Chapter 11

Antibody Pull-Down Experiments in Fission Yeast

Qianhua Dong and Fei Li

Abstract

Proteins act as executors for almost all kinds of cellular processes. The majority of proteins achieve their proper functions through interacting with other proteins. Knowing the binding partners of a protein is instrumental for understanding its function. The antibody pull-down method is a powerful and common approach to detect protein-protein interactions. Here, an antibody pull-down protocol is described for detecting protein-protein interactions in fission yeast *Schizosaccharomyces pombe*.

Key words Pull-down, Antibody, Co-immunoprecipitation, In vivo, Protein-protein interaction, Fission yeast, *Schizosaccharomyces pombe*

1 Introduction

Proteins are the fundamental workhorses for cellular activities. Proteins are involved in plethora of biological processes within the cell, such as DNA replication, gene expression, epigenetic regulation, development, and signal transduction. However, proteins rarely act alone. They usually associate with other proteins to form intricate network to mediate cellular functions. Characterization of protein-protein interactions is thus a critical step toward deciphering complicated molecular functions in living organisms.

The protein-protein interactions can be classified as either stable or transient interactions. These interactions are mediated by a combination of a variety of bonds, such as charge-charge interaction, hydrophobic bonding, van der Waal's forces, and salt bridges of each protein. While many methods have been developed for studying the interaction between protein and protein, antibody pull-down is one of the most commonly used methods to analyze protein-protein interactions. This method is based on the binding affinity between antigen and antibody and the subsequent isolation of the capture/target complex by precipitation.

Co-immunoprecipitation (Co-IP) is a popular antibody pull-down method for protein interaction discovery in vivo.

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The protein of interest, termed "bait," is precipitated from lysate by the antibody that is immobilized to the support, such as glass beads. The binding partners, or "preys," are co-precipitated with the bait, and can be detected by western blot analysis. A specific antibody against the protein of interest itself would be ideal for the Co-IP experiments, but tagged versions of the protein of interest also work well and are routinely used. One advantage of the Co-IP experiments is that protein interactions detected by the approach are in near native physiological conditions. However, it does not necessarily mean that these interactions are direct; there might be other molecules (protein, DNA, or RNA) that link the interactions.

Here, we described a Co-IP protocol based on immunoaffinity purification in fission yeast *Schizosaccharomyces pombe*. *S. pombe* is easy to grow at low cost. Importantly, the ease to genetic manipulation and a large mutant collection available allow its use for efficiently dissecting the requirements for specific protein interactions. In addition, generation of a tagged version of the protein of interest expressing at the endogenous level is straightforward in *S. pombe*. Tags, such as GFP, HA, Myc, S, and TAP, have been successfully used in *S. pombe* in our lab to study different aspects of cellular biology, such as heterochromatin silencing [1–3] or centromere regulation [4, 5]. The protocol can be divided into five steps (Fig. 1).

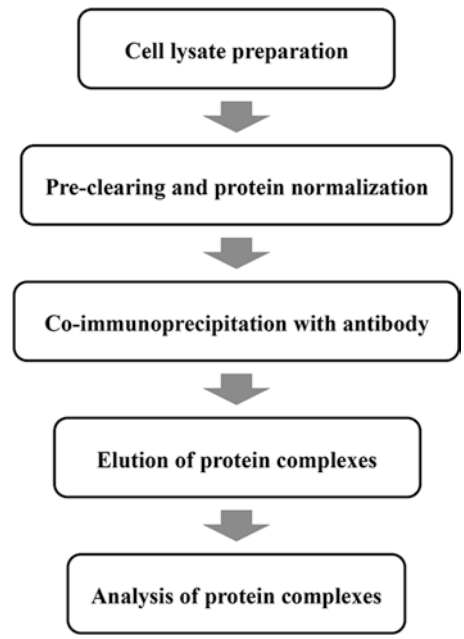

Fig. 1 Flowchart of co-immunoprecipitation protocol in fission yeast

2 Materials

2.1 Equipment

1. Micropipettes.
2. Microscopy.
3. Hemocytometer.
4. Micro Centrifuge (refrigerated).
5. Benchtop centrifuge (refrigerated).
6. Benchtop Disruptor (Scientific Industries Disruptor Genie).
7. Spectrophotometer.
8. End-over-end rotator.
9. Heating block.
10. Acid-washed Glass beads (Sigma, 425–600 μm).
11. 27-gauge needles.
12. 50 mL Tubes.
13. 1.5 mL Tubes.
14. 1.5 mL Protein low-retention microcentrifuge tubes.
15. 1 mL Disposable cuvettes.

2.2 Reagents

Prepare all the solutions using ultrapure water (Milli-Q, 18.2 MΩ·cm resistivity at 25 °C) and analytical grade reagents. All the reagents are stored at room temperature unless specified otherwise.

1. 1 M Tris–HCl pH 7.5.
2. 1 M Tris–HCl pH 6.8.
3. 0.5 M Ethylenediaminetetraacetic acid (EDTA) pH 8.0.
4. Nonidet P-40 (NP40).
5. Bradford reagent, store at 4 °C.
6. 1 M Dithiothreitol (DTT), store at −20 °C.
7. 100 mM Phenylmethanesulfonyl fluoride (PMSF), store at −20 °C.
8. 100× Protease inhibitor cocktail (Sigma), store at −20 °C.
9. Protein A agarose (KPL), store at 4 °C.
10. β-Mercaptoethanol, store at 4 °C.
11. Sodium dodecyl sulfate (SDS).
12. Bromophenol blue (BPB).
13. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, adjust pH to 7.4.
14. Lysis buffer: 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% Glycerol. Immediately prior to use,

the buffer needs to be supplemented with 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor cocktail (*see* **Note 1**).

15. Wash buffer: 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% Glycerol. Prior to use, buffer needs to be supplemented with 1 mM DTT and 1 mM PMSF (*see* **Note 2**).
16. 5× Laemmli buffer [6]: 250 mM Tris–HCl pH 6.8, 10% (w/v) SDS, 50% (w/v) glycerol, 0.05% BPB, and 5% β-mercaptoethanol (*see* **Note 3**).

3 Methods

Keep all the buffers and reagents on ice in advance.

3.1 Cell Lysate Preparation

1. Grow 100 mL yeast cells to early log phase (~1 × 10^7/mL) (*see* **Note 4**).
2. Collect cells by centrifugation at 4 °C, 3000 × *g* for 2 min using 50 mL tubes (*see* **Note 5**).
3. Wash cell pellet once with 50 mL ice-cold 1× PBS. Resuspend the cells in 1 mL ice-cold lysis buffer without protease inhibitors and transfer yeast cells to 1.5 mL tube.
4. Centrifuge at 5000 × *g* for 30 s; discard the supernatant (*see* **Note 6**) and measure the cell wet weight.
5. Resuspend the cells pellet with 100 μL lysis buffer (supplemented with 1× protease inhibitor cocktail) and add 0.9 g chilled glass beads to each tube (*see* **Note 7**).
6. Break the cells with a disruptor for 3 min at 4 °C. Place all the tubes immediately on ice for 3 min. Repeat three times (*see* **Note 8**).
7. Invert the tube and puncture the bottom of each tube with a 27-gauge needle. Place each punctured tube into a new 1.5 mL tube.
8. Spin with benchtop centrifuge for 1 min at 500 × *g* (*see* **Note 9**). Add 900 μL lysis buffer (supplemented with 1× protease inhibitor cocktail) to each tube (*see* **Note 10**). Resuspend the pellet and incubate on ice for 10 min.

3.2 Pre-Clearing and Protein Normalization

1. Centrifuge at 20,000 × *g* for 10 min at 4 °C to remove cell debris.
2. Collect the supernatant and transfer into a 1.5 mL protein low-retention microcentrifuge tube.
3. Prepare the Bradford reagent according to the manufacturer.
4. Provide two cuvettes containing the Bradford reagent for each sample, and add 1 and 2 μL whole cell extract to each cuvette,

respectively (*see* **Note 11**). Prepare blanks by adding equivalent amounts of lysis buffer.

5. Measure the OD 595 for each cuvette.
6. Calculate the average OD 595 reading for each sample and adjust all the samples to the same concentration by adding lysis buffer.

3.3 Co-Immunoprecipitation with Antibody

1. Transfer 900 µL cell extract into a 1.5 mL protein low-retention microcentrifuge tube and add appropriate amount of antibody (*see* **Note 12**) as recommended by the supplier. Save the remaining cell extract for **step 3**.
2. Rotate sample tubes for 1–2 h at 4 °C (*see* **Note 13**).
3. Transfer 50 µL normalized remaining cell extract from **step 1** to a new 1.5 mL tube and add 50 µL freshly prepared 2× Laemmli buffer. Mix and heat the samples for 5 min at 95 °C. Incubate on ice for 2 min. This is the "Input."
4. For each pull-down, take 30 µL protein A agarose slurry (equivalent to 20 µL of packed beads) (*see* **Note 14**) and add into 1.5 mL protein low-retention microcentrifuge tubes that contain 1 mL lysis buffer (without protease inhibitors). Wash beads three times with lysis buffer. Remove the supernatant after each wash.
5. Spin the tubes carrying cell lysates briefly to collect any liquid in the cap, and transfer all the lysate into the tubes containing washed beads.
6. Incubate all the samples on a rotation wheel for 1–2 h at 4 °C.

3.4 Elution of Protein Complexes

1. After incubation, spin the samples in a microcentrifuge at 500 × *g*, 4 °C, for 1 min. Carefully remove the supernatant as much as possible (*see* **Note 15**). Add 1 mL ice-cold wash buffer and resuspend the beads by gently inverting each tube several times. Incubate all the samples with rotation for 5 min at 4 °C.
2. Repeat wash three times (*see* **Note 16**).
3. After the last wash, discard the supernatant. Spin the tubes briefly and use pipettor to remove all the excess wash buffer. Make sure not to remove any beads.
4. Add 40 µL freshly prepared 1× Laemmli buffer and mix gently. Heat for 5 min at 95 °C. Incubate on ice for 2 min. These are the "IP" samples.

3.5 Analysis of Protein Complexes

1. Centrifuge the "Input" and "IP" samples at 12,000 × *g* for 1 min.
2. Load the samples on a SDS-PAGE gel, and analyze them by Western blotting.

4 Notes

1. For weak interaction, salt and NP-40 concentration can be reduced to 60 mM and 0.05% respectively. Cross-linker might be used for weak and transient interaction, such as formaldehyde [7], and dithiobis-succinimidyl propionate (DSP) [8, 9]. Some of the protein-protein interactions can be indirectly linked by DNA or RNA. In that case, DNase or RNase should be added into the lysis buffer during cell lysis and binding steps. The DNase or RNase untreated samples can serve as a control.
2. More stringent washes can be performed by increasing the salt concentration of the wash buffer up to 500 mM.
3. 5× Laemmli buffer can be diluted with water to make 2× and 1× Laemmli buffers. Add β-mercaptoethanol right before use.
4. Count the cell number of each sample by hemocytometer. Collect similar amount of cells for the strains of interest and corresponding controls.
5. Collect cells into the same tubes through two sequential centrifugations.
6. Cell pellet can be frozen in liquid nitrogen and stored at −80 °C before lysis.
7. Appropriate amount of glass beads should be added; otherwise, the lysis efficiency may be affected. ~3 g of chilled glass beads per gram of cell wet weight works well for most occasions.
8. Check lysis efficiency under microscopy and make sure over 95% of the cells are broken. Break the cells more times using the same conditions if necessary.
9. Make sure that the punctured tube tightly adheres to the new 1.5 mL tube, and centrifuge at low speed without rotor lid.
10. Total lysis buffer added to each sample can be reduced to 600 μL. This makes low abundant protein much easier to detect by western blot. But be cautious that less than 600 μL lysis buffer might reduce protein solubility.
11. Dilute the whole cell extract ten times if OD 595 value is more than 1.0.
12. Antibody should be stored in small aliquots to prevent contamination and repeated freezing and thawing. Some antibodies are bead-conjugated. For those antibodies, wash the beads three times with lysis buffer, then add 900 μL cell extract, goes directly to **step 6**.
13. Some of the primary monoclonal antibodies have low affinity with beads conjugated protein A, such as mouse IgG1.

Secondary antibody can be used during immunoprecipitation to improve the binding efficiency.

14. Cut pipette tip when handling the beads.
15. Discard the supernatant carefully and make sure not to remove any beads.
16. Washing more times can reduce the background, but may also reduce the yield of the pull-down proteins.

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