Enzymatic Assay of PHOSPHORYLASE b
(EC 2.4.1.1)

PRINCIPLE:

$$(\text{Glycogen})_n + P_i \xrightarrow{\text{Phosphorylase a}^1} (\text{Glycogen})_{n-1} + \alpha\text{-D-Glucose 1-Phosphate}$$

$$(\text{Glycogen})_n + P_i \xrightarrow[\text{5'AMP}]{\text{Phosphorylase b}} (\text{Glycogen})_{n-1} + \alpha\text{-D-Glucose 1-Phosphate}$$

$$\alpha\text{-D-Glucose 1-Phosphate} \xrightarrow{\text{Phosphoglucomutase}} \alpha\text{-D-Glucose 6-Phosphate}$$

$$\alpha\text{-D-Glucose 6-Phosphate} + \beta\text{-NADP} \xrightarrow{\text{G-6-PDH}} \text{6-Phosphogluconate} + \beta\text{-NADPH}$$

Abbreviations used:
5'-AMP = Adenosine 5'-Monophosphate
P_i = Inorganic Phosphate
PGLUM = Phosphoglucomutase
β-NADP = β-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form
G-6-PDH = Glucose-6-Phosphate Dehydrogenase
β-NADPH = β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form

CONDITIONS: T = 30°C, pH = 6.8, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 500 mM Potassium Phosphate Buffer, pH 6.8 at 30°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.8 at 30°C with 1 M KOH.)

B. 4% (w/v) Glycogen Solution (Glycogen)
(Prepare 10 ml in deionized water using Glycogen Type III, Sigma Prod. No. G-8876. This may require stirring and heat to solubilize.)

C. 300 mM Magnesium Chloride Solution ($MgCl_2$)
(Prepare 5 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

REAGENTS: (continued)

D. 100 mM Ethylenediaminetetraacetic Acid Solution (EDTA)
(Prepare 2 ml in deionized water using Ethylenediaminetetraacetic Acid, Tetrasodium Salt, Sigma Stock No. ED4SS.)

E. 6.5 mM β-Nicotinamide Adenine Dinucleotide Phosphate Solution (β-NADP)
(Prepare 15 ml in deionized water using β-Nicotinamide Adenine Dinucleotide Phosphate, Sodium Salt, Sigma Prod. No. N-0505. **PREPARE FRESH.**)

F. 0.1% (w/v) α-D-Glucose 1,6-Diphosphate Solution[2] (G 1,6-DiP)
(Prepare 1 ml in deionized water using α-D-Glucose 1,6-Diphosphate, Cyclohexylammonium Salt, Hydrate, Sigma Prod. No. G-5875.)[2]

G. Glucose-6-Phosphate Dehydrogenase Solution (G-6-PDH)
(Immediately before use, prepare a solution containing 10 units/ml in cold deionized water using Glucose-6-Phosphate Dehydrogenase, Sigma Prod. No. G-6378.)

H. Phosphoglucomutase Solution (PGLUM)
(Immediately before use, prepare a solution containing 10 units/ml in cold deionized water using Phosphoglucomutase, Sigma Prod. No. P-3397.)

I. 40 mM β-Glycerophosphate with 80 mM Cysteine, pH 6.8 at 30°C (Enzyme Diluent)
(Prepare 50 ml in deionized water using β-Glycerophosphate Disodium Salt, Hydrate, Sigma Prod. No. G-6251 and L-Cysteine, Hydrochloride, Monohydrate, Sigma Prod. No. C-7880. Adjust to pH 6.8 with 1 M NaOH.)

J. Phosphorylase b Enzyme Solution (Phosphor b)
(Immediately before Step 1: Prepare a solution containing 5 - 10 mg solid/ml of Phosphorylase b in cold Reagent I.)

K. Diluted Phosphorylase b Enzyme Solution (Dil Phosphor b)
(Immediately before Step 2, prepare a solution containing 0.20 - 0.40 unit/ml of Phosphorylase B in cold Reagent I.)

L. 100 mM Adenosine 5'-Monophosphate Solution (5'-AMP)[3]
(Prepare 1 ml in deionized water using Adenosine 5'-Monophosphate, Sodium Salt, Sigma Prod. No. A-1752. **PREPARE FRESH**.)

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PROCEDURE:

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

Deionized water	99.50
Reagent A (Buffer)	15.00
Reagent B (Glycogen)	7.50
Reagent C ($MgCl_2$)	0.67
Reagent D (EDTA)	0.15
Reagent E (β-NADP)	10.00
Reagent F (G 1,6-DiP)	0.50

Mix by stirring and adjust to pH 6.8 at 30°C with 100 mM HCl or 100 mM NaOH, if necessary.

Step 1:

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

	Test	Blank
Reaction Cocktail	2.70	2.70
Reagent G (G-6-PDH)	0.10	0.10
Reagent H (PGLUM)	0.10	0.10

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

Reagent J (Phosphor b)	0.10	------
Reagent I (Enzyme Diluent)	------	0.10

Mix by inversion and record the increase in A_{340nm} for approximately 10 minutes. Obtain the ΔA_{340nm}/minute using the maximum linear rate for both the Test and Blank.[4]

Step 2:

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

Reaction Cocktail	2.70	2.70
Reagent G (G-6-PDH)	0.10	0.10
Reagent H (PGLUM)	0.10	0.10
Reagent K (5'-AMP)	0.050	0.050

PROCEDURE: (continued)

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

	Test	Blank
Reagent K (Dil Phosphor b)	0.10	------
Reagent I (Enzyme Diluent)	------	0.10

Immediately mix by inversion and record the increase in A_{340nm} for approximately 5 minutes. Obtain the ΔA_{340nm}/minute using the maximum linear rate[5] for both the Test and Blank.

CALCULATIONS:

Phosphorylase a units/ml enzyme =

$$\frac{(\Delta A_{340}\text{/min Test without 5'AMP} - \Delta A_{340}\text{/min Blank without 5'AMP})(3.00)(df)}{(6.22)(0.1)}$$

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

Phosphorylase a and b units/ml enzyme =

$$\frac{(\Delta A_{340}\text{/min Test with 5'AMP} - \Delta A_{340}\text{/min Blank with 5'AMP})(3.05)(df)}{(6.22)(0.1)}$$

$$\text{Phosphorylase a and b units/mg solid} = \frac{\text{Phosphorylase a and b units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

Phosphorylase b units/mg solid = Phosphorylase a and b units/mg solid - Phosphorylase a units/mg solid

3.00 = Total volume (in milliliters) of Phosphorylase A assay
3.05 = Total volume (in milliliters) of Phosphorylase A and B assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of β-NADPH at 340 nm
0.1 = Volume (in milliliter) of enzyme used

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

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UNIT DEFINITION:

One unit will form 1.0 μmole of α-D-glucose 1-phosphate from glycogen and orthophosphate in the presence of adenosine 5'-monophosphate per minute at pH 6.8 at 30°C, measured in a system containing phosphoglucomutase, β-nicotinamide adenine dinucleotide phosphate, and glucose-6-phosphate dehydrogenase.

FINAL ASSAY CONCENTRATION:

In a 3.05 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.2% (w/v) glycogen, 1.3 mM magnesium chloride, 0.10 mM ethylenediaminetetraacetic acid, 0.43 mM β-nicotinamide adenine dinucleotide phosphate, 0.0003% (w/v) α-D-glucose 1,6-diphosphate, 1 unit glucose-6-phosphate dehydrogenase, 1 unit phosphoglucomutase, 1.6 mM adenosine 5'-monophosphate, and 0.02 - 0.04 unit phosphorylase b.

REFERENCES:

Bergmeyer, H.U., Gawehn, K., and Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U. ed.) 2nd ed., Volume I, 505-507, Academic Press, Inc., New York, NY

Fischer, E.H. and Krebs, E.G. (1962) *Methods in Enzymology*, Volume V, 369-373

NOTES:

1. Phosphorylase a activity is present in the phosphorylase b preparation and must be accounted for in the enzyme assay.

2. α-D-Glucose 1,6-Diphosphate is an activator of phosphoglucomutase.

3. Phosphorylase b is enzymatically inactive in the absence of 5'-AMP.

4. The maximal rate is reached within approximately 3 minutes. This maximal rate is due to any phosphorylase a activity which may be present.

5. The maximum linear rate should not exceed a ΔA_{340nm} of 0.1 absorbance unit/minute.

NOTES: (continued)

6. 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 β-NADP at pH 7.4 at 25°C.

7. Phosphoglucomutase Unit Definition: One unit will convert 1.0 µmole of α-D-glucose 1-phosphate to α-D-glucose 6-phosphate per minute at pH 7.4 at 30°C.

8. This assay is based on the cited references.

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

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