Enzymatic Assay of NITRATE REDUCTASE
(EC 1.6.6.1)

PRINCIPLE:

Nitrate + ß-NADH $\xrightarrow{\text{Nitrate Reductase}}$ Nitrite + ß-NAD + H_2O

Nitrite + Sulfanilamide + NED ——> Nitrite Color Complex

Abbreviations used:
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form
NED = N-(1-Naphthyl)ethylenediamine Dihydrochloride

CONDITIONS: T = 30°C, pH = 7.3, A_{540nm}, Light path = 1 cm

METHOD: Spectrophotometric Stop Rate Determination

REAGENTS:

A. 25 mM Potassium Phosphate Buffer with 10 mM Potassium Nitrate and 0.05 mM Ethylenediaminetetraacetic Acid, pH 7.3 at 30°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379, Potassium Nitrate, Sigma Prod. No. P-6162, and Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED2SS.)

B. 2.0 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH)
(Dissolve the contents of one 10 mg vial of ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-110, in the appropriate volume of deionized water. **PREPARE FRESH**.)

C. 3 M Hydrochloric Acid Solution (HCl)
(Prepare 100 ml in deionized water using Hydrochloric Acid, Sigma Prod. No. H-7020.)

D. 58 mM Sulfanilamide Solution (Sulf)
(Prepare 100 ml in Reagent C using Sulfanilamide, Sigma Prod. No. S-9251.)

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REAGENTS: (continued)

E. 0.77 mM N-(1-Naphthyl)ethylenediamine Dihydrochloride Solution (NED)
(Prepare 100 ml in deionized water using N-(1-Naphthyl)ethylenediamine Dihydrochloride, Sigma Prod. No. N-9125.)

F. 1.45 mM Nitrate Standard Solution (Std Soln)
(Prepare 50 ml in Reagent A using Sodium Nitrite, Sigma Prod. No. S-2252.)

G. Nitrate Reductase Enzyme Solution
(Immediately before use, prepare a soluiton containing 0.2-0.6 unit/ml of Nitrate Reductase in cold Reagent A.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers[1]:

	Test	Blank	Std1	Std2	Std3	Std4	Std5
Reagent A (Buffer)	1.80	1.90	1.89	1.87	1.85	1.83	1.80
Reagent B (ß-NADH)	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Reagent F (Std Soln)	----	----	0.01	0.03	0.05	0.07	0.10

Mix by swirling and equilibrate to 30°C. Then add:

Reagent G (Enz Soln)	0.10	----	----	----	----	----	----

Immediately mix by swirling and incubate at 30°C for exactly 2 minutes. Stop the reaction by adding:

Reagent D (Sulf)	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Mix thoroughly by swirling, then add:

Reagent E (NED)	1.00	1.00	1.00	1.00	1.00	1.00
						1.00

Mix by swirling and incubate for 10 minutes at 25°C. Transfer the solutions to suitable cuvettes and record the A_{540nm} for the Test, Blank and Standards using a suitable spectrophotometer.

CALCULATIONS:

Standard Curve:

r A_{540nm} Standard = A_{540nm} Std - A_{540nm} Std Blank

CALCULATIONS: (continued)

Prepare a standard curve by plotting the $?A_{540nm}$ of the Standard versus µmoles of nitrite.

Sample Determination:

$r\ A_{540nm}$ Sample = A_{540nm} Test - A_{540nm} Test Blank

Determine the µmoles of nitrite formed using the standard curve.

$$\text{Units/ml enzyme} = \frac{(\mu\text{mole Nitrite formed})(df)}{(2)(0.1)}$$

df = Dilution factor
2 = Time of assay (in minutes)
0.1 = Volume of enzyme (in milliliter) used

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

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will reduce 1.0 µmole of nitrate to nitrite per minute in a ß-NADH system at pH 7.3 at 30°C.

FINAL ASSAY CONCENTRATION:

In a 2.00 ml reaction mix, the final concentrations are 24 mM potassium phosphate, 0.05 mM ethylenediaminetetraacetic acid, 9.5 mM potassium nitrate, 0.10 mM ß-nicotinamide adenine dinucleotide, reduced form, and 0.02 - 0.06 unit nitrate reductase.

REFERENCE:

Redinbaugh, M.G. and Campbell, W.H. (1985) *Journal of Biological Chemistry* **260**, 3380-3385

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REFERENCES:

Smarrelli, Jr., J. and Campbell, W.H. (1983) *Biochimica et Biophysica Acta* **742**, 435-445

NOTES:

1. Perform the color development of the Test and Standards at the same time in order to prevent deviations in color development from the standard curve.

2. This assay is based on the cited references.

3. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.