Jun 09, 2025 Version 1

Direct In Vitro Pulldowns of Hexahistidine-Tagged Proteins and Peptides with Interactors V.1

DOI
https://dx.doi.org/10.17504/protocols.io.dm6gpqk91lzp/v1

Dieter Waschbüsch[1], Amir R Khan[2]

[1]School of Biochemistry and Immunology, Trinity Biomedical Sciences, Institute Trinity College Dublin, Dublin 2, Ireland;
[2]School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland

AKhanLab

Dieter Waschbuesch
School ofBiochemistry and Immunology, Trinity College Dublin

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Protocol Citation: Dieter Waschbüsch, Amir R Khan 2025. Direct In Vitro Pulldowns of Hexahistidine-Tagged Proteins and Peptides with Interactors. **protocols.io https://dx.doi.org/10.17504/protocols.io.dm6gpqk91lzp/v1**

Protocol status: Working
We use this protocol and it's working

Created: May 18, 2025

Last Modified: June 24, 2025

Protocol Integer ID: 218513

Keywords: ASAPCRN, untagged proteins as prey, tagged protein, peptides with interactor, tagged peptide, untagged protein, direct in vitro pulldowns of hexahistidine, strong two protein, protein, peptide, purified protein, molar amounts of protein, known interactor, direct in vitro pulldown, hexahistidine, interactor, mutational analysis, direct binding, direct pulldown

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. We use Hexahistidine-tagged peptides and proteins as bait and untagged proteins as prey in our experiments.

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.
  - 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)

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.

1.3 **Note:** Depending on the size, a concentration between 1-5 mg/ml is ideal.

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

1.5 Note: Volumes below 500 µl are possible but not recommended.

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

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

1.8 Formula to calculate the molar concentration:

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

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

Example:

- Bait: c=2 mg/ml, MW=20000 g/mol → 100 µM. 10 µM in 1 ml = 100 µl
- Prey: 3.6 mg/ml, MW=40000 g/mol → 90 µM. 10 µM in 1 ml = 111 µl

1.9 **Table 1:** Example pipetting scheme

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

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 (binding buffer) Ni-NTA agarose beads 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. 15m

2.4 Spin down samples at 1000 x g for 2 minutes. 2m

2.5 Remove supernatant. If required, collect a 15 µl sample of the 'unbound' fraction (U).

2.6 Resuspend in 1 ml of extraction buffer for ~1 minute. 1m

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

2.8 Remove supernatant and wash cells with extraction 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.