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Low throughput protocol for immunoprecipitation followed by mass spectrometry of cells stably expressing an HA-tagged protein

Low throughput IP
protocol for mass
spectrometry

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We use this protocol and it's working

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Abstract

Analysis of protein complexes by mass spectrometry provides a powerful approach for identifying proteins that associate with other proteins. Frequently, this can be done by expressing the protein of interest with an epitope tag, such as a Hemagglutinin-A (HA) epitope, using either a stably expressed lentivirus or by gene editing the HA epitope into the gene of interest. The protocol has been used extensively to create the Bioplex protein interaction network [Huttlin et al Nature. 545:505-509 (2017); Huttlin et al Cell, 162: 425-440 (2015)].

Attachments



[Low throughput IP pr...](#)

138KB



Materials

MCLB Stock:

- [M] 50 millimolar (mM) Tris, pH 7.5
- [M] 150 millimolar (mM) NaCl
- [M] 0.5 % NP40

*Note: Made in batches of 0.5 L at 1x, filtered and stored at 4 °C

MCLB + Roche protease inhibitor tablets + DTT:

- cOmplete™ Protease Inhibitor Cocktail **Roche Catalog #4693116001**
- [M] 1 millimolar (mM) DTT
- Make 30 mL MCLB with inhibitors and DTT for first wash or diluting samples if necessary.
30 mL 1M DTT , 3 mini protease inhibitor tablets.

50 % Bead slurry:

- Prepare slurry of anti-HA beads, mouse monoclonal 12CA5 from Sigma, in a 1.5 mL tube.
- Spin beads gently at 3000 rpm, 00:00:30 to pellet.
- Remove buffer and wash with 3x 1 mL MCLB (no inhibitors) .
- Can store at 4 °C for several days.

HA elution buffer:

- Can use either [M] 50 millimolar (mM) Tris pH 7.5 / [M] 150 millimolar (mM) NaCl or use PBS.
- PBS has been used for high throughput purposes.

HA peptide for elution:

- [M] 250 µg/mL HA peptide dissolved in HA elution buffer.
- Crude HA peptide from Bio-Synthesis Inc: Sequence: H₂N-YPYDVPDYA-CO₂H

TCA precipitation:

- Neat TCA
- [M] 10 % TCA in HPLC grade water
- Acetone

Trypsin Digestion:

- Digest Buffer: [M] 200 millimolar (mM) EPPS, pH 8.5 / [M] 10 % Acetonitrile
- Trypsin (Thermo)
- [M] 5 % formic acid in HPLC grade water



Safety warnings

 For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Before start

This protocol is for 293T cells but has been used broadly for many cell types. Typically, cells used have the proteins to be immunoprecipitated have stably expressed HA-tagged proteins via lentivirus vectors, or proteins fused with an HA epitope using gene editing.

Cell harvest:

- 2 × 15cm plate or 5 × 10cm plates per IP.
- Include HA-tagged GFP bait as a control.



Cell Harvest

17h 15m 3s

- 1 Wash plates 2x with cold PBS, then add  5 mL PBS per plate. 
- 2 Gently pipette up and down to dislodge cells and homogenize or gently scrape. 
- 3 Transfer to 15 mL conical tube and pellet cells  3000 rpm, 00:05:00 , discard sup.
- 4 Add  1 mL PBS and transfer to 2 mL tube.
- 5 Spin, aspirate PBS, and snap freeze pellet. 
- 6 Store at  -80 °C or use immediately. 
- 7 Quick thaw frozen 293T pellets in 2mL tubes in  37 °C water bath for approximately  00:00:03 . 
- 8 Transfer to ice metal block and add  1.2 mL MCLB .
- 9 Once pellet is thawed, pipette up and down to resuspend and tumble tubes for  00:20:00 in the cold room at  4 °C to lyse cells. 

- 10 Spin at  16.1 rcf, 00:20:00 in pre-chilled bench-top centrifuges to clear lysate. 
- 11 Reserve  25 µL -  50 µL lysate for QC protein assay and western blot of input, if desired.
- 12 Carefully transfer remaining supernatant to fresh 1.5 mL tube containing  40 µL washed HA bead slurry .



Note

Use 1.5 mL tubes, not 2 mL for the IP, as the conical shape is more ideal for pelleting the beads/washing in subsequent steps.

13 Incubate cleared lysate with beads for  03:00:00 with tumbling in the cold room at  4 °C .

3h



14 Spin samples  3000 rpm, 4°C, 00:01:00 to pellet beads in centrifuge.



15 Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!)



16 Add  1 mL MCLB to each tube, and gently resuspend beads by shaking.

17 Repeat spin/wash step 3 more times for a total of 4 × 1 mL washes with detergent present:



17.1 (Wash 1/3): Spin samples  3000 rpm, 4°C, 00:01:00 to pellet beads in centrifuge. Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!). Add  1 mL MCLB to each tube, and gently resuspend beads by shaking.

17.2 (Wash 2/3): Spin samples  3000 rpm, 4°C, 00:01:00 to pellet beads in centrifuge. Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!). Add  1 mL MCLB to each tube, and gently resuspend beads by shaking.

17.3 (Wash 3/3): Spin samples  3000 rpm, 4°C, 00:01:00 to pellet beads in centrifuge. Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!). Add  1 mL MCLB to each tube, and gently resuspend beads by shaking.

18 Perform 3 × 1 mL washes in the absence of detergent 50mM Tris/150mM NaCl, without NP-40:





- 18.1 (Wash 1/3): Spin samples  3000 rpm, 4°C, 00:01:00 to pellet beads in centrifuge. Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!). Add  1 mL MCLB (without NP-40) to each tube, and gently resuspend beads by shaking.
- 18.2 (Wash 2/3): Spin samples  3000 rpm, 4°C, 00:01:00 to pellet beads in centrifuge. Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!). Add  1 mL MCLB (without NP-40) to each tube, and gently resuspend beads by shaking.
- 18.3 (Wash 3/3): Spin samples  3000 rpm, 4°C, 00:01:00 to pellet beads in centrifuge. Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!). Add  1 mL MCLB (without NP-40) to each tube, and gently resuspend beads by shaking.
- 19 Carefully aspirate remaining wash buffer. Use gel loading tip and pipettor to remove as close to beads as possible.
- Note**
- Can use P3111 capillary tips which are smaller than the agarose resin, but not entirely necessary, beads do not have to be dry.
- 20 Add  100 µL elution buffer (HA peptide elution buffer +  250 µg/mL HA peptide).
- 21 Incubate in shaker at  37 °C (gentle shaking) for  00:30:00 .
- 22 Collect bead eluate by centrifuging  3000 rpm, 00:01:00 .
- 23 Repeat elution with equal volume of HA peptide; incubate second elution for 15 min:



30m





23.1 Add  100 μ L elution buffer (HA peptide elution buffer +  250 μ g/mL HA peptide).

23.2 Incubate in shaker at  37 $^{\circ}$ C (gentle shaking) for  00:15:00 .

15m

23.3 Collect bead eluate by centrifuging  3000 rpm, 00:01:00 .

24 Transfer eluate to labeled 1.5mL tubes; freeze at  -80 $^{\circ}$ C .

25 Proceed to TCA precipitation.

TCA Precipitation

17h 15m 3s

26 **The following steps constitute the TCA precipitation/acetone wash and trypsinization in preparation for analysis by mass spectrometry:** Can perform TCA precipitation

45m

 Overnight at  4 $^{\circ}$ C or for  00:45:00  On ice .



27 Add  55 μ L neat TCA to samples (assuming 2 x  100 μ L elution), vortex to mix, then gently spin to ensure TCA is not in tube caps.

28 Spin max speed  13000 rpm, 4 $^{\circ}$ C, 00:30:00 ; carefully aspirate all but  30 μ L sample .

29 Wash pellet with  1 mL cold  10 % TCA made in HPLC grade water.



30 Spin max speed  00:15:00 , vacuum as in Step 2.

15m



31 Wash with  1 mL cold Acetone .





32 Spin max speed  00:10:00 , vacuum.

10m



33 Repeat Acetone wash 2 more times (3 acetone washes total):



Note

Do not reduce to 2 washes. TCA tracks along and samples do not reach basic pH in 200mM EPPS digest buffer)

33.1 (Wash 1/2): Wash with  1 mL cold Acetone . Spin max speed  00:10:00 , vacuum.

10m

33.2 (Wash 2/2): Wash with  1 mL cold Acetone . Spin max speed  00:10:00 , vacuum.

10m

34 Air dry or use speedvac to dry pellet for digest, must be completely dry, as acetone can cause peptide modifications.

Note

DO NOT HEAT.

Trypsin Digestion

17h 15m 3s

35 Resuspend dried pellet in  40 μ L 200mM EPPS (pH 8.5)/10% Acetonitrile (digest buffer).

36 Spot check with  0.2 μ L sample for a couple of samples to ensure  8.5 .



37 Add  100 ng trypsin per sample (Thermo). Stock is  20 ng in  20 μ L (measure to confirm 20 μ L for each tube). This is  1 μ g/ μ L , make a master mix of trypsin digest buffer and add  40 μ L to each sample.



Note

Do not vortex, as this can dislodge the pellet.

38 Incubate at 37 °C for 06:00:00 (warm room or thermomixer, can shake gently).

6h



39 Acidify with 2 digest volumes of 5 % formic acid in HPLC grade water. (For 40µL digest, add 80 µL 5% formic acid .)

40 Spot check pH for a couple of samples to ensure 2 .



41 Proceed to stage tip followed by analysis by mass spectrometry.

