**Determination of nitrate-nitrite concentrations in resin bag extracts**

**Reaction details**

The following protocol details a single reagent vanadium (III) chloride reaction for determining nitrate-nitrite concentrations in resin bag extracts. The reaction moves forward by allowing vanadium (III) chloride to affect the quantitative reduction of nitrate to nitrite to nitric oxide. Nitrate and nitrite are captured by Griess reagents sulfanilamide and NED to produce a pale pink pigment. The concentration of pink pigment is proportional to the nitrate-nitrite concentrations in each extract

**Reagent Preparation**

1. **1M HCl:**

In a 500 mL volumetric flask, bring to volume with ultrapure water (MilliQ):

* + - 42 mL concentrated HCl

1. **2% sulfanilamide solution:**

In a 15 mL centrifuge tube:

* + - 0.2 g sulfanilamide
    - 10 mL 1M HCl solution

1. **0.2% NEDD solution:**

In a 15 mL centrifuge tube:

* + - 0.2 g N-(1-naphthyl)-ethylenediamine dihydrochloride
    - 10 mL ultrapure water

1. **Saturated vanadium chloride solution (make weekly):**

In a 50 mL volumetric flask:

* + - 0.35 g vanadium (III) chloride **CAUTION: REACTIVE WITH AIR, WORK QUICKLY**
    - 50 mL 1M HCl solution

1. **Reagant ‘cocktail’ solution (make after reagent 1-4):**

In a 500 mL volumetric beaker:

* + - 50 mL saturated vanadium chloride solution
    - 3.3 mL 2% sulfanilamide solution
    - 3.3 mL 0.2 NEDD solution
    - 400 mL ultrapure water

1. **Sample matrix (2.0 M NaCl/0.1 M HCl):**

In a 1 L volumetric flask, bring to volume:

* + - 116.88 g sodium chloride
    - 8.4 mL concentrated hydrochloric acid

1. **100 ppm NO3- stock solution (make weekly):**

In a 500 mL volumetric flask, bring to volume with the sample matrix

* + - 360.9 mg potassium nitrate

**Standard Curve**

1. Dilute the 100 ppm NO3- stock solution to 10 ppm by adding 150 μL stock solution to 1350 μL sample matrix in a centrifuge tube
2. Create a 7-point standard curve by diluting the 10 ppm NO3- solution in sample matrix using the dilutions in Table 1

**Table 1**: Dilutions for the 7-point standard curve

|  |  |  |
| --- | --- | --- |
| [NO3-NO2] | 10 ppm NO3 stock (μL) | Matrix (μL) |
| 0 | 0 | 1000 |
| 0.3 | 30 | 970 |
| 0.5 | 50 | 950 |
| 1.0 | 100 | 900 |
| 2.5 | 250 | 750 |
| 5.0 | 500 | 500 |
| 10.0 | 1000 | 0 |

**Assay Procedure**

1. Add 60 μL of each standard in triplicate in wells A1-A7, B1-B7, and C1-C7
2. Add 60 μL of random standards in wells H1-H3. This will serve as a positive control and will be calculated as unknowns to test for the accuracy of the standard curve
3. Add 60 μL of the sample matrix in wells H10-H12. This will serve as a negative control
4. Add 60 μL of unknowns into any empty wells. Be sure to add unknowns in duplicates (i.e. in two separate wells). Sample replicates will be averaged to account for any unaccounted heterogeneity in samples
5. Add 140 μL of Vanadium ‘cocktail’ solution to each well
6. Place a clean lid on the 96-well plate, shake for 60 seconds, and incubate for 5 hours (preferred) or overnight (16-18 hours) at room temperature
7. Turn on the microplate reader and open the computer software. Select a project, or create a new one, and select the “nitrate\_resin” protocol. This protocol will shake the well plate once a plate is loaded and measure each well at 540nm given the plate design in Figure 1

**A few notes**

* Vortex each standard and unknown before pipetting into wells to homogenize sample
* Work quickly and efficiently to reduce evaporation error. Use a multichannel pipette with a solution reservoir to speed up Vanadium ‘cocktail’ assaying process
* The standard curve may need to be altered to avoid extrapolating values from the curve. If a plate has any unknown concentration (not absorbance) values that are >10% larger than the largest concentration value in a standard curve, the standard curve should be expanded and plate should be assayed again
  + To avoid unnecessary labor, run a sample standard curve with a single replicate and problematic unknowns to see if any additional modifications to the curve are needed before re-assaying entire plate
* Samples will lose pigment if the concentration is too high. If this occurs, problematic unknowns need to be re-assayed along with an additional standard curve using a diluted sample : cocktail ratio
  + Steve Allison’s protocol recommends that “high” nitrate-nitrite concentrations be diluted such that 10 μL of sample is assayed against 160 μL vanadium cocktail solution
  + The exact sample : cocktail ratio may need to be further diluted if a particular sample has especially high nitrate-nitrite concentrations
  + Note: Evan’s resin bags from Ithaca required a 1:199 sample: cocktail ratio
* While the microplate reader software creates a standard curve, linear regression for the standard curve, and determines concentrations of all unknown samples, this is not necessarily in a reproducible format, so it is recommended that the user export the plate data with the well plate id and absorbance values to do calculations on their own (see “Microplate analysis” below). There is an R script titled “NO3\_plate\_analysis.R” in the “lab\_protocols” GitHub repository that should help streamline these calculations

**Figure 1**

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**Figure 1** Layout of 96-well plate with standards, positive controls, blanks, and sample loadings

**Microplate analysis**

* Calculate the mean of the blanks (wells H9-H11) and subtract all absorbance measurements by this value
* Construct a linear regression with known standard concentration as the independent variable and blank-corrected absorbance value as the dependent variable and check the R2 value. A passable R2 value is anything greater than 0.98. Individual wells can be removed to achieve a better fit
* Calculate the concentration of each well by subtracting the blank-corrected absorbance by the intercept of the linear regression equation and dividing this value by the slope of the linear regression equation:
* Calculate the coefficient of variation for each duplicate positive control. The coefficient of variation is calculated as the standard deviation divided by the mean. Acceptable controls are <10%
* Calculate nitrate-nitrite concentrations of each unknown sample by quantifying the mean of each duplicate
* The unknown samples are expressed in ppm, which is equivalent to mg L-1. If measuring with soil extracts, units can be converted to mg NO3-NO2 kg-1 by multiplying the amount of extractant used in L (40 μL = 4.0×10-5 L), then dividing by the dry mass of the soil extracted (kg)

**Further protocol and reaction details**

Protocol modified from protocols supplied from Aimée Classen ([https://classenlab.com/)](https://classenlab.com/) and Steve Allison (<https://allisonlab.bio.uci.edu/>). Protocol is based on a single reagent vanadium (III) chloride reaction for determining nitrate concentrations in soil extracts described in Doane & Horwáth (2003).

**References**

Doane, T. A., & Horwáth, W. R. (2003). Spectrophotometric determination of nitrate with a single reagent. *Analytical Letters*, *36*(12), 2713–2722. https://doi.org/10.1081/AL-120024647