Cytoplasmic Extract Preparation

Forty 15 cm plates of 90 % confluent cells were washed twice with 1x PBS, scraped off the plates in 10 ml of 1x PBS and collected into 50 ml Falcon tubes (4 plates per falcon) in the cold room. Cells were spun down at 1900 g, 4 °C for 10 min. PBS was decanted until about 5ml were remaining, and the pellet was swirled until it detached from the tube. The corresponding pellets were combined into one falcon and spun down again. PBS was decanted and pellets were frozen.

- resuspend cells in 5pcv hypotonic buffer
- spin 5min, 2000g, 4°C
- discard supernatant and resuspend cells in 3pcv hypotonic buffer
- let cells swell on ice for 20min.
- use glass homogenizer (douncer) to disrupt cell membranes → about 30 strokes with loose pestle
- spin 15min, 3300g, 4°C in 15ml-Falcons to pellet nuclei
- remove SN = cytoplasmic extract and put into a new 15mL-Falcon
- supplement extract with 0.11 Volumes of 10xcytoplasmic buffer
- determine protein concentration via BCA assay (use 1:10 dilution of extracts) (Prepare BSA standard curve for reference.)
- store extracts on ice for further use in IP

Buffers:

<u>1M Hepes-KOH, pH 7.9</u>

47.66 g of Hepes (238.3 g/mol) in 200 ml ddH2O

The solution is placed on ice to cool down and an ice water bath was used during pH adjustment to 7.9 with KOH (about 4-5 ml of 9 M KOH needed). The buffer was sterile filtered (0-0.22 µm filter) and stored at 4 °C.

<u>4M KCl</u>

29.824 g of KCl (74.56 g/mol) are solved in 100 ml of ddH2O in a 50 °C water bath, sterile filtered (0-0.22 µm filter) and stored at 4 °C prior to cytoplasmic extract preparation.

<u>0.2M PMSF</u>

0.6968 g of PMSF (174.2 g/mol) are solved in 20 ml of isopropanol and stored at -20 °C. (Tip: Thaw 1-2 hours before starting the extraction and then keep on ice, very unstable in aqueous solution)

10x Cytoplasmic Extract Buffer

0.3 M Hepes pH7.9, 1.4 M KCl, 0.03 M MgCl2 in ddH_20. Stored at 4 °C for up to two weeks.

Hypotonic Buffer

10 mM Hepes ph7.9, 1.5 mM MgCl2, 10 mM KCl in ddH_20. Store at 4 °C for up to two weeks. Just before use add: 0.2 mM PMSF and 0.5 mM DTT. Buffer can also be supplemented with 1 tablet of protease inhibitor cocktail (Roche) per 50 ml.

RNA Affinity Purification with biotinylated RNA

1. Biotinylation of RNA

Material: Megascript T7 *in vitro* transcription kit (Ambion)
Biotin-16-UTP (Epicentre)

In the following, the reaction mixture for labeling a 500nt long RNA is depicted. This yields in this specific case in an incorporation of 5-6 modified UTPs into the full-length transcript. Use longer incubation times for short transcripts! The transcription yields more products with T7 polymerase (~100µg per reaction). Do not use transcripts longer than 2000nt for IP!

Components	Volume
ATP 75mM	2 µl
GTP 75mM	2 µl
CTP 75mM	2 µl
UTP 75mM	1.9 µl
Biotin-16-UTP 5mM	1.5 µl
H_2O	4.48 µl
10x Buffer	2 µl
1 µg Vector or ~100ng PCR product	2.12 µl
Enzyme mix	2 µl
Total	20 µl

The ingredients of the *in vitro* transcription reaction are mixed in the order shown above and mixed by pipetting up and down. Be careful to mix the solution well before adding the vector as the 10x buffer contains Spermidine that may precipitate the vector. The transcription reaction is incubated at 37 °C for 14-16h o/n.

The next day, DNA is removed by adding 1 µl Turbo DNase (Megascript kit, Ambion) and incubating it for 15 min at 37 °C. RNA is precipitated by adding 30 µl of H_2O and 30 µl LiCl precipitation solution (Megascript kit, Ambion) and freezing the mixture for 30-60min at -20 °C. RNA is pelleted (16.000g,

10min,4°C), washed with 70 % Ethanol and dissolved in 50-100 µl ddH_2O. Usually, we get >100µg RNA per Rxn.

2. Blocking beads, coupling RNA and IP of RNA-binding proteins

Day 1

- mix Streptavidin beads (GE Healthcare; 17-5113-01) by shaking
- use 50µL slurry per reaction(=25µL packed) (and ~200µL for pre-clearing)
- wash 2x 500 µl LS-WB at RT and spin each time 5000rpm, 1min
- add 500 µl SAg-BB and blocked for ~2-3 hours at 4 °C on the turning wheel
- spin 5000rpm, 1min
- wash 3x 5min with 500 µl HS-WB-300
- add 260pmol RNA per reaction to ~25µL packed beads and 250µL HS-WB-300
- coupling for 4-5h at 4°C on turning wheel
- in the meantime: pre-clear protein extract with ~100µL packed, pre-blocked beads
- spin 5000rpm, 4°C, 1min
- wash beads 3x 1mL HS-WB-400 and use these beads for IP
- use pre-cleared extracts for IP (~3mg per reaction), supplemented with RNase-Inhibitor
- IP o/n @ 4°C on turning wheel

Day 2

- wash beads 7x 5min. with 1mL HS-WB-400 on a turning wheel
- with last wash: change/transfer into new Eppi and spin down; remove SN completely
- elute proteins with 50µL 6M Urea, 0.01% NP40, 1mM DTT → shaking 900rpm, RT, 30min
- spin 5000rpm, 1min, RT
- transfer SN in new 1,5mL Eppi and add 5Vol (250µL) cold Acetone (-20°C) →invert
- precipitate proteins at -20 °C for at least 1 hour
- pellet proteins at max. rpm at RT for 30min.
- wash pellets 2x 1mL 80 % ethanol and spin down at maximum rpm for 10 minutes after each wash.
- resuspend pellets in 20-30µl 2x Sample buffer.

For Mass Spec analysis, 10µL of each sample are loaded on SDS-PAGE. For Western blot validation, 1µL each is sufficient (fill up with Sample buffer)

Buffer

LS-WB: 20mM HEPES-KOH, pH 7.9 (4°C)
100mM KCl
10mM $MgCl_2$
1mM DTT
0.01% NP-40

SAg-BB: 20mM HEPES-KOH, pH 7.9 (4°C)
100mM KCl
10mM $MgCl_2$
1mM DTT
0.01% NP-40
1mg/mL BSA
40µg/mL Glycogen
40µg/mL yeast tRNA

HS-WB300: 20mM HEPES-KOH, pH 7.9 (4°C)
300mM KCl
10mM $MgCl_2$
1mM DTT
0.01% NP-40

HS-WB400: 20mM HEPES-KOH, pH 7.9 (4°C)
400mM KCl
10mM $MgCl_2$
1mM DTT
0.01% NP-40

Staining of SDS-PAGE gel

We use mass-spec compatible silver staining or sensitive Coomassie stainings. Please visit DKFZ Proteomics Core Facility (Tore Kempf, Martina Schnölzer) for protocols. Here is a protocol for sensitive coomassie:

Sensitive Coomassie Staining

The protein gel was removed from the gel cast and rinsed with deionized water three times for 30min. Proteins were fixed using 30 % ethanol with 2 % phosphoric acid (w/v) for 30 min. The gel was then incubated in staining solution for 3 h to overnight. The staining solution has a green color and might contain dye particles. The gel was destained with ddH_2O, documented and bands were excised.

The sensitive coomassie stain is compatible with mass-spec analysis of protein bands.

Buffer:

Staining solution

5 % Aluminiumsulfate

10 % Ethanol

0,02 % Coomassie Brilliant Blue G250

2 % (w/v) phosphoric acid

Reagents were added to water in the given order, mixed well and dissolved completely before adding the next one and volume was adjusted with water at the end.