Chapter 20

Protein–Protein Interactions: Pull-Down Assays

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Abstract

Determining protein partners is an essential step toward understanding protein function and identifying relevant biological pathways. Many methods exist for investigating protein–protein interactions. The pull-down assay is an in vitro technique used to detect physical interactions between two or more proteins and an invaluable tool for confirming a predicted protein–protein interaction or identifying novel interacting partners. This method typically involves the use of affinity purification with various wash and elution steps. In this chapter, we describe how an interaction between two purified bacterial proteins or between bacterial and eukaryotic proteins can be detected by pull-down experiments.

Key words Pull-down, Protein–protein interactions, Tagged protein, Affinity purification

1 Introduction

Pathogenic bacteria produce virulence factors that usually help the pathogen to survive in an environmental niche, to promote colonization and invasion of host tissues, or to modulate the immune system. Virulence factors are toxins or effector proteins than can be transported by diverse secretion machineries in bacteria [1, 2]. Once secreted, these proteins can be assembled on the bacterial cell surface, released in the extracellular space, or secreted directly into a host cell or a neighboring bacterium. Once in host cells, effectors often target key proteins to hijack the host cellular machinery to remodel signaling cascades. The yeast two-hybrid system is often used to screen a large number of host proteins that potentially interact with bacterial effectors [3]. Regarding the mechanism of the secretion systems, a bacterial two-hybrid system is frequently employed to identify interaction networks between components of the secretory apparatus, as well as interaction between effectors and proteins of the machinery [4]. However, protein–protein interactions that have been determined by two-hybrid assay must be confirmed by other methods [5].

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Pull-down is an in vitro method widely used to detect or confirm interactions among multiple proteins. This assay is similar in methodology to co-immunoprecipitation experiments in its use of an affinity ligand to capture interacting proteins. The difference between these two methods is that while co-immunoprecipitation uses immobilized antibodies to capture protein complexes, the pull-down approach uses a purified and tagged protein as a "bait" to bind any interacting proteins. The method consists of first immobilizing the tagged protein (bait) on an affinity ligand specific to the tag, creating an affinity support to capture and purify other proteins (prey) that interact with the bait. The bait and prey proteins can be obtained from multiple sources, such as cell lysates, purified proteins, expression systems, and in vitro transcription/translation systems. Once the prey proteins have been incubated with an immobilized bait protein, interacting complexes are eluted using an eluting buffer depending on the affinity ligand. Each experiment needs proper controls to demonstrate that characterized interactions are not an artifact. For example, a positive control consisting of an immobilized bait protein alone is necessary to verify proper attachment of the tagged bait protein to the affinity support. To identify and eliminate false positives caused by nonspecific binding of prey proteins to the affinity support, cell lysates or purified proteins can be analyzed after being passed through a minus bait support. Following a pull-down experiment, protein fractions are resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then visualized by gel staining or western-blotting detection.

In this chapter, we describe detailed pull-down assay procedures that allow the identification of interacting proteins. First, we focus on how to perform a pull-down experiment to identify an interaction between a bacterial bait protein and eukaryotic prey proteins expressed in host cells (Subheadings 3.1 and 3.2). Next, we present how the interaction between two purified proteins can be visualized by a pull-down assay (Subheading 3.3). In these procedures, pull-down experiments have been performed using specific bait proteins fused to a 6× histidine tag. As a consequence, we selected Ni-NTA agarose beads as the affinity support used to immobilize these recombinant proteins.

2 Materials

Prepare all solutions with distilled water at room temperature and keep them at the indicated temperatures.

2.1 Preparation of Cell Lysate

1. Eukaryotic cells.
2. Cell culture dish, treated for optimal cell attachment, with growth surface area around 55 cm^2, sterile.

3. Plasmid containing the gene of interest fused to a specific tag (obtained from a EndoFree maxipreparation).
4. Transfection reagent.
5. Phosphate buffered saline (PBS): Prepare a 10× solution with bidistilled water (18.2 MΩ cm) containing 10.6 mM KH_2PO_4, 30 mM Na_2HPO_4, $2H_2O$, and 1.54 M NaCl, and sterilize with a 0.2 μm filter. The 1× solution obtained following dilution with bidistilled water will have a pH of around 7.4.
6. Radioimmunoprecipitation assay (RIPA) buffer: Ready-to-use solution containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.
7. Antiprotease cocktail: Mix 1% (v/v) of protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail 2 (Sigma-Aldrich), phosphatase inhibitor cocktail 3 (Sigma-Aldrich), and phenylmethylsulfonyl fluoride (PMSF).

2.2 Pull-Down Assays

1. 1 M Tris–HCl, pH 7.5 stock solution. Weigh 121.1 g Tris base and transfer to a 1 L graduated cylinder. Add water to 800 mL, mix, adjust pH with HCl, and make up to 1 L with water. Store at room temperature (*see* **Note 1**).
2. 5 M NaCl stock solution. Weigh 292.2 g NaCl and transfer to a 1 L graduated cylinder. Add water to 800 mL, stir, and adjust volume to 1 L with water (*see* **Note 1**).
3. Equilibrium buffer (*see* **Note 2**): 20 mM Tris–HCl, pH 7.5, 250 mM NaCl. Mix 1 mL 1 M Tris–HCl, pH 7.5 stock solution with 2.5 mL 5 M NaCl stock solution in a 50 mL centrifuge tube, and add water to a volume of 50 mL. Keep at 4 °C (*see* **Note 3**).
4. Elution buffer (*see* **Note 2**): 20 mM Tris–HCl, pH 7.5, 250 mM NaCl, 500 mM imidazole. Weigh 1.7 g imidazole in 50 mL solution of equilibrium buffer. Keep at 4 °C (*see* **Note 3**).
5. Purified His-tagged protein (bait).
6. Ni-NTA agarose beads: 6% beaded agarose (cross-linked), precharged with Ni^{2+} (Protino® Ni-NTA Agarose, Macherey Nagel, or equivalent). Store at 4 °C (*see* **Note 4**).
7. 0.8 mL empty columns for gravity flow (Pierce™ Centrifuge Columns, Thermo Fisher Scientific, or equivalent).
8. Refrigerated microcentrifuge.

2.3 Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Components

1. Resolving gel: 1.5 M Tris–HCl, pH 8.8. Weigh 90.8 g, transfer to 500 mL graduated cylinder, and add 300 mL water. Adjust pH with HCl and fill with water to 500 mL. Store at room temperature.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh 30.275 g, transfer to 500 mL graduated cylinder, and add 300 mL water.

Adjust pH with HCl and fill with water to 500 mL. Store at room temperature.

3. 30% acrylamide/Bis solution (37.5:1 acrylamide:Bis). Store at 4 °C.
4. Ammonium persulfate (APS): 20% solution in water. Store at −20 °C (*see* **Note 5**).
5. *N*,*N*,*N*′,*N*′-tétraméthyléthylènediamine (TEMED). Store at room temperature.
6. SDS-PAGE running buffer: 25 mM Tris–HCl, 192 mM glycine, 0.1% SDS. Prepare 10× running buffer solution: Weigh 30 g Tris base, 144 g glycine, and 10 g SDS and add distilled water to 1 L. Store at room temperature. Prepare fresh 1× solution before gel electrophoresis.
7. Laemmli lysis buffer [6], 4× concentrate: 62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol. Store at −20 °C (*see* **Note 6**).
8. Protein ladder.

3 Methods

3.1 Preparation of Cell Lysate

1. Seed eukaryotic cells at 5.10^5 in 10 cm cell culture dish (*see* **Note 7**) and incubate overnight at 37 °C in CO_2.
2. Transfect cells with plasmid containing gene of interest fused to a specific tag with appropriate transfection reagent for time necessary for optimal expression of protein (16–24 h is usually a good range).
3. Cool cells by placing plates on ice, wash cells with 1× PBS. Add 2 mL cold PBS and harvest cells using cell scraper.
4. Centrifuge 5 min at 80 × *g* at 4 °C.
5. Resuspend cells with 200 µL RIPA buffer supplemented with antiprotease cocktail.
6. Incubate on ice 20 min and mix gently every 5 min with a P200 micropipette.
7. Stock prepared cells at −80 °C (*see* **Note 8**).
8. Right before pull-down experiment, thaw prepared cell extract. Centrifuge at 17,000 × *g* at 4 °C for 20 min. Use the supernatant as prey by following **step 9** in Subheading 3.2 (*see* **Note 9**).

3.2 Pull-Down Assay Using Cell Lysate as Prey (See Notes 10 and 11)

1. Transfer 120 µL Ni-NTA agarose beads to gravity flow column (*see* **Note 12**).
2. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Discard flow-through.

3. Add 400 µL distilled water to column (*see* **Note 13**).
4. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Discard flow-through.
5. Mix carefully 50 µg His-tagged protein (bait) with 400 µL equilibrium buffer and load onto column (*see* **Notes 14** and **15**).
6. Incubate 1 h (*see* **Note 16**) with agitation at 4 °C (*see* **Note 17**) and 10 min on ice without agitation (*see* **Note 18**).
7. Centrifuge column for 1 min at 1000 × *g* at 4 °C and keep flow-through.
8. Load flow-through to column, and centrifuge column for 1 min at 1000 × *g* at 4 °C (*see* **Note 19**). Keep flow-through at 4 °C for analysis.
9. Mix 200 µL cell extract (*see* **Note 20**) with 200 µL equilibrium buffer and load onto column (*see* **Note 21**).
10. Incubate 1 h at 4 °C under agitation (*see* **Note 22**) then 10 min on ice without agitation (*see* **Note 18**).
11. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Keep flow-through for analysis.
12. Wash column by adding to column 400 µL equilibrium buffer.
13. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Discard flow-through.
14. Wash column by adding to column 400 µL equilibrium buffer containing 50 mM imidazole. Keep the first washing for analysis.
15. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Discard flow-through.
16. Repeat **steps 14** and **15** three times and go to **step 17**. Keep last washing fraction at 4 °C for analysis.
17. Elute by loading 80 µL elution buffer to column and incubate 10 min at 4 °C (*see* **Note 18**).
18. Centrifuge column for 1 min at 1000 × *g* at 4 °C and keep eluted fraction.
19. Repeat **steps 17** and **18** with eluted fraction (*see* **Note 22**). Keep eluted fraction at 4 °C for analysis.

3.3 Pull-Down Assay Using Purified Protein as Prey (See Note 11)

1. Incubate 50 µg His-tagged bait protein with 50 µg purified prey protein in total volume of 400 µL equilibrium buffer (*see* **Note 23**) 2 h 30 min at 4 °C under agitation (*see* **Notes 17** and **24**).
2. Add 80 µL Ni-NTA agarose beads to gravity flow column and follow **steps 1–4** of Subheading 3.2.

3. Equilibrate column by adding 400 µL equilibrium buffer supplemented with 20 mM imidazole.
4. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Discard flow-through.
5. Load 400 µL incubated bait and prey proteins onto column. Incubate 10 min on ice without agitation (*see* **Note 18**).
6. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Keep flow-through at 4 °C for analysis.
7. Wash by adding to column 400 µL equilibrium buffer supplemented with 20 mM imidazole.
8. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Save first washing at 4 °C for analysis.
9. Repeat washing **steps 7** and **8** four times and keep last washing fraction at 4 °C for analysis.
10. Add 200 µL elution buffer to column and incubate on ice 10 min.
11. Centrifuge column for 1 min at 1000 × *g* at 4 °C. Keep eluted fraction at 4 °C for analysis.

3.4 SDS-PAGE and Analysis of Protein Fractions

1. To 15 µL protein fraction add 5 µL Laemmli lysis buffer, 4× concentrate. Heat for 3 min at 100 °C and centrifuge 30 s using a microcentrifuge to bring down condensate.
2. Load 10 µL protein fraction and 5 µL protein ladder on SDS-polyacrylamide gel.
3. Electrophorese proteins in running buffer at 100 V for 15 min then 180 V until dye front has reached bottom of gel.
4. Identify interacting proteins by immunodetection or blue coomassie coloration (*see* **Note 25**).

4 Notes

1. We prefer not to use the solutions after 6 months of storage.
2. A different buffer, such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MES (2-(*N*-morpholino) ethanesulfonic acid), or phosphate buffers, may be required for your specific protein–protein interaction. Additionally, different pH values may be tested as these are specific and dependent on the interaction between proteins.
3. We found that pull-down experiments work better with fresh equilibrium and elution buffers.
4. The bait proteins used in this protocol are tagged with 6× His that bind the nickel agarose affinity support. The choice of the matrix-associated antibody depends on the fusion tag. The His

tag is composed of a peptide motif that consists of six histidine residues with a high affinity towards metals like nickel that composes the used Ni-NTA agarose but also the Ni-NDA, Ni-TED, or Ni-TALON resins. The 6× His tag is very small (~1 kDa), which renders it less immunogenic than other larger tags, is shown not to affect the native conformation of bait proteins, and maintains its partner binding activity. Few naturally occurring proteins also bind to Ni-NTA matrices, making this tag the most commonly used affinity tag. In pull-down assays, the choice of the matrix-associated antibody depends on the fusion tag. What follow are some examples of tags with their advantages and disadvantages. The FLAG tag is an octapeptide that is likely located on the surface of the fusion protein due to the hydrophilic nature of amino acid residues and has affinity to anti-FLAG resin. Like the His tag, the FLAG tag is small, but a disadvantage is that the monoclonal antibody matrix is not as stable as Ni-NTA. Glutathione S-transferase (GST) tag binds to glutathione-associated support with high affinity and specificity. This tag has the advantage that GST isoforms are not normally found in bacteria, so purified bacterial prey proteins normally do not have affinity with glutathione resin. However, GST tag is large (26 kDa), exists as a dimer, is prone to nonspecific interaction, is expensive, and affinity to its support depends on certain reagents. The maltose-binding protein (MBP) tag from an *Escherichia coli* periplasmic protein has affinity for matrix consisting of sugars or anti-MBP. This tag is used for the purposes of overcoming problems associated with the expression and purification of recombinant proteins [7]. However, the disadvantage of the MBP tag is its large size, its immunogenicity, and the mild elution of MBP-tagged proteins, which complicate pull-down experiments.

5. Make an aliquot of 1 mL before −20 °C storage. This will prevent the degradation caused by repeated thawing.
6. Make an aliquot of 500 μL before −20 °C storage. The used Laemmli lysis buffer can be kept at 4 °C for 1 month.
7. As negative control, prepare a cell lysate without expressing bait protein (negative cell lysate). This will eliminate false positives resulting from nonspecific interactions of cell lysate proteins with the Ni-NTA agarose beads. Additional negative controls can include an irrelevant protein with the same tag or expression of the tag alone, as in the case of the GFP.
8. Before stocking the cells, remove an aliquot and control by western blot the production of the prey protein.
9. Whole-cell lysate instead of the supernatant fraction can also be used to test whether the prey protein of interest localizes in the pellet fraction.

10. Pull-down experiments using cell lysates will not demonstrate that interaction between the bait and prey proteins is direct but only determine that they are part of the same complex. To prove a direct interaction, the prey protein must be purified and used in pull-down experiments as described in Subheading 3.3.
11. Try to work mostly on ice or at 4 °C to prevent the degradation or the denaturation of the proteins.
12. Break the end cap of the gravity flow column and place it on a 1.5 mL Eppendorf tube. Thoroughly resuspend the Ni-NTA resin by inverting the bottle several times to obtain a uniform suspension. Pipette tips must be cut to allow the Ni-NTA agarose beads to get into.
13. This step eliminates the left 30% ethanol present in the Ni-NTA resin.
14. Before loading the bait protein, plug the gravity flow column using a piece of parafilm before replacing it on a 2 mL Eppendorf tube.
15. Prepare a supplementary column by mixing 50 μg of a known noninteracting bait fused to 6× His tag with 400 μL equilibrium buffer to an empty column. Additionally, prepare a column by adding 400 μL equilibrium buffer to an empty column. These negative bait columns will be used in combination with cell lysates to eliminate false positives resulting from nonspecific interactions.
16. The incubation time can be increased from several hours to overnight at 4 °C under agitation depending on the strength of the interaction between bait and prey proteins.
17. Rotate on roller or rotating platform.
18. The column should stand straight on the ice. This step allows the resin to flow by gravity before centrifugation.
19. We found that loading two times the flow-through increases the capacity of the binding.
20. The volume is dependent on the protein concentration of the cell extract. As a guide, 125–150 μg of protein of a cell extract is usually incubated per microgram of bait protein. Alternatively, cell extract samples can be normalized by visualization of transfected proteins to ensure equivalent expression of the prey and the relevant controls (*see* **Note 7**).
21. Several controls should be added at this step. Load 400 μL equilibrium buffer without prey protein to analyze the efficiency of the immobilization of the bait protein. As negative controls, load onto the negative column (*see* **Note 12**) 200 μL cell lysate containing the prey protein or the negative cell lysate (*see* **Note 7**) mixed with 200 μL equilibrium buffer. Additionally,

load 200 μL negative cell lysate mixed with 200 μL equilibrium buffer onto the column associated with the bait protein.

22. We found that loading two times the eluted fraction increased its quantity.
23. As negative control, incubate 50 μg bait protein (minus prey) or prey protein alone (minus bait) in 400 μL equilibrium buffer. The minus prey control will ensure that the Ni-NTA agarose resin will correctly capture the His-tagged bait protein alone. The minus bait control will eliminate false positives resulting from an interaction between affinity support and prey protein.
24. A different incubation temperature and time may be required for your specific protein–protein interaction.
25. A prey protein that interacts with the bait protein will be found in the eluted fraction. In contrast, a noninteracting protein will not be retained by the bait protein, will pass through the column, and will be found in the flow-through protein fraction.

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