1.0 Procedure title

Measuring Nitrate and Nitrite in Algae Culture using Griess Reagent in 96-well Plates

2.0 Procedure impacts and concerns

2.1 Safety

The reagents used in this assay are potentially harmful to human health. Especially important are the use of chemical resistant gloves, and safety glasses for eye protection.

Sulfonamide: LD50 = 3900 mg/kg (Rat)

Review the MSDS for each of the reagents used before starting the assay.

2.2 Quality

Wiping the pipette tip before delivering small volumes, < 10 ul, was essential for accuracy, as verified by the validation.

The effect of reading the color developed plate after 60 minutes has not been studied. To assure valid results read the plate before 60 minutes of color development.

2.3 Delivery

N/A

2.4 Environmental

All waste and unused reagents must be collected in a common container for professional disposal.

2.5 Cost

Cost of reagents per plate was estimated to be about $46.25, when applying the procedure described here applied to four plates. Each kit costs about $185.

2.6 Compliance

Use of the Water Chemistry Template Macro helps to ensure valid data results, as well as expedite the analysis process.

3.0 Related Procedures

TBD — TBD

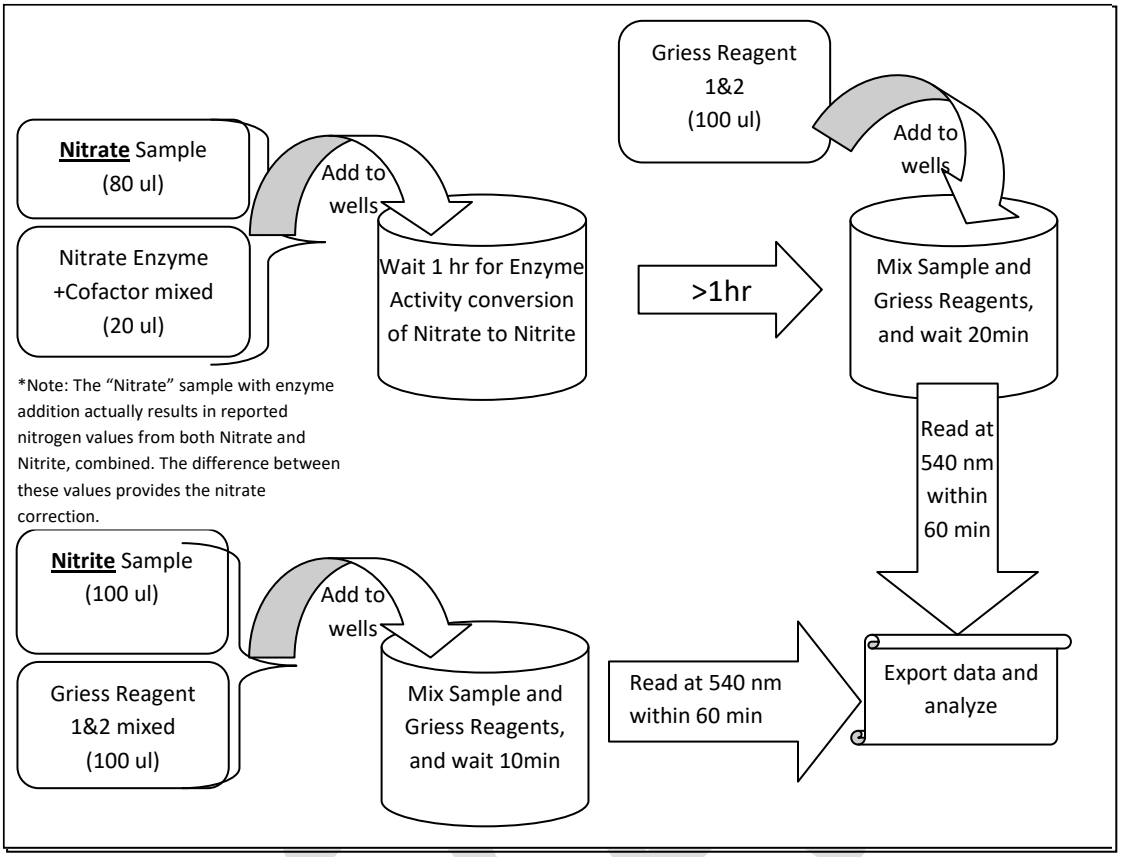
4.0 Responsibilities

Document Owner	Manage content and distribution	Jeremy Ferrara
Process Owner	Responsible for content and process validation	Jeremy Ferrara
Plant Manager	Responsible for implementation and conformance	Nicole Heaps

5.0 Process

5.1 Process description

The method was based on the Caymen Chemical “Nitrate/Nitrite Colorimetric Assay Kit” Item # 780001 The assay works in the following way. Nitrite reacts with sulfanilamide and naphthylethylenediamine, Griess reagents 1&2, to form a deep purple azo compound. Nitrate can be converted to nitrite using nitrate reductase enzyme. The difference between results obtained using enzyme and without using enzyme calculates the amount of nitrate in a sample.



A small amount of buffer is used to dilute the reagents to allow for accurate multichannel pipetting. The reagents for this assay are provided in the Caymen kit. Proper dilutions of the Nitrate and Nitrite standards provided are essential to getting precise results.

The schematic shown in the figure can be accomplished in a standard 96-well culture plate using a final volume of 200 ul per well. The "Nitrate" plate applies the Nitrate Reductase enzyme activity to convert all nitrate to nitrite. For a Nitrate plate, 80 ul of diluted sample is mixed with 20 ul of enzyme, and allowed to react for 1 hour. For Nitrate plate, 100 ul of sample is added to the required wells. Then, 100 ul of Griess reagent mix is added to each well. Color development is allowed to proceed 10 to 60 minutes before reading at 540 nm. The entire process from start to finish can be completed for one full plate applying both Nitrate and Nitrite assay in about 2 hours and 15 minutes; averaging about 1.5 minutes per sample where most of the time would be spent mixing the samples at various steps. Any given mixing event can take about 5 to 10 seconds. Cost of reagents per plate was estimated to be about $46.25, when applying the procedure described here. Each kit costs about $185. Since Row A and Row B of the plate has been designated for standards and QC check samples, each plate would accept up to 72 dilutions of samples, or 36 dilutions of samples in duplicate.

5.2 Process diagram

5.3 Equipment

Vortexer
15 ml PP Conical Tube (Fisher Cat# 05-539-12)
Filter Unit, 0.22 um Cellulose Nitrate (Fisher Cat# 09-761-102)
Pipette aid
Pipette and Pipette tips (20, 200, and 1000 ul)
Pipettes (5, 10, 25 ml)
1.5 ml micro centrifuge tubes (Fisher Cat# 05-408-129)
Clear 96-well culture plates (BD Falcon Cat# 353072)
Kimwipes (Fisher Cat# 06-666-11C)
Spectrophotometer (Abs 540 nm) to read 96-well plates, Molecular Diagnostics SpectraMax M2

5.4 Reagents

Nitrate/Nitrite Colorimetric Assay Kit (Caymen Chemical Cat # 780001)

Potassium phosphate, monobasic buffer (13.61%, 1M) 1 ea
Nitrate Reductase Enzyme Preparation 2 × 1 ea
Nitrate Reductase Cofactor Preparation 2 × 1 ea
Sodium Nitrate Standard 1 ea
Sodium Nitrite Standard 1 ea
Griess Reagent (sulfanilamide) R1 2 × 1 ea
Griess Reagent (naphthylethylenediamine) R2 2 × 1 ea

Ultra Pure water (milliQ or equivalent)

Nitrate QC Standard, 100 ppm NO_3^- (Ricca Chemical Cat # r5307100) (Stable 12 months)
Nitrite QC Standard, 100 ppm NO_2^- (Ricca Chemical Cat # r5444000) (Stable 12 months)

5.5 Process steps

5.5.1 Preparation an algae culture filtrate.

i. Refer to procedure TBD, Filtering algae pond samples for water chemistry. Ref: TBD

5.5.2 Prepare the Assay Buffer/Diluent — Stable 2 months at 4°C.

i. Allow the buffer vial to thaw. Use running water and vortexing to expedite the thaw process.
ii. Dilute the contents of the vial to 100 ml using UltraPure water or equivalent.
iii. Mix thoroughly.

5.5.3 Dissolve the Nitrate and Nitrite kit standards — Vial contents of standard diluted to 1.0 ml creates a 2 mM stock of either NO_3^- or NO_2^-.

i. Remove the metal seal from one each of the Nitrate and Nitrate standard vials.
ii. Carefully, slowly remove the rubber stopper from each vial. Avoid touching the inside of the stopper.
iii. Add 1.0 ml of Assay buffer to each of the standard vials, and recap.
iv. Vortex gently to dissolve.

5.5.4 Dilute the Nitrate and Nitrite standards 1:10 in assay buffer

i. Add 100 ul of nitrate vial contents to 900 ul of assay buffer in a microcentrifuge tube.
ii. Cap and vortex to mix.
iii. Add 100 ul of nitrite vial contents to 900 ul of assay buffer in a microcentrifuge tube.
iv. Cap and vortex to mix.

The 1:10 dilution is required for use in dilutions for the standard curve. The concentration of this 1:10 dilution is 200 uM of either NO_3^- or NO_2^-.

5.5.5 Prepare serial dilutions of standard in microcentrifuge tubes.

i. Use the 1:10 dilution from the previous step to make the dilutions of the standards for the standard curve.
ii. Refer to the table to make the 8 required concentrations of either *Nitrate* or *Nitrite*.
iii. Deliver the required volumes of Stock to Kit Buffer/Diluent.
iv. Cap and vortex thoroughly to mix.

There will be enough volume of each standard to accommodate 2 plates each for both nitrate and nitrate.

	Nitrate **Standard Curve**							***Nitrite*** **Standard Curve**					
Cal#	Vol Stock (ul)	Vol Diluent (ul)	Conc uM in 80 ul	Conc uM in 200ul	ppm NO3 in 80 ul	ppm N in 80 ul		Vol Stock (ul)	Vol Diluent (ul)	Conc uM in 100 ul	Conc uM in 200ul	ppm NO2 in 100 ul	ppm N in 100 ul
1	2	1000	0.40	0.16	0.02	0.0056		2	1000	0.40	0.20	0.0	0.0056
2	25	375	12.5	5	0.8	0.18		25	475	10	5	0.5	0.14
3	50	350	25	10	1.6	0.35		50	450	20	10	0.9	0.28
4	75	325	37.5	15	2.3	0.53		75	425	30	15	1.4	0.42
5	100	300	50	20	3.1	0.70		100	400	40	20	1.8	0.56
6	125	275	62.5	25	3.9	0.88		125	375	50	25	2.3	0.70
7	150	250	75	30	4.7	1.05		150	350	60	30	2.8	0.84
8	175	225	87.5	35	5.4	1.23		175	325	70	35	3.2	0.98

Be sure to wipe the pipette tip before adding the 2 ul stock to 1000 ul of diluent for accurate transfer.

5.5.6 Prepare dilutions (serially) of the algae culture filtrates in Kit Buffer/Diluent

Recommended dilutions for pond samples expected to be between 2 to 100 ppm nitrate and 30 to 500 ppm nitrite are 1:20 and 1:200 dilutions.

i. 1:20 – Add 25 ul of sample to 475 ul of Kit Buffer/Diluent – Rinse tip.
ii. Cap the 1:20 microcentrifuge tube, and vortex thoroughly to mix.
iii. 1:200 – Add 50 ul of the 1:20 dilution to 450 ul of Kit Buffer/Diluent – Rinse tip.
iv. Cap the 1:200 microcentrifuge tube, and vortex thoroughly to mix.

Because the required dilutions tend to exceed 1:40, it is recommended to perform the dilutions in microcentrifuge tubes, serially.

All dilutions are made using some of the 100ml of assay kit buffer provided. If additional buffer is required to make sample dilutions, then use (100 mM KH_2PO_4, pH 7.4).

5.5.7 Prepare the Enzyme+Cofactor Mix to 2.5 ml in Assay Kit Buffer/Diluent.

i. Remove the metal seal from one each of Enzyme and Cofactor vials provided in the kit.
ii. Carefully, slowly remove the rubber stopper from each vial. Avoid touching the inside of the stopper.
iii. Add 2.5 ml of Assay buffer to Enzyme vial.
iv. Recap the enzyme vial and gently agitate to dissolve.
v. Transfer the 2.5 ml from the Enzyme vial to the Cofactor vial.
vi. Recap the cofactor vial and gently agitate to dissolve, and mix the enzyme and cofactor. You may vortex gently to agitate while you do not observe any bubble formation.

Each kit comes with 2 vials each of Enzyme and Cofactor. One set can accommodate 1 *Nitrate* plate when using 20 ul of mix per well.

5.5.8 Deliver Kit Buffer/Diluent Blank and Media Source to A1/B1 and A2/B2, respectively.

i. Add 80 ul of Kit Buffer/Diluent to wells A1 and B1 of the *Nitrate* plate for a quality control check against contaminated water source.

80 ul => Nitrate Plate

ii. Add 80 ul of the media source used in the algae culture to wells A2 and B2 of the *Nitrate* plate for a quality control check on the media.

iii. Add 100 ul of Kit Buffer/Diluent to wells A1 and B1 of the *Nitrite* plate for a quality control check against contaminated water source.

100 ul => Nitrite Plate

iv. Add 100 ul of the media source used in the algae culture to wells A2 and B2 of the *Nitrite* plate for a quality control check on the media.

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Kit Buffer	Media	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6	Cal7	Cal8	QC Lo	QC Hi
B	Kit Buffer	Media	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6	Cal7	Cal8	QC Lo	QC Hi
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
G	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
H	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36

Reference the Plate Map during the next steps.

5.5.9 Deliver calibration standards to Row A and Row B.

i. Add 80 ul of each of the 12 calibration standards to wells A3 through B10 of the *Nitrate* plate.

ii. Add 100 ul of each of the 12 calibration standards to wells A3 through B10 of the *Nitrite* plate

5.5.10 Deliver QC Lo and QC Hi Check Standards.

i. Add 80 ul of each of the QC Lo and QC Hi Check standards to wells A11/B11 and A12/B12 of the *Nitrate* plate, respectively.

ii. Add 100 ul of each of the QC Lo and QC Hi Check standards to wells A11/B11 and A12/B12 of the *Nitrite* plate, respectively.

iii. Concentrations of the QC check standards are shown in the table.

	QC Lo	QC Hi	Units
Nitrate	0.25	5.00	mg NO_3^-/L
	0.056	1.13	ppm N
Nitrite	0.25	3.23	mg NO_2^-/L
	0.98	0.076	ppm N

5.5.11 Deliver the diluted algae culture samples to the plates.

i. Add 80 ul of each of the sample dilutions to the required wells of the *Nitrate* plate.

ii. Add 100 ul of each of the sample dilutions to the required wells of the *Nitrite* plate.

a. Cover the *Nitrite* plate with a 96-well lid.

5.5.12 Deliver the Enzyme+Cofactor Mix to the *Nitrate* plate.

20 ul of mix to each well in the nitrate plate.

i. Transfer the Enzyme+Cofactor mixture to a multichannel pipette trough.
ii. Deliver 20 ul of the mixture to each well in the *Nitrate* plate.
iii. Rinse the tip in the well 3X upon delivery.
iv. Cover the *Nitrate* plate with a 96-well lid.

5.5.13 Prepare the Griess Color Reagent mix

Using the addition of the 4 ml buffer provides enough reagent for 2 full plates without compromising the result.

i. Pour the contents of one bottle each of Griess Reagent 1&2 into a 50 ml conical tube.
ii. Rinse the Griess bottles with 4 ml of Kit Buffer.
iii. Add 4 ml to Griess 1 empty bottle, and swirl.
iv. Transfer the 4 ml from Griess 1 bottle to the Griess 2 empty bottle, and swirl.
v. Transfer the 4 ml from Griess 2 to the 50 ml conical tube containing the mix.
vi. Cap and vortex the Griess Reagent 1&2 mix in the 50 ml conical tube.

5.5.14 Wait 1 hour for enzyme activity – conversion of *Nitrate* to *Nitrite*.

5.5.15 Add the Griess Color Reagent Mix.

i. Add 100 uL of Griess Color Reagent Mix to each of the assay wells in both the *Nitrate* AND *Nitrite* plates.
ii. Pipette up and down briefly, 3X, to mix.

5.5.16 Allow color development to proceed for between 10 and 60 minutes.

5.5.17 Measure Absorbance 540 nm.

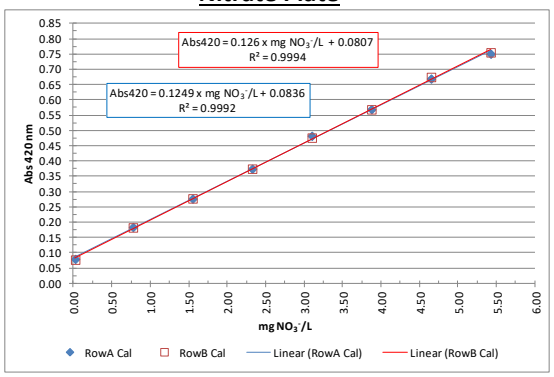
i. Measure the absorbance. Ref: TBD
ii. Export data to text file.

5.5.18 Workup the data using the Water Chemistry Template Macro. Ref: TBD

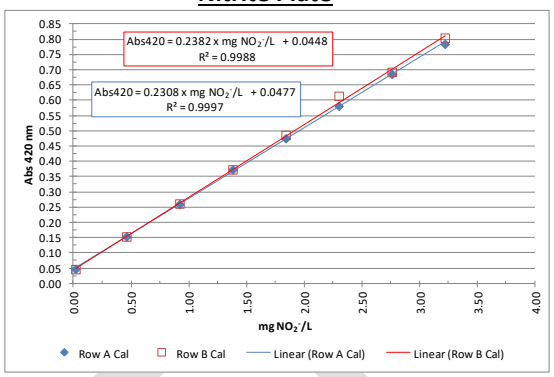
5.6 Example Data

5.6.1 Example of Calibration Standard Curve

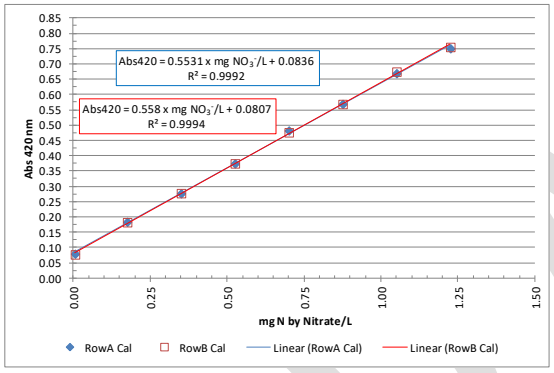
Nitrate Plate

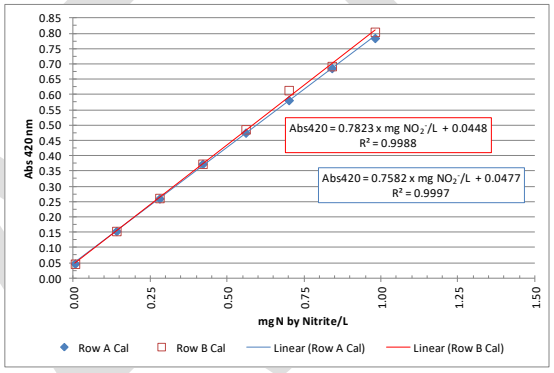


Nitrite Plate



mg Nitrate/L
mg Nitrite/L





mg N/L

5.6.2 Example of QC check standard results with Upper and Lower Bound at +/-3s, respectively.

The QC check standards are used to validate that the experiment was performed accurately using quality reagents. QC check standard results must be "within spec", between the Upper and Lower Bound determined during quality control studies.

Nitrate						Nitrite					
QC-Lo			QC-Hi			QC-Lo			QC-Hi		
	mg NO_3^-/L	mg N/L		mg NO_3^-/L	mg N/L		mg NO_2^-/L	mg N/L		mg NO_2^-/L	mg N/L
Target	0.249	0.056	Target	5.0	1.129	Target	0.249	0.076	Target	**3.0**	0.913
Mean	0.228	0.051	Mean	5.160	1.166	Mean	0.247	0.075	Mean	3.015	0.918
Std Dev, s	0.016	0.004	Std Dev	0.056	0.013	Std Dev, s	0.016	0.005	Std Dev, s	0.025	0.007
3s	0.048	0.011	3s	0.169	0.038	3s	0.047	0.014	3s	0.074	0.022
Upper Bound	0.276	0.062	Upper Bound	5.329	1.204	Upper Bound	0.294	0.090	Upper Bound	3.089	0.940
Lower Bound	0.179	0.040	Lower Bound	4.992	1.128	Lower Bound	0.200	0.061	Lower Bound	2.942	0.896
%Err-Avg	-8.6	-8.6	%Err-Avg	3.2	3.2	%Err-Avg	-0.8	-0.8	%Err-Avg	0.5	0.5
%Err-Hi	10.8	10.8	%Err-Hi	6.6	6.6	%Err-Hi	18.3	18.3	%Err-Hi	3.0	3.0
%Err-Lo	-28.0	-28.0	%Err-Lo	-0.2	-0.2	%Err-Lo	-19.8	-19.8	%Err-Lo	-1.9	-1.9

5.7 Reagent Recipes

5.7.1 Assay Kit Buffer/Diluent [Potassium Phosphate (100mM, pH 7.4)

Dilute the contents of the vial provided in the kit to 100 ml using UltraPure water or equivalent. Stable 2 months

5.7.2 Nitrate QC Check Standards

Use the volumes presented in the tables to dilute the 100 ppm QC stock sources in microcentrifuge tubes.

Nitrate	Stock Source (ppm)	Vol Stock (ul)	Vol Dil (ul)	ppm NO_3^-	ppm N
QC Hi	100	50	950	5.0	1.13
QC Lo	100	3	1200	0.249	0.056

Nitrite	Stock Source (ppm)	Vol Stock (ul)	Vol Dil (ul)	ppm NO_2^-	ppm N
QC Hi	100	30	970	3.0	0.91
QC Lo	100	3	1200	0.249	0.076

6.0 Waste and Safety

6.1 Disposal of reagents

i. All unused reagents and reagent waste is to be collected into a single waste container for professional removal offsite.
ii. Fluid in plates is to be removed and collected with other unused reagents and reagent waste.
iii. Tips should be completely expelled into the assay wells, or into waste collection. When the tips are clear of any fluid, they can be placed in the trash.

6.2 Required PPE

i. Gloves and safety glasses are to be worn for the entire duration of the experiment.

6.3 Potential Health Effect of Reagents

6.3.1 Potential Health Effects of Potassium Dihydrogen Phosphate, Anhydrous, KH_2PO_4

Inhalation: May be harmful if inhaled. May cause respiratory tract irritation.
Ingestion: Ingestion may cause gastrointestinal irritation and diarrhea. NFPA (1,0,0)
Skin Contact: May cause skin irritation.
Eye Contact: May cause eye irritation. Kit Buffer/Diluent
Chronic Exposure: No information found.

6.3.2 Potential Health Effects of Sodium Nitrate, $NaNO_3$

Inhalation: Causes respiratory tract irritation. May cause methemoglobinemia, cyanosis (bluish discoloration of skin due to deficient oxygenation of the blood), convulsions, tachycardia, dyspnea (labored breathing), and death. Danger! Strong oxidizer.
Ingestion: Harmful if swallowed. May cause irritation of the digestive tract. May cause methemoglobinemia, cyanosis (bluish discoloration of skin due to deficient oxygenation of the blood), convulsions, and death. Methemoglobinemia is characterized by dizziness, drowsiness, headache, shortness of breath, cyanosis (bluish discoloration of skin due to deficient oxygenation of the blood), rapid heart rate and chocolate-brown colored blood. NFPA (1,0,0,Ox)
Skin Contact: Causes skin irritation.
Eye Contact: Causes eye irritation.
Chronic Exposure: Sodium nitrate may react with secondary or tertiary amines to form nitrosamines (certain nitrosamines are cancer suspect agents).

6.3.3 Potential Health Effects of Sodium Nitrate, $NaNO_2$

Inhalation: Inhalation of Sodium Nitrite produces irritation of respiratory tract, irregular breathing, sore throat, cough and vomiting. Symptoms might be similar to those of ingestion.
Ingestion: Sodium Nitrite is toxic. As little as 1 grams may be fatal. Ingestion may cause Gastroenteritis, nausea, dizziness, vomiting, rapid heart beat, irregular breathing, coma, convulsions, and death due to circulatory collapse.
Skin Contact: Skin contact may cause irritation with symptoms of redness, swelling, itching and pain.
Eye Contact: Eye contact may cause irritation with symptoms of redness, itching, tearing and pain.
Chronic Exposure: May lead to symptoms of acute toxicity.

Danger! Toxic if swallowed or dust is inhaled.
Oxidizer: May ignite organic materials and react with other materials.

NFPA (2,0,1,Ox)

6.3.4 Potential Health Effects of Sulfanilamide, $C_6H_8N_2O_2S$

Inhalation Irritating to respiratory system. May be harmful if inhaled.
Ingestion May be harmful if swallowed. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.
Skin Contact: Irritating to skin. May be harmful in contact with skin.
Eye Contact: Irritating to eyes.
Chronic Exposure: No data available.

Griess Reagent 1

NFPA (2,1,1)

6.3.5 Potential Health Effects of N-(1-Naphthyl)ethylenediamine, dihydrochloride (Naphthylethylenediamine), $C_{12}H_{14}N_2 \bullet 2\ HCl$

Inhalation: Irritating to respiratory system. May be harmful if inhaled.
Ingestion: Harmful if swallowed. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.
Skin Contact: Irritating to skin. May be harmful in contact with skin.
Eye Contact: Irritating to eyes.
Chronic Exposure: No data available.

Griess Reagent 2

NFPA (2,1,1)

7.0 Required documents

7.1 Input documents

Assay protocol.

7.2 Output documents

i. Absorbance data file in text format with timestamp of the read associated with the file. TBD
ii. Saved data workup from Water Chemistry Template Macro. TBD

8.0 Document control

8.1 Revision history

R0 – Initial Release – <Editor name> <Date>
R1 – <Editor name> <Date>

8.2 Document approval

<Name> <Approval date>

8.3 Document reviewers

<Name> <Name>	<Last reviewed date> <Last reviewed date>

9.0 Risk analysis

<Owner> <RPN>