

# Pierce<sup>®</sup> Mammalian Co-Immunoprecipitation Kit

23605

1452.4

| Number | Description   |
|--------|---|
| 23605  | <p><b>Pierce Mammalian Co-Immunoprecipitation (Co-IP) Kit</b>, contains sufficient reagents to immobilize 10 primary antibodies and to perform 40 Co-IPs when using 25 µl of Antibody Coupling Gel</p> <p><b>Kit Contents:</b></p> <p><b>Antibody Coupling Gel (AminoLink<sup>®</sup> Plus Resin)</b>, 2 ml of settled gel supplied as a 50% slurry (e.g., 400 µl of 50% slurry is equivalent to 200 µl of settled gel)</p> <p><b>Control Gel (Crosslinked 4% Beaded Agarose)</b>, 2 ml of settled gel supplied as a 50% slurry (e.g., 400 µl of 50% slurry is equivalent to 200 µl of settled gel)</p> <p><b>Coupling Buffer and Co-IP Buffer (BupH<sup>™</sup> Modified Dulbecco's PBS)</b>, 2 packs, each pack results in 0.14 M NaCl, 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</p> <p><b>Quenching Buffer</b>, 50 ml, 1 M Tris•HCl, pH 7.4</p> <p><b>Wash Solution</b>, 50 ml, 1 M NaCl</p> <p><b>Sodium Cyanoborohydride Solution (5 M)</b>, 0.5 ml</p> <p><b>Spin Cup Columns</b>, 50 each, columns contain 0.45 µm cellulose acetate filters</p> <p><b>Microcentrifuge Collection Tubes</b>, 144 each</p> <p><b>Elution Buffer (IgG Elution Buffer)</b>, 50 ml, pH 2.8</p> <p><b>Lane Marker Sample Buffer, Non-reducing (5X)</b>, 5 ml, contains 0.3 M Tris•HCl, 5% SDS, 50% glycerol, lane marker tracking dye; pH 6.8</p> <p><b>M-PER<sup>®</sup> Mammalian Protein Extraction Reagent</b>, 25 ml</p> |

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

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## Introduction

The 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 gel, reagents for direct covalent immobilization of the primary antibody, reagents to perform control experiments, buffers for protein binding and recovery, and Pierce 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 along with the proteins of interest. Because the antibody heavy and light chains may co-migrate with one of the relevant bands, important data may be masked. The Pierce Co-IP Kit solves this problem by retaining the antibody on the gel support. Furthermore the entire procedure is performed in a Spin Cup Column, isolating the protein complex and allowing the Co-IP to be completed quickly with consistent results as no resin is lost during the procedure.

This kit includes the M-PER Mammalian Protein Extraction Reagent for lysing eukaryotic cells quickly and gently. The M-PER Reagent is more efficient at extracting proteins than the freeze-thaw method and is gentler than most other common lysis methods yielding higher amounts of native protein.

## General Guidelines for Mammalian Cell Lysis using M-PER Reagent

- **Adherent Cells vs. Cell Pellets:** M-PER Reagent effectively lyses both plated cells and cells pelleted from suspension cultures or scraped cells. For direct, in-plate lysis of adherent cells, protein extraction efficiency is similar to freeze/thaw methods. For lysis of pelleted cells (from cell suspension or scraped adherent cells), protein extraction efficiency is typically 25% higher than that achieved with freeze-thaw (three cycles) and 20% higher than sonication (2 minutes with 50% pulse) methods.
- **Cell Lines:** M-PER Reagent has been tested on representatives of several different cell types. Complete lysis of adherent cells was observed with, but is not limited to, the following cell lines: COS-7, NIH 3T3, Hepa 1-6, 293, CHO, MDA, MB 231 and FM2 cells.
- **Additives:** M-PER Reagent does not include protease inhibitors. If desired, add Halt™ Protease Inhibitor Cocktail Kit (Product No. 78410) to the reagent. For immunoassays such as ELISA or RIA, extracts prepared in M-PER Reagent alone will generate satisfactory results. However, adding 150 mM NaCl to the cell lysate often improves results.
- **Volume for Cell Lysis:** The volumes listed in Table 2 are optimal volumes for cell lysis without scraping cells. If more concentrated extracts are preferred, a smaller volume may be used; however, scraping the cells is necessary for maximal recovery.
- **Compatibility with Protein Assays:** A feature that distinguishes M-PER Reagent from other detergent-based lysis methods is its compatibility with both Coomassie Plus (Product No. 23236) and BCA Protein Assay Reagent (Product No. 23225) to determine the efficiency of protein extraction.

## 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 Slide-A-Lyzer® Cassettes (Product No. 66382), Slide-A-Lyzer MINI Dialysis Units (Product No. 69576) or by desalting thoroughly with Zeba™ Desalt 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/desalting 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 × g). Centrifuging at greater speeds may cause the gel to clump and make resuspending the gel difficult.

- SurfactAmps<sup>®</sup> X-100 (10% Triton<sup>®</sup> X-100, Product No. 28314) may be added to the Coupling Buffer at a concentration of 0.1-1% to reduce nonspecific binding. Note that SurfactAmps X-100 is not supplied with the kit.
- For optimal results, first-time users should couple at least 200 µg of purified antibody to 100 µl of settled gel and perform Co-IP reactions with decreasing amounts of antibody-coupled gel. Gel amounts to use are indicated in Table 1.

**Table 1.** Amounts of Antibody Coupling Gel and antibody to use.\*

| <u>Coupling Gel Slurry</u> | <u>Coupling Gel</u> | <u>Antibody</u>    | <u>Antibody</u>    |
|----------------------------|---------------------|--------------------|--------------------|
| <u>Volume (µl)</u>         | <u>Volume (µl)</u>  | <u>Amount (µg)</u> | <u>Volume (µl)</u> |
| 200                        | 100                 | 200-1,000          | 400                |
| 100                        | 50                  | 100-500            | 100-200            |

\*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 the Pierce Mammalian Co-Immunoprecipitation Kit

### A. Antibody Immobilization

1. Equilibrate the Antibody Coupling Gel 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 Gel to obtain an even suspension. Add the Antibody Coupling Gel (50% 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 gel by adding 0.4 ml of Coupling Buffer to the spin cup containing the gel. Cap the microcentrifuge tube and resuspend gel 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 gel.  
**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 gel does not resuspend easily, swirl the gel with a small pipette tip being careful not to puncture the filter.
10. Place spin cup into a new microcentrifuge tube and add 0.4 ml of Coupling Buffer. Cap and invert tube end-over-end 10 times. Centrifuge the tube.  
**Note:** If desired, save the wash to approximate the 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 gel.
12. In a fume hood, add 4 µl 5 M sodium cyanoborohydride, close the caps and invert five times. Incubate for 30 minutes with end-over-end mixing. Centrifuge the tubes.
13. Wash the gel four times (as described in Step 4) with 0.4 ml Wash Solution and two times with 0.4 ml Coupling Buffer.
14. Store antibody-coupled gel 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. Mammalian Cell Lysis

### Protocol I: Lysis of Adherent Mammalian Cells

- Carefully remove (decant) culture medium from adherent cells.
- Optional wash: If the culture medium contained phenol red or other reagents that could interfere with subsequent protein analysis, wash the cells once with PBS (Product No. 28372; 0.1 M phosphate, 0.15 M NaCl, pH 7.2) or the Coupling Buffer supplied in this kit.
- Add the volume of M-PER Reagent as indicated in Table 2 to the plate or to each well. Gently shake the reaction for 5 minutes.

**Table 2.** Volume of M-PER Reagent to use for different culture plates.

| <u>Plate Type</u>    | <u>Volume of M-PER Reagent</u> |
|----------------------|--------------------------------|
| 60-100 mm (diameter) | 250–500 $\mu$ l                |
| 6-well plate         | 200–400 $\mu$ l per well       |
| 24-well plate        | 100–200 $\mu$ l per well       |
| 96-well plate        | 50–100 $\mu$ l per well        |

- Collect lysate and transfer to a microcentrifuge tube. Centrifuge samples at  $\sim 13,000 \times g$  for 5-10 minutes to pellet the cell debris.
- Transfer supernatant to a new tube for further analysis.

### Protocol II: Lysis of a Mammalian Cell Suspension

- Centrifuge the cell suspension at  $2,500 \times g$  for 10 minutes to pellet the cells. Discard the supernatant.
- Optional wash: If the culture medium contained phenol red or other reagents that could interfere with subsequent protein analysis, wash the cells once by resuspending the cell pellet in a desired wash buffer (e.g., PBS). Centrifuge at  $2,500 \times g$  for 10 minutes to pellet the cells.
- Add M-PER Reagent to the cell pellet (500  $\mu$ l of M-PER Reagent is sufficient for lysing 50 mg of wet cell pellet). For optimal results use a 10:1 v/w ratio.

**Note:** If a large amount of cells is used, first add 1/10 the final recommended volume of M-PER Reagent to the cell pellet. Pipette mixture up and down to suspend the pellet. Add the rest of the recommended final volume of M-PER Reagent to the cell suspension.

- Gently shake the reaction for 10 minutes. Remove cell debris by centrifugation at  $27,000 \times g$  for 15 minutes.
- Transfer supernatant to a new tube for further analysis.

## C. Co-Immunoprecipitation of Protein Complex

**Note:** The amount of bait:prey complex required and incubation time depend upon the antibody-bait protein system used and affinity of the protein:protein interaction and must be optimized for each system.

- Prepare appropriate experimental controls (see Appendix) with the bait and prey proteins.
- Dilute the cell lysate prepared in Section B (i.e., bait:prey complex) and controls in Coupling Buffer if necessary. Recommended total sample volume in the spin cup is 0.1-0.3 ml.
- Centrifuge to remove the Coupling Buffer used to store the antibody-coupled support.
- Place spin cups into new microcentrifuge tubes. Add the bait:prey complex and controls to the appropriate gel 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 gel 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.

- Centrifuge the tubes. Discard (or save for future analysis) the flow-through collected in the microcentrifuge tubes.

- Place the spin cups into new microcentrifuge tubes and add 0.4 ml of Co-IP Buffer. Invert the tubes 10 times and centrifuge.
- Repeat Step 6 two more times.

**Note:** Evaluate the washes (e.g.,  $A_{280}$ , 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.

#### D. Elution of Co-Immunoprecipitation Complex

- Add the appropriate volume of Elution Buffer to the gel 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  $\mu$ l of 1 M Tris, pH 9.5 per 200  $\mu$ l 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 2.** Amount of Elution Buffer to use.

| <u>Elution Buffer</u><br><u>Volume (<math>\mu</math>l)</u> | <u>Antibody-Coupled Gel Slurry</u><br><u>Volume (<math>\mu</math>l)</u> | <u>Antibody-Coupled Settled Gel</u><br><u>Volume (<math>\mu</math>l)</u> |
|--|---|--|
| 200  | 400   | 200  |
| 100  | 200   | 100  |
| 50   | 100   | 50   |
| 50   | 50  | 25   |
| 50   | 20  | 10   |

- 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 Imperial™ Stain Reagent (Product No. 24615 or 24617) for fast results without the need to destain.
- Immediately following the last elution step, proceed to Section E, Regeneration of Gel and Storage Conditions. Proceeding immediately to Section E will extend the life of the antibody-coupled gel.

#### E. Regeneration of Gel and Storage Conditions

- Add 0.4 ml of Coupling Buffer to the spin cup columns. Cap the tubes and gently invert 10 times.
- Centrifuge the tubes. Empty the microcentrifuge tubes.
- Repeat Steps 1 and 2 two additional times.
- 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 gel from drying.

#### F. Preparation of Samples for SDS-PAGE Analysis

- Add 20  $\mu$ l of the sample to a microcentrifuge tube.
- Equilibrate Sample Buffer (dark pink solution) to room temperature. Gently mix the Sample Buffer by inverting the tube 5-10 times. Add 5  $\mu$ l of the 5X Sample Buffer to the sample.

**Note:** The 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  $\mu$ l of 1 M DTT (154.25 Da) to the 25  $\mu$ l sample containing Sample Buffer and mix well. Other sample buffers may also be used.

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

## Troubleshooting

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

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

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

## Appendix

### A. Control Experiments

When the Co-IP results are analyzed by SDS-PAGE, several protein bands may appear on the gel indicating possible interacting proteins. However, these bands may also be caused by nonspecific interactions with the gel 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 Gel:** The supplied Control Gel is composed of the same support material as the Co-IP gel, but it is not activated. This gel provides an excellent negative control when processed the same as the Antibody Coupling gel.
- **Quenched Antibody Coupling Gel:** Create a quenched gel control by adding 400  $\mu$ l of Quenching Buffer to the Antibody Coupling Gel instead of the antibody then continue with the standard procedure.
- **Non-relevant antibody:** Couple a non-related antibody to the Antibody Coupling Gel then 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 may be co-purified after they are allowed to interact with each other in solution, or the bait protein may be coupled directly to the antibody coupling gel 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 gel 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 may 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.

## Related Products

|       |  |
|-------|--|
| 28314 | <b>SurfactAmps X-100 (10% solution of Triton X-100), 6 <math>\times</math> 10 ml</b>                       |
| 20291 | <b>No-Weigh™ DTT, 48 microtubes each containing 7.7 mg of DTT</b>  |
| 69702 | <b>Pierce Spin Cup Columns, 50 units, columns contain 0.45 <math>\mu</math>m cellulose acetate filters</b> |
| 69720 | <b>Pierce Microcentrifuge Tubes, 2 ml, 72 tubes</b>  |
| 21004 | <b>IgG Elution Buffer, 1 L</b>   |
| 21027 | <b>Gentle Ag/Ab Elution Buffer, 500 ml</b>   |
| 20501 | <b>AminoLink Plus Coupling Resin, 10 ml</b>  |
| 28372 | <b>Coupling Buffer: BupH Phosphate Buffered Saline Pack, 40 packs</b>                                      |
| 44892 | <b>AminoLink Reductant: Sodium Cyanoborohydride, 2 <math>\times</math> 1 g</b>                             |
| 28374 | <b>Binding Buffer: BupH Modified Dulbecco's PBS Pack, 40 packs</b>   |
| 28376 | <b>BupH Tris Buffered Saline Pack, 40 packs</b>  |
| 39001 | <b>Lane Marker Non-Reducing Sample Buffer (5X), 5 ml</b>   |
| 78410 | <b>Halt Protease Inhibitor Cocktail Kit</b>  |

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Slide-A-Lyzer<sup>®</sup> Dialysis Cassette Technology is protected by U.S. Patent # 5,503,741 and 7,056,440.

Slide-A-Lyzer<sup>®</sup> MINI Dialysis Unit Technology is protected by U.S. Patent # 6,039,871.

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Current versions of product instructions are available at [www.thermo.com/pierce](http://www.thermo.com/pierce). For a faxed copy, call 800-874-3723 or contact your local distributor.

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