Published in final edited form as:

Curr Protoc Mol Biol. 2010 July ; CHAPTER: Unit-18.14. doi:10.1002/0471142727.mb1814s91.

Analysis of protein kinase specificity using arrayed positional scanning peptide libraries

Catherine Chen and Benjamin E. Turk*

Department of Pharmacology, Yale University, New Haven, Connecticut

Abstract

Protein kinases vary substantially in their consensus phosphorylation motifs, the residues that are either preferred or deselected by the kinase at specific positions surrounding the phosphorylation site. The protocol described here is used to rapidly determine phosphorylation motifs for serine-threonine kinases. The procedure involves screening an arrayed combinatorial peptide library consisting of 198 biotinylated substrates. Peptides are phosphorylated by the kinase of interest in the presence of radiolabeled ATP and then captured on streptavidin membrane. The membrane is subsequently washed, dried and exposed to a phosphor screen to visualize and quantify incorporation of radiolabel into the peptides. The phosphorylation motif is thereby derived from the relative extent of phosphorylation of each peptide in the array.

Keywords

phosphorylation motif; kinase specificity; peptide array

Unit introduction

Protein kinases phosphorylate their target substrates in the context of specific amino acid sequences surrounding the site of phosphorylation (Ubersax and Ferrell, 2007; Turk, 2008). Kinase phosphorylation site selectivity has an important role in mediating substrate recognition and ultimately ensuring activation of correct downstream signaling pathways in cells. This unit presents a method in which a combinatorial peptide library is rapidly screened to determine the phosphorylation site motif for a protein kinase (Hutti et al., 2004). The method involves simultaneous assay of hundreds of peptide substrates in which the amino acid sequence is systematically varied across multiple positions surrounding the phosphorylation site, providing a comprehensive view of kinase specificity.

The general workflow for peptide library screening is shown in Figure 1. The procedure is carried out using either a 384-plate format (Basic Protocol) or a 1536-well format (Alternate Protocol), depending on the needs of the investigator (as described below). In advance of performing either protocol, stock solutions of the 198 peptide substrates must be made and arrayed into storage plates (Support Protocol 1). Both formats involve setting up reactions in multiwell plates consisting of the protein kinase of interest, radiolabeled ATP, and a set of biotinylated peptide substrates. Once the reaction is complete, aliquots of each reaction are transferred to a streptavidin-coated membrane, which is then washed extensively, dried, and exposed to a phosphor storage screen (Support Protocol 2).

*Corresponding author: ben.turk@yale.edu, Phone: (203) 737-2494, Fax: (203) 785-7670.

Strategic planning

It is important to carefully consider the source from which the kinase is purified to ensure that it is both catalytically active and available in sufficient quantity. The most common expression systems for producing recombinant proteins use *E. coli*, insect cells, or mammalian cells. Bacterial expression systems have the advantage of producing large quantities of kinase. In addition, because bacteria have much lower levels of endogenous protein kinase activity than eukaryotes, potential co-purifying kinases are not generally a concern. However, the success rate for producing highly active kinases from bacteria is low (probably around 10%; see Park et al., 2005), and hence eukaryotic cell expression systems are generally preferred. Though these systems produce lower quantities of protein than bacteria, proper folding and post-translational modification generally provide the kinase in active form. While baculoviral expression systems have the advantage of higher yield over mammalian cell expression, they are more difficult to establish, particularly for laboratories with no experience with insect cell culture. We have had good experience using transiently transfected HEK293T cells as a system for expression followed by GST or FLAG epitope-based purification to produce sufficient yield of active kinase for peptide library screening. We recommend this system in cases for which a method for producing active kinase has not already been established. It is important to note that kinases purified from insect or mammalian cells may be contaminated with endogenous kinases from the expression host. Though such contaminating kinases will almost certainly be found at lower levels than the kinase of interest, common abundant cellular kinases (e.g. protein kinase A and casein kinase 1) are highly active on peptide substrates (Kemp et al., 1977; Flotow et al., 1990), and thus can potentially dominate the signal on the peptide array. Accordingly, it is strongly recommended that a kinase inactive mutant of the protein kinase of interest be expressed and purified in parallel to the wild-type version and also subjected to peptide library screening to identify potential background signals. Such kinase inactive mutants are generally made by site-directed mutagenesis of either a conserved lysine or aspartate residue that is essential for catalysis (Gibbs and Zoller, 1991).

It is useful to establish optimal reaction conditions for the kinase of interest prior to performing the peptide library screen. This can be done by varying the buffer conditions while assaying the kinase using a generic protein or peptide substrate as described in Unit 18.7. Most kinases appear to have maximal activity at a pH around neutral and at low ionic strength, and the effect of varying these parameters can be dramatic. The identity and concentration of the divalent metal ion cofactor can have a major impact on kinase activity. It is recommended that a kinase be tested in the presence of Mg^{2+} or Mn^{2+} beforehand to determine which is optimal for activity.

Basic Protocol

Peptide Array Screening in 384-Well Plates

This method uses a peptide library consisting of 198 biotinylated peptides (Figure 2, top panel; Hutti et al., 2004). Each peptide contains a central phosphoacceptor residue (an even mix of Ser and Thr) flanked by degenerate positions (indicated by an "X" in Figure 2) comprising an equimolar mixture of the 17 amino acids excluding Cys, Ser, and Thr. In each peptide, one position (indicated with a "Z") is fixed as one of the 20 naturally occurring unmodified amino acids, phosphothreonine (pT) or phosphotyrosine (pY). The entire library of peptides is subjected to phosphorylation by the kinase in a reaction containing ^{33}P - or ^{32}P -labeled ATP in a single 384-well plate. At the end of the incubation time, aliquots of all reactions are simultaneously transferred onto a streptavidin membrane. Washing of the membrane and visualization of the results by exposure to a phosphor screen (described in Support Protocol 2) provides an array of spots of varying intensity reflecting the relative

rates of phosphorylation of the different peptides (Figure 2, bottom panel). The Basic Protocol is appropriate for most researchers who are interested in determining the specificity of a small number of kinases of interest. For those interested in screening larger numbers of kinases, the smaller scale reaction setup described in the Alternate Protocol is most appropriate.

Materials

- Clear polystyrene 384-well plates
- Lint-free blotting paper (V&P Scientific)
- Two 384 slot pin replicators, 2 μ l volume (V&P Scientific, VP384S2)
- Two alignment frames for slot pin replicator (V&P Scientific, VP381 Library Copier)
- Ser/Thr peptide library set (Anaspec, catalog no. 62017), 0.6 mM aqueous stocks arrayed in a 384-well plate (see Support Protocol 1)
- 0.1% Tween 20
- Pin-cleaning solution (V&P Scientific)
- Isopropanol
- Clear adhesive seals for multiwell plates
- Reagent reservoirs for the slot pin replicator
- Multichannel pipette, 20 μ l
- Plate sealer (optional but recommended)
- 30°C incubator
- Centrifuge with microplate carriers
- SAM² streptavidin membrane (Promega)
- Kinase reaction buffer (optimized for the kinase of interest; the following buffer works for many kinases: 50 mM HEPES/10 mM MgCl₂/1 mM DTT/0.1% Tween 20, pH 7.4)
- 4 $\times$ Kinase solution (typically 10 – 50 μ g/ml, diluted freshly in kinase reaction buffer and kept on ice)
- 10 mM ATP (adjusted to pH 7.0 and stored at -20°C)
- 4 $\times$ ATP solution (200 μ M cold ATP/0.1 μ Ci/ μ l [γ -³²P]ATP or [γ -³²P]ATP, diluted in kinase reaction buffer and kept on ice)
- 4" $\times$ 6" Rubber mat (available from an art supply store)
- SDS wash buffer: 0.1% SDS/10 mM Tris-HCl/140 mM NaCl, pH 7.5

Transferring the peptide library from the aqueous stock plate into the reaction plate

- 1 Remove a sealed peptide library aqueous stock plate from the freezer and allow it to thaw on the benchtop for approximately 20 minutes. Mix the plate by gently shaking, and then place it, still sealed, on ice.

- 2 Using a multichannel pipette, transfer 10 μ l of reaction buffer into each well of rows A through K of a 384-well reaction plate (see plate template in Figure 3).
- 3 Briefly centrifuge the peptide library and reaction plates to ensure that the liquid is at the bottom of the wells. Return both peptide library and reaction plates to ice for 5 minutes, and unseal the peptide library stock plate.
- 4 Move both plates to the benchtop and place an alignment frame over each plate.
Ensure that the alignment frames are firmly placed so that the plate is centered and cannot move side to side within the frame.
- 5 Prime the slot pin replicator by dipping the pins in 0.1% Tween 20 and then blotting onto lint-free blotting paper. Using the alignment frame to guide the pin-tool device into the peptide library stock plate, lower the pins into the peptide solution. Dip the pins in the peptide solution 3 to 5 times by raising and lowering the entire replicator.
This step transfers 2 μ l aliquots of each peptide solution to the slotted pins. The priming step with 0.1% Tween 20 is needed for efficient capillary action. To ensure that every pin becomes filled, it is necessary to dip the pins in the peptide solution multiple times. Take care that the pins are placed in the center of the wells and do not come into contact with the edges. This is important for ensuring accurate transfer and avoiding cross-contamination between wells. If the pins are not directed to the center of the wells, the plate needs to be readjusted in the alignment frame.
- 6 Transfer the peptide library from the slot pin replicator to the reaction plate by aligning the pin-tool device with the alignment frame and lowering the pins into the wells. Dip the pins in the reaction buffer 3 to 5 times by raising and lowering the entire replicator.
- 7 Blot the excess liquid from the slot pin replicators onto lint-free blotting paper and then wash the slot-pin replicator by dipping the pins 3 to 5 times in 0.1% Tween 20, blotting excess liquid onto blotting paper, and then washing the pins twice in ddH₂O and once in isopropanol in the same manner.
- 8 Return the reaction plate to ice.

Initiating the reaction by addition of the kinase and ATP

- 9 Add 5 μ l of 4 $\times$ kinase solution to each well of rows B through J (see Figure 3) of the plate using a multichannel pipette.
- 10 Add 5 μ l of 4 $\times$ ATP solution to each well of rows B through J using a multichannel pipette.
Either ³²P or ³³P-labeled ATP may be used for this procedure, and the appropriate safety precautions should be taken for the isotope used. If using ³²P-labeled ATP, all steps should be performed using acrylic shielding to protect the user. Contaminated disposables and liquid should be discarded as appropriate for the user's institution.
- 11 Seal the reaction plate, mix by shaking gently, and then incubate at 30°C for 2 hours.

Transferring the reactions onto streptavidin membrane

- 12 Remove the reaction plate from the incubator and chill on ice.

- 13 Tape a piece of streptavidin membrane of adequate size to the rubber mat. Mark one corner with a pencil so that the membrane can be oriented later.
- 14 Unseal the reaction plate and place it on the benchtop. Place an alignment frame onto the plate.
- 15 Prime the second 384 slot pin-tool by dipping the pins in 0.1% Tween 20 and blotting out the excess liquid. Align the device onto the alignment frame and carefully lower the pins into the wells containing the reaction. Dip the pins 3 – 5 times by raising and lowering the entire pin-tool device.

We recommend having two 384 pin tool devices: one dedicated for radioactive use, and a separate one used to transfer peptides from the stock plates to the reaction plates. This ensures that the peptide stock plates do not become contaminated with radioactivity or traces of protein kinase.

- 16 Transfer the liquid onto the streptavidin membrane by carefully applying the pins to the membrane and holding for several seconds. Gently rock the array back and forth while having pins still in contact with the membrane so that the liquid from each pin is blotted onto the membrane. Transfer the pins to 0.1% Tween 20 to soak.

The array of spots should be visible to the eye. Inspect that the entire array has been transferred onto the membrane. If a few spots are missing, individual spots can be applied manually using a pipettor.

- 17 Allow approximately 20 seconds for the peptides to bind to the membrane, and then carefully detach the membrane from the surface and immerse it in 200 ml of SDS wash buffer to quench the reaction.
- 18 Blot the slot pin replicator onto lint-free blotting paper and then wash the pins by dipping 3 -5 times once with 0.1% Tween 20, twice with ddH₂O and once with isopropanol, blotting the contents of the pins on lint-free blotting paper in between washes.
- 19 Follow the steps described in Support Protocol 2 to wash the membrane and collect data.

Alternate Protocol

Peptide Array Screening in 1536-Well Plates

This method is conceptually similar to the one described in the basic protocol, and employs the same set of peptides. The difference between the two is in the scale of the reactions, with the basic protocol involving 20 μ l reactions in 384-well plates, and the following alternate protocol using 2 μ l reactions in 1536-well plates (Mok et al.). The advantage of using smaller scale reactions is that they consume ten-fold less peptide, protein kinase, and radionuclide, and can be readily multiplexed (four kinases can be analyzed in parallel). The disadvantages are that the startup costs are higher, and that the method requires more practice to perform reproducibly. For this reason, the 1536-well method is best suited to investigators that plan to analyze large numbers of kinases (>20), while for most users interested in a small number of specific kinases the basic 384-well protocol is the best choice.

Additional Materials

- Clear polystyrene 1536 well plates

- Two 1536-well slot pin replicators with 5 rows of 48 floating 200 nl pins (FP3S200, V&P Scientific)
- Pin-tool strip with one row of 48 floating 200 nl pins (V&P Scientific)
- Two alignment frames with 4 pairs of guide holes for 1536-well replicator (V&P Scientific)
- One alignment frame with guide holes for each row of a 1536 well plate for the pin tool strip (V&P Scientific)
- Ser/Thr peptide library set (Anaspec, catalog no. 62017), 0.6 mM aqueous stocks arrayed in a 384-well plate (see Support Protocol 1)
- 10 mM ATP stock (adjusted to pH 7.0 and stored at -20°C)
- 10× kinase/ATP solution, 120 µl (typically 40 – 200 µg/ml kinase, 550 µM cold ATP, 0.3 µCi/µl [γ -³³P]-ATP in kinase reaction buffer).

Transferring the peptide library from the aqueous stock plate into the reaction plate

- 1 Remove a sealed peptide library aqueous stock plate from the freezer and allow to thaw on the benchtop for approximately 20 minutes. Mix the plate by gently shaking, and then place it, still sealed, on ice.
- 2 Using a multichannel pipette, transfer 2 µl of ice-cold reaction buffer into each well in the first seven rows of a 1536 well plate. Briefly centrifuge the peptide stock and reaction plates to ensure that the liquid brought to the bottom of the wells. Return plates to ice to chill for several minutes.

The stock and reaction plates should be kept on ice as much as possible to avoid evaporation. For screening a single kinase, 7 rows in the top quadrant of the plate are used. Up to four kinases can be screened at once by using the remaining three quadrants of the plate.

- 3 Move both plates to the benchtop and place an alignment frame over each plate.
- 4 Prime the slot pin replicator by dipping the pins in 0.1% Tween 20 and then blotting onto lint-free blotting paper. Using the alignment frame to guide the pin-tool device into the peptide library stock plate, lower the pins into the peptide solution. Dip the pins in the peptide solution 3 to 5 times by raising and lowering the entire replicator.

This step transfers ~200 nl aliquots of each peptide solution to the slotted pins.

- 5 Transfer the peptide library from the slot pin replicator to the reaction plate by first aligning the pin-tool device with the pair of guide holes on the alignment frame and lowering the pins into the wells. Dip the pins in the reaction buffer 3 to 5 times by raising and lowering the entire replicator.
- 6 Blot the excess liquid from the slot pin replicators onto lint-free blotting paper and then wash the slot-pin replicator by dipping the pins 3 to 5 times in one tray of 0.1% Tween 20, blotting excess liquid onto blotting paper, and then washing the pins twice in ddH₂O and once in isopropanol in the same manner.
- 7 Return the reaction plate to ice, and seal the stock plate with aluminum adhesive and return to the -20°C freezer.

Initiating the reaction by addition of the kinase and ATP

- 8 Evenly pipet the 10× kinase solution along the length of a reagent reservoir on ice.

We recommend that this procedure be performed using or ³³P- rather than ³²P-labeled ATP, since it is difficult to perform the liquid handling steps using 1536-well plates with acrylic shielding as protection. All contaminated disposables and liquid should be discarded in accordance with institutional requirements.

- 9 One row at a time, add kinase/ATP to all rows that contain peptide solution (rows B through F) as follows:
- Prime the pin tool strip with 0.1% Tween 20, blot excess liquid onto blotting paper, and lower the row of pins into the 10× kinase solution, making sure that all pins come in contact with the liquid.
 - Transfer the kinase into a single row of the reaction plate containing reaction buffer by first aligning the pin-tool device with the guide holes corresponding to that row, lowering the pins into the wells, and dipping the pins in the reaction solution 3 – 5 times.
 - Blot the excess liquid from the pin-tool device and wash the pins by immersing the tips twice with 0.1% Tween 20 and twice with ddH₂O, blotting the pins between washes.
 - Repeat these steps for the remaining four rows that contain peptide solution.
- 10 Leave the pin tool with the tips soaking in 0.1% Tween 20. Seal the reaction plate and then incubate at 30°C for 2 hours.
- 11 Blot the excess liquid from the pin-tool device and wash the pins by twice with 0.1% Tween 20, and twice with ddH₂O. Then, soak the pins for about 1 minute in pin cleaner solution, blot excess liquid, and then wash pins twice in ddH₂O, and once with isopropanol and allow the pins to air-dry.

Use of pin cleaner between kinases is highly recommended to inactivate and remove residual kinase from the pins.

Transferring the reactions onto streptavidin membrane

- 12 Remove the reaction plate from the incubator and chill on ice.
- 13 Tape a piece of streptavidin membrane of adequate size to the rubber mat. Mark one corner with a pencil so that the membrane can be oriented later.
- 14 Un-seal the reaction plate and place it on the benchtop. Place an alignment frame onto the plate.
- 15 Prime the second 1536-well pin-tool device in 0.1% Tween 20 and blot excess liquid. Align the pin-tool device with the guide holes of the alignment frame and carefully lower the pins into the wells containing the reaction. Dip the pins 3 – 5 times by raising and lowering the entire pin-tool device.
- 16 Transfer the liquid to the streptavidin membrane by carefully lowering the entire array, striving to have all of the pins contact the membrane simultaneously. Gently rock the array back and forth while having pins still in contact with the membrane to maximize the efficiency of liquid transfer. Lift the pin tool

vertically away from the membrane, and let the pins soak in 0.1% Tween 20 during the following step.

Make sure that the entire array has been successfully transferred. If a few spots are missing, individual spots can be filled in by manually transferring with a spare 200 nl pin or by pipetting.

- 17 Allow approximately 20 seconds for the peptides to bind to the streptavidin membrane and then carefully detach the membrane from the surface and immerse it in 200 ml SDS wash buffer to quench the reaction.
- 18 Follow the steps described in Support Protocol 2 to wash the membrane and collect data.
- 19 Blot the slot pin replicator onto lint-free blotting paper and then wash the pins by dipping 3 -5 times once with 0.1% Tween 20, twice with ddH₂O and once with isopropanol, blotting the contents of the pins on lint-free blotting paper in between washes.

Support protocol 1

Preparation of Peptide Stock Solutions and Plates

Peptides are reconstituted in DMSO to 10 mM concentration and kept at -20 °C for long term storage in microcentrifuge tubes. Because peptides are hygroscopic, stocks of defined concentration cannot be made by using a fixed weight of dry peptide powder. Rather, peptides are first dissolved in DMSO, and the exact peptide concentration is determined by measuring the absorbance of a diluted sample at 280 nm from Tyr and Trp residues. The concentration is then adjusted to 10 mM by adding the appropriate volume of DMSO. These DMSO stocks are diluted in buffered water to a concentration of 0.6 mM and arrayed into stock plates. Aqueous stock plates can be stored at -20 °C for up to one year. Both DMSO and aqueous peptide solutions should be subjected to a minimum number of freeze-thaw cycles (generally no more than 10).

Materials

- 180 member peptide library, 1 mg per peptide (Anaspec, catalog no. 62017-1)
- 18 member phosphopeptide library, 1 mg per peptide (Anaspec, catalog no. 62335)
- DMSO, degassed by bubbling with argon for 5 min
- 20 mM HEPES, pH 7.4
- 1.5 ml or 0.5 ml polypropylene microcentrifuge tubes
- Multiwell storage plates, either 384-well polypropylene plates (basic protocol) or 1536-well polystyrene plates (alternate protocol)
- Aluminum multiwell plate seals suitable for storage at -20°C

Prepare DMSO peptide stock solutions

- 1 Add a volume DMSO to each peptide stock vial sufficient to prepare a 50 mg/ml solution, and mix well to completely dissolve the peptide.
- 2 Prepare a 200-fold dilution of each peptide solution by mixing a small aliquot rapidly into 20 mM HEPES, pH 7.4.
- 3 Measure the A_{280} of each diluted peptide, using diluted DMSO as a blank.

- 4 Calculate the peptide concentration of each solution according to Beer's law: $A_{280} = \epsilon_{280} \cdot b \cdot c$, where ϵ is the molar extinction coefficient for the peptide, b is the path length in cm, and c is the peptide concentration in molar. Use the following extinction coefficients: 4380 M⁻¹ for peptides without fixed Tyr or Trp residues, 5580 M⁻¹ for peptides with fixed Tyr residues, and 9940 M⁻¹ for peptides with fixed Trp residues.
- 5 Multiply each calculated concentration by 200 to get the concentration of the original DMSO 6. solution.
- 6 Adjust the concentration of the DMSO stock to 10 mM by adding an appropriate volume of DMSO. The volume of DMSO to add is given by $V = [\text{peptide}] \cdot V_i / (10 - V_i)$, where $[\text{peptide}]$ is in mM and V_i is the initial volume of the solution.
- 7 Transfer the adjusted 10 mM DMSO stock solutions to microcentrifuge tubes and store at -20 °C until stock plates are needed.

Prepare aqueous peptide dilutions and array into stock plates

- 8 Thaw DMSO stocks at room temperature and mix thoroughly by vortexing.
- 9 Aliquot either 169.2 µl (for basic 384 well protocol) or 23.5µl (for alternate 1536 well protocol) of 20 mM HEPES, pH 7.4 into each of a series of microcentrifuge tubes (one for each peptide), and label tubes to indicate the identity of the peptide (e.g. -5P, -5A, -5S, etc.).
- 10 Add 10.8 µl (basic protocol) or 1.5 µl (alternate protocol) of each DMSO stock to the appropriate tube and vortex rapidly to mix. This generates 0.6 mM diluted aqueous solutions for transfer into stock plates.
- 11 Chill 0.6 mM aqueous peptide solutions and four empty storage plates on ice.
- 12 Aliquot either 20 mM HEPES buffer or 0.6 mM peptide solution into the appropriate wells of multiwell plates (5 µl per well for 1536 well plates or 40 µl per well for 384 well plates). Use the template shown in Figure 3.
Filling the peripheral wells with buffer is helpful for decreasing evaporation of the peptide solutions, which would lead to variable peptide concentrations in the reaction plates and thus spurious results.
- 13 Cover plates with aluminum adhesive seals and store at -20 °C.

Support protocol 2

Washing and Imaging of Peptides Bound to Streptavidin Membrane

Materials

SDS wash buffer: 0.1% SDS/10 mM Tris-HCl/140 mM NaCl, pH 7.5

2M NaCl

2M NaCl/1% H₃PO₄

Distilled or deionized H₂O

Benchtop orbital or rocking platform shaker

Storage phosphor system with image analysis software (BioRad Personal Molecular Imager with ImageQuant software or the equivalent)

- 1 Decant the buffer from the streptavidin membrane strip. Perform the following washes by adding 200 ml of the appropriate solution, agitating the solution on a

benchtop shaker for 3 min, decanting the solution, and replacing with the succeeding wash solution:

One additional wash with 0.1% SDS/TBS

Two washes with 2M NaCl

Two washes with 2M NaCl/1% H₃PO₄

Radioactively contaminated liquids should be disposed of in accordance with institutional procedures.

- 2 Rinse the membrane twice briefly with 200 ml of distilled water.
- 3 Allow the membrane to air dry on a piece of aluminum foil. Wrap the membrane in saran wrap and expose to a phosphor screen at least overnight.

The results can also be visualized by autoradiography, but phosphor storage is preferable for quantitative analysis of the data. While visual inspection of the array gives a qualitative sense of the major features of the phosphorylation motif, quantification of the data can indicate more subtle preferences that a kinase may have for specific amino acids at a given position. In addition, database scanning software used to identify candidate protein substrates requires quantified, normalized data be used as an input.

- 4 Scan the phosphor screen on the imager. Quantify spot intensities as appropriate for the software accompanying the imaging system. For QuantityOne (BioRad), we quantify the signal volume using an array of circles. Be sure to include a circle corresponding to a well containing kinase without peptide to use as a background signal. Export raw volume data into a spreadsheet.
- 5 In the spreadsheet, subtract the value arising from the well containing kinase only from each of the signals to provide background corrected data.
- 6 Normalize the data by dividing each value by the average of all values corresponding to a single position in the peptide. For the normalized data, the sum of all values within a position should thus be 22, and the average value should be 1. Residues that are positively selected by the kinase at a particular position will give rise to values >1, while those that are negatively selective will have values <1.

Commentary

Background information

The peptide library screen described in this unit reveals the specific amino acid preferences for a kinase at multiple positions surrounding the phosphorylation site in substrates. The method has the advantage of allowing for comprehensive systematic analysis of all potential residues at each position to be performed rapidly. Because the reaction volumes are on the order of microliters only small amounts of purified protein kinase and peptide are required. A disadvantage of the method is that because the substrates are mixtures rather than individual peptides, it assumes that the various positions within the substrate behave independently from one another. In other words, the presence of a particular amino acid at one position does not influence the preference of the kinase at another position. For example, negative interactions between two positions for a particular kinase (positive selection of a given amino acid at either one position or another but not both) would manifest as positive selection at both positions. Such positional interdependence can be revealed through follow up studies using individual consensus peptides.

Knowing the key determinants for substrate recognition by protein kinases is important for understanding fundamentally how these enzymes achieve specificity *in vivo*. Proper targeting to a small repertoire of protein substrates among thousands of other intracellular proteins requires is achieved through multiple mechanisms. In addition to kinase preferences for specific sequence motifs at the site of phosphorylation, these mechanisms include localization to specific cellular compartments and the use of scaffolds and protein interaction domains to enhance affinity for substrates (Remenyi et al., 2006; Ubersax and Ferrell, 2007; Turk, 2008). Phosphorylation site specificity can also be important for targeting specific sites from among dozens of Ser, Thr or Tyr residues within a single protein.

Data from peptide library screening can be applied in a number of ways. Probably the most widespread is in helping to map sites of phosphorylation in known protein substrates for a kinase. Rather than needing to systematically mutate every potential phosphoacceptor residue in a given protein, sites that best match the consensus phosphorylation motif can be selectively mutated, thus saving time and labor. Knowledge of potential phosphorylation sites can also facilitate mapping sites by mass spectrometry by hypothesis-driven approaches (Chang et al., 2004). A greater challenge is to search protein sequence databases for matches to the kinase consensus motif as a means to identify new candidate protein substrates. A number of online programs are available for database searching. The Swiss-Prot and TrEMBL databases can be searched for simple sequence patterns using ScanProsite tool (de Castro et al., 2006). However, ScanProsite searches typically return a large number of hits, which can be difficult to prioritize. Programs such as Scansite (Yaffe et al., 2001; Obenauer et al., 2003) allow searches to be carried out with a positional weight matrix (a list of specificity scores for every amino acid residue at multiple positions surrounding the phosphorylation site). Scansite returns a list of phosphorylation sites ranked by how well they match the input matrix, which should reflect how well a site (at the peptide level) is phosphorylated by the kinase. Scansite thus takes into account weak preferences at multiple positions that when combined can have a substantial effect on phosphorylation efficiency.

Peptide library screening data can also be used to generate customized consensus peptide substrates for a given kinase that are typically more efficient and specific than commercially available generic substrates (Hutti et al., 2004). These substrates are important tools for measuring protein kinase activity *in vitro* (see Unit 18.7), and can form the basis of assays suitable for high-throughput screening to identify kinase inhibitors. In addition, such substrates can be used to follow changes in the activity of a kinase isolated from cultured cells treated with particular stimuli (typically by immunoprecipitation). Recent work has even incorporated such peptides into biosensors for monitoring kinase activity in living cells with spatiotemporal resolution (Kunkel et al., 2004; Allen et al., 2006).

Critical parameters

When working with small volumes of liquid in multiwell plates, sample evaporation can be a major problem leading to experimental artifacts and even a failed procedure. For 384-well plate assays, evaporation tends to be partial and is most pronounced for peripheral wells. This can lead to undesired concentration of peptide and kinase in some wells but not others, leading to spurious enhancement of the signal. For 1536-well plates, evaporation of 2 μ l samples can occur rapidly, particularly if a plate at 30°C is left open to the atmosphere. Keeping the plates on ice as much as possible can minimize evaporation, and it is particularly important to chill the plate completely before unsealing it after removal from the incubator at the end of the reaction time. The plate layout shown in Figure 3 is designed to minimize evaporation by filling peripheral wells with buffer. If users choose to design their own layout, it is important to avoid using wells on the edge of the plate for kinase reactions.

Use of the pin tool liquid handling devices can be challenging at first, but we have found that even beginning researchers can become proficient with a little practice. We strongly recommend practicing both plate-to-plate liquid transfers and plate-to-membrane liquid transfers using a dye solution before attempting the procedure with radioactive samples. For users of the alternate protocol, we also recommend practicing filling the 1536-well plate with buffer, since pipetting into the small wells can be difficult at first.

For the method to be successful, the kinase must be able to phosphorylate short peptide substrates with some efficiency. Many protein kinases prefer proteins over peptides as substrates, probably due to the pervasive utilization of docking interactions for substrate targeting. However, we have found this approach to be successful for most kinases, even those for which docking and scaffolding interactions are critical for their function in vivo (such as MAP kinases; see (Sheridan et al., 2008).

Troubleshooting

The most likely problem that a user will encounter is a weak phosphorylation of the peptides. The most common solution is to simply increase the amount of kinase used in the assay. The reaction time can also be increased, though we do not recommend overnight incubations due to problems with evaporation within the wells. Alternatively, the amount of cold ATP in the reaction can be decreased (to a final concentration of 10 μ M), which will increase the specific activity of the radiolabel and typically increase sensitivity.

Optimization of buffer conditions (see Strategic Planning above) can also help increase the signal. If these simple modifications to the protocol do not solve the problem, then it is likely that the kinase is not sufficiently active to perform the procedure. This will require changing the expression and purification protocol for the kinase. If a bacterial expression system is being used, switching to a eukaryotic system is recommended. In some cases generating fully active kinase requires phosphorylation by an upstream kinase, which can be sometimes done in vitro if the activating kinase is known and available. In some cases expressing the catalytic domain of the kinase as opposed to the full-length kinase may increase activity if the kinase has an autoinhibitory region. If the protein kinase was purified from mammalian cells, be sure that phosphatase inhibitors are present in the lysis buffer. In addition, providing the appropriate stimulus prior to cell lysis can dramatically increase the activity of the isolated kinase. Bear in mind however that manipulations commonly used to boost kinase activity (e.g. growth factors or phorbol esters) also increase the risk of having contaminating kinases co-purify with the kinase of interest.

The phosphorylation profile can also be hampered by high background signals. This may be alleviated by being careful to minimize the amount of time the membrane spends following the spotting of the kinase reactions prior to its immersion in the first wash buffer. Increasing the wash times may also help reduce the background. A specific type of background signal can arise due to contamination with highly abundant kinases such as PKA, which may occur when assaying kinases purified from insect or mammalian cells. The PKA pattern is characterized by strong signals for Arg at the -2 and -3 positions (Gibbs and Zoller, 1991). Adding PKI, a potent and specific peptide inhibitor of PKA (Scott et al., 1986), at 0.5 μ M to the reaction buffer will completely eliminate this background signal. It is also common to observe apparent selectivity for Ser and/or Thr residues at several positions. Most of these signals are artifacts arising from the presence of two potential sites of phosphorylation in peptides with fixed Ser and Thr residues. While some kinases do authentically select Ser or Thr as part of their phosphorylation motif, caution is warranted in interpreting any apparent selectivity for these residues.

It is also possible to have too much kinase activity, which can result in high signals for all of the peptides in the array. An overactive kinase can be misinterpreted as having little or

no selectivity. In this case, decreasing the amount of protein kinase used may help, and performing the peptide screen at several kinase concentrations is recommended to find optimal conditions.

Anticipated results

Protein kinases are typically highly selective at one or two positions within the peptide sequence, either for a single residue (e.g. Pro) or for a type of residue (e.g. aliphatic). Quantitatively, the normalized selectivity value (or the sum of the values for a type of residue) is typically >7.0. In addition, there are usually several other positions that display some preference for certain residues without being stringently selective (e.g. normalized selectivity values in the range of 1.5 – 2.5). The bottom panel of Figure 2 shows sample data for the mammalian kinase Pim1, which is highly selective for substrates with Arg residues at both the -3 and -5 positions (Bullock et al., 2005). Significant though less stringent preferences are also apparent at the -2 and +1 positions

Time considerations

Preparation and arraying of the peptide stock solutions takes approximately two days, but will provide enough peptide library to screen dozens of kinases. Once the stock plates are ready, the peptide library screen itself is fairly rapid, taking about 6 hours from start to finish (including a 2 hour incubation time for the reaction). Initial phosphorimager data can be acquired by the next day to visualize the results. We find that the signal to noise ratio improves with prolonged exposure (generally 3 days to 1 week) and this is recommended for obtaining quantitative data.

Acknowledgments

We thank Grace Jeschke for performing the Pim-1 peptide library screen shown in Figure 2. This work was supported by National Institutes of Health grant R01 GM079498 to B.E.T. C.C. was supported by National Institutes of Health Training Grant T32 CA009085.

Literature Cited

- Allen MD, DiPilato LM, Rahdar M, Ren YR, Chong C, Liu JO, Zhang J. Reading dynamic kinase activity in living cells for high-throughput screening. *ACS Chem Biol*. 2006; 1:371–376. [PubMed: 17163774]
- Bullock AN, Debreczeni J, Amos AL, Knapp S, Turk BE. Structure and substrate specificity of the Pim-1 kinase. *J Biol Chem*. 2005; 280:41675–41682. [PubMed: 16227208]
- Chang EJ, Archambault V, McLachlin DT, Krutchinsky AN, Chait BT. Analysis of protein phosphorylation by hypothesis-driven multiple-stage mass spectrometry. *Anal Chem*. 2004; 76:4472–4483. [PubMed: 15283590]
- de Castro E, Sigrist CJ, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Res*. 2006; 34:W362–365. [PubMed: 16845026]
- Flotow H, Graves PR, Wang AQ, Fiol CJ, Roeske RW, Roach PJ. Phosphate groups as substrate determinants for casein kinase I action. *J Biol Chem*. 1990; 265:14264–14269. [PubMed: 2117608]
- Gibbs CS, Zoller MJ. Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. *J Biol Chem*. 1991; 266:8923–8931. [PubMed: 2026604]
- Hutti JE, Jarrell ET, Chang JD, Abbott DW, Storz P, Toker A, Cantley LC, Turk BE. A rapid method for determining protein kinase phosphorylation specificity. *Nat Methods*. 2004; 1:27–29. [PubMed: 15782149]

- Kemp BE, Graves DJ, Benjamini E, Krebs EG. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J Biol Chem.* 1977; 252:4888–4894. [PubMed: 194899]
- Kunkel MT, Ni Q, Tsien RY, Zhang J, Newton AC. Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically-encoded fluorescent reporter. *J Biol Chem.* 2004; 280:5581–5587. [PubMed: 15583002]
- Mok J, Kim PK, Lam HYK, Piccirillo S, Zhou X, Jeschke GR, Sheridan DL, Parker SA, Desai V, Jwa M, Cameroni E, Niu H, Good M, Remenyi A, Ma JN, Sheu YJ, Sassi HE, Sopko R, Chan CSM, De Virgilio C, Hollingsworth NM, Lim WA, Stern DF, Stillman B, Andrews BJ, Gerstein MB, Snyder M, Turk BE. Deciphering protein kinase specificity through large-scale analysis of yeast phosphorylation motifs. Submitted.
- Obenauer JC, Cantley LC, Yaffe MB. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res.* 2003; 31:3635–3641. [PubMed: 12824383]
- Park J, Hu Y, Murthy TV, Vannberg F, Shen B, Rolfs A, Hutt JE, Cantley LC, Labaer J, Harlow E, Brizuela L. Building a human kinase gene repository: bioinformatics, molecular cloning, and functional validation. *Proc Natl Acad Sci USA.* 2005; 102:8114–8119. [PubMed: 15928075]
- Remenyi A, Good MC, Lim WA. Docking interactions in protein kinase and phosphatase networks. *Curr Opin Struct Biol.* 2006; 16:676–685. [PubMed: 17079133]
- Scott JD, Glaccum MB, Fischer EH, Krebs EG. Primary-structure requirements for inhibition by the heat-stable inhibitor of the cAMP-dependent protein kinase. *Proc Natl Acad Sci USA.* 1986; 83:1613–1616. [PubMed: 3456605]
- Sheridan DL, Kong Y, Parker SA, Dalby KN, Turk BE. Substrate discrimination among mitogen-activated protein kinases through distinct docking sequence motifs. *J Biol Chem.* 2008; 283:19511–19520. [PubMed: 18482985]
- Turk BE. Understanding and exploiting substrate recognition by protein kinases. *Curr Opin Chem Biol.* 2008; 12:4–10. [PubMed: 18282484]
- Ubersax JA, Ferrell JE Jr. Mechanisms of specificity in protein phosphorylation. *Nat Rev Mol Cell Biol.* 2007; 8:530–541. [PubMed: 17585314]
- Yaffe MB, Leparo GG, Lai J, Obata T, Volinia S, Cantley LC. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat Biotechnol.* 2001; 19:348–353. [PubMed: 11283593]

Figure 1. Workflow for peptide library screening procedure. Stock solutions (Support Protocol 1) should be made in advance of assaying the kinase on the peptides (Basic or Alternative Protocol). The procedure for processing membranes immediately subsequent to peptide binding (Support Protocol 2) is identical for both the Basic and Alternative Protocols.

Figure 2.

Peptide library and sample results. The top panel shows the set of peptides used to determine phosphorylation site motifs for serine-threonine kinases. Fixed positions can be any of the 20 unmodified amino acids, phosphothreonine (pT) or phosphotyrosine (pY). The bottom panel shows results obtained using the mammalian Pim-1 kinase.

Figure 3. Peptide stock plate layout. Recommended arrangements of peptide stocks in 384-well (top) or 1536-well (bottom) plates are shown. Wells containing buffer with no peptide are marked with a dash. For clarity, only the top seven rows of the 1536-well plate are displayed.