IP Juni 08, S.L.

Protocol Immunprecipitation

- Before starting an IP on should be certain, that one can clearly detect the Protein to precipitate in a standard Western Blot with the lysis conditions used in the following IP.
- Optimal antibody concentration (and incubation time/temperature) should be determined by titration. Note detergent concentration might disturb antibody-antigen recognition.
- You can check the antibody datasheet for recommended antibody concentration. As a guideline use:
  - 1 – 5 µl polyclonal antiserum
  - 1 µg affinity-purified polyclonal antibody
  - 0.2 to 1 µl ascites fluid (monoclonal antibody)
  - 20 to 100 µl culture supernatant (monoclonal antibody)
- Choose the right sepharose (protein G or A) according to the host species of the primary antibody (see list)
- Depending on the secondary antibody that is used, bands may appear on the blot at 55 and 27 kDa which are the heavy and the light IgG chains of the primary antibody. (These bands are less pronounced if primary antibody agarose conjugates is used)
- As control you have to include normal IgG (corresponding to the host species of the primary antibody)
- Also inform yourself on what kind of protein you are working, are the conditions of its solubility known (especially working with keratins this is an important question), is it phosphorylated and so on.
- Depending on these information you have to pick an appropriate buffer.
- The Standard Buffer for IPs is the RIPA dilution Buffer as listed below, however buffer conditions are dependent on the protein to be immunoprecipitated and thus may vary.
- You should include the following controls: 1. IgG control
  2. without first antibody
  3. with the specific first antibody

1. Grow 1-2 10cm^2 dishes of cells to near confluence (want ~ 1 x 10^7 cells per antibody).
2. From now on work on ice!
3. Rinse twice in PBS (10ml) and once in Aqua dest.
4. Add 1ml (per 10 cm2 dish) IP buffer to each dish and scrape off cells
5. Shear cells 15-30x with 30 gauge needle until homogenous on ice
   Optional: you can also sonicate the cells on ice or cells can be lysed by using the dounce/pestel)
6. Incubate 10 min on ice
7. For control (total lysate) remove 30µl add appropriate volume of 5xLaemmli buffer and boil 10 min 98°C, then freeze down at -20°C
8. Microfuge at 20,000g, 20 min at 4 deg (to remove cell debris)
9. Recover supernatant
   For control (Input) remove 30µl of supernatant, add appropriate volume of 5xLaemmli Buffer and boil 10min at 98°C, then freeze down at –20°C
10. **Optional: Preclearing** of the lysate with immobilized recomb protein G/A agarose (**Pierce #20365)**
    Apply lysate to the protein G/A sepharose and incubate at 4 deg. for 1 hour with rotation.
11. Centrifuge lysate at 2500xg for 3 min at 4 deg
12. Recover supernatant and add specific antibody. Incubate 1 hour to o.n. (depending on the antibody) at 4 deg.
13. Prepare immobilized recomb protein G/A agarose in a new tube
14. **Complexation:** Apply lysate to the protein G/A sepharose and incubate at RT for 2 hour with rotation.
15. ***Alternatively you can first bind the antibody to he protein G/A sepharose and then add your lysate***
16. Collect immunoprecipitates (IP) by centrifugation at 2500 x g for 3 min at 4 deg.

17. Take off 30µl supernatant and add appropriate volume of 5xLaemmli Buffer (final concentration 1x), 5min 95°C then freeze down. Discard the rest of the supernatant.
16. Wash IP 5x in IP-Buffer (more stringent) or PBS (less stringend), with spins of 3 min, 2500xg

    Use detergent-free buffer for the last washing step. Non-ionic detergents disturb denaturation and charge of the proteins by SDS.
18. Elute and neutralize the protein of interest from the immobilized recomb protein G/A agarose according to the Pierce instructions (**Pierce #20365)**

Lysisbuffer:

Nonionic detergents such as NP-40 and Triton X-100 are less harsh than ionic detergents such as SDS and sodium deoxycholate. Instead of NP40 you can use Igepal. For keratins you can also use Empigen.

Other variables that can affect the success of IP include salt concentration, divalent cation concentration, and pH. To optimize, these should be tested within the following ranges:

Salts: 0 -1 M
Detergent, non-ionic: 0.1 – 2%
Detergent, ionic: 0.01 – 0.5%
Divalent cations: 0 – 10 mM
EDTA: 0 – 5 mM
pH: 6 – 9

Protease inhibitors

As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added **fresh** to the lysis buffer. Mixtures (“cocktails”) of protease and phosphatase inhibitors are commercially available. If not using a cocktail, two of the most commonly used protease inhibitors for IP are PMSF (50 ug/ml) and aprotinin (1 ug/ml).

Protease inhibitors:

Complete Protease-Inhibitor-Cocktail (Roche)

Oder:

1,8 µg/ml Aprotinin (Serinproteaseinhibitor)

1 mM PMSF (Serinproteaseinhibitor)

10 µg/ml Leupeptin (Serin-/Cysteinproteinaseinhibitor)

Phosphatase inhibitors:

10 mM NaF

1 mM Na3VO5 (Tyrosinphosphataseinhibitor)

0.5µg/ml Okadarsäure

RIPA (Lysisbuffer/ IP) Buffer: for keratins

50mM Tris HCl, pH 7.4

150mM NaCl

5mM EDTA

5mM EGTA

0.5% Triton-X (1% nach Omary)

Complete Protease-Inhibitor-Cocktail (Roche), 1/ 10ml

1mM PMSF or Pefablock (this is stabil at 4°C)

Optional: if lysate is viscous (due to nucleic acids) you can use RNase in the buffer

(The kind and amount of detergent varies with the proteins you want to precipitate. Alternatives to Triton are Desoxycholat and Empigen.)

To counteract against the oxidative conditions during the procedure one can add 2mM DTT to the buffer.

As Phosphatase Inhibitor 2mM Na Vanadat can be added, if the protein of interest is phosphorylation sensitive.

Protein A/G affinities for antibodies from various species:

Species	Affinity for Protein A	Affinity for Protein G
Chicken	-	+
Cow	++	++++

Goat	-	++
Guinea Pig	++++	++
Hamster	+	++
Horse	++	++++
Human IgG_1	++++	++++
Human IgG_2	++++	++++
Human IgG_3	-	++++
Human IgG_4	++++	++++
Mouse	++	++
Mouse IgG_1	+	++++
Mouse IgG_{2a}	++++	++++
Mouse IgG_{2b}	+++	+++
Mouse IgG_3	++	+++
Pig	+++	+++
Rabbit	++++	+++
Rat	+/-	++
Rat IgG_1	-	+
Rat IgG_{2a}	-	++++
Rat IgG_{2b}	-	++
Rat IgG_{2c}	+	++
Sheep	+/-	++

Affinity for beads also always dependent on the salt concentration