

## Anti-Ubiquitin western blot

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### INTRODUCTION

This protocol was set-up to increase the sensitivity of anti-Ub western-blot. It consists in a denaturing step of the proteins directly on the filter, which renders the epitopes more accessible to the antibodies increasing the ratio signal to noise.

### MATERIALS

#### Denaturing solution

- 6 M guanidium chloride
- 20 mM Tris pH 7.5
- 1 mM PMSF (freshly added)
- 5 mM  $\beta$ -mercaptoethanol (freshly added)

### METHODS

1. Proteins are transferred on a PVDF (polyvinylidene fluoride) membrane (Immobilion P, Millipore), previously activated by incubation in 100% methanol for 5 minutes at room temperature.
2. Pre-stained molecular weight markers are used in this case, since colouring the filter with Ponceau solution is not recommended, as this might interfere with antibody recognition.
3. After transfer, filters are subjected to a treatment in denaturing solution for 30 minutes at 4°C (the membrane should become transparent during this treatment).
4. After extensive washing in TBS-T buffer (TBS Tween 0,1%), filters are blocked overnight at 4°C in 5% BSA (dissolved in TBS-T).
  - **It is particularly important to avoid drying the membrane during these treatments.**
5. After blocking, filters are incubated with the P4D1 antibody (Santa Cruz, 1:1000) or FK2 (Biomol, 1:1000) against Ub, diluted 1:1000 in TBS-T 5% BSA, for one hour at room temperature
6. Wash three times for 10 minutes each time in TBS-T.
7. Incubate with the anti-mouse horseradish peroxidase-conjugated secondary antibody diluted in TBS-T 3% BSA for 30 minutes at RT.
8. Wash the filter three times in TBS-T for 5 minutes each time.
9. The bound secondary antibody is revealed using the ECL (Enhanced Chemiluminescence) method (Amersham).

### BIBLIOGRAPHY

Penengo L, Mapelli M, Murachelli AG, Confalonieri S, Magri L, Musacchio A, Di Fiore PP, Polo S and Schneider TR. (2006) Crystal structure of the ubiquitin binding domains of rabex-5 reveals two modes of interaction with ubiquitin. *Cell*. 124:1183-95.