**Enzymatic Assay of PYRUVATE KINASE**  
*(EC 2.7.1.40)*  
*From Rabbit Liver*

**PRINCIPLE:**

Phospho(enol)pyruvate + ADP $\xrightarrow{\text{Pyruvate Kinase}}$ Pyruvate + ATP $\xrightarrow{\text{Mg}^2+}$

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

Abbreviations used:
- **ADP**: Adenosine 5'-Diphosphate
- **ATP**: Adenosine 5'-Triphosphate
- **$\beta$-NADH**: $\beta$-Nicotinamide Adenine Dinucleotide, Reduced Form
- **$\beta$-NAD**: $\beta$-Nicotinamide Adenine Dinucleotide, Oxidized Form

**CONDITIONS:**  
- $T = 37^\circ C$, pH = 7.6, $A_{340\text{nm}}$, Light path = 1 cm

**METHOD:**  
Continuous Spectrophotometric Rate Determination

**REAGENTS:**

A. 100 mM Potassium Phosphate Buffer, pH 7.6 at 37°C.  
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous.  
Adjust to pH 7.6 at 37°C with 1 M KOH.)

B. 8.0 mM Phospho(enol)pyruvate Solution (PEP)  
(Prepare 1 ml in deionized water using Phospho(enol)Pyruvate, Monopotassium Salt.)

C. 3 mM $\beta$-Nicotinamide Adenine Dinucleotide, Reduced Form Solution ($\beta$-NADH)  
(Dissolve the contents of a 10 mg vial of $\beta$-Nicotinamide Adenine Dinucleotide, Reduced Form,  
Disodium Salt, in the appropriate volume of Reagent A.  
**PREPARE FRESH.**)  

D. 100 mM Magnesium Sulfate Solution  
(Prepare 1 ml in deionized water using Magnesium Sulfate, Heptahydrate.)
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REAGENTS: (continued)

E. 40 mM Adenosine Diphosphate Solution (ADP)
(Prepare 1 ml in deionized water using Adenosine 5'-Diphosphate, Di(Monocyclohexylammonium) Salt.)

F. L-Lactic Dehydrogenase Solution (LDH)
(Immediately before use, prepare a solution containing 500 units/ml of L-Lactic Dehydrogenase, in cold Reagent A.)

G. 30 mM Fructose 1,6-Diphosphate Solution (F 1,6-P)
(Prepare 2 ml in deionized water using Fructose 1,6-Diphosphate, Sodium Salt.)

H. Pyruvate Kinase
(Immediately before use, prepare a solution containing 0.3 - 0.6 unit/ml of Pyruvate Kinase in cold Reagent A.)

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>Deionized Water</td>
<td>1.30</td>
<td>1.30</td>
</tr>
<tr>
<td>Reagent A (Buffer)</td>
<td>0.80</td>
<td>0.90</td>
</tr>
<tr>
<td>Reagent B (PEP)</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Reagent C (β-NADH)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Reagent D (Magnesium Sulfate)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Reagent E (ADP)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent F (LDH)</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Reagent G (F 1,6-P)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 37°C. Monitor the A\textsubscript{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent H (Enzyme Solution) 0.10

Immediately mix by inversion and record the decrease in A\textsubscript{340nm} for approximately 5 minutes. Obtain the \( r \) A\textsubscript{340nm}/minute using the maximum linear rate for both the Test and Blank.
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CALCULATIONS:

\[
\text{Units/ml enzyme} = \frac{(r_{A_{340nm/min Test}} - r_{A_{340nm/min Blank}})(3)(df)}{(6.22)(0.1)}
\]

3 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of β-NADH at 340 nm
0.1 = Volume (in milliliters) of enzyme used

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

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

UNIT DEFINITION:

One unit will convert 1.0 µmole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C in the presence of 1.0 mM of fructose 1,6-diphosphate. In the absence of added fructose 1,6-diphosphate which acts as an (activator), considerably lower activity will be observed.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 38 mM potassium phosphate, 0.43 mM phospho(enol)pyruvate, 0.2 mM β-nicotinamide adenine dinucleotide, 6.7 mM magnesium sulfate, 1.3 mM adenosine 5'-diphosphate, 20 units lactic dehydrogenase, 1 mM fructose 1,6-diphosphate and 0.03 to 0.06 unit pyruvate kinase.

REFERENCE:


NOTES:

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

2. This assay is based on the cited reference.

This procedure is for informational purposes.