

Protein Kinase Assay Kit, Universal Cat. No. 539551

Introduction

Protein kinases catalyze the transfer of gamma phosphate from adenosine triphosphate (ATP) to a serine, threonine, or tyrosine residue on a protein substrate. They play a critical role in cellular regulation, and hence are a subject of intense research in both basic biomedical science and drug discovery endeavors. Protein kinases discovered so far are roughly divided into three subtypes: (1) protein kinase C (PKC) that requires calcium and/or phospholipid for its activity; (2) cAMP-dependent protein kinase (PKA) whose activity is dependent on cyclic AMP; and (3) protein tyrosine kinase (PTK) that phosphorylates tyrosine residues only. Another group of protein kinases known as MAP kinases is characterized by their requirement for dual phosphorylation. Each protein kinase subtype also has a broad spectrum of substrate specificity. Therefore, a short synthetic peptide with defined sequence is commonly used as a substrate for assaying a specific type of protein kinase activity.

Assay of protein kinase activity is traditionally carried out by phosphorylating a peptide substrate with γ - ^{32}P -ATP followed by separation of the ^{32}P -peptide product from the unreacted γ - ^{32}P -ATP on a phosphocellulose membrane. This method requires at least one basic amino acid residue in the peptide substrate, which imposes an obvious limitation on the peptide sequence design for assay specificity and optimization. Another serious drawback is the relatively weak binding of the peptide substrate and the phosphocellulose membrane that often results in loss of the ^{32}P -peptide product during the wash step. To overcome these problems, the peptide substrate can be tagged with a biotin group so that the biotinylated ^{32}P -peptide product consistently binds to a streptavidin membrane in a manner independent of the peptide sequence (Goueli, et al. 1995. *Analytical Biochemistry* **225**, 10). However, the separation of the ^{32}P -peptide product from the free γ - ^{32}P -ATP via the streptavidin membrane still demands an intensive and inconvenient washing procedure.

The Calbiochem® Universal Protein Kinase Assay Kit is based on a process that combines affinity binding and ultrafiltration separation to analyze a mixture sample.

The protein kinase assay begins with a reaction of a protein kinase sample with a biotinylated peptide substrate and γ - ^{32}P -ATP. After the reaction, free avidin in solution is added for binding to the biotinylated ^{32}P -peptide product. The sample is then subjected to a centrifugal ultrafiltration process with a membrane that retains the product-avidin complex and passes the free γ - ^{32}P -ATP. Separation by such affinity ultrafiltration process is easy, gentle, and efficient. Separation occurs in solution relatively free of nonspecific background. In addition, the ultrafiltration separation is performed in a contained and compact system that eases the handling of the radioactive sample and minimizes the radioactive waste. The sample reservoir of the centrifugal ultrafiltration unit containing the ^{32}P -peptide product as retentate can be directly placed into a liquid scintillation vial for counting.

CALBIOCHEM® offers several protein kinase assay kits that are carefully formatted to ensure their assay performance and user-friendliness. They are designed for protein kinase activity assays with greater flexibility. These kits allow the user to choose their own protein kinase, peptide substrate, and kinase reaction buffer. The kits may be used to assay protein kinase activity or potency of an activator or inhibitor of the protein kinase. Furthermore, the high efficiency and consistency of the separation process across a large concentration range of the biotinylated peptide substrate makes it an ideal for quantitative kinetic studies such as measuring the enzymatic turnover rate and Michaelis-Menten constant (K_m).

USA and Canada
Tel (800) 628-8470
technical@calbiochem.com

Germany
Tel 0800 100 3496
techservice@merckbiosciences.de

United Kingdom and Ireland
UK Freephone 0800 622935
Ireland Toll Free 1800 409445
customer.service@
merckbiosciences.co.uk

All Other Countries
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www.calbiochem.com
technical@calbiochem.com



Kit Components and Storage

| Materials Provided | Quantity |
|---|-----------------|
| <u>Store at -20° C – Ship on Dry Ice</u> | |
| 10X (0.15 mM) ATP Solution | 300 µl |
| <u>Store at +4° C – Ship on Blue Ice</u> | |
| Stop Solution (8.0 M guanidine hydrochloride) | 1200 µl |
| 50% Trichloroacetic Acid Solution | 360 µl |
| 1% Bovine Serum Albumin Solution | 240 µl |
| Neutralization Solution | 600 µl |
| Avidin Solution | 1000 µl |
| Wash Solution | 50 ml |
| <u>Store at Room Temperature</u> | |
| Centrifugal Ultrafiltration Units | 100 units |

All the kit components are stable for 1 year from the date of purchase if they are handled and stored correctly.

Items Needed but Not Provided with the Kit

- (1) **Protein Kinase Sample:** This can be a purified enzyme preparation or cellular/tissue extract. It is advisable to avoid or limit other protein kinases in the sample that may act on the peptide substrate. Caution should also be exercised with regards to the effects of impure proteases and phosphatases in the sample. Purified protein kinases that may serve as positive controls for the assay are also available from CALBIOCHEM® (Please see our Signal Transduction Catalog, Chapter 5).
- (2) **Test Substance:** This is an optional item depending on whether an activator or inhibitor is to be tested. The concentration of Test Substance should be made as appropriate according to its potency in modulating the activity of Protein Kinase.
- (3) **Biotinylated Peptide Substrate:** The sequence of this peptide substrate is determined by the need to match the specific type of protein kinase of assay interest. The best way to biotinylate the peptide is to covalently couple a biotin group to the amino terminus of the peptide automatically synthesized on the solid phase before it is cleaved from the solid phase.
- (4) **10X Protein Kinase Reaction Buffer:** The composition of this buffer is determined by the need to match the specific type of protein kinase and by the specific reaction condition of the user's interest. For example, the buffer may have variables such as the pH value, divalent metal ions, or essential activators as appropriate.
- (5) **γ-³²P-ATP Stock Solution:** A typical commercial source of γ-³²P-ATP should have a specific activity of about 3,000 Ci/mmol and a concentration of about 10 µCi/µl.
- (6) Deionized Water
- (7) Microcentrifuge
- (8) Scintillation vials with an inner diameter larger than 17 mm to accommodate the sample reservoir of Centrifugal Ultrafiltration Unit.

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Assay Protocol

I. Preparation of Solutions

- (1) **Thaw 10X ATP Solution.**
- (2) **Kinase/Substance Mix:** This is an option exercised only when the effect of Test Substance requires its pre-incubation with Protein Kinase Sample. Make the pre-mix of Test Substance and Protein Kinase Sample with appropriate concentrations and for specific incubation time.
- (3) **10X Biotinylated Peptide Substrate Solution:** Use Deionized Water and Biotinylated Peptide Substrate to prepare 10X (0.25 mM) Biotinylated Peptide Substrate Solution. The concentration of peptide may be varied as needed (e.g. in kinetic study requiring a series of substrate concentrations). However, the maximal peptide concentration should not exceed 2 mM so that all the peptide substrate and product in the kinase reaction can be bound by avidin provided in this kit.
- (4) **γ -³²P-ATP Working Solution:** Use Deionized Water to dilute γ -³²P-ATP Stock Solution to prepare a working solution (about 0.2 μ Ci/ μ l).
- (5) **Protein Kinase Reaction Mix:** The following formulation is designed to make enough volume of each Protein Kinase Reaction Mix for a given multiplier, N. To compensate for the liquid loss during the pipetting steps, N should be about 110% of the actual test number to be conducted with each Protein Kinase Reaction Mix. For example, N is 11 for 10 actual tests. The actual tests should include a negative control (without the enzyme) and other controls as required. In a microcentrifuge tube, the following components are added and mixed carefully:

| Component | Reaction Volume |
|---|---------------------------------|
| 10X ATP Solution | 2.5 x N μ l |
| 10X Biotinylated Peptide Substrate Solution | 2.5 x N μ l |
| 10X Protein Kinase Reaction Buffer | 2.5 x N μ l |
| γ - ³² P-ATP Working Solution | 2.5 x N μ l |
| Deionized Water or optional Test Substance | 10 x N μ l |
| Total | 20 x N μl |

- (6) **Control Mix Without the Peptide Substrate:** This control is for determining the nonspecific phosphorylation of proteins in Protein Kinase Sample by γ -³²P-ATP. In a microcentrifuge tube, the following components are added and mixed.

| Component | Reaction Volume |
|---|-----------------------------|
| 10X ATP Solution | 2.5 μ l |
| 10X Protein Kinase Reaction Buffer | 2.5 μ l |
| γ - ³² P-ATP Working Solution | 2.5 μ l |
| Deionized Water | 2.5 μ l |
| Deionized Water or optionally Test Substance | 10 μ l |
| Total | 20 μl |

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Germany
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II. Reaction and Termination

Pipette 20 µl of Protein Kinase Reaction Mix into microcentrifuge tubes. Initiate the reaction by adding 5 µl of Protein Kinase Sample (or optional Kinase/Substance Mix) to the microcentrifuge tubes containing 20 µl of Protein Kinase Reaction Mix and to the microcentrifuge tube containing 20 µl of control mix without the peptide substrate. Also set up the negative control, with 5 µl of Deionized Water instead of Protein Kinase Sample, in one of the microcentrifuge tubes containing 20 µl of Protein Kinase Reaction Mix. Incubate at an appropriate temperature and for a desired period of time.

In the first termination method, terminate all the reactions by adding 10 µl of Stop Solution Mix. After a brief centrifugation, the terminated reactions are ready for Step III (see below).

If, after Step III, the counts in the control mix without the peptide substrate are substantially higher than the negative control (with the first termination method), the nonspecific phosphorylation of Protein Kinase Sample may be too high. To remove this nonspecific phosphorylation, the second termination method should be used.

In the second termination method, all the reactions are terminated by adding and mixing with 3 µl of 50% Trichloroacetic Acid and 2 µl of 1% Bovine Serum Albumin Solution. Place the terminated reactions on ice for 10 minutes. After a centrifugation for 5 minutes at 14,000 x g, take out 25 µl of the supernatant and transfer into another microcentrifuge tube. Neutralize the supernatant samples by adding and mixing 5 µl of Neutralization Solution. The neutralized samples are ready for Step III.

III. Affinity Ultrafiltration Separation and Measurement

NOTE: The Wash Solution may contain some crystalline precipitate after storage at +4°C. To re-dissolve the crystals, warm the Wash Solution in a bath of lukewarm water for 10 to 15 minutes.

Add 8 µl of Avidin Solution to all the terminated reaction samples obtained in Step II. After an incubation of 5 minutes at room temperature, sequentially transfer 50 µl of Wash Solution and 20 µl of the reaction samples into the sample reservoirs of Centrifugal Ultrafiltration Units (care must be taken not to touch the membrane in the sample reservoir with the pipette tip). Cap the reservoirs and place the ultrafiltration units in a microcentrifuge. Centrifugation for 5 minutes at 14,000 x g should remove most of the liquid. Add 100 µl of Wash Solution to the sample reservoir followed by another spin of 5 minutes at 14,000 x g. Repeat this washing step twice. In the final spin, allow sufficient time to remove most of the sample liquid.

To obtain total cpm in a reaction sample, set up a reference sample by transferring 4 µl of liquid sample from any of the reaction samples into a blank sample reservoir of Centrifugal Ultrafiltration Unit.

Transfer the sample reservoirs containing the reaction and reference samples into Scintillation vials. Add appropriate amount of liquid scintillation cocktail to the vials. Count for radioactivity with a channel set for ³²P.

IV. Calculations

Protein Kinase Specific Activity (pmole phosphate incorporated per minute per sample amount) is as follows:

$$\frac{375[\text{cpm}(\text{Enzyme} + \text{Substrate}) - \text{cpm}(\text{Enzyme alone})]}{[(20/4)\text{cpm}(\text{Reference})(\text{Time})(\text{Sample Amount})]}$$

or

$$\frac{75[\text{cpm}(\text{Enzyme} + \text{Substrate}) - \text{cpm}(\text{Enzyme alone})]}{[\text{cpm}(\text{Reference})(\text{Time})(\text{Sample Amount})]}$$

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Where

- 4 is the reference sample volume (in μl);
- 20 is the reaction sample volume (in μl) taken to the sample reservoir;
- 375 is the total picomoles of ATP in Protein Kinase Reaction Mix;
- $\text{cpm}(\text{Reference})$ is the reference sample count;
- $\text{cpm}(\text{Enzyme} + \text{Substrate})$ is the reaction sample count for protein Protein Kinase Sample;
- $\text{cpm}(\text{Enzyme Alone})$ is the reaction sample count for Control without the Peptide Substrate;
- Time is the incubation period (in minutes);
- Sample Amount is the amount of Protein Kinase Sample (in μg protein or μl volume).

Related Products

| Description | Cat. No. |
|---|----------|
| Protein Kinase Assay Kit, Non-Radioactive | 539484 |
| Protein Kinase A Assay Kit | 539490 |
| Protein Kinase C Assay Kit | 539491 |
| Protein Tyrosine Kinase Assay Kit | 539700 |
| K-LISA™ PTK Screening Kit | 539701 |
| ProteoEnrich™ ATP-Binders™ Kit | 71438 |

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