011) A New Reduction Device for the Microplate Determination of Nitrate in Water, Soil, Plant Tissue, and Physiological Fluids. Journal of AOAC International. Vol. 94. No. 6, pp 1896-1905. http://dx.doi.org/10.5740/jaoacint.10-454

CAUTION: Cadmium is a toxic heavy metal. Due care should be exercised when handling the Reductor, and proper techniques should be used to dispose of the device after its useful life.

Purchase does not include or carry any right to resell or transfer this product either as a stand-alone product or as a component of another product.

PARATECHS COROPORATION
LIMITED WARRANTY

ParaTechs warrants that, at the time of shipment, the Product will conform to the specifications that accompany the Product. This warranty limits ParaTechs' liability to replacement of the Product. **PARATECHS MAKES NO OTHER WARRANTIES, EXPRESS OR IMPLIED, WITH RESPECT TO THE PRODUCT; INCLUDING ANY WARRANTIES OF MERCHANTABILITY OR FITNESS FOR ANY PARTICULAR PURPOSE OR THAT THE PRODUCT DOES NOT INFRINGE ANY PROPRIETARY RIGHTS OF ANY THIRD PARTY.**

For further information on other ParaTechs products contact us at info@paratechs.com or call (859) 317-9213. See also our website at www.paratechs.com.

Revised 07/15/2016