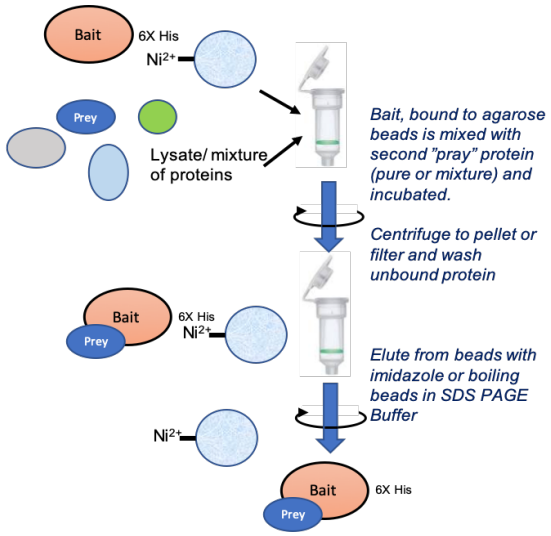
Biochemistry Lab

MDH-CS Interaction Pull Down
Experimental Design

Introduction: Testing for protein-protein interactions using an *in-vivo* pull-down assay (also known as co-precipitation or bait and prey capture assay) is a simple and semi-quantitative approach to showing protein binding. 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. Depending on the strength and transient nature of the protein interaction, one may need to include crowding agents. As some proteins may aggregate, inclusion of weak non-ionic detergents such as NP-40 or Tween-20 may be needed. 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. This protocol relies on capture of the bait protein by His tag and the prey protein to be free of the same affinity tag used to capture the bait.



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 0.25 µg of protein, a reasonable sized and easily detected band of single protein on a gel, will be seen between for 1 and 5 µg 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 and total binding capacity for His-tag proteins in the spin column will be 1000-1250 µg of protein. We will be loading ~100 µg of bait (MDH) His-tag protein to

the beads. Assuming a 1:1 binding ratio bait (MDH) to pray (CS), which will be impacted by many factors, and an elution volume of 100 µl will result in a bait concentration of ~0.5 – 1.0 µg/µl protein in the eluted fraction. Thus, running 5-10 µl 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.

Affinity (6XHis Tags): Only one of the proteins, the bait can be "his tagged". The prey protein if possessing a His tag, must have the tag removed. The CS clone has been created with a TEV protease recognition sight between the His tag and the remaining CS peptide. TEV protease is a specific protease from Tobacco Etch Virus (TEV) that cleaves between Gln and Gly of a seven amino acid sequence (Glu-Asn-Leu-Tyr-Phe-Gln-/Gly). At this time, the wgMDH and hMDH1 (cytoplasmic MDH) clones do not have a TEV site. The hMDH2 and the hMDH2 mutants do poses a TEV site. Therefore you will need to remove the TEV site from CS and MDH will be bound to columns.

Also VERY important is both the bait and prey proteins must be extensively dialyzed to remove imidazole as residual imidazole will interfere with binding to beads causing false negative results.

Helpful values for calculations.

	@ 1µM µg/µl	Holoenzyme MW (Da)
wgMDH	0.069	69,000
hMDH1v3	0.077	77,200
hMDH2	0.066	66,000
CS	0.98	98,012

EXPERIMENTAL DESIGN

Part I: TEV cleavage and prey protein preparation. Here we will cleave the his tag from CS, then flow the TEV-CS mixture through Ni-Agarose beads to separate any cleaved His tag, uncut His-CS and TEV from cleaved CS.

- For each 1:1 interaction, transfer 100 µg of purified and dialyzed recombinant CS into a 1.7 ml microcentrifuge tube. The first experiment will use 9 tubes.
- To the tube containing CS add:
  - 1 µl of Invitrogen AcTEV Protease 10 U/µl for each 100 µg of CS. This is a His tagged TEV for easy TEV removal.
  - DTT (stock conc = 100 mM) Use CV=CV to calculate how much DTT to reach 0.5 mM
  - Mix by inversion and incubate at 30°C for 1 hour.
  - After 1-2 hour, remove 2 µl and save for SDS PAGE

- Repeat but NO TEV replacing with buffer only. This will show untreated CS will bind to beads

- Prepare two x 100 µl of Ni-Agarose beads in a spin column (do this while TEV treating your protein)
  - Suspend a 50% slurry of Ni-Agarose beads and transfer 200 µl (use a cut pipet tip) of the slurry into 2.0 ml microcentrifuge tube and centrifuge at 1,250 x g for 1 min.
  - Using a 1ml pipet, remove all of liquid and leave beads in the tube.

- Wash the spin column by adding 1000 µl Elution buffer, invert to mix beads and centrifuge as above. Then remove the supernatant from the beads (keep the beads)
- Wash the beads with Binding Buffer, centrifuge, remove supernatant (keep the beads) and repeat a total of five times. Remove all liquid and the beads are ready to bind to CS.

- Add the TEV treated CS solution (don't forget to save 2 µl first) to the "dry" washed bead pellet and mix by flicking the tube with your finger. Do not invert or vortex.
- Repeat for untreated CS preparation.
- Incubate for 10-20 min with mixing (room temp).
- Centrifuge the beads/TEV-CS mixture and carefully remove (AND SAVE) the supernatant. THIS IS THE CLEAVED CS!!!! Label as Non-Binding fraction.
- Wash the beads with 1 ml binding buffer, centrifuge and remove supernatant (don't bother saving this). Repeat a total of two times. Beads should be "dry" when finished.
- Add 100 µl of Elution Buffer to the beads and incubate with mixing for 2 min.
- Centrifuge and KEEP SUPERNATE – this is the His tag uncleaved CS, TEV and His tag separated from CS.

SDS PAGE Analysis.

- Add 3 µl of 5X SDS PAGE Sample buffer to the 15 µl digested CS.
- Transfer 3 µl of 5X SDS PAGE Sample buffer to 15 µl of non-binding fraction.
- Transfer 3 µl of 5X SDS PAGE Sample buffer to 15 µl of Elution fraction.
- Same for non-treated CS beads.

Run an SDS PAGE gel. Lane 1: 10 µl MW Standards. Lanes 2-4: 15 µl of the samples prepared above in order listed.

Stain and analyze for % of CS protein cleaved in non-binding fraction vs uncleaved and bound to beads.

IF most of the CS is cleaved, conduct a protein assay and proceed to Pull Down Experiment Part II.

PartI I: Pull Down Wild-Type MDH-CS. Now we will check for the interaction between CS and MDH. Beads will be prepared for MDH binding. MDH will then be bound to the beads and washed for non-binding protein. The beads will be spun dry Then His-tag "less" cleaved CS will be introduced to MDH for Bait-Prey binding. Beads will be mixed and interactions happen. Spin the beads to separate non-binding CS from MDH and then elute anything bound to the beads. We will detect the protein by SDS PAGE.

The first set of experiments will be do conduct the assay with CS and mitochondrial, cytosolic and watermelon glycoxyl MDH in with and without a crowding agents -PEG. This will show the base conditions by which CS and MDH can interact. The best condition will be used to measure the mutant protein and wild-type MDH-CS interaction using increasing concentration of CS. Later, follow-on experiments will be designed using these conditions with different concentrations of CS on each wild-type and mutant MDH. If time permits, we can use conditions described in the Joy Omini paper using metabolites to alter the interaction.

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 – PREPARE THREE TUBES

- 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 (MDH) Protein Immobilization –

- With caps applied, add bait protein to washed beads in spin column (bead only, no buffer).
  - add 100 µg of purified MDH protein. Up to 800 µl volume.
- Incubate at 4°C 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. MDH is ON THE BEADS!!!!

Pray (His tag removed CS) Protein Preparation –

- Using up to 800 µl of volume, calculate the volume of digested prey protein necessary to load µg of prey protein.
- Calculate the volume of PEG or Glycerol to achieve 10% PEG in tube 2, or 20% glycerol. One tube is left using dialysis buffer only – no addition.

Prey Protein (CS) Capture –

- Apply bottom cap and remove top cap from spin columns containing bait bound to beads (no buffer just beads).
- Add 100 µg of prey (CS) protein in up to 800 µl of prey protein as calculated in the prey protein preparation section.
- Add the calculated volume of PEG or Glycerol into tubes 2 and 3.
- Incubate at 4°C 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 Binding Buffer solution (with PEG or Glycerol as needed), and mix by inversion. Centrifuge as before and repeat for 2 total washes. Finish with "dry" beads, then continue to the elution step. SAVE each flow through.

Bait-Prey Protein Elution –

- Add 100 µl of His elution buffer to the spin column and incubate with rocking (users' choice of music) 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 + 4 µl 2X or 5X SDS Sample Buffer.
- Boil and load 15 µl of each fraction.

Binding Buffer:

- 50 mM phosphate buffer, pH 7.4
- With added PEG or Glycerol. Prepare 10 ml of each.

Elution Buffer:

- 50 mM phosphate buffer, pH 7.4
- 50 mM NaCl
- 300 mM Imidazol