Enzymatic Assay of DIAPHORASE\(^1\)
(EC 1.8.1.4)

**PRINCIPLE:**

\[
\text{DPIP} + \beta\text{-NADH} \xrightarrow{\text{Diaphorase}} \beta\text{-NAD} + \text{reduced DPIP}
\]

Abbreviations used:
- DPIP = 2,6-Dichlorophenol-Indophenol
- \(\beta\text{-NADH}\) = \(\beta\text{-Nicotinamide Adenine Dinucleotide, Reduced Form}\)
- \(\beta\text{-NAD}\) = \(\beta\text{-Nicotinamide Adenine Dinucleotide, Oxidized Form}\)

**CONDITIONS:** \(T = 25^\circ \text{C}, \text{pH} = 7.5, A_{600nm}, \text{Light path} = 1 \text{ cm}\)

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

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)
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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:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B</td>
<td>2.80</td>
<td>2.80</td>
</tr>
<tr>
<td>Reagent C</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent D</td>
<td>-----</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent E</td>
<td>0.10</td>
<td>-----</td>
</tr>
</tbody>
</table>

Immediately mix by inversion and record the decrease in the A$_{600\text{nm}}$ for 5-10 minutes. Obtain the ΔA$_{600\text{nm}}$/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

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

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:


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.