INSTRUCTIONS

Pierce Classic IP Kit

26146

2137.7

Number	Description
26146	**Pierce Classic IP Kit,** contains sufficient reagents to perform 50 reactions using 10µL of immobilized antibody support

Kit Contents:

Pierce Protein A/G Plus Agarose, 0.55mL of settled resin supplied as a 50% slurry (e.g., 100µL of 50% slurry is equivalent to 50µL of settled resin)

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

100X Conditioning Buffer, 5mL, neutral-pH buffer

20X Tris-Buffered Saline, 25mL, when diluted results in 0.025M Tris, 0.15M NaCl; pH 7.2

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 columns, includes column accessories

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™ Classic IP Kit enables highly effective and efficient antigen immunoprecipitations using less than 10 µg of antibody. The specific antibody is first added to the sample to form an immune complex that is then added to the Protein A/G Plus Agarose. The complex is washed to remove non-bound material, and a low pH elution buffer dissociates the bound immune complex from the Protein A/G. The kit includes optimized buffers for high antigen yield and efficient spin columns and collection tubes, which shortens the protocol by minimizing handling and mixing.

Important Product Information

- Perform all steps at 4°C unless otherwise indicated.
- Perform all resin centrifugation steps for 30-60 seconds at low speed (i.e., 1000 × *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 may result in back pressure in the column and incomplete washing or elution.
- Co-elution of antibody with the immunoprecipitated antigen occurs with this kit. Consequently, there could be at least three protein bands on a reducing SDS-PAGE gel or Western blot: the antibody heavy chain (50kDa), light chain (25kDa) and the antigen. If an antibody masks the immunoprecipitated antigen, use the Thermo Scientific Clean-Blot IP Detection Reagent (Product No. 21230 and 21233) or eliminate co-elution using the Thermo Scientific Pierce IP Direct Kit (Product No. 26148).

- For optimal results, use an affinity-purified antibody. Although serum may be used, the antibody that is specific for the antigen of interest may comprise only 1-2% of the total IgG in the serum sample and will result in low antigen yields.
- 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 yields ~10mg of cell pellet and ~3µg/µL (or 300µg) when lysed with 100µL of IP Lysis/Wash Buffer.
- For best results, add Thermo Scientific Halt Protease (Product No. 78429) and Phosphatase (Product No. 78420) Inhibitor Cocktails to minimize degradation and dephosphorylation of cell lysate proteins.
- The IP Lysis/Wash buffer is compatible with the Thermo Scientific Pierce BCA Protein Assay (Product No. 23225).
- Proper controls are vital for identifying relevant interactions. The supplied Pierce Control Agarose Resin is composed of a similar support material used for Pierce Protein A/G Agarose Resin and can be used as a negative control.
- The Pierce Spin Columns package includes spin columns, 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. When scaling-up (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).

Additional Materials Required

- Phosphate-buffered saline (PBS; Product No. 28372)
- Dithiothreitol (DTT; Product No. 20290 or 20291)
- Microcentrifuge collection tubes, 2mL

Procedure or Protocol for the Pierce Classic IP Kit

A. Mammalian Cell Lysis

Protocol I: Lysis of Cell Monolayer (Adherent) Cultures

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

Table 1. 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 × 100mm	500-1000µL
100 × 60mm	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 50mg 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 pellet and pipette the mixture up and down to mix. Add the remaining volume 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.
5. Transfer supernatant to a new tube for protein concentration determination and further analysis.

B. 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 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 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.

C. Preparation of Immune Complex

Note: The amount of sample needed and the incubation time are dependent upon each specific antibody-antigen system and may require optimization for maximum yield. The following protocol is for 2-10µg of affinity-purified antibody and can be scaled up as needed; see the Additional Information Section at the end of the protocol for suggested antibody and resin amounts.

1. Combine 2-10µg of affinity purified antibody with the pre-cleared cell lysate in a microcentrifuge tube. The suggested amount of total protein per IP reaction is 500-1000µg, as determined by the Pierce BCA Protein Assay.
2. Dilute the antibody/lysate solution to 300-600µL with IP Lysis/Wash Buffer.
3. Incubate for 1 hour to overnight at 4ºC to form the immune complex.

D. Capture of the Immune Complex

1. Gently swirl the bottle of Pierce Protein A/G Agarose to obtain an even suspension. Using a wide-bore or cut pipette tip, add 20µL 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.
2. Wash resin twice with 100µL of cold IP Lysis/Wash Buffer. Discard the flow-through after each wash.
3. Gently tap the bottom of the spin column on a paper towel to remove excess liquid and insert the bottom plug.
4. Add the antibody/lysate sample to Protein A/G Plus Agarose in the spin column. Attach the screw cap and incubate column with gentle end-over-end mixing or shaking for 1 hour.
5. Remove bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge column and save the flow-through. Do not discard flow-through until confirming that the IP was successful.
6. Remove the screw cap, place the column into a new collection tube, add 200µL of IP Lysis/Wash Buffer and centrifuge.

   Note: An alternative wash buffer (20X TBS Buffer) is supplied if a detergent-free wash is required. Dilute buffer to 1X before use.
7. Wash the resin three times with 200µL IP Lysis/Wash Buffer and centrifuge after each wash.
8. Wash the resin once with 100µL of 1X Conditioning Buffer (diluted from 100X with ultrapure water).

E. Elution of the Immune Complex

Note: There are two options for recovering the immune complex. The sample-buffer elution is ideal for Western blot analysis. The low-pH elution is ideal for enzymatic or functional assays, once the low pH is neutralized.

- **Sample-buffer elution:** 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. Keep the column unplugged and in the collection tube, and incubate at 100ºC for 5-10 minutes. Centrifuge to collect eluate. Allow the sample to cool to room temperature before applying to the SDS-PAGE gel.

  Note: After heating the resin with SDS sample buffer, the resin cannot be reused and must be discarded.
- **Low-pH elution:** Place the spin column into a new collection tube and add 50µL of Elution Buffer. Incubate for 10 minutes at room temperature. The column does not need to be closed or mixed. Centrifuge the tube and collect the

flow-through. Perform additional elutions as needed. Analyze each eluate separately to ensure that the antigen has completely eluted.

Optional**:** To neutralize the low pH of the Elution Buffer (e.g., for downstream enzymatic or functional assays), add 5µL of 1M Tris, pH 9.5 to the collection tube, which will neutralize the pH upon centrifugation. Alternatively, use a neutral pH elution buffer (i.e., Gentle Elution Buffer, Product No. 21027).

Note: For a more concentrated eluate, less Elution Buffer may be used; however, overall yield might be reduced.

Troubleshooting

Problem	Possible Cause	Solution
Antigen did not immunoprecipitate	Sample did not contain enough antigen to detect	Verify protein expression and/or lysis efficiency of the lysate by SDS-PAGE or Western blot
	Antibody did not bind antigen	Use a recent source of the antibody or a different antibody that recognizes a different epitope
	Component in the IP Lysis/Wash Buffer interfered with antibody-antigen binding	Perform the IP and washes using 1X Tris-Buffered Saline
Antigen is non-functional in the downstream application	Antigen is sensitive to low pH and became inactive during the elution process	Repeat the IP and use a high-salt, neutral pH elution buffer, such as the Gentle Elution Buffer (Product No. 21027)
Eluted antibody bands masks antigen of interest	Antigen has a molecular weight of approximately 50kDa or 25kDa	Use Clean-Blot IP Detection Reagents (Product No. 21230 or 21233) for Western blot detection
		Use a different antibody species for Western blot detection from the antibody species used for IP (i.e., immunoprecipitate with mouse IgG and detect with rabbit IgG)
		Use Pierce Direct IP Kit (Product No. 26148) or Pierce Crosslink IP Kit (Product No. 26147) to immobilize antibody to the resin
		Do not reduce samples before SDS-PAGE so the antibody migrates at 150 kDa

Additional Information

A. Alternative Immunoprecipitation Protocols

The Pierce Classic IP Protocol achieves optimal results when the immune complex is formed first and then captured with the Protein A/G resin; however, there are two alternative approaches for capturing the immune complex.

- If a shorter IP method is required, add the antibody and cell lysate to the Protein A/G resin to form and capture the immune complex in one step.
- If using an unpurified antibody such as serum or ascites, first bind the antibody to the Protein A/G resin, wash and then add the cell lysate.

B. Large-scale Antibody Coupling

The Pierce Classic IP Protocol can be scaled up as needed. Guidelines for scaling up the amounts of antibody and resin are listed in Table 2. Adjust volumes for coupling reagents and washes accordingly.

Table 2. Amount of Thermo Scientific Pierce Protein A/G Plus Agarose and antibody to use for larger scale IPs.

Antibody Amount (µg)	Resin Slurry Amount (µL)
2-20	20
25-50	40
50-250	100

C. Visit the website for additional information including the following:

- 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)

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 100 µl 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.

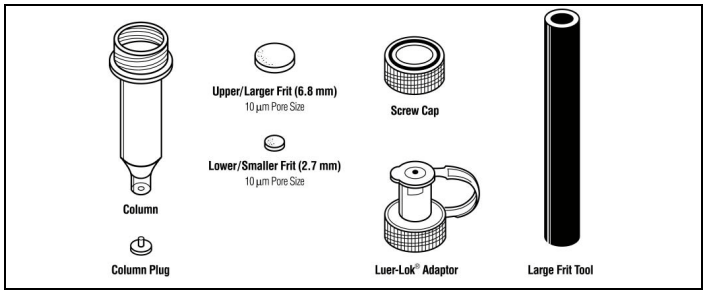

Figure 1. Schematic of the Pierce Spin Column contents.

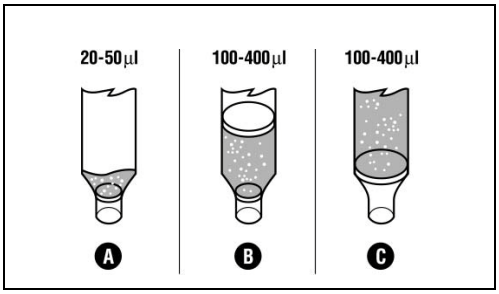

Figure 2. Three formats for frit placement.

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
28358	**20X TBS Buffer,** 500mL
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
20423	**Pierce Protein A/G Plus Agarose,** 2mL
39001	**Lane Marker Sample Buffer, Non-Reducing (5X),** 5mL
21027	**Gentle Ag/Ab Elution Buffer,** pH 6.6, 500mL
28372	**BupH™ Phosphate Buffered Saline (PBS),** 40 packs each pack makes 500mL
28374	**BupH Modified Dulbecco's Phosphate Buffered Saline Packs,** 40 packs, each pack makes 500mL
21004	**IgG Elution Buffer,** low-pH elution buffer for general protein affinity purifications, 1L
89897	**Pierce Centrifuge Columns, 5mL** (resin bed capacity), gravity or centrifuge compatible, 25 units
21230	**Clean-Blot IP Detection Reagent (HRP),** 2.5mL
21233	**Clean-Blot IP Detection Reagent (AP),** 2.5mL
26148	**Pierce Direct IP Kit**

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