IP: Immunoprecipitation Protocol - Denaturing

In immunoprecipitation (IP) approaches, antibodies are employed to specifically pull down the corresponding target molecule from a complex sample like a tissue or cell lysate. Isolated proteins or complexes can be analyzed further to identify potential interaction partners of the target molecule or to determine the specificity of an antibody.
Some proteins are not efficiently solubilized by Triton X-100. For these proteins, the denaturing solubilization protocol is recommended. For further details have a look at: Geumann C, Grønborg M, Hellwig M, Martens H & Jahn R (2010). A sandwich enzyme-linked immunosorbent assay for the quantification of insoluble membrane and scaffold proteins. Analytical Biochemistry 402: 161-9.

Materials and reagents

- **PBS:** Phosphate buffered saline (pH 7.4)
- **Solubilization buffer A:** Phosphate buffered saline (PBS), pH 7.4, 2% SDS, 5 mM EDTA, protease inhibitors
- **Solubilization buffer B**: PBS, 1.2% Triton X-100, 5 mM EDTA, protease inhibitors
- **Washing buffer**: PBS, 0.2% Triton X-100, 5 mM EDTA, protease inhibitors
- **Blocking buffer:** PBS, 2% BSA

Procedure

All steps should be carried out at 4°C. Protein solubilization (solubilization step 1) and binding of the antibodies to the beads (immunoprecipitation step 1) can be carried out in parallel.
Protease inhibitors should be included.

Denaturing solubilization with SDS

Some proteins or protein complexes are insoluble in Triton X-100 containing buffers and require an SDS based denaturing step.

1. Adjust protein samples to 3 mg/ml total protein and a final SDS concentration of 1.2% with **solubilization buffer A** and rotate 15 min at RT.
2. Add 5 volumes of ice-cold **solubilization buffer B** to each sample and rotate 15 min at 4°C.
3. Pellet the insoluble fraction at 100,000x g for 30 min (acceptable alternative: 13,000 rpm for 30 min at 4°C in a tabletop centrifuge) and transfer the supernatant to a new tube.
   Note: If complete tissue samples are used, DNase should be added as 0.1 µg/µl together with protease inhibitors, and SDS should be added as last component after mixing everything else.

Immunoprecipitation

1. Incubate 5-10 µg of antibody or 5 µl antiserum with 10 µl Protein G or A slurry in 200 µl **PBS** for 1 h to bind.
2. Centrifuge beads for 5 min at 2400x g and discard the supernatant.
3. Block beads with 200 µl of **blocking buffer** for 30 min.
4. Centrifuge beads for 5 min at 2400x g and discard the supernatant.
5. Wash beads with **washing buffer,** centrifuge beads for 5 min at 2400x g, and carefully remove **washing buffer.**
6. Add 100-200 µl of the sample (supernatant from step 3) and incubate for 2 h at 4°C rotating head over tail.
7. Centrifuge beads for 5 min at 2400x g and collect supernatant for subsequent analysis.
8. Wash suspension twice with **washing buffer.** Centrifuge beads for 5 min at 2400x g and remove **washing buffer.**
9. For SDS-PAGE analysis, incubate the pellet with SDS loading buffer and apply to SDS-PAGE. Apply starting material and supernatant from immunoprecipitation step 7 for comparison.

Remarks

- If membrane proteins are immunoprecipitated, make sure that detergent is included in all steps that contain your target protein.
- Not all IgG subtypes from all species bind equally well to protein A or protein G. It is therefore important to choose the right resin (for details see table 1).

Species	Subclass	Protein A binding	Protein G binding
Human	IgA	variable	-
	IgD	-	-
	IgE	-	-
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	-	++++
	IgG_4	++++	++++

Species	Subclass	Protein A binding	Protein G binding
	IgM	variable	-
Avian egg yolk	IgY	-	-
Cow	IgG	++	++++
Dog	IgG	++	+
Goat	IgG	-	++
Guinea pig	IgG	++++	++
Hamster	IgG	+	++
Horse	IgG	++	++++
Lama	IgG	-	+
Monkey (rhesus)	IgG	++++	++++
Mouse	IgG_1	+	++++
	IgG_{2a}	++++	++++
	IgG_{2b}	+++	+++
	IgG_3	++	+++
	IgM	-	-
Pig	IgG	+++	+++
Rabbit	IgG_1	++++	+++
Rat	IgG_1	-	+
	IgG_{2a}	-	++++
	IgG_{2b}	-	++
	IgG_3	+	++
Sheep	IgG	+/-	++

Table 1