Enzymatic Assay of HEXOKINASE INSOLUBLE
(EC 2.7.1.1)

PRINCIPLE:

$_D$-Glucose + ATP $\xrightarrow{\text{Hexokinase}}$ $_D$-Glucose 6-Phosphate + ADP

$_D$-Glucose 6-Phosphate + ß-NADP $\xrightarrow{\text{G-6-PDH}}$ 6-PG + ß-NADPH

Abbreviations used:
ATP = Adenosine 5'-Triphosphate
ADP = Adenosine 5'-Diphosphate
G-6-PDH = Glucose-6-Phosphate Dehydrogenase
ß-NADP = ß-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form
ß-NADPH = ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form
6-PG = 6-Phospho-$_D$-Gluconate

CONDITIONS: T = 30°C, pH = 7.4, A_{340nm}, Light path = 1 cm

METHOD: Spectrophotometric Rate Determination

REAGENTS:

A. 250 mM Glycylglycine Buffer, pH 7.4 at 30°C
(Prepare 100 ml in deionized water using Gly-Gly, Hydrochloride, Sigma Prod. No. G-1127. Adjust to pH 7.4 at 30°C with 1 M NaOH.)

B. 1 M Glucose Solution (Glucose)
(Prepare 25 ml in deionized water using ß-$_D$(+)Glucose, Sigma Prod. No. G-5250.)

C. 20 mM ß-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form, Solution (ß-NADP)
(Prepare 10 ml in deionized water using ß-Nicotinamide Adenine Dinucleotide Phosphate, Sodium Salt, Sigma Prod. No. N-0505. **PREPARE FRESH**.)

D. 300 mM Magnesium Chloride Solution ($MgCl_2$)
(Prepare 10 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

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REAGENTS: (continued)

E. 30 mM Adenosine 5'-Triphosphate Solution (ATP)
(Prepare 10 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-5394.)

F. Glucose-6-Phosphate Dehydrogenase Enzyme Solution (G-6-PDH)
(Immediately before use, prepare a solution containing 50 units/ml of Glucose-6-Phosphate Dehydrogenase, Sigma Prod. No. G-6378 in cold Reagent A.)

G. Hexokinase Insoluble Enzyme Suspension (Hex-Insol)
(Immediately before use, prepare a suspension containing 0.5 - 1.0 unit/ml of Hexokinase, Insoluble Enzyme Attached to Beaded Agarose in cold deionized water.)

PROCEDURE:

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

Deionized Water	41.40
Reagent A (Buffer)	10.00
Reagent E (ATP)	2.00
Reagent B (Glucose)	4.00
Reagent C (ß-NADP)	2.00
Reagent D ($MgCl_2$)	2.00
Reagent F (G-6-PDH)	2.00

Mix by swirling and adjust to pH 7.4 at 30°C with either 1 M HCl or 1 M NaOH if necessary. Incubate at 30°C for 5 minutes and then pipette (in milliliters) the following reagents into 50 ml beakers:

	Test	Blank
Reaction Cocktail	20.00	20.00

Equilibrate to 30°C. Then add:

Reagent G (Hex-Insol)	0.15	------
Deionized Water	------	0.15

Mix continuously by stirring and incubate at 30°C for exactly
5 minutes. Immediately filter the samples through Whatman #54 filter paper and transfer the filtrates from both the

Test and Blank to suitable cuvettes and record the A_{340nm} for approximately 3 minutes using a suitable spectrophotometer.

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PROCEDURE: (continued)

The final A_{340} after the 5 minute reaction time (prior to filtration) is the result of the total Hexokinase activity (which is the sum of the soluble and insoluble Hexokinase activities). The soluble activity is indicated by the increasing linear change in A_{340} after filtration. The insoluble activity is the difference between the total activity and the soluble activity.

CALCULATIONS:

Use the initial spectrophotometric readings of the filtered Test and Blank to calculate the total activity.

Total Activity (Units/ml enzyme) =

$$\frac{(A_{340nm}\ \text{Test} - A_{340nm}\ \text{Blank})(20.15)(df)}{(5)(6.22)(0.15)}$$

Use the $r\ A_{340}$/min determined for the filtered Test and Blank to calculate the soluble activity.

Soluble Activity (Units/ml enzyme) =

$$\frac{(r\ A_{340nm}/\text{min Test} - r\ A_{340nm}/\text{min Blank})(20.15)(df)}{(6.22)(0.15)}$$

20.15 = Total volume (in milliliters) of assay
df = Dilution factor
5 = Time (in minutes) of assay
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm
0.15 = Volume (in milliliter) of enzyme used

Insoluble Activity (Units/ml enzyme) = Total Activity - Soluble Activity

$$\text{Insoluble Activity (Units/g)} = \frac{\text{units/ml enzyme (1000)}}{\text{mg dry agarose/ml enzyme solution}}$$

UNIT DEFINITION:

One unit will phosphorylate 1.0 µmole of glucose per minute at pH 7.4 at 30°C.

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FINAL ASSAY CONCENTRATION:

In a 20.15 ml reaction mix, the final concentrations are 39 mM glycylglycine, 63 mM glucose, 0.63 mM ß-nicotinamide adenine dinucleotide phosphate, 9.4 mM magnesium chloride, 0.94 mM adenosine 5'-triphosphate, 32 units glucose-6-phosphate dehydrogenase, and 0.075 - 0.15 unit hexokinase, insoluble enzyme attached to beaded agarose.

REFERENCE:

Bergmeyer, H.U., Gawehn, K., and Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U. ed.) 2nd ed., Volume I, 473-474

NOTES:

1. Sigma currently uses a computer program written by Research Instruments International for use with UVIKON spectrophotometers which determines the amount of soluble enzyme activity present.

2. Glucose-6-Phosphate Dehydrogenase Unit Definition: One unit will oxidize 1.0 µmole of D-glucose 6-phosphate to 6-phospho-D-gluconate per minute in the presence of ß-NADP at pH 7.4 at 25°C.

3. This assay is based on the cited reference.

4. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.