

A novel method for non-radioactive assays of specific protein kinases

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We report the development of a novel, non-radioactive protein kinase assay which employs dye-conjugated peptide substrates for specific protein kinases. The peptides have been designed so that phosphorylation causes a change from a +1 to a -1 net charge, allowing rapid separation and identification of the reaction products. Unlike assays utilizing ^{32}P incorporation, this assay is not subject to interference from phosphorylation of other substrates present in crude samples.

Introduction

Changes in the level of phosphorylation in the cell have been shown to affect a wide variety of cellular processes, including cell division, cell motility, signal transduction and oncogenic responses. In addition, phosphorylation of specific target proteins has been shown to affect processes such as *in vitro* protein translation in cell-free systems and transcription of eukaryotic genes. Therefore, it is not surprising that protein kinases have become one of the most studied classes of enzymes in the world.

Serine-threonine kinases

In general, protein kinases in mammalian cells are classified by the types of amino acids they phosphorylate. The bulk of phosphorylation found in cells is mediated by serine-threonine protein kinases such as calcium- and phospholipid-dependent protein kinase C, cyclic nucleotide-dependent protein kinases, calmodulin-dependent protein kinases and many others. The method used to assay these enzymes is dependent on the system studied. In whole cells, incorporation of radioactive phosphate from the media into protein is monitored. In crude extracts, the extent of ^{32}P incorporation is measured following the addition of [γ - ^{32}P]ATP and activators of specific kinases. In systems involving pure proteins, phosphorylation can be studied using purified protein kinases obtained either from commercial sources or by direct purification of the desired kinase.

Tyrosine kinases

Tyrosine kinases, the second general class of protein kinases, are predominantly membrane proteins or proteins associated with membrane proteins. Tyrosine kinases such as the EGF receptor, PDGF receptor, insulin receptor and others, are thought to be involved in signal transmission from the outside of the cell. However, the route involved with signal transmission is often thought to involve the activation of serine-threonine kinases present in the cell. Thus, studies of the physiological effects of tyrosine kinases frequently involve measurement of the activity of serine-threonine protein kinases.

Limitations of radioactive protein kinase assays

Most existing assays of protein kinases depend upon the measurement of the amount of radioactive phosphate which has been transferred from [γ - ^{32}P]ATP to an acceptor substrate added to the assay. These assays have two intrinsic drawbacks: 1) they use a radioactive nucleotide which must be replaced regularly and which requires special safety precautions; and 2) their accuracy may be dependent upon the particular substrate used and on the amount of natural substrate available in the sample for phosphorylation by other kinases. Two types of substrates are used: general substrates such as casein or histone, and peptide substrates such as Kemptide. Using purified enzymes and peptides, peptide substrates have been shown in some cases to be modified by only one protein kinase.

Radioactive protein kinase activity measurements made in crude samples using either peptide or protein substrates are complicated by the phosphorylation of natural substrates, which occurs due to the presence of other kinases in the sample. Accurate measurement of the activity of a specific kinase under these circumstances relies on measurement of the *differential* level of phosphorylation seen when specific activators of the protein kinase under study are added to the assay. A method that could measure the phosphorylation of a specific peptide added to the assay, even in a crude sample, would eliminate this complication.

Novel non-radioactive protein kinase assays

Promega has recently developed the PepTagTM Non-Radioactive Protein Kinase Assays* for the measurement of cAMP-Dependent Protein Kinase (cAMP-PK) and Protein Kinase C (PKC). These assays utilize brightly colored peptide substrates, created by the addition of a dye molecule which imparts a "hot pink" color. The amino acid compositions of these peptides are designed such that they have a +1 net charge at neutral pH. Following modification by a specific protein kinase, the peptides acquire a -1 net charge due to the addition of a phosphate group. This change in the net charge of the substrate allows the modified and unmodified versions of the substrate to be separated by agarose gel electrophoresis at neutral pH in any standard flat bed apparatus. The phosphorylated peptide migrates quickly towards the positive electrode while the unmodified substrate migrates towards the negative electrode ([Figure 1](#)). The activity of the kinase in the sample can then be estimated based upon the amount of phosphorylated peptide formed.

*Patent pending.

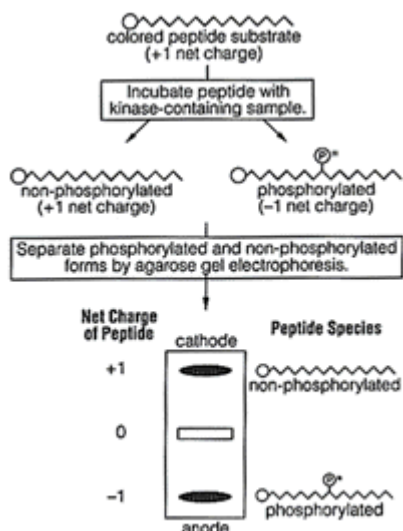


Figure 1. Schematic diagram of the PepTag Non-Radioactive Protein Kinase Assay procedure.

Included with each system are the appropriate colored peptide substrate, the solutions required for the kinase reaction and quantitation steps, and the appropriate positive control protein kinase and kinase activator.

Specific quantitation of protein kinases

Because the colored peptides supplied with the PepTag Systems are highly specific for the particular kinase to be assayed, phosphorylation of other substrates which occur naturally in the sample does not add to the kinase activity measured. When it is important to confirm the specificity of a particular kinase, differential activity should be measured in the presence or absence of specific activators such as phosphatidyl serine (for PKC) or cAMP (for cAMP-PK). A qualitative estimate of the activity of a particular kinase in a sample can be obtained in about 1.5 hours by directly observing the amount of product which migrates towards the positive electrode ([Figure 2](#)).

While quantitation of specific kinase activity can be performed by measuring incorporation of ^{32}P into the labeled substrates, as for typical kinase assays, kinase activity also can be quantitated spectrophotometrically. To do this, the peptide substrate is simply excised from the gel and released by melting the agarose plug in the Clarifying Solution supplied, which prevents the rapid re-solidification of the agarose. The absorbance at 570nm is then measured in a spectrophotometer. These additional steps for spectrophotometric quantitation require only 30-60 minutes ([Figure 2](#)).

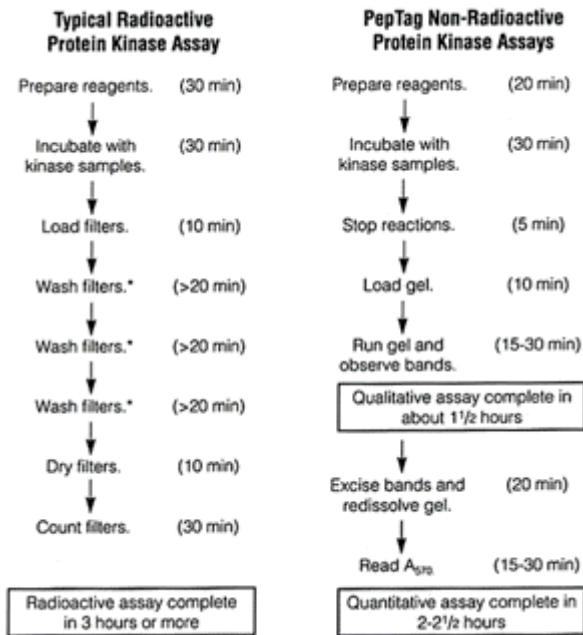


Figure 2. Comparison of a typical radioactive protein kinase assay with the PepTag Non-Radioactive Protein Kinase Assay. The assay times listed are based on performing a batch of 30 assays simultaneously. *Twenty-minute wash times are adequate for phosphocellulose filters, but 1-2 hour wash times are typically needed for glass fiber filters.

Alternatively, the intact agarose gel can be analyzed in a scanning densitometer with light of about 570nm. Because differences may exist between commercial densitometers, however, we cannot recommend a specific protocol for scanning.

Fluorescent detection to 10ng sensitivity

The dye coupled to the peptide substrate, in addition to having a high molar absorptivity, is also fluorescent. This property allows very small amounts of the phosphorylated target peptide to be visualized, and thus very small amounts of kinase activity to be detected. Using fluorescence visualization, the PepTag assays have been used to detect the activity of as little as 100 attomoles (about 8ng) of Protein Kinase C and 30 attomoles (1-2ng) of cAMP-Dependent Protein Kinase catalytic subunit (Figure 3). Using a spectrofluorometer to measure fluorescence in excised gel bands, quantitation in this sensitivity range also should be possible.

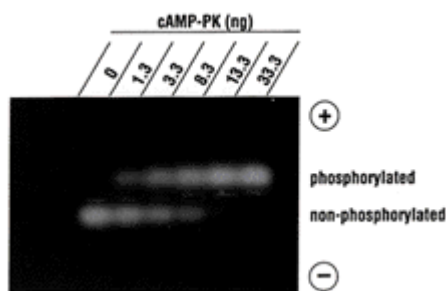


Figure 3. Detection of cAMP-Dependent Protein Kinase. One microgram of Promega

Peptide A (provided in the system) was incubated in a solution containing 20mM HEPES (pH 7.4), 1mM ATP, 10mM MgCl₂ and varying amounts (1.3-33.3ng) of the catalytic subunit of bovine cAMP-Dependent Protein Kinase (Promega) in a final volume of 20μl for 30 minutes at room temperature. The reactions were stopped by heating to 95°C for 10 minutes. The samples were loaded on a 0.8% agarose gel and run at 100V for 15 minutes. Phosphorylated peptide migrated towards the anode (+), while non-phosphorylated peptide migrated towards the cathode (-). The gel was photographed on a transilluminator.

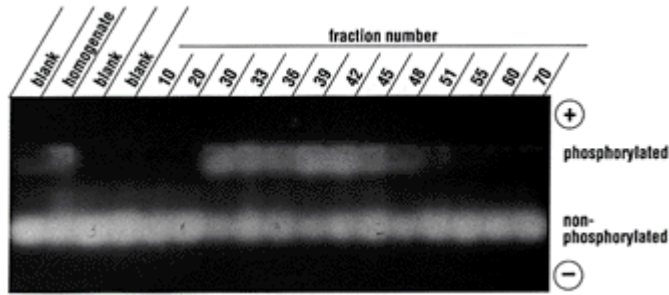


Figure 4. Detection of Protein Kinase C in crude samples fractionated by DEAE-cellulose chromatography. The brains from 100 adult male rats were homogenized, centrifuged, and filtered as described by Walton *et al.* (4). The filtrate was loaded on to a 250ml DEAE anion exchange column. After washing, protein was eluted in a 0.0-0.4M NaCl gradient. Fractions were then assayed using both a radioactive protein kinase assay (4) and the PepTag Non-Radioactive Protein Kinase Assay as described below. Ten microliters of column fraction was added to a solution containing 20mM HEPES (pH 7.4), 1.3mM CaCl₂, 1mM DTT, 200μg/ml phosphatidylserine, 10mM MgCl₂, 1mM ATP, and 2μg of Promega Peptide C in a final volume of 40μl for 30 minutes at room temperature. The reaction was stopped by heating to 95°C for 10 minutes. The samples were loaded onto a 0.8% agarose gel which was run and photographed as described in [Figure 3](#). Peptide phosphorylated by Protein Kinase C migrated towards the anode (+), while non-phosphorylated peptide migrated towards the cathode (-). The column fractions from which the samples were taken are indicated in the figure. Fraction 42 contained the peak of Protein Kinase C activity. Comparison of the results from this experiment with that of the radioactive assay showed that Protein Kinase C was reliably detected in the same fractions by both methods (data not shown).

Importance of a rapid assay for protein kinases

Purified kinases are important tools for studies which measure the effect of protein kinases on a variety of different systems. The purification of protein kinases is complicated, however, by the fact that some kinases, such as the calcium- and phospholipid-dependent Protein Kinase C, are unstable, especially in crude preparations where they quickly undergo degradation by cellular proteases. For these applications, it would be very helpful to have a rapid, qualitative kinase assay which would allow kinase activity in column fractions to be quickly detected.

Because the electrophoretic separation of the phosphorylated and the unmodified substrate can be visualized in only 10-15 minutes from the start of the run, the PepTag Assay can be used to rapidly determine which fractions from a protein kinase purification contain the desired activity. [Figure 4](#) illustrates how this assay was used to monitor the fractionation of a DEAE-cellulose column which

contained Protein Kinase C. The ability to rapidly assay protein kinases through the initial steps of purification could be crucial to the success of the purification, given the instability commonly observed for these enzymes in crude cellular extracts.

Application to phosphatase assays

We have also found that the phosphorylated peptides which are made in the PepTag Assay Systems can be used to measure protein phosphatase activity. To test this, we used cAMP-Dependent Protein Kinase to phosphorylate its specific peptide substrate. Following inactivation of the kinase, alkaline was able to dephosphorylate the substrate and restore its original +1 net charge (data not shown). We do not yet know if this particular substrate will show specificity for a particular cellular protein phosphatase.

The basic assay format lends itself well to phosphatase assays. For these assays, however, the modified form of the peptide would now be the *unphosphorylated* form of the peptide, not the phosphorylated form of the peptide.

Summary

In conclusion, the PepTag Systems introduce a novel, rapid and non-radioactive method to detect and quantitate the activity of cAMP Dependent Protein Kinase or Protein Kinase C. The assays are specific for the protein kinase in question and are as rapid as the more commonly used radioactive assays. Furthermore, because they do not use radioactivity, these new assays can be performed without shielding, health risks or concern about the disposal of radioactive reagents.

References

1. Hunter, A. (1987) *Cell* **50**, 823.
2. Ullrich, A. and Schlessinger, J. (1990) *Cell* **61**, 203.
3. Roskoski, R., Jr. (1983) *Meth. Enzymol.* **99**, 3.
4. Walton, G.M. *et al.* (1987) *Anal. Biochem.* **161**, 425.

Ordering information:

Product	Size	Cat.#
Protein Kinase C	1µg (2 x 0.5µg)	V5261
cAMP-Dependent Protein Kinase, Catalytic Subunit	2,500u	V5161
	12,500u (5 x 2,500u)	V5162
cAMP-Dependent Protein Kinase, Regulatory Subunit (Type II)	2,500u	V5221

cGMP-Dependent Protein Kinase (alpha Isozyme)	6,000u	V5171
	30,000u (5 x 6,000u)	V5172

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