Enzymatic Assay of HEXOKINASE

PRINCIPLE:

Glucose + ATP $\xrightarrow{\text{Hexokinase}}$ Glucose 6-Phosphate + ADP + H⁺
Cresol Red + H⁺ $\xrightarrow{}$ Reduced Cresol Red

Abbreviations used:
ATP = Adenosine 5′-Triphosphate
ADP = Adenosine 5′-Diphosphate

CONDITIONS:  T = 25°C, pH 8.5, A$_{560\text{nm}}$, Light path = 1 cm

METHOD:  Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 100 mM Glycylglycine Buffer, pH 8.5 at 25°C.
(Prepare 100 ml in deionized water using Glycylglycine, Free Base. Adjust to pH 8.5 at 25°C with 1 M HCl.)

B. 200 mM Adenosine 5′-Triphosphate Solution (ATP)
(Prepare 10 ml in deionized water using Adenosine 5′-Triphosphate, Disodium Salt. PREPARE FRESH.)

C. 200 mM Glucose Solution (Gluc)
(Prepare 10 ml in deionized water using β-(+)-Glucose.)

D. 0.01% Cresol Red with 128 mM Magnesium Chloride Solution (Cresol Red)
(Prepare 200 ml in deionized water using Cresol Red, Sodium Salt and Magnesium Chloride, Hexahydrate. Facilitate by first dissolving Cresol Red into 6.6 ml of 95% ethanol. Transfer this solution to a 200 ml graduated cylinder and add 5.2 g of Magnesium Chloride, Hexahydrate. Dilute to 200 ml with deionized water.)
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REAGENTS: (continued)

E. 100 mM Hydrochloric Acid Standardized Solution (HCl) (Prepare 1 liter in deionized water using Concentrated Hydrochloric Acid. Standardize against Tris Base with 121 indicator. Color change is from orange to pink.)

F. 0.5% Glucose Solution (Prepare 50 ml using β-D-Glucose.)

G. Hexokinase Enzyme Solution (Immediately before use, prepare a solution containing 10 units/ml of Hexokinase in Reagent F.)

PROCEDURE:

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

Reagent B (ATP)  5.00
Reagent D (Cresol Red)  6.60

Mix and slowly add 0.1 M NaOH until the solution just turns from red to purple (pH about 8.2). Then add:

Deionized Water  33.40
Reagent A (Buffer)  5.00

Mix. Adjust to pH 8.5 at 25°C with 100 mM HCl or 100 mM NaOH, if necessary.

Titer Determination:

Determine titer of reaction cocktail by pipetting (in milliliters) the following reagents into a suitable cuvette:

Reaction Cocktail  2.50
Reagent C (Glucose)  0.40

Mix by inversion and equilibrate to 25°C. Monitor the A_{560nm} until constant, using a suitably thermostatted spectrophotometer. Record the initial A_{560nm}. Then add:

Reagent D (HCl)  0.10

Mix and immediately record final A_{560nm}. 
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PROCEDURE: (continued)

\[
\text{Titer} = \frac{(A_{560nm \text{ initial}} - A_{560nm \text{ final}}) \times 2.9}{(1000) \times (0.1) \times \text{(Molarity of Reagent D)}}
\]

2.9 = Volume of Titer reaction Mix
1000 = Conversion from millimolar to micromolar
0.1 = Volume of Reagent D used

Sample:

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

<table>
<thead>
<tr>
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<th>Test</th>
<th>Blank</th>
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<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Reagent C (Gluc)</td>
<td>0.40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 25°C. Monitor the \(A_{560nm}\) until constant, using a suitably thermostatted spectrophotometer. Then add:

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<thead>
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<tr>
<td>Reagent F (Diluent)</td>
<td>------</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent G (Enzyme Solution)</td>
<td>0.10</td>
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Immediately mix by inversion and record the decrease in \(A_{560nm}\) for approximately 5 minutes. Obtain the \(\frac{A_{560nm}}{\text{min}}\) using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

\[
\text{Units/mg enzyme} = \frac{A_{560nm/\text{min Test}} - A_{560nm/\text{min Blank}}}{(\text{titer}) \times (\text{mg enzyme/ml RM})}
\]

RM = Reaction Mix

UNIT DEFINITION:

One unit will phosphorylate 1.0 µmole of glucose per minute at pH 8.5 (±0.5) at 25°C.
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FINAL ASSAY CONCENTRATIONS:

In a 3 ml reaction mix, the final concentrations are 8.3 mM glycylglycine, 17 mM ATP, 0.0011% cresol red, 14 mM magnesium chloride, 27 mM glucose and 1 unit hexokinase.

REFERENCE


NOTES:

1. 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.