INSTRUCTIONS

Pierce Crosslink Magnetic IP/Co-IP Kit

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88805

Number	Description
88805	**Pierce Crosslink Magnetic IP/Co-IP Kit**, contains sufficient reagents to perform 40 reactions using 25µL of magnetic beads

Kit Contents:

Pierce Protein A/G Magnetic Beads, 1mL supplied at 10mg/mL in water containing 0.05% NaN_3

IP Lysis/Wash Buffer, 2 × 50mL, pH 7.4, 25mM Tris, 150mM NaCl, 1mM EDTA, 1% NP40, 5% glycerol

20X Coupling Buffer, 25mL, when diluted results in 10mM sodium phosphate, 150mM NaCl; pH 7.2

DSS (disuccinimidyl suberate), No-Weigh Format, 8 × 2mg microtubes

Neutralization Buffer, 1.0mL, pH 8.5

Elution Buffer, 25mL, pH 2.0

Lane Marker Sample Buffer, Non-reducing, (5X), 5mL, 300mM Tris·HCl, 5% SDS, 50% glycerol, lane marker tracking dye; pH 6.8

Storage: Upon receipt store products at 4°C. Store DSS desiccated at 4ºC. Product is shipped with an ice pack.

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Introduction

The Thermo Scientific™ Pierce™ Crosslink Magnetic IP/Co-IP Kit enables highly efficient antigen immunoprecipitations (IP) and co-immunoprecipitations (co-IP) by covalently crosslinking antibodies onto Thermo Scientific™ Pierce™ Protein A/G Magnetic Beads. Using the crosslinking chemistry of disuccinimidyl suberate (DSS), the kit provides an effective method for performing IPs and co-IPs by coupling antibody to the beads and then covalently crosslinking to the beads with DSS. The antibody-crosslinked beads are then washed to remove antibody that is not covalently bound. The prepared beads are incubated with antigen sample, washed to remove non-bound material and eluted in a low-pH elution buffer that dissociates bound antigen from the antibody-crosslinked beads. The kit includes optimized buffers for high antigen yield using a minimum of 2µg of antibody for IP or 5-10µg of antibody for co-IP, as well as sample buffer for preparing samples or SDS-PAGE analysis. Coupling and crosslinking are performed manually with a magnetic stand, but both IP and co-IP can be performed manually or automatically with an instrument such as the Thermo Scientific™ KingFisher™ Flex System.

Procedure Summary

1. Prewash beads two times with 1X Modified Coupling Buffer.
2. Bind antibody to beads for 15 minutes.
3. Wash beads three times with 1X Modified Coupling Buffer.
4. Crosslink antibody to beads with DSS for 30 minutes.
5. Wash beads three times with Elution Buffer followed by two washes with IP Lysis/Wash Buffer.
6. Incubate cell lysate with antibody-crosslinked beads for 1-2 hours at room temperature or overnight at 4°C.
7. Wash beads two times with IP Lysis/Wash Buffer and one time with ultrapure water.
8. Elute bound antigen.

Important Product Information

- The antibody coupling and crosslinking steps are performed manually with a magnetic stand. The IP can be performed manually or with an automated instrument platform such as the KingFisher Flex Instrument.
- Do not centrifuge, dry or freeze the magnetic beads, as this can cause the beads to aggregate and lose binding activity.
- 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 recovery.
- 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 ~10mg of cell pellet and ~3µg/µL (or 300µg) when lysed with 100µL of IP Lysis/Wash Buffer.
- To minimize protein degradation, include protease inhibitors (e.g., Thermo Scientific™ Halt™ Protease Inhibitor Single-Use Cocktail, EDTA-free, Product No. 78425) when preparing cell lysates.
- The IP Lysis/Wash Buffer is compatible with the Thermo Scientific™ Pierce™ BCA Protein Assay (Product No. 23225).
- Optimal time for low-pH elution is 5 minutes. If the desired amount of antigen is not obtained, then a second 5-minute low-pH elution may be performed to remove additional bound antigen.

Additional Materials Required

- 1.5mL microcentrifuge tubes
- Antibody for coupling/crosslinking
- Antigen sample for IP
- Magnetic stand (e.g., DynaMag™-2 Magnet, Product No. 12321D)
- Phosphate-buffered saline (PBS, 100mM sodium phosphate, 100mM NaCl, pH 7.2, Product No. 28372)

For Automated IP:

- KingFisher Flex System with 96 Deep Well Head (Product No. 5400630)
- Thermo Scientific™ Microtiter Deep Well 96 Plate, V-bottom, polypropylene (100-1000µL, Product No. 95040450)
- KingFisher Flex 96 Tip Comb for Deep Well Magnets (Product No. 97002534)

Procedure for the Pierce Crosslink Magnetic IP/Co-IP Kit

A. Binding of Antibody to Protein A/G Magnetic Beads

Note: The following protocol is optimized for coupling 2-10µg of antibody.

1. Prepare 2mL of 1X Modified Coupling Buffer for each IP reaction by diluting 0.1mL of 20X Coupling Buffer and 0.1mL of IP Lysis/Wash Buffer with 1.8mL of ultrapure water.
2. Vortex the bottle of Pierce Protein A/G Magnetic Beads to obtain a homogeneous suspension. Add 25µL of beads into a microcentrifuge tube. Place tube on a magnetic stand to collect beads for 1 minute. Remove and discard the storage solution.
3. Add 500µL of 1X Modified Coupling Buffer prepared in Step 1 to the tube. Gently mix and incubate for 1 minute at room temperature on a rotating platform. Collect the beads on a magnetic stand, then remove and discard the supernatant. Repeat this step one time.
4. Dilute antibody 1:20 with 20X Coupling Buffer and 1:20 with IP Lysis/Wash Buffer, so the final concentration of antibody is 2-10µg per 100µL. For example, to prepare 100µL of antibody solution, dilute antibody stock into 5µL of 20X Coupling Buffer, 5µL of IP Lysis/Wash Buffer and add ultrapure water to bring the final volume to 100µL.
5. Add 100µL of prepared antibody solution to the beads, gently mix and incubate on a rotating platform for 15 minutes at room temperature. Gently vortex the beads every 5-10 minutes during incubation to ensure that the beads stay in suspension.
6. Collect the beads with a magnetic stand. Remove and discard the supernatant
7. Add 100µL of 1X Modified Coupling Buffer and gently vortex or invert the tube to mix. Collect the beads with a magnetic stand, then remove and discard the supernatant.
8. Add 300µL of 1X Modified Coupling Buffer and gently vortex or invert the tube to mix. Collect the beads with a magnetic stand, then remove and discard the supernatant. Repeat this step one time.

B. Crosslinking the Bound Antibody

Note: Conventional IP can be performed by omitting crosslinking; however, if crosslinking is omitted, the antibody will co-elute with the antigen during the elution steps.

Note: The DSS crosslinker is moisture-sensitive. Keep unused DSS in foil pouch. Dissolve DSS in DMSO or DMF immediately before use. DSS is not compatible with amine-containing buffers (e.g., Tris, glycine).

1. Puncture the foil cover of a single tube of DSS with a pipette tip and add 217µL of DMSO or DMF to prepare a 10X solution (25mM). Use the pipette to thoroughly mix the solution (i.e., draw up and expel the solution) until the DSS is dissolved.
2. Dilute the DSS 1:100 in DMSO or DMF (10µL of 10X DSS with 990µL solvent) to make 0.25mM DSS.
3. Add 2.5µL of 20X Coupling Buffer, 4µL of 0.25mM DSS and 43.5µL of ultrapure water to the beads. The total solution volume will be 50µL. The DSS is added at 10X molar excess to the Pierce Protein A/G Magnetic Beads at a working concentration of 20µM.
4. Incubate the crosslinking reaction for 30 minutes at room temperature on a rotator or mixer. Gently vortex the beads every 10-15 minutes during incubation to ensure that the beads stay in suspension.
5. Collect the beads with a magnetic stand. Remove and save the flow-through to verify antibody crosslinking.
6. Add 100µL of Elution Buffer to the beads and gently mix for 5 minutes at room temperature on a rotating platform to remove non-crosslinked antibody and quench the crosslinking reaction. Collect the beads with a magnetic stand, then remove and discard the supernatant.
7. Add 100µL of Elution Buffer to the beads and gently vortex or invert to mix. Collect the beads with a magnetic stand, then remove and discard the supernatant. Repeat one time.
8. Add 200µL of cold IP Lysis/Wash Buffer to the beads and gently vortex or invert to mix. Collect the beads with a magnetic stand, then remove and discard the supernatant. Repeat one time.
9. Proceed to the Manual or Automated Antigen Immunoprecipitation Protocols. If desired, the antibody-crosslinked beads can be stored at 4°C.

C. Mammalian Cell Lysis

Protocol I: Lysis of Cell Monolayer (Adherent) Cultures

1. Carefully remove culture medium from confluent cells.
2. Wash cells one time 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.

D. Manual Antigen Immunoprecipitation

1. Dilute the lysate solution to 500µL with IP Lysis/Wash Buffer.
2. Add 500µL of diluted lysate solution to the tube containing crosslinked magnetic beads and incubate for 1 hour at room temperature on a rotator or mixer. Gently vortex the beads every 10-15 minutes during incubation to ensure that the beads stay in suspension.
3. Collect the beads with a magnetic stand, remove the unbound sample and save for analysis.
4. Add 500µL of IP Lysis/Wash Buffer to the tube and gently mix. Collect the beads and discard the supernatant. Repeat this step one time.
5. Add 500µL of ultrapure water to the tube and gently mix. Collect the beads on a magnetic stand and discard the supernatant.
6. Add 100µL of Elution Buffer to the tube. Incubate for 5 minutes at room temperature on a rotator or mixer. Magnetically separate the beads and save the supernatant containing the target antigen. To neutralize the low pH add 10µL of Neutralization Buffer for each 100µL of eluate. For optimal antigen recovery, repeat this elution one time.

E. Automated Antigen Immunoprecipitation

Note: The following protocol is designed for use with the KingFisher Flex Instruments. The protocol can be modified according to your needs using the Thermo Scientific™ BindIt™ Software provided with the instrument.

1. Download the "Crosslink Immunopreciptation" protocol from the Thermo Fisher Scientific website into the BindIt Software on an external computer.
2. Transfer the protocol to the KingFisher Flex Instrument from an external computer. See the BindIt Software User Manual for detailed instructions on importing protocols.
3. Set up plates according to Table 2.

Table 2. Pipetting instructions for the IP protocol using Microtiter Deep Well 96 Plates.

Plate #	Plate Name	Content	Volume	Time/Speed
1	Bind	Protein A/G Beads	25µL	1 hour/Slow
		Antigen Sample	500µL	
2	Wash 1	IP Lysis/Wash Buffer	500µL	30 seconds/Slow
3	Wash 2	IP Lysis/Wash Buffer	500µL	30 seconds/Slow
4	Wash 3	Ultrapure Water	500µL	30 seconds/Slow
5	Elution 1	Elution Buffer	100µL	5 minutes/Slow
6	Elution 2	Elution Buffer	100µL	5 minutes/Slow
7	Tip Plate	KingFisher Flex 96 Tip Comb for Deep Well Magnets	-	10 seconds/Fast

4. Select the protocol using the arrow keys on the instrument keypad and press Start. See the KingFisher Flex Instrument User Manual for detailed information.
5. Slide open the door of the instrument's protective cover.
6. Load plates into the instrument according to the protocol requests, placing each plate in the same orientation. Confirm each action by pressing Start.
7. After the samples are processed, remove the plates as instructed by the instrument's display. Press Start after each plate. Press Stop after all the plates are removed.

Notes:

- The low-pH elutions need to be neutralized by adding 10µL of Neutralization Buffer for each 100µL of eluate directly to each well immediately after incubation takes place.
- If fewer than 96 wells are used, fill the same wells in each plate. For example, if using wells A1 through A12, use these same wells in all plates.
- Combine the Tip Comb with a Deep Well 96 Plate. See the instrument user manual for detailed instructions.
- To limit evaporation, select "Mix" and "Slow" speed under the subheading "Heating Action."

Troubleshooting

Problem	Possible Cause	Solution
Antibody detected with the eluted antigen	Non-crosslinked antibody was not removed sufficiently with the washes following the crosslinking procedure	Increase the number of Elution Buffer washes after crosslinking
	The antibody-coupled beads were treated with a reducing agent (e.g., DTT) during the IP or elution steps, reducing the antibody and eluted antibody fragments or subunits that were not covalently linked to the beads	Use buffers not containing reducing agents
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
		Add more sample
	Antibody could not bind antigen	Use a recent source of the specific antibody or an alternative antibody that recognizes a different epitope
	Component in the IP Lysis/Wash Buffer interfered with antibody-antigen binding	Perform the IP and washes using alternate buffer (e.g., 0.5% CHAPS in TBS)
Low amount of recovered protein	The protein degraded	Add protease inhibitors
	Insufficient amount of magnetic beads was used	Increase the amount of magnetic beads used for capture
	Sample had an insufficient amount of target protein	Increase amount of antigen sample
Protein does not elute	Elution conditions were too mild	Increase incubation time with elution buffer to 10 minutes
		Use more stringent elution buffer
Multiple nonspecific bands	Nonspecific protein bound to the magnetic beads	Add 50-350mM NaCl to the Binding/Wash and Elution Buffers
Recovered protein was inactive	Elution conditions were too stringent	Use a milder elution buffer (e.g., Pierce Gentle Ag/Ab Elution Buffer, Product No. 21027)
Magnetic beads aggregated	Magnetic beads were frozen or centrifuged	Handle beads as directed in the instructions
	Beads were not thoroughly mixed before and/or during incubation	

Additional Information Available on Our Website

• Frequently Asked Questions

• Tech Tip #43: Protein stability and storage

• Tech Tip #34: Binding characteristics of Protein A, Protein G, Protein A/G and Protein L

• Visit thermofisher.com for information on KingFisher Products

Frequently Asked Questions for the Thermo Scientific KingFisher Instrument

Question	Answer
Which plates are compatible with KingFisher Flex and KingFisher 96 Instruments?	The KingFisher Flex Instrument is compatible with KingFisher 24 Deep Well Plates, Microtiter Deep Well 96 Plates, KingFisher 96 and 96 PCR Plates.
Is it possible to concentrate samples during the run?	Both deep-well plates and KingFisher 96 Plates can be used during the same run. Therefore, it is possible to start the processing using larger volumes (in a deep-well plate) and elute the purified sample to a smaller volume (in a KingFisher 96 Plate).
Is it possible to heat the samples during the run?	The heating block is located inside the instrument and can be used automatically during the sample process. All plates compatible with the KingFisher Flex Instrument can be heated using specially designed, interchangeable heating blocks.
Are the reagent volumes in each well critical?	For best results, keep the specified volumes within defined limits to avoid spillover.

Related Products

88802-3 **Pierce Protein A/G Magnetic Beads**
88804 **Pierce Classic Magnetic IP/Co-IP Kit**
88816-7 **Pierce Streptavidin Magnetic Beads**
88826-7 **Pierce NHS-Activated Magnetic Beads**
88828 **Pierce Direct Magnetic IP/Co-IP Kit**
24615 **Imperial™ Protein Stain,** 1L
34075 **SuperSignal™ West Dura Extended Duration Substrate**
78440 **Halt™ Protease and Phosphatase Inhibitor Cocktail (100X)**
78430 **Halt Protease Inhibitor Single-Use Cocktail (100X)**
28348 **Phosphate Buffered Saline (20X)**
87787 **Pierce IP Lysis Buffer**
LC2673 **Novex™ Tris-Glycine Native Sample Buffer**
21658 **DSS (disuccinimidyl suberate), No-Weigh™ Format**
XP04200BOX **Novex Tris-Glycine protein gels** (see thermofisher.com/proteingels for a complete listing)

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