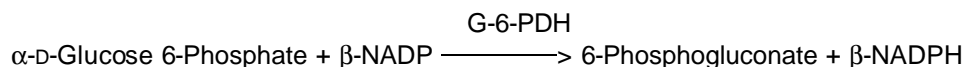
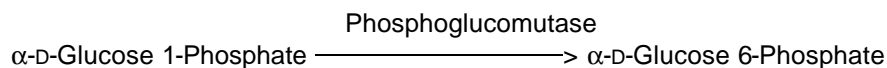
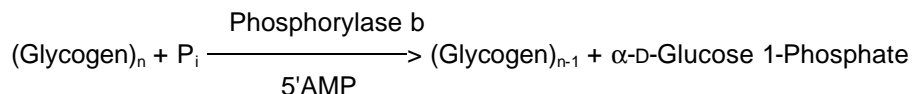
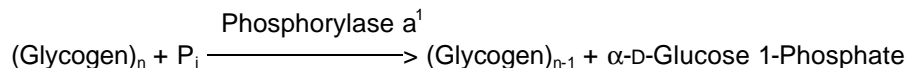


## Enzymatic Assay of PHOSPHORYLASE b (EC 2.4.1.1)

### PRINCIPLE:



Abbreviations used:

5'-AMP = Adenosine 5'-Monophosphate

P<sub>i</sub> = 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<sub>340nm</sub>, 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<sub>2</sub>)  
(Prepare 5 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

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**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  $\beta$ -Nicotinamide Adenine Dinucleotide Phosphate Solution ( $\beta$ -NADP)  
(Prepare 15 ml in deionized water using  $\beta$ -Nicotinamide Adenine Dinucleotide Phosphate, Sodium Salt, Sigma Prod. No. N-0505. **PREPARE FRESH.**)
- F. 0.1% (w/v)  $\alpha$ -D-Glucose 1,6-Diphosphate Solution<sup>2</sup> (G 1,6-DiP)  
(Prepare 1 ml in deionized water using  $\alpha$ -D-Glucose 1,6-Diphosphate, Cyclohexylammonium Salt, Hydrate, Sigma Prod. No. G-5875.)<sup>2</sup>
- 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  $\beta$ -Glycerophosphate with 80 mM Cysteine, pH 6.8 at 30°C (Enzyme Diluent)  
(Prepare 50 ml in deionized water using  $\beta$ -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)<sup>3</sup>  
(Prepare 1 ml in deionized water using Adenosine 5'-Monophosphate, Sodium Salt, Sigma Prod. No. A-1752. **PREPARE FRESH.**)

## Enzymatic Assay of PHOSPHORYLASE b (EC 2.4.1.1)

### 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 <sub>2</sub> )	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:

	<u>Test</u>	<u>Blank</u>
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  $A_{340\text{nm}}$  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  $A_{340\text{nm}}$  for approximately 10 minutes. Obtain the  $\Delta A_{340\text{nm}}/\text{minute}$  using the maximum linear rate for both the Test and Blank.<sup>4</sup>

#### 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

## Enzymatic Assay of PHOSPHORYLASE b (EC 2.4.1.1)

### PROCEDURE: (continued)

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

	<u>Test</u>	<u>Blank</u>
Reagent K (Dil Phosphor b)	0.10	-----
Reagent I (Enzyme Diluent)	-----	0.10

Immediately mix by inversion and record the increase in  $A_{340\text{nm}}$  for approximately 5 minutes. Obtain the  $\Delta A_{340\text{nm}}/\text{minute}$  using the maximum linear rate<sup>5</sup> for both the Test and Blank.

### CALCULATIONS:

Phosphorylase a units/ml enzyme =

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

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

Phosphorylase a and b units/ml enzyme =

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

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

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  $\beta$ -NADPH at 340 nm

0.1 = Volume (in milliliter) of enzyme used

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

## Enzymatic Assay of PHOSPHORYLASE b (EC 2.4.1.1)

### UNIT DEFINITION:

One unit will form 1.0  $\mu$ mole of  $\alpha$ -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,  $\beta$ -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  $\beta$ -nicotinamide adenine dinucleotide phosphate, 0.0003% (w/v)  $\alpha$ -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.  $\alpha$ -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  $\Delta A_{340\text{nm}}$  of 0.1 absorbance unit/minute.

**Enzymatic Assay of PHOSPHORYLASE b  
(EC 2.4.1.1)**

**NOTES:** (continued)

6. Glucose-6-Phosphate Dehydrogenase Unit Definition: One unit will oxidize 1.0  $\mu$ mole of D-glucose 6-phosphate to 6-phospho-D-gluconate per minute in the presence of  $\beta$ -NADP at pH 7.4 at 25°C.
7. Phosphoglucomutase Unit Definition: One unit will convert 1.0  $\mu$ mole of  $\alpha$ -D-glucose 1-phosphate to  $\alpha$ -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.**