Anti-HA magnetic beads

Materials Needed

* Pierce Anti-HA magnetic beads (88837)
* Magnetic rack (ThermoFisher 12321D DynaMag 2)
* Elution buffer
  + SDS-PAGE loading buffer
  + ThermoFisher HA Peptide (26184)
  + ThermoFisher IgG elution buffer, pH 2.0 (21028)
* PBS or TBS
* Wash buffer:
  + TBST
  + IP lysis buffer diluted 1:10 (Thermo 87788)
* 1.5 mL centrifuge tubes
* Tube rotator or tube shaker at 4°C
* Pipettes

Prep

**See Notes section at end for more info on controls.**

1. **Lysates can be pre-cleared with unconjugated plain or Protein A/G magnetic beads (such as ThermoFisher 88803) to eliminate nonspecific binding proteins.**
2. Quantify total protein present in lysates with a BCA assay before affinity purification. Adjust volumes to normalize protein concentrations across fractions.
   1. The BCA assay can also be used after affinity purification if 0.1 M glycine was used for elution.
   2. The Bio-Rad RC-DC assay or the Pierce 660 nm protein assay reagent (22660) with ionic detergent compatibility reagent (22663) can be used if total protein quantification is desired after elution in non-reducing SDS-PAGE loading buffer.
3. Label 1.5 mL tubes for each sample:
   1. 1 tube for input
   2. 3-6 for eluate: gels, blots, and mass spectrometry
4. Gently vortex beads to mix before aspirating from container.

Procedure for IP of HA-tagged protein

Immunoprecipitation

1. Place 100 μL Pierce Anti-HA Magnetic Beads per 1 mg total protein into a 1.5mL tube.
   1. **BWS:** Adjust volumes as needed. ThermoFisher recommends 25 µL (0.25 mg), but I have seen increased yields when using 100 μL beads for Nrf1-HA.
2. Wash 3x with 0.05% TBST 500 µL 0.05% TBST/100 μL beads. Invert or gently vortex each time.
   1. **BWS:** Lift plastic tube rack off magnet and invert 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.
   1. **BWS:** Be careful when opening tubes in the plastic stand. It can break.
   2. **BWS:** the same tip can be used to add TBST to all tubes if the tip does not contact the tubes. When aspirating supernatant, discard the tip each time to avoid transfer of beads among tubes.
4. Add sample to pre-washed beads for 30 min RT on tube rotator.
   1. **BWS:** Overnight 4°C is typical, but ThermoFisher found no difference in yield between 30, 60, or 120 min RT and overnight at 4°C. See ThermoFisher email 02/10/2016. Shorter incubation times may prevent dissociation of protein complexes or degradation by proteases.
5. Collect the beads with a magnetic stand. **Remove the unbound sample and save for analysis.**
6. Wash 3x with 500 µL TBST. Each time, invert to mix, separate beads, and discard supernatant.
7. Wash 1x with 500 µL ultrapure water. Invert to mix, separate beads, and discard supernatant.

Elution

SDS-PAGE loading buffer elution

1. Add 2-4x bead volume 2x SDS-PAGE loading buffer to the tube.
   1. **BWS:** 4x loading buffer can be diluted in IP lysis buffer or npH2O.
2. Gently vortex to mix and incubate the sample at 65-100°C for 5-10 minutes.
   1. **BWS: 65-75°C 10 min or 95°C 5 min. 10 min 95°C may elute antibody and increase background. I have found SDS-PAGE elution to have the highest yield.**
3. Magnetically separate the beads and save the supernatant containing the target antigen.
   1. Notes: Using non-reducing sample buffer can minimize interference from co-eluting antibody fragments. For elution under reducing conditions, add 2.5µL of 2M DTT/100µL sample.

HA peptide elution

1. Prepare Pierce HA Peptide (26184) at 2 mg/mL in TBS. Aliquot and store at -20°C.
2. Add 2x bead volume of 2 mg/mL Pierce HA Peptide to the beads, gently vortex to mix and incubate the sample at 37°C on a rotator for 5-10 minutes. Elution may be performed at reduced temperatures, but lower yields may result.
   1. **BWS:** 200 μL/100 μL beads (2x bead volume as recommended in 26184 datasheet). Use the Eppendorf ThermoMixer F1.5 to elute at 37°C with mixing. Elution is not as effective at 30°C, and would be less effective at RT. See email from Funmilayo Suleman at ThermoFisher 02/10/2016.
3. Separate the beads on a magnetic stand and save the supernatant containing the target antigen.
4. Repeat elution step once for higher recovery.
   1. **If the eluted HA-tagged protein will be used for function applications or is sensitive to pH extremes or sodium thiocyanate, then elute the protein with Pierce HA Peptide.**
   2. **BWS:** The peptide out-competes HA-tagged proteins for binding to the antibody, displacing the HA-tagged proteins, which will then appear in the eluate.
   3. **BWS:** According to Pierce tech support, elution with 0.1 M Glycine pH 2.0 results in double the yield over HA peptide elution (phone call 12/07/2015), but HA peptide elution will be the cleanest (phone call 12/09/2015). **Pierce HA Peptide may interfere with protein determination assays and absorbance at 280nm. It may also generate a signal when the sample is analyzed by mass spectrometry. Remove the HA Peptide with a desalting column, such as Thermo Zeba 89882 (30-120 μL) or 89889 (200-700 μL).**

Acidic elution

1. Add 100µL of IgG Elution Buffer, pH 2.0 or 0.1M glycine, pH 2.0.
   1. Glycine FW: 70.07. 1 M=75.07 g/L, 0.1 M=7.5 g/L x0.375 g/50 mL
2. Gently vortex to mix and incubate the sample at room temperature on a rotator for 5-10 minutes.
3. Magnetically separate the beads and save the supernatant containing the target antigen.
4. To neutralize low pH, add 15 µL Neutralization Buffer (1 M Tris, pH 8.5) for each 100 µL of eluate.
   1. Notes: A low-pH elution may be used for single-use applications. Optimal incubation time for low-pH elution is 5-10 minutes; exceeding 10 minutes may result in nonspecific binding and yield reduction. The HA antibody will not leach from the beads when eluting with the recommended acidic elution buffer (0.1M glycine, pH 2.0).

Basic elution

1. Add 100µL of 50mM NaOH to the tube.
2. Gently vortex to mix and incubate the sample at room temperature on a rotator for 5 minutes.
3. Magnetically separate the beads and save the supernatant containing the target antigen.
4. Neutralize the sample by adding 50µL of Neutralization Buffer for each 100µL of eluate.
   1. Notes: Basic elution buffer (e.g., 50mM NaOH) may be used to elute HA-tagged protein; however, the stringency of the buffer will cause the HA antibody to leach from the beads.

Important product information

* **BWS:** see product documentation for full info
* **Do not centrifuge, dry or freeze the Pierce Anti-HA Magnetic Beads.** Centrifuging, drying or freezing will cause the beads to aggregate and lose binding activity. To ensure good dispersal of beads for optimal antibody binding, it is important to include 0.025% to 0.1% non-ionic (e.g., Tween-20 Detergent) or zwitterionic (e.g., CHAPS) detergent in the binding and wash buffers and to mix the beads during incubation.
* **Use lysis buffer to adjust the IP reaction volume.**
* **Do not use cell lysate containing dithiothreitol (DTT). DTT may cause the HA antibody to leach from the beads.**
* If desired, a reference HA-tagged positive control is available (Product No. 26180X).

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| **Table 1. Characteristics of the Thermo Scientific Pierce Anti-HA Magnetic Beads.** | |
| **Composition:** | High-affinity mouse IgG1 monoclonal antibody covalently coupled to a blocked magnetic bead surface |
| **Magnetization:** | Superparamagnetic (no magnetic memory) |
| **Mean Diameter:** | 1µm (nominal) |
| **Density:** | 2.0g/cm3 |
| **Bead Concentration:** | 10mg/mL |
| **Binding: Capacity:** | ≥ 10µg GST-ERK-HA (70kDa fusion protein)/mg of beads |

Anti-HA agarose

Materials Needed

* Pierce Anti-HA Agarose resin (26182)
* PBS or TBS
* TBST or RIPA buffer
* SDS-PAGE loading buffer
* 1.5 mL centrifuge tubes
* Tube rotator or tube shaker at 4°C
* Pipettes

Prep

1. **Lysates can be pre-cleared with control agarose resin (26150) to eliminate nonspecific binding proteins.**
2. Quantify total protein present in cell fractions with a BCA assay before affinity purification.
   1. Adjust volumes to normalize protein concentrations across fractions.
   2. The BCA assay can also be used after affinity purification if 0.1 M glycine was used for elution.
   3. The Bio-Rad RC-DC assay or the Pierce 660 nm protein assay reagent (22660) with ionic detergent compatibility reagent (22663) can be used if total protein quantification is desired after elution in non-reducing SDS-PAGE loading buffer.
3. Retain ~10% of the total lysate volume as an input control.
4. An IgG isotype control purification can also be performed.
5. Cell fractionation can be verified by Western blot for fraction-specific proteins.
6. Label 1.5 mL tubes for each sample
   1. 1 tube for pre-elution supernatant
   2. 3-6 for eluate: gels, blots, and mass spectrometry
7. Thoroughly resuspend the Pierce Anti-HA Agarose by inverting the bottle several times before dispensing. Do not vortex.

Procedure for IP of HA-tagged protein

A. Immunoprecipitation using spin columns or microcentrifuge tubes

1. Add 100 μL Pierce Anti-HA Agarose slurry per sample to 1.5 mL tubes. Centrifuge 1 min max speed to pellet resin. Discard supernatant.
   1. The total original amount of resin slurry is the **resin volume**.
   2. The resin pellet volume after discarding liquid is the **bed volume**.
   3. A repeater pipette can be used to ensure consistent amounts of resin across samples.
2. Add one resin volume PBS or TBS. Centrifuge 1 min max speed. Discard supernatant.
   1. Alternatively, the total amount of resin needed can be added to a single tube, washed, returned to original resin volume with buffer, and aliquotted into tubes with samples. Note that more resin will be lost in tubes and tips with this method.
3. Add sample lysates to tubes **(1-2 mg total protein, ≥200 μL or 2x resin volume).** Pipette 5x to mix.
4. Incubate 4°C overnight on tube rotator in cold room.
5. Centrifuge 1 min max speed to pellet resin. Remove supernatant for analysis of binding efficiency.
6. Prepare wash solution (0.05% TBST).
7. Wash resin 3x with TBST.
   1. Add 1 mL TBST per sample. Pipette 5x to mix.
   2. Wash on tube rotator or tube shaker 5 min.
   3. Centrifuge 1 min max speed.
   4. Discard supernatant.
   5. Repeat.

B. Elution of HA-tagged protein

Chemical elution protocol

1. Add one bed volume non-reducing **2x** SDS-PAGE loading buffer. Pipette 5x to mix.
   1. Dilute 4x loading buffer in TBS or RIPA buffer.
   2. **SDS-PAGE loading buffer will denature the antibody and inactivate the resin.**
   3. **Do not add β-mercaptoethanol.** It will elute the antibody from the resin.
   4. Optional: heat to 65°C 5 min and vortex before centrifugation to maximize elution.
2. Centrifuge 1 min max speed to pellet resin.
3. Aliquot elution fraction and store at -80°C.
4. Repeat 2 more times.
   1. If using glycine or NaOH elution, neutralize fraction with 1:10 - 1:20 of 1M Tris, pH 9.5.
   2. Do not keep the elution buffers on the column for an extended period of time.
5. To reuse resin, wash column with five bed volumes of 3M NaSCN followed by 10 bed washes of TBS.

**Elution with SDS-PAGE loading buffer will result in the highest yield but also the highest background, because it will elute non-specific proteins.**

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| **Table 2. Advantages and disadvantages of the chemical elution options.** | | |
| **Solution** | **Advantage** | **Disadvantage** |
| 0.1M Glycine, pH 2-2.8 | Useful if protein is resistant to low pH  Preserves resin binding activity | May denature protein  Elution capacity is generally lower |
| 50mM NaOH | High elution capacity | May denature protein  Reduces resin life |
| 3M NaSCN | High elution capacity  Preserves resin binding capacity | May denature protein |
| **Note:** No loss of binding capacity occurs after 10 binding/elution steps of 0.1M glycine or 3M NaSCN; however, loss of resin activity can occur with exposure to 50mM NaOH. | | |

Gentle elution protocol

1. Prepare Pierce HA Peptide at 1mg/mL in TBS.
2. Add one bed volume of 1mg/mL Pierce HA Peptide and incubate for 10-15 minutes at 30°C. Elution may be performed at reduced temperatures; however, lower yields may result.
3. Pellet resin with a 5-10 second pulse at 12,000 × *g*. Collect eluate.
4. Repeat steps 2 and 3 two to three additional times.
5. If resin is to be reused, wash five times with one bed volume 3M NaSCN to remove bound HA peptide.

**Note:** Pierce HA peptide (26184) can be used to competitively elute HA-tagged fusion proteins from immobilized anti-HA affinity resin. The peptide out-competes HA-tagged proteins for binding to the antibody, displacing the HA-tagged proteins, which will then appear in the eluate. **If the eluted HA-tagged protein will be used for function applications or is sensitive to pH extremes or sodium thiocyanate, then elute the protein with Pierce HA Peptide.** According to Pierce tech support, elution with 0.1 M Glycine pH 2.0 results in double the yield over HA peptide elution (phone call 12/07/2015), but HA peptide elution will be the cleanest (phone call 12/09/2015).

**Pierce HA Peptide may interfere with protein determination assays and absorbance at 280nm. It may also generate a signal when the sample is analyzed by mass spectrometry. Remove the HA Peptide with a desalting column, such as Thermo Zeba 89882 (30-120 uL) or 89889 (200-700 uL).**

Procedure for Column Purification of HA-tagged Protein

A. Column Set-up

1. Pre-equilibrate the resin and buffers and perform all steps at room temperature. If the protein is temperature-sensitive, the procedure may be performed at 4ºC.
2. Obtain a spin or gravity-flow column. The flow rate of the gravity flow column can be controlled by adding tubing at the bottom opening of the column. Use the recommended centrifuge force if using a spin column.
3. Resuspend resin and add 1-4 mL of the slurry to the column. Allow the bed to drain. Wash the column with 2-5 bed volumes of TBS. Do not allow the resin to become dry.

B. Binding of HA Fusion Protein to Column

1. Add cell lysate to column. Lysate volume should be at least equal to the bed volume. Adjust volume with TBS if needed.
2. Adjust the flow rate to 0.5mL/min. Multiple binding passes may be required for complete binding. Capping the column and incubating on an end-over-end rocker may improve binding.
3. Collect flow-through and save for analyzing binding efficiency.
4. Wash the column with 10 bed volumes of TBS containing 0.05% Tween-20 (TBS-T). Washes can be analyzed by measuring the absorption at 280nm or by protein assay to confirm if the final washes contain no protein.

C. Elution of HA Fusion Protein from Column

**Note:** If the eluted HA-tagged protein will be used for functional applications or is sensitive to pH extremes or sodium thiocyanate, elute with Pierce HA Peptide.

Gentle elution protocol

1. Add the bottom plug to the column and add one bed volume of 1mg/mL Pierce HA Peptide in TBS. Incubate at 30°C for 10-15 minutes. Elutions may be performed at lower temperatures, but elution efficiency may be reduced.
2. Remove column plug and cap and collect elution fraction.
3. Repeat steps 1 and 2 two to three more times.
4. If the resin is to be reused, wash the column with five bed volumes of 3M NaSCN, followed by 10 bed washes of TBS.
5. For storage of the column, add two bed volumes of TBS containing 0.05% azide. Store column at 4°C.

Chemical elution protocol

1. Add one bed volume of 0.1M glycine pH 2.0-2.8, 3M NaSCN, or 50mM NaOH three times.
2. Repeat step 1 two additional times for a total of three elution fractions.
3. Collect elution fraction. If using glycine or NaOH elution, neutralize fraction with 1:10 - 1:20 1M Tris, pH 9.5. Do not keep elution buffers on column for an extended period of time.
4. To reuse resin, wash column with five bed volumes of 3M NaSCN followed by 10 bed washes of TBS.
5. For storage of the column, add two bed volumes of TBS containing 0.05% azide. Store column at 4°C.

Notes

Controls

* **Pre-clearing control: unconjugated magnetic beads or agarose.**
* **Antibody control:** IP with IgG beads on cell lysate expressing tagged protein.
* **Input control:** Retain ~10% of the total pre-IP lysate volume. Run 2-5% total protein input on blots.
* **Tag control:** 
  + If a tagged protein is used, cells expressing the tag alone
  + HA IP on cells not expressing the tag1

General

* Ribosomal pathways are frequently enriched because ribosome proteins have strong affinity for magnetic beads2,3.
* Note that trypsin digestion can also be performed “on bead” (prior to elution from magnetic beads)3,4.
* Using multiple tags can improve protein enrichment (like 3x FLAG5).
* High affinity tags, like 3x FLAG, can be difficult to elute from beads. This is not an issue if doing on-bead digestion for mass spec.
* Cell fractionation can be verified by Western blot for fraction-specific proteins.
* See [Life Technologies Pierce Anti-HA Agarose User Guide](http://tools.lifetechnologies.com/content/sfs/manuals/MAN0011739_Pierce_AntiHA_Agarose_UG.pdf) for more info.
* The binding capacity is 60-150 nmol HA-tagged fusion protein per 1mL of settled resin. Elution capacity is at least 6- 15nmol HA-tagged fusion protein per 1mL of settled resin using 3M NaSCN. Binding and elution capacity will vary depending on the HA-fusion protein and the method of elution. Binding and elution capacities are based on a 37kDa HA- tagged protein.
* Elution in 0.1 M glycine is recommended if non-reducing SDS-PAGE loading buffer is not used.
* The column purification procedure can be performed with 5 mL centrifuge columns (Pierce 89897). Gravity filtration can be slower than microtube IP. It is mainly useful for large sample volumes.
* **It is not advisable to wash with RIPA buffer.** We are eluting with SDS-PAGE loading buffer that contains SDS. RIPA buffer, though it contains less SDS (0.1% SDS vs. 1.1% in loading buffer), could elute some of the protein when used to wash. I have detected Nrf1 by Western blot in RIPA buffer used to wash the resin (see 9/1/15 blot for an example).
* Magnetic beads (Pierce 88837) are easier to work with, because the beads move to the walls of the tube instead of being pelleted, but agarose may have better binding capacity. Note that magnetic beads are not compatible with DTT that may be used in STMDPS buffer for subcellular fractionation.

Troubleshooting

**If yield is low, the sample or the resin could be limiting. The resin has a high binding capacity, so increasing sample should be the first step.**

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| **Problem** | **Possible Cause** | **Solution** |
| HA-tagged protein is in the flow- through | Column was overloaded | Reduce amount of lysate added to column or increase the amount of resin |
|  | Fusion tag was not accessible to resin | Denature protein or switch HA tag to the other terminus of the protein |
|  | Column was not regenerated after use | Regenerate column with 3M NaSCN |
| Minimal or no HA-tagged protein present in the elution fractions | Protein degraded | Perform purifications at 4ºC and include protease inhibitors during the binding step |
|  | Protein was not fully eluted | Prepare additional elution fractions or use a different elution buffer (see Table 2 for recommendations) |
|  | Protein was not expressed | Check protein lysate for presence of HA- fusion protein by Western blot before purification |
|  | Protein expression was very low | Add more lysate or optimize expression conditions to increase yield |
| HA-tagged protein appears as multiple bands on stained gels | Protease activity occurred during purification | Add protease inhibitors to lysate and wash buffers |
|  | Wash step was insufficient | Add additional wash steps or increase detergent or sodium chloride concentration in the wash buffer |
| 1. **Elution with SDS-PAGE loading buffer produces multiple bands on stained gels** | 1. **Reducing sample buffer was used and the antibody’s 25kDa light chain and 50kDa heavy chain are visible** | 1. **Omit reducing agent from the sample buffer** |

References

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