INSTRUCTIONS

Pierce Co-Immunoprecipitation (Co-IP) Kit

26149

2181.7

Number	Description
26149	**Pierce Co-Immunoprecipitation (Co-IP) Kit,** contains sufficient reagents to perform 50 reactions using 25µL of immobilized antibody support

Kit Contents:

AminoLink Plus Coupling Resin, 2mL of settled resin supplied as a 50% slurry (e.g., 100µL of 50% slurry is equivalent to 50µL of settled resin)

Coupling Buffer (20X), 25mL, when diluted results in 0.01M sodium phosphate, 0.15M NaCl; pH 7.2

Sodium Cyanoborohydride Solution (5M), 0.5mL

Quenching Buffer, 50mL, 1M Tris•HCl

Wash Solution, 60mL, 1M NaCl

IP Lysis/Wash Buffer, 2 × 50mL, 0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4

Modified Dulbecco's PBS (20X), 25mL, when diluted results in 0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride and 0.01M KCl; pH 7

Conditioning Buffer (100X), 5mL, neutral pH buffer

Elution Buffer, 50mL, pH 2.8, contains primary amine

Lane Marker Sample Buffer, Non-reducing, (5X), 5mL, 0.3M Tris•HCl, 5% SDS, 50% glycerol, lane marker tracking dye; pH 6.8

Pierce Spin Columns – Screw Cap, 100 each

Microcentrifuge Collection Tubes, 2mL, 100 each

Microcentrifuge Sample Tubes, 1.5mL, 50 each

Pierce Control Agarose Resin (crosslinked 4% beaded agarose), 2mL of settled resin supplied as a 50% slurry (e.g., 100µL of 50% slurry is equivalent to 50µL of settled resin)

Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific™ Pierce™ Co-Immunoprecipitation (Co-IP) Kit enables isolation of native protein complexes from a lysate or other complex mixture by directly immobilizing purified antibodies onto an agarose support. Co-IP is a common approach to study protein:protein interactions that uses an antibody to immunoprecipitate the antigen (bait protein) and co-immunoprecipitate any interacting proteins (prey proteins). Traditional co-IP methods that use Protein A or G result in co-elution of the antibody heavy and light chains that may co-migrate with relevant bands, masking important results. The Pierce Co-IP Kit resolves this issue by covalently coupling antibodies onto an amine-reactive resin. The kit includes optimized buffers for protein binding and recovery, reagents to perform control experiments and efficient spin columns and collection tubes, which shorten the protocol and minimize handling and mixing.

Important Product Information

- Perform all resin centrifugation steps for 30-60 seconds at low speed (i.e., 1000-3000 × *g*). Centrifuging at speeds greater than 5000 × *g* may cause the resin to clump and make resuspension difficult.
- When centrifuging spin columns, the flow-through volume should not exceed 600µL when using a 2mL collection tube and 300µL when using a 1.5mL collection tube. Exceeding these volumes can cause back pressure in the column and incomplete washing or elution.
- Primary amines (e.g., Tris, glycine) in the antibody solution will compete for coupling sites on the resin. Remove primary amines before antibody immobilization using Thermo Scientific Zeba Spin Desalting Columns or Slide-A-Lyzer Dialysis Cassettes.
- Gelatin or carrier proteins in the antibody solution will compete for coupling sites on the resin. Remove gelatin and carrier proteins using the Pierce Antibody Clean-up Kit (Product No. 44600) or by performing Protein A/G purification (Product No. 20423) and dialysis.
- To reduce nonspecific protein binding in immunoprecipitations, pre-clear the lysate using the Control Agarose Resin or add Surfact-Amps™ X-100 (10% Triton™ X-100, Product No. 28314) to the Modified Dulbecco's PBS at 0.1-1%. **Note:** Surfact-Amps X-100 is not supplied with the kit.
- The IP Lysis/Wash Buffer has been tested on representative cell types including but not limited to the following cell lines: HeLa, Jurkat, A431, A549, MOPC, NIH 3T3 and U2OS. Typically, 10^6 HeLa cells yield ~10 mg of cell pellet and ~3µg/µL (or 300µg) when lysed with 100µL of IP Lysis/Wash Buffer.
- For best results, add Halt™ Protease (Product No. 78430) and Phosphatase (Product No. 78428) Inhibitor Cocktails to minimize degradation and dephosphorylation of cell lysate proteins. These inhibitors are also available as a combined cocktail (Product No. 78440). See the Related Thermo Scientific Products Section for more information.
- The IP Lysis/Wash Buffer and Elution Buffer are compatible with the Pierce BCA Protein Assay (Product No. 23225).
- Proper controls are vital for identifying relevant interactions. The supplied Pierce Control Agarose Resin is composed of the same support material as the AminoLink Plus™ Coupling Resin but is not amine-reactive. This resin provides an excellent negative control.
- The Pierce Spin Columns package includes columns, screw caps, 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., > 200µL of resin), the large frit can be inserted 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. (See the Additional Information Section.)
- The Pierce Co-Immunoprecipitation Kit can be scaled as needed. For optimal results, perform co-IP reactions with resin, antibody, elution and wash quantities indicated in Table 1. These conditions result in a coupling efficiency of ~95% after 2 hours. Coupling efficiency can be approximated by measuring the absorbance of the antibody solution at 280nm before and after coupling.

Table 1. Amounts of antibody, coupling resin, wash buffer and elution buffer to use.

Antibody Amount (µg)	Coupling Resin Volume (µL) (volume of 50% slurry)	Wash Volume (µL)	Elution Buffer Volume (µL)
250-1000	100 (200)	400	100-150
75-250	50 (100)	300	75-100
10-75	25 (50)	200	50-75
≤ 10	10 (20)	200	50

Additional Material Required

- Microcentrifuge collection tubes, 2mL

Procedure for Co-Immunoprecipitation

A. Antibody Immobilization

Note: The following protocol is for coupling 10-75µg of affinity-purified antibody in a solution free of amines and carrier proteins (see the Important Product Information Section). Scale this protocol as needed; see Table 1 in the Important Product Information Section for suggested antibody and resin volumes.

1. Equilibrate the AminoLink Plus Coupling Resin and reagents to room temperature.
2. Prepare 2mL of 1X Coupling Buffer for each co-IP reaction by diluting the 20X Coupling Buffer with ultrapure water.
3. Gently swirl the bottle of AminoLink Plus Coupling Resin to obtain an even suspension. Using a wide-bore or cut pipette tip, add 50µL of the resin slurry into a Pierce Spin Column. Place column into a microcentrifuge tube and centrifuge at 1000 × *g* for 1 minute. Discard the flow-through.
4. Wash resin twice by adding 200µL of 1X Coupling Buffer, centrifuge and discard the flow-through.
5. Gently tap the bottom of the spin column on a paper towel to remove any excess liquid and insert the bottom plug.
6. Prepare 10-75µg of affinity-purified antibody for coupling by adjusting the volume to 200µL, using sufficient ultrapure water and 20X Coupling Buffer to produce 1X Coupling Buffer. For example add 10µL of 20X Coupling Buffer, 180µL of ultrapure water and 10µL of antibody at 1µg/1µL. Add the ultrapure water, 20X Coupling Buffer and affinity-purified antibody directly to the resin in the spin column.
7. In a fume hood, add 3µL of the Sodium Cyanoborohydride Solution for every 200µL of reaction volume.

   Note: Sodium cyanoborohydride is highly toxic. Wear gloves and use caution when handling.
8. Attach the screw cap to the column and incubate on a rotator or mixer at room temperature for 90-120 minutes, ensuring that the slurry remains suspended during incubation.
9. Remove and retain the bottom plug and loosen the screw cap. Place the spin column into a collection tube and centrifuge. Save the flow-through to verify antibody coupling.
10. Remove the screw cap, add 200µL of 1X Coupling Buffer, centrifuge and discard the flow-through. Repeat this step once.
11. Add 200µL of Quenching Buffer to the column, centrifuge and discard the flow-through.
12. Tap the bottom of the column on a paper towel to remove excess liquid and insert the bottom plug. Add 200µL of Quenching Buffer to the resin.
13. In a fume hood, add 3µL of Sodium Cyanoborohydride Solution and attach the screw cap. Incubate for 15 minutes with gentle shaking or end-over-end mixing.
14. Remove plug and loosen the screw cap. Place spin column in a collection tube, centrifuge and discard the flow-through.
15. Remove screw cap, wash the resin twice with 200µL of 1X Coupling Buffer, centrifuging after each wash.
16. Wash the resin six times with 150µL of Wash Solution, centrifuging after each wash.
17. Either proceed to cell lysis and co-IP or, if storing the resin, proceed to the next step.
18. Wash the resin twice with 200µL of 1X Coupling Buffer, centrifuging after each wash.
19. Tap the column on a paper towel to remove excess liquid and place plug in bottom of spin column. Add 200µL of 1X Coupling Buffer, attach the screw cap and store column at 4°C. For long-term storage, add sodium azide to a final concentration of 0.02%.

B. Mammalian Cell Lysis

Protocol I: Lysis of Cell Monolayer (Adherent) Cultures

1. Carefully remove culture medium from cells.
2. Wash the cells once with 1X Modified Dulbecco's PBS.
3. Add ice-cold IP Lysis/Wash Buffer (Table 2) to the cells. Incubate on ice for 5 minutes with periodic mixing.

Table 2. Suggested volume of IP Lysis/Wash Buffer to use for different standard culture plates.

Plate Size/Surface Area	Volume of IP Lysis/Wash Buffer
100 × 100 mm	500-1,000µL
100 × 60 mm	250-500µL
6-well plate	200-400µL per well
24-well plate	100-200µL per well

4. Transfer the lysate to a microcentrifuge tube and centrifuge at ~ 13,000 × *g* for 10 minutes to pellet the cell debris.
5. Transfer supernatant to a new tube for protein concentration determination and further analysis.

Protocol II: Lysis of Cell Suspension Cultures

1. Centrifuge the cell suspension at 1000 × *g* for 5 minutes to pellet the cells. Discard the supernatant.
2. Wash cells once by suspending the cell pellet in PBS. Centrifuge at 1000 × *g* for 5 minutes to pellet cells.
3. Add ice-cold IP Lysis/Wash Buffer to the cell pellet. Use 500µL of IP Lysis/Wash Buffer per 50 mg of wet cell pellet (i.e., 10:1 v/w). If using a large amount of cells, first add 10% of the final volume of IP Lysis/Wash Buffer to the cell pellet and pipette the mixture up and down to mix. Add the remaining volume of IP Lysis/Wash Buffer to the cell suspension.
4. Incubate lysate on ice for 5 minutes with periodic mixing. Remove cell debris by centrifugation at ~ 13,000 × *g* for 10 minutes. Transfer supernatant to a new tube for protein concentration determination and further analysis.

C. Pre-clear lysate using the Control Agarose Resin

1. For 1mg of lysate, add 80µL of the Control Agarose Resin slurry (40µL of settled resin) into a spin column.
2. Centrifuge column to remove storage buffer.
3. Add 100µL of 1X Coupling Buffer to the column, centrifuge and discard the flow-through.
4. Add 1mg of lysate to the column containing the resin and incubate at 4ºC for 30 minutes to 1 hour with gentle end-over-end mixing.
5. Centrifuge column at 1000 × *g* for 1 minute. Discard the column containing the resin and save the flow-through, which will be added to the immobilized antibody for the co-IP.

D. Co-IP

- Perform all co-IP steps at 4°C unless otherwise indicated.
- The amount of bait:prey complex required and incubation time depends upon the system used and the affinity of the antibody, bait and prey interactions and must be optimized for each system.
- The protocol uses the IP Lysis/Wash Buffer for coupling and washing the immune complex. The 20X Modified Dulbecco's PBS is supplied in the kit as an alternative binding/wash buffer. Use this buffer for complexes that might be disrupted by detergents. For each co-IP reaction, prepare 2mL of 1X Modified Dulbecco's PBS by diluting with ultrapure water.

1. Mix the bait and prey proteins (if separate) and prepare appropriate experimental controls.
2. Dilute the bait:prey protein mixture and controls in IP Lysis/Wash Buffer, or other suitable buffer. Recommended total sample volume in the spin column is 100-500µL.
3. Wash the resin twice by adding 200µL of IP Lysis/Wash Buffer to the spin column containing the antibody-coupled resin, centrifuge and discard the flow-through.
4. Gently tap the bottom of the spin column on a paper towel to remove excess liquid and insert the bottom plug.
5. Add the bait:prey protein mixture and controls to the appropriate resin. Attach cap and incubate with gentle mixing or rocking for 1-2 hours or overnight at 4ºC.

   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 0.5mL aliquots through the spin cup until the entire sample has been processed.

6. Remove the bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge the spin columns. Save the flow-through for future analysis.
7. Remove the screw cap, place the column into a new tube, add 200µL of IP Lysis/Wash Buffer and centrifuge.
8. Wash the sample two more times with 200µL IP Lysis/Wash Buffer and centrifuge after each wash.

   Note: Evaluate the washes (e.g., A_{280}, SDS-PAGE or Thermo Scientific 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 washes might be necessary for samples containing high protein concentrations.

   Optional Wash: To improve elution, perform an additional wash with 100µL of 1X Conditioning Buffer (diluted from 100X using ultrapure water). This buffer contains low salt and minimal buffer; therefore, test the flow-through to confirm that the bait:prey complex remained intact.

E. Elution of Co-IP

Note: If the protein or antibody is sensitive to low pH, use a neutral pH system (i.e., Thermo Scientific Gentle Elution Buffer, Product No. 21027). For downstream enzymatic or functional assays, add 5µL of 1M Tris, pH 9.5 to the collection tube, which will neutralize the pH of the Elution Buffer upon centrifugation.

1. Place the spin column into a new collection tube. Add 10µL of Elution Buffer and centrifuge.
2. Keep the column in the tube and add 50µL of Elution Buffer. Incubate for 5 minutes at room temperature. The column does not need to be closed or mixed.

   Note: For a more concentrated eluate, use less Elution Buffer; however, overall yield may be reduced.
3. Centrifuge the tube and collect the flow-through. Analyze the flow-through for protein. Perform additional elutions (i.e., Steps E1-E3) as needed. Analyze each flow-through fraction separately to ensure that the antigen has completely eluted.
4. To preserve activity of the antibody-coupled resin, immediately proceed to Section F, Resin Regeneration and Storage.

F. Resin Regeneration and Storage

1. Add 100µL of 1X Coupling Buffer to the spin column, centrifuge and discard the flow-through. Repeat this step once.
2. Replace the bottom plug on the spin column. Add 200µL of 1X Coupling Buffer to spin column. Replace screw cap. Wrap the bottom of the tube with laboratory film to prevent resin from drying. For long-term storage (i.e., > 2 weeks) add sodium azide at a final concentration of 0.02%.

G. Sample Preparation for SDS-PAGE Analysis

1. Equilibrate the 5X Lane Marker Sample Buffer to room temperature. Gently mix the sample buffer by inverting 5-10 times. For a reducing gel, add 1M DTT to a final concentration of 100mM in the 5X Sample Buffer.
2. Add 5X Sample Buffer to sample to make a 1X final solution (i.e., add 5µL 5X Sample Buffer to 20µL of sample).
3. Heat the sample at 95-100°C for ~5 minutes. Allow the sample to cool to room temperature before applying to the gel.

Troubleshooting

Problem	Possible Cause	Solution
Antibody is detected with the eluted protein complex	Non-coupled antibody was not removed sufficiently with the Wash Solution during the coupling procedure	Wash the antibody-coupled resin with Elution Buffer until no additional antibody elutes from the resin (as determined by protein assay or A_{280})
	A reducing agent was added to the antibody-coupled resin during the IP or elution steps, which eluted antibody fragments not directly linked to the resin	Use buffers that do not contain reducing agents (i.e., DTT or β-mercaptoethanol)
Proteins are detected in the negative control resin experiment	Proteins bind nonspecifically to the coupling resin	Increase the number of washes before eluting or add Triton X-100 to the co-IP buffer to decrease nonspecific binding
		Pre-adsorb sample to the Control Resin before performing the co-IP*
Bait protein is not captured from sample	An insufficient amount of antibody is coupled to the resin causing low protein binding	Check flow-through and wash fractions to verify that antibody is coupled to the resin
		Perform a sample-buffer elution of the resin to evaluate antibody coupling (see the Additional Information Section)
		Increase antibody amount to ensure that sufficient antibody is coupled to the resin
		Use a more sensitive detection method
Bait protein is not captured from sample	Antibody does not recognize native bait protein (common with antibodies made against peptides)	Use a different antibody
	Antibody is sensitive to amine coupling	Increase antibody amount, reduce coupling time or try a different antibody
	The bait protein does not elute from the antibody using acidic conditions	Optimize the elution conditions (see Tech Tip #27 on our website)
Bait protein is captured but no interacting prey proteins are detected	Protein:protein interactions are weak and cannot withstand the washing protocol	Use a different wash buffer or a slower centrifugation speed
		Use a crosslinker to reinforce weak or transient interactions
	Antibody binds only to the non-complexed protein because of shared binding sites or conformational changes	Use an antibody that recognizes a different epitope on the bait protein
	Co-IP buffer conditions do not promote protein:protein interaction	Specific ions, cofactors, etc. might be required to promote the interactions
	There is no protein complex involving the bait protein	None
Resin leaks from the spin column	Frit was displaced from bottom of spin column	When placing plug in the column, tap the bottom of the spin column on a paper towel to remove excess liquid – liquid present below the frit can produce back pressure and frit displacement when the bottom plug is inserted

*Interacting protein might be obscured in the gel analysis by nonspecific protein bands. Incubating the sample with Control Resin to capture proteins that bind to the resin before performing the co-IP reaction can eliminate this interference.

Additional Information

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 200µL 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. Sample-buffer elution

To troubleshoot if the antibody is coupled to the gel, perform an elution using the sample buffer. This elution method may also be used if you are certain that antibody fragments will not interfere with Western blot target detection.

1. Prepare 50µL of 2X Non-reducing Lane Marker Sample Buffer (i.e., dilute the 5X sample buffer to 2X with ultrapure water) and add DTT to a final concentration of 20mM. Place the spin column containing the resin into a new collection tube and add the 2X reducing sample buffer.
2. Keep the column unplugged and in the collection tube, and incubate at 100ºC for 5-10 minutes. Centrifuge tube to collect eluate. Allow the sample to cool to room temperature before performing the SDS-PAGE.

   Note: After heating the resin with SDS sample buffer, the resin cannot be reused and must be discarded.

C. 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 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.

D. Pierce Spin Columns

Pierce Spin Columns can hold up to 900µL. Columns can be placed in 1.5mL or 2mL microcentrifuge tubes or used with a Luer-lok Adapter (see Figure 1) for processing samples with a syringe. When using a syringe, sample size and wash volumes are only limited by the volume capacity of the syringe. For small volumes of resin, use columns with only the small, pre-inserted frit (Figure 2A). For applications requiring more than 100µL of resin, the large frit may be used at either the top or bottom (Figure 2B and 2C). Resins may be used repeatedly when the resin is in-between the small and large frit (Figure 2B).

- To remove a frit from a column, use an unfolded paper clip and insert the wire through the column tip and push the frit.
- To insert a frit, place the frit inside the column and use the frit tool to push the frit into position.
- To remove the top frit from an already-packed column with a top and bottom frit, use an unfolded paper clip to tip the top frit up. The top frit can be then removed with tweezers.

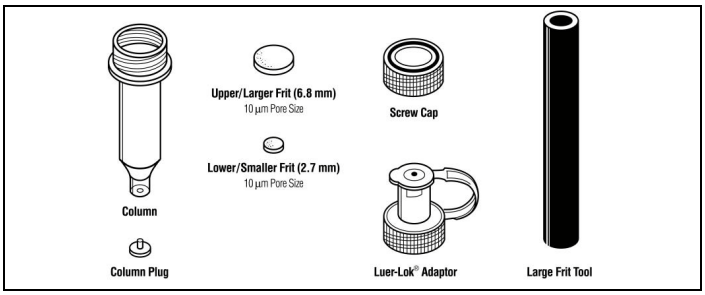

Figure 1. Schematic of the Pierce Spin Column contents.

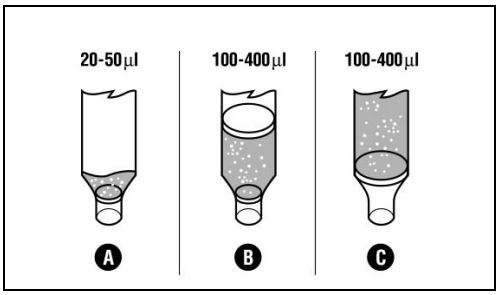

Figure 2. Three formats for frit placement.

E. Information available from our website

- Tech Tip #27: Optimize elution conditions for immunoaffinity purification
- Tech Tip #43: Protein stability and storage
- Tech Tip #40: Convert between times gravity (× *g*) and centrifuge rotor speed (RPM)

Related Thermo Scientific Products

78428	**Halt Phosphatase Inhibitor Single-Use Cocktail** (100X), 100µL × 24 microtubes
78440	**Halt Protease and Phosphatase Inhibitor Cocktail** (100X), 1mL
78430	**Halt Protease Inhibitor Single-Use Cocktail (100X),** 24 × 100µL
69705	**Pierce Spin Columns – Screw Cap,** 25/pkg
69720	**Pierce Microcentrifuge Tubes,** 2mL, 72/pkg
69715	**Pierce Microcentrifuge Tubes,** 1.5mL, 72/pkg
89879	**Pierce Micro-Spin Columns,** 50/pkg
28314	**Surfact-Amps X-100 (10% solution of Triton X-100),** 6 × 10mL
20291	**DTT No-Weigh™ Format,** 48 microtubes each containing 7.7mg
39001	**Lane Marker Sample Buffer, Non-Reducing (5X),** 5mL
21027	**Gentle Ag/Ab Elution Buffer,** pH 6.6, 500mL
28374	**BupH Modified Dulbecco's Phosphate Buffered Saline Packs**, 40 packs
21004	**IgG Elution Buffer,** 1L
26150	**Pierce Control Agarose Resin,** 10mL