Application Note

Immunoprecipitation with Dynabeads® Protein A or Protein G

Introduction

Immunoprecipitation (IP) is the technique by which a target protein is precipitated from a solution using an antibody specific to the target. In addition to isolating single proteins from a sample, this process can also be used to isolate protein complexes present in cell extracts, by targeting a protein believed to be in the complex. The immune complexes are captured by Dynabeads® Protein A or Dynabeads® Protein G and easily removed from the supernatant by magnetic separation. After washing, the precipitate can be eluted off the beads and analyzed by western blotting or mass spectrometry.

Applications of immunoprecipitation

- Determination of the molecular weight, identity or quantity of protein of interest
- Assess for protein-protein interactions
- Concentration of proteins
- Expression levels of a protein of interest

The procedure can be divided into several stages

1. Sample preparation
2. Pre-clearing (optional)
3. Immunoprecipitation
4. Elution

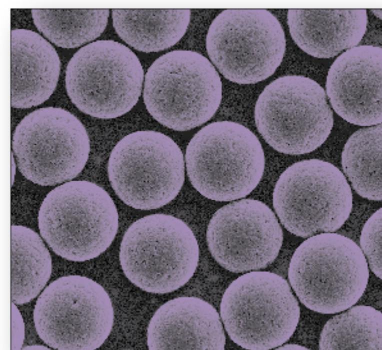

Fig.1. Monosized and super-paramagnetic Dynabeads®.

Sample Preparation

Depending on location of the antigen within the cell (nuclear, cytosolic, membrane-bound, etc.) and application of immunoprecipitation, an appropriate lysis strategy should be determined with minimal effect on the structural integrity of antigen. The ionic strength (salt concentration), choice of detergent and pH of the lysis buffer may significantly affect the efficiency of extraction and integrity of the antigen.

- **Slightly alkaline pH and low ionic strength buffers typically favor protein solubilization.**
- **High salt concentration and low pH may cause the antigen to become denatured and precipitate out of solution.**

The choice of detergents is crucial and may be influenced by many factors including (among others) the subcellular location of the antigen and whether one would like to preserve subunit associations and other protein-protein interactions.

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- **Non-ionic (e.g., Triton® X-100, NP-40) or zwitterionic (e.g., CHAPS) detergents tend to preserve non-covalent protein-protein interactions.**
- **Ionic detergents (e.g., SDS, sodium deoxycholate) tend to have a denaturing effect on protein-protein interactions and may adversely affect the ability of the antibody to recognize the target antigen.**

Proteolytic digestion can occur when cells are lysed and contents of lysosomes are mixed with other compartments of the cell. Therefore, regardless of the lysis strategy employed, the lysis buffer should always contain a cocktail of protease inhibitors.

Following preparation of the lysate, determine the protein concentration of the lysate and adjust it to between 1 to 5 mg/mL with lysis buffer or PBS.

Two of the most commons buffers used for cell lysis are:

- **RIPA buffer** gives lower background in immunoprecipitation. However, RIPA can denature some proteins. If you are conducting immunoprecipitation experiments to study protein-protein interactions, RIPA should not be used as it can disrupt protein:protein interactions.
- **NP-40 buffer** denatures to a lesser extent, and is thus used for phosphorylation experiments such as looking at kinase activity. Also NP-40 is used for protein-protein interactions. NP40 is a non-ionic detergent, and is the most commonly used detergent in cell lysis buffers for immunoprecipitation and westerns.

Pre-clearing

The pre-clearing step is incorporated into the procedure to lower the amount of non-specific binders in the cell lysate and to remove proteins with high affinity for Protein A or Protein G. Dynabeads® Protein A or Dynabeads® Protein G exhibit low nonspecific binding in many sample types, however, certain samples may still require pre-clearing to remove molecules which have high non-specific binding activity. In cases where pre-clearing is required, mix uncoated Dynabeads® Protein A or Dynabeads® Protein G with the sample under equivalent conditions for subsequent IP. Pre-clearing should be kept to minimum to retain the reproducibility and quantitative nature of the procedure.

Some researchers find pre-clearing unnecessary and skip this step.

Immunoprecipitation

Immunoprecipitation is performed using one of the methods described below.

Direct Method

Primary antibody is bound to the Dynabeads® Protein A or Dynabeads® Protein G according to Ig capture procedure in the product insert protocol. Just prior to mixing the Dynabeads® with IP sample, apply magnet to remove all the supernatant. Then add the IP sample to the bead pellet and resuspend the beads with a pipette. Incubate the mixture at 2-8°C with tilting and rotation (in vertical plan). The incubation time depends upon the concentration of target protein and the specificity of the antibody toward its target. When concentration of the target protein is high, a short incubation time of 10 min should be sufficient to capture the target. Otherwise the incubation can be prolonged up to 30 min-1 hour.

The Indirect Method

In this method, the antibody is first incubated with the cell lysate to form an antibody-antigen complex. This complex is then captured by adding Dynabeads® Protein A or Dynabeads® Protein G added to the sample.

Care must be taken to avoid using an excess amount of antibody, as free antibodies will be captured by the beads much faster and may reduce the protein yield by occupying the binding sites.

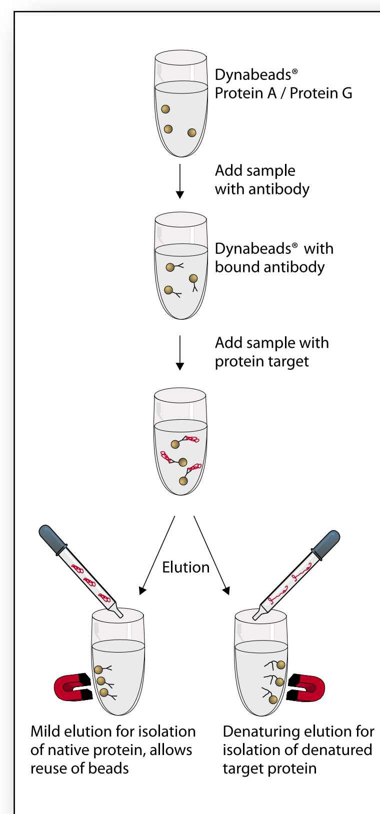

Fig. 2: An antibody, specific for the target protein, is added to Dynabeads® Protein A or Protein G. The separated antibodies are eluted in a small volume, and the beads can be reused. Dynabeads® with captured antibody facilitate small-scale precipitation and co-precipitation within minutes. Target proteins/antigens are eluted in a small volume. Depending on your elution regime, the beads can be reused.

Difference between direct and indirect method.

Direct method:

- **Use when the target protein is abundant.**
- **Can make up a stock of the beads.**
- **Uses less amount of primary antibody.**

Indirect method:

- **May perform better for antibodies with poor affinity or where the target.**
- **Use to minimize exposure of Dynabeads® to the sample, hence can be used to control level of background binding.**

The amount of primary antibody required for IP should be determined empirically. The antibody should be titrated to determine the optimal concentration. Based on Dynabeads® Protein A and Dynabeads® Protein G binding capacity, calculate the amount of beads required to capture the primary antibody. However, to ensure rapid kinetics, keep the sample concentrated otherwise full protein capture may not occur during incubation time. The volume of the beads added should not be less than 1/10 of the sample volume.

Washing Steps

After the complexes have been captured on the Dynabeads®, RIPA, PBS or other buffers can be used to wash the beads. The choice is dependendt on the downstream application. RIPA buffer is more stringent whereas PBS is less stringent. When PBS is used, non-ionic detergents such as Tween-20 (0.01-0.1%) should be added to reduce the level of non-specific binding. For washing, use large quantities of washing buffer, at least twice the sample volume. Wash the Dynabeads® at least 3 times with constant rotation during each wash.

Nonspecific binding

The most common challenge with immunoprecipitation is trying to lower the number and type of background proteins. Background problems can arise from many different sources and can be either specific or nonspecific. The following are suggestions dealing with nonspecific background problems:

- **To reduce nonspecific binding, immunoprecipitation buffers usually have some detergent to reduce hydrophobic interactions and high salt to reduce ionic interactions.**
- **Increase the number of washes**
- **Prolong the washing steps**
- **Use more stringent washing buffers for washing**
- **Decrease the incubation time if using the direct method or try the indirect method.**
- **Decrease the primary antibody concentration.**
- **A pre-clearing step may be performed to remove molecules that nonspecifically bind to the Protein A or Protein G or Dynabeads® themselves.**

Despite these precautions, nonspecific binding can occur.

In a control reaction, primary antibody is replaced by a non-relevant immunoglobulin.

Note: Dynabeads® Protein A or Dynabeads® Protein G mixed with sample can not be used as a control reaction, as some bio-molecules will inevitably interact with either protein A, protein G or Dynabeads® matrix itself.

Elution

Components of the bound immune complex (both antigen and antibody) are eluted from the Dynabeads® and analyzed by SDS-PAGE, often followed by western blot detection to verify the identity of the antigen. Boiling in SDS-PAGE buffer will results in co-elution of the antibody along with the target protein. If co-elution of antibody fragments with antigen results in bands interfering with detection of any co-precipitated proteins on SDS-PAGE, the antibody has to be cross-linked to the beads. Cross-linkers like DMP (dimethyl pimelimidate) or DSS (disuccinimidyl suberate) can be used for cross-linking.

Note: cross-linking is not 100% efficient and a small amount of IgG will never get cross-linked to the beads. In order to prevent contamination from the antibodies which are not cross.linked, perform an elution step using low pH buffer to elute them off the beads before immunoprecipitation. Remember to bring the pH of the bead suspension back to the normal level immediately after elution and before immunoprecipitation.

Note: some loss of antibody affinity toward its target might be observed as some of the binding sites within the antibody may also get cross-linked and affect the binding. If loss of affinity is observed after cross-linking, either do not cross-link or use one of our Surface Activated Dynabeads® for covalent binding of antibody to the beads.

Note: even after cross-linking, use of reducing agents like DTT or β-mercaptoethanol in SDS-PAGE can reduce the disulfide bridges of antibodies and cause the light chains of antibody to be released into the eluate.

Except SDS-PAGE buffer, other conventional elution methods like change of pH can also be used to elute target protein off the beads such as 0.1M citrate (pH 2-3).

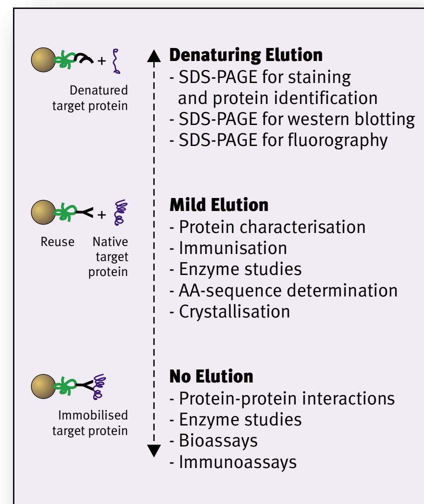

Fig. 3: Different elution possibilities for captured proteins.

Other Dynabeads® for Immunoprecipitation:

Several other types of Dynabeads® in addition to Dynabeads® Protein A or Dynabeads® Protein G can also be used for immunoprecipitation.

- **Dynabeads® Streptavidin:** these beads can be used with any biotinylated antibody.
- **Dynabeads® Surface Activated:** for covalent coupling of primary antibody directly to the beads. When co-elution of primary antibody with target protein is not desired, covalent coupling of antibody can prevent that. Dynabeads® can also be coated with antibody and stored for further use. Add 0.02% sodium azide (NaN_3) and keep refrigerated.
- **Secondary Coated Dynabeads®:** these beads can be coated with a specific type of primary antibody depending on the origin of the secondary antibody.

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