Enzymatic Assay of PYRUVATE KINASE
(EC 2.7.1.40)

PRINCIPLE:

\[ ADP + PEP \xrightarrow{\text{Pyruvate Kinase}} ATP + \text{Pyruvate} \]

\[ \text{Pyruvate} + \beta\text{-NADH} \xrightarrow{\text{Lactic Dehydrogenase}} \text{Lactate} + \beta\text{-NAD} \]

Abbreviations used:
- ADP = Adenosine 5'-Diphosphate
- PEP = Phospho(enol)Pyruvate
- ATP = Adenosine 5'-Triphosphate
- \( \beta\text{-NADH} \) = \( \beta\text{-Nicotinamide Adenine Dinucleotide, Reduced Form} \)
- \( \beta\text{-NAD} \) = \( \beta\text{-Nicotinamide Adenine Dinucleotide, Oxidized Form} \)

CONDITIONS: \( T = 30^\circ C \), \( pH = 7.2 \), \( A_{340nm} \), Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 100 mM Imidazole HCl Buffer, pH 7.2 at 30°C.  
   (Prepare 50 ml in deionized water using Imidazole, Sigma Prod. No. I-0250. Adjust to pH 7.2 with 1 M HCl.)

B. 100 mM Adenosine 5'-Diphosphate Solution (ADP)  
   (Prepare 10 ml in deionized water using Adenosine 5'-Diphosphate, Sodium Salt, Sigma Prod. No. A-2754. PREPARE FRESH.)

C. 1000 mM Magnesium Chloride Solution (MgCl\(_2\))  
   (Prepare 10 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

D. 2500 mM Potassium Chloride Solution (KCl)  
   (Prepare 10 ml in deionized water using Potassium Chloride, Sigma Prod. No. P-4504.)
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REAGENTS: (continued)

E.  155 mM Phospho(enol)pyruvate Solution (PEP)  
(Prepare 10 ml in deionized water using Phospho(enol)pyruvate, Mono(Cyclohexylammonium) Salt, Sigma Prod. No. P-3637.  **PREPARE FRESH.**)

F.  13.1 mM \( \beta \)-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (\( \beta \)-NADH)  
(Dissolve the contents of one 10 mg vial of \( \beta \)-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Stock No. 340-110, in the appropriate volume of deionized water or prepare 1 ml in deionized water using \( \beta \)-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Prod. No. N-8129. **PREPARE FRESH.**)

G.  L-Lactic Dehydrogenase Enzyme Solution (LDH)  
(Immediately before use, prepare a solution containing 400 units/ml in deionized water using L-Lactic Dehydrogenase, Sigma Prod. No. L-2500.)

H.  Pyruvate Kinase Enzyme Solution (PK)  
(Immedately before use, prepare a solution containing 0.15 - 0.30 unit/ml of Pyruvate Kinase in cold deionized water.)

PROCEDURE:

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

<table>
<thead>
<tr>
<th>Reagent A (Buffer)</th>
<th>19.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B (ADP)</td>
<td>2.00</td>
</tr>
<tr>
<td>Reagent C (MgCl(_2))</td>
<td>0.40</td>
</tr>
<tr>
<td>Reagent D (KCl)</td>
<td>0.75</td>
</tr>
<tr>
<td>Deionized water</td>
<td>2.50</td>
</tr>
<tr>
<td>Reagent F (( \beta )-NADH)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Mix and adjust to pH 7.2 at 30°C with 100 mM HCl or 100 mM KOH, if necessary.
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PROCEDURE: (continued)

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>Reaction Cocktail</td>
<td>2.75</td>
<td>2.85</td>
</tr>
<tr>
<td>Reagent G (LDH)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Reagent E (PEP)</td>
<td>0.10</td>
<td>-----</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 30°C. Monitor the $A_{340nm}$ 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>Deionized Water</td>
<td>-----</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent H (PK)</td>
<td>0.10</td>
<td>-----</td>
</tr>
</tbody>
</table>

Immediately mix by inversion and record the decrease in $A_{340nm}$ for approximately 10 minutes. Obtain the $\Delta A_{340nm}$/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 $\beta$-NADH at 340nm
0.1 = Volume (in milliliters) of enzyme used

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

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

UNIT DEFINITION:

One unit will convert 1.0 µmole of phospho(enol)pyruvate to pyruvate per minute at pH 7.2 at 30°C.
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FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 72 mM imidazole, 7.6 mM adenosine 5'-diphosphate, 15.2 mM magnesium chloride, 71.2 mM potassium chloride, 5.2 mM phospho(enol)pyruvate, 0.19 mM β-nicotinamide adenine dinucleotide, reduced form, 20 units L-lactic dehydrogenase and 0.015 - 0.030 unit pyruvate kinase.

NOTES:

1. L-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 µmole of pyruvate to L-lactate per minute at pH 7.5 at 37°C.

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.