Pierce® Co-Immunoprecipitation (Co-IP) Kit

Number Description
23600 Pierce Co-Immunoprecipitation (Co-IP) Kit, contains sufficient reagents to immobilize 10 primary antibodies and to perform 40 Co-IPs when using 25 μl of Antibody Coupling Resin

Kit Contents:
- Antibody Coupling Resin (AminoLink® Plus Resin), 2 ml of settled resin supplied as a 50% slurry (e.g., 400 μl of 50% slurry is equivalent to 200 μl of settled resin)
- Pierce Control Agarose Resin, Crosslinked 4% Beaded Agarose, 2 ml of settled resin supplied as a 50% slurry (e.g., 400 μl of 50% slurry is equivalent to 200 μl of settled resin)
- Coupling Buffer (BupH™ Modified Dulbecco’s PBS), 2 packs, each pack results in 0.14 M sodium chloride, 0.008 M sodium phosphate, 0.002 M potassium phosphate and 0.01 M KCl, pH 7.4 when reconstituted with 500 ml of ultrapure water
- Quenching Buffer, 50 ml, 1 M Tris•HCl, pH 7.4
- Wash Solution, 50 ml, 1 M NaCl
- Sodium Cyanoborohydride Solution (5 M), 0.5 ml
- Spin Cup Columns, 50 each, columns contain 0.45 μm cellulose acetate filters
- Microcentrifuge Collection Tubes, 144 each
- IgG Elution Buffer, 50 ml, pH 2.8
- Lane Marker Sample Buffer, Non-reducing (5X), 5 ml, contains 0.3 M Tris•HCl, 5% SDS, 50% glycerol, lane marker tracking dye; pH 6.8

Storage: Upon receipt store kit at 4°C. This kit is shipped at ambient temperature.

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Introduction

The Thermo Scientific Pierce Co-Immunoprecipitation (Co-IP) Kit allows for the isolation of native protein complexes from a lysate or other complex mixture. Co-IP is a common approach to the study of protein:protein interactions that uses an antibody to immunoprecipitate the antigen (bait protein) and co-immunoprecipitate any interacting proteins (prey proteins). The kit contains an amine-reactive resin, reagents for direct covalent immobilization of the primary antibody, reagents to perform control experiments, buffers for protein binding and recovery, and Spin Cup Columns for ease of use and maximum recovery of captured proteins without resin loss.

Traditional Co-IP methods that use Protein A or G result in detection of the antibody with the target proteins. Because the antibody heavy and light chains may co-migrate with one of the relevant bands, important results can be masked. The Pierce Co-IP Kit resolves this issue by retaining the antibody on the resin. Furthermore, the entire procedure is performed in a spin column, isolating the protein complex and allowing quick Co-IP completion with consistent results.

Important Product Information

- The antibody solution must not contain amines (e.g., Tris or glycine) as they will compete for coupling sites. Remove amines before coupling by dialysis using Thermo Scientific Slide-A-Lyzer Cassettes (Product No. 66382) or MINI Dialysis Units (Product No. 69576) or by desalting thoroughly with desalting spin columns (Product No. 89889).
- The antibody solution must not contain gelatin or other carrier proteins as they compete for coupling sites, thereby reducing binding capacity and causing nonspecific binding. Remove gelatin and carrier proteins using Protein A or Protein G purification and subsequent dialysis against PBS. Alternatively, inquire with the antibody vendor if a carrier-free antibody is available.
- Use of proper controls is vital for identifying relevant interactions. This kit provides the necessary components to perform several types of controls. For a discussion of control experiments, refer to the Appendix.
- Perform all steps at room temperature unless otherwise indicated. The steps may be performed at 4°C but will require more time for completion.
- Perform all centrifugation steps for 1 minute at medium speed (i.e., 3,000-5,000 \( \times \) g). Centrifuging at greater speeds may cause the resin to clump and make resuspending the resin difficult.
- To reduce nonspecific binding add Thermo Scientific SurfactAmps X-100 (10% Triton® X-100, Product No. 28314) to the Coupling Buffer at 0.1-1%. Note: SurfactAmps® X-100 is not supplied with the kit.
- The spin columns are supplied with screw caps, column plugs, Luer-Lok™ Adapter Caps, large frits and a large frit tool. The large frit is not needed for the standard IP protocol. When scaling-up the IP reaction (i.e., > 200 \( \mu l \) of resin), the large frit can be inserted into the column to facilitate washing. The Luer-Lok Caps have a flip top that may be used during wash steps. Use the screw caps for sealing the spin columns during storage.
- For optimal results for first-time use, couple at least 200 \( \mu g \) of purified antibody to 100 \( \mu l \) of settled resin and perform Co-IP reactions with decreasing amounts of antibody-coupled resin. Resin amounts to use are indicated in Table 1.

<table>
<thead>
<tr>
<th>Coupling Resin Slurry Volume (( \mu l ))</th>
<th>Coupling Resin Volume (( \mu l ))</th>
<th>Antibody Amount (( \mu g ))</th>
<th>Antibody Volume (( \mu l ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>100</td>
<td>200-1,000</td>
<td>400</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>100-500</td>
<td>100-200</td>
</tr>
</tbody>
</table>

*Using these guidelines results in a coupling efficiency of \(~85\%\) after 4 hours. Coupling efficiency can be approximated by measuring the absorbance of the antibody solution at 280 nm before and after coupling.

Procedure for Co-Immunoprecipitation

A. Antibody Immobilization

1. Equilibrate the Antibody Coupling Resin and reagents to room temperature. Dissolve Coupling Buffer in the appropriate quantity of ultrapure water. To store excess buffer, add a preservative such as 0.02% sodium azide and store at 4°C.

2. Gently swirl the bottle of Antibody Coupling Resin to obtain an even suspension. Add the 50% resin slurry into a Spin Cup Column (see Table 1 for amount guidelines). Place column inside a microcentrifuge tube and centrifuge.
3. Remove the spin cup from the microcentrifuge tube and empty the tube. Place spin cup back into the tube.
4. Wash resin by adding 0.4 ml of Coupling Buffer to the spin cup containing the resin. Cap the tube and suspend resin by inverting and gently shaking the tube. Centrifuge the tube.
5. Remove spin cup from the tube and empty the tube. Place spin cup back into the microcentrifuge tube.
6. Repeat Steps 4 and 5.
7. Place spin cup into new microcentrifuge tube. Dilute the purified antibody specific for the bait protein in Coupling Buffer (see Table 1 for coupling guidelines). Add the purified antibody to the spin cup containing the resin.
   **Note:** The antibody solution must not contain primary amines (e.g., Tris or glycine) or carrier proteins (see Important Product Information section).
8. In a fume hood, add 1 µl of 5 M Sodium Cyanoborohydride for every 100 µl of diluted purified antibody being coupled. Close the cap and invert tube five times.
   **Note:** Sodium cyanoborohydride is toxic. Wear gloves and use caution when handling.
9. Incubate tube for 4 hours to overnight with gentle end-over-end mixing. Centrifuge the tubes.
   **Note:** When using more antibody than recommended or if the resin does not suspend easily, swirl resin with a small pipette tip being careful not to puncture the filter.
10. Place spin cup into a new tube and add 0.4 ml of Coupling Buffer. Cap and invert tube end-over-end 10 times. Centrifuge the tube. If desired, save the wash to approximate binding efficiency.
11. Add 0.4 ml Quenching Buffer, invert 10 times and centrifuge. Discard the flow-through buffer. Add 0.4 ml Quenching Buffer to the resin.
12. In a fume hood, add 4 µl 5 M Sodium Cyanoborohydride, close caps and invert five times. Incubate for 30 minutes with end-over-end mixing. Centrifuge the tubes.
13. Wash resin four times with 0.4 ml Wash Solution and two times with 0.4 ml Coupling Buffer.
14. Store antibody-coupled resin and any controls in 0.4 ml Coupling Buffer at 4°C. For long-term storage (i.e., more than two weeks), add sodium azide to a final concentration of 0.02%.

**B. Co-Immunoprecipitation of Protein Complex**

**Note:** The amount of bait:prey complex required and incubation time depends upon the antibody-bait protein system used and affinity of the protein interaction and must be optimized for each system.
1. Create the bait:prey complex and appropriate experimental controls (see Appendix) with the bait and prey proteins.
2. Dilute the bait:prey complex and controls in Coupling Buffer if necessary. Recommended total sample volume in the spin cup is 0.1-0.3 ml.
3. Centrifuge to remove the Coupling Buffer used to store the antibody-coupled support.
4. Place spin cups into new microcentrifuge tubes. Add the bait:prey complex and controls to the appropriate resin in the spin cups. Incubate with gentle end-over-end mixing or rocking for 1-2 hours.
   **Note:** It may be necessary to optimize the binding time for each application. For large sample volumes, transfer the antibody-coupled resin to a separate tube containing the protein complex. After incubation, centrifuge 0.5 ml aliquots through the spin cup until the entire sample has been processed.
5. Centrifuge the tubes. Discard (or save for future analysis) the flow-through collected in the microcentrifuge tubes.
6. Place spin cups into new tubes and add 0.4 ml of Coupling Buffer. Invert tubes 10 times and centrifuge. Repeat this step two more times.
   **Note:** Evaluate the washes (e.g., A280, SDS-PAGE or Micro BCA™ Protein Assay) to determine the optimal number of washes for the specific system. There should be no protein in the final wash fraction. Additional washing may be necessary for samples containing high-protein concentrations.
C. Elution of Co-Immunoprecipitation Complex

1. Add the appropriate volume of Elution Buffer to the resin in the spin cup (Table 2), cap the tube and gently tap to mix. Centrifuge the tube.

   **Note:** Before using the purified material in functional applications, neutralize the pH of the eluted sample. The Elution Buffer has a pH of 2.5-3.0 and can be neutralized by adding 10 µl of 1 M Tris, pH 9.5 per 200 µl of Elution Buffer. Alternatively, if the protein or antibody is sensitive to low pH, use a neutral pH system such as Gentle Elution Buffer (Product No. 21027).

<table>
<thead>
<tr>
<th>Elution Buffer</th>
<th>Antibody-Coupled Resin Slurry</th>
<th>Antibody-Coupled Settled Resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (µl)</td>
<td>Volume (µl)</td>
<td>Volume (µl)</td>
</tr>
<tr>
<td>200</td>
<td>400</td>
<td>200</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

2. Repeat Step 1 until the samples are eluted. Protein generally elutes within the first three fractions. Do not pool fractions. Assess the amount of protein in the first three fractions by SDS-PAGE. Use Thermo Scientific Imperial Stain Reagent (Product No. 24615 or 24617) for fast results without the need to destain.

3. Immediately following the last elution step, proceed to Section D, Regeneration of Resin and Storage Conditions. Proceeding immediately to Section D will extend the life of the antibody-coupled resin.

D. Regeneration of Resin and Storage Conditions

1. Add 0.4 ml of Coupling Buffer to the spin cup columns. Cap the tubes and gently invert 10 times.
2. Centrifuge the tubes. Empty the microcentrifuge tubes.
3. Repeat Steps 1 and 2 two additional times.
4. Add 0.4 ml of Coupling Buffer to the spin cup. Place the capped spin cups in a microcentrifuge tube and store at 4°C. For long-term storage (i.e., > two weeks) add sodium azide to a final concentration of 0.02% and wrap the capped spin cups with laboratory film to prevent the resin from drying.

E. Preparation of Samples for SDS-PAGE Analysis

1. Add 20 µl of the sample to a microcentrifuge tube.
2. Equilibrate Lane Marker Sample Buffer (dark pink solution) to room temperature. Gently mix the sample buffer by inverting the tube 5-10 times. Add 5 µl of the 5X sample buffer to the sample.

   **Note:** The Lane Marker Sample Buffer is viscous and may require that the pipette tip be “snipped” to allow the solution to be drawn up into the tip. Move pipette up and down to mix. The sample buffer does not contain reducing agents. To prepare the sample for a reducing gel, add 2-3 µl of 1 M DTT (154.25 Da) to the 25 µl sample containing sample buffer and mix well. Other sample buffers may also be used.

3. Heat sample at 95-100°C for ~5 minutes.
4. Allow sample to cool to room temperature and apply to the gel for electrophoresis.

**Related Thermo Scientific Products**

- 25200-25244 Precise™ Protein Gels (see catalog or web site for a complete listing)
- 28314 SurfactAmps X-100 (10% solution of Triton X-100), 6 x 10 ml
- 20291 DTT, No-Weigh™ Format, 48 microtubes each containing 7.7 mg of DTT
- 69702 Spin Cup Columns, 50 units, columns contain 0.45 µm cellulose acetate filters
- 69720 Microcentrifuge Tubes, 2 ml, 72 tubes
- 21004 IgG Elution Buffer, 1 L
**Troubleshooting**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody is detected along with eluted protein complex</td>
<td>Uncoupled antibody was not removed sufficiently with Wash Solution during the coupling procedure</td>
<td>Wash the antibody-coupled resin with Elution Buffer until no additional antibody elutes from the resin (as determined by protein assay or measuring the absorbance at 280 nm)</td>
</tr>
<tr>
<td>Proteins are detected in the control experiment</td>
<td>Proteins bind nonspecifically to the coupling resin</td>
<td>Increase the number of washes before eluting or add Triton X-100 to the Co-IP buffer to decrease nonspecific binding</td>
</tr>
<tr>
<td>Bait protein is not captured from sample</td>
<td>Antibody is not coupled to the resin or an insufficient amount of antibody is coupled to the resin causing low protein binding</td>
<td>Check flow-through and wash fractions by measuring the absorbance at 280 nm to verify that antibody is coupled to the resin</td>
</tr>
<tr>
<td>Antibody is sensitive to low pH and has become inactive during the elution steps (rare)</td>
<td></td>
<td>Use a more sensitive detection method</td>
</tr>
<tr>
<td>Antibody does not recognize native bait protein (common with antibodies made against peptides)</td>
<td></td>
<td>Prepare more antibody-coupled resin and use a high-salt, neutral pH elution buffer such as Gentle Ag/Ab Elution Buffer (Product No. 21027)</td>
</tr>
<tr>
<td>Antibody is sensitive to amine coupling (occasionally a problem with monoclonal antibodies)</td>
<td></td>
<td>Increase antibody amount, reduce coupling time or try a different antibody</td>
</tr>
<tr>
<td>The bait protein does not elute from the antibody using acidic conditions</td>
<td></td>
<td>Use Gentle Elution Buffer (Product No 21027), lithium bromide, guanidine-HCl**, urea**, potassium thiocyanate** or nonionic detergents to elute antigen</td>
</tr>
<tr>
<td>Bait protein is captured but no interacting prey proteins are detected</td>
<td>Protein:protein interactions are weak and cannot withstand the washing protocol</td>
<td>Use another method, such as label transfer (Product No 33033), to capture weak or transient interactions</td>
</tr>
<tr>
<td>Antibody binds only to the non-complexed protein because of shared binding sites or conformational changes</td>
<td></td>
<td>Try Co-IP using antibodies that recognize different epitopes on the bait protein</td>
</tr>
<tr>
<td>Co-IP buffer conditions do not promote protein:protein interaction</td>
<td></td>
<td>Specific ions, cofactors, etc. may need to be added to the Co-IP Buffer to promote the interaction</td>
</tr>
<tr>
<td>There is no protein complex involving the bait protein</td>
<td></td>
<td>None</td>
</tr>
</tbody>
</table>

*Interacting protein may be obscured in the gel analysis by nonspecific protein bands. Incubating the sample with Control Resin to capture any proteins that bind to the resin before performing the Co-IP reaction can often eliminate this potential source of interference.

**Using denaturants disrupts antibody structure and, therefore, the antibody-coupled resin cannot be reused.*
Appendix

A. Control Experiments

When the Co-IP results are analyzed by SDS-PAGE, several protein bands may appear indicating possible interacting proteins; however, these bands may also be caused by nonspecific interactions with the resin matrix. Use of proper controls is vital for identifying relevant interactions. This kit provides the necessary components to perform several types of controls.

Any of the following controls may be performed along side the Co-IP. When results are analyzed by SDS-PAGE, bands that appear in both the Co-IP and control lanes represent nonspecific interactions and may be disregarded.

- **Control Resin:** The supplied Control Resin is composed of the same support material as the Co-IP resin, but it is not activated. This resin provides an excellent negative control when processed the same as the Antibody Coupling resin.

- **Quenched Antibody Coupling Resin:** Create a quenched resin control by adding 400 μl of Quenching Buffer to the Antibody Coupling Resin instead of the antibody and continue with the standard procedure.

- **Non-relevant antibody:** Couple an unrelated antibody to the Antibody Coupling Resin and continue with the standard procedure.

B. Additional Applications

- **Using prey and bait proteins from different samples:** In addition to studying native protein complexes, this kit can be used for Co-IP using a bait protein from one sample and a prey protein from another sample. In this case, the bait and prey proteins are co-purified after they are allowed to interact with each other in solution, or the bait protein is coupled directly to the antibody coupling resin and then used to capture the prey protein. When the bait and prey proteins exist in separate samples a different control experiment may be used; the prey protein may be incubated with the antibody-coupled resin in the absence of bait protein. Because the prey protein should not bind in the absence of bait protein, any protein bands recovered in this control experiment can be considered a nonspecific interaction and disregarded.

- **Protein:protein interaction disruption:** Attempts to disrupt protein:protein interactions are often performed. The ability to disrupt a complex indicates the degree of specificity to the interaction and provides some insight into the mode of interaction of the two proteins. Disrupting a protein complex is often accomplished by increasing the ionic strength of the buffer, adding a detergent to the buffer or removing a cofactor that is required for the binding interaction.

References


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