Immunoprecipitation protocol

1. Wash culture dishes (150mm) twice with cold PBS.
2. Harvest: in 1.3 ml ice-cold PBS (with freshly added protease inhibitors: Roche cocktail + 1 μm DTT) and collect cells using a scraper. Immediately spin: 2000 rpm, 5', 4c.
3. Resuspend pellet in 0.35 ml of lysis buffer (with freshly added protease inhibitors & DTT). Quickly place on ice.
4. Incubate on ice for 30' and transfer to 1.5ml tubes vortexing often. But do not shake vigorously as this could result in loss of protein due to foaming.
5. Centrifuge at max speed (12000-16000 rpm) for 10',4c. When using 15 ml tubes and centrifuging at about 6000 rpm do it for 30'.
6. Transfer supernatant to a new tube – DO NOT disturb pellet to avoid high nonspecific background.
7. Take out 50ul aliquot for whole lysate (input).
8. Dilute supernatant with Dilution Buffer to obtain 150mM NaCl final conc.
9. Preclearing of supernatant : add 20μl of nonspecific antibody-conjugated beads - rabbit purified IgG (or just beads). IgG-AC (Santa-Cruz). Incubate for 1 hours on rocking plate in 4c.
10. Spin: 5' at max speed in 4c. Remove SN into fresh tube.
11. Immunoprecipitation: Add 1μg purified (1μl) antibody to the cleared lysate and incubate overnight (only with the antibody, without beads). Leave one sample as negative control (add nonspecific antibody or incubate without antibodies).
12. The next day, add 60μl of beads (Protein A Sepharose or Protein A agarose/Salmon Sperm DNA) and incubate additional 2 hours.
13. Spin: 1500 rpm for 2' at 4c. keep the SN for WB (unbound).
14. Wash pellet x3 in 1ml of Wash Buffer (plus protease inhibitors & DTT) .
15. Resuspend in 20μl of 2x SDS-PAGE loading buffer and boil for 5' before loading on the gel.
16. Separation: 4%-20% gradient or 10% TG gel, in 1xSDS protein gel buffer (Tris-Glycine-SDS), 120V 1,5h.
17. Transfer: black-black (gel) - clear-red (membrane) (on the clear side: sponge, whatman paper, membrane, gel, whatman paper, sponge). run 1,5hour in cold room, 100V.

    Mark the side of membrane that touches the gel (the side its blotted on).

Western Blot protocol

All washes and dilutions are done in TBS buffer or in 5% milk in TBS.

1. Blocking: 5% low fat milk in TBS, 1hr, RT.
2. Primary Ab: o/n at 4c (or 2h in RT) in 5% milk TBS. Usually Ab dilution is 1:500 or 1:1000
3. 4 X Wash in 5% milk TBS for 15'
4. Secondary Ab: 1 hour with anti-rabbit HRP antibody diluted 1:5000 in 5% milk TBS in RT.
5. 4 X Wash in TBS for 15'
6. Incubation with substrate: Pierce's SuperSignal 1:1, incubate for 1min, then insert into a plastic bag and watch the signal using the imaging system.
7. If you wish to keep the membrane: seal it and place it in -20°C or 4 °C]

LYSIS Buffer:

FINAL conc.	STOCK conc.	Buffer 50mL
20mM Hepes pH 7.3	1M	1 ml
20% glycerol	100%	10 ml
500mM NaCl	5M	5 ml
1.5mM MgCl2	100mM *	0.75 ml
0.2mM EDTA	500mM	20 μl
0.1% Triton X-100 **	100%	50 μl
diH2O		32.55 ml

DILUTION Buffer:

FINAL conc.	STOCK conc.	Buffer 50mL
20mM Hepes pH 7.3	1M	1 ml
20% glycerol	100%	10 ml
0mM NaCl	____	____
1.5mM MgCl2	100mM *	0.75 ml
0.2mM EDTA	500mM	20 μl
0.1% Triton X-100 **	100%	50 μl
diH2O		37.55 ml

WASH Buffer:

FINAL conc.	STOCK conc.	Buffer 50mL
20mM Hepes pH 7.3	1M	1 ml
0.5% NP-40**	100%	0.25 ml
250mM NaCl	5M	2.5 ml
1.5mM MgCl2	100mM *	0.75 ml
0.2mM EDTA	500mM	20 μl
diH2O		ml

* Note that the stock conc. is 100mM here

** cut the end of the tip when pipetting detergents