Enzymatic Assay of HEXOKINASE

PRINCIPLE:

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

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, Prod. No. G-1002. 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, Prod. No. A-5394. PREPARE FRESH.)

C. 200 mM Glucose Solution (Gluc)
   (Prepare 10 ml in deionized water using β-(+)-Glucose, Prod. No. G-5250.)

D. 0.01% Cresol Red with 128 mM Magnesium Chloride Solution (Cresol Red)
   (Prepare 200 ml in deionized water using Cresol Red, Sodium Salt, Prod. No. C-9877, and Magnesium Chloride, Hexahydrate, Prod. No. M-0250. 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 Sigma 121 indicator. Color change is from orange to pink.)

F. 0.5% Glucose Solution  
(Prepare 50 ml using β-D-Glucose, Prod. No. G-5250.)

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 $A_{560\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Record the initial $A_{560\text{nm}}$. Then add:

| Reagent D (HCl) | 0.10 |

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

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

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>
<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 C (Gluc)</td>
<td>0.40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

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

| Reagent F (Diluent) | -------- | 0.10 |
| Reagent G (Enzyme Solution) | 0.10 | ------ |

Immediately mix by inversion and record the decrease in \(A_{560\text{nm}}\) for approximately 5 minutes. Obtain the \(\frac{A_{560\text{nm}}}{\text{minute}}\) for both the Test and Blank.

CALCULATIONS:

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

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 Sigma product and stock numbers.

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