MabAffinity® Protein L High Flow Beads

MabAffinity Protein L High Flow Beads, with rProtein L coupled to high flow Agarose matrix, has been successfully used for a wide range of antibodies and antibody fragments capture for its strong affinity to the variable region of antibody kappa light chains. Although it binds to the Fab portion of the immunoglobulin, Protein L does not interfere with the antigen-binding site of the antibody. Therefore, Protein L potentially can be used in immunoprecipitation (IP) procedures. As a good complementary choice for Protein A and Protein G, besides IgG, Protein L has been used for efficient purification of IgM, IgE, IgD, IgA and IgY. MabAffinity Protein L High Flow Beads also shows great advantages in purification of antibody fragments such as Fabs, single-chain variable fragments (scFv), and domain antibodies (Dabs).

- Binding specificities that complement Protein A and G media
- Suitable for different applications, including poly or monoclonal antibodies and antibody fragments from different species and subclasses for its broad binding specificity
- Purification of monoclonal antibodies from FCS supplemented media without interference of bovine immunoglobulin
- Single point attachment coupling chemistry gives better ligand accessibility for higher binding capacity
1. Description

MabAffinity Protein L High Flow Beads, as part of Acrbiosystems’ MabAffinity Family, is manufactured by immobilizing recombinant Protein L ligand to highly cross-linked agarose matrix through stable bond formed by epoxy coupling chemistry. Protein L ligand is derived from E Coli fermentation and interacts with the variable region of kappa light chain of a wide range of immunoglobulins. Protein L can bind to kappa types I, III, and IV of human IgG and kappa type I of mouse IgG. Besides IgG, Protein L has been used for efficient purification of IgM, IgE, IgD, IgA and IgY containing kappa light chains. MabAffinity Protein L High Flow Beads also shows great advantages in purification of antibody fragments such as Fabs, single-chain variable fragments (scFv), and domain antibodies (Dabs). Therefore, as a good complementary choice for Protein A and Protein G, MabAffinity Protein L High Flow Beads has been used for both excellent chromatography purification of antibodies and fragments from several species of mammals in one step, but also immunoprecipitation to purify and detect proteins or protein complexes successfully.

Please refer to the following Table 1 for specifications of MabAffinity Protein L High Flow Beads in details.

Table 1. Characteristics of MabAffinity Protein L High Flow Beads

<table>
<thead>
<tr>
<th>Composition</th>
<th>Highly cross-linked Agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average particle size</td>
<td>90 micron</td>
</tr>
<tr>
<td>Form</td>
<td>Slurry in 20% Ethanol</td>
</tr>
<tr>
<td></td>
<td>1ml/5ml prepacked column</td>
</tr>
<tr>
<td></td>
<td>Spin column kit (5 cycles)</td>
</tr>
<tr>
<td>Ligand</td>
<td>rProtein L</td>
</tr>
<tr>
<td>Coupling chemistry</td>
<td>Epoxy</td>
</tr>
<tr>
<td>Dynamic binding capacity(^1)</td>
<td>&gt; 5 - 10 mg human IgG /ml media</td>
</tr>
<tr>
<td>Recommended flow rate</td>
<td>100-300cm/hr</td>
</tr>
<tr>
<td>Recommended column height</td>
<td>5-20cm</td>
</tr>
<tr>
<td>Maximum Pressure Drop</td>
<td>0.3MPa</td>
</tr>
<tr>
<td>Chemical stability</td>
<td>Stable in all aqueous buffers commonly used in Protein L chromatography</td>
</tr>
<tr>
<td>pH working range</td>
<td>2-10</td>
</tr>
<tr>
<td>pH CIP range (short term)</td>
<td>2-11</td>
</tr>
<tr>
<td>CIP stability</td>
<td>8M Urea, 10mM NaOH + 1M NaCl, 0.1M acetic acid</td>
</tr>
<tr>
<td>Temperature stability(^2)</td>
<td>2-40 °C</td>
</tr>
<tr>
<td>Storage</td>
<td>20% Ethanol</td>
</tr>
<tr>
<td>Shelf life</td>
<td>5 years</td>
</tr>
</tbody>
</table>
Table 2. Characteristics of MabAffinity Protein L Prepacked Column

<table>
<thead>
<tr>
<th>Composition</th>
<th>Highly cross-linked Agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average particle size</td>
<td>90 micron</td>
</tr>
<tr>
<td>Form</td>
<td>1ml/5ml prepacked column;</td>
</tr>
<tr>
<td>Ligand</td>
<td>rProtein L</td>
</tr>
<tr>
<td>Coupling chemistry</td>
<td>Epoxy</td>
</tr>
<tr>
<td>Dynamic binding capacity&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&gt; 5 - 10mg human IgG /1ml prepacked column;</td>
</tr>
<tr>
<td></td>
<td>&gt; 25 - 50mg human IgG /5ml prepacked column</td>
</tr>
<tr>
<td>Recommended flow rate</td>
<td>0.2 - 1 ml/min for 1ml prepacked column</td>
</tr>
<tr>
<td></td>
<td>1 - 5ml/min for 5ml prepacked column</td>
</tr>
<tr>
<td>Column dimension</td>
<td>0.7x2.5 cm for 1ml prepacked column</td>
</tr>
<tr>
<td></td>
<td>1.6x2.5 cm for 5ml prepacked column</td>
</tr>
<tr>
<td>Maximum Pressure Drop</td>
<td>0.3Mpa</td>
</tr>
<tr>
<td>Chemical stability</td>
<td>Stable in all aqueous buffers commonly used in protein G chromatography</td>
</tr>
<tr>
<td>pH working range</td>
<td>2-10</td>
</tr>
<tr>
<td>pH CIP range (short term)</td>
<td>2-11</td>
</tr>
<tr>
<td>CIP stability</td>
<td>8M Urea, 10mM NaOH + 1M NaCl, 0.1M acetic acid</td>
</tr>
<tr>
<td>Temperature stability&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2-40 °C</td>
</tr>
<tr>
<td>Storage</td>
<td>20% Ethanol</td>
</tr>
<tr>
<td>Shelf life</td>
<td>5 years</td>
</tr>
</tbody>
</table>

1, Determined at 10% breakthrough at 4min residence time
2, Delivered at room temperature, and recommended long-term storage at 2-8 °C

2, Instructions for chromatography purification

2.1 Column packing

MabAffinity Protein L High Flow Beads is supplied as a suspension in 20% ethanol. Decant the 20% ethanol solution and exchange it with water or other packing buffer required before use. Then follow the procedures below:

1. Equilibrate all material to the temperature at which the purification will be performed. Assemble the column (and packing device, if necessary).
2. Remove air from the column dead spaces by flushing the end-piece and adapter.
with packing buffer. Make sure no air has been trapped under the column net. Close the column outlet leaving the net covered with packing buffer.

3. Resuspend the medium stored in its container by shaking (avoid stirring the sedimented medium). Mix the packing buffer with the medium to form a 50% to 70% slurry (sedimented bed volume/total slurry volume = 0.5 to 0.7).

4. Pour the homogeneous slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will help to minimize the introduction of air bubbles.

5. If using a packing device, immediately fill the remainder of the column and packing device with packing buffer. Mount the adapter or lid of the packing device and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.

6. Open the bottom outlet of the column and turn on the pump to run at the desired flow rate. Ideally, MabAffinity Protein L resin is packed at a constant pressure of approximately 1 bar (0.1 MPa). If the packing equipment does not include a pressure gauge, use a packing flow rate of approximately 400 cm/h (10 cm bed height, 25°C, water as packing buffer).

   If the recommended pressure or flow rate can not be obtained, use the maximum flow the pump can deliver.

7. When the bed height has stabilized, mark the compressed bed height and close the bottom outlet and stop the pump.

8. If using a packing device, disconnect the packing device and mount the adapter to the column.

9. With the adapter inlet open, push the adapter down, approximately 2 mm below previous compressed bed height, and the packing buffer will flush the adapter inlet. Close the adapter inlet.

10. The column is now ready to use for antibody purification.

Note:
Do not exceed 75% of the packing flow rate in subsequent chromatographic purification procedures.

2.2 Purification

2.2.1 Binding Affinity
Binding characteristics of Protein L ligand are summarized in Table 3, which can be used as a general guide for affinity separation media selection of antibody purification.
Table 3 Binding characteristics property of rProtein L ligand

<table>
<thead>
<tr>
<th>Species</th>
<th>Subclass</th>
<th>rProtein L</th>
<th>Species</th>
<th>Subclass</th>
<th>rProtein L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Total IgG</td>
<td>+++</td>
<td>Mouse</td>
<td>Total IgG</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td>+++</td>
<td></td>
<td>IgG1</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgG2</td>
<td>+++</td>
<td></td>
<td>IgG2a</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgG3</td>
<td>+++</td>
<td></td>
<td>IgG2b</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgG4</td>
<td>+++</td>
<td></td>
<td>IgG3</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgA</td>
<td>+++</td>
<td></td>
<td>IgM</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgA1</td>
<td>+++</td>
<td>Rat</td>
<td>Total IgG</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgA2</td>
<td>+++</td>
<td></td>
<td>IgG1</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgD</td>
<td>+++</td>
<td></td>
<td>IgG2a</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgE</td>
<td>+++</td>
<td></td>
<td>IgG2b</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>+++</td>
<td></td>
<td>IgG2c</td>
<td>+++</td>
</tr>
<tr>
<td>Cow</td>
<td>Total IgG</td>
<td>-</td>
<td>IgG3</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td>-</td>
<td>Hamster</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG2</td>
<td>-</td>
<td>Rabbit</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>Total IgG</td>
<td>?</td>
<td>Chicken</td>
<td>IgY</td>
<td>+</td>
</tr>
<tr>
<td>Goat</td>
<td>IgG1</td>
<td>-</td>
<td>Cat</td>
<td>Total IgG</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td>-</td>
<td>Dog</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG2</td>
<td>-</td>
<td>Pig</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Total IgG</td>
<td>-</td>
<td>Guinea-pig</td>
<td>IgG1</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td>-</td>
<td></td>
<td>IgG2</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>IgG2</td>
<td>-</td>
<td>Koala</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Monkey(rhesus)</td>
<td>IgG</td>
<td>?</td>
<td>Llama</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

Strong binding ++, medium interaction +, weak or no interaction —

For purification of antibody fragments containing variable region of kappa light chain (VL-kappa), Protein L ligand also have been used successfully. Please refer to the following Figure 1 and Table 3 for separation media selection. For more detail information about KBP, LBP and FabAffinity family resins, please visit our website.

Figure 1 Structure of antibodies and fragments

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Table 4 Guide to affinity media for antibody fragments purification

<table>
<thead>
<tr>
<th>Antibody fragment</th>
<th>Fab</th>
<th>ScFv</th>
<th>Domain Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chain Subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kappa light chain</td>
<td></td>
<td></td>
<td>VH3 heavy chain</td>
</tr>
<tr>
<td>Lambda light chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kappa light chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kappa light chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH3 heavy chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recommended products</td>
<td>Protein L or Protein A</td>
<td>Protein L</td>
<td>Protein L</td>
</tr>
<tr>
<td>Binding site</td>
<td>V kappa or CL-kappa</td>
<td>CL-Lambda</td>
<td>V kappa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>V kappa</td>
</tr>
</tbody>
</table>

The dynamic binding capacity is a function of the sample residence time. It is necessary to use appropriate linear flow rate during sample application to ensure that residence time is in 4 to 8 min range at optimal column height of 5 to 20 cm. The residence time is equal to the packed bed height (cm) divided by the linear flow rate (cm/h) applied during sample loading.

2.2.2 Recommended purification parameters

Generally, antibodies and fragments bind MabAffinity Protein L High Flow Beads at neutral pH and physiological ionic strength, and are eluted at low pH. The recommended buffers for purification listed below can be used as good starting conditions for your experiments:

Recommended buffers:
- **Binding buffer:**
  - 20mM Sodium phosphate, 150mM NaCl, pH 7.2
  - 20mM Tris, 100mM NaCl, pH 7.8
  - Phosphate buffered saline (PBS), pH 7.4 (0.01M phosphate buffer, 0.0027M KCl, 0.14M NaCl)
- **Elution buffer:**
  - 100mM Glycine, pH 2.5-3.0
- **Neutralize buffer:** 1M Tris pH 8-9

Purification procedures:
1. Pack the column as described in “Column Packing” section. The recommended column height is within 5-20cm.
2. Equilibrate the column at recommended flow rate with 5-10 column volumes of binding buffer to get a stable baseline.
3. Calculate appropriate sample amount for loading. In principle, dynamic capacity
is related to lots of parameters, such as antibody type, residence time, sample concentration, binding buffer and so on. Therefore, the maximum loading volume can be obtained by frontal analysis for individual sample under specific binding conditions. Generally, the dynamic binding capacity is around 15-25mg Fab/ml medium for 4-8min residence time.

**Note:**
Please note that there might be considerable deviations in binding capacity for different immunoglobulins derived from the same species, even if they are of the same subclass.

4. Apply clarified sample of antibody onto column. Samples need to be clarified by 0.45micron filter to remove any particles and colloids before application. It is recommended to dilute samples of high protein concentration, such as anti-serum, with equal volume of binding buffer to reduce sample viscosity.

5. Wash column with 5 column volumes of binding buffer until UV level drop to baseline. Though not necessary for most of the cases, optional intermediate washing step with salts or detergents may help to remove impurities to some extent.

6. Elute the column with 10 column volumes elution buffer. The most commonly used elution buffer is pH3.0; however, pH 2.5-3.0 is required for efficient elution of some kind of very strong binding antibodies with high recovery. Non-ionic detergents, arginine and urea have been reported to improve antibody stability and avoid aggregation during elution.

7. Neutralize the elution peak immediately with 1M Tris buffer of pH 8.0-9.0.

8. Re-equilibrate the column with 5-10 column volumes of neutral binding buffer.

**Purification cases:**
- **Sample:** Clarified Hybridoma cell culture containing mouse IgM
- **Binding buffer:** PBS pH 7.3
- **Elution buffer:** 100mM Glycine pH 3.0
- **Column:** MabAffinity Protein L High Flow Beads packed into a 0.5x 5 (cm, i.d. x Height) 1ml column
- **Load sample volume:** 100ml
- **Flow rate:** 1ml/min (300cm/hr) for loading and elution
- **UV280nm detection**
- **Peak neutralized immediately with 1/20(v/v) 1M Tris pH8.0 after elution**
- **Purity assay by 10-20% reduced gradient SDS-PAGE**
100mL hybridoma cell culture was loaded onto MabAffinity Protein L column for mouse IgM capture. And finally IgM with >95% purity was obtained successfully within one step.

### 2.2.3 Microspin Column Purification
A simplified 500ug to 1mg antibody purification protocol from 200-400ul sample with 100ul micro-spin column is described as below:

1. **Equilibration**
   - Add 400 µl binding buffer
   - 3000xg, 1-2min

2. **Sample Load**
   - All samples should be clarified by high speed centrifugation or 0.45micron filtration to remove any particles and cell debris before loading.

   - Add 200 µl sample in binding buffer
   - Incubate 5-60min end-over-end
   - 3000xg, 1-2min

3. **Wash**
   - Add 400 µl binding buffer
   - 3000xg, 1-2min

4. **Elution**
   - Add 200 µl elution buffer
   - 3000xg, 1-2min

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Eluted fractions should be neutralized immediately after elution to avoid any precipitation or denaturation under low pH buffer. Three fractions can be subjected to analysis and characterization prior to pooling.

2.3 Clean in place (CIP)

Clean in place (CIP) is the important procedures for removing very tightly bound, precipitated or denatured proteins, DNA and lipids, so as to maintain performance and capacity of the column. MabAffinity Protein L High Flow Beads allows the use of low concentration of NaOH as CIP agent, and recommended CIP procedures are as below:

**CIP procedures:**
1. Wash the column with 3 to 5 column volumes of binding buffer.
2. Backflush with 1 to 2 column volumes of CIP buffer with contact time of 10 minutes, and three commonly used CIP buffers are listed below for selection:
   - 0.1M glycine pH 2.5
   - 8M Urea
   - 10mM NaOH
3. Wash immediately with 5-10 column volumes of binding buffer at pH 7-8 to remove CIP reagents.

CIP is usually performed immediately after the elution. Before applying the alkaline NaOH CIP solution, we recommend equilibrating the column with a solution of neutral pH in order to avoid the direct contact between low-pH elution buffer and high pH NaOH solution on the column. Mixing acid and alkaline solutions might cause a rise in temperature in the column. Cleaning reagents concentration, contact time and frequency are typically the main parameters to vary during the optimization of the CIP. The nature of the feed material will ultimately determine the final CIP. However, the general recommendation is to clean the column at least every 5 cycles during normal use. Depending on the nature of the contaminants, different protocols may have to be combined, for example 10mM NaOH every cycle, and 8M Urea or 0.1M glycine pH 2.5 every 10-20 cycles. 8M Urea can remove the precipitated proteins to restore the performance for resin. 1M NaCl can be introduced into CIP regents for stabilizing the ligand under alkaline conditions.

2.4 Sanitization
Sanitization reduces microbial contamination of the chromatography column to a minimum. MabAffinity Protein L High Flow Beads allows the use of 0.1M acetate acid in 20% ethanol as sanitizing agent for sanitization.

**Sanitization procedures:**
1. Wash the column with 3 column volumes of binding buffer.
2. Wash with 0.1 M acetic acid in 20% ethanol for sanitization. Contact time of one hour is recommended.
3. Wash immediately with at least 5 column volumes of sterile and filtered binding buffer at pH 7-8.

**2.5 Trouble shooting**

**1. High column backpressure during purification**
- Disconnect the column with system and make sure no tubings or connectors in the system caused the high system pressure; always use tubings and connectors of right inner diameters
- Remove flow restrictor from systems if possible
- Calibrate the pressure sensor in your systems
- Make sure all buffers and samples be filtered through 0.22 or 0.45 micron disc membrane for clarification. For small volume sample, 10000g@10-20min centrifugation is an alternative solution
- Lower flow rate when use buffers of high viscosity or working at cold temperature, especially during sample loading
- Replace top screen net of column adapter in case of clogging
- Lower the column bed height to 20 cm or less, too high beds will cause high pressure
- Perform a thorough CIP procedure to restore the initial back pressure if column bed clogs. Unpack the column and wash media batch wise
- Increase the CIP frequency and optimize the CIP regent formulations
- Avoid freeze the medium or column during storage

**2. Poor binding or low capacity**
- Check the binding affinity of your antibodies of interest to the ligand
- Make sure the pH values of binding buffer and sample are pH 7-8
- Check if there exist some interference substances in binding buffer or samples, such as high concentration of chaotropic substances
- Lower flow rate to give a residence time of 4-8min for sample loading
- Check the history of the medium about how it has been cleaned and stored.

**3. Inefficient elution**
- Check the pH value and composition for elution buffer
- Try elution buffer of lower pH, for example pH 2.3
3. Instructions for immunoprecipitation (IP)

3.1 Introduction

Immunoprecipitation is a purification method for detection or analysis of a particular antigen by precipitation of a sedimentable matrix (e.g. Protein L Agarose resin) with the complex of the antigen and its antibody. The protein can then be examined for quantity or physical characteristics (molecular weight, isoelectric point, etc.) by SDS-PAGE and immunoblotting.

Protein L family resins play very critical role in immunoprecipitation of immune complexes formed between an antigen and its specific antibody. Immunoprecipitation procedure comprises several steps which should be optimized for specific application according to actual conditions. The protocol should be developed empirically to obtain optimal results. The following protocol is just a generic reference for immunoprecipitation using Protein L Agarose in single protein precipitation. Co-immunoprecipitation or chromatin immunoprecipitation is not included.

3.2 Buffers and solutions

Before starting immunoprecipitation, the target protein (antigen) must be extracted from the cells by proper lysis buffer, which should be prepared in advance. The lysis buffer can be mild or harsh depending on the nature of the protein.

Release of soluble proteins within cells (e.g., cytosolic or luminal organellar proteins) may
not require the use of detergents to be released from cells. Instead, cells can be mechanically disrupted by repeated passage through a needle, and soluble proteins can be separated from insoluble material by centrifugation. A PBS-based detergent-free lysis buffer is appropriate for these proteins.

Soluble and membrane proteins can be released by non-denaturing lysis buffer containing the non-ionic detergent such as Triton X-100, Nonidet P-40, CHAPS, digitonin, or octyl glucoside. But many cytoskeletal and nuclear proteins, as well as a fraction of membrane proteins are not efficiently extracted under these conditions. Extraction with non-denaturing lysis buffer allows immunoprecipitation with antibodies to epitopes that are exposed on native proteins.

If epitopes of native proteins are not accessible to antibodies, or if the antigen cannot be extracted from the cell with nonionic detergents, cells should be solubilized under denaturing conditions. Denaturation is achieved by heating the cells in a denaturing lysis buffer that contains an ionic detergent such as SDS or Sarkosyl (N-lauroylsarcosine). The denaturing lysis buffer also contains DNase I to digest DNA released from the nucleus.

ACRObiosystems’ BenzNuclease (alternative to Benzonase) is recommended to replace Dnase I to get the optimal IP results together with MabAffinity Protein L Agarose.

Table 5 Summary of Lysis buffers

<table>
<thead>
<tr>
<th>Components</th>
<th>Detergent-free Soluble</th>
<th>Soluble (low salt)</th>
<th>Detergent-soluble native</th>
<th>Soluble cytoplasmic or nuclear (RIPA)</th>
<th>Detergent-free denaturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer/Salt: 0-1M</td>
<td>PBS</td>
<td>137mM NaCl</td>
<td>150mM NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA: 0-5mM</td>
<td>5mM</td>
<td>2mM</td>
<td>5mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-ionic detergent: 0.1-2%</td>
<td>1% NP-40</td>
<td>1% NP-40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ionic detergent: 0.01-0.5%</td>
<td></td>
<td></td>
<td></td>
<td>0.1% SDS</td>
<td>1% SDS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5% sodium deoxycholate</td>
<td></td>
</tr>
<tr>
<td>Reducing reagent</td>
<td></td>
<td></td>
<td></td>
<td>10mM DTT or beta-mercaptoethanol</td>
<td></td>
</tr>
<tr>
<td>Protease inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additives</td>
<td>0.02% sodium Azid</td>
<td>10% glycerol</td>
<td>Benzonase (optional)</td>
<td>15U/ml DNase I or BenzNuclease</td>
<td></td>
</tr>
</tbody>
</table>

Reagents in lysis buffer

Protease inhibitors

In addition to place the lysis procedure at 4°C or on ice, protease inhibitor should be added freshly to the lysis buffer to slow down proteolysis, dephosphorylation and denaturation along with lysis. Some researchers strongly recommended lysing the cells in a cold room. The following protease inhibitors are commonly used. Select whatever is
effective and convenient for you to obtain.

Cocktail is commercially available. You can also prepare it yourself following the recipe.

Protease Inhibitor Cocktail (100x):
- PMSF, 5mg (50μg/ml)
- Aprotinin, 100ug (1μg/ml)
- Leupeptin, 100ug (1μg/ml)
- Pepstatin, 100ug (1μg/ml)

100% Ethanol bring up to 1ml, aliquot and keep at -20°C.

Sodium vanadate inhibits all tyrosine protein phosphatases. 200 micromolar is generally recommended. Add it fresh for each experiment from a stock made in water and store in a plastic tube at room temperature. Storage in glass leads to the appearance of precipitates.

50mM sodium fluoride is recommended as an inhibitor of Serine/Threonine protein phosphatases.

**Other reagents or solutions**
EDTA prevents phosphorylation in the lysate. 100 mM EDTA stock solution is made with 1.86 g into 40ml H₂O. Add NaOH to dissolve and adjust pH to 7.4. Finally, adjust the total volume to 50 ml.

Sterile PBS pH 7.4
Sterile PBS-BSA 1% (filtered)
Loading/sample buffer for Western blotting

Phosphate is a good buffer substance at pH 7.2 and also functions as an inhibitor of phosphatases. Tris is a poor buffer at pH 7.2 and does not inhibit phosphatases. Tris is helpful if you need to add calcium or manganese, both of which will precipitate phosphate. Otherwise, phosphate is preferable.

### 3.3 Selection of antibodies

**Polyclonal antibodies**
In terms of antigen, polyclonal antibodies can be categorized into antibodies to whole proteins (native or recombinant) and antibodies to peptides. Antibodies to whole proteins have the advantage that they frequently recognize multiple epitopes on the target antigen, enhance the avidity of the interactions and increase the efficiency of immunoprecipitation. However, the features lead to a disadvantage of higher backgrounds and possible misidentification of antigens due to cross-reaction with epitopes on other proteins. Anti-peptide polyclonal antibodies have much lower chance to cross-react with other
proteins. But their immunogenicity is limited to the specific epitopes. Moreover, the purity of polyclonal antibodies also affects immunoprecipitation results. Unfractionated antisera contain serum proteins and antibodies to other antigens, which respectively will cause non-specific binding to resin and cross-reaction with cellular proteins. Polyclonal antibodies purified from MabAffinity Family resins usually performs well. For some cases, antigen-purified antibodies are required to get better IP results.

**Monoclonal antibodies**

Monoclonal antibodies are more specific, which help to reduce background. But their less affinity may lead to failure of immunoprecipitation. Ascites may also contain endogenous antibodies to other antigens and proteins such as transferrin that can bind to other proteins in the lysate.

In conclusion, selection of antibodies for immunoprecipitation should be determined empirically or by requirement of individual experiment.

### 3.4 Procedures for IP

#### 3.4.1 Preparation of lysates

**3.4.1.1 Non-denaturing condition**

- **Cell culture: suspension cell**
  1) Collect cells in suspension by low-speed centrifugation (e.g. 200g or 400g) for 5 min at 4°C, in a 15- or 50-ml capped conical tube. Place tube on ice. Carefully pipette supernatant.

  *Approximately 0.5–2 × 10⁷ cells are required to yield 1 ml lysate, which is the amount generally used for each immunoprecipitation. Labeled cells are likely to have been pelleted earlier as part of the labeling procedure. If the cells are frozen, they should be thawed on ice before solubilization.***

  2) Wash cells twice with ice-cold PBS by resuspension and centrifugation, using the same volume of PBS as in the initial culture.

  3) Add 1 ml ice-cold lysis buffer per ~0.5–2 × 10⁷ cells and resuspend pellet by gentle agitation for 3 sec with a vortex mixer set at medium speed.

  *Do not shake vigorously, as this could result in loss of material or protein denaturation due to foaming.*

  4) Keep suspension on ice 15 to 30 min (maintain constant agitation or keep cells still according to the cell type and lysis buffer) and transfer to a 1.5-ml conical microcentrifuge tube.

  See steps 5)~6) described in the part of adherent cell.

- **Cell culture: Adherent cell**
  1) Wash cells attached to a tissue culture plate twice with ice-cold PBS. Remove the PBS with a pipette.

  *CAUTION: Dispose of radioactive materials following applicable safety regulations.*

  2) Place the tissue culture plate on ice or operate in a cold room.

  3) Add ice-cold lysis buffer to the tissue culture plate to a concentration of 10⁶–10⁷ cells/ml.
4) Scrape the cells off the plate with a cell scraper, and transfer the suspension to a 1.5-ml conical microcentrifuge tube with a pipette. Vortex gently for 3 sec and keep tubes on ice for 15 to 30 min.

Optional: Maintain constant agitation for 30 minutes at 4°C. Sonicate each sample on a 70% duty cycle or less by placing only the very tip of the pin into the vial, then slowly lowering it into the lysate until it foams completely and then stop. Alternatively, pass the lysate through a 21 gauge needle to shear the DNA & incubate 30–60 minutes on ice.

5) Clear the lysate by microcentrifuging 15 min or longer at 16,000 × g (maximum speed), 4°C.

Centrifugation can be carried out in a microcentrifuge placed in a cold room or in a refrigerated microcentrifuge. Take precautions to ensure that the 4°C is maintained during the spin (e.g., use a fixed-angle rotor with a lid, as the aerodynamics of this type of rotor reduces generation of heat by friction). The microfuges in the cold room are not satisfactory because they heat up to room temperature in 15 minutes. If it is necessary to reduce background, the lysate can be spun for 1 hr at 100,000 × g in an ultracentrifuge.

6) Transfer the supernatant to a fresh microcentrifuge tube with a pipette. Do not disturb the pellet, and leave the last 20 to 40 μl of supernatant in the centrifuge tube. Keep the cleared lysate on ice until preclearing or addition of antibody beads.

- Tissue

1) Dissect the desired tissue with clean tools on ice or in a cold room if possible, and as quickly as possible to prevent degradation by proteases.

2) Place the tissue slice in microfuge tubes and freeze it by immersing in liquid nitrogen. Preserve samples at -80°C for future use or keep at 4°C (on ice or in a cold room) for immediate extraction.

3) Add proper volume of lysis buffer rapidly to achieve a concentration of 5-20mg tissue/ml. Homogenize with a dounce homogenizer or a sonicator, maintaining temperature at 4°C throughout the treatment.

4) Centrifuge the lysis mixture for 10~20 min at 12,000 rpm at 4°C in a microcentrifuge. Pipette the supernatant and transfer to a new tube, discard the pellet. A longer centrifugation may be necessary to obtain a clear lysate. Make sure that the procedure is performed at 4°C on ice or in a cold room and the pellet is not resuspended.

3.4.1.2 Denaturing condition

- Cell culture

1) Collect cells in suspension culture or adherent culture (see the steps above). Place tubes on ice.

2) Add 100 μl denaturing lysis buffer per ~0.5-2 × 10⁷ cells in the pellet.

3) Resuspend the cells by vortexing vigorously 2 to 3 sec at maximum speed. Transfer suspension to a 1.5 ml conical microcentrifuge tube.

The suspension may be very viscous due to release of nuclear DNA. Addition of BenzNuclease is recommended to effectively reduce viscosity.
4) Heat samples for 5 min at 95°C.
5) Dilute the suspension with 0.9 ml non-denaturing lysis buffer. Mix gently.
The excess 1% Triton X-100 in the non-denaturing lysis buffer sequesters SDS into Triton X-100
micelles.
6) Shear DNA by passing the suspension five to ten times through a 25-G needle
attached to a 1-ml syringe.
If the DNA is not digested by DNase I or BenzNuclease in the denaturing lysis buffer or thoroughly
sheared mechanically, it will interfere with the separation of pellet and supernatant after centrifugation.
Repeat mechanical disruption until the viscosity is reduced to manageable levels.
7) Incubate 5 min on ice.
8) Clear the lysate and perform immunoprecipitation

3.4.2 Preparation of MabAffinity Protein L Resin
1) Centrifuge the resin at 12 000 x g for 20 seconds and discard the supernatant. Add
lysis buffer or PBS to the resin to exchange 20% ethanol. Repeat 3 times for buffer
exchange.
2) Prepare a 50% slurry by mixing equal volumes of resin and lysis buffer or PBS.
3) Store at 4 ºC and mix well before use.

3.4.3 Antibody binding to Protein L resin
In other protocols, antibody is first added to the lysate. Binding of antibody to Protein L
resin prior to lysate enables better control of the amount of antibody bound to Protein L
resin. In addition, it allows for removal of unbound antibodies and other proteins in the
antibody sample, which may interfere with the recovery of the antigen.
1) In a 1.5-ml conical microcentrifuge tube, combine 30 μl of 50% Protein L resin slurry,
0.5 ml ice-cold PBS, and the following quantity of specific antibody.
   1 to 5 μl polyclonal antiserum
   1 μg affinity-purified polyclonal antibody
   0.2 to 1 μl ascitic fluid containing monoclonal antibody
   1 μg purified monoclonal antibody
   20 to 100 μl culture supernatant containing monoclonal antibody
The quantities of antibody are estimated by expectation of normal antibody preparation. It is advisable
to determine the quantities by trial experiment.
Antibody-conjugated beads can be prepared prior to preparation of the cell lysate, to minimize the time
the cell extract is kept on ice.
2) Set up a nonspecific immunoprecipitation control in a 1.5-ml conical microcentrifuge
tube by incubating 30 μl of 50% Protein L resin slurry, 0.5 ml ice-cold PBS, and the
appropriate control antibody (select one).
   1 to 5 μl preimmune serum as a control for a polyclonal antiserum
   1 μg purified irrelevant polyclonal antibody (an antibody to an epitope that is not present in the cell
   lysate) as a control for a purified polyclonal antibody
   0.2 to 1 μl ascitic fluid containing irrelevant monoclonal antibody (an antibody to an epitope that is not
**preparation in the cell lysate and of the same species and immunoglobulin subclass as the specific antibody** as a control for an ascitic fluid containing specific monoclonal antibody

1 μg purified irrelevant monoclonal antibody as a control for a purified monoclonal antibody

20 to 100 μl hybridoma culture supernatant containing irrelevant monoclonal antibody as a control for a hybridoma culture supernatant containing specific monoclonal antibody.

3) Mix suspensions thoroughly. Tumble incubation mixtures end over end ≥1 hr (up to 24hr) at 4°C in a tube rotator.

4) Microcentrifuge 2 sec at 16,000 × g (maximum speed), 4°C.

5) Pipet the supernatant (containing unbound antibodies) and discard it.

6) Add 1 ml lysis buffer and resuspend the resins by inverting the tube three or four times.

7) Wash twice by resuspension and centrifugation with lysis buffer.

### 3.4.4 Pre-clearing (optional)

Preclearing removes nonspecifically adsorbing material by binding to Protein L resin prepared without antibody or coupled with irrelevant (nonspecific) antibody. Irrelevant antibody is an antibody directed against an unrelated protein, and could also be whole IgG; it must not cross-react with the protein being immunoprecipitated. The following steps only use Protein L resin for preclearing.

1) Add 50–100 μl of prepared Protein L resin suspension (50% slurry) to 1 ml cell lysate in an Eppendorf tube.

2) Gently mix for 1 hour at 4°C. Prolong the time if necessary.

3) Centrifuge at 12 000 x g for at least 5 min. Save the supernatant.

### 3.4.5 Immunoprecipitation

1) Add 10 μl of 10% BSA and then the clear lysate to the tube containing specific antibody bound to Protein L resin. If a nonspecific immunoprecipitation control is performed, divide lysate in two ~0.4-ml aliquots, one for the specific antibody and the other for the nonspecific control.

In order to avoid carryover of beads with precleared material, leave 20 to 40 μl of supernatant on top of the pellets in the preclearing tubes. Discard beads and remaining supernatant. The BSA blocks nonspecific binding sites on the antibody-conjugated beads during incubation with the cell lysate.

2) Incubate 1 to 2 hr at 4°C while mixing end over end in a tube rotator.

3) Microcentrifuge 5 sec at 16,000 × g, 4°C.

4) Pipet the supernatant (containing unbound proteins).

5) Add 1 ml ice-cold wash buffer, cap the tubes, and resuspend the beads by inverting the tube 3 or 4 times.

6) Microcentrifuge 2 sec at 16,000 × g, 4°C.

7) Aspirate the supernatant, leaving 20 μl supernatant on top of the beads.

8) Wash beads three more times as the above method.

9) Wash beads once more using 1 ml ice-cold PBS and aspirate supernatant completely with a pipette.
10) Analyze immunoprecipitates by one- or two-dimensional electrophoresis, or immunoblotting.

3.4.6. Dissociation and analysis
1) Suspend the final pellet in 30 μl SDS-PAGE sample buffer.
2) Heat to 95 °C for 3 minutes.
3) Centrifuge at 12,000 x g for 20 seconds to remove the resins. Carefully remove the supernatant.
4) Analyse the supernatant by SDS-PAGE, followed by protein staining and/or immunoblotting for detection. Radiolabelled antigens are detected by autoradiography.

3.5 Trouble shooting for IP

1. No or scarce target antigen is detected.
Possible reasons:
Antigen expressed at very low levels.
Cell lysis is incomplete due to inappropriate conditions.
Epitope is not exposed in native antigen.
Antibody does not bind to denatured antigen.
Antigen is degraded during the process.
Antibody concentration is too low to precipitate antigen.
Antibody does not bind to the resin.
Problems occur in the detection assay.

Solutions:
- Transfect cells for higher expression level by replacing the previous cell or optimization.
- Adjust the components of the lysis buffer.
- Use denaturing lysis buffer instead of non-denaturing buffer and vice versa.
- Ensure that fresh protease inhibitor is added and perform the procedure at 4°C on ice or in a cold room.
- Increase concentration of precipitating antibody.
- Identify and use antibody that precipitates antigen. Otherwise, change the resin.
- Check the steps of the detection assay.

2. High background of nonspecific bands
Possible reasons:
Incomplete washing or preclearing
Incomplete removal of detergent-insoluble proteins
Antibody contains aggregates.
Antibody solution contains nonspecific antibodies.
Too much antibody
Nonspecifically immunoprecipitated proteins

**Solutions:**
- Prolong the washing time after immunoprecipitation. Increase the stringency of the washes. For example, wash with a buffer containing a higher concentration of detergent or salt.
- Preclear with Protein L resin or the resin coupled with irrelevant antibody.
- Centrifuge lysate 30 min to 1 hr at 100,000 × g to remove aggregated proteins before binding to the resin.
- Use affinity-purified antibodies; adsorb antibody with acetone extract of cultured cells that do not express antigen; for yeast cells, adsorb antibody with null mutant cells. Use less antibody.
- Add saturating amount of competitive protein (i.e. BSA, gelatine, acetone powders). Fractionate cell lysate (e.g. ammonium sulfate precipitation, lectin absorption, or gel filtration) prior to immunoprecipitation; after washes in wash buffer, wash beads once with 0.1% SDS in wash buffer or 0.1% SDS/0.1% sodium deoxycholate.

**4, Storage**

Store unused media in its container at a temperature of 2 to 8°C for long term storage. Ensure that the container is closed and fully tightened.

Equilibrate packed columns with 5-10 column volumes of 20% ethanol to prevent microbial growth.

A thoroughly CIP procedure is recommended before long term storage. After storage, equilibrate with binding buffer and ready for use.

Never freeze the media in case of generation of fine particles to increase the back pressure.

**5, Ordering Information**

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