Enzymatic Assay of PYRUVATE OXIDASE
(EC 1.2.3.3)

PRINCIPLE:

Pyruvate + O₂ + Pᵢ → Acetylphosphate + CO₂ + H₂O
Pyruvate Oxidase

FAD, TPP, Mg²⁺

2H₂O₂ + 4-Aminoantipyrine + EHSPT → Quinoneimine dye + 4H₂O

Abbreviations used:
Pᵢ = Inorganic Phosphate
FAD = Flavin Adenine Dinucleotide
TPP = Thiamine Pyrophosphate
EHSPT = N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)-m-Toluidine
POD = Peroxidase

CONDITIONS:  T = 37°C, pH 5.7, A₅₅₀nm, Light path = 1 cm

METHOD:  Spectrophotometric Rate Determination

REAGENTS:

A. 150 mM Potassium Phosphate Buffer, pH 5.9 at 37°C
   (Prepare 100 ml in deionized water using Potassium
   Phosphate, Monobasic, Anhydrous,
   Adjust to pH 5.9 at 37°C with 1 M KOH.)

B. 0.15 mM Flavin Adenine Dinucleotide Solution (FAD)
   (Prepare 10 ml in deionized water using Flavin Adenine
   Dinucleotide, Disodium Salt
   PREPARE FRESH.)

C. 3 mM Cocarboxylase (Thiamine Pyrophosphate) Solution
   (TPP)
   (Prepare 10 ml in deionized water using Cocarboxylase,
   PREPARE FRESH.)

D. 7.4 mM 4-Aminoantipyrine Solution (4-AAP)
   (Prepare 25 ml in deionized water using
   4-Aminoantipyrine, Free Base.)

E. Peroxidase Enzyme Solution (POD)
   (Immediately before use, prepare a solution containing
   50 purpurogallin units/ml in deionized water using
   Peroxidase.)
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REAGENTS:  (continued)

F.  150 mM Magnesium Sulfate Solution (MgSO$_4$)  
(Prepare 5 ml in deionized water using Magnesium Sulfate, Heptahydrate.)

G.  300 mM Sodium Pyruvate Solution (Pyr)  
(Prepare 1 ml in deionized water using Pyruvic Acid, Sodium Salt.)

H.  0.3% (w/v) N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)-m-Toluidine Solution (EHSPT) 
(Prepare 25 ml in deionized water using N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)-m-Toluidine, Sodium Salt)

I.  50 mM Potassium Phosphate Buffer, pH 5.7 at 37°C  
(Enzyme Diluent) 
(Prepare 50 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Adjust to pH 5.7 at 37°C with 1 M KOH.)

J.  15 mM Ethylenediaminetetraacetic Acid Solution (EDTA)  
(Prepare 25 ml in deionized water using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate)

K.  Pyruvate Oxidase Enzyme Solution  
(Immediately before use, prepare a solution containing 0.2 - 0.4 unit/ml of Pyruvate Oxidase in cold Reagent I.)

PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

Reagent A (Buffer)  10.00
Reagent D (4-AAP)  2.00
Reagent H (EHSPT)  2.00
Reagent C (TPP)  2.00
Reagent B (FAD)  2.00
Reagent J (EDTA)  2.00
Reagent F (MgSO$_4$)  2.00
Reagent E (POD)  3.00

Mix by swirling.
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PROCEDURE: (continued)

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Reagent G (Pyr)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 37°C. Monitor the A$_{550\text{nm}}$ 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 I (Enzyme Diluent)</td>
<td>------</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent K (Enzyme Solution)</td>
<td>0.10</td>
<td>-------</td>
</tr>
</tbody>
</table>

Immediately mix by inversion and record the increase in A$_{550\text{nm}}$ for approximately 5 minutes. Obtain the $r$ A$_{550\text{nm}}$/min using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

\[
\text{Units/ml enzyme} = \frac{(r \ A_{550\text{nm}}/\text{min Test} - r \ A_{550\text{nm}}/\text{min Blank})(3.1)(df)}{(36.88)(0.1)(0.5)}
\]

3.1 = Volume (in milliliters) of assay
df = Dilution factor
36.88 = Millimolar extinction coefficient of quinoneimine dye under the assay conditions
0.1 = Volume (in milliliter) of enzyme used
0.5 = Factor based on the equation that one mole of H$_2$O$_2$ produces half a mole of quinoneimine dye

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

UNIT DEFINITION:

One unit will produce 1.0 µmole of H$_2$O$_2$ per minute at pH 5.7 at 37°C during the conversion of pyruvate and phosphate to acetylphosphate and CO$_2$. 
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FINAL ASSAY CONCENTRATIONS:

In a 3.10 ml reaction mix, the final concentrations are 
50 mM potassium phosphate, 0.48 mM 4-aminoantipyrene,  
0.02% (w/v) N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-  
toluidine, 0.2 mM cocarboxylase, 0.0097 mM flavin adenine  
dinucleotide, 0.97 mM ethylenediaminetetraacetic acid, 9.7  
mM magnesium sulfate, 48 mM sodium pyruvate, 15 units  
peroxidase, and  
0.02 - 0.04 unit pyruvate oxidase.

REFERENCES:

of Bacteriology 160, 273-278.

NOTES:

1. This assay is based on the cited reference.

2. Peroxidase Unit Definition: One unit will form 1.0 mg  
purpurogallin from pyrogallol in 20 seconds at pH 6.0  
at 25°C.

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

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