Enzymatic Assay of NITRATE REDUCTASE (NAD[P]H) (EC 1.6.6.2)

PRINCIPLE:

ß-NADPH + Nitrate $\xrightarrow{\text{Nitrate Reductase}}$ ß-NADP + Nitrite + H_2O

Abbreviations used:
ß-NADPH = ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form
ß-NADP = ß-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form

CONDITIONS: T = 25°C, pH = 7.5, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 100 mM Potassium Phosphate Solution
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379.)

B. 100 mM Potassium Phosphate Buffer, pH 7.5 at 25°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Dibasic, Trihydrate, Sigma Prod. No. P-5504. Adjust to pH 7.5 at 25°C with Reagent A.)

C. 0.1 mM Flavin Adenine Dinucleotide Solution (FAD)
(Prepare 100 ml in deionized water using Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625. **PREPARE FRESH and PROTECT FROM LIGHT.**)

D. 12 mM ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form Solution (ß-NADPH)
(Dissolve the contents of one 10 mg vial of
ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form, Tetrasodium Salt, Sigma Stock No. 201-210, in the appropriate volume of deionized water. **PREPARE FRESH**.)

E. 100 mM Potassium Nitrate Solution (KNO_3)
(Prepare 1 ml in deionized water using Potassium Nitrate, Sigma Prod. No. P-8394.)

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REAGENTS: (continued)

F. Nitrate Reductase Enzyme Solution
(Immediately before use, prepare a solution containing 0.13 - 0.25 unit/ml of Nitrate Reductase in cold Reagent B. Store on ice; use within 5 minutes.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	Blank
Deionized Water	0.80	1.10
Reagent B (Buffer)	1.60	1.60
Reagent C (FAD)	0.15	0.15
Reagent D (ß-NADPH)	0.05	0.05
Reagent E (KNO_3)	0.30	------

Mix by inversion and equilibrate to 25°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent F (Enzyme Solution)	0.10	0.10

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 5 minutes. Obtain the ΔA_{340nm}/ minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})\,(3)\,(df)}{(6.22)\,(0.1)}$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADPH at 340 nm
0.1 = Volume (in milliliter) of enzyme used

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

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CALCULATIONS: (continued)

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

UNIT DEFINITION:

One unit will reduce 1.0 µmole of nitrate per minute in the presence of ß-NADPH at pH 7.5 at 25°C.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are
57 mM potassium phosphate, 0.005 mM flavin adenine dinucleotide, 0.2 mM ß-nicotinamide adenine dinucleotide phosphate, reduced form, 10 mM potassium nitrate and
0.013 - 0.025 unit nitrate reductase.

REFERENCE:

Gilliam, M.B., Sherman, M.P., Griscavage, J.M., and Ignarro, L.J. (1993) *Analytical Biochemistry* **212**, 359-365

NOTES:

1. This assay is based on the cited reference.

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

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