Low throughput IP protocol for mass spectrometry

Sep 06, 2021

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

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dx.doi.org/10.17504/protocols.io.bqpfmvjn

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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 protocol for mass spectrometry.pdf

DOI

dx.doi.org/10.17504/protocols.io.bqpfmvjn

PROTOCOL CITATION

Harper JW 2021. Low throughput protocol for immunoprecipitation followed by mass spectrometry of cells stably expressing an HA-tagged protein. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bqpfmvjn

KEYWORDS

immunoprecipitation, mass spectrometry, protein complexes, HA-tagged protein

CREATED

Dec 13, 2020

LAST MODIFIED

Sep 06, 2021

OWNERSHIP HISTORY

Dec 13, 2020 dominikchimienti

Feb 23, 2021 Harper JW

PROTOCOL INTEGER ID

45511

Citation: Harper JW (09/06/2021). Low throughput protocol for immunoprecipitation followed by mass spectrometry of cells stably expressing an HA-tagged protein. https://dx.doi.org/10.17504/protocols.io.bqpfmvjn

MATERIALS TEXT

MCLB Stock:

- [M]**50 Milimolar (mM) Tris, pH 7.5**
- [M]**150 Milimolar (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 Milimolar (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 Milimolar (mM) Tris pH 7.5** / [M]**150 Milimolar (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: H_2N-YPYDVPDYA-CO_2H

TCA precipitation:

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

Trypsin Digestion:

- Digest Buffer: [M]**200 Milimolar (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).

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BEFORE STARTING

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 x 15cm plate or 5 x 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** . 3s

8 Transfer to ice metal block and add **1.2 mL MCLB** .

9 20m

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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** .

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 3h

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

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 x 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.

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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 x 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.

> 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 

30m

Incubate in shaker at **37 °C** (gentle shaking) for **00:30:00** .

22 

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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:

23.1 Add **100 µl elution buffer** (HA peptide elution buffer + **250 µg/mL HA peptide**).

23.2 Incubate in shaker at **37 °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 °C** .

25 Proceed to TCA precipitation.

TCA Precipitation 17h 15m 3s

26 45m

The following steps constitute the TCA precipitation/acetone wash and trypsinization in preparation for analysis by mass spectrometry: Can perform TCA precipitation **Overnight** at **4 °C** or for **00:45:00** **On ice** .

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

28 Spin max speed **13000 rpm, 4°C, 00:30:00** ; carefully aspirate all but **30 µl sample** .

29

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

30 15m

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

31

Wash with **1 mL cold Acetone** .

32 10m

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

33

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

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.

DO NOT HEAT.

Trypsin Digestion 17h 15m 3s

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

36

Spot check with **0.2 µl sample** for a couple of samples to ensure pH **8.5** .

37 Add **100 ng trypsin per sample** (Thermo). Stock is **20 ng** in **20 µl** (measure to confirm 20µL for each tube). This is [M] **1 µg/µl** , make a master mix of trypsin digest buffer and add **40 µl** to each sample.

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Do not vortex, as this can dislodge the pellet.

38 6h

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

39 Acidify with 2 digest volumes of [M]**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 pH**2** .

41

Proceed to stage tip followed by analysis by mass spectrometry.

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