PREPARATION OF IF- ENRICHED CYTOSKELETAL PROTEINS

Ice-cold means cooled in ice water. In order to prevent proteolysis, make sure to perform all steps on ice. Pre-cool glass homogenizers, buffers etc.

1. Cells

1.1 Carry cells to your bench. Remove media, wash cells 2 x with ice-cold PBS. Scrape off cells with rubber policeman and collect on ice. Spin down cells 5 min. at 1.200 rpm and resuspend in 3-5 ml of ice-cold low-salt buffer per 9 cm dish. Homogenize with Dounce homogenizer (L pestle) ~5 times. Add PMSF to 2 mM final concentration. Incubate 5 min on ice. Continue with 1.3
Alternatively, quick-freeze cell pellet in EtOH/dry ice and store at –20°C.

1.2 If there are only one or a few dishes, cells can be lysed on the dish: after PBS wash, add ice-cold low-salt buffer to the cells and transfer straight into Dounce, using rubber policeman and blue tip. Wash dish once more with ice-cold low salt buffer and add into Dounce. Homogenize with Dounce homogenizer (L pestle) ~5 times. Add PMSF to 2 mM final concentration and incubate 5 min on ice.

1.3 Spin down lysate 10 min at 5 000 x g (4°C). Discard spnt. (or keep for further analysis if required!) and resuspend sediment in 5-10 ml of ice-cold high-salt buffer per 9 cm dish (more buffer is ok.). Add PMSF to 2 mM final concentration. Homogenize thoroughly (20-50 x) with Dounce homogenizer (S pestle) until no longer viscous and incubate for 30 min on ice. Homogenize a few times during this incubation period.
Spin 10 min at 15 000 x g (4° C). Supernatant should appear translucent and not viscous. If not, repeat extraction with high-salt buffer. Resuspend pellet once with ice-cold TE and re-spin as before.
Freeze or resuspend in appropriate buffer and store at -20° C.
If quantitation of proteins by Western blotting is planned, take a small aliquot of cells (200-500.000) and lyse immediately in SDS sample buffer. This represents total (SDS-soluble) proteins.

2. Tissues

2.1 Homogenize tissue (washed in PBS if necessary) in ~ 20 ml per g of tissue of ice-cold low-salt buffer using manual Dounce (L pestle, 5-10 times) or motor-driven Teflon pestle (low speed, 10-60 sec; tissue should be disrupted and appear as homogeneous solution). Incubate 5 min on ice.
Important: Very hard tissue like human epidermis cannot be extracted this way. In such instances, use a *Warring Blendor* to mince tissue cooled with liquid N_2.
Add PMSF to 2 mM final concentration. Remove particulate material by filtering through cloth if necessary. Homogenize a few times with Dounce (L pestle) on ice and spin 10 min at 5 000 x g (4°C). Discard spnt. (or analyze if required!).

2.2 Resuspend pelleted material in at least 10-20 ml (per g of tissue) in ice-cold high-salt buffer. Add PMSF to 2 mM final concentration. Homogenize thoroughly with electric homogenizer or Dounce (S pestle) and incubate on ice for 30 min. During this incubation, homogenize once for 20 sec with Polytron and 2-3 x with Dounce to achieve optimal extraction.
Spin 10 min at 15 000 x g (4°C). Resuspend pellet in same vol. of high-salt buffer and repeat extraction and centrifugation step. If the homogenate is still viscous, a third extraction step is required. The protein pellet should be homogeneously white and the supernatant should appear translucent and not viscous. It might be necessary to extract several times or to increase the volume of the extraction buffer. For many tissues, 1 extraction step is sufficient (indicated by clear and non-viscous spnt.).
After final spin, resuspend pellet in ice-cold TE and re-spin as before.
Freeze or resuspend in appropriate buffer and store at -20°C.

3. Removal of nucleic acids from cytoskeletal preparations

For some purposes, it is necessary to remove DNA and RNA completely from cytoskeletal preparations. To achieve that, add 50-500 µl of TE to final protein pellet. Add 1/10 Vol of 10 x DNAse I buffer and 0.5 µl of ultrapure DNAse I (RNAse and protease-free) and 0.2 µl of a 10 x dilution of RNAse. Resuspend by pipetting up and down a few times (cytoskeleton will not dissolve but DNA/RNA will!). Incubate at 37°C for 15 min. Spin down 5 min at 14.000 rpm. Discard spnt. and add sample buffer.

4. Remarks / Solutions

Extraction principle:

Low salt/detergent buffer disrupts plasma and vesicle membrane and weak macromolecular interactions but keeps nuclei and attached cytoskeleton intact so that they can be sedimented at low speed.

High salt/detergent together with shear force disrupt most non-covalent interactions and breaks down chromatin. After 15.000 x g, except intermediate filament proteins and desmosomal proteins, most other proteins and polymers are soluble and remain in supernatant.

If necessary, include a cocktail of protease inhibitors (eg from Pierce or Roche). To inhibit phosphatases, use 1 mM Na-vanadate or others.

Low-salt buffer: 10 mM Tris-HCl pH 7.6, 140 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5 % Triton X-100.

High-salt buffer: 10 mM Tris-HCl pH 7.6, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 5 mM EGTA, 1 % Triton X-100.

PBS: 140 mM NaCl, 2.7 mM KCl, 1.5 mM K_2PO4, 8.1 mM Na_2HPO4, final pH: 7.4.

10X DNase I Buffer: 400 mM Tris-HCl pH 7.5, 100 mM NaCl, 60 mM $MgCl_2$. Make up with RNAse-free water and store at -20°C in aliquots.

Preparation of cytoskeletal fractions from CK plus and CK minus cells (Cornelia, Romina)

- seed 2Mil cells in 10 cm dishes 2x WT and 2x KO
- after 24 h add 1.2mM $CaCl_2$ for 48 h
- remove media

Total Cell lysates:

- wash cells 1x ice cold PBS, 1x water
- add 500 µl of Laemmli Lysis buffer, scrabe with rubber man und pipette into 1.5 ml Eppi
- cook 5 min 98°C, 3 x 30 s sonification
- cook 5 min 98°C, 3 x 30 s sonification
- cook 5 min, centrifuge full speed 5 min, freeze

Cytoskeletal fraction:

- wash cell 2 x with ice-cold PBS
- add 2 ml of ice-cold low salt buffer per 10 cm dish, scrabe off with rubber policeman and collect in dounce
- wash dish once more with 2 ml ice-cold low salt buffer and add into Dounce. Homogenize with Dounce homogenizer (L pestle) 12 times on ice!
- Incubate 5 min on ice
- Distribute into 2 2 ml-Eppis, spin down lysate 10 min at 5 000 x g (4°C)
- Collect supernatant in separate tube, freeze → TCA, resuspend in 250 µl
- Add 2 ml of ice-cold high salt buffer into dounce
- Resuspend each pellet in 1 ml of ice-cold high-salt buffer (total volume again 4 ml)
- Homogenize 10 x with Dounce homogenizer (S pestle)
- 10 min incubation on ice, repeat 3 x
- One more time homogenize 10 x with Dounce homogenizer (S pestle) (total 4 x), until no longer viscous
- Distribute into 2 2 ml-Eppis, spin down lysate 10 min at 15 000 x g (4°C)
- Collect supernatant in separate tube, freeze → TCA, rsuspend in 250 µl
- Add 250 µl of Laemmli Lysis buffer and pool pellets (pellets were black? KO much smaller than WT pellet)
- Cook 10 min 98°C, 3x30 s sonification, always pipet in between first with blue and then yellow tip to check for complete suspension
- Cook 10 min 98°C, 3x30 s sonification
- Cook 10 min, centrifuge full speed 5 min, freeze

Laemmli Lysis buffer: 5x Laemmli buffer (Probenpuffer)

Low-salt buffer: 10 mM Tris-HCl pH 7.6, 140 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5 % Triton X-100, 1x Halt protease Inhibitor, 20 mM NEM, 1x Phosphatase-Inhibitor, 1 µl RNase, 1 µl DNase

High-salt buffer: 10 mM Tris-HCl pH 7.6, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 5 mM EGTA, 1 % Triton X-100, 1x Halt protease Inhibitor, 20 mM NEM, 1x Phosphatase-Inhibitor, 1 µl RNase, 1 µl DNase