

ProductInformation

UNIVERSAL PROTEIN KINASE ASSAY KIT

Product Number **KA-1**

Storage Temperature 2 °C to 8 °C

TECHNICAL BULLETIN

Product Description

Protein kinases catalyze the transfer of γ -phosphate from adenosine triphosphate (ATP) to a serine, threonine or tyrosine residue in a protein substrate. Due to the critical roles they play in cellular regulation, these enzymes are of interest to researchers in both basic biomedical science and drug discovery. Protein kinases have been grouped into a number of subtypes, which include:

1. Protein Kinase C (PKC) which requires calcium and phospholipid for its activity,
2. cAMP-dependent Protein Kinase A (PKA) whose activity is dependent on cAMP, and
3. Protein Tyrosine Kinase (PTK) which phosphorylates tyrosine residues only.

Each protein kinase subtype 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.

A conventional assay of protein kinase activity involves phosphorylation of a peptide substrate by 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, imposing a limitation on the peptide sequence design, and possibly also limiting specificity and optimization of the assay. Another serious drawback to this method is that the relatively weak binding of the peptide substrate to the phosphocellulose membrane often results in the loss of the ^{32}P -peptide product during the wash step. To overcome these problems, the peptide substrate may be tagged with a biotin group so the biotinylated ^{32}P -peptide product consistently binds to a streptavidin membrane in a manner independent of the peptide sequence¹. However, the separation of ^{32}P -peptide product from the free γ - ^{32}P -ATP through the streptavidin membrane still requires an extensive and cumbersome washing procedure.

The Universal Protein Kinase Assay Kit uses affinity binding and ultrafiltration separation to analyze a sample simply, safely, efficiently, and consistently.

In the first step of the protein kinase assay, a protein kinase sample is combined with a biotinylated peptide substrate and γ - ^{32}P -ATP. The components are allowed to react, then free avidin is added which binds to the biotinylated ^{32}P -peptide product. The sample is then subjected to a centrifugal ultrafiltration process using 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 because the separation binding occurs in a solution that is 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 reduces 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.

The Universal Protein Kinase Assay Kit is designed for flexibility in protein kinase activity assays. This kit allows the user to choose his or her own protein kinase, peptide substrate and kinase reaction buffer. The kit may be used to assay activity of a protein kinase sample, or potency of a test substance or drug candidate in activating or inhibiting a protein kinase. Furthermore, the high efficiency and consistency of the ultrafiltration separation process across a large concentration range of the biotinylated peptide substrate makes it ideal for quantitative kinetic studies such as measuring the enzymatic turnover rate and Michaelis-Menten constant (K_m).

Kit Components

- U 5380: 10X (0.15 mM) ATP Solution; 10mg: to be resuspended in 150 μL dH₂O by user (-20 °C)
- U 2630: 600 μL Stop Solution (8.0 M Guanidine Hydrochloride) (4 °C)
- U 3505: 180 μL 50% Trichloroacetic Acid solution (4 °C)

- U 4505: 120 µl 1% Bovine Serum Albumin (4 °C)
- U 4755: 300 µl Neutralization Solution (4 °C)
- U 5130: 500 µl Avidin Solution (4 °C)
- U 5255: 25 µl Wash Solution (4 °C)
- U 5505: 50 Centrifugal Ultrafiltration Units

**Store filtration units at room temperature;
PROTECT FROM FREEZING**

Additional materials to be provided by the user:

1. Protein Kinase Sample: This sample may be a purified preparation or a 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 regard to the effects of impure proteases and phosphatases in the sample
2. Test Substance: This is an optional component, depending on whether an activator or inhibitor is being tested. The test substance concentration is calculated according to its potency in modulating the activity of the protein kinase sample.
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 specific type of protein kinase and the specific reaction condition of interest to the user. Buffer component variables to consider include pH, divalent metal ions, and essential activators.
5. γ -³²P -ATP Stock Solution: A typical commercial source of γ -³²P -ATP has a specific activity of about 3000 Ci/mmol and a concentration of about 10 µCi/µl.
6. Deionized Water
7. Microcentrifuge
8. Scintillation Vials: Each scintillation vial should have an inner diameter greater than 17 mm in order to accommodate the sample reservoir of the Centrifugal Ultrafiltration Unit.

Storage/Stability

After resuspending in 150 µl dH₂O, store ATP solution at -20 °C. Store remaining components at 4 °C. Filters may be stored at RT. **Protect ultrafiltration units from freezing. Freezing will damage them.**

Preparation Instructions

1. Resuspend the ATP Solution (Product No. U 5380) in 150 µl dH₂O. Keep on ice (After resuspension, the ATP solution should be stored at -20 °C).
2. Kinase/Substance Mix: This option is exercised when the effect of the Test Substance requires pre-incubation with the Protein Kinase Sample. Pre-mix the Test Substance and Protein Kinase Sample using appropriate concentrations and 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 peptide concentration may be varied as needed (e. g. in kinetic studies 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 the kit.

4. γ -³²P -ATP Working Solution: Use deionized water and γ -³²P -ATP Stock Solution to prepare γ -³²P -ATP Working Solution (about 0.2 µCi/µl).

5. Protein Kinase Reaction Mix: The following formulation is designed to provide the appropriate amount of Protein Kinase Reaction Mix for a given number of reactions, N. To compensate for liquid loss during pipetting, N should be 110% of the actual test number to be conducted with each protein kinase reaction mix (e. g. N is set to 11 for 10 tests). The tests should include a negative control (no enzyme added) as well as other appropriate controls. Mix the following components in a microcentrifuge tube:

2.5 x N µl of 10X ATP Solution
 2.5 x N µl of 10X Biotinylated Peptide Substrate Solution
 2.5 x N µl of 10X Protein Kinase Reaction Buffer
 2.5 x N µl of γ -³²P -ATP Working Solution
10 x N µl of Deionized Water or Optional Test Substance
 Total: 20 x N µl

6. Control Mix (Without Peptide Substrate): This control measures endogenous phosphorylation of proteins in the Protein Kinase Sample by γ -³²P -ATP. Mix the following components in a microcentrifuge tube:

2.5 µl of 10X ATP Solution
 2.5 µl of Deionized Water
 2.5 µl of 10X Protein Kinase Reaction Buffer
 2.5 µl of γ -³²P -ATP Working Solution
10 µl of Deionized Water or Optional Test Substance
 Total: 20 µl

Procedure

1. Pipette 20 μ l of Protein Kinase Reaction Mix into the appropriate number (N) of microcentrifuge tubes.
2. Initiate reactions by adding 5 μ l of Protein Kinase Sample (or optional kinase/substance mix) to each of these microcentrifuge tubes containing 20 μ l of Protein Kinase Reaction Mix and the microcentrifuge tube containing 20 μ l of Control Mix (without peptide substrate).
3. Prepare the negative control by adding 5 μ l of deionized water instead of Protein Kinase Sample to one of the microcentrifuge tubes containing 20 μ l of Protein Kinase Reaction Mix.
4. Incubate all the reactions at the appropriate temperature for the desired period of time.

Termination Step

5. In the first termination method, terminate all the reactions by adding and mixing with 10 μ l of Stop Solution. After a brief centrifugation, the terminated reactions are ready for Step 6. If, after Step 6, the count of peptide minus control is substantially higher than that of kinase minus control (using 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, add and mix 3 μ l of 50 % Trichloroacetic Acid Solution and 2 μ l of 1 % Bovine Serum Albumin Solution to each sample. Place the samples on ice for 10 minutes. Centrifuge the samples for 5 minutes at 14,000 x g, then transfer 25 μ l of the supernatant of each sample to another microcentrifuge tube. Neutralize the supernatant samples by adding and mixing 5 μ l of Neutralization Solution. The neutralized samples are now ready for Step 6.

Affinity Ultrafiltration Separation and Measurement

6. Add 8 μ l of Avidin Solution to each of the terminated reaction samples obtained in Step 5.
7. After a 5 minute incubation at room temperature, sequentially transfer 50 μ l of Wash Solution and 20 μ l of the reaction samples into the sample reservoirs of the Centrifugal Ultrafiltration Units (care must be taken not to touch the membrane in the sample reservoir with the pipette tip).
8. Cap the reservoirs and place the ultrafiltration units in a microcentrifuge.
9. Centrifugation for 5 minutes at 14,000 x g should remove most of the liquid.

10. Add 100 μ l of Wash Solution to the sample reservoir followed by another spin of 5 minutes at 14,000 x g.
11. Repeat this wash step twice. In the final spin, allow sufficient time to remove most of the sample liquid.
12. To measure the total cpm in each reaction sample, set up a reference sample by transferring 4 μ l of the liquid from any of the reaction samples into a blank sample reservoir of a Centrifugal Ultrafiltration Unit.
13. Transfer the sample reservoirs containing the reaction and reference samples into scintillation vials.
14. Add the appropriate amount of liquid scintillation cocktail to each vial.
15. Count for radioactivity with a channel set for ^{32}P .

Results

Protein Kinase specific activity (pmole phosphate per minute per sample amount) is calculated as follows:

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

or

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

Where:

4 is the reference sample volume (in μ l);
20 is the reaction sample volume (in μ l) taken to the sample reservoir;
375 is the total picomoles of ATP in Protein Kinase Reaction Mix;
cpm (Reference) is the reference sample count;
cpm (Enzyme + Substrate) is the reaction sample count for the Protein Kinase Sample;
cpm (Enzyme Alone) is the reaction sample count for Control Mix (without peptide substrate);
Time is the reaction incubation time (in minutes);
Sample Amount is the amount of Protein Kinase Sample (in μ g protein or μ l volume).

Reference

1. Goueli, B.S. et al., Anal. Biochem. **225**, 10-17 (1995).

Related Products

- Sigma-Genosys offers custom synthesis of biotinylated peptides of any user-specified sequence.

- Purified protein kinases that may be used as positive controls for the assay are available from Sigma.

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