Enzymatic Assay of FRUCTOSE-6-PHOSPHATE KINASE
(EC 2.7.1.11)
from Rabbit Liver

PRINCIPLE:

$$\text{ATP} + {}_{D}\text{-Fructose 6-Phosphate} \xrightarrow[Mg^{2+}]{\text{PFK}} \text{F-1,6-DP} + \text{ADP}$$

$$\text{F-1,6-DP} \xrightarrow{\text{Aldolase}} \text{GAP} + \text{DHAP}$$

$$\text{GAP} \xrightarrow{\text{TPI}} \text{DHAP}$$

$$2\text{DHAP} + 2\ \text{ß-NADH} \xrightarrow{\text{GDH}} 2\ \text{Glycerol-3-Phosphate} + 2\ \text{ß-NAD}$$

Abbreviations used:
ATP = Adenosine 5'-Triphosphate
PFK = Fructose-6-Phosphate Kinase
F-1,6-DP = $_{D}$-Fructose 1,6-Diphosphate
ADP = Adenosine 5'-Diphosphate
GAP = D-Glyceraldehyde 3-Phosphate
DHAP = Dihydroxyacetone Phosphate
TPI = Triosephosphate Isomerase
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
GDH = a-Glycerophosphate Dehydrogenase
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 30°C, pH = 8.0, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 60 mM Tris HCl Buffer, pH 8.0 at 30°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.0 at 30°C with 1 M HCl.)

B. 30 mM $_{DL}$-Dithiothreitol Solution (DTT)
(Prepare 10 ml in deionized water using $_{DL}$-Dithiothreitol, Sigma Prod. No. D-0632.)

C. 60 mM Adenosine 5'-Triphosphate Solution (ATP)
(Prepare 10 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-6144.)

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REAGENTS: (continued)

D. 6 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH)
(Dissolve the contents of one 5 mg vial of ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-105, in the appropriate volume of deionized water.)

E. 750 mM Magnesium Chloride Solution ($MgCl_2$)
(Prepare 10 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

F. Aldolase Enzyme Solution (Aldolase)
(Immediately before use, prepare a solution containing 100 units/ml of Aldolase, Sigma Prod. No. A-1893, in cold deionized water.)

G. a-Glycerophosphate Dehydrogenase/Triosephosphate Isomerase Enzyme Solution[1] (GDH/TPI)
(Immediately before use, prepare a solution containing 100 GDH units/ml using a-Glycerophosphate Dehydrogenase-Triosephosphate Isomerase, Sigma Prod. No. G-6755, in cold deionized water.)

H. 120 mM D-Fructose 6-Phosphate Solution (F-6P)
(Prepare 10 ml in deionized water using D-Fructose 6-Phosphate, Disodium Salt, Sigma Prod. No. F-3627.)

I. Fructose-6-Phosphate Kinase Enzyme Solution (PKF)
(Immediately before use, prepare a solution containing 0.5 - 1.0 unit/ml in cold deionized water.)

PROCEDURE:

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

	Test	Blank
Reagent A (Buffer)	2.50	2.50
Reagent B (DTT)	0.10	0.10
Reagent C (ATP)	0.10	0.10
Reagent D (ß-NADH)	0.10	0.10
Reagent E ($MgCl_2$)	0.02	0.02
Reagent F (Aldolase)	0.02	0.02
Reagent G (GDH/TPI)	0.01	0.01
Reagent I (PFK)	0.05	------
Deionized Water	------	0.05

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PROCEDURE: (continued)

Mix by inversion and equilibrate to 30°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

	Test	Blank
Reagent H (F-6P)	0.10	0.10

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 5 minutes. Obtain the $r\ A_{340nm}$/min using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(r\ A_{340nm}/\text{min Test} - r\ A_{340nm}/\text{min Blank})(3)(df)}{(6.22)(0.05)(2)}$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm
0.05 = Volume (in milliliter) of enzyme used
2 = Conversion factor accounting for 2 moles of ß-NADH oxidized per mole of D-fructose-1,6-diphosphate produced

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

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

UNIT DEFINITION:

One unit will convert 1.0 µmole of fructose 6-phosphate and ATP to fructose 1,6-diphosphate and ADP per minute at pH 8.0 at 30°C in a coupled system with aldolase, glycerophosphate dehydrogenase and triosephosphate isomerase.

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FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 50 mM Tris, 1 mM DL-dithiothreitol, 2 mM adenosine 5'-triphosphate, 0.2 mM ß-nicotinamide adenine dinucleotide, reduced form, 5 mM magnesium chloride, 2 units aldolase, 1 unit a-glycerophosphate dehydrogenase, 8 units triosephosphate isomerase, 4 mM fructose 6-phosphate, and 0.025 - 0.05 unit fructose-6-phosphate kinase.

REFERENCE:

Massey, T.H. and Deal, Jr., W.C. (1973) *Journal of Biological Chemistry* **248**, 56-62

NOTES:

1. The Triosephosphate Isomerase activity is approximately 8 fold that of the a-Glycerophosphate Dehydrogenase activity.

2. Aldolase Unit Definition: One unit will convert 1.0 µmole of fructose 1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate per minute at pH 7.4 at 25°C.

3. a-Glycerophosphate Dehydrogenase Unit Definition: One unit will convert 1.0 µmole of dihydroxyacetone phosphate to a-glycerophosphate per minute at pH 7.4 at 25°C.

4. Triosephosphate Isomerase Unit Definition: One unit will convert 1.0 µmole of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate per minute at pH 7.6 at 25°C.

5. This assay is based on the cited reference.

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