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Direct In Vitro Pulldowns of Hexahistidine-Tagged Proteins and Peptides with Interactors V.2

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We use this protocol and it's working

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Abstract

Direct pulldowns of two purified proteins are a standard tool to determine whether they bind directly to each other. Mutational analyses allow us to find out which residues are involved in the direct binding of known interactors. By using defined molar amounts of proteins, it is even possible to roughly estimate how strong two proteins interact with each other. Here, we describe a protocol that uses hexahistidine-tagged peptides and proteins as bait and untagged proteins as prey in vitro, with subsequent pulldown by Ni^{2+} -agarose beads.



Materials

Consumables

- 1.5 ml Reaction tubes (Greiner, 616201)

Reagents

- Purified bait and prey proteins/peptides
- Nickel-NTA agarose resin (e.g., Thermo Fisher R90110, etc.) equilibrated in binding buffer
- Binding buffer (i.e., 20 mM Tris pH 8.0 at 4°C, 300 mM NaCl, 10 mM β -Mercaptoethanol, 20 mM Imidazole)

Note

The amount of Imidazole can be varied depending on the proteins, as some are more or less sticky to the Nickel-agarose resin. In the vast majority of experiments, the above-mentioned buffer worked perfectly fine, providing a clean background in negative (=no bait) controls.

- Elution buffer (20 mM Tris pH 8.0 at 4°C, 300 mM NaCl, 200 mM Imidazole)
- Coomassie Staining solution (e.g., as described here: <https://app.jove.com/t/1350/staining-proteins-gels-with-coomassie-g-250-without-organic-solvent>)

Equipment

- Overhead rotator (LD-79, LABINCO BV)
- SDS-PAGE gels and electrophoresis system (ATTO)
- Tabletop microcentrifuge (Ohaus or VWR MicroStar 12)

Protocol

25m

1 Pipetting scheme and general considerations

1.1 Perform all steps on ice or in the cold room.



1.2 Determine the concentrations of your proteins or peptides.

Note

Depending on the molecular weight of the protein/peptide, a concentration between 1-5 mg/ml is ideal.

1.3 Typically, 10 μ M bait and prey in 1 ml, but according to the amount and availability of the protein, lower concentrations and/or volume work fine.

Note

Volumes below 500 μ l are possible but not recommended

1.4 Always include a no-bait control to confirm that the non-His-tagged protein does not bind to the resin.

1.5 Triplicates are better than duplicates, which are better than just one condition.

1.6 Formula to calculate the molar concentration:

$$\frac{c[\frac{g}{l}]}{MW[\frac{g}{mol}]} \times 1000000 = cm[\mu M]$$

Here c = concentration in gram/liter (or, mg/ml), MW = Molecular Weight (gram/ mol)

Example:



- Bait: $c=2 \text{ mg/ml}$, $MW=20000 \text{ g/mol} \rightarrow 100 \text{ } \mu\text{M}$. $10 \text{ } \mu\text{M}$ in $1 \text{ ml} = 100 \text{ } \mu\text{l}$
- Prey: 3.6 mg/ml , $MW=40000 \text{ g/mol} \rightarrow 90 \text{ } \mu\text{M}$. $10 \text{ } \mu\text{M}$ in $1 \text{ ml} = 111 \text{ } \mu\text{l}$

1.7 **Table 1:** Example pipetting scheme

		1	2	3	4	5	6
Bait		100 μl	100 μl	100 μl	100 μl	100 μl	100 μl
Prey					111 μl	111 μl	111 μl
Buffer		850 μl	850 μl	850 μl	739 μl	739 μl	739 μl

2 **Pulldown**

- 2.1 Pipette your binding buffer, bait, and prey protein according to the pipetting scheme.
- 2.2 Add 50 μl of 50% slurry Ni-NTA agarose beads equilibrated in binding buffer to the Eppendorf tubes.
- 2.3 Incubate in an overhead rotator for 15 minutes. If no rotator is available, invert tubes every minute by hand.
- 2.4 Spin down samples at 1000 x g for 2 minutes.
- 2.5 Remove supernatant. If required, collect a 15 μl sample of the 'unbound' fraction (U).
- 2.6 Resuspend in 1 ml of binding buffer for ~1 minute.

15m

2m



1m



2.7 Spin down samples at 1000 x g for 2 minutes.

2m



2.8 Remove supernatant and wash cells with binding buffer.

2.9 Repeat the wash step two times.

2.10 Remove supernatant carefully and completely after the last wash.

Note

Best done in two steps: remove most of the supernatant with a 1 ml pipette, wait ~1 minute, then remove the remaining supernatant with a 200 µl pipette, so that the Ni-agarose resin falls dry.

2.11 Elute proteins with 50 µl of elution buffer by vortexing briefly.

2.12 Spin down at full speed in an Eppendorf centrifuge.

2.13 Mix 36 µl supernatant with 12 µl 4x SDS-PAGE loading buffer.

2.14 Incubate at 95°C for 5 minutes.

5m



2.15 Vortex, spin down samples.

2.16 Make input samples: Dilute 40 µg of your bait and prey proteins in 100 µl (75 µl protein sample + dH₂O and 25 µl 4x SDS-PAGE loading buffer). A load of 5 µl per lane contains 2 µg of protein, which gives a well-visible band in a conventional SDS-PAGE (Laemmli).



Note

Samples can now be stored at -20°C or directly be analysed by SDS-PAGE.