Enzymatic Assay of DIAPHORASE[1] (EC 1.8.1.4)

PRINCIPLE:

DPIP + β-NADH $\xrightarrow{\text{Diaphorase}}$ β-NAD + reduced DPIP

Abbreviations used:
DPIP = 2,6-Dichlorophenol-Indophenol
β-NADH = β-Nicotinamide Adenine Dinucleotide, Reduced Form
β-NAD = β-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 25°C, pH = 7.5, A_{600nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 20 mM Tris HCl Buffer, pH 7.5 at 25°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.5 at 25°C with 1 M HCl.)

B. 0.23 mM β-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β-NADH)
(Dissolve the contents of a 1 mg vial of β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-110, in 60 ml of Reagent A or prepare 6 ml in Reagent A using β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Prod. No. N-8129. **PREPARE FRESH**.)

C. 1.17 mM 2,6-Dichlorophenol-Indophenol Solution (DPIP)
(Prepare 100 ml in deionized water using 2,6-Dichlorophenol-Indophenol, Sodium Salt, Sigma Prod. No. D-1878.)

D. 200 mM Tris HCl Buffer with 294 mM Potassium Chloride, 0.54 mM Flavin Mononucleotide and 0.025% (w/v) Bovine Serum Albumin, pH 7.5 at 25°C (Enzyme Diluent)
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503, Potassium Chloride, Sigma Prod. No. P-4504, Flavin Mononucleotide, Sodium Salt, Sigma Prod. No. F-6750, and Albumin, Bovine, Sigma Prod. No. A-4503. Adjust to pH 7.5 at 25°C with 1 M HCl.)

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REAGENTS: (continued)

E. Diaphorase Enzyme Solution
(Immediately before use, prepare a solution containing 0.05 - 0.1 unit/ml of Diaphorase in cold Reagent D.)

PROCEDURE:

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

	Test	Blank
Reagent B (β-NADH)	2.80	2.80
Reagent C (DPIP)	0.10	0.10

Mix by inversion and equilibrate to 25°C. Monitor the A_{600nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent D (Enzyme Diluent)	------	0.10
Reagent E (Enzyme Solution)	0.10	------

Immediately mix by inversion and record the decrease in the A_{600nm} for 5-10 minutes. Obtain the ΔA_{600nm}/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{600nm}/\text{min Test} - \Delta A_{600nm}/\text{min Blank})(3)(df)}{(21)\,(0.1)}$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
21 = Millimolar extinction coefficient of DPIP at 600 nm
0.1 = Volume (in milliliters) of enzyme used

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

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

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UNIT DEFINITION:

One unit of either "diaphorase" or "lipoyl" dehydrogenase will oxidize 1.0 µmole of β-NADH per minute at pH 7.5 at 25°C, with the corresponding reduction of the appropriate electron acceptor.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 25 mM Tris, 0.22 mM β-nicotinamide adenine dinucleotide, reduced form, 0.039 mM 2,6-dichlorophenol-indophenol, 9.8 mM potassium chloride, 0.018 mM flavin mononucleotide, 0.00083% (w/v) bovine serum albumin, and 0.005 - 0.01 unit diaphorase.

REFERENCES:

Savage, N. (1957) *Biochem. J.* **67**, 146-155.

NOTES:

1. This assay is not to be used for Diaphorase, from Porcine Heart, Sigma Prod. No. D-3752.
2. This assay is based on the cited reference.
3. 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.