Immunoprecipitation of Proteins in *Caenorhabditis elegans*

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[Abstract] Immunoprecipitation (IP) is a biochemical technique to precipitate a protein out of solution using an antigen that can specifically bind to that protein. IP can be performed to isolate and concentrate one particular protein from a sample of thousands of different proteins. IP is also readily performed to pull down interacting proteins of complexes out of solution. This protocol outlines the methods used to IP proteins in whole worm lysates and their preparation for detection on Western blots using denaturing conditions.

Materials and Reagents

1. Transgenic *Caenohabditis elegans* (*C. elegans*) strain with over-expressed protein of interest
   Note: Depending on protein expression and specificity of antibody, this protocol can also be used to immunoprecipitate endogenous proteins.
2. OP50 *Escherichia coli* (*E. coli*) (*C. elegans* Genetic Center)
3. Trizma hydrochloride (Sigma-Aldrich, catalog number: T5941)
4. Trizma base (Sigma-Aldrich, catalog number: T1503)
5. Tris base (BioShop, catalog number: TRS001)
6. Bacto-tryptone (BD, catalog number: 211705)
7. NaCl (BioShop, catalog number: SOD002)
8. Cholesterol (95%) (Sigma-Aldrich, catalog number: C8503)
9. Agar (Sigma-Aldrich, catalog number: A1296)
10. Na_2HPO_4 (Sigma-Aldrich, catalog number: S0876)
11. KH_2PO_4 (Sigma-Aldrich, catalog number: P0662)
12. $MgSO_4 \cdot 7H_2O$ (BioShop, catalog number: MAG521)
13. EDTA (BioBasic, catalog number: EB0185)
14. NP-40 (BioShop, catalog number: NON.505)
15. PMSF (Roche, catalog number: 10837091001)
16. Na_3VO_4 (Sigma-Aldrich, catalog number: S6508)

17. Pepstatin-A (Sigma-Aldrich, catalog number: P4265)
18. NaF (Sigma-Aldrich, catalog number: S7920)
19. cOmplete protease inhibitor cocktail tablets (Roche, catalog number: 4693159001)
20. SDS (Caledon, catalog number: 7771)
21. Glycerol (BioShop, catalog number: GLY001)
22. β-mercaptoethanol (Sigma-Aldrich, catalog number: M7154)
23. Bromophenol blue (Sigma-Aldrich, catalog number: B5525)
24. BCA protein assay kit (Thermo Fisher Scientific, catalog number: 23225)
25. Siliconized low-binding tips 200-1,000 µl (Denville Scientific Inc., catalog number: P3193-S)
26. Siliconized low-binding tips 200 µl (Denville Scientific Inc., catalog number: P3010-S)
27. Protein A/G PLUS-Agarose non-conjugated beads (Santa Cruz Biotechnology, catalog number: SC-2003)
28. EZview Red ANTI-FLAG M2 Affinity Gel conjugated beads (Sigma-Aldrich, catalog number: F2426)
29. GFP polyclonal antibody, rabbit (Genscript, catalog number: A01388)
30. GFP monoclonal antibody (B-2) (mouse) (Santa Cruz Biotechnology, catalog number: SC-9996)
31. Donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology, catalog number: SC-2313)
32. Goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, catalog number: SC-2005)
33. MYOB dry mix (see Recipes)
34. MYOB plates (see Recipes)
35. M9 buffer (see Recipes)
36. Lysis buffer (see Recipes)
37. SDS-PAGE sample buffer (see Recipes)

Equipment

1. Refrigerated microcentrifuge (Eppendorf, model: 5417R)
2. Large centrifuge (Eppendorf, model: 5810R)
3. Rotisserie agitator (Barnstead Thermolyne LabQuake, model: C415110)
4. Sonicator (Branson Sonifier, model: 450)

Procedure

A. Collection of worms

1. Propagate desired strain of worms using the OP50 *E. coli* seeded on MYOB plates. 15 fully confluent (not starved) 10 cm plates of worms gives enough total protein for two IPs (10-25 mg).
2. Collect all worms off of plates with 15 ml of M9 buffer into a 15 ml conical tube.

3. Spin down worms at 2,000 rpm for 1 min and replace supernatant with fresh M9 buffer.
   Note: Repeat this step until worms are relatively clean of bacteria (clear supernatant).
4. Resuspend worms by gentle inversion in 15 ml PBS.
5. Spin down worms and remove as much supernatant as possible.
6. Transfer up to 500 µl of packed worms using siliconized tips into a microcentrifuge tube.
   Note: Siliconized tips are recommended to prevent adherence of worms.
7. Add up to 1 ml of ice cold lysis buffer (2x worm volume) and incubate on ice for 30 min.
   Note: Worms can be flash frozen using liquid nitrogen and stored at -80 °C up to one week. For best results, continue with lysis immediately.

B. Lysis of worms

1. If performing lysis on same day, flash freeze worms in liquid nitrogen for 10 sec. If performing lysis on previously stored worm samples, continue to step B2.
2. Thaw frozen worms in room temperature water until a third of the frozen sample has melted.
3. Insert tip of sonicator halfway into sample and sonicate for 5-7 sec using 8 W power. Immediately flash freeze samples for 10 sec in liquid nitrogen.
   Note: During sonication, do not allow samples to completely thaw during this process. It is important to keep the sample cold at all times to avoid protein degradation. Incubating samples on ice during sonication is optimal. One alternative that has been previously reported (Ding et al., 2005) is to use a pestle and mortar to pulverize the worm pellet in the presence of liquid nitrogen to disrupt the cuticle, which may help increase protein yield and preserve the integrity of proteins.
4. Repeat steps B2-3 two times. After final flash freeze, let samples thaw in room temperature water until half melted.
5. Rotate samples at 4 °C by end-over-end agitation for 30 min.
6. Spin down samples at 13,000 rpm for 30 min at 4 °C.
7. Transfer supernatant (solubilized proteins) into a new microcentrifuge tube and measure protein concentration using a standard kit (*e.g.* BCA protein assay kit).

C. Immunoprecipitation

1. At least 4 mg of total protein was used for each IP. 8-10 mg of protein is recommended. Adjust the volume of protein sample to 1 ml using ice cold lysis buffer.
   Note: It is not recommended to store samples for freezing at this step.
2. Equilibrate agarose beads by washing in lysis buffer three times using 2x volume of bead slurry.
   a. If using antibody-conjugated beads, add 20-30 µl of equilibrated beads to protein sample and incubate at 4 °C with agitation for 2-4 h.
      Note: Incubation of beads beyond 4 h did not change immunoprecipitation efficacy in our hands.

b. If using non-conjugated beads, first add antibody to sample and incubate at 4 °C with agitation for 1 h. Then add 20-30 µl of equilibrated beads and further incubate for another 1-3 h.

Note: We have had experience using both antibody-conjugated agarose beads and non-conjugated agarose beads. We did not detect any notable differences between IP efficiency.

4. After incubation, wash beads 5 times using 1 ml ice cold lysis buffer, allowing samples to incubate with agitation at 4 °C for 2-5 min between washes.
5. Elute proteins with 50-100 µl of SDS-PAGE sample buffer and boiling for 5 min, analyze by Western blot.

Notes:

a. When performing Western blot, another 5-10 minutes of boiling is recommended to elute proteins from beads.

b. For several examples of Western blots whose samples were prepared using this protocol, please refer to Chan et al. (2014). The specificity of the immunoprecipitation will depend upon the degree of specificity of the antibody being used. For best results, pre-clear the lysates with an irrelevant antibody to improve signal-to-noise ratio. Regardless, several different types of controls can be performed to evaluate the specificity of the immunoprecipitation, including performing the immunoprecipitation with a sample that does not contain the epitope of interest (Chan et al., 2014), or including a mock immunoprecipitation control, whereby antibody is not added to sample before the immunoprecipitation.

Recipes

Note: Recipes for MYOB and M9 were taken from Burns et al. (2006).

1. MYOB dry mix (for 370 g)
   27.5 g trizma HCl
   12 g trizma base
   230 g Bacto-tryptone
   100 g NaCl
   0.4 g cholesterol (95%)
2. MYOB plates (for 1 L)
   7.4 g MYOB dry mix
   24 g agar
   Add up to 1 L ddH_2O
   Autoclave and pour 35 ml per 10 cm plate
3. M9 buffer (for 1 L)
   6 g Na_2HPO_4

3 g KH_2PO_4
5 g NaCl
Add up to 1 L ddH_2O
After cooling to room temperature, add 2 ml of 1 M $MgSO_4 \cdot 7H_2O$

4. Lysis buffer
   25 mM Tris-HCl (pH 7.5)
   100 mM NaCl
   1 mM EDTA
   0.5% NP-40
   1 mM PMSF
   1 mM Na_3VO_4
   1 µg/ml Pepstatin-A
   10 mM NaF
   1 tablet/50 ml buffer cOmplete protease inhibitor cocktail tablets
   ddH_2O to desired final volume
5. SDS-PAGE sample buffer
   100 mM Tris-HCl (pH 6.8)
   2% SDS
   20% glycerol
   4% β-mercaptoethanol
   0.02% bromophenol blue

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