IP-Kinase Assay Protocol

* This protocol is applicable only to kinases whose activity is not altered by cell lysis or immunoprecipitation procedures and do not require soluble cofactors for activity.
* Verify that the antibody used quantitatively immunoprecipitates the kinase from lysates without affecting its activity, either positively or negatively.
* Plan out the necessary components (antibody, buffers, isotope, substrate, and P81 paper/gel lanes) in advance by determining the number of reactions required. Plan on including negative controls (lysate of unstimulated cells; immunoprecipitation with non-immune antibody; sample with no enzyme) as well as a positive control (purified active enzyme).
* Establish conditions for the kinase assay to ensure that activity is measured well within the linear portion of the reaction (the limiting component should be the availability of active kinase, with substrate and ATP in excess). The most highly active samples are the first to deplete reactants, slowing activity in those samples.

1. Prepare cell lysate using stimulated cells and perform immunoprecipitation according to procedures and using buffers described in this protocol section.

2. Centrifuge down the immunoprecipitate and remove as much of the supernatant as possible without disturbing the pellet.

   Note: Do not boil the immunoprecipitate after the final washing step. Keep samples on ice.

3. Prepare the solutions for the kinase assay:

   * 5 x Assay Buffer: 100 mM MOPS; 125 mM beta-glycerol phosphate, pH 7.2; 5 mM EGTA; 5 mM sodium orthovanadate; 5 mM dithiothreitol
   * Magnesium/ATP Cocktail: 100 microM non-radioactive ATP and 75 mM magnesium chloride in Assay Buffer (If kinase is known to prefer manganese ions for activation, manganese chloride can be substituted for magnesium chloride)
   * [gamma-32P] ATP: dilute stock solution to final concentration of 1 microCi/microliter using Magnesium/ATP Cocktail.
   * Substrate: (e.g., myelin basic protein) prepare a 2 mg/ml solution using Assay Buffer

4. Prepare reaction buffer mixture by adding 10 microliters of each component times the number of assay samples plus one to a single large tube. This will ensure uniformity of the reactants as well

as allowing for sufficient amounts for all samples. Mix well.

5. Measure the specific activity of the radioactive label counting a 1:10 and 1:100 dilution of the [gamma-32P] ATP solution in a scintillation counter. Specific activity is expressed as cpm/pmol (the concentration of the radioactive ATP is trivial, so it is assumed zero)

6. Dispense the appropriate amount of reaction buffer mixture to each tube containing the immunoprecipitate, still on ice, mix well, and transfer all the tubes to a shaking water bath set at the correct temperature. Incubation temperatures and times should be optimized for each kinase.

7. Termination of the assay at the appropriate time can be carried out by adding SDS-sample buffer if the samples are to be electrophoresed, or by returning the tubes to an ice bucket. When dealing with large numbers of samples, it is advisable to first transfer to ice and then add any termination mix, because of the time between addition of termination mix to the first and last tubes.

8. Either spot samples of 30 microliters on P81 paper or electrophorese, transfer to nitrocellulose, and expose to film. If using the latter procedure, also excise radioactive bands and determine cpm.

9. Express kinase activity quantitatively using the specific activity of the ATP solution measured above. When assaying a purified kinase, express the catalytic rate in its linear range in mol phosphate transferred from ATP to substrate/min/mg of kinase. Highly active kinases transfer on the order of micromol phosphate/min/mg of kinase.

10. When assaying kinase activity immunoprecipitated from a lysate, it is not possible to express activity per mg of kinase. Instead, determine the relative activation by subtracting counts incorporated in assays of non-immune immunoprecipitations from gross counts to determine net cpm. If non-immune cpm are not insignificant, it may be that the immunoprecipitates are contaminated with other kinases. Using net cpm, plot results as fold-activation over unstimulated, or as a percentage of maximal activity.