Enzymatic Assay of PHOSPHO(ENOL)PYRUVATE CARBOXYLASE
(EC 4.1.1.31)

PRINCIPLE:

PEP + CO₂ + H₂O  \textit{PEP Carboxylase}  \rightarrow \text{Oxalacetate} + P_i

Oxalacetate + β-NADH  \textit{Malic Dehydrogenase}  \rightarrow \text{Malate} + β-NAD

Abbreviations used:
PEP = Phospho(enol)pyruvate
PEP Carboxylase = Phospho(enol)pyruvate Carboxylase
P_i = Inorganic Phosphate
β-NADH = β-Nicotinamide Adenine Dinucleotide, Reduced Form
β-NAD = β-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS:  T = 25°C, pH = 8.5, A₃₄₀nm, Light path = 1 cm

METHOD:  Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 110 mM Tris Sulfate Buffer, pH 8.5 at 25°C
(Prepare 100 ml by dissolving Trizma Base, in 110 mM Sulfuric Acid which was prepared in deionized water with Sulfuric Acid. Adjust to pH 8.5 at 25°C with 1 M NaOH.)

B. 300 mM Magnesium Sulfate Solution (MgSO₄)
(Prepare 10 ml in deionized water using Magnesium Sulfate, Heptahydrate.)

C. 6 mM β-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β-NADH)
(Dissolve the contents of one 10 mg vial of β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, in the appropriate volume of Reagent A. PREPARE FRESH.)
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REAGENTS: (continued)

D. Malic Dehydrogenase Enzyme Solution (MDH)  
(Immediately before use, prepare a solution containing 
600 units/ml in Reagent A using Malic Dehydrogenase.)

E. 100 mM Sodium Bicarbonate Solution (Bicarb)  
(Prepare 10 ml in deionized water using Sodium 
Bicarbonate.)

F. 30 mM Phospho(enol)pyruvate Solution (PEP)  
(Prepare 1 ml in Reagent A using 
Phospho(enol)pyruvate, Monopotassium Salt.)

G. Dioxane  
(Use low peroxide grade)

H. 300 mM Dithioerythritol Solution (DTE)  
(Prepare 1 ml in Reagent A using Dithioerythritol. 
PREPARE FRESH.)

I. 5.0 mM Magnesium Sulfate Solution (Enzyme Diluent)  
(Prepare 20 ml in Reagent A using Magnesium Sulfate, 
Heptahydrate.)

J. Phospho(enol)pyruvate Carboxylase Enzyme Solution  
(Immediately before use, prepare a solution containing 
0.5 - 1 mg/ml of Phospho(enol)pyruvate Carboxylase in 
Reagent I.)

PROCEDURE:

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A (Buffer)</td>
<td>1.80</td>
<td>1.80</td>
</tr>
<tr>
<td>Reagent B (MgSO₄)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent C (β-NADH)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent E (Bicarb)</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Reagent G (Dioxane)</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Reagent H (DTE)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>
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PROCEDURE: (continued)

Mix by inversion and equilibrate to 25°C. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent D (MDH)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Reagent I (Enzyme Diluent)</td>
<td>------</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent J (Enzyme Solution)</td>
<td>0.10</td>
<td>------</td>
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</tbody>
</table>

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

<p>| | | |</p>
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<tbody>
<tr>
<td>Reagent F (PEP)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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

CALCULATIONS:

$$\text{Units/mg enzyme} = \frac{r_{A_{340nm}}/\text{min Test} - r_{A_{340nm}}/\text{min Blank}}{(6.22) \ (\text{mg enzyme/ml RM})}$$

6.22 = Millimolar extinction coefficient of β-NADH at 340 nm
RM = Reaction Mix

UNIT DEFINITION:

One unit will form 1.0 μmole of oxalacetate from phospho(enol)pyruvate and CO$_2$ per minute at pH 8.5 at 25°C.

FINAL ASSAY CONCENTRATION:

In a 2.91 ml reaction mix, the final concentrations are 80 mM Tris sulfate, 10.5 mM magnesium sulfate, 0.21 mM β-NADH, 10 mM sodium bicarbonate, 10% (v/v) dioxane, 10 mM DTE, 1.0 mM PEP, 6 units malic dehydrogenase and 0.05 - 0.1 mg of phospho(enol)pyruvate carboxylase.
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REFERENCE:


NOTES:

1. Malic Dehydrogenase Unit Definition: One unit will convert 1.0 µmole of oxalacetate and β-NADH to L-malate and β-NAD per minute at pH 7.5 at 25°C.

2. All product and stock numbers, unless otherwise indicated, are OUR product and stock numbers.

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