Batch absorption of Urm1-Flag conjugates from Drosophila tissues for mass spectrometry analysis

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Abstract

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Protocol

Step 1.

1. Grow flies expressing transgenic constructs under control of the UAS/GAL4 system at 25°

2. Procedure to prepare protein lysate from different developmental stages in *Drosophila*;

1. Embryo collection

- To retrieve a collection of all embryonic stages, harvest 0-22 hour old embryos in a mesh-basket and wash away the yeast paste with deionized H_2
- Dechorinate the embryos in a 1:1 dilution of Sodium hypochlorite 14% (f.c. 7%) and deionized H_2O for 2-4 minutes, rinse embryos for 1 min in deionized water.
- Transfer the embryos in 100 µl portions to 1.5 ml microtubes and mince with pestle in 200 µl Lysis buffer A (50 mM HEPES pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% Glycerol, 25 mM NAF, 10 µM ZnCl2), supplemented with 1 tablet Complete Inhibitors (Roche) per 50 ml, 1mM PMSF and 10 mM N-ethyl maleimide (NEM).
- Add 800 ul Lysis buffer A to each embryo-portion and continue grinding.
- Incubate all samples on ice for 30 minutes.
- Spin 14 000 x g for 15 min at 4°C and transfer and pool supernatants to fresh tubes.
- Measure protein concentration.

1. Larvae collection

- Collect wandering third instar *Drosophila* larva and rinse them twice in 1xPBS.
- Add 25 larva per aliquot to 1.5 ml microtubes with 125 µl Lysis buffer A (see above) and grind the larvae with a pestle.

- Add 375 µl fresh Lysis buffer A and continue grinding.
- Incubate all samples on ice for 30 minutes.
- Spin 14 000 x g for 15 min at 4°C and transfer and pool supernatants to fresh tubes.
- Measure protein concentration.

- Adult fly collection
- Collect 0-4 day old adults flies under CO_2 anesthesia, 40 flies per aliquot, in 1.5 ml microtubes.
- Grind the flies in 200 µl Lysis buffer A (see above), add another 200 µl and continue grinding.
- Incubate all samples on ice for 30 minutes.
- Spin 14 000 x g for 15 min at 4°C and transfer and pool supernatants to fresh tubes.
- Measure protein concentration.

3. Take out 60 µl from the lysate, mix with 60 µl 2x Sample buffer and incubate 7 min at 98°C (=INPUT).

4. Immunoprecipitation with Flag M2 magnetic beads

- Wash the Flag M2 magnetic beads (Sigma M8823) 3 times in Lysis Buffer A before usage. Make sure that the beads are completely defrosted before aliquoting the slurry with a cut pipet tip. Collect magnetic beads with a magnetic separator.
- Set up the absorption with Flag M2 magnetic beads at 4°C, by mixing 9 mg total protein in 4.5 ml volume + 150 ml magnetic beads (slurry, equals 75 µl packed beads) in 5 ml Falcon polypropylene tubes (352063).
- Incubate for 2 hours at 4°C on rotator.
- Collect beads and remove the supernatant.
- Wash beads 4 times with about 4 ml Lysis buffer A per sample (collect beads with magnetic separator between each wash).
- Wash 2 times with 4 ml Flag M2 Magnetic rinsing buffer (20 mM Tris-HCl, pH 8.0, 1mM $CaCl_2$).
- Save just a little buffer for shipment, secure with Parafilm and keep at 4°C until shipment on wet ice to the mass spectrometry facility.

Analysis of FlagM2-conjugates by mass spectrometry

1. The proteins captured after affinity purification using Flag M2 beads were first subjected to an on-bead trypsin digestion.

2. Digested peptides were injected onto the LC-MS/MS system (UltimateTM 3000 RSLCnano chromatography system and Q Exactive Plus Orbitrap mass spectrometer, Thermo Scientific).

3. The peptides were separated on a homemade C18 column, 25 cm (Silica Tip 360µm OD, 75µm ID, New Objective, Woburn, MA, USA) with a 60 min gradient at a flow rate of 300nl/min. The gradient went from 5-26% of buffer B (2% acetonitrile, 0.1% formic acid) in 120 min and up to 95% of buffer B in 5 min.

4. The digested peptides were subsequently analysed by standard bioinformatics methods.

Source: Procedure as described by the SciLifeLab facility for mass-spectrometry based proteomics in Stockholm/Uppsala, Sweden.