

TNF-alpha Immunoprecipitation

INTRODUCTION

When a protein is expressed at low levels and is difficult to detect with western blot analysis, immunoprecipitation, or pull down, may be the method of choice. An immunoprecipitating reagent has to be specific in order to avoid precipitation of unwanted protein and sufficient affinity is required to pull down the protein. In addition, it has to withstand stringent washing steps. The Anti-TNF-alpha Affibody[®] molecule is a highly specific affinity ligand and has been proven well suited for pull down experiments of TNF-alpha proteins.

Figure 1a and 1b show that TNF-alpha was precipitated from LPS stimulated, but not from PBS stimulated THP-1 cell extract, using agarose immobilized Anti-TNF-alpha Affibody[®] molecule. With increased amount of total protein, the TNF-alpha band became more intense. TNF-alpha was not detected in cell extracts prior to immunoprecipitation proving that an immunoprecipitation step is needed for TNF-alpha detection in this model system.

These results show that the Anti-TNF-alpha Affibody[®] molecule efficiently precipitates TNF-alpha proteins from a complex mix of proteins. When performing immunoprecipitation experiments with antibodies, there is often a problem with cross reactions between the enzyme conjugated second step reagent and the precipitating antibody. This type of cross reaction is avoided with an Affibody[®] molecule as the precipitating reagent.

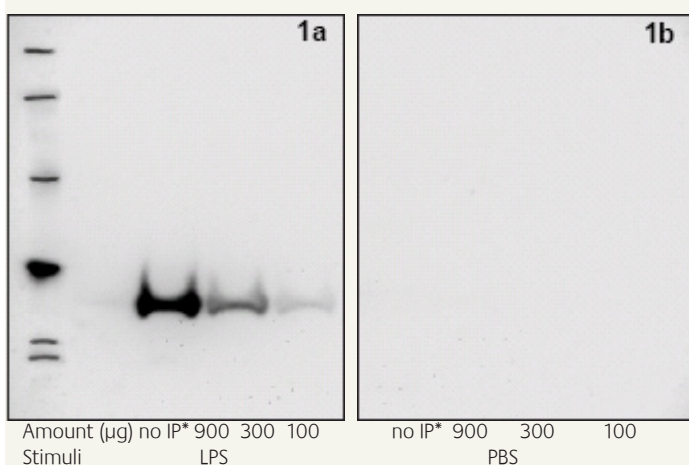


Fig. 1. The agarose immobilized Anti-TNF-alpha Affibody[®] molecule precipitates TNF-alpha protein from cell extracts derived from LPS stimulated but not from unstimulated THP-1 cells.

RESULTS

IMMUNOPRECIATION

The human pro-monocytic cell line THP-1 was primed with PMA (200 nM) over night and stimulated with LPS (200 ng/ml) or with PBS (control) for 2 hours prior to termination of culture. Cell extracts prepared from LPSstimulated cultures and from control cells were incubated with agarose immobilized Anti-TNF-alpha Affibody[®] molecule for 2 hours. After incubation, the unbound proteins were washed away and the bound protein was eluted with SDS-PAGE separation and blotted onto a PVDF filter. The filter was stained with an antibody against full length TNF-alpha protein (25 kD).

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MATERIALS AND BUFFERS REQUIRED

Anti-TNF-alpha Affibody® IP kit: Cat no 10.0841.04.9002 (Affibody), containing TNF-alpha Affibody® molecule and cysteine blocked agarose

PBS: 2.68 mM KCl, 1.47 mM KH₂PO₄, 137 mM NaCl, 8.1 mM Na₂HPO₄, pH 7.4

Lysis buffer (RIPA): 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA

Protease inhibitors: Complete Mini, EDTA free (Roche Diagnostics)

Tubes: 1.5 ml conical tube (Eppendorf), 50 ml tubes (Falcon)

PREPARATION OF CELL EXTRACT

Note: Work on ice or in a cold room.

1. Wash the cells with ice cold PBS.
2. Count the cells.
3. Wash the cells with ice cold PBS and remove excess PBS from the cell pellet.
4. Lyse cells with RIPA buffer containing protease inhibitors. Prepare cell extract with at least 10×10^6 cells per ml lysis buffer.
5. Pipette the cell pellet and transfer the lysate to an Eppendorf tube. If low TNF-alpha protein expression is anticipated, use a higher concentration extract by increasing the number of cells per ml.
6. Incubate the cell extract at +4°C by gently rocking or by end over end rotation for 30 minutes.
7. Spin the lysate for 15 minutes at $10\,000 \times g$ at +4°C. Use a cooled centrifuge.
8. Remove the supernatant (cell extract) carefully to a new Eppendorf tube.
9. The cell extract is now ready for pre-clearing or alternatively, storage at -80°C.

PRE-CLEARING OF CELL EXTRACT

Note: To reduce unspecific binding of proteins to the agarose matrix, a pre-clearing step may be needed. Work on ice or in a cold room.

1. Wash the cysteine blocked agarose twice with lysis buffer.
2. Add 1 ml of cell extract per 100 µl cysteine blocked agarose matrix.
3. Incubate with end over end rotation for 1 hour at +4°C.
4. Spin for 30 seconds at a maximum of $5000 \times g$. Use a

cooled centrifuge.

5. Collect the supernatant, i.e. the pre-cleared extract, and transfer it to a new Eppendorf tube. Discard the cysteine blocked agarose. Measure the total protein content with a BCA test or similar.
6. The pre-cleared cell extract is now ready for precipitation experiments or store at -80°C.

PULL DOWN

1. Mix the pre-cleared cell extract with 15-30 µl of packed agarose immobilized Anti-TNF-alpha Affibody® molecule. Note: The volume of cell extract and the amount of matrix should be determined by the user. The combination of 30 µl packed matrix with an end volume of 300 µl is recommended. A lower volume may make mixing difficult.
2. Incubate the mix for 2 hours at +4°C by end over end rotation.
3. Spin the mix at a maximum of $5000 \times g$ for 30 seconds. Use a cooled centrifuge.
4. Discard the supernatant.
5. Wash by adding 1 ml RIPA buffer containing protease inhibitors and spin the mix at a maximum of $5000 \times g$ for 30 seconds. Use a cooled centrifuge.
6. Discard the supernatant. Repeat the washing steps 4 times.
7. Resuspend the mix in 30 µl of sample buffer. Boil for 10 minutes.
8. Analyze the sample immediately by Western blot, or store at -20°C.

WESTERN BLOT

For SDS-PAGE separation and transfer, the Novex NuPAGE system (4-12% gels) and the Novex transfer system have been used. Immobilon PVDF membranes from Millipore for blotting and PBST+0.5-2% casein as blocking solution is recommended. TNF-alpha staining was performed with the mouse anti-TNF-alpha antibody (ab9813, Abcam), followed by HRP conjugated anti-mouse IgG (Dako). For chemiluminescence, Super Signal, West-Dura (Pierce) was used as substrate.

LIMITATIONS

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