Direct Immunoprecipitation

Covalent Couple Antibody to Agarose
to Prevent Interference

(Cat. # 786-636)
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INTRODUCTION

Immunoprecipitation (IP) is one of the most useful immunochemical techniques. IPs are routinely used to determine the presence and quantity of an antigen, molecular weight of a polypeptide, rate of synthesis or degradation, identify certain post translational modifications and interactions with other proteins, nucleic acids and ligands. IPs consist of four main steps:

1. Labeling of the antigen (Optional step)
2. Release of antigen by cell lysis
3. Formation of antibody-antigen complexes

G-Biosciences Direct Immunoprecipitation kit contains all the reagents necessary to complete all aspects of immunoprecipitation, with the exception of labeling. The kit utilizes our Amine Reactive HOOK™ Activated Agarose to covalently couple the antibody. The covalently coupled antibody is then retained on the agarose during elution preventing contamination of the immunoprecipitated antigen by the antibody. The kit is designed for effective immunoprecipitations using <10µg antibody. The kit is suitable for 50 reactions using 10µl immobilized antibody.

ITEM(S) SUPPLIED (Cat. # 786-636)

<table>
<thead>
<tr>
<th>Description</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection Tube, 2ml</td>
<td>100</td>
</tr>
<tr>
<td>Control Agarose (4% agarose)</td>
<td>2ml</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>5ml</td>
</tr>
<tr>
<td>HOOK Activated Agarose (Amine Reactive)</td>
<td>2ml</td>
</tr>
<tr>
<td>Mammalian Cell PE LB™</td>
<td>100ml</td>
</tr>
<tr>
<td>IP Coupling Buffer [20X]</td>
<td>25ml</td>
</tr>
<tr>
<td>Quenching Buffer</td>
<td>25ml</td>
</tr>
<tr>
<td>Sodium Cyanoborohydride (NaCNBH₃) [5M]</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Wash Solution</td>
<td>2 x 60ml</td>
</tr>
<tr>
<td>Spin Column</td>
<td>50</td>
</tr>
<tr>
<td>Caps (Micro, Screw Cap)</td>
<td>50</td>
</tr>
</tbody>
</table>

STORAGE CONDITIONS

It is shipped at ambient temperature. Upon arrival, store at 4°C. Once opened, store the sodium cyanoborohydride at room temperature with a dessicant.
ADDITIONAL ITEM(S) REQUIRED

- 1X PBS
- Centrifuge tubes (1.5ml)

IMPORTANT INFORMATION

- Perform all immunoprecipitation steps at 4°C, unless stated otherwise. Coupling of antibody to resin to performed at room temperature.
- Amines present in the buffers will compete for the amine reactive binding sites on the HOOK™ Activated Agarose. Avoid buffers that have primary amines, including Tris and glycine. These can be removed by buffer-exchange with our SpinOUT™ columns or Tube-O-DIALYZER™ dialysis devices.
- Gelatin, BSA or other proteins in the antibody solution will also compete. We recommend cleaning up the antibody with our Pearl™ Antibody Clean Up kit (Cat. # 786-803).
- Perform centrifugations for 60 seconds at 1,000-2,000xg. Excessive speeds or times may result in resin clumping making it difficult to resuspend the resin.
- We recommend using an affinity purified antibody as serum, although compatible with this kit, will result in a lower antigen yield due to reduced specific antibody binding.
- For optimal results, we recommend including a protease inhibitor and phosphatase inhibitor cocktail in the lysis buffer. ProteaseArrest™ (Cat. # 786-108) and PhosphataseArrest™ (Cat. # 786-450) are recommended.
PROTOCOL

Coupling Antibody to HOOK™ Activated Agarose

- **CAUTION:** The pH of all buffers used should be greater than pH 7.0 to prevent the release of toxic gas. **DO NOT ACIDIFY SOLUTIONS.**
- **WARNING:** Sodium cyanoborohydride is toxic, open tubes and prepare solutions in a fume hood. Wear gloves.
- To ensure complete coupling, we advise using the recommended quantities of reagents, as these provide a molar excess of protein or peptide to the reactive groups on the agarose. Ensure complete and thorough mixing during shaking of the agarose to prevent beads from drying out.

1. Equilibrate the resin and reagents to room temperature.
2. Prepare 2ml 1X IP Coupling Buffer for each immunoprecipitation (0.1ml 20X IP Coupling Buffer in 1.9ml deionized water).
3. Gently resuspend the HOOK™ Agarose by inverting the bottle 2-3 times. Once a homogeneous slurry is formed, use a wide bore pipette tip to remove 20µl slurry and place in a 1.5ml centrifuge tube. Briefly centrifuge for 60 seconds at 1,000-2,000xg and remove the storage buffer.
4. Add 200µl 1X IP Coupling Buffer to the column, centrifuge for 60 seconds at 1,000-2,000xg and discard the supernatant. Repeat wash step one more time.
5. Prepare 2-10µg affinity purified antibody by adjusting the volume to 200µl with 20X IP Coupling Buffer and deionized water. For example, for 10µl antibody add 10µl 20X IP Coupling Buffer and 180µl deionized water. Add directly to the resin. **CAUTION:** THE FOLLOWING STEP MUST BE PERFORMED IN A FUME HOOD
6. Add 3µl sodium cyanoborohydride solution for every 200µl antibody: resin mix.
7. Seal the tube and incubate at room temperature for 2 hours with end-over-end mixing.
8. Centrifuge for 60 seconds at 1,000-2,000xg and remove and save the supernatant to determine antibody coupling.
9. Add 200µl 1X IP Coupling Buffer to the resin, centrifuge for 60 seconds at 1,000-2,000xg and then discard the supernatant. Repeat wash step one more time.
10. Add 200µl Quenching Buffer to the resin, centrifuge for 60 seconds at 1,000-2,000xg and discard the flow through.
11. Add 200µl Quenching Buffer to the resin. **CAUTION:** THE FOLLOWING STEP MUST BE PERFORMED IN A FUME HOOD
12. Add 3µl sodium cyanoborohydride to every 200µl Quenching Buffer. Seal the tube and incubate at room temperature for 15 minutes with end-over-end mixing.
13. Centrifuge for 60 seconds at 1,000-2,000xg and remove the supernatant.
14. Add 200µl 1X IP Coupling Buffer to the resin, centrifuge for 60 seconds at 1,000-2,000xg and discard the supernatant. Repeat wash step one more time.
15. Add 400µl Wash Solution to the resin and transfer to a spin column.
16. Snap off the bottom end cap and retain for sealing the spin column. Centrifuge for 60 seconds at 1,000-2,000xg and discard the flow through. Repeat wash step five more times. Proceed to the immunoprecipitation step.

**NOTE:** For storage of resin, wash twice with 200µl 1X IP Coupling Buffer and store at 4°C in 200µl 1X IP Coupling Buffer. For long term storage supplement with 0.02% sodium azide.

**Lysis of Cell Suspensions**

1. Pellet the cells by centrifugation at 3,000x g for 5 minutes. Remove and discard the supernatant
2. Wash the cell pellet once with 5-10ml PBS. Pellet the cells again by centrifugation. Remove and discard the PBS wash.
3. Add ice cold Mammalian Cell PE LB™ and suspend the cell pellet. Incubate on ice for 10-30 minutes with periodic mixing. Add 10µl Mammalian Cell PE LB™ for every 1mg wet cell pellet. For large wet cell pellets add ~10% the final volume of Mammalian Cell PE LB™, vortex to suspend the pellet and then add the remaining Mammalian Cell PE LB™.

**NOTE:** Freeze/thaw cycles are not necessary for lysis; however, one or two freeze/thaw cycles are not detrimental to the cell extract, and often ensure complete lysis.

4. Centrifuge the suspension at 20,000x g for 30 minutes in a refrigerated centrifuge to pellet the cell debris. Collect the clear supernatant in a fresh tube and perform a protein concentration assay.

**Lysis of Adherent Mammalian Cells**

1. Remove the culture medium from the adherent cells.
2. Wash the cells once with PBS. Remove the PBS wash.
3. Add an appropriate volume of ice cold Mammalian Cell PE LB™ (see table) to cover the culture surface area.

<table>
<thead>
<tr>
<th>Plate Size</th>
<th>Volume of Mammalian Cell PE LB™</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>50-100µl/well</td>
</tr>
<tr>
<td>24-well plate</td>
<td>100-200µl/well</td>
</tr>
<tr>
<td>6-well plate</td>
<td>200-400µl/well</td>
</tr>
<tr>
<td>60mm culture plate</td>
<td>250-500µl</td>
</tr>
<tr>
<td>100mm culture plate</td>
<td>500-1,000µl</td>
</tr>
</tbody>
</table>

4. Incubate on ice for 5-10 minutes with periodic mixing.
5. Transfer the lysate to a micro centrifuge tube. Centrifuge the suspension at 20,000x g for 30 minutes in a refrigerated centrifuge to pellet the cell debris. Collect the clear supernatant in a fresh tube and perform a protein concentration assay.
Preclear the Lysates

1. For every 1mg lysate, transfer 80µl homogenous Control Agarose Slurry to the lysate and seal the tube.
2. Incubate at 4°C for 30-60 minutes with mixing.
3. Centrifuge the tube for 60 seconds at 1,000-2,000xg and transfer the supernatant to a clean tube.

Immunoprecipitation Procedure

NOTE: The amount of lysate required and the length of incubation require optimization for each specific antibody-antigen system used. The following protocol uses the recommend 2-10µg affinity purified antibody.

1. Wash the antibody coupled resin with 200µl ice cold Mammalian Cell PE LB™ and discard the flow through. Repeat the wash step once. Seal the bottom of the spin column.
2. Dilute 500-1000µg cell extract with ice cold Mammalian Cell PE LB™ to a final volume of 300-600µl.
3. Add the diluted cell extract to the spin column. Cap the top of the column and incubate at 4°C for 1 hour to overnight with end-over-end mixing.
4. Remove the bottom stopper and remove the cap from the spin column. Place the column in a 2ml collection tube and centrifuge for 60 seconds at 1,000-2,000xg. Collect the flow through and save until successful IP has been achieved.
5. Transfer the spin column to a clean tube and add 200µl Mammalian Cell PE LB™ and centrifuge for 60 seconds at 1,000-2,000xg. Repeat the wash three more times.

Immune Complex Elution

1. Place the spin column in a fresh collection tube and add 25µl Elution Buffer and centrifuge for 60 seconds at 1,000-2,000xg to collect the eluate.
2. Keep the column in the same collection tube and add 75µl Elution Buffer. Incubate at room temperature for 10 minutes.
3. Centrifuge for 60 seconds at 1,000-2,000xg to collect the eluate. Perform additional elutions as needed and analyze eluates separately to ensure complete elution of antigen.
4. To neutralize the eluates, add 5µl 1M Tris, pH9.5 to the collection tubes.
**Resin Regeneration**

1. Add 100µl 1X IP Coupling Buffer to the column, centrifuge for 60 seconds at 1,000-2,000xg and discard the flow through. Repeat wash step one more time. Seal the bottom of the column with the supplied plug.

2. For storage of resin, add 200µl 1X IP Coupling Buffer supplement with 0.02% sodium azide and store at 4°C.

3. Keep the column in the same collection tube and add 75µl Elution Buffer. Incubate at room temperature for 10 minutes.

4. Centrifuge for 60 seconds at 1,000-2,000xg to collect the eluate. Perform additional elutions as needed and analyze eluates separately to ensure complete elution of antigen.

5. To neutralize the eluates, add 5µl 1M Tris, pH9.5 to the collection tubes.
## TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Issue</th>
<th>Possible Cause</th>
<th>Suggested Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody or antibody fragments detected with antigen</td>
<td>Insufficient washing resulting in unbound antibody remaining.</td>
<td>Increase number of washes and wash the antibody bound resin with elution buffer until no protein is detected.</td>
</tr>
<tr>
<td></td>
<td>Presence of reducing agents in lysis or wash buffers fragmented the antibody</td>
<td>Do not use buffers containing reducing agents.</td>
</tr>
<tr>
<td>No immunoprecipitation of antigen</td>
<td>Antigen levels to low to detect</td>
<td>Check protein expression and lysis efficiency by Western blotting</td>
</tr>
<tr>
<td></td>
<td>Antibody not binding to the antigen</td>
<td>Try fresh aliquot of antibody, or a different antibody against the same antigen</td>
</tr>
<tr>
<td></td>
<td>Components in Mammalian Cell PE LB™ may interfere with antigen-antibody interaction</td>
<td>Perform IP and washes in 1X TBS or 1X PBS</td>
</tr>
<tr>
<td>Antigen failed to elute</td>
<td>Elution buffer not sufficient to disrupt antigen:antibody interaction</td>
<td>Optimize and vary the elution conditions. Vary salt concentrations and pH.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elute using 1X Non-Reducing SDS PAGE sample loading buffer. Add to spin column, incubate 100°C for 5-10 minutes. Spin to recover. Note: Resins must be discarded after this elution type.</td>
</tr>
<tr>
<td>No functional activity of antigen in downstream applications</td>
<td>The low pH elution may have inactivated the antigen</td>
<td>Elute with a high salt and neutral pH elution buffer.</td>
</tr>
</tbody>
</table>
RELATED PRODUCTS

1. **Classical Immunoprecipitation** (Cat. # 786-637) Utilizes Protein A/G immobilized on agarose to bind antibody to immunoprecipitated proteins of interest.

2. **Cross-Link Immunoprecipitation** (Cat. # 786-639) Utilizes Protein A/G immobilized on agarose to bind antibody to immunoprecipitated proteins of interest. The supplied DSS protein cross-linker covalently couples the antibody to the resin removing downstream interference by the antibody.

3. **Co-Immunoprecipitation** (Cat. # 786-638) Utilizes our Amine Reactive HOOK™ Activated Agarose to covalently couple the antibody. The covalently coupled antibody is then retained on the agarose during elution preventing contamination of the immunoprecipitated antigen by the antibody. Used to study protein-to-protein interactions.

For other related products, visit our website at [www.GBiosciences.com](http://www.GBiosciences.com) or contact us.

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