

## Immunoprecipitation

Version 1.0

**Background** This is a general immunoprecipitation protocol. The lysis buffer successfully solubilises membrane proteins (and has been used to IP the insulin receptor from a range of cells). However, the conditions of the immunoprecipitation reaction are close to physiological (except for the presence of a small amount of detergent) so this procedure is useful to co-immunoprecipitate protein complexes. Also, phosphatase inhibitors are included to preserve phosphorylation patterns.

### *Procedure*

#### A: Preparation of cell lysate

- 24h after seeding, cells are prepared for lysis as follows:-
  - suspension cells: wash two times by spinning cells (700rpm, 4min 4°C) and resuspending in 5ml cold full PBS
  - adherent cells: rinse flask with 5ml cold full PBS  
rinse 3ml cold 0.02% EDTA  
incubate with 3ml cold 0.02% EDTA for 3min on ice  
slap flasks to dislodge cells, add 3ml full PBS, pipette up and down and transfer to 15ml falcon tubes
- Lysing cells:-
  - add 20 $\mu$ l protease inhibitor cocktail to 5ml lysis buffer containing Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>
  - spin cells and resuspend with 100 $\mu$ l ice cold lysis buffer per million cells
  - incubate on ice for 10min, flicking every minute or so
  - shear using a 23G needle
  - clarify lysate by spinning at 13,000rpm for 10min at 4°C

#### B: Immunoprecipitation

- Add 100 $\mu$ l lysate + 1 $\mu$ l purified antibody (1-2 $\mu$ g) + 20 $\mu$ l Protein A/G  
or 20 $\mu$ l rabbit serum + 40 $\mu$ l Protein A/G  
and incubate (with tumbling) overnight at 4°C

2. Pellet beads by pulsing in a lab-top centrifuge at up to 13,000 xg. Wash 1x 200 $\mu$ l ice cold lysis buffer
3. Pellet beads and wash a further twice with 200 $\mu$ l ice cold PBS

### C: Analysis of immunoprecipitate by Western Blotting

**NOTE :** All our gel work is performed using the Novex system in accordance with the manufacturer's protocols

1. Add Novex gel sample buffer to lysate to a final concentration of 1x sample buffer. Typically, we add 5 $\mu$ l of sample buffer to 15 $\mu$ l of lysate. Add Novex reducing agent (typically 2 $\mu$ l to 20 $\mu$ l).
2. Place in waterbath for 10min at 70°C immediately before loading
3. Load an run on Novex Gel in accordance with manufacturer's instructions.
4. Blot onto PVDF membrane in accordance with manufacturer's protocol. Remember to pre-wet the PVDF membrane with 100% methanol prior to immersing in blot buffer.
5. Perform Western blotting in accordance with our Western Blotting protocol.

### **KEY POINTS:**

- Keep all buffers ice cold, and spins are at 4°C to reduce risk of proteolytic degradation or phosphatase activity
- The procedure must not be interrupted at any stage until the blot is completed
- This general procedure works for most proteins, but does NOT work for certain difficult-to-immunoprecipitate proteins, including chemokine receptors such as CXCR4. Different lysis buffers may be required for such proteins.
- If examining cell surface proteins, EDTA must be used to detach adherent cells from the flask rather than trypsin. Trypsin will non-specifically cleave surface proteins.
- The procedure is not quantitative.
- Always determine the relative specificity of the IP by performing a parallel immunoprecipitation using a control immunoglobulin of the same species. Also run the lysate and the depleted lysate side-by-side with the IP for troubleshooting purposes.

### *Materials*

#### Full PBS

137mM NaCl (8g), 2.7mM KCl (0.1g), 8.1mM Disodium hydrogen phosphate (1.14g anhydrous), 1.5mM Potassium Dihydrogen phosphate (0.1g), 0.9mM Calcium chloride (900 $\mu$ l of 1M), 0.5mM Magnesium chloride (500 $\mu$ l of 1M).

Dissolve solids in 1 litre MilliQ, adjust to pH 7.2 if necessary.

### Lysis Buffer

50mM HEPES, pH 7.4, 150mM NaCl, 1% Triton X-100, 10mM EDTA  
Add 30mM NaF, 1mM Na<sub>3</sub>Vo<sub>4</sub>, 1μg/ml leupeptin immediately before use

Protein A/G beads are Santa Cruz sc-2003, distributed in the UK by Insight Biotechnologies

All gel apparatus and buffers are from Novex, distributed in the UK by Invitrogen

### *Source References*

This procedure is based on the protocol provided by Santa Cruz with the Protein A.G beads which we routinely use for immunoprecipitation.

### *References where we have used this protocol*

At present this procedure has not been used in any of our published papers

### **DISCLAIMER**

**Please note that this protocol is provided for information only. While we believe it to be accurate, we do not warrant as to its validity or suitability for any particular purpose. In the event that you wish to use this protocol, we accept no liability for any direct or indirect losses howsoever caused. It is your responsibility to ensure that any use of this protocol is performed safely and in accordance with any local laws, regulations or guidelines applicable to you.**