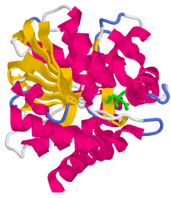
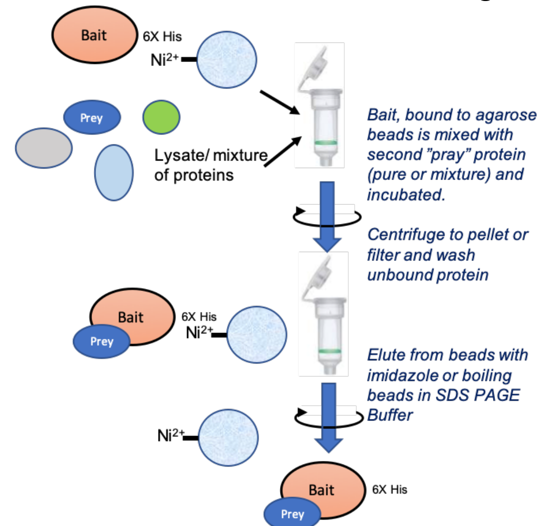


His Tag Pulldown

Co-Precipitation Interaction Assay Protocol

Introduction: Protein-protein interactions are often detected or confirmed in an *in-vitro* method. Pull-down or co-precipitation (called immunoprecipitation if using antibodies to capture one of the proteins). In general, one protein, called the bait is immobilized on an agarose bead. After incubation, beads are centrifuged and supernatant solution saved to determine loss in bait and prey. After washing, the bait and any bound "prey" protein is eluted and analyzed. Controls include proteins that do not bind (a GFP-His protein for example), controls with bait but no pray, and controls that do not have bait but include prey. These last two controls account for background aka non-specific binding proteins and protein "trapped" in the voids of the beads.

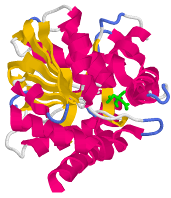


Detect bait and prey protein in lysate and elution by enzyme activity, SDS PAGE, western blot or MS

Format: Pulldowns can be done using a slurry of beads in a microfuge tube where careful removal of supernatant solutions is important to avoid loss of beads. Another approach is depicted in the image shown here, where beads are in a spin column format, making the washing and elution faster and less likely for cross contamination and retrieval of flow-through non-binding and eluted samples relatively easy. Finally, a third approach is to use magnetic beads and a strong magnate is used to isolate the beads on the side of the tube allowing for easy sample retrieval and bead washing with minimal sample dilution or bead loss. Some formats use a cobalt instead of nickel as both will chelate a His-tagged protein, but colbalt with lower binding capacity has better specificity in binding.

Protein Interaction/Binding Detection: A simple, semi-quantitative approach is to perform a SDS PAGE gel to identify the binding and elution of bait-pray interactions. A more sensitive analysis would include western-blotting. When using lysate, where potential prey interactors are unknown, trypsinizing gel slices followed by MS analysis often works well. If using two know proteins from a purified source, enzyme assays can be conducted in the load, supernatant and after washing, eluted fractions. Depending on the strength of the interactions, crowding agents such as PEG may need to be included in the binding incubation and wash periods. Obtaining specific interactions may require adjusting ionic strength and use of a non-ionic detergent in the wash steps.

SDS-PAGE Coomassie Detection Sensitivity – while a single protein can be detected with as little as 5-25 ng of protein, a reasonable sized and easily detected band of protein on a gel, will be seen between 500 and 1000 ng of protein. Thus, if loading and binding a bait-pray mixture and detection is SDS PAGE one should ensure the bait is in excess of binding capacity. Many Ni-Agarose beads will bind 25-50 µg of protein per µl of gel beads. A typical spin or microfuge tube pull-down assay will have ~25 µl of packed beads. Using the protocol given here, saturated, beads will contain 625 – 1,250 µg of protein. Elution volumes of 200 µl will result in a bait concentration of ~3 – 6 µg/µl protein in the eluted fraction. Thus, running 1-5 ul of sample (plus SDS PAGE sample buffer) on a gel should provide enough protein to visualize using Coomassie staining. Detection of pray, if strong binding and 1:1 bait to pray should also be seen. Weak binding may require more elution loaded onto the gel to be detected.



His Tag Pulldown

Co-Precipitation Interaction Assay Protocol

The following protocol is adapted from Pierce Pull-Down PolyHis interaction kit (21277) from ThermoFisher using 25 µl of packed beads in a spin column format. For a non-spin, traditional microfuge tube format, modest modifications can be used. If using magnetic beads, such as Dynabeads or Pierce HisPur NiNTA Magnetic beads, the protocol must be modified further based on manufacture's recommendation.

Bead Preparation –

- Starting with a 50% bead to buffer slurry, transfer 50 µl of beads (use a pippet with the tip cut off for the transfer) to a spin column. The final bead volume should be 25 µl.
- Add 400 µl of His-elution buffer and gently resuspend beads by inversion. Place spin tube in 2 ml microfuge tube and centrifuge at 1250 x g for 30-60 seconds. Discard solution in 2 ml tube.
- Repeat with His-binding buffer for a total of 5 times.

Bait Protein Preparation from Lysate –

- Induce and express protein as appropriate for bait protein. Culture 10 ml for each interaction tube and control.
    - If you are doing an experiment with a total of five tubes (including controls) then you have five experimental pull-down assays. You should prepare enough lysate for this may tubes plus one or two to ensure you have enough lysate to add to the last tube.
- For each experimental condition (pull-down assay), centrifuge 10 ml of induced culture at 5000 x g for 10 min. Do this as one solution. Keep pellet, discard supernatant solution
- Resuspend pellet in 500 µl His-binding buffer with lysozyme (1.0-0.5 mg/ml) for each experimental condition/pull down assay. Incubate on ice for 15-30 min with rocking, followed by sonication.
- Centrifuge at 8,000 x g for 20 min at 4ºC and save supernatant. This should be clear without particulate/cloudy appearance.
- This is bait lysate. Use 200 µl of this lysate for each pull-down assay tube. 400 µl if the bait protein is poorly expressed.

Bait Protein Preparation from Purified Protein –

- Induce, express and purify His-tagged bait protein.
- Dialyze imidazole away with 2-4 changes of buffer. Use a large ratio of dialyzed sample to dialysate (~1:50 or 100) in His-binding buffer.
- Determine protein concentration after dialyzing sample. 0.5-2.0 mg/ml is optimal.

Bait Protein Immobilization –

- With caps applied, add bait protein to washed beads in spin column (bead only, no buffer).
    - If using lysate, use either 200 µl or 400 µl bait lysate protein
    - If using purified protein, add 150 µg of purified protein. Up to 800 µl volume. e.g. for a 1.0 mg/ml purified and dialyzed His-tag bait protein, 150 µl.
- Incubate at 4ºC for at least 30 min with gentle rocking (not Metalica but perhaps nice 80's soft rock like 10cc or air supply).
- Remove top and bottom caps and centrifuge in a 2.0 ml tube at 1250 x g for 30-60 sec. Label as "bait flow through" and place on ice for later analysis.

- Replace bottom cap, add 400 µl His-wash buffer and resuspend by inversion. Centrifuge as above. Do not save wash.
- Repeat 4-5 times, more if using lysate.

Pray Protein Preparation –
- Express, purify and dialyze prey protein in pull-down assay buffer.
- If prey protein is His-tagged, remove using appropriate protease tag (TEV or Thrombin) and remove un-digested protein and His-tag fragments using the TEV protease protocol.
- Determine protein concentration.
- Using up to 800 µl of volume, calculate the volume of digested prey protein necessary to load 150 µg of prey protein.

Prey Protein Capture –
- Apply bottom cap and remove top cap from spin column containing bait bound to beads (no buffer just beads).
- Add up to 800 µl of prey protein as calculated in the prey protein preparation section.
- Incubate at 4ºC with gentle rocking (this time, Billy Joel or the Eagles greatest hits often works well) for 1 hour.
- Remove caps and centrifuge at 1250 x g for 30-60 sec. SAVE the flow through as "prey-flow through".
- Replace bottom cap, add 400 µl of wash solution, and mix by inversion. Centrifuge as before and repeat for 5 total washes. Finish with "dry" beads, then continue to the elution step.

Bait-Prey Protein Elution –
- Add 250 µl of His elution buffer to the spin column and incubate with rocking (users' choice) for 5 min.
- Centrifuge at 1250 x g for 30-60 seconds. Save the flow through as "Elution 1".
- One elution should be sufficient, repeat if necessary.

Analyze of samples. Measure control and experimental "load" (samples without beads), the prey-flow through and the elution 1 fraction. Samples can be measured using SDS PAGE or enzyme assay.

Sensitivity depends on protein concentrations and protein type. A suggested starting approach is:
- 20 µl fraction + 10 µl 2X or 5X SDS Sample Buffer.
- Boil and load 20 µl of each fraction.

His Binding Buffer:
- 50 mM Tris-Cl, pH 8.0
- 5 mM Imidazole
- 100 mM NaCl
- 0.1 mM EDTA
- 1 mM PMSF

added fresh for lysate bait