Enzymatic Assay of FRUCTOSE-6-PHOSPHATE KINASE
(EC 2.7.1.11)
from Bacillus stearothermophilus

PRINCIPLE:

F-6-P + ATP $\xrightarrow{\text{F-6-PK}}$ F-1,6-DP + ADP

ADP + PEP $\xrightarrow{\text{Pyruvate Kinase}}$ ATP + Pyruvate

Pyruvate + ß-NADH $\xrightarrow{\text{Lactic Dehydrogenase}}$ Lactate + ß-NAD

Abbreviations used:
F-6-P = Fructose 6-Phosphate
ATP = Adenosine 5'-Triphosphate
F-6-PK = Fructose-6-Phosphate Kinase
F-1,6-DP = Fructose 1,6-Diphosphate
ADP = Adenosine 5'-Diphosphate
PEP = Phospho(enol)Pyruvate
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 30°C, pH = 9.0, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 100 mM Tris Buffer, pH 9.0 at 30°C.
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 9.0 at 30°C with 1 M HCl.)

B. 100 mM Magnesium Sulfate Solution ($MgSO_4$)
(Prepare 10 ml in deionized water using Magnesium Sulfate, Heptahydrate, Sigma Prod. No. M-1880.)

C. 56 mM Phospho(enol)pyruvate Solution (PEP)
(Prepare 5 ml in deionized water using Phospho(enol)pyruvate, Trisodium Salt, Hydrate, Sigma Prod. No. P-7002. **PREPARE FRESH.**)

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REAGENTS: (continued)

D. 2.5 M Potassium Chloride Solution (KCl)
(Prepare 5 ml in deionized water using Potassium Chloride, Sigma Prod. No. P-4504.)

E. 500 mM Fructose 6-Phosphate Solution (F-6-P)
(Prepare 10 ml in deionized water using D-Fructose 6-Phosphate, Disodium Salt, Sigma Prod. No. F-3627. **PREPARE FRESH**.)

F. 100 mM Adenosine 5'-Triphosphate Solution (ATP)
(Prepare 8.2 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-5394 and then add 1.8 ml of 1 N NaOH.[1] **PREPARE FRESH**.)

G. 13.1 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH)
(Prepare 1 ml in deionized water using ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Prod No. N-8129 or dissolve the contents of one 10 mg vial of ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-110, in the appropriate volume of deionized water. **PREPARE FRESH.**)

H. PK/LDH Enzyme Suspension[2] (PK/LDH)
(Use PK/LDH Enzymes Suspension, Stock No. 40-7.)

I. 50 mM Tris HCl Buffer, pH 8.5 at 30°C (Enz Dil)
(Prepare 50 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.5 at 30°C with 1 M HCl.)

J. Fructose-6-Phosphate Kinase Enzyme Solution
(Immediately before use, prepare a solution containing 0.2 - 0.4 unit/ml of Fructose-6-Phosphate Kinase in cold Reagent I.)

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PROCEDURE:

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

Reagent A (Buffer)	27.36
Reagent F (ATP)	0.30
Reagent C (PEP)	0.39
Reagent G (ß-NADH)	0.60
Reagent E (F-6-P)	0.60
Reagent D (KCl)	0.06
Reagent B ($MgSO_4$)	0.60
Reagent H (PK/LDH)	0.10

Mix by swirling and equilibrate to 30°C.

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

	Test	Blank
Reaction Cocktail	3.00	3.00

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

Reagent I (Enz Dil)	------	0.10
Reagent J (Enzyme Solution)	0.10	------

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 10 minutes. Obtain the $r\ A_{340nm}$/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(r\ A_{340nm}/\text{min Test} - r\ A_{340nm}/\text{min Blank})(3.1)(df)}{(6.22)(0.1)}$$

3.1 = Volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm
0.1 = Volume (in milliliter) of enzyme used

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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 9.0 at 30°C.

FINAL ASSAY CONCENTRATION:

In a 3.10 ml reaction mix, the final concentrations are 90 mM Tris, 1.9 mM magnesium sulfate, 4.8 mM potassium chloride, 0.70 mM phospho(enol)pyruvate, 9.7 mM fructose 6-phosphate, 0.97 mM adenosine 5'-triphosphate, 0.25 mM ß-nicotinamide adenine dinucleotide, 7 units pyruvate kinase, 10 units lactic dehydrogenase, and 0.02 - 0.04 unit fructose-6-phosphate kinase.

NOTES:

1. The NaOH is added in order to increase the pH of the adenosine 5'-triphosphate solution.

2. Contains not less than 700 units/ml of Pyruvate Kinase and 1000 units/ml of Lactic Dehydrogenase.

3. Pyruvate Kinase Unit Definition: One unit will convert 1.0 µmole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C.

4. L-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 µmole of pyruvate to L-lactate per minute at pH 7.5 at 37°C.

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