运用肽库筛选磷酸化激酶 motif 方法 Screening Kinase Phosphorylation Motifs Using Peptide Libraries

Screening Kinase Phosphorylation Motifs Using Peptide Libraries

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ABSTRACT

Determining which protein kinases are responsible for phosphorylating which protein substrates is critical for mapping signal transduction cascades that regulate cell growth, division, differentiation, and death. Oriented peptide library screening, combined with bioinformatics-based searches of protein sequence databases, offers a strategy for identifying substrates of particular protein kinases.

MATERIALS

Buffers, Solutions, and Reagents

[γ-32P]ATP (specific activity 3000 Ci/mmole, 10 mCi/ml)

Acetic acid, 0.1 M in water

Acetic acid, 30% in water

Acetone

Acetonitrile

Ammonium acetate, 0.1% (pH 11.5), freshly prepared and stored at 4°C

Ammonium acetate, 0.1% (pH 8.0), freshly prepared and stored at 4°C

Ammonium acetate, 0.1% (pH 9.5), freshly prepared and stored at 4°C

ATP, 10 mM (pH 7.0) (store at -20°C)

DEAE Sephacel (Sigma I6505)

Degenerate Ser- or Thr-containing peptide libraries

EDTA, 0.1 M (pH 8.0)

Enzymatically active kinase of interest to be screened

FeCl3, 100 mM in 0.1 M acetic acid (prepare fresh)

Immobilized iminodiacetic acid agarose (IDA agarose) with 1,4 butanediol diglycidyl ether spacer (Pierce 20277)

Methanol, dry

Phosphoric acid, 85%

Solution 1: 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.5) containing 1 M NaCl

Solution 2: Methanol:acetonitrile:acetone (1:1:1)

Standard reagents for peptide synthesis and protein sequencing (if this is being done in-house)

Thionyl chloride

Special Equipment

Centrifuge tubes, disposable, polypropylene (50 ml and 15 ml)

Columns, polypropylene columns (12-ml and 3-ml capacity) with tip plugs

Geiger counter, hand-held

Glass storage vials

Microcentrifuge tubes, labeled 1-7, and 1-10

P81 paper

Peptide synthesizer and protein sequencer. These pieces of equipment are only necessary if library synthesis and sequencing are being done in-house rather than through a commercial provider.

React-Vials, 3-5 ml, with Teflon seals and caps and stir bars

Ring stand with clamps

Scintillation counter

SpeedVac concentrator

Test tubes, glass 13 x 100 mm

METHOD

Stage 1: Kinase-directed Phosphorylation of the Peptide Library

Place 30 µl of 10x kinase buffer in a 1.5-ml microfuge tube. It is best to use whichever buffer, including cofactors, the kinase prefers for phosphorylation of a known substrate. For many kinases, a satisfactory 10x buffer is 500 mM Tris-HCl (pH 7.5), 100 mM MgCl2, 10 mM dithiothreitol (DTT).

To the 30 µl of 10x kinase buffer, add the following reagents in the order stated

degenerate peptide library mixture (10 mg/ml) 100 µl
10 mM ATP 3 µl
[γ-32P]ATP (10 mCi/ml) 0.1 µl
ddH2O to 275-295 µl
purified active kinase to 300 µl

Incubate the kinase reaction at 30°C and monitor its course as follows:
Remove 5- µl aliquots of the reaction mixture at 0, 1, and 2 hr and spot these onto labeled 2-cm x 2-cm squares of P81 paper
Allow the squares to air-dry, then wash them four times in a beaker containing 50 ml of 0.45% phosphoric acid for 2 min/wash.
Place the squares in scintillation vials, cover with 5-10 ml of scintillation fluid, and count.
Using the counts from the radioactive peptides bound to the P81 paper and the specific activity of the [γ-32P]ATP on the day of the assay, determine the amount of phosphorylated peptide.

Each 300- µl reaction contains a total of 30 nmole of ATP into which the trace amount of radioactive ATP was distributed. Subtract the cpm incorporated in the 0-hr

background control from the 1- and 2-hr samples, and multiply each by 60 (300 µl total / 5 µl counted) to get the total cpm incorporated into the peptide library. To determine the total nmole of phosphorylated peptide library, calculate

$$\frac{\text{cpm incorporated}}{\text{total } ^{32}\text{P cpm in reaction}} \times 30$$

Typically, a well-behaved kinase will yield somewhere between 2,000 and 10,000 cpm after 2 hr. The kinase reaction can be performed overnight if necessary to obtain a sufficient amount of phosphorylated peptide library.

When 0.2-2% of the peptide library has been phosphorylated, stop the reaction by adding 300 µl of 30% acetic acid to bring the final acetic acid concentration to 15% (v/v).

Stage 2: Purification of the Phosphorylated Peptides

Prepare the DEAE-Sephacel resin by placing 2 ml of the packed resin (typically 4 ml of a 50:50 slurry from the bottle as purchased) and 10 ml of 30% acetic acid in a 15-ml disposable polypropylene centrifuge tube. Mix gently, then collect the resin by low-speed centrifugation (<1000 g for 2 min). Discard the supernatant and resuspend the washed resin in 2 ml of 30% acetic acid.

Clamp a 12-ml disposable chromatography column in a ring stand, keeping the tip plugged. Fill the column with 8 ml of 30% acetic acid and add 2 ml of the washed resuspended 50:50 DEAE-Sephacel slurry. Allow the resin to settle for 20 min by gravity, to provide a packed bed volume of ~1 ml.

It is important that the bed volume is very close to 1 ml because fraction collection is

based on a 1-ml column volume.

Label 7 microfuge tubes 1-7. Use a marker pen to indicate the position of 600 µl on the side of tube 1, 1 ml on tube 2, and 500 µl on tubes 3-7.

Remove the plug from the column and allow it to run until the top of the resin is just exposed. Apply all 600 µl of the phosphorylated library mixture to the top of the resin.

Allow the column to run by gravity and collect the first 600 µl of eluate (the dead volume) in tube 1. Switch to tube 2 and immediately add 600 µl of 30% acetic acid to the top of the column. Collect the eluate in tube 2. Finally, add 3 ml of 30% acetic acid to the top of the column. Collect the next 400 µl in tube 2 (so that the final volume in this tube is 1 ml) and subsequent fractions of 500 µl in tubes 3-7.

Subject 5 µl from each tube to scintillation counting and calculate the total cpm contained in each fraction. Plot the cpm as a function of fraction number.

The phosphorylated peptide library should elute in fraction 2, whereas the unincorporated ATP generally elutes in fractions 5-7 and later. In addition to directly counting 5 µl of each fraction, it is also helpful to spot 5 µl of each fraction onto P81 paper and count the bound peptide as described in step 3. This allows an estimate of the amount of recovered phosphopeptide independent of any co-eluting radiolabeled ATP or free phosphate.

Dry down the fraction containing the peptide library (fraction 2) overnight on a SpeedVac apparatus. At this point, the sample can be stored at -20°C prior to proceeding to the next step (either step 12 or step 15). Methyl Esterification (Optional)

The methyl esterification (described in steps 12-14) is optional, although strongly recommended. This process masks the free carboxyl groups on the peptide by converting them into methyl esters, which reduces the background observed from nonphosphorylated peptides following IMAC purification.

Prepare for esterification of the phosphopeptides by making a fresh solution of methanolic HCl in a chemical fume hood. Note: This reaction is exothermic!

For each peptide library to be purified, carefully add 40 µl of thionyl chloride, drop-wise, to 1 ml of dry methanol.
After opening a fresh bottle of thionyl chloride, aliquot the remainder into small screw-top glass vials to shield it as much as possible from air and water.

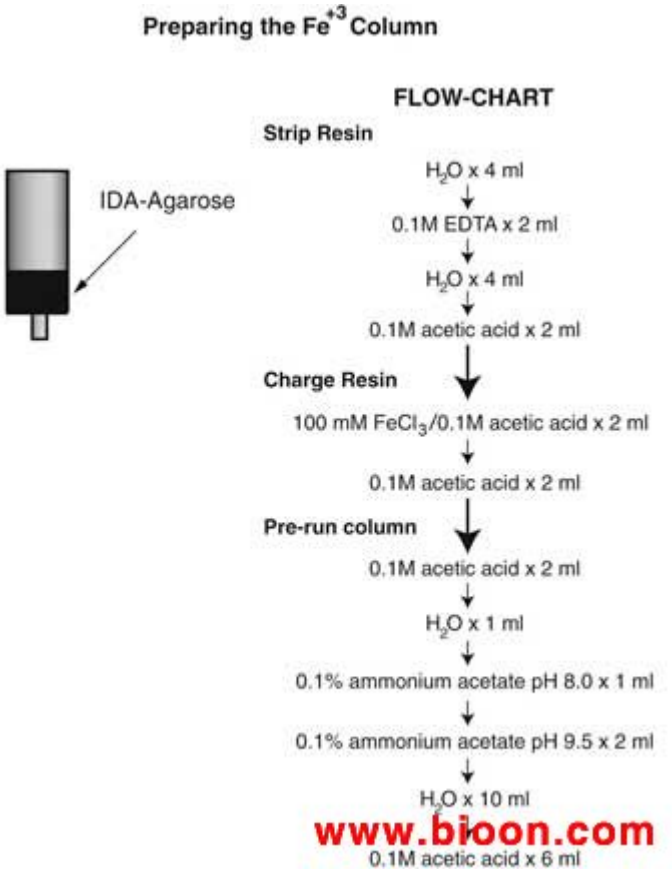
Immediately dissolve the peptide, from step 11, in the 1.04 ml of methanolic HCl solution prepared in step 12. After the peptide has dissolved, transfer the solution to a glass React-Vial containing a stir bar, and cap the container. Stir the mixture at room temperature for 1 hr.

Transfer the solution to a 13 x 100-mm test tube and dry the peptide library solution for several hours to overnight on a SpeedVac apparatus. As soon as the sample is dry (i.e., after concentration overnight on the SpeedVac), proceed to step 15. Do not store the dried peptide at -20°C; proceed immediately to step 15.

To make the IMACcolumn: Clamp a 3-ml disposable chromatography column in a ring stand, keeping the tip plugged. Fill the column with 2-3 ml of water, then add 900 µl of a 1:1 slurry of the IDA agarose, and allow the resin to settle by gravity.

Alternatively, use nitrilotriacetic acid agarose (NTA agarose), metal-free. See step 19 for changes to the procedure if NTA agarose is used.

Remove the tip plug from the column and allow the covering water to drain until the surface of the resin is exposed. Strip the resin of any contaminating metal ions by washing the column sequentially with 4 ml of water, 2 ml of 0.1 M EDTA, 4 ml of water, and 2 ml of 0.1 M acetic acid. (A flowchart of steps 16-18 is shown below.)



Charge the resin by adding 2 ml of freshly prepared 100 mM FeCl3 in 0.1 M acetic acid, followed by a single wash using 2 ml of 0.1 M acetic acid. The column should be yellow in color.

Prerun the column to elute any contaminants prior to sample loading. Wash the column sequentially with 2 ml of 0.1 M acetic acid, 1 ml of water, 1 ml of 0.1% ammonium acetate (pH 8.0), 2 ml of 0.1% ammonium acetate (pH 9.5), 10 ml of water, and 6 ml of 0.1 M acetic acid.

The color of the column will change progressively from yellow to brown, and then back to light yellow again.

While carrying out the column washes in step 18, resuspend the dried peptide library sample from step 14 in either 100 µl of solution 1 by aggressive vortexing (if using IDA agarose and no methyl esterification [steps 12-14]) or 100 µl of solution 2 (if using IDA agarose and methyl esterification).

If using NTA agarose and methyl esterification, resuspend the sample from step 14 in solution 2. If NTA agarose is used and methyl esterification was not performed, resuspend the sample from step 11 in 50 l of 0.1 M acetic acid, adjust pH to 3.5 with 30% acetic acid, and vortex aggressively.

If methyl esterification was used, wash the column with an additional 6 ml of water immediately prior to adding the peptide.

Apply the peptide sample to the Fe+3 column. Rinse the sample tube with another 50 µl of solution 1 or 2, as appropriate, and apply this to the column as well.

A hand-held Geiger counter is useful to ensure that most of the radioactivity, corresponding to the phosphorylated peptide library, has been adequately transferred to the column.

Label a new set of ten microcentrifuge tubes 1-10. Develop the column, collecting 0.5-ml fractions, by eluting with 2 ml of water (fractions 1-4), 1 ml of 0.1% ammonium acetate (pH 8.0) (fractions 5,6), and 2 ml of 0.1% ammonium acetate (pH 11.5) (fractions 7-10).

Count 5 µl of each fraction in a scintillation counter and plot the cpm as a function of fraction number.

Most of the radioactivity should elute in fractions 1 and 2, and 7-10. Fractions 1 and 2 correspond to non-peptide products containing 32P, and fractions 7-10 correspond to the phosphorylated peptides. In addition to directly counting 5 µl of each fraction, it is also helpful to spot 5 µl of each fraction onto P81 paper and count the bound peptide as in step 3. This will help to estimate the final amount of recovered phosphopeptide library.

Pool the tubes containing the phosphorylated peptide library (generally fractions 7-9) into a single tube and dry down in a SpeedVac apparatus. Redissolve the final pellet in 80 µl of water.

It is often necessary to add small amounts (generally less than 10 µl) of 30% acetic acid to neutralize the ammonium acetate and fully dissolve the pellet, as evidenced by release of radioactivity into the liquid phase.

Count 2 µl of the sample by scintillation counting. Based on the amount of radioactivity measured, and the total radioactivity and nmole of phosphorylated peptide library calculated in step 3, determine the volume of sample that corresponds to 200-1000 pmole of phosphorylated peptide library.

Sequence 200-1000 pmole of phosphorylated peptide library by automated Edman degradation.

Typically, we find that empirically sequencing 40 µl (half the final yield) usually works well at this step. If there are problems with peptide sequence (see below), it may be necessary to remove excess volatile salt from the peptide solution. To do this,

dry the remaining peptide solution in a SpeedVac and redissolve the resulting peptide pellet in 250-500 µl of water and re-lyophilize several times.

Stage 3: Analysis of the Sequencing Data

Arrange the sequencing data in spreadsheet format using a program such as Microsoft Excel or Lotus 1-2-3.

The columns of the spreadsheet should correspond to the amino acids (Ala, Asp, Glu, etc.), and the rows of the spreadsheet should correspond to the different cycle of Edman sequencing (Fig. 2). For example, cell Ai,j in the spreadsheet represents the amount (in pmole) of amino acid i, reported for the phosphorylated library sample in sequencing cycle j. AGlu,4 might represent the amount of Glu in sequencing cycle 4.

Calculate the mole percentage of each amino acid in each sequencing cycle (fixed j). This value, denoted MPij (for sample mole percentile), is given by

$$MP_{ij} = \frac{A_{ij}}{\sum_{i=1}^{i=16} A_{ij}}$$

where the sum of Aij in the denominator is performed over all 16 amino acids in that sequencing cycle j (i.e., the sum of all values in that row of the spreadsheet).

Perform this same calculation for all remaining sequencing cycles j+1, j+2, etc. (rows in the spreadsheet) that contain degenerate amino positions.

Perform the identical calculations for the mole percentages of each amino acid present in the degenerate positions in the initial starting (unphosphorylated) peptide library

mixture. Let Bij represent the amount, in pmole, of amino acid i reported for the starting library mixture in sequencing cycle j. The mole percentage of amino acid i in cycle j for the control, denoted by CPij (control percentile), is similarly given by

$$CP_{ij} = \frac{B_{ij}}{\sum_{i=1}^{i=16} B_{ij}}$$

Calculate the raw selectivity value for a given amino acid i in a particular sequencing cycle j, denoted:

S_{ij}^{raw} where $S_{ij}^{raw} = M_{ij} / CP_{ij}$.

Normalize the raw selectivity values so that the sum of all selectivity values in a given sequencing cycle j is equal to the total number of possible amino acids. If the library has been synthesized without Cys, Ser, Thr, or Tyr in the degenerate positions, then the sum of all selectivity values should be normalized to 16, by defining

$$S_{ij}^{norm} = \left[\frac{S_{ij}^{norm}}{\sum_{i} S_{ij}^{raw}} \right] \times 16$$

where the calculation is performed for individual fixed values of j.

Construct bar graphs showing normalized preference values versus amino acid for each sequencing cycle, as shown in Figure 2. Scan these plots by eye to pick out residues showing high selectivity for particular amino acids in different sequencing cycles, as well as amino acids showing significant cycle-to-cycle variation in selectivity

PROBLEMS, PITFALLS, AND SOLUTIONS

The most common problems that occur when determining protein kinase phosphorylation motifs by oriented peptide library screening are failure to obtain sufficient phosphorylation of the starting peptide library mixture, and difficulties in separating the phosphorylated peptides from the nonphosphorylated peptide background. Difficulties with peptide phosphorylation can sometimes be remedied by substituting Mn2+ for Mg2+ in the kinase reaction in step 1, or by using a combination of Mn2+ and Mg2+, depending on the particular kinase. In addition, certain kinases may require additional cofactors to increase their intrinsic activity, such as the presence of phosphatidyl serine and diacyl glycerol for maximal activity of typical protein kinase Cs. Alternatively, the activity of the kinase domain in a full-length protein kinase is sometimes masked by other parts of the molecule. In these cases, expression of the isolated kinase domain, free from any other modular domain(s) in the protein, can sometimes improve the activity. Finally, certain bacterial- or baculoviral-expressed kinases do not appear to have significant activity against peptides. In these cases, immunoprecipitating an overexpressed epitope-tagged version of the kinase from mammalian cells may yield sufficient kinase activity for the assay. Clean separation of phosphopeptides from the nonphosphorylated peptide library background is critical to the success of the technique. The addition of methyl esterification described in optional steps 12-14 will usually dramatically improve this separation if problems are encountered in the absence of this procedure.

References

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