INSTRUCTIONS

Pierce Magnetic HA-Tag IP/Co-IP Kit

88838 2516.2

Number	Description
88838	**Pierce Magnetic HA-Tag IP/Co-IP Kit,** contains sufficient reagents to perform 40 reactions using 25µL of magnetic beads

Kit Contents:

HA-tagged Positive Control (26180X), 500µL, 1mg/mL, *Escherichia coli* extract containing HA-tagged GST-PI3K-SH2 domain

Application Set (88838X):

Pierce Anti-HA Magnetic Beads, 1mL, supplied at 10mg/mL in PBS containing 0.05% Tween™-20 Detergent and 0.05% sodium azide

Pierce IP Lysis/Wash Buffer, 2 × 50mL, pH 7.4, 0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP40, 5% glycerol

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

Elution Buffer, 5mL, pH 2.0

Neutralization Buffer, 1mL, pH 8.5

Storage: HA-tagged Positive Control ships separately on dry ice; upon receipt store at -70°C. Application Set shipped at ambient temperature; upon receipt store at 4°C.

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Introduction

The Thermo Scientific™ Pierce™ Magnetic HA-Tag IP/Co-IP Kit provides a simple and fast method to study HA-tagged proteins with advantages over the traditional immunoprecipitation (IP) procedure using Protein A/G magnetic beads. The high-affinity, anti-HA antibody-coupled beads enable IP of HA-tagged proteins or co-immunoprecipitation (co-IP) of their interacting partners without antibody contamination. The Thermo Scientific™ Pierce™ Anti-HA Magnetic Beads are used for the IP of specific HA-tagged proteins expressed in human *in vitro* expression systems and mammalian and bacterial cell lysates. The anti-HA antibody coupled to the blocked magnetic beads is a high-affinity mouse IgG_1 monoclonal antibody that recognizes the HA-epitope tag (YPYDVPDYA) derived from the human influenza hemagglutinin (HA) protein. For immunoprecipitation, the beads are added to a sample containing HA-tagged proteins. The bound HA-tagged proteins are dissociated from the beads using a low-pH elution buffer and removed from the solution manually using a magnetic stand or by automation using an instrument such as the Thermo Scientific™ KingFisher™ Flex or KingFisher Duo Instrument. Automated instruments are especially useful for large-scale screening of multiple samples.

Table 1. Characteristics of the Thermo Scientific Pierce Anti-HA Magnetic Beads.

Composition:	High-affinity mouse IgG_1 monoclonal antibody covalently coupled to a blocked magnetic bead surface
Magnetization:	Superparamagnetic (no magnetic memory)
Mean Diameter:	1µm (nominal)
Density:	$2.0 g/cm^3$
Bead Concentration:	10mg/mL
Binding Capacity:	≥ 10µg GST-ERK-HA (70kDa fusion protein)/mg of beads

Important Product Information

- Do not centrifuge, dry or freeze the Pierce Magnetic Beads. Centrifuging, drying or freezing will cause the beads to aggregate and lose binding activity.
- For best results, determine optimal conditions for expression of HA-tagged fusion protein before attempting IP.
- Include a non-transfected lysate as a negative control to identify nonspecific binding of proteins to the anti-HA magnetic beads. The HA-tagged Positive Control assists in verifying whether the anti-HA magnetic beads can successfully capture the HA-tagged protein.
- Cell lysate with expressed HA-tagged protein may be prepared from mammalian cells using the procedure provided or from bacterial cells using a bacterial lysis protocol (e.g., Thermo Scientific™ B-PER™ II Bacterial Protein Extraction Reagent, Product No. 78260). The buffers provided in this kit are compatible with samples prepared from bacterial lysate.
- To minimize protein degradation, include protease inhibitors (e.g., Thermo Scientific™ Halt™ Protease Inhibitor Single-Use Cocktail, EDTA-free, Product No. 78425) in the preparation of cell lysates.
- Binding capacity and elution recovery will vary depending on the HA-fusion protein and the method of elution.
- A low-pH elution may be used for single-use applications. Optimal time for low-pH elution is 10 minutes; exceeding 10 minutes may result in nonspecific binding and yield reduction. The HA antibody will not leach from the beads when eluting with IgG Elution Buffer, pH 2.0.
- Pierce Anti-HA Magnetic Beads are compatible with IP and analyses by Western blot.
- Do not use cell lysate containing dithiothreitol (DTT). DTT may cause the HA antibody to leach from the beads.

Additional Materials Required

- Phosphate-buffered saline (PBS, 100mM sodium phosphate, 100mM NaCl, pH 7.2; Product No. 28372)
- DTT (Product No. 20290)
- Sample containing HA-tagged protein

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)

For Manual IP:

- 1.5mL microcentrifuge tubes
- Magnetic stand (e.g., Thermo Scientific™ MagnaBind™ Magnet for 6 × 1.5mL microcentrifuge tubes, Product No. 21359)
- End-over-end rocker or rotator

Procedure for Lysis of Mammalian Cells

Note: For optimal results, use a protease inhibitor cocktail such as Halt Protease Inhibitor Cocktail (Product No. 87786) when preparing cell lysate.

Protocol I: Lysis of Cell Monolayer (Adherent) Mammalian Cells

1. Carefully remove culture medium from confluent cells.
2. Wash the cells once with ice-cold PBS.
3. Add the appropriate volume of IP Lysis/Wash Buffer (Table 2) to the cells. Incubate on ice for 5 minutes with periodic mixing.
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 (e.g., Thermo Scientific™ BCA Protein Assay Kit, Product No. 23225) and further analysis.

Table 2. Recommended 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

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 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.
5. Transfer supernatant to a new tube for protein concentration determination (e.g., BCA Protein Assay Kit, Product No. 23225) and further analysis.

Procedure for IP of HA-Tagged Proteins

A. Manual IP/Co-IP

Note: The amount of lysate needed and incubation time are dependent upon the expression level of the HA-tagged protein and require optimization for each specific system. For co-IP experiments, the buffer system must be optimized to maintain the protein:protein interaction.

Note: To ensure homogeneity, mix the beads thoroughly before use by repeated inversion, gentle vortexing or using a rotation platform.

1. Place 25µL (0.25mg) of Pierce Anti-HA Magnetic Beads into a 1.5mL microcentrifuge tube.
2. Add 175µL of IP Lysis/Wash Buffer to the beads and gently vortex to mix.
3. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
4. Add 1mL of IP Lysis/Wash Buffer to the tube. Invert the tube several times or gently vortex to mix for 1 minute. Collect beads with a magnetic stand. Remove and discard the supernatant.
5. Add the sample containing the HA-tagged protein to the pre-washed magnetic beads and incubate at room temperature for 30 minutes with mixing.

   Note: To prepare the positive control, dilute 30µL into 270µL of IP Lysis/Wash Buffer.
6. Collect the beads with a magnetic stand, remove the unbound sample and save for analysis.
7. Add 300µL of IP Lysis/Wash Buffer to the tube and gently mix. Collect the beads and discard the supernatant. Repeat this wash twice.
8. Add 300µL of ultrapure water to the tube and gently mix. Collect the beads on a magnetic stand and discard the supernatant.

B. Elution of HA-Tagged Protein

Note: If the eluted HA-tagged protein will be used for functional applications, use Elution Protocol 1 to elute the protein. If the protein is sensitive to the low pH, use Thermo Scientific™ Pierce™ HA Peptide (Product No. 26184) to competitively elute the HA-tagged protein. Elute in 100µL of Pierce HA Peptide (2mg/mL in Tris-buffered saline containing 0.05% Tween-20 Detergent) for 5-10 minutes at 37°C. For electrophoretic analysis, use Elution Protocol 2.

- **Elution Protocol 1**

1. Add 100µL of Elution Buffer to the beads. Incubate the beads at room temperature with mixing for 5-10 minutes.
2. Separate the beads on a magnetic stand and save the supernatant containing the target antigen. To neutralize the low pH, add 15µL of Neutralization Buffer for each 100µL of eluate.
3. For reducing-gel analysis, prepare Reducing Sample Buffer by adding 2.5µL of 2M DTT and 20µL of Lane Marker Non-reducing Sample Buffer (5X).
4. Add 77.5µL of elution sample to the 22.5µL of prepared Reducing Sample Buffer. Heat sample at 95-100°C for 5-10 minutes in a heat block.

- **Elution Protocol 2**

1. Prepare Non-reducing Sample Buffer by mixing 200µL of 5X Lane Marker Sample Buffer with 800µL of ultrapure water. The 1X solution may be stored at 4°C for up to one year.
2. Add 100µL of 1X Non-reducing Sample Buffer to the beads. Vortex briefly to resuspend the beads and then incubate in a heat block at 95-100°C for 5-10 minutes.

   Note: Using Non-reducing Sample Buffer can minimize interference from co-eluting antibody fragments.
3. For reducing-gel analysis, add 2.5µL of 2M DTT to the 100µL sample.

C. Automated IP/Co-IP

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

1. Download the "HA_Tag_Immunoprecipitation" protocol from the Thermo Scientific website (http://www.thermoscientific.com/bindit-protocols) 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 3.

Table 3. Pipetting instructions for the IP protocol using the Thermo Scientific Microtiter Deep Well 96 Plates.

Plate #	Plate Name	Content	Volume	Time/Speed
1	Beads	Anti-HA Magnetic Beads	25µL	5 seconds
		IP Lysis/Wash Buffer	175µL	
2	Bead Wash	IP Lysis/Wash Buffer	1000µL	1 minute/slow
3	Bind	Sample containing HA-tagged Protein	300µL	30 minutes/slow
4	Wash 1	IP Lysis/Wash Buffer	300µL	30 seconds/slow
5	Wash 2	IP Lysis/Wash Buffer	300µL	30 seconds/slow
6	Wash 3	Ultrapure Water	300µL	30 seconds/slow
7	Elution	Elution Buffer	100µL	10 minutes/slow
8	Tip Plate	KingFisher 96 Tip Comb for Deep Well Magnets	-	10 seconds/fast

Notes:

- If less 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.
- To ensure bead homogeneity, mix the vial thoroughly by repeated inversion, gentle vortexing or rotating platform before adding the beads to Plate 1.
- If low-pH Elution Buffer is selected for elution, neutralize the pH by adding 15µL of Neutralization Buffer for each 100µL of eluate upon run completion.
- If using SDS-PAGE Sample Buffer in a heated elution, install the KingFisher Flex Heating Block (see manual for proper installation) to heat samples at 95-100°C for 5-10 minutes.

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 request, 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 removing each plate. Press Stop after all the plates are removed.

Troubleshooting

Problem	Possible Cause	Solution
Little or no HA-tagged protein is detected	Tagged protein degraded	Include protease inhibitors (e.g., Halt Protease Inhibitor Single-Use Cocktail, EDTA-free, Product No. 78425) in the Lysis Buffer
		Use new lysate or lysate stored at -80°C
	No or minimal tagged protein was expressed	Verify protein expression by SDS-PAGE or Western blot analysis of the lysate using HA-tagged Positive Control as a reference
		Increase the amount of lysate used for IP/co-IP
		Use a more sensitive detection system such as Thermo Scientific SuperSignal West Femto Chemiluminescent Substrate (Product No. 34095)
Failure to co-IP interacting protein	Wash conditions were too stringent for the weak or transient interaction	Reduce the number of washes and/or lower the ionic strength of wash buffer
	Interacting protein was expressed at a low level	Apply additional protein sample
		Use a more sensitive detection system
	Buffer system was not optimal for the protein:protein interaction	Optimize the co-IP buffer
	Insufficient sample loaded on the gel to detect by Western blot	Elute sample in 30% acetonitrile/0.5% formic acid, then dry down using a Thermo Scientific™ Speedvac™ Vacuum Concentrator. Bring the sample back up in SDS-PAGE Sample Buffer and load entire elution fraction on to gel
Magnetic beads aggregated	Magnetic beads were frozen or centrifuged	Handle the beads as directed in the instructions
	Buffer was incompatible with magnetic beads	
	Detergent was not added to the wash and bind solutions	

Additional Information Available on Our Website

- Frequently Asked Questions
- Tech Tip #43: Protein stability and storage
- Visit *www.thermoscientific.com/kingfisher* for information on the KingFisher Products
- In the U.S.A., purchase KingFisher Supplies from Fisher Scientific. Contact your local Thermo Fisher Scientific office to purchase KingFisher Supplies outside the U.S.A.

Frequently Asked Questions for the KingFisher Instrument

Question	Answer
Which plates are compatible with the KingFisher Flex Instrument?	The KingFisher Flex Instrument is compatible with the KingFisher 24 Deep Well Plates, Microtiter Deep Well 96 Plates and KingFisher 96 and 96 PCR Plates
Is it possible to concentrate samples during the run?	Both deep-well plates and KingFisher 96 Plates may 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
Why do the beads stick to the plastic tips and wells or the eluted proteins stick to the wells?	Eluted proteins and proteins conjugated to beads can nonspecifically bind to plastics. Adding detergent to the Binding/Wash Buffer prevents the protein conjugated to the bead from sticking (e.g., 0.05%-0.1% Tween-20 Detergent). Also include a small amount of detergent in the elution buffer (e.g., 0.05% Tween-20 Detergent) or silanize the elution plate
Are the reagent volumes in each well critical?	For best results, keep the specified volumes within defined limits to avoid spillover

Related Thermo Scientific Products

88836-7	**Pierce Anti-HA Magnetic Beads**
88832-3	**HisPur™ Ni-NTA Magnetic Beads**
88821-2	**Pierce Glutathione Magnetic Beads**
26180	**Pierce HA-Tag IP/Co-IP Kit**
26181-2	**Pierce Anti-HA Agarose**
26183	**Anti-HA Antibody**
26184	**Pierce HA Peptide**
20290	**DTT (Dithiothreitol)**
78260	**B-PER II Bacterial Protein Extraction Reagent**