PhosFree™ Phosphate Assay Biochem Kit

(Cat. # BK050)

ORDERING INFORMATION

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PhosFree™ Phosphate Assay Biochem Kit (Cat. # BK050)

The PhosFree™ Phosphate Assay Biochem Kit is an extremely quick and economical way to measure phosphate in solution. There is minimal interference from other common ions and reagents used in molecular biology and biochemistry. This kit is designed to guide you through the process of measuring end-point and kinetic assays. There is special consideration for ATPase and GTPase assays which can be performed easily with this kit. The kit manual is separated into six sections:

Section I: Overview and Uses of the Kit
Section II: Equipment required
Section III: Kit Contents
Section IV: Assay Methods
Section V: Troubleshooting
Section VI: Associated Products

Section I: Overview

The PhosFree™ Phosphate Assay is a simple two or three step assay which measures phosphate down to a level of 0.02 nmole per 20 µl of reaction mix. The assay is based on a malachite green complex which specifically binds to phosphate ions, resulting in an increase in absorbance at 650 nm. This unparalleled sensitivity make the assay a first choice for determining phosphate concentrations in biological fluids and buffers, as well as a platform for measuring phosphate released from hydrolysis of ATP, GTP or other nucleotide or another phosphate releasing reactions. There are special considerations for phosphate releasing reactions which are usually sensitive to pH. Also there is an extra step for biological solutions containing more than 10 µg/ml of protein which require clarification after quenching the reaction.

Uses:

1) To measure phosphate in a biological solution or buffer.
2) To measure ATP or GTP hydrolysis from an enzyme reaction.
3) To measure phosphate release from phosphatase catalyzed reactions.

Section II: Required Equipment

1) Spectrophotometer capable of measuring absorbance at 650 nm wavelength.
2) Small capacity (100-1000 µl) cuvettes or 96-well microtitre plates.
3) Pipettors 20, 200 and 1000 µl capacity.
4) Microfuge.
5) Ice supply, and 4^{o}C storage area.

Section III: Kit Contents

1) 1 x 100 ml Activator. Store at room temperature
2) 1 x 100 ml Quencher. Store at room temperature.
3) 1 x 1 ml 1 mM Phosphate Standard. Store at room temperature

Section IV: Assay Methods

Preliminaries:

In order to set up your PhosFree™ Phosphate Assay you need to determine what format is best for you. There are end-point or kinetic assays, and high or low protein content assays to choose from. If your test solution has less than 10 µg/ml of protein (see Associated Products section for the Advanced Protein Assay, Cat. # ADV01) then this is called a low protein content assay. If more than 10 µg/ml then this is a high protein content assay. The end-point assay determines the level of phosphate in a solution such as a buffer or a sample of urine, which is not expected to change during the time taken to measure the phosphate concentration. The kinetic assay is useful for reactions which hydrolyze ATP or GTP to form ADP or GDP and free phosphate over a time course. Finally, even if you decide to perform a kinetic assay it is useful to use the endpoint method as a rapid method for determining the most suitable enzyme concentration and buffer composition. The assay's range is 20 to 1000 µM phosphate, so consider this when designing your assay. We suggest performing the Standard Curve first to get acquainted with how the assay fits into your laboratory setting (e.g. spectrophotometer settings and cuvettes or 96-well plates etc). When you have performed the Standard Curve then decide which assay type you would like to perform, then follow the corresponding method described after the Standard Curve section. It is recommended to perform a Standard Curve with each experiment in order to ensure reproducibility between experiments: If you require a one step assay and improved protein compatibility, try the CytoPhos™ Phosphate Assay kit (Cat. # BK054).

Phosphate Standard Curve:

This method is for 220 µl final volume, if your cuvette or well of a 96-well plate requires a different volume then adjust all volumes accordingly. Furthermore, this method is time sensitive, try to measure all samples between 10 and 15 min after addition of Activator and Color developer.

1. Pipette 20, 10, 8, 6, 4, 2, 1 and 0 µl of 1 mM Phosphate Standard in duplicate tubes or wells, and make each tube or well to 20 µl with phosphate free Milli-Q water.

2. Pipette 100 µl of Quencher (0.6 M Perchloric Acid) into each tube or well.

3. Pipette 100 µl of Activator (Malachite Green Reagent) into each tube or well.

4. Incubate at room temperature (24°C) for exactly 10 min and read absorbance at 650 nm. Use the zero phosphate concentration sample as a blank to zero the spectrophotometer.

5. Calculate the average absorbance values of duplicates, and plot them as a standard curve (concentration on the x-axis and absorbance on the y-axis). See Figure 1.

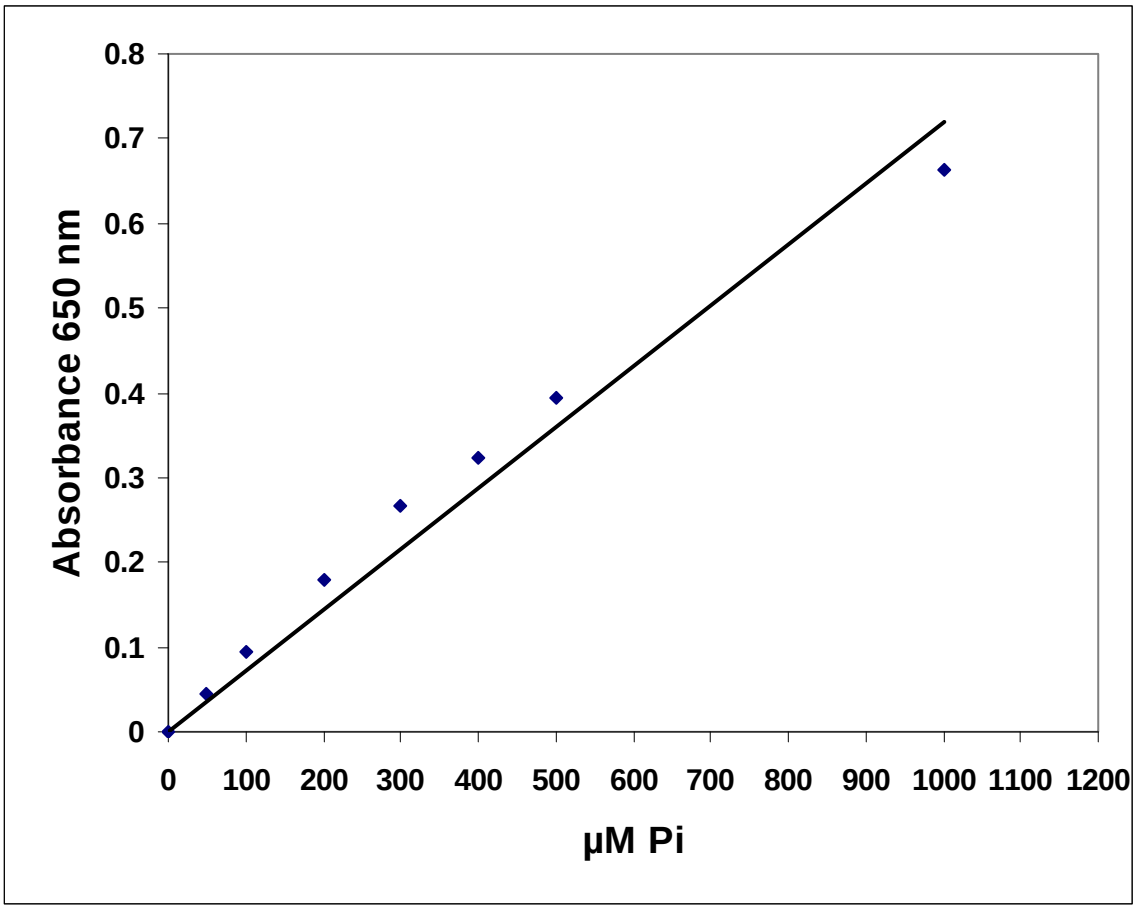

Figure 1. Phosphate Standard Curve*. 1 mM Phosphate standard solution was pipetted into wells of a 96-well plate in volumes of 20, 10, 8, 6, 4, 2, 1, 0 µl and the final volume was made up to 20 µl with Milli-Q water. 100 µl Quencher was added, followed by 100 µl of Activator. After a 10 min incubation at room temperature the absorbance was read at 650 nm. Note: The spectrophotometer path length is 0.8 cm*

Endpoint assay with low protein content:

This method is for 220 µl final volume, if your cuvette or well of a 96-well plate requires a different volume then adjust all volumes accordingly. Furthermore, this method is time sensitive, try to measure all samples between 10 and 15 min after addition of Activator and Color developer.

1. Make serial two-fold dilutions of your sample in Milli-Q water until you are sure there is less than 1 mM phosphate in at least one dilution. If you know approximately the range of phosphate expected in your sample, then use this as a guideline to aim for 500 µM final concentration of phosphate.

2. Pipette 20 µl of each dilution in duplicate into microfuge tubes or wells of a 96-well plate.

3. Pipette 100 µl of Quencher (0.6 M Perchloric Acid) into each tube or well.

4. Pipette 100 µl of Activator (malachite green reagent) into each tube or well.

5. Incubate at room temperature (24°C) for 10 min and read absorbance at 650 nm.

6. Calculate the average absorbance values of duplicates, and read the concentrations of phosphate using the Standard Curve.

7. Multiply the concentration of phosphate in your dilutions by the dilution factor, this will give the final concentration of phosphate in your original sample.

Endpoint assay with high protein content:

This method is for 220 µl final volume, if your cuvette or well of a 96-well plate requires a different volume then adjust all volumes accordingly. Furthermore, this method is time sensitive, try to measure all samples between 10 and 15 min after addition of Activator and Color developer.

1. Make serial two-fold dilutions of your sample in Milli-Q water until you are sure there is less than 1 mM phosphate in at least one dilution. If you know approximately the range of phosphate expected in your sample, then use this as a guideline to aim for 500 µM final concentration of phosphate.

2. Pipette 20 µl of each dilution in duplicate into microfuge tubes.

3. Pipette 100 µl of Quencher (0.6 M Perchloric Acid) into each tube or well.

4. Centrifuge for 5 min at 14000 x g at 4°C.

5. Pipette the supernatant into clean tubes or wells of a 96-well plate; discard the pellet which contains the denatured protein.

6. Pipette 100 µl of Activator (Malachite Green Reagent) into all tubes or wells.

7. Incubate at room temperature (24°C) for 10 min and read absorbance at 650 nm.

8. Calculate the average absorbance values of your samples and read off the concentrations of phosphate from the Standard Curve.

9. Multiply the concentration of phosphate in your dilutions by the dilution factor, this will give the final concentration of phosphate in your original sample.

Kinetic assay with low protein content:

This method is for 220 µl final volume, if your cuvette or well of a 96-well plate requires a different volume then adjust all volumes accordingly. Furthermore, this method is time sensitive, try to measure all samples between 10 and 15 min after addition of Activator and Color developer.

Note. The kinetic assay is performed by taking timed aliquots of a master reaction which are quenched on ice before final analysis for free phosphate. DO NOT quench the reactions into the Activator because this solution is acidic which will cause hydrolysis of the phosphodiester bonds in ATP or GTP or other phosphate containing substrate, thus increasing your background signal in the earlier time points.

1. Label duplicate tubes T=0, T=5, T=10, T=15, T=20, T=25, T=30, T=35, T=40, T=45, T=50, T=55 and T=60, and place on ice. Alternatively place a 96-well plate on ice and label the edges of the plate to correspond to the same time points, the T=60 time point can be omitted for simplicity because then the samples would fill one row.

2. Make duplicate 300 µl reactions on ice, noting the following:

   a) pH and Buffer: Use a strong enough buffer to hold the pH constant even when 1 mM ATP is hydrolyzed. For example 20 mM Hepes pH 7.4 or 20 mM Tris-HCl pH 8.0 is adequate. However, 3.0 mM Tris-HCl pH 7.0 would not be adequate. Definitely DO NOT USE phosphate based buffers as these will lead to excessive background signals

   b) Substrate: Use 1.0 mM substrate if possible. ATP and GTP or other phosphate containing substrates should be prepared at pH 7.0 to reduce endogenous hydrolysis. Both ATP and GTP can be obtained from Cytoskeleton Inc. Cat. # BSA04-001 and BST06-001 respectively.
   c) Divalent cation: Most phosphate releasing enzyme catalyzed reactions require a divalent cation. The most commonly used one is magnesium (chloride salt) at a concentration of 1.0 to 10 mM other divalent cations may be required depending on the enzyme. Myosin is a special case in which KCl can activate its ATPase activity.
   d) Enzyme or protein extract: Purified enzymes with high activity can be used at less than 10 µg protein per ml of reaction. However, extracts, impure proteins or low turnover enzymes may require up to 1 mg protein per ml reaction. Un-stimulated small G-proteins, tubulin and actin are special cases where the turnover of GTP or ATP hydrolysis is very low compared to other proteins (e.g. alkaline phosphatase), so extended assay times maybe required.
   e) Salt concentration: This depends on the reaction; usually 50 mM is a good start. Cytoskeletal motor proteins are sensitive to salt concentration in that microtubule or F-actin stimulated NTP hydrolysis activity may be inhibited at >75 mM KCl or NaCl because of polymer/motor dissociation.

An example reaction with alkaline phosphatase and ATP hydrolysis would contain the following:

1. 289 µl Milli-Q water
2. 6.0 µl 1.0 M Tris-HCl pH 8.0
3. 1.5 µl 1.0 M $MgCl_2$
4. 3.0 µl 100 mM ATP pH 7.0-10.0
5. 1.0 µl 10 units/ml Alkaline phosphatase

3. Place the reactions in a 30 or 37°C waterbath and start a timer for 60 min.

4. After 10 s take 20 µl from each tube and quench by pipetting into the T=0 tubes on ice, keep these on ice until all the samples have been collected.

5. After 5 min take another 20 µl and quench into the T=5 tubes on ice, repeat every 5 min until all the quench tubes have been used.

6. Pipette 100 µl of Quencher (0.6 M Perchloric Acid) into each tube or well.

7. Pipette 100 µl of Activator (Malachite Green Reagent) into each tube or well.

8. Incubate at room temperature (24°C) for 10 min and read absorbance at 650 nm.

9. Calculate the average absorbance value from the duplicates and read off the concentrations of phosphate using your Standard Curve.

10. Use linear regression analysis or a hand drawn graph of µM phosphate [y-axis] versus time [x-axis] to calculate the rate of Pi released per min, this will be in µM/min.

11. Multiply by 0.02 to make the units nmoles / min / 20 µl of reaction.

12. Divide the rate of Pi release (nmoles/min/20 µl of reaction) by the amount of protein (in mg) in a 20 µl sample of reaction mix. This will give you your Specific Activity in nmoles/min/mg protein.

Kinetic assay with high protein content:

This method is for 220 µl final volume, if your cuvette or well of a 96-well plate requires a different volume then adjust all volumes accordingly. Furthermore, this method is time sensitive, try to measure all samples between 10 and 15 min after addition of Activator and Color developer.

Note. The kinetic assay is performed by taking timed aliquots of a master reaction which are quenched on ice before final analysis for free phosphate. DO NOT quench the reactions into the Activator because this solution is acidic which will cause hydrolysis of the phosphodiester bonds in ATP or GTP or other phosphate containing substrate, thus increasing your background signal in the earlier time points.

1. Label duplicate tubes T=0, T=5, T=10, T=15, T=20, T=25, T=30, T=35, T=40, T=45, T=50, T=55 and T=60, and place on ice.

2. Make duplicate 300 µl reactions on ice (see notes in "Kinetic assay with low protein content" section).

3. Place the reactions in a 30 or 37°C waterbath and start a timer for 60 min.

4. After 10 s take 20 µl from each tube and quench by pipetting into the T=0 tubes on ice, keep these on ice until all the samples have been collected.

5. After 5 min take another 20 µl and quench into the T=5 tubes on ice, repeat every 5 min until all the quench tubes have been used.

6. Pipette 100 µl of Quencher (Perchloric Acid) into each tube and centrifuge for 5 min at 14000 x g at 4°C.

7. Pipette supernatants into clean tubes or wells of a 96-well plate.

8. Pipette 100 µl of Activator (Malachite Green Reagent) into each tube or well.

9. Incubate at room temperature (24°C) for 10 min and read absorbance at 650 nm.

10. Calculate the average absorbance value from the duplicates and read off the concentrations of phosphate using your Standard Curve.

11. Use linear regression analysis or a hand drawn graph of µM phosphate [y-axis] versus time [x-axis] to calculate the rate of Pi released per min, this will be in µM/min.

12. Multiply by 0.02 to make the units nmoles / min / 20 µl of reaction.

13. Divide the rate of Pi release (nmoles/min/20 µl of reaction) by the amount of protein (in mg, see Associated Products) in a 20 µl sample of reaction mix. This will give you your Specific Activity in nmoles/min/mg protein.

Section V: Troubleshooting

Observation	Possible cause	Remedy
1. No increase in absorbance in the most concentrated sample.	1. Sensitivity of spectrophotometer set too low. 2. Incorrect labeling of tubes. 3. Sample too dilute. 4. Sample contains too little phosphate.	1. Increase sensitivity (read machine manual). 2. Repeat experiment. 3. Use more concentrated samples. 4. Use more sample.
2. In the kinetic assay, plotted phosphate versus time is not linear	1. Enzyme is denaturing during assay. 2. Enzyme is hydrolyzing all the substrate.	1. Change buffer conditions to stabilize enzyme, try reducing agent DTT at 0.5 mM and glycerol 5% v/v, or immobilize the protein to stabilize the enzyme. 2. Use less enzyme.
3. In the kinetic assay, the large number of samples is difficult to process quickly enough to reduce the background hydrolysis.	1. Perchloric acid solution (Quencher) is hydrolyzing the phosphate substrate.	1. Use a higher through-put method e.g. Multichannel pipettors and 96-well plates. 2. Perform a "buffer plus substrate" parallel reaction and subtract the background of this control from the master reaction's measurements.
4. In the kinetic assay, there is very little phosphate increase from T=0 to T=60 min.	1. Enzyme does not catalyze a phosphate generating reaction. 2. Enzyme is too dilute. 3. Enzyme is inactive or denatured in the reaction. 4. Enzyme has a low turnover. 5. Substrate is not appropriate.	1. NA 2. Increase enzyme concentration. 3. Test various conditions (divalent cations, pH, buffers, salt concentration and sugars in order to stabilize the enzyme. 4. Extend reaction time or increase concentration of enzyme. 5. Try other substrates
5. In kinetic assay, high absorbance at T=0 and little increase towards T=60 min.	1. Phosphate in reaction at T=0. 2. Interfering substances in reaction. 3. Reaction is not stopped on ice.	1. Use phosphate free chemicals and prepare nucleotide triphosphate stocks at pH 7.0. 2. Test each component of your reaction in an endpoint assay and remove or replace the offending chemical. 3. Use alternative method for stopping reaction time points. e.g. Snap freeze time point samples in liquid nitrogen.

Section VI: Related Phosphate and Protein Assays

HTS Kinesin ATPase Endpoint Assay	Cat. # BK053	KIT 1000 assays
CytoPhos™ Phosphate Assay (1-500 µg/ml protein reactions)	Cat. # BK054	KIT 1000 assays
EasyRad Phosphate Assay (Radioactive based)	Cat. # BK055	KIT 1000 assays
Kinesin ELIPA (Enzyme Linked Inorganic Phosphate Assay)	Cat. # BK060	KIT 96 assays
Advanced Protein Assay	Cat. # ADV01	500 ml
Precision Red™ Advanced Protein Assay	Cat. # ADV02	500 ml