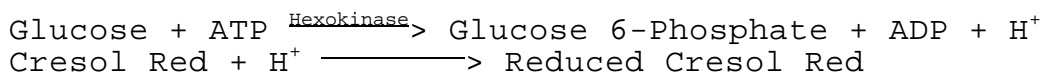


Enzymatic Assay of HEXOKINASE

PRINCIPLE:



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
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Mix and immediately record final $A_{560\text{nm}}$.

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PROCEDURE: (continued)

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

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:

	<u>Test</u>	<u>Blank</u>
Reaction Cocktail	2.50	2.50
Reagent C (Gluc)	0.40	0.40

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 $r A_{560\text{nm}}/\text{minute}$ using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

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

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

Darrow, R.A., and Colowick, S.P. (1962) *Methods in Enzymology* **V**, 226-227.

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.