INSTRUCTIONS

Phosphoprotein Phosphate Estimation Assay Kit

23270

0934.2

Number	Description
23270	**Phosphoprotein Phosphate Estimation Assay Kit,** sufficient materials for performing 500 test tube assays or twenty 96-well microplate assays

Kit Contents:

Ammonium Molybdate Solution, 25mL

Malachite Green Solution, 75mL

Lyophilized Protein Standard, Phosvitin, 1mg

BupH™ Tris Buffered Saline, 1 pack

Storage: Upon receipt store components at room temperature. Once it is dissolved, store the Phosvitin at 4°C for one month or -20°C for long-term.

Introduction

The Thermo Scientific Phosphoprotein Phosphate Estimation Assay Kit is designed to aid in determining the extent of phosphorylation of a purified protein. The assay is based on the alkaline hydrolysis of phosphate from seryl and threonyl residues in phosphoproteins and quantification of the released phosphate with Malachite Green and ammonium molybdate. The assay is rapid and easy to perform.

The kit can be used to identify an unknown protein of interest such as a phosphoprotein containing either phosphoserine or phosphothreonine as well as to estimate the level of phosphorylation. The test protein sample is compared with a standard set of specific concentrations of Phosvitin, a phosphoprotein of known phosphorylation level. The alkaline hydrolysis step does not release phosphate from phosphotyrosine residues in peptide linkage. Therefore, a negative result for an unknown purified protein preparation indicates that the protein is either (1) not a phosphoprotein or (2) is phosphorylated exclusively at tyrosine residues. In the latter case, Western blot analysis using an anti-phosphotyrosine antibody will be necessary to distinguish between these two possibilities.

The Phosphoprotein Phosphate Estimation Assay Kit can also be used to determine the amount of a purified preparation of a known phosphoprotein in a sample. This is done by constructing a standard curve using solutions of known concentration of the same protein and comparing the absorbance of the unknown sample against the resulting standard curve.

Additional Materials Required

- 2.0N NaOH (prepared by dissolving 8.0gm of sodium hydroxide pellets in 100mL of ultrapure water)
- 4.7N HCl (prepared by mixing 39mL of concentrated HCl with 61mL of ultrapure water)
- 96-well microplates (e.g., Product No. 15041 or 15031) and sealing tape for 96-well microplates (Product No. 15036), if intending to perform the assay in microplate format
- 12 × 75mm test tubes, if intending to perform the assay in test tube format
- Microplate reader capable of measuring absorbance at 650nm or UV/visible spectrophotometer capable of measuring absorbance at 620nm
- Glassware must be clean and phosphate-free. Use Thermo Scientific RBS pF Detergent Concentrate (Product No. 27959, 27960) for cleaning glassware without the risk of phosphate contamination.

Material Preparation

A. Tris Buffered Saline (TBS)

Prepare TBS by dissolving contents of the Thermo Scientific BupH Tris Buffered Saline Pack in 500mL of ultrapure water.

B. Reconstitute Lyophilized Phosvitin to Prepare Stock Solution

1. During shipment, the lyophilized phosvitin may come in contact with the grey vial septum. Before opening, verify that the lyophilized protein is settled to the bottom of the vial. If necessary, tap the side of the vial gently to settle the lyophilized protein. Carefully remove the septum to avoid disturbing any protein that may have settled on the under side of the septum.
2. Add 1mL of prepared TBS to the vial of lyophilized phosvitin. Carefully replace the septum into the vial opening. Holding the septum firmly in place with your thumb, and dissolve the protein by gently rocking the vial so that the buffer contacts all inside surfaces.
3. Store the phosvitin stock solution (1mg/mL) at 4°C for one month. If the solution will not be used within one month, store 100µL aliquots at -20°C.

C. Dilute Phosvitin Stock Solution to Prepare Phosphoprotein Standards

1. Standard A (100µg/mL): Prepare a 100µg/mL phosvitin solution by transferring 100µL of the Phosvitin Stock Solution (1mg/mL) to a new tube and adding 900µL of TBS. Mix well.
2. Standard B (15µg/mL): Transfer 150µL of Standard A to a new tube and and add 850µL of TBS. Mix well.
3. Standard C (10µg/mL): Transfer 100µL of Standard A to a new tube and and add 900µL of TBS. Mix well.
4. Standard D (5µg/mL): Transfer 50µL of Standard A to a new tube and and add 950µL of TBS. Mix well.
5. Standard E (2.5µg/mL): Transfer 25µL of Standard A to a new tube and and add 975µL of TBS. Mix well.

D. Protein Sample

Dissolve the sample to be assayed in TBS at a concentration of 100µg/mL. If the sample is already in solution (it must not be in phosphate buffer), dilute the sample in TBS to a concentration of 100µg/mL.

E. Phosphate Reagent

Mix one volume of Ammonium Molybdate Solution with three volumes of Malachite Green solution.
Note: This solution must be prepared new immediately before performing the assay.

Assay Procedure

For best results, assay each standard and sample point in triplicate.

A. Microplate Protocol

1. Pipette 50µL of each Phosphoprotein Standard and test sample into appropriate wells of a microplate. For the blank (e.g., zero phosphate standard), use 50µL of TBS.
2. Add 50µL of 2.0N NaOH to each well.
3. Cover the microplate with sealing tape, mix the plate for 30 seconds in a microplate shaker and incubate at 65°C for 30 minutes.
4. Add 50µL of 4.7N HCl to each well and mix the plate for 30 seconds in a microplate shaker.
5. Add 50µL of Phosphate Reagent to each well and mix the plate for 30 seconds in a microplate shaker.
6. Incubate the plate at room temperature for 30 minutes.
7. Read the absorbance at 650nm in a microplate reader.

B. Test Tube Protocol

1. Pipette 0.2mL of each Phosphoprotein Standard and test sample in appropriate test tubes. For the blank (e.g., zero phosphate standard), use 50µL of TBS.
2. Add 0.2mL of 2.0N NaOH to each tube.
3. Cover the tubes with caps, vortex to mix, and incubate at 65°C for 30 minutes in an oven or water bath.
4. Add 0.2mL of 4.7N HCl to each tube and vortex to mix.
5. Add 0.2mL of Phosphate Reagent to each tube and vortex to mix
6. Incubate the tubes at room temperature for 30 minutes.
7. Measure the absorbance of each standard and sample at 620nm in a spectrophotometer.

C. Estimate of Phosphorus Content

1. Plot the absorbances of the Phosphoprotein Standards against their corresponding concentration phosvitin protein (see Table 1 and Figure 1).
2. Estimate of the amount of phosphorylation by interpolating the test sample absorbance values on the Phosvitin Standard curve. Follow the step-by-step calculations outlined below to calculate the number of moles of phosphorus/phosphate per mole of protein for your sample.

D. Calculate Moles of Phosphorus per Mole of Protein

1. Calculate the slope and y-intercept values from the slope of the standards graph.

   Note: If using Microsoft Excel, click on the chart and then select 'Chart' and 'Add trendline' from the top menu. Under 'Add a trendline', click on 'Options' and then 'Display equation on chart'. This will automatically calculate an equation for the line.

2. Using the slope and y-intercept values, plug in your sample absorbance for [y] and solve for [x]:

   y = mx + b

3. Multiply your [x] value by 0.10 to get the amount of phosphorus (µg) in the unknown sample. (This step is to account for the fact that phosvitin protein is 10% phosphate by weight.)
4. Divide [x] by the total amount of sample (µg) per tube or microplate well.

   (x / total µg of sample per tube or microplate well) = amount of phosphorus per sample

5. Multiply the amount of phosphorus per sample by the molecular weight of your sample.

   (MW of sample) × (amount of phosphorus per sample) = weight of phosphorus per mole of protein

6. Divide the weight contributed by phosphorus by the molecular weight of phosphorus (31) to get the phosphorus molecules per molecule of your protein sample.

   $$\frac{\text{Weight contributed by phosphorus}}{\text{31 (MW of phosphorus)}} = \text{Number of phosphorus molecules per molecule of protein}$$

E. Example Data and Calculations

Table 1. Typical data from Phosvitin Standard set assayed using the microplate protocol of the Thermo Scientific Phosphoprotein Phosphate Estimation Assay Kit.

Sample	Protein Amount (µg/well)	Absorbance (650 nm)
Blank	0	0.056
Standard E	0.125	0.102
Standard D	0.25	0.161
Standard C	0.5	0.283
Standard B	0.75	0.392
Unknown Sample	5.0	0.077

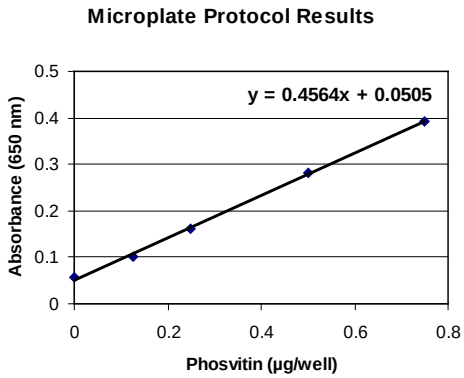

Figure 1. Typical standard curve achieved with the microplate protocol using the Thermo Scientific Phosphoprotein Phosphate Estimation Assay Kit.

1. Calculate the slope and y-intercept values from the slope of the standards graph.

   y = 0.4564x + 0.0505

2. Using the slope and y-intercept values, plug in your sample absorbance for [y] and solve for [x]:

   y = mx + b

   0.077 = 0.4564x + 0.0505

   x = 0.058µg Phosvitin

3. Multiply your [x] value by 0.10 to get the amount of phosphorus (µg) in the unknown sample. (This step is to account for the fact that Phosvitin protein is 10% phosphate by weight.)

   (0.058µg Phosvitin)(0.10) = 0.0058µg phosphorus in sample

4. Divide [x] by the total amount of sample (µg) per tube or microplate well.

   0.0058µg phosphorus / 5µg protein microplate well = 0.00116µg of phosphorus per µg sample protein

5. Multiply the amount of phosphorus per sample by the molecular weight of your sample (e.g., MW = 44,000).

   (44,000) × (0.00116µg) = 55µg phosphorus per mole of protein

6. Divide the weight contributed by phosphorus by the molecular weight of phosphorus (31) to get the phosphorus molecules per molecule of your protein sample.

   55µg phosphorus / 31 MW of phosphorus = 1.6 phosphorus molecules per molecule of protein

Additional Information

A. Example Application

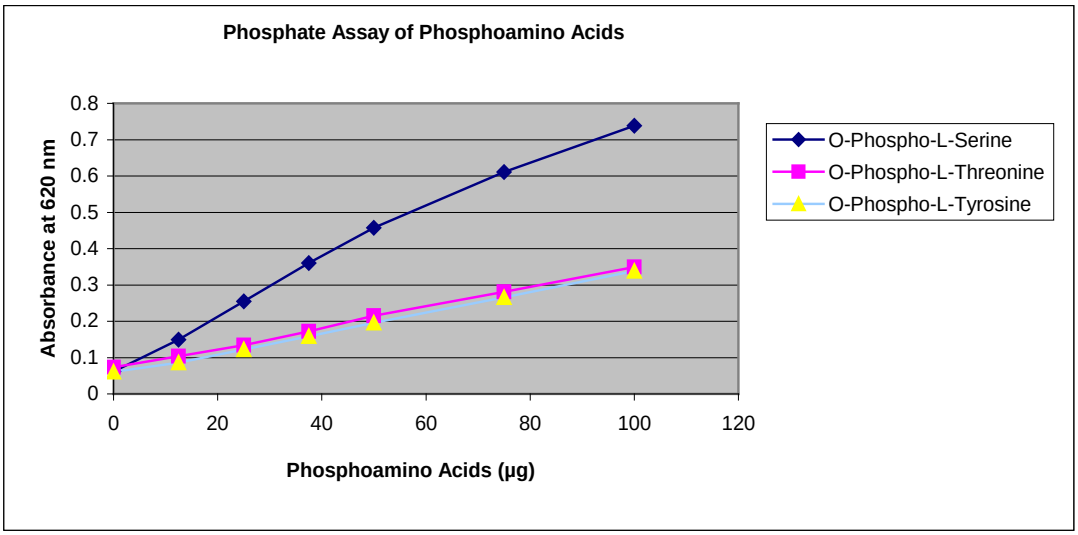

Figure 2. Phosphate assay of phosphoamino acids. Note: Although it was possible to assay phosphate in O-phospho-L-tyrosine (i.e., the free amino acid), it is not possible to assay tyrosyl phosphate groups present in phosphopeptides and phosphoproteins. Phosphate groups of O-phospho-L-tyrosine present in phosphopeptides and phosphoproteins are resistant to hydrolysis by 2.0N NaOH or 4.7N HCl.

B. Incompatible Substances with the Phosphoprotein Phosphate Estimation Assay Kit

- 0.1% SDS
- 10mM $Cu(NO_3)_2$
- 10mM $FeCl_3$
- 10mM $CoCl_2$

C. Compatible Buffers and Substances with the Phosphoprotein Phosphate Estimation Assay Kit

- 25mM Tris + 0.15M NaCl, pH 7.2
- 0.2M Sodium carbonate-bicarbonate buffer, pH 9.4
- 50mM HEPES, pH 7.2
- 0.1M Sodium citrate buffer, pH 7.5
- 1.0M NaCl
- 0.2M Tris
- 10 mM DTT
- 10 mM EDTA
- 0.1 % Triton™ X-100
- 0.1 % Tween™-20
- 0.1 % NP-40

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