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SOP25v2_TGD_Immunoprecipitation

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We use this protocol and it's working

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Abstract

This protocol describes how to perform immunoprecipitation in protein lysates with the intention of sending the samples for mass spectrometry.

The main concept is described below:

1) To pull down a specific protein in a lysate, you need an antibody that has been validated specifically for IP. Any other antibody might not be specific enough, resulting in an impure IP sample.

2) The antibody will bind to the protein of interest in the lysate during incubation.

3) Once the antibody-protein complexes have formed, they need to be separated from the rest of the sample. This is where the magnetic beads come in - here, Protein G magnetic beads are used. These are specific to IgG antibody and will bind to the antibody part of the antibody-protein complex.

4) The bead-antibody-protein complex is then washed multiple times and the stored for downstream experiments.

5) The sample needs to be validated by immunoblotting, which just visually confirms the protein of interest is actually in the IP sample and has been pulled down.

Guidelines

Bench work should be completed on the **Protein bench** (unless you are washing cells in tissue culture hood). **Orange labeled protein-only** materials should be used for this protocol to avoid cross-contamination.

Lab attire: gloves, lab coat, and safety glasses/goggles.

Materials

	A	B	C
	Item	Supplier	Catalog
	Protein G Magnetic Beads	Fisher Scientific	50812662
	Pierce IP lysis buffer	Thermo Fisher	87787
	IgG control Polyclonal antibody, human	VWR International	76206-074
	PBS 1X	Thermo Fisher	10010049
	Magnetic Rack		
	1.5 mL tubes		
	Rotator with adapter for 1.5 mL tubes		

For additional reagents for immunoblotting and protein quantification, please see the corresponding protocols.

Troubleshooting

Safety warnings

Waste Disposal: all cell pellets should be disposed of in biohazardous. Anything else can be disposed of in the trash.

Before start

Get ice.

Overview

1 The goal of immunoprecipitation is to use an IP validated antibody for your protein of interest to pulldown that protein from a lysate solution and subsequently any of its binding partners. The pulldown will be sent for mass spectrometry to confirm its identity and discover its binding partners. This can inform further research.

Preparation of Lysate

2 This protocol has only been validated with adherent cells.

The first step is to calculate how many cells you will need to lyse to get the protein concentration you need for Mass spectrometry. It is recommended that every immunoprecipitation (IP) has a starting input of 1 mg of protein. Therefore, when you lyse the cells, you will need to ensure you have at least 2 mg of total protein for 1 immunoprecipitation run - one for your protein, and one for your negative IgG control.

Based on previous experimentation, it has been observed that 10×10^6 cells (10 million) gives about 0.4 mg of protein. This is a very rough approximation but can give you an idea of how many cells to lyse to get the protein amount that you need for your experiments.

3 Lyse your sample (tissue, cells, etc) with lysis buffer. We use Pierce IP lysis buffer because it does not interfere with the protein quantification reagents, and therefore allows for accurate concentration determination.

4 Take your snap frozen or fresh cell pellet and wash with an appropriate amount of PBS 1x time. Spin down and remove the PBS.

5 Add the Pierce IP lysis buffer (cold) to your cell pellet, and resuspend well. Keep on ice as much as possible.

See this example for the calculations:
Say I want to perform 4 IPs at the same time - 3 for my 3 proteins of interest, and 1 for my IgG negative control. I will need at least 4 mg of protein.
If 10 million cells = ~0.4 mg protein, I need 100 million cells to get <4 mg protein. Always go higher than what you need to ensure you hit your target.
IP requires bead and antibody end-over-end incubation. In order for this to be effective, there needs to be at least 200 uL of lysate in each sample. So based on this, you can calculate how much IP buffer to add. If I want to perform 4 IPs, I will need at least 800 uL of IP lysis buffer added to my 100 million cells. I can always add more if I want a more efficient lysis, but this ensures I get at least 200 uL per IP.

Also, if you have multiple, considerable cell pellets to lyse, you want to ensure that each one gets a good amount of IP lysis buffer for effective lysing. So best to go above what you actually need for IP.

6 Let sample incubate on shaker (on ice) for 30 minutes.

7 Spin down sample in centrifuge at 12,000 x g, for 10 minutes, at 4C.

8 Transfer supernatant (which is your lysate) to a new tube. You can discard the remaining cell pellet in red biohazardous bin.

Protein Lysate Quantification

9 Now that you have your lysate, it has to be accurately quantified. Use the BCA kit to do this. Protocol linked here: https://protocols.io/view/sop22v1-tgd-bca-b8mgru3w

Tips: Be sure to include some dilutions (1/2, 1/10, etc) because we expect the final concentration of the neat sample to be above the standard curve.

Once you have quantified the lysate, it is best to go immediately into IP to ensure no protein degradation occurs from storage.

Immunoprecipitation Day 1

10 Once the lysate has been quantified, we can start the IP. The first step is to preclear the lysate with IgG antibody. This step is to basically remove any proteins or unwanted particles in the lysate that would ordinarily bind to your antibody during IP BEFORE you even start the incubation with your antibody for your protein of interest.

Since your antibody will be an IgG antibody, the IgG control antibody mimics the antibody for your protein of interest but just doesn't have a specific binding site for any protein. Therefore, it can potentially trap any proteins/particles that would unspecifically bind to your actual antibody during IP.

11 Calculate how many beads you need per sample to preclear the lysate.

For protein G magnetic beads: 1 mL of beads binds to 280 ug of human IgG

We want a ratio of 1:1 of beads to IgG control antibody, and we need 1 ug of IgG antibody to preclear lysate.

1 mL beads/280 ug IgG ⟶ 3.57 uL beads/1 ug IgG

12 Measure out 3.57 uL of beads into tubes for each IP that you have to do. Add 300 uL of Pierce IP lysis buffer to each aliquot of beads, resuspend well, and place tubes on a magnetic rack. Once beads have fully pulled away, remove the lysis buffer. Repeat two more times.

13 Take 60 ug worth of protein from your lysate and set aside. This is your **INPUT** sample and represents the whole protein catalogue in your lysate, as well as a positive control in your validation steps later on.

Continuing the example from above, if my lysate concentration from my 100 million cells is 2 mg/mL, and my total lysate is 3 mL. To get 60 ug, I need 30 uL of lysate into my **INPUT** tube.

14 Now, you have to add the correct amount of lysate to each aliquot of beads. We want 1 mg of protein per IP. Based on the BCA concentration, calculate how much volume you need to add to each sample to get 1 mg final.

Example: My lysate concentration is 2 mg/mL, and my total lysate is 2.97 mL (I removed 30 uL for **INPUT**). I have 5.94 mg of protein total. I can do a max of 5 IPs with this lysate and each IP will need 500 uL of lysate.

15 Resuspend the beads in the lysate well for each tube, and add 1 ug of IgG antibody to each tube, Rotate end over end in the cold room for 30 minutes.

16 While the lysate is being pre-cleared, calculate how much antibody you will need for the actual IP.
When performing an IP for the first time with a particular antibody, start with the manufacturer's recommendation on working concentrations for 1 mg of lysate. If the recommendation is unknown, you can start with 5 ug per 1 mg of lysate. For the IgG negative control, you can go with 5 ug.

17 After 30 min, place samples containing lysate, IgG and beads on a magnetic rack. The beads should have pulled down the IgG antibody and anything that nonspecifically bound. Move the lysate to a new tube and save the beads as "**PRECLEAR BEADS**" just in case.

18 To each lysate, add the appropriate amount of antibody for your protein of interest based on the company or literature recommendations. If you have 4 reactions, 3 of them would be for each protein of interest and the fourth will be for your IgG negative control. This is just to ensure that nothing unwanted is sticking to your antibody, even after preclearing the lysate.

19 Incubate the samples end over end overnight in the Cold Room at 4 C.

Immunoprecipitation Day 2

20 The remaining steps should occur at 4 C, in the cold room.

21 First, measure out the beads you need to pull down your antibody-protein complex that has been incubating overnight. You need to wash them first with PBS before using them.

Based on previous experimentation, it is best to go with a 5:1 beads to antibody ratio. That means, say we used 5 ug of antibody in our IP, we want beads for 5x more antibody than we have, so we want beads for 25 ug IgG. This just allows for greater capture, without going too high and picking up unwanted nonspecific proteins/particles.

For example:
3.57 uL beads = 1 ug IgG
17.85 uL beads = 5 ug IgG
But we want beads for 5x more IgG than we have.
5 ug IgG x 5 = 25 ug IgG.
25 ug IgG = 89.25 uL beads (17.85 × 5)

22 Measure out the beads in separate tubes, one for each IP reaction that you have. Wash with 200 uL of cold PBS and resuspend well, then place the tubes on a magnetic rack to pull the beads away. You can discard the supernatant. Repeat two more times.

23 Move each lysate+antibody mix (which has been incubating overnight) to a corresponding tube of washed beads. Mix and resuspend well.

24 Incubate the beads+antibody+lysate mix for 1 hr, end over end, in the cold room at 4 C. During this time, the beads will be binding to the antibody-protein complexes

25 After 1 hr, place the samples on the magnetic rack and wait for a few minutes until the supernatant has cleared and all the beads have pulled away. Move the supernatant to a tube called **UNBOUND.** This contains all the protein that did not bind to your antibody or to the beads.

26 Now, we have to wash the beads to remove any impurities. The first wash should be with 200 uL of cold IP lysis buffer. Resuspend well and rotate end over end for 2 minutes. Then briefly spin down, and place on magnetic rack and move the supernatant into a new tube labeled **WASH 1.**

27 The second through fifth wash steps are the same as above, however use 200 uL of cold PBS instead of IP lysis buffer. Collect supernatant along the way as **WASH 2, 3, 4,** and **5.**

28 Once Wash 5 is complete, resuspend the beads with a final 60 uL of PBS. Take 30 uL of this resuspension into a new tube and add 170 uL of PBS. This will be your final **IP, diluted.** The other 30 uL will be your **IP, concentrated.** The diluted IP is for mass spec and should be immediately stored in -80 C, without any freeze-thawing cycles. The concentrated IP is for western blot validation.

At the end, you should have these samples for every IP that you performed:
UNBOUND
WASH 1-5
IP diluted
IP concentrated
Plus 1 separate **INPUT** sample to represent the lysate used for all IPs.

Store all samples at -80 C for long term.

Western Blot Validation

29 Now, we have to use immunoblotting to confirm that our IP actually worked and our antibody was effective in pulling down our proteins of interest. We will load our samples into the gel, transfer to blot, and then incubate that blot in the primary antibody for that protein (which may or may not be the same IP antibody that was used) to detect if its there.

30 For general western blotting protocol, see the following:
https://protocols.io/view/sop24v2-tgd-western-blotting-with-sds-page-gels-b52tq8en

31 For specific tips for immunoprecipitation samples:
- Load at least 20 ug of **INPUT,** based on the BCA concentration
- We assume that the amount of protein in **UNBOUND** is the same as **INPUT,** because very little is being pulled down. So load the same volume for **UNBOUND** as you did for **INPUT,** this will ensure you aim at 20 ug of protein in the **UNBOUND** samples as well
- Load the same volume for the **IP concentrated** sample. You want to make sure the amounts loaded are all relative to each other for good comparison
- Load the same volume for the **WASHES.** You can just load **WASH 1, 3 and 5.**

Loading order:
1) Input
2) IgG IP, concentrated
3) Antibody IP, concentrated
4) IgG Unbound

5) Antibody Unbound
6) IgG Wash 1
7) Antibody Wash 1
8) IgG Wash 3
9) Antibody Wash 3
10) IgG Wash 5
11) Antibody Wash 5

Detection of Bands:

During IP and sample preparation for western blotting, the IP antibodies that you use have a potential to degrade into their heavy and light chain components. This means, when you load your IP samples into the gel, you are loading not only the protein of interest but also the IP antibody in its whole form or degraded components. The heavy chain is 50 kD and the light chain is 25 kD.

When you detect the primary WB antibody with the secondary, the secondary can potentially bind to the primary but also any heavy or light chains of the antibody used in IP. This means you might see extra bands at 50 or 25 kD, which can be a problem if your protein of interest is that size.

To avoid this problem, we can do one of two things:

1) Use TidyBlot HRP detection reagent - this secondary specifically does not bind to heavy or light chains. Use at concentration of 1:400 in 5% milk in PBS-T.

2) Use a different antibody to the one used for IP, with a different host species. For example if the IP antibody was grown in rabbit, the WB antibody should be raised in mouse or another animal. This ensures that the HRP secondary, which will be mouse or another animal (not rabbit) only binds to the WB antibody in the blot.