Horwitz lab IP procedures for mass spectrometry

This discussion outlines the strategies and procedures that we use for immuo-purification in our analyses of binding partners and phoshorylation sites using an ion trap mass spectrometer. The critical issues, from the point of view of mass spectrometry, are the dynamic range and the sensitivity. The dynamic range is particularly important since it is ~100 on typical machines, although new developments have increased this about 50 fold. With complex protein mixtures, this range can be compromised. The sensitivity of the mass spectrometer is relatively high, which makes contamination a particular problem.

Our procedures address these issues by attempting to decrease the "non-specific" binding and lower the expression of the "bait" protein to near endogenous levels when ectopically expressed proteins are used. Most of our studies have used FLAG tagged proteins expressed in HEK293 cells. The FLAG bait uses a peptide elution which affects a relatively simple and rapid purification procedure. We use HEK cells because they can be transfected with a high efficiency.

We have found that the expression level of the ectopic protein is critical. When low expression (comparable to endogenous levels) is used with paxillin, for example, most of the known binding partners are observed, with high peptide coverage, in analyses by mass spectrometry. When expression is too high relative to endogenous expression, the dynamic range is determined by the highly expressed "bait" protein, and the relative concentration of (localized) bait and binding partners can be too low to detect. In assessing expression, it is important to ensure that all of the cells are expressing at a relatively low level rather than a few cells expressing high amounts. Both would look the same when the cultures are analyzed by western blots but would appear different if observed by staining or using a fluorescent tag, for example. There are several strategies for achieving low but efficient transfection. We generally use a carrier plasmid (pBluescript, pUC19) as a co-tranfectant to bait cDNA plasmid, at a maximum concentration of 3μg/100mm dish. A transfection time of 24 rather than 48 hours further serves to reduce expression in the HEK cells. For 3 different proteins (paxillin, GIT, and βPIX), we found near endogenous expression levels using between 1-10ng of cDNA/100mm dish, depending on the protein of interest. Using a FLAG-GFP fusion, we

find that most of the cells express the bait at relatively low levels. While we use transfection, other strategies include infection using retrovirus (with compromised promoters/enhancers to reduce expression, if necessary), FACS analyses of lines co-expressing GFP, and "knock out" (and "knock in") cell lines.

Before doing MS analyses, we routinely analyze our samples by SDS-PAGE. We typically analyze 20% of the wash and 10% of eluate on SDS-PAGE and silver stain for the presence of the bait proteins in comparison to the negative control. This is followed by blotting and probing for anti FLAG immunoreactivity, or a suitable antibody for the tag being used. The goal is for the bait protein to be one of the major bands on the silver stain. However, putative binding partners may be visible as well (recall the dynamic range issue outlined above). As expected, when high amounts of DNA (3-4ug) are transfected, the bait protein is the predominant band on the gel. However, at lower DNA concentrations (1-10ng) the bait protein may no longer be the predominant band, as putative binding partners become more prominent.

There are many strategies for washing IPs prior to elution. If the immunoprecipitates are insufficiently washed, the gels can be dominated by nonspecific interactions. When the immunoprecipitates are washed too aggressively, binding partners can be removed and the bait protein eluted prematurely. The details of the washing procedure need to be determined empirically since it is complex dependent. For our analyses, we use a series of NaCl washes using concentrations in the range of 25-200mM. The use of other agents including weak detergents or chaotropic agents can also be useful, although the detergent must be removed before analysis by mass spectrometry. For analyses of phosphorylation, we use relatively aggressive wash procedures to ensure that the protein of interest is in fact the major protein present in the eluate when analyzed by silver staining of SDS-gels.

We generally elute our columns with a FLAG peptide since it elutes efficiently after relatively short incubation times (~10-20 minutes). Elution times longer than 30 minutes can result in an increase in non-specific proteins, thus masking the bait protein and its binding partners. If ectopically expressed proteins are used as the "bait", the location of the tag can also be important.

The bottom line from our experience is that low, uniform expression, high purity and sufficient sample concentration are the keys to success in MS analyses. We typically require clean gels by silver stain with the bait protein as a major component before samples can be sent for MS analyses. For binding partners, we use western blots AND silver stain to document the presence of known binding proteins. In our experience, if you can see them on a silver stain, you will see them by mass spectrometry. On the other hand, detection by western blotting does not ensure detection by mass spectrometry, since the concentration of binding partner might either lie outside the dynamic range of the instrument or not be high enough to be detected by MS. Thus, the need to develop biochemical criteria for purity and the presence of binding partners, which must be present at concentrations within the dynamic range of the instrument, cannot be over emphasized. While instruments with increased sensitivity, mass accuracy, and dynamic range are becoming available, they do not decrease the need for low expression and IP protocols optimized for purity and the presence of binding partners, when they are known.

Representative IP Protocol for Mass Spectrometry Analyses

Transfect 80% confluent HEK cells (4X10cm plates) with lipofectamine (17.5 μl) and 4 μg total DNA/plate (10 ng-4 μg of the bait protein cDNA; use the Bluescript vector to bring total DNA concentration to 4 μg).

Incubate for 24-48 hours in incubator.

Lyse cells for 30 min on ice with 0.5% NP-40 in 25mM Tris, 100 mM NaCl, with proteinase inhibitors, pH 7.4

For phosphorylation analysis, incubate cells for 30 min before lysis with 1 mM pervanadate and 10 nM calyculin and include these phosphatase inhibitors (leupeptin and E-64 10ug/ml, aprotinin 5ug/ml) in the lysis buffer.

Scrape the plates in a total volume of 1 ml (for the 4 plates)and pass through a 3/8 inch, 26 gauge needle

Centrifuge at 12,000 x g for 5 min at 4°C

Preclear (supernatant) with mouse IgG agarose (30μl) for 1.5 h on rocker at 4°C
Repeat (overnight at 4°C often helps)

IP for 1-2 h with anti-FLAG-agarose (60 μl; Sigma) on rocker at 4°C

Wash the bead pellet two times (200μl) with 25 mM Tris, 100 mM NaCl, pH 7.4

Elute with 100μl of 25 mM Tris, 0.2 mg/ml FLAG peptide, pH 7.4 for 20 min at RT.

For gel analyses, run 20% of wash volume and 10% of elution, silver stain and blot for FLAG.

Turn in 40μl for MS/MS analysis