Enzymatic Assay of PHOSPHORYLASE b
(enzyme 2.4.1.1)

**PRINCIPLE:**

Phosphorylase a

\[(\text{Glycogen})_n + P_i \rightarrow (\text{Glycogen})_{n-1} + \alpha-D-\text{Glucose 1-Phosphate}\]

Phosphorylase b

\[(\text{Glycogen})_n + P_i \rightarrow (\text{Glycogen})_{n-1} + \alpha-D-\text{Glucose 1-Phosphate} \]

5'AMP

\[
\alpha-D-\text{Glucose 1-Phosphate} \rightarrow \alpha-D-\text{Glucose 6-Phosphate}
\]

Phosphoglucomutase

\[
\alpha-D-\text{Glucose 6-Phosphate} + \beta-NADP \rightarrow 6-\text{Phosphogluconate} + \beta-NADPH
\]

G-6-PDH

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.)
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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 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 Solution² (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)

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
- Reagent A (Buffer)
- Reagent B (Glycogen)
- Reagent C (MgCl$_2$)
- Reagent D (EDTA)
- Reagent E ($\beta$-NADP)
- Reagent F (G 1,6-DiP)

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:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>2.70</td>
<td>2.70</td>
</tr>
<tr>
<td>Reagent G (G-6-PDH)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent H (PGLUM)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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)
- Reagent I (Enzyme Diluent)

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

Step 2:

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

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>2.70</td>
<td>2.70</td>
</tr>
<tr>
<td>Reagent G (G-6-PDH)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent H (PGLUM)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent K (5'-AMP)</td>
<td>0.050</td>
<td>0.050</td>
</tr>
</tbody>
</table>
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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:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent K (Dil Phosphor b)</td>
<td>0.10</td>
<td>------</td>
</tr>
<tr>
<td>Reagent I (Enzyme Diluent)</td>
<td>------</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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

CALCULATIONS:

Phosphorylase a units/ml enzyme =

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

Phosphorylase a units/mg solid =

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

Phosphorylase a and b units/ml enzyme =

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

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

Units/mg protein = $$\frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$
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’-monophosphosphate 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:


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 ΔA₃₄₀nm of 0.1 absorbance unit/minute.
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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.