

Peptide Competition Assay (PCA) Protocol

The peptide competition assay (PCA) is a recommended procedure for confirming the specific band reactivity of anti-peptide polyclonal antibodies, especially domain specific antibodies like phospho-specific antibodies. It is not uncommon to see more than one band on immunoblots of lysates when probing with a primary antibody. The PCA provides a means of determining which band or staining pattern is specific for the antibody. Typically, the experiment should be run in parallel, once with the phosphorylated form of the peptide immunogen and once with the non-phosphorylated form of the immunizing peptide. In the PCA, the antibody is pre-incubated with the peptide (or peptides) prior to use in immunoblotting assays. The immunoblotting experiment is run in duplicate, once with antibody pre-incubated with peptide and once with a control antibody not pre-incubated with peptide. All other parameters of the immunoblotting experiment should remain constant, i.e., use the same dilutions and buffer conditions throughout the experiment.

I. Reagents Required

Reagent
Whole Cell Lysates
Blocking Buffer
Secondary Antibodies
HRP and ALP Substrates
Molecular Biology Grade UltraPure Water (MB-010-1000)

II. Procedure

1. Prior to performing the PCA, optimize conditions for immunoblotting for both the antibody and antigen, including the amount of lysate, dilution of primary and secondary antibodies, dilution buffer, blocking buffer, and other experimental conditions. Optimized conditions should be replicated in the PCA without change.
2. This procedure describes the PCA using phosphorylated and non-phosphorylated peptides and a phospho-specific primary antibody, where an antibody concentration of 1 $\mu\text{g}/\text{mL}$ and a 200-fold molar excess of peptide are used in a total reaction volume of 2 mL. Molar ratios are based on the molar mass of IgG as 150,000 Daltons.
3. Transfer and immobilize the antigen on nitrocellulose or PVDF membrane and prepare three identical test samples for analysis by PCA. The test samples should be blocked as previously established in step 1.
4. Slowly thaw the primary antibody (i.e., phospho-specific antibody) on ice.
5. Prepare a 3 mL solution of primary antibody at a 2X concentration in dilution buffer relative to the optimum dilution as previously established in step 1.
6. If desired, aliquot the diluted primary antibody and store any unused solution at -80°C for future use.
7. Slowly warm the lyophilized peptides (both the phosphorylated and non-phosphorylated forms of the immunizing peptide) to room temperature, ideally under desiccation.
8. Reconstitute each of the peptides to a concentration of 100 μM using Molecular Biology Grade UltraPure Water (1 x 1 L) (MB-010-1000) at room temperature. Refer to the molecular weight of the peptide for the exact amount and volume. For a peptide with a molecular mass of 1500, reconstitution of 100 μg with 0.67 mL water yields a solution with a concentration of 100 μM .
9. Allow the peptides to dissolve at room temperature, and then gently triturate several times using a pipette. Avoid introducing air bubbles.
10. Label 3 test samples as follows:
 - Sample (a): water only. No peptide control.
 - Sample (b): phosphorylated peptide.
 - Sample (c): non-phosphorylated peptide.
11. Prepare 2X peptide stock solutions (2.66 μM) and water control by pipetting the following:
 - Sample (a): no peptide control: 973 μL dilution buffer (antibody diluent as previously established in step 1) + 27 μL water.
 - Sample (b): phosphorylated peptide 2X stock: 973 μL dilution buffer + 27 μL reconstituted (100 μM) phosphorylated peptide.
 - Sample (c): non-phosphorylated peptide 2X stock: 973 μL dilution buffer + 27 μL reconstituted (100 μM) non-phosphorylated peptide.If desired, aliquot any unused reconstituted peptide solution and store at -80°C for future use.
12. Pipette 1 mL of the 2X antibody stock into each of the samples marked (a), (b), and (c). The tubes should be incubated for 30 minutes at room temperature with gentle rocking.
Note: some antibody-peptide combinations require incubation for extended periods of time at alternative temperatures. If desired results are not obtained, try incubating the mixture for 1–2 hours at 37°C or for 2–24 hours at 4°C .

13. Centrifuge the samples for 15 minutes at 4°C in a microfuge (10,000–15,000 rpm) to pellet any immune complexes. Carefully remove the supernatant. If no visible pellet is seen, then just leave approx. 5–10 µL at the bottom to avoid disturbing invisible immune complexes.
Note: If you do not centrifuge the solution, you may see high background staining.
14. The pre-incubated antibody in each of the three samples is ready for use. Pipette the contents of each sample onto the three identical test samples for immunoblotting (i.e. Western blotting strips).
 - a. Incubate each strip for 2 hours at room temperature, followed by several washes to remove unbound antibody.
 - b. Transfer each strip to a new solution containing a labeled secondary antibody (i.e., goat anti-rabbit IgG alkaline phosphatase conjugate).
 - c. Remove unbound secondary antibody by thorough washing and develop bands.
15. The signals obtained with antibody incubated with sample.
 - a. (water only, no peptide control) represent the maximum signal. Signals obtained with sample.
 - b. (phosphorylated peptide) and sample.
 - c. (non-phosphorylated peptide) are compared to the signal from sample (a) to determine if the peptide(s) compete for antigen binding. A positive PCA result shows no or little binding to a specific band for sample (b).

III. Notes

Ideally an excess of peptide is added to the antibody solution, where 200- to 500-fold more peptide is added on a molar basis relative to antibody. The method stated above makes certain assumptions given our experience that most antibodies show bivalent binding, and most peptides fall within the range of excess stated above. There does not appear to be any significant difference between a 200-fold and 500-fold excess. If the results of the PCA are less than optimum, then try altering the concentration of peptide upward or downward (i.e., fix the antibody concentration) to improve the results of the PCA. The best results are often determined empirically.

The higher the antibody titer or initial volume of antibody taken, the higher will be the antigen/peptide necessary to completely block the antibody activity.

A partial inhibition of antibody activity is an indication that more antigen/peptide will be needed to completely block the antibody.

For PCA experiments that do not test phospho-specific antibodies, only two experimental samples need to be prepared: one no-peptide control and one peptide as stated in procedure step 11.

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