Tandem affinity purification of a bait host protein in presence of a viral protein

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Abstract

1st purification: a-FLAG (A2220; Sigma-Aldrich)

2nd purification: GFP TRAP_A (ChromoTek)

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Protocol

Day 1 - plate cells

Step 1.

1. Plate 5. 10^6 (± 1/6,5 of a confluent T150) HeLa cells in 20 mL of DMEM 10% FBS, on 75cm flask. 10 flasks/condition.
2. Incubate at 37°C, 5% CO

Day 2 - transfection (per flask)

Step 2.

1. Lipofectamine 2000 mix: 60 ul of Lipo + 940 ul of DMEM.
2. DNA Mix: 10 ug of DNA (3,34 µg for V5-tagged viral protein + 6,66 µg for FLAG-GFP tagged bait) in 1mL of DMEM (final volume).
3. Add 1 to 2, mix.
4. Incubate 30 min at room temperature.
5. Remove 5 mL of DMEM from the flask and add the 2 mL of transfection (final volume of 17 mL)
6. Incubate 24 hours at 37°C, 5% CO

Day 3 - buffer preparation

Step 3.

50 mL IP buffer (1 week, 4°C)

45 mL of H_2O

3mL from NaCl 5M stock (300 mM)

1,5 mL from Tris-HCl pH 7,4, 1M stock (30 mM)

0,5 g of Triton (1 %)

1 mM EDTA (supplemented with the tablet of protease inhibitor)

* Add 1 tablet of protease inhibitor Complete (Roche) / 50 mL lysis buffer.

* Add 1 tablet PhosStop (Roche) / 10 mL lysis buffer.

Day 3 - harvesting cells

Step 4.

1. Remove culture medium.
2. Wash 2X with 1X PBS.
3. Add 2 mL of PBS per flask.
4. Scrap cells with cell scraper.
5. Collect all in one 50 mL tube.
6. Spin 3000 g, 10 min, 4°C
7. Remove supernatant.
8. Add 5 mL of IP buffer.
9. Incubate 30 min, 4°C, under gentle rotation.
10. Transfer in 2 mL tubes.
11. Spin 13,000 g, 30 min, 4°C.
12. Transfer supernatants to 15 mL tube
13. Determine protein concentration with a BCA kit. Follow instructions from provider.

Day 3 - Anti-FLAG IP

Step 5.

1. Incubate 30 mg protein with 200 µL agarose A/G beads (50% beads) in 15 mL tube.
2. Incubate 1 hour, 4°C, under gentle rotation.

Prepare anti-FLAG

1. Wash 200 µL beads ANTI-FLAG M2 beads **(A2220; Sigma-Aldrich)** with 1 mL IP buffer in 1,5 mL tubes.
2. Spin 1 minute, 5000 g, 4°
3. Repeat 2X.

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3. Spin 2500 g, 5 min, 4°
4. Transfer supernatant to 200 µL ANTI-FLAG M2 beads in 15 mL tube.

5. Incubate overnight, 4°C, under gentle rotation.

Day 4 - buffer preparation

Step 6.

100 mL TBS

92 mL of H2O

5 mL from Tris-HCl, pH 7.4, 1M stock (50 mM)

3 mL from NaCl 5M stock (150 mM)

TBS-ETDA

50 / 10 mL TBS

20 / 4 µL from EDTA 0,5 M (0,2 mM)

10 mL TBS-inhibitors

TBS

* Add 1 tablet of protease inhibitor Complete EDTA-free (Roche) / 10 mL TBS.

* Add 1 tablet PhosStop (Roche) / 10 mL TBS.

Prepare 3X Flag peptide

Dissolve 3X flag peptide **(F 4799; Sigma-Aldrich)** in TBS-inhibitors at concentration 5 mg/mL.

Aliquot and store at -20°C. Repeated freezing and thawing is not recommended.

Day 4 - FLAG elution

Step 7.

1. Spin 5000 g, 4°C, 1 min.
2. Discard supernatant.
3. Wash 3X with 1000 µL IP buffer (spin 1 min at 5000 g, 4°C).
4. For elution, add 100 µL of 3X flag elution solution (1 mg/mL) to each sample (dilution with 10 mL TBS-inhibitors).
5. Rotate 1 hour, 4°

Day 4 - Anti-

Step 8.

Prepare GFP TRAP

1. Wash 100 µL beads with 1 mL TBS-EDTA.
2. Spin 5 minutes, 2500 g, 4°C.
3. Repeat 3X.

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1. Centrifuge resin for 5 minutes at 2500 g, 4°
2. Transfer supernatant to fresh tubes. **E1, E2, E3**
3. Repeat steps 8 to 11, 3X.
4. Transfer E1-3 with 100 µL GFP TRAP_A beads (ChromoTek).
5. Top up to 1 mL with TBS-EDTA.
6. Rotate 1 hour, 4°
7. Centrifuge resin for 2 minutes at 2500 g, 4°
8. Remove supernatant.
9. Wash 3X with 1000 µL TBS-EDTA.
10. Re-suspend beads in 100 µL 2X LDS (NUPAGE, 4X), 1X Reducing agent (NUPAGE, 10X). 95°C, 10 min.
11. 12500 g, 2 min.
12. Freeze protein elution at -20°
13. Use 20 µL to run a western blot and check the presence/absence of controls.
14. Run a keratin-free SDS-PAGE with 2 x 40 µL.
15. Proceed to mass spectrometry.