INSTRUCTIONS

Pierce® Direct IP Kit

26148

2121.0

Number	Description
26148	**Pierce Direct IP Kit,** contains sufficient reagents to perform 50 reactions using 10 µl of immobilized antibody support

Kit Contents:

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

20X Coupling Buffer, 25 ml, when diluted results in 0.01 M sodium phosphate, 0.15 M sodium chloride; pH 7.2

Sodium Cyanoborohydride Solution (5 M)**,** 0.5 ml

Quenching Buffer, 50 ml, 1 M Tris•HCl

Wash Solution, 60 ml, 1 M NaCl

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

100X Conditioning Buffer, 5 ml, neutral pH buffer

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

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

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

Pierce Spin Columns – Screw Cap, 50 each

Microcentrifuge Collection Tubes, 2 ml, 100 each

Microcentrifuge Sample Tubes, 1.5 ml, 50 each

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

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

Introduction

The Thermo Scientific Pierce Direct IP Kit enables highly effective and efficient antigen immunoprecipitations by directly immobilizing purified antibodies onto an agarose support. Immobilizing the antibody provides faster and easier immunoprecipitations, enables reuse of the immobilized antibody and results in purified antigen free from antibody contamination. Immunoprecipitation is achieved using less than 10 µg of antibody and a short coupling protocol. After the antibody is coupled to the AminoLink Resin, the antigen sample is incubated with the immobilized antibody to form the immune complex. The complex is washed to remove non-bound material, and a low pH elution buffer is used to dissociate the bound antigen from the antibody.

In contrast to traditional methods, the Direct IP Kit uses an amine-reactive support that does not contain Protein A or Protein G and does not need a crosslinker for covalent immobilization. Furthermore, this method couples any primary amine-containing molecule, unlike methods requiring an antibody species and subclass that binds strongly to Protein A or Protein 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

- Amines (e.g., Tris, glycine) in the antibody solution will compete for coupling sites on the resin. Remove amines before coupling 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 Thermo Scientific Pierce Antibody Clean-up Kit (Product No. 44600) or by performing Protein A/G purification (Product No. 20423) and dialysis.
- Perform antibody coupling at room temperature. Perform cell lysis and antigen IP at 4°C.
- Perform all resin centrifugation steps for 30-60 seconds at low speed (i.e., 1,000 × *g*). Centrifuging at speeds greater than 5,000 × *g* may cause the resin to clump and make resuspending difficult.
- When centrifuging spin columns, the flow-through volume should not exceed 600 µl when using a 2 ml collection tube and 300 µl when using a 1.5 ml collection tube. Exceeding these volumes may result in back pressure in the column and incomplete washing or elution.
- 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 ~10 mg of cell pellet and ~3 µg/µl (or 300 µg) when lysed with 100 µl 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 the same support material as the AminoLink Plus Coupling Resin but is not amine-reactive. This resin provides an excellent negative control.

Procedure for the Pierce Direct IP Kit

A. Coupling of Antibody to AminoLink Plus Coupling Resin

Note: The following protocol is for coupling 2-10 µg of affinity-purified antibody in a solution that is free of amines (e.g., Tris, glycine) and carrier proteins (see the Important Product Information Section). This protocol can be scaled up as needed; see the Additional Information Section at the end of the protocol for suggested antibody and resin volumes.

1. Equilibrate the AminoLink Plus Coupling Resin and reagents to room temperature.
2. Prepare 2 ml of 1X Coupling Buffer for each 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 20 µl of the resin slurry into a Pierce Spin Column. Place column into a microcentrifuge tube and centrifuge at 1,000 × *g* for 1 minute. Discard the flow-through.
4. Wash resin twice by adding 200 µl 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 2-10 µg of affinity-purified antibody for coupling by adjusting the volume to 200 µl, using sufficient ultrapure water and 20X Coupling Buffer to produce 1X Coupling Buffer. For example, for 20 µl of antibody add 10 µl of 20X Coupling Buffer and 170 µl of water. 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 3 µl of the Sodium Cyanoborohydride Solution for every 200 µl 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 200 µl of 1X Coupling Buffer, centrifuge and discard the flow-through. Repeat this step once.

11. Add 200 µl 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 200 µl of Quenching Buffer to the resin.
13. In a fume hood, add 3 µl 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 200 µl of 1X Coupling Buffer, centrifuging after each wash.
16. Wash the resin six times with 150 µl of Wash Solution, centrifuging after each wash.
17. Either proceed to immunoprecipitation or, if storing the resin, proceed to the next step.
18. Wash the resin twice with 200 µl 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 200 µl 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 (decant) culture medium from cells.
2. Wash the cells once with 1X Coupling Buffer.
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 × 100 mm	500 µl-1 ml
100 × 60 mm	250-500 µl
6-well plate	200-400 µl per well
24-well plate	100-200 µl 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 1,000 × *g* for 5 minutes to pellet the cells. Discard the supernatant.
2. Wash cells once by suspending the cell pellet in 1X Coupling Buffer. Centrifuge at 1,000 × *g* for 5 minutes to pellet cells.
3. Add ice-cold IP Lysis/Wash Buffer to the cell pellet. Use 500 µl 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% 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 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 to determine the concentration and further analysis.

C. Antigen Immmunoprecipitation General Protocol

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.

1. Remove the bottom plug and loosen the screw cap of the spin column containing the antibody-coupled resin. Place the spin column in a collection tube and centrifuge to remove storage buffer. Discard the flow-through.
2. Remove screw cap and place column into a collection tube. Wash resin twice with 200 µl of ice-cold IP Lysis/Wash Buffer. Discard the flow-through after each wash.
3. Tap bottom of the spin column on a paper towel to remove excess liquid. Replace bottom plug.
4. Dilute the cell extract in IP Lysis/Wash Buffer. The recommended sample volume in the spin column is 300-600 µl. The suggested amount of total protein per IP reaction is 500-1,000 µg, as determined by the Pierce BCA Protein Assay.
5. Add the sample to the antibody-coupled resin in the spin column. Attach the screw cap and incubate column with gentle end-over-end mixing or shaking for 1 hour to overnight at 4°C.
6. 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.
7. Remove the screw cap, place the column into a new collection tube, add 200 µl 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.
8. Wash the sample three times with 200 µl IP Lysis/Wash Buffer and centrifuge after each wash.
9. Wash the sample once with 100 µl of 1X Conditioning Buffer.

D. Antigen Elution

1. Place the spin column into a new collection tube, add 25 µl of Elution Buffer and centrifuge.

   Optional**:** To neutralize the low pH of the Elution Buffer (e.g., for downstream enzymatic or functional assays), add 5 µl of 1 M Tris, pH 9.5 to the collection tube, which will neutralize the pH upon centrifugation (Step D3). Alternatively, use a neutral pH elution buffer (i.e., Gentle Elution Buffer, Product No. 21027).
2. Keep the column in the tube and add 75 µl of Elution Buffer. Incubate for 10 minutes at room temperature. The column does not need to be closed or mixed.

   Note: For a more concentrated eluate, less Elution Buffer may be used; however, overall yield might be reduced.
3. Centrifuge the tube and collect the flow-through. Analyze the eluate for presence of antigen. Perform additional elutions (i.e., Steps D1-D3) as needed. Analyze each eluate separately to ensure that the antigen has completely eluted.
4. To preserve activity of the antibody-coupled resin, immediately proceed to Section E, Resin Regeneration and Storage.

E. Resin Regeneration and Storage

1. Add 100 µl 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 200 µl 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%.

F. 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 1 M DTT to a final concentration of 100 mM in the 5X Sample Buffer.
2. Add 5X Sample Buffer to sample to make a 1X final solution (i.e., add 5 µl of 5X Sample Buffer to 20 µl 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 detected with the eluted antigen	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 measuring the absorbance at 280 nm
	The antibody-coupled resin was treated with a reducing agent (i.e., DTT or β-mercaptoethanol) during the IP or elution steps, which reduced the antibody and eluted antibody fragments or subunits that were not covalently linked to the resin	Use buffers that do not contain reducing agents
Antigen did not immunoprecipitate	Sample does 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 couple to the resin	Make sure the antibody solution does not contain amines or carrier proteins; verify the antibody coupling by monitoring the flow-through and wash fractions (i.e., measure the absorbance at 280 nm or analyze by SDS-PAGE)
	Component in the IP Lysis/Wash Buffer interfered with antibody-antigen binding	Perform the IP and washes using 1X Tris-Buffered Saline
Antigen did not elute	The antibody-antigen interaction was not disrupted by the Elution Buffer	Optimize the elution conditions (see Tech Tip #27 on our web site)
		Elute the antigen as follows: add 100 µl of 1X non-reducing SDS sample buffer to the column and incubate at 100°C for 5-10 minutes – keep the spin column in the tube while heating and do not plug or cap the column*
Antigen is non-functional in the downstream application	Antigen is sensitive to low pH and has become 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)

*After heating the antibody-coupled resin with SDS sample buffer, the resin cannot be reused and must be discarded.

Additional Information

A. Large-scale Antibody Coupling

The Pierce Direct 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 AminoLink Plus Coupling Resin and antibody to use for larger scale IPs.

Antibody Amount	Resin Slurry Amount (µl)	Reaction Volume (µl)
25-100 µg	100	200
50-200 µg	200	300
100-400 µg	400	400

B. Please visit the web site 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)

Related Thermo Scientific Products

78428	**Halt Phosphatase Inhibitor Single-Use Cocktail** (100X), 100 µl × 24 microtubes
78440	**Halt Protease and Phosphatase Inhibitor Cocktail** (100X), 1 ml
44600	**Pierce Antibody Clean-up Kit**
78430	**Halt Protease Inhibitor Single-Use Cocktail (100X),** 24 × 100 µl
89882	**Zeba Spin Desalting Columns,** 0.5 ml
28348	**20X Phosphate Buffered Saline** (Coupling Buffer), 500 ml
28358	**20X TBS Buffer,** 500 ml
69705	**Pierce Spin Columns – Screw Cap,** 25/pkg
69720	**Pierce Microcentrifuge Tubes,** 2 ml, 72/pkg
69715	**Pierce Microcentrifuge Tubes,** 1.5 ml, 72/pkg
89879	**Pierce Micro-Spin Columns,** 50/pkg
20501	**AminoLink Plus Coupling Resin,** 10 ml
39001	**Lane Marker Sample Buffer, Non-Reducing (5X),** 5 ml
21027	**Gentle Ag/Ab Elution Buffer,** pH 6.6, 500 ml
28372	**BupH™ Phosphate Buffered Saline (PBS),** each dry-blend pack makes 500 ml with water, 40 packs
21004	**IgG Elution Buffer,** low-pH elution buffer for general protein affinity purifications, 1 L
89897	**Pierce Centrifuge Columns, 5 ml** (resin bed capacity), gravity or centrifuge compatible, 25 units

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

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermo.com/pierce