Enzymatic Assay of NITRATE REDUCTASE (CYTOCHROME)
(EC 1.9.6.1)

PRINCIPLE:

$$\text{Methyl Viologen(red)}^{1} + \text{Nitrate} \xrightarrow{\text{Nitrate Reductase}} \text{Methyl Viologen(ox)} + \text{Nitrite} + H_2O$$

Abbreviations used:
red = reduced
ox = oxidized

CONDITIONS: T = 30°C, pH = 7.0, A_{540nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:[2]

A. 150 mM Potassium Phosphate Buffer, pH 7.0 at 30°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 7.0 at 30°C with 1 M KOH. **PREPARE FRESH**.)

B. 100 mM Sodium Nitrate Solution ($NaNO_3$)
(Prepare 10 ml in deionized water using Sodium Nitrate, Sigma Prod. No. S-5506. **PREPARE FRESH**.)

C. 0.02% (w/v) Methyl Viologen Solution (Viologen)
(Prepare 10 ml in deionized water using Methyl Viologen, Sigma Prod. No. M-2254. **PREPARE FRESH**.)

D. 0.8% (w/v) Sodium Hydrosulfite Solution (HS)
(Prepare 10 ml in deionized water using Sodium Hydrosulfite, Sigma Prod. No. S-1256. **PREPARE FRESH**.)

E. 0.8% (w/v) Sodium Bicarbonate Solution (Bicarb)
(Prepare 10 ml in deionized water using Sodium Bicarbonate, Sigma Prod. No. S-8875.)

REAGENTS: (continued)

F. Sodium Hydrosulfite/Sodium Bicarbonate Solution (Hydro/Bicarb)
(Immediately before use, prepare by adding 5 ml of Reagent D and 5 ml of Reagent E to a suitable container. Do not shake.)

G. 3 M Hydrochloric Acid Solution (HCl)
(Prepare 10 ml in deionized water using Hydrochloric Acid, Sigma Prod. No. H-7020. **PREPARE FRESH**.)

H. 1% (w/v) Sulfanilamide Solution (Sulfa)
(Prepare 5 ml in Reagent G using Sulfanilamide, Sigma Prod. No. S-9251. **PREPARE FRESH**.)

I. 0.01% (w/v) N-(1-Naphthyl)ethylenediamine Solution (Naphthyl)
(Prepare 100 ml in deionized water using N-(1-Naphthyl)ethylenediamine Dihydrochloride, Sigma Prod. No. N-9125. **PREPARE FRESH**.)

J. Nitrate Reductase Enzyme Solution
(Immediately before use, prepare a solution containing approximately 0.075 unit/ml of Nitrate Reductase in cold
deionized water.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers[3]:

	Test	Blank
Deionized Water	------	0.10
Reagent A (Buffer)	0.10	0.10
Reagent B ($NaNO_3$)	0.10	0.10
Reagent C (Viologen)	0.10	0.10
Reagent J (Enzyme Solution)	0.10	------

Mix by inversion and equilibrate to 30°C. Then add[4]:

Reagent F (Hydro/Bicarb)	0.10	0.10

PROCEDURE: (continued)

Mix carefully (do not shake) until the blue color is uniform and incubate at 30°C for exactly 10 minutes. Shake vigorously until the blue color disappears. Then quickly add:

	Test	Blank
Reagent H (Sulfa)	0.50	0.50
Reagent I (Naphthyl)	0.50	0.50

Mix by inversion and incubate at 30°C for 10 minutes. Then add:

Deionized Water	1.50	1.50

Mix by inversion and record the A_{540nm} for both the Test and Blank using a suitable spectrophotometer.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(A_{540nm}\ \text{Test} - A_{540nm}\ \text{Blank})(3.0)(df)}{(10)(17.2)(0.1)}$$

3.0 = Total volume of Colorimetric Assay
df = Dilution factor
10 = Time (in minutes) of the assay as per the Unit Definition
17.2 = Millimolar extinction coefficient[5] of the Diazo-nitrite compound at 540 nm
0.1 = Volume (in milliliter) of enzyme used in the assay

$$\text{Unit/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

Unit/g solid = units/mg solid x 1000

UNIT DEFINITION:

One unit will reduce 1.0 µmole of nitrate to nitrite per minute at pH 7.0 at 30°C in a methyl viologen system.

Enzymatic Assay of NITRATE REDUCTASE (CYTOCHROME) (EC 1.9.6.1)

FINAL ASSAY CONCENTRATION:

In a 0.50 ml reaction mix, the final concentrations are 30 mM potassium phosphate, 0.08% (w/v) sodium hydrosulfite, 0.08% (w/v) sodium bicarbonate, 0.004% (w/v) methyl viologen, 20 mM sodium nitrate, and 0.0075 unit nitrate reductase.

REFERENCE:

Worthington, C.E. (1988) in *Worthington Enzyme Manual* **240-241**, Worthington Biochemical Corporation, Freehold, NJ

Lowe, R.H. and Evans, H.J. (1964) *Biochim. Biophys. Acta.* **85**, 377-389

NOTES:

1. Methyl violgen is utilized as an electron donor in this reaction.

2. Dissolve all reagents in deionized water which have been boiled for 10 minutes and then cooled. After cooling, degas the water to further ensure the removal of oxygen. To dissolve the reagents mix gently to avoid aeration. Never shake any of the reagents. After preparing the different reagents, store in vials which are tightly capped. Store on ice at 0-5°C. It is best to have a minimum volume of air in these containers.

3. Allow the reagents to flow down the side of the vial. It is essential that all of the reagents be virtually free of oxygen in order to prevent the oxidation of the reduced methyl viologen, the substrate for the reaction. If the substrate is oxidized, no blue color will appear and no enzymatic reaction will occur.

4. Before the addition of Reagent F (Hydro/Bicarb) gently invert the container. Do not agitate or mix the solution since this may introduce some oxygen into the mixture.

5. The millimolar extinction coefficient was determined experimentally by Sigma.

6. This assay is based on the cited references.

7. Where Sigma Product or Stock numbers are specified,

equivalent reagents may be substituted.

Enzymatic Assay of NITRATE REDUCTASE (CYTOCHROME) (EC 1.9.6.1)

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.