

The peptide competition assay (PCA) is a recommended procedure for confirming the specific band reactivity of an antibody, 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. The experiment should be run in parallel; with the peptide immunogen (e.g. phosphorylated form peptide) and with or without the irrelevant peptide (e.g. non-phosphorylated peptide).

## Procedure in WB

In the PCA, the antibody is pre-incubated with the peptide prior to use in immunoblotting assays. The immunoblotting experiment is run in duplicate; one with antibody pre-incubated with peptide and another one with a control antibody not pre-incubated with peptide. All other parameters of the immunoblotting experiment should remain constant i.e. the same dilutions and buffer conditions throughout the experiment.

The following example uses phosphorylated and non-phosphorylated peptides with phospho-specific primary antibody. The primary antibody concentration is used at 1 µg/ml with a 200-fold molar excess of peptide.

*Molar ratios are based on the molar mass of IgG as 150 000 Daltons. Based on weight, the primary antibody can be incubated with a 5-fold excess of peptide.*



*We recommend first optimizing conditions for immunoblotting for both the antibody and antigen, including the amount of lysate, dilution of primary and secondary antibodies, dilution buffer, and blocking buffer.*

1. Transfer and immobilize the antigen on nitrocellulose or PVDF membrane and prepare 3 identical test samples for analysis by PCA.
2. Prepare a solution of primary antibody at a 2X concentration in dilution buffer.

*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.*



3. We usually recommend storing each peptide stock at concentration of 100 µM (the phosphorylated and non-phosphorylated forms of the immunizing peptide) in the same dilution buffer as the primary antibody, 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.*



4. Label 3 antibody test samples as follows:
  - Sample (a): dilution buffer only. No peptide control.
  - Sample (b): phosphorylated peptide.
  - Sample (c): non-phosphorylated peptide.
5. Prepare 2X peptide solutions (2.66 µM) and the 3 samples as following:
  - Sample (a): dilution buffer only. No peptide control.
  - Sample (b): phosphorylated peptide 2X solution.
  - Sample (c): non-phosphorylated peptide 2X solution.
6. Mix the related volume of the 2X peptide solutions into each antibody sample marked (a), (b) and (c) in glass test tube in order to prevent the coating of the antibody. The tubes should be incubated for 30 min at room temperature with gentle rocking.

*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.*



7. The pre-incubated antibody in each of the three samples is ready for use. Pipette the contents of each antibody-peptide sample onto the three identical test samples for immunoblotting (e.g. strips in WB, wells in ELISA).
  - Incubate each strip for 2 hours at room temperature, followed by several washes to remove unbound antibody.
  - Transfer each strip to a new solution containing a labeled secondary antibody
  - Remove unbound secondary antibody by thorough washing and develop bands.
8. The signals obtained with antibody incubated with sample (a) (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).

*Most antibodies show bivalent binding and most peptides fall within the range of excess between 200 and 500 fold. If the results of the PCA are less than optimum, we recommend altering the concentration of peptide upward or downward.*



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