Co-immunoprecipitaton of polyribosomes that possess a FLAG-tagged ribosomal protein with αFLAG agarose beads

Maria Eugenia Zanetti/Bailey-Serres Lab
Center for Plant Cell Biology, UC Riverside

This protocol is a modification of the version provided from SIGMA in the technical bulletin of *FLAG-tagged protein immunoprecipitation kit* (Product number: FLAGIPT-1). Extraction buffer and binding buffer were optimized to preserve polyribosomal integrity without affecting antigen-antibody binding reaction. This protocol can be used for isolation of polysomes of leaves of mature *Arabidopsis thaliana* that are transgenic for tagged ribosomal proteins.

Polysomes extraction

1- Homogenize the tissue (previously pulverized to a fine powder with a mortar and pestle under liquid nitrogen) with 2 volumes of Polysome Extraction Buffer (PEB). For preparative immunoprecipitation use 2.5 ml of packed tissue and 5 ml of PEB. Incubate mixture on ice for 10 min.
2- Transfer to Corex centrifugation tubes and Centrifuge at 16,000 x g for 10 min at 4 ^{0}C.
3- Transfer the supernatant to a new Corex tube and centrifuge at 16,000 x g for 10 min at 4 ^{0}C.
4- Transfer the supernatant to a new tube. This fraction is SN 16,000 x g. Determine A_{260}.

Preparation of the α-FLAG M2 agarose beads (Sigma)

1- Thoroughly suspend the αFLAG M2 agarose gel (Sigma, product number A 2220) in the vial to make a uniform suspension of the resin. The ratio of suspension to packed volume should be 2:1.Transfer 200 μl of suspension (100 μl of packed gel) to a 15ml-Falcon tube.
2- Centrifuge at 8,200 x g for 30 sec.
3- Remove the supernatant with a pipette and add 2 ml of wash buffer.
4- Centrifuge at 8,200 x g for 30 sec.
5- Remove the supernatant with pipette and wash one more time with 2 ml of wash buffer before to continue with the co-immunoprecipitation.

Co-immunoprecipitation

1- Mix 250 to 300 units of A_{260} of SN 16,000 x g with 100 μl (packed volume) of α-FLAG M2 agarose beads in a 15 ml- Falcon tube. Bring volume to 5 ml with binding buffer.
2- Incubate for 2 h at 4 ^{0}C with gently shaking (Binding Step)
3- Centrifuge for 30 sec at 8,200 x g at 4 ^{0}C.

4- Transfer the supernatant to a new tube. This is the Supernatant of the Co-IP or unbound fraction.
5- Add 4 ml of binding buffer to the beads, mix by inverting the tube and centrifuge for 30 sec at 8,200 x g at 4 ^{0}C (First wash).
6- Remove the supernatant with pipette and add 4 ml of binding buffer. Incubate at 4 ^{0}C for 5 min. with gently shaking (Second wash).
7- Centrifuge for 30 sec at 8,200 x g at 4 ^{0}C.
8- Remove the supernatant with pipette and add 4 ml of wash buffer. Incubate at 4 ^{0}C for 5 min. with shaking (Third wash)
9- Centrifuge for 30 sec at 8,200 x g at 4 ^{0}C.
10- Remove the supernatant and add to the beads 300 to 400 µl of wash buffer containing 200 ng/µl of 3X-FLAG peptide (Sigma, product number F 4799). Incubate for 30 min at 4 ^{0}C with shaking (Elution Step).
11- Centrifuge for 30 se at 8,200 x g at 4 ^{0}C. Transfer the supernatant to a new tube. This is the elution of the Co-IP.

RNA Extraction

Note: this RNA extraction protocol uses Qiagen RNeasy kit (Cat Num.74904)

1- Add 1 vol of 8 M guanidine-HCl to the Elution of the Co-IP and vortex for 3 min.
2- Add 1.5 vol of 100% Ethanol and vortex for 1 min.
3- Precipitate the RNA at –20 ^{0}C overnight.
4- Centrifuge at 16,000 x g for 45 min
5- Remove supernatant and let the pellet dry for 20 min.
6- Prepare extraction buffer adding 10 µl of β-mercaptoethanol to 1 ml of RLT buffer (from Qiagen RNeasy kit).
7- Resuspend the pellet in 450 µl of extraction buffer and vortex for 1 min.
8- Add 250 µl of 100% ethanol and mix by inverting the tube. Do not Vortex
9-Apply the sample into an RNeasy mini spin column (pink). Incubate for 3 min.
10-Centrifuge for 15 sec at 16,000 x g
11- Add 700 µl of RW1 buffer and centrifuge for 15 sec at 16,000 x g. Discharge the flow through.
12- Add 500 µl of RPE buffer (Four volumes of ethanol are already added) and centrifuge for 15 sec at 16,000 x g. Discharge the flow through.
13- Add 500 µl of RPE buffer to the column and centrifuge for 2 min at16,000 x g.
14- Transfer the column to a new 2 ml microfuge tube, and centrifuge for 1 min at 16,000 x g.
15-Transfer the column to a new 1.7 ml microfuge tube and add 50 µl of RNAse free water. Incubate for 5 min.
16-Elute RNA centrifuging for 1 min. at 16,000 x g.

Buffers and Solutions

Polysomes Extraction Buffer (PEB)
100 mM Tris-HCl pH 8.5
200 mM KCl
25 mM EGTA
36 mM $MgCl_2$
1% Detergent mix
1% PTE (Polyoxyethylene 10 trydecyl ether)
2% DOC (Deoxychollic acid)
5 mM DTT
1 mM PMSF
50 μg/ml Cycloheximide
50 μg/ml Chloramphenicol
1 mg/ml Heparin (Sigma, molecular biology grade)

[For PEB and Wash buffers, the buffers are prepare from stock solutions of Tris, KCl, EGTA, $MgCl_2$ and the detergents that have been autoclaved. Add DTT, PMSF, cycloheximide, chloramphenicol, and heparin to buffer immediately before use].

Wash buffer
100 mM TRis-HCl pH 8.5
200 mM KCl
25 mM EGTA
36 mM $MgCl_2$
5 mM DTT
1 mM PMSF
50 μg/ml Cycloheximide
50 μg/ml Chloramphenicol
50U/ml RNasin (Promega)

Wash buffer to be used in the preparation of the α-FLAG M2 agarose beads
100 mM TRis-HCl pH 8.5
200 mM KCl
25 mM EGTA
36 mM $MgCl_2$

20% Detergent mix
20% Brij-35
20% Triton X-100
20% Nonidet P-40
20% Tween-20