Microplate measurement of nitrite + nitrate

DISCLAIMER
Before using this or any other analytical method it is imperative that you check that it works with your samples. The bare minimum is to test accuracy and precision.

- Test accuracy by creating a standard curve by serial dilution of a sample and/or via spike and recovery tests. Both tests will show if the analysis is affected by the sample matrix.
- Test precision by repeated analysis of the same sample. It's best to do separate precision tests for the analytical method (replicate analyses of the same extract) and for the entire extraction and analysis procedure (extract the same sample several times and carry each extract through the analysis procedure). These tests will show you where poor precision is creeping into your analysis.

Remember that your results are qualitative if you rely on a standard curve with a purified analyte.

Principle
The principle of this assay is reduction of nitrate by vanadium(III) combined with detection by the acidic Griess reaction.

Method adapted from:
Katrina M. Miranda,1 Michael G. Espey, and David A. Wink (2001) A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. NITRIC OXIDE: Biology and Chemistry. Vol. 5, No. 1, pp. 62–71

Prepare stock solutions
Make saturated V(III) solution:

- Read the MSDS for VCl(III). It is corrosive (wear gloves + other PPE). Work quickly because it reacts with air
- Dissolve 400 mg of VCl3 in 50 mL of 1 M HCl
- Remove excess solid
- Store in dark at 4ºC (this reagent should last a few weeks)

Make 0.1% NEED (or NEDD) solution:

- Dissolve 0.1 g N-(1-Naphthyl)ethylenediamine dihydrochloride in 100 mL of H2O (may require heat + stirring)
- Store in dark at 4ºC (this reagent should last a few weeks)

Make 2% sulphanilamide solution

- Dissolve 2 g of sulphanilamide in 100 mL of 5% HCl [1](may require heat + stirring)
- Store in dark at 4ºC (this reagent should last a few weeks)

[1] 5% HCl = 15.63 mL of 32% HCl made up to 100 mL

Procedure for KCl and H2O extracts (<u>not for persulfate digests</u>)

Make Vanadium "cocktail"

- Only make enough to last for a day (e.g. 50 mL)
- To a 100-mL beaker add:
    - 5 mL of saturated V(III)
    - 0.33 mL of 2% sulphanilamide
    - 0.66 mL of 0.1% NEED
    - 40 mL of H2O
    - Purge with N2 and keep in closed container

Make 10 mM nitrate stock solution

- Dissolve 0.101 g of KNO3 in 100 mL of DI water
- Store in dark at 4ºC

Make 200 µM nitrate standard

- Pipette 2mL of 10 mM stock solution into 100 mL vol flask
- Make up to 100.0 mL using extractant or sample matrix

Make nitrate std curve solutions

- Table below is for 10 mL final volume. Scale appropriately for other volumes

Conc (µM)	mL of 200 µM	mL of matrix
0	0.0	10.0
10	0.5	9.5
20	1.0	9.0
40	2.0	8.0
80	4.0	6.0
120	6.0	4.0
160	8.0	2.0
200	10.0	0.0

Analysis procedure

- Pipette 100 µL of sample or standard (0-200 µM) into 96-well plate
- Add 100 µL of vanadium cocktail solution
- Shake microplate and/or somehow mix
- Incubate at room temperature for 2-24 hours
- Read absorbance at 540 nm

Notes

- Solutions should turn a pale pink colour. If samples are too concentrated the reaction goes too far and colour is lost.

- If samples are concentrated, either dilute them, or increase amount of vanadium solution and decrease amount of sample (e.g. use 20 µL of sample and 180 µL of vanadium)

	1 std	2 std	3	4	5	6	7	8	9	10	11	12
A	0.00	0.00	Sam 1	Sam 2								
B	10.00	10.00	Sam 1	Sam 2								
C	20.00	20.00										
D	40.00	40.00										
E	80.00	80.00										
F	120.00	120.00										
G	160.00	160.00										Sam 40
H	200.00	200.00										Sam 40

Procedure for persulfate digests

This procedure dilutes everything 5-fold and can cope with the low pH of persulate digests. Because everything is diluted 5-fold there should be no need for further dilution of samples. Normal concentration of nitrate in persulate digests: Unfumigated soil: 50-300 µM; fumigated 200-800 µM

Make 1000 µM nitrate standard

- Pipette 10mL of 10 mM stock solution into 100 mL vol flask
- Make up to 100.0 mL using extractant or sample matrix

Analysis procedure

- Pipette 20 µL of sample or standard (0-1000 µM) into 96-well plate
- Add 80 µL of H2O to each well
- Add 100 µL of vanadium (the saturated V solution, not the cockatil)
- Add 50 µL of sulphanilamide
- Add 50 µL of NEED, and pump up and down to mix
- Incubate at room temperature for 2-3 hours (this gives max. absorbance)
- Read absorbance at 540 nm