

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

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. The protocol is also good for seedlings and floral tissues. Reference for protocol: Zanetti, M.E., Chang I.-F, Galbraith D.W., Bailey-Serres, J. (2005) Immuno-affinity purification of polyribosomal complexes of Arabidopsis for global analysis of gene expression. *Plant Physiology*. 138:624-635.

Polysome extraction

All glassware should be heat sterilized. Avoid introduction of RNases. All procedures should be done on ice or at 4°C in a cold room.

- 1- Homogenize the tissue (previously pulverized to a fine powder with a mortar and pestle under liquid nitrogen) with 2 volumes of freshly prepared Polysome Extraction Buffer (PEB). For preparative immunoprecipitation use 2.5 ml of packed leaf tissue and 5 ml of PEB. Preparative immunoprecipitation from seedlings requires more tissue than for leaves. Incubate mixture on ice for 10 min. [Caution: The tissue should only be allowed to thaw in the buffer. All extraction procedures need to be performed at 4°C or on ice]
- 2- Transfer to Corex centrifugation tubes and Centrifuge at 16,000 x g for 10 min at 4°C.
- 3- Transfer the supernatant to a new Corex tube and centrifuge at 16,000 x g for 10 min at 4°C.
- 4- Transfer the supernatant to a new tube. This fraction is SN 16,000 x g. Determine A_{260} .

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

- 1- Thoroughly suspend the a-FLAG M2 agarose gel (Sigma, product number A 2220) in the vial to make a uniform suspension of the resin. Use wash buffer#1. The ratio of suspension to packed volume should be 2:1. Transfer 200 ml of suspension (100 ml of packed gel) to a new 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 #1.
- 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#2 before continuing with the co-immunoprecipitation.

Immunoprecipitation of polysomes

- 1- Mix 250 to 300 units of A₂₆₀ of SN 16,000 x g with 100 ml (packed volume) of a-FLAG M2 agarose beads in a 15 ml- Falcon tube. Bring volume to 5 ml with binding buffer (Wash Buffer #2 = polysome extraction buffer without detergents. It is also okay to use PEB w/ detergents).
- 2- Incubate for 2 h at 4 °C with gently back-and-forth shaking (Binding Step)
- 3- Centrifuge for 30 sec at 8,200 x g at 4 °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 gently inverting the tube and centrifuge for 30 sec at 8,200 x g at 4 °C (First wash).
- 6- Remove the supernatant with pipette and add 4 ml of binding buffer (WashBuffer#2 = PEB w/o detergents). Incubate at 4 °C for 5 min. with gently shaking (Second wash).
- 7- Centrifuge for 30 sec at 8,200 x g at 4 °C.
- 8- Remove the supernatant with pipette and add 4 ml of wash buffer #2. Incubate at 4 °C for 5 min. with shaking (Third wash)
- 9- Centrifuge for 30 sec at 8,200 x g at 4 °C.
- 10- Remove the supernatant and add to the beads 300 to 400 ml of wash buffer #2 containing 200 ng/ml of 3X-FLAG peptide (Sigma, product number F 4799). Incubate for 30 min at 4 °C with shaking (Elution Step).
- 11- Centrifuge for 30 sec at 8,200 x g at 4 °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 °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 ml of β-mercaptoethanol to 1 ml of RLT buffer (from Qiagen RNeasy kit).
- 7- Resuspend the pellet in 450 ml of extraction buffer and vortex for 1 min.
- 8- Add 250 ml 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 ml of RW1 buffer and centrifuge for 15 sec at 16,000 x g. Discharge the flow through.
- 12- Add 500 ml 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 ml of RPE buffer to the column and centrifuge for 2 min at 16,000 x g.
- 14- Transfer the column to a new 2 ml micro tube; centrifuge for 1 min at 16,000 x g.
- 15- Transfer the column to a new 1.7 ml microfuge tube and add 50 ml of RNase free water. Incubate for 5 min.
- 16- Elute RNA centrifuging for 1 min. at 16,000 x g

Buffers and Solutions

These buffers are prepared from stock solutions of Tris, KCl, EGTA, MgCl₂ and the detergents that have been autoclaved. Add DTT, PMSF, cycloheximide, chloramphenicol, heparin and RNasin to buffers immediately before use.

Polysome Extraction Buffer (PEB)

100 mM Tris-HCl pH 9.0
200 mM KCl
25 mM EGTA
36 mM MgCl₂
1% Detergent mix (see below)
1% PTE (Polyoxyethylene 10 trydecyl ether)
2% DOC (Deoxycholic acid)
5 mM DTT
1 mM PMSF
50 mg/ml Cycloheximide
50 mg/ml Chloramphenicol
1 mg/ml Heparin (Sigma, molecular biology grade)

20% Detergent mix for PEB

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

Wash buffer #2 (For washing beads after binding to polysomes)

100 mM Tris-HCl pH 9.0
200 mM KCl
25 mM EGTA
36 mM MgCl₂
5 mM DTT
1 mM PMSF
50 mg/ml Cycloheximide
50 mg/ml Chloramphenicol
50U/ml RNasin (Promega)

Wash buffer #1 (Used in the initial preparation of the α-FLAG M2 agarose beads)

100 mM Tris-HCl pH 8.5 (pH 9.0 also acceptable)
200 mM KCl
25 mM EGTA
36 mM MgCl₂