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Fluorescent Peptide Assays For Protein Kinases

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Abstract

Protein kinases are enzymes that regulate many cellular events in eukaryotic cells, such as cell cycle progression, transcription, metabolism and apoptosis. Protein kinases each have a conserved ATP binding site, as well as one or more substrate-binding site(s) that exhibit recognition features for a protein substrate. By thus bringing ATP and a substrate into close proximity, each protein kinase can modify its substrate by transferring the γ -phosphate of the ATP molecule to a serine, threonine or tyrosine residue on the substrate. In such a way, signaling pathways downstream from the substrate can be regulated, dependent on the phosphorylated *versus* dephosphorylated forms of the substrate.

Keywords

Peptide Assays; Kinases; fluorescent peptides

The biochemical activity of most purified protein kinases can be measured by monitoring the incorporation of phosphate into a peptide substrate. A frequently used assay involves the use of radiolabelled [γ - ^{32}P]-ATP to evidence the transfer of the γ -phosphate from ATP to the substrate. The following protocol describes an alternative assay using a class of fluorescent peptide substrate to measure the incorporation of non-radiolabelled phosphate. While this protocol pertains to the Sox fluorophore the basic approach may be applied to other fluorescent reagents. The assay described here is based on the principle that in the presence of magnesium, the phosphorylation of the peptide substrate leads to an increase in the fluorescence emission intensity of an appended Sox fluorophore.

Basic Protocol

Measuring protein kinase activity using fluorescent peptides

A fluorescence-based assay may be performed to determine the activity of a given protein kinase. This involves incubating the protein kinase with a suitable fluorescent peptide substrate and the co-substrate MgATP in an appropriate buffer and monitoring the change in fluorescence over time. Generally, a reaction mixture is prepared in the absence of a substrate peptide, which is then added to initiate the assay. The assay may be performed in a fluorimeter, where usually a single reaction is monitored at a time, or in a plate reader where multiple reactions may be monitored simultaneously. The assay described here is sensitive, continuous in nature and does not require a second coupled enzyme assay.

Buffer Components—To initiate studies, a solution of HEPES buffer at pH 7.5 containing 10 mM magnesium is used. Such a solution is chosen for its historical reliability in fostering a satisfactory protein kinase activity level. A typical protein kinase has a K_M for

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MgATP that is in the range of 10-100 μM in the presence of 10 mM magnesium. Thus, a concentration of 1 mM MgATP can be used for initial assessment of the activity of an uncharacterized kinase. Bovine serum albumin (BSA) and a low concentration of detergent (such as 0.03% Brij-35) can be used to deter denaturation of protein kinases that nonspecifically adhere to the sides of their containers (i.e., test tubes and cuvettes). Solutions should be prepared fresh using degassed water, to minimize oxidation of the fluorescent peptide during the course of the experiments.

Instrument settings—Reactions of Sox peptides are optimally monitored at 482 nm with excitation at 360 nm (please refer to individual instrument instructions for specific settings).

Degassing water—There are several methods of removing dissolved gases from water. A simple method is to subject the water to vacuum for 5-10 mins, subject the water to ultrasonics for 10-15 mins, or using a 7 μM filter, gently sparge the solvent for 5 minutes with an inert gas, such as helium, which has a very low solubility compared to oxygen and nitrogen.

Materials: Always use distilled, deionized water, which has been degassed.

Always use the highest-grade chemicals where possible

Stock solutions (see recipes under “Reagents and Solutions”)

5x Assay Buffer, pH 7.5

10 mg/mL Bovine Serum Albumin (BSA)

250 μM peptide (5x peptide) (see below for a description of available peptides)

5 mM non-radiolabelled ATP (5x ATP)

10-100 nM enzyme in 1x assay buffer

Instruments: Fluorimeter [any standard fluorimeter fitted with temperature control and a normal set of excitation and emission filters will be more than adequate; e.g. Fluorolog model FL3-11 fluorometer (Jobin Yvon, Edison, NJ)]

Spectrophotometer [e.g. Cary 50 Scan UV-visible Spectrophotometer (Varian Inc, CA)]

Apparatus: Fluorescence grade cuvette [e.g. a three-window fluorescence grade quartz cuvette with a path length of 10 mm and aqueous volume of 100 μL , Hellma, Plainview, NY)]

pH meter equipped with electrode [e.g. Corning, NY, catalog number: 37-475305, or InLab®Micro micro combination pH electrode]

pH paper [e.g. Fisher Scientific, catalog number 19-120-829]

1. Prepare Solutions (A) and (B) (as described below) and incubate on ice:

A. Assay Reaction mixture

180 μL of 5x assay buffer

4 μL of 10 mg/mL BSA

200 μL of 5x (5 mM) ATP

100 μ L of protein kinase solution (20-100 nM) (in 1x assay buffer)

Bring to 800 μ L with water

B. Control (no enzyme) reaction mixture

200 μ L of 5x assay buffer

4 μ L of 10 mg/mL BSA

200 μ L of 5x (5 mM) ATP

Bring to 800 μ L with water

Note:

- i. the above solutions are calculated for ten reactions at a volume of 100 μ L each.
 - ii. final volumes can be adjusted to the volume of the cuvette. The above are for a 100- μ L cuvette.
2. Transfer 80 μ L of Solution A into the cuvette and place the cuvette in the fluorimeter. Note it is good practice to pre-equilibrate the cuvette at 30 °C in the fluorimeter before adding the solution.
3. Incubate the cuvette at 30 °C for 5 minutes prior to initiating the reaction.
4. Initiate the reaction by adding a pre-warmed peptide solution (20 μ L) to the cuvette, with stirring.
5. Monitor the change in fluorescence emission continuously at 485 nm during the linear phase (the first 5-10% of the reaction) (most fluorimeters have a setting that allows measurements to be quantified at set time intervals).
6. Determine the endpoint of the reaction (see below).
7. Repeat steps 2-6 for the control assay

Note:

- i. several trials are usually necessary to determine the optimum concentration of protein kinase to use in the assays. Typically, concentrations in the range of 2-20 nM may be used, depending on the activity of the protein kinase towards the peptide substrate.
- ii. the total change in fluorescence will vary between peptides, but is typically between 2 and 6-fold.
- iii. wash and dry the cuvette between each assay to avoid carrying over solutions between assays.
- iv. protein kinases often have significant ATPase activity (i.e. they hydrolyze ATP), therefore it is important not to incubate the protein kinase with ATP for long periods of time. Alternatively, swap the 5x ATP in the 'Assay Reaction mixture' for a 5x peptide solution and initiate reactions by the addition of 20 μ L of 5x ATP.
- v. When determining the rate for a protein kinase catalyzed reaction using a fluorescent substrate it is necessary to be able to relate the change in fluorescence to the progression of the reaction (Substrate, S $\rightarrow$ product, P). In essence a conversion factor, f, is required to allow one to convert a

change in fluorescence with time ($\Delta F/\Delta t$) to a change in product formation with time ($\Delta P/\Delta t$). A very important measurement therefore is the determination of the overall change in fluorescence that corresponds to the complete conversion of $S \rightarrow P$. One way to achieve this determination is to monitor the reaction directly for approximately 5 half lives (where the reaction has proceeded 96.8% to completion). The endpoint may then be determined by fitting the observed change in fluorescence F with time to the first-order rate equation, eqn. 1, where F_{∞} is the fluorescence of the product, F_0 is the fluorescence of the substrate and k_{obs} is the observed rate constant for the reaction. In most cases enough kinase can be added to a reaction to achieve a convenient rate of conversion. Thus, in Figure 1 we monitor the conversion $S \rightarrow P$ of a reaction over the course of five half lives (20 minutes).

$$F = (F_{\infty} - F_0)(1 - e^{-k_{\text{obs}}t}) + F_0 \quad \text{eqn. 1}$$

- vi. We illustrate the approach with the example shown in Figure 1A where the phosphorylation of a 10 μM solution of a fluorescent peptide substrate, by a protein kinase, was examined. To determine the change in fluorescence that accompanies the phosphorylation the reaction was allowed to proceed to approximately 5 half lives. The best non-linear fit of the progress curve in Figure 1A to eqn. 1 furnished a value for $\Delta F_{\infty} = F_{\infty} - F_0$ of 206300 ± 1200 fluorescence units (FU), which corresponds to a conversion factor of $f = 20630 \text{ FU per } \mu\text{M of product formed}$.
- vii. Typically the rate of a protein kinase catalyzed reaction is determined under conditions where the conversion of substrate to product is not allowed to exceed 10%. Over this range the appearance of product with time should be approximately linear. In Figure 1B the same concentration of substrate peptide (10 μM) as used in Figure 1A was incubated with a smaller amount of the same protein kinase and monitored for 300 seconds. The best linear fit through the progress curve gives a slope of 74 FU/second, which when divided by the conversion factor f of 20630 FU per μM of product furnished a rate of 0.0036 $\mu\text{M/s}$.

Measuring protein kinase activity using a fluorescence plate reader

A large number of assays can be performed and read in a more time efficient manner using 96 well plates and a plate reader.

Additional materials for Alternate Protocol

Instruments: Plate reader (e.g. Victor³ V multi-label plate reader (Perkin Elmer) connected to a computer)

Apparatus: 96 well plate (e.g. Corning, catalog # 3992))

Multi-channel pipette (e.g. P200 Rainin)

Solution reservoir (e.g. Eppendorf, catalog # 022265806)

1. Prepare the Reaction Mixtures as described above. Keep the mixtures on ice until the time of assay.

2. Add 80 μL of the reaction mixture to each well of the 96-well plate, pre-warmed at 30 $^{\circ}\text{C}$.
3. Incubate reactions at 30 $^{\circ}\text{C}$ in the plate reader for 5 minutes.
4. Initiate the reactions with 20 μL of the 5x peptide solution, pre-warmed to 30 $^{\circ}\text{C}$.
5. Monitor the change in fluorescence emission continuously at 485 nm during the linear phase (the first 5-10% of the reaction) (most plate readers have a setting that allows measurements to be quantified at set time intervals).
6. Determine the endpoint of the reaction (see below).

Note:

- i. reactions and controls may be monitored simultaneously.
- ii. It is important to be aware that slight variations in temperature can occur between the wells of a 96-well plate, especially between the center at the edges of a plate.

REAGENTS AND SOLUTIONS

Always use distilled, deionized water

Always use the highest-grade chemicals when possible

For accurate preparation of solutions use grade A volumetric flasks, an analytical balance and calibrated pipettes

5x Assay Buffer

125 mM HEPES, pH 7.5

250 mM KCl

0.5 mM EDTA

0.5 mM EGTA

50 mM MgCl_2

10 mM DTT

Buffer made without DTT can be stored at 4 $^{\circ}\text{C}$ for a month. DTT should be added to the assay buffer fresh every time on the day of the assay.

10 mg/mL BSA

Dissolve 100 mg of BSA in 10 mL of water. Aliquot, freeze and store up to a year at -80°C .

5x (5 mM) ATP

Dissolve 60 mgs of ATP [$(\text{Na}^+)_2\text{ATP}^{2-} \cdot 3\text{H}_2\text{O}$, Roche catalog #: 10519987001] in 10 mL water. Adjust the pH to 7.5 using a 1 mM solution of potassium hydroxide. Adjust the concentration of the ATP to 5 mM by adding the appropriate volume of water and monitoring the absorbance at 259 nm (OD_{259}) using a spectrophotometer. The concentration may be calculated from the relationship $A = \epsilon lc$, where A = absorbance, $\epsilon = 15,400$ ($\text{cm}^{-1}\text{M}^{-1}$), l = path length in (cm), and c = concentration (M). Store at -20°C for up to a year. It is recommended to freeze stock of ATP in 100 μL aliquots to avoid excessive handling.

5x (250 μ M) peptide

Peptides are available commercially or can be synthesized (Luković E, et. al., 2008). Dissolve the peptide in water and adjust the pH to an approximate value of 7.5 (pH paper can be used to measure pH) (see below for solubility issues).

Determine the concentration by measuring the absorbance at 355 nm (OD_{355}) using a spectrophotometer. The concentration may be calculated from the relationship $A = \epsilon lc$, where A = absorbance, $\epsilon = 8,247 \text{ (cm}^{-1}\text{M}^{-1}\text{)}$ (based on the extinction coefficient of the Sox moiety at 355 nm in solution of 0.1 M NaOH and 1 mM Na_2EDTA), l = path length in (cm), and c = concentration (M).

Store in 250 μ l aliquots for up to a year or more at -20°C .

Note: Peptides with low solubility in aqueous solution should be dissolved in other solvents, such as 10% ammonium bicarbonate solution for a negatively charged peptide, or 30% acetic acid for a positively charged peptide (note a basic solution should not be used with cysteine-containing peptides). Several organic solvents such as acetonitrile, DMSO, DMF, or isopropanol may also be used. In each case the minimum quantity of the non-aqueous solvent should be used, followed by the addition of water, or buffer to make up the desired volume. If a peptide shows a tendency to aggregate add 6 M guanidine-HCl, 6 M urea, or 6 M urea with 10-20% acetic acid to the peptide and dilute accordingly.

COMMENTARY

Background Information

Protein kinases are enzymes that control diverse cellular processes in eukaryotic cells by phosphorylation of key substrates in biochemical pathways. The human genome encodes 518 separate protein kinase genes, accounting for nearly 1.7% of all human genes (Manning et. al., 2002). While protein kinases contain a highly conserved ATP-binding site, protein substrates are recognized through a variety of strategies, often involving multiple weak interactions, which support recognition of a consensus sequence at the active site. Protein kinases catalyze the transfer of the γ -phosphate from ATP to the hydroxyl group of a serine, threonine or tyrosine on a substrate protein. This phosphorylation alters the physical properties of a substrate, leading to a change in its catalytic activity or ligand-binding ability. Such changes influence downstream cell signaling pathways, thereby affecting processes such as cell growth, proliferation, differentiation and apoptosis.

The biochemical activity of recombinant kinases can be quantified *in vitro* by monitoring the amount of phosphate incorporation from ATP into a substrate. Common approaches for measuring protein kinase activities involve either radioactive labels or the coupling of a second colorimetric reaction to the protein kinase reaction. The radioactive labeling method monitors the transfer of the gamma (γ) phosphate of [γ - ^{32}P] ATP to a substrate and is not continuous in nature. This method also requires special handling and precautions, which can be technically challenging and is generally difficult to use in high throughput format. Coupled assays require careful controls to ensure the coupled assay has no influence on the observed kinetics.

Fluorescence assays may be sensitive and amenable to high-throughput. Some of the common approaches include the use of environmentally sensitive fluorophores positioned adjacent to the phosphorylated residue, or fluorescence resonance energy transfer (FRET) technology to detect conformation changes in the substrate upon phosphorylation. Though widely used, these methods often require specific reagents and significant development, to optimize the sensitivity of the reaction.

The use of the fluorescent Sox moiety was introduced by Shults and Imperiali (Shults and Imperiali, 2003; Shults et. al, 2005). The original approach required the incorporation of an unnatural, Sox-containing, amino acid into a peptide using standard solid phase peptide chemistry. However, it was shown recently that the introduction of the Sox moiety can be achieved through cysteine labeling, greatly simplifying substrate preparation (Luković E et. al 2008). The basis for the assay is the observed increase in fluorescence of a suitably positioned Sox moiety, in the presence of Mg^{2+} , which accompanies the phosphorylation of a Ser, Thr or Tyr residue. This increased fluorescence can be measured at 485 nm when excited at 360 nm (Shults and Imperiali, 2003). In general, a Sox moiety placed at the -2 or +2 position from the phosphorylatable residue in a peptide confers optimum sensitivity. This ability to place the Sox moiety either *N*-terminal or *C*-terminal to the phosphorylation site represents a significant advantage of the approach as most protein kinases appear to be relatively insensitive to substitution at one of the positions. Currently, a range of Sox-based fluorescent peptide substrates are available commercially from Invitrogen. Alternatively, when an appropriate substrate is not commercially available, Sox peptides can be synthesized following the methods reported by (Luković E et. al 2008).

Critical Parameters and Troubleshooting

Many protein kinases are capable of phosphorylating peptide substrates, with K_M values in the range of 10-100 μM . We have found in most cases that the incorporation of a Sox moiety into a peptide has only a minor (less than 5-fold) effect on peptide turnover and therefore knowledge of the kinetic parameters of a protein kinase for a particular peptide substrate is generally a good place from which to design an assay. We have routinely used the peptide up to concentrations of 200 μM and as low as 10 μM when determining the activity of a protein kinase using the initial rate approach.

It is important to monitor for any change in fluorescence of the peptide in the absence of enzyme using the control assay. If a significant change does occur, it may be due to the slow decomposition of the Sox fluorophore due to chemical reaction or oxidation. If this occurs, consider degassing the solvents more effectively and using higher-grade chemicals in the buffers. Small changes of the control fluorescence with time can be subtracted from the assay readings.

Care should be taken when handling any protein kinase. Mixing should be gentle to prevent denaturation. To avoid adherence of a protein kinase to the sides of tubes, plates or cuvettes it is recommended that BSA (40-100 $\mu g/mL$), and/or 0.03% Brij-35 be used in the reaction. Most protein kinases are stable for several weeks when frozen at $-80^\circ C$ in a solution containing glycerol (usually 10%). To avoid excessive handling enzyme preparations should be aliquoted, snap-frozen in liquid nitrogen, and stored at $-80^\circ C$.

Determination of an accurate endpoint for the reaction is critical for an accurate determination of a reaction rate. While a kinase reaction will go to completion in many cases, considerable care must be taken to ensure the correct endpoint is obtained. One potential problem is the decomposition of the fluorescent peptide over time due to oxidation or bleaching. Another possible issue, in some cases, may be that product inhibition of the enzymatic reaction significantly slows down the attainment of the endpoint. Bleaching may be avoided by incubating the reaction in the dark until the endpoint is reached. While it may not be necessary to determine the endpoint for each individual assay it is important to note that the overall change in fluorescence that accompanies substrate conversion may vary between reaction conditions. In cases where the attainment of the endpoint is slow, the concentration of the protein kinase may be increased by 10-fold to facilitate substrate conversion.

Anticipated Results

Upon peptide phosphorylation, an increase in signal intensity of 3 to 6 fold is expected.

Time Considerations

Aside from the initial preparation time (preparation of solutions, reagents, mixtures, etc), each individual reaction assay can be completed and recorded within minutes.

Additional Notes

For a good mathematical description of simple kinetic expressions as well as practical considerations the reader is referred to *Catalysis in Chemistry and Enzymology* by W.P. Jencks. Dover Edition, 1987.

Sox-based peptides can be used to screen collections of small molecules for inhibitors of a particular protein kinase. However, it should be noted that a small percentage of a typical library of small molecules may exhibit significant autofluorescence, which may interfere with the kinetic measurements.

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Figure 1.

Time courses for the phosphorylation of a fluorescent substrate by a protein kinase at A) 10x protein kinase and B) 1x protein kinase