APPLICATION NOTE

EGFR Immunoprecipitation

INTRODUCTION

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

RESULTS IMMUNOPRECIPITATION

Cell extracts were prepared from high EGFR expressing human squamos carcinoma cell line A431, from low EFGR expressing SH-SY5Y (human neuroblastoma) cells and from the EGFR negative RAMOS (human B-cell lymphoma) cell line. The three extracts were incubated with agarose immobilized Anti-EGFR Affibody® molecule for 2 hours. After incubation, the unbound proteins were washed away and the bound protein was eluted and separated with SDS-PAGE and blotted onto a PVDF filter. The filter was stained with an antibody against full length EGFR, approximately 140 kDa.

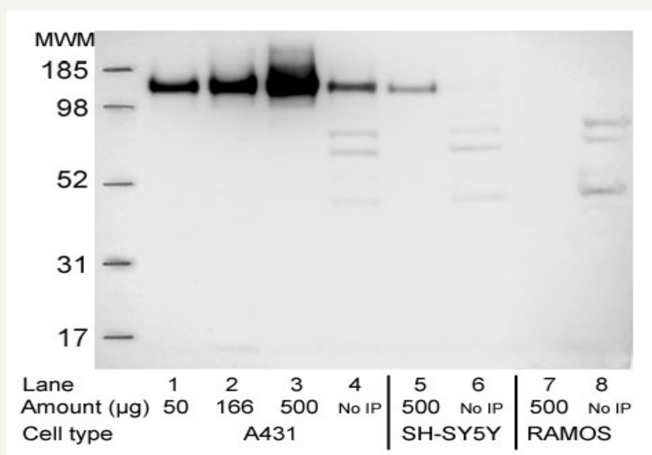

Fig. 1. The agarose immobilized Anti-EGFR Affibody® molecule precipitates EGFR protein from cell extracts derived from A431 and SH-SY5Y, but not from RAMOS cells.

A single protein with MW of approximately 140 kDa was precipitated from A431 and SH-SY5Y cells but not from RAMOS cells, as shown in figure 1. With increased amount of A431 proteins, the 140 kDa EGFR band got more intense on the Western blot (lanes 1-3). The EGFR band was also detected in A431 cell extracts prior to precipitation together with bands of lower MW that were not present after precipitation (lane 4). SH-SY5Y cells also express EGFR but to a lower level than A431, as shown by a relatively weak EGFR band (lane 5). There were no EGFR detected in SH-SY5Y cell extracts prior to precipitation (lane 6) and there were no EFGR precipitated from RAMOS cells or present in the cell extracts (lanes 7-8).

In summary, the Anti-EGFR Affibody® molecule efficiently precipitates EGFR from a complex protein mix. When performing immunoprecipitation experiments with antibodies, there is often a problem with cross reaction between the enzyme conjugated second step reagent and the precipitating antibody but this type of cross reaction is elegantly avoided using an Affibody® molecule as the precipitating reagent.

EGFR Immunoprecipitation

MATERIALS AND BUFFERS REQUIRED

Precipitating agent: Anti-EGFR Affibody® IP kit (Agarose immobilized Anti-EGFR Affibody® molecule and cysteine blocked agarose (Affibody cat no 10.1246.04.9002))
PBS: 2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 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 x 10^6 cells per ml lysis buffer.
5. Pipette the cell pellet and transfer the lysate to an Eppendorf tube. If low EGFR 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 1-2 hours.
7. Spin the lysate for 15 minutes at 10 000**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 RIPA buffer.
2. Add 1 ml of cell extract per 100 µl packed cysteine blocked agarose.
3. Incubate with end over end rotation for 1 hour at +4°C.
4. Spin for 30 seconds at a maximum of 5000**g*. Use a cooled centrifuge.
5. Collect the supernatant (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-EGFR 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 smaller volume may make mixing difficult.
2. Incubate the mix for 1-2 hours at +4°C by end over end rotation.
3. Spin the mix at a maximum of 5000*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**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 were used. The proteins were blotted onto Immobilon PVDF membranes (Millipore) and the membranes were blocked with PBST + 0.5% casein. Western Blot staining of EGFR was done with rabbit anti-EGFR antibody (Abcam cat no ab2430-1), followed by HRP conjugated anti-rabbit IgG (Dako). Super Signal West-Dura (Pierce) was used as substrate for chemiluminescence. Images of the blot was taken with a ChemiImager (Alpha Innotech Corp.).

LIMITATIONS
Warranty: Affibody® products are warranted to meet stated product specifications and to confirm to label descriptions when used and stored properly. Unless otherwise stated, this warranty is limited to one year from date of sales for products used, handled and stored according to Affibody AB's instructions. Affibody AB's sole liability is limited to replacement of the product or refund of the purchase price. Affibody® products are supplied for research use only. They are not intended for medicinal, diagnostic or therapeutic use. Affibody® products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Affibody AB.
Affibody AB, P.O. Box 20137, SE-161 02 Bromma, Sweden. Phone: +46-8-598 838 00, Fax: +46-8-598 838 01, E-mail: biotechnology@affibody.com, Web: www.affibody.com/shop.
Rev 060607