SILAC Protocol

(2/23/06 version)

To identify and better quantitate the repertoire of proteins that associate with different bait proteins, we have developed methodologies to exploit differential isotopic labeling of proteins using SILAC or Stable Isotope Labeling with Amino acids in Cell culture. Using SILAC, cells expressing epitope-tagged bait are labeled with stable heavy isotopes of arginine and/or lysine, and cell lysates mixed with lysates prepared from control cells not expressing FLAG-tagged bait and grown in normal (light) media. Immune complexes of the bait are purified from the mixed lysates, washed with 1M NaCl (High Salt fraction) to remove weakly associated proteins and then eluted with the FLAG peptide (Flag-peptide fraction). Using FT ICR MS/MS analysis, the ratio of heavy and light peptides can be determined for most of the proteins present in the mixture of captured proteins. Thus proteins that are predominantly "light" are likely derived from the control cell lysates, proteins that are predominantly "heavy" are derived from an interaction with the bait, and those proteins that are both "light" and "heavy" arise from both control and bait expressing lysates. This approach has allowed the identification of a number of proteins that appear to be selectively enriched in the FLAG-FAK-expressing and FLAG-cortactin expressing lysates (Table 1 & 2).

Protocol:

Cells: FLAG-FAK expressing HEK293; FLAG-cortactin expressing HEK293 or untransfected HEK293 cells.

SILAC medium: Light medium: D-MEM (Gibco-Invitrogen) containing 0.8mM L-lysine and 0.5 mM L-arginine (SIGMA) and 10% dialyzed fetal bovine serum (dFBS, Gibco-Invitrogen); Heavy medium: D-MEM containing 0.8 mM [^{13}C] L-lysine (K+6) and 0.5 mM [$^{13}C^{15}N$] L-arginine (R+10) from Cambridge Isotope Laboratories (CIL) and 10% d FBS.

Buffers:

Lysis Buffer: CSK/NP:

20 mM Tris-HCl, pH: 7.4, 100 mM NaCl, 1 mM Mg_2Cl, 10% glycerol, 2.5 mM Na vanadate, 2.5 mM PPi,1mM NaF, RNAse 10µg/ml, EDTA-free protease inhibitor cocktail (Sigma)

Wash buffers:
MS: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA.

HS: 20 mM Tris-HCl, pH 7.4, 1M NaCl , 1 mM EDTA

Flag-peptide elution buffer
FpeB: 20 mM Tris-HCl, 100 mM NaCl, 1mM EDTA, + 1.5 mg/ml Flag peptide DYKDDDDK (Sigma)

Procedure:

Lysate preparation: Bait expressing-HEK cells in 3 plates (140mm) were grown for 5-6 generations in “heavy” medium. In parallel untransfected HEK cells are grown in “light” medium. Cell monolayers from both heavy and light cultures are washed once with PBS and scrapped into CSK/NP buffer at 0-4 °C. The cell lysates from heavy and light cells are clarified by centrifugation for 15' at 15,000 rpm and equivalent amounts of extract (determined by BCA assay, Pierce) are mixed to yield approximately 4-5mg of total protein/ml and a final volume of 4ml.

Affinity beads: 500µl of M2 agarose beads suspension is washed once with MS buffer and mixed with 1ml of lysate (app 5-7mg/ml) from light HEK 293 lysate. The beads are incubated (rotation in Eppendorf tube) for 1hr at room temp and washed with 2 x MS buffer and 1 x HS. They are resuspended in the original volume of MS buffer and ready to use with the heavy/light lysate prepared as above.

Affinity purification: 100µl of pre-blocked M2 agarose beads suspension is then added to 4ml of the heavy/light mixed lysates followed by rotation at 4°C for 2h.

The beads are centrifuged for 2 minutes at 2000 rpm and washed once with 15-20 volumes of 1 x CSK-NP and twice with 15-20 volumes of MS buffer. This is followed by resuspension of the beads in 300µl of HS buffer for 20 minutes at 0-4°C and centrifugation (supernatant of this wash is designared the High Salt (HS) fraction and contains proteins weakly associated with the bait). Elution of the bait is carried out by incubation of the beads with 150-200µl of FpeB.(rotation at room temperature for 30 minutes). The supernatant is the Flag peptide eluate fraction (Flag peptide fraction).

Both, HS and Flag-peptide fractions are subjected to MS analysis.

Mass spectrometry analysis and data analysis.

Samples were reduced with 10µL of 10mM dithiolthreitol in 0.1M ammonium bicarbonate for 30 minutes at room temperature (RT) and alkylated with 30µL of 50mM iodoacetamide in 0.1M ammonium bicarbonate for 30 minutes at RT before being digested with 1µg of Promega modified trypsin for 24 hrs at RT. A second 1µg of trypsin was added and digestion was allowed to proceed for an additional 24 hours. The sample was then desalted on a self-packed 6cm x 150µm id C18 column. Approximately 25% of the digest was introduced into the mass spectrometer for analysis. The mass spectrometry system consisted of a Thermo Electron LTQ-FT mass spectrometer (hybrid linear ion trap – FTICR) with a Protana nanospray ion source interfaced to a self-packed 8 cm x 75µm id Phenomenex Jupiter 10µm C18 reversed-phase capillary column. The peptides were eluted from the column by an acetonitrile/0.1 M acetic acid gradient over two hours at a flow rate of 0.3µL/min. The nanospray ion source was operated at 2.8kV. The digest was analyzed by acquiring one full scan mass spectrum to determine peptide molecular weights (FTICR – 100K resolution) followed by ten product ion spectra (linear ion trap) to determine amino acid sequence.

This mode of analysis produces approximately 10,000 MS/MS spectra of ions ranging in abundance over several orders of magnitude. MS/MS spectra were searched using the Sequest algorithm against the NCBI non-redundant (NR) database. Search results were validated using in-house software (Proteofarm). his software examines parameters such as Xcorr, percent ions found, percent ion explained, species, etc to validate individual search results. Those proteins that have at least one spectrum pass this step are grouped into a custom database. The spectra are then searched again against the custom database and the verification process repeated with more stringent criteria (modifications such as oxidized Met, acrylo Cys [gel bands], phospho Ser/Thr/Tyr, etc can be added to the search at this step). At this point, spectra with marginal validation scores or those with possible post-translational modifications can be manually verified. In SILAC experiments, the data is first searched against the normal database and then against the same database with Lys (~ +6 or +8 Da) and Arg (~ +6, +8 or +10 Da) statically modified to reflect the heavy isotopes. Unlike other experiments, the data are uploaded as both 'light' (normal search) and 'heavy' (statically modified search) results. For a protein of interest, the ratio of light to heavy (L:H) is determined by manual calculation of the areas for each isotopic species of each peptide of that protein. The final ratio for the protein is the average ratio for all peptides determined.