TRAP purification of polysomes

Bench version updated by Mauricio Reynoso and Julia Bailey-Serres, December 2016, Center for Plant Cell Biology, University of CA, Riverside. Mustroph et al., 2009 (below) has more details.

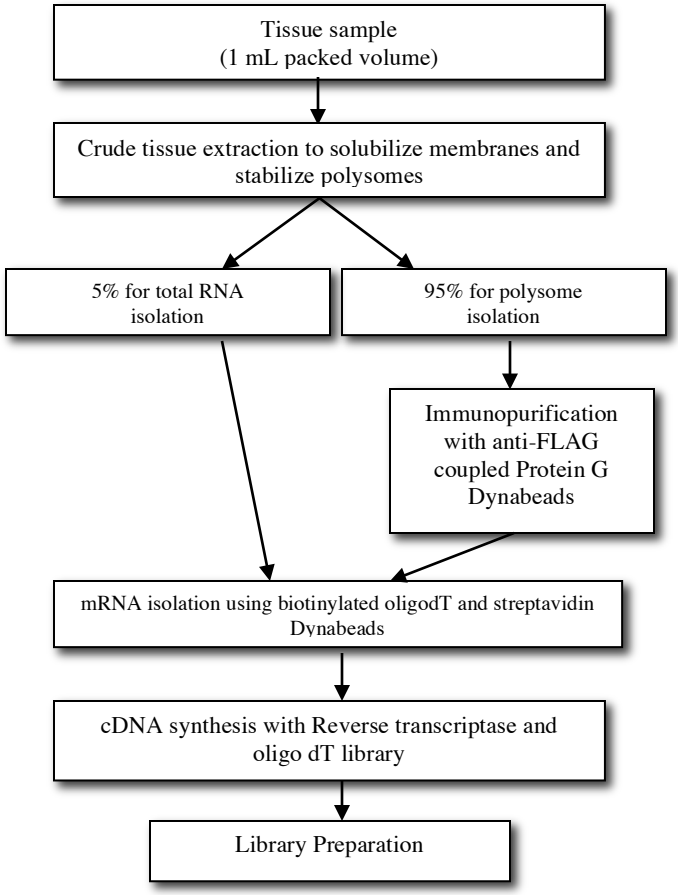
Overview:
This protocol describes how ribosomes and associated mRNA are isolated by TRAP (**T**ranslating **R**ibosome **A**ffinity **P**urification). Polyribosomes (polysomes) are multiple ribosomes engaged in translation on a single mRNA. Ribosomes have been traditionally isolated from detergent-treated cell extracts by a series of high-speed centrifugations that require specialized equipment. The expression of an epitope-tagged version of ribosomal protein L18 (RPL18) in a transgenic plant allows for the rapid immunoprecipitation of ribosomes from crude cell extracts.

Method and Concept References:

1. Zanetti, M.E., Chang I.-F., Gong, F.-C., 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.
2. Mustroph, A., Juntawong, P. and Bailey-Serres, J. (2009) Isolation of plant polysomal mRNA by differential centrifugation and ribosome immunopurification methods. Methods Mol Biol. Plant Systems Biology (D. Belostotsky, ed.) 553:109-26.
3. Bailey-Serres, J. (2013) Microgenomics: Genome-scale, cell-specific monitoring of multiple gene regulation tiers. Annual Review of Plant Biology. 64:293-325.

Perform polysome immunopurifications (TRAP) to obtain these subpopulations of cellular mRNAs. Isolate total mRNA from the same samples.

TRAP experimental overview



Safety note
This protocol uses toxic chemicals. Read the SOPs for Cycloheximide and PMSF before starting. All buffer waste should be collected and disposed of appropriately.

Materials for TRAP

- All solutions and equipment used in this protocol need to be free of RNAse. Glassware, if used, must be sterilized by autoclaving for 15 min.
- **All steps are carried out on ice or at 4 °C.**
- Unless otherwise stated, all solutions are prepared with sterile deionized water.
- The plant material must be harvested directly into liquid nitrogen, ground to a fine powder using sufficient liquid nitrogen to maintain a frozen state. Pulverization can be accomplished with a porcelain mortar and pestle. The pulverized tissue is stored at -80 °C until use.

Solutions and chemicals

- Miracloth for filtering, autoclaved
- Monoclonal ANTI-FLAG® M2 antibody produced in mouse (F1804 Sigma)
- Dynabeads Protein G for immunoprecipitation (10003D Life technologies)
- 99 % (v/v) ethanol

The following stock solutions are autoclaved and stored at room temperature

2 *M*	Tris, adjust to pH 9.0 with HCl
2 *M*	KCl
0.5 *M*	ethylene glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid (EGTA), adjust to pH 8.0 with 10 *M* NaOH. Note, EGTA only dissolves after the pH has been adjusted.
1 *M*	$MgCl_2$

Following stock solutions are not autoclaved but are stored at RT:

20 % (v/v)	Polyoxyethylene 10 tridecyl ether (PTE). Note, shake before use.	
10 %	Sodium Deoxycholate (DOC), Use lung protection while weighing.	
10 % (v/v)	polyoxyethylene sorbitan monolaurate 20 (Tween 20)	
20 % Detergent mix, Dissolve while heating to about 60 °C; protect lungs and hands.		
	20 % (w/v)	polyoxyethylene(23)lauryl ether (Brij-35)
	20 % (v/v)	Triton X-100
	20 % (v/v)	Octylphenyl-polyethylene glycol (Igepal CA 630)
	20 % (v/v)	polyoxyethylene sorbitan monolaurate 20 (Tween 20)

Solutions NOT to be autoclaved, stored at -20 °C in aliquots

0.5 *M*	Dithiothreitol (DTT)
50 mg/mL	Chloramphenicol, dissolved in ethanol
0.1 *M*	Phenylmethylsulfonyl fluoride (PMSF), dissolved in isopropanol
100 mg/mL	Cycloheximide, dissolved in ethanol, prepared fresh *just before use*

Equipment

- This technique is based on the usage of transgenic *Oryza sativa* or other plants expressing a FLAG-tagged ribosomal protein. These stable transgenic lines are essential for this protocol.
- Preparative centrifuge with fixed angle or swinging bucket rotor accommodating 30 mL tubes (*i.e.* Beckman J2-21 highspeed centrifuge and JA-20 rotor, fitted with rubber inserts to accommodate 15 or 30 mL Corex tubes or Oakridge Polycarbonate tubes, e.g. Thermo 3118-0050
- Rocking shaker, capable of shaking at about 60 rpm/min
- Magnetic rack for 15 ml tubes

Isolation of Epitope-Tagged Polysomes

Prepare Buffers (estimated time, 45 minutes)

Bead wash and binding buffer (BB): prepared on the day of each experiment and kept on ice

Final concentration		Amount of stock solution for 10 mL
0.2 *M*	Tris, pH 9.0	1 mL
0.2 *M*	KCl	1 mL
0.025 *M*	EGTA	0.5 mL
0.035 *M*	$MgCl_2$	0.35 mL
0.02%	Tween-20	20 µl

Polysome Extraction buffer (PEB): prepared on the day of each experiment and kept on ice

Final concentration		Amount of stock solution for 50 mL
0.2 *M*	Tris, pH 9.0	5 mL
0.2 *M*	KCl	5 mL
0.025 *M*	EGTA	2.5 mL
0.035 *M*	$MgCl_2$	1.75 mL
1 %	Detergent mix[1]	2.5 mL
1 %	PTE	2.5 mL
1 m*M*	DTT (0.5 M)	0.1 mL
1 m*M*	PMSF	0.5 mL
100 µg/mL	Cycloheximide	50 µL
50 µg/mL	Chloramphenicol	50 µL

[1] Pipette with a 1000 mL tip enlarged by cutting 0.5 cm from the end. It takes time for the detergents to go into solution. DOC is needed at 1% for mature leaves and seed endosperm.

Wash buffer (WB): prepared on the day of each experiment and kept on ice

Final concentration		Amount of stock solution for 100 mL
0.2 *M*	Tris, pH 9.0	10 mL
0.2 *M*	KCl	10 mL
0.025 *M*	EGTA	5 mL
0.035 *M*	$MgCl_2$	3.5 mL
1 m*M*	DTT (0.5 M)	0.2 mL
1 m*M*	PMSF	1 mL
100 µg/mL	Cycloheximide	100 µL
50 µg/mL	Chloramphenicol	100 µL

* This **Wash Buffer** will be used in the final elution step when using 3X FLAG peptide.

Preparation of the α-FLAG M2 coupled Protein G Dynabeads (Estimated time, 1h 25 min)

- Aliquot 50 µl of resuspended Protein G Dynabeads in 1.7 ml microfuge tube per sample.
- Separate the beads by placing to a magnet for 3 min.
- Discard supernatant by pipetting.
- Wash beads one time with 400 ul of BB by pipetting and repeat steps 2 and 3.
- Place 5 µl (1 µg/µl) of Anti-FLAG monoclonal antibody (**α-**FLAG-M2) in a 1.7 ml microfuge tube and dilute it in 400 µl of BB.
- Add the diluted antibody to the washed beads and resuspend by pipetting.
- Incubate with gentle agitation for 1 h at room temperature. **[**Perform the **Tissue extraction** during this incubation**]**
- Magnetically separate the beads and discard carefully the supernatant by pipetting.
- Wash the beads with 400 µl of BB by pipetting as in steps 2 and 3.
- Precool the beads on ice and resuspend the beads in a small volume of the clarified extract containing polysomes (after 16,000 xg centrifugation).
- Remove the supernatant with a pipette and wash one more time with 1.5 mL of WB before continuing with the immunoprecipitation. These are the **washed α-FLAG-M2**

coupled Protein G Dynabeads. You will add the **clarified extract** (supernatant) to the beads (remember to reserve some clarified supernatant for total RNA).

Tissue extraction (Estimated time, 45 min or longer depending on sample number)

- The pulverized tissue is placed into a 15 mL Falcon tube. Keep tissue frozen.
- Estimate volume of pulverized tissue powder (**1 mL** root tissue) and add it to a 50 mL Falcon tube with five times (**5 mL**) the volume of freshly prepared Polysome Extraction buffer (PEB). Use a plastic transfer pipette. Use at least **5 mL** of packed leaf tissue and keep a ratio of buffer:pulverized-tissue > 3 (5 is best).
- Let the mixture thaw on ice. During thawing, stir gently with a glass rod. Keep cold!
- Transfer the mixture to a glass homogenizer with a plastic transfer pipette.
- Homogenize the mixture by use of a glass homogenizer; five strokes; keep on ice. Note[2].
- Let the mixture stand on ice for 10 min (or until all samples are prepared)
- Pour into 15 mL Corex tube on ice (tubes will need to be balanced to within 0.3 g)
- Centrifuge the samples at 4°C, 16,000 *g*, for 15 min in a preparative centrifuge; use a fixed angle or swinging bucket rotor with rubber Corex tube adapters.
- Put a fresh Corex tube on ice. Place a piece of Miracloth at the top to form a small funnel.
- Using a transfer pipette, filter the supernatant into the new tube on ice, avoiding the pellet. If any of the pellet has been transferred, then repeat the centrifugation step to ensure removal of material that pellets at 16,000 *g*. This is the **clarified extract**.
- Save 0.25 mL of the extract; this 5 % of the clarified extract is used to isolate total RNA. Keep this reserve on ice until processing RNA isolations of TRAP samples.

Note[2]. Recommended step that can be replaced by homogenization with a bead beater (TissueLyser, QIAGEN for example) for lines expressing FLAG-RPL18 under semi- or constitutive promoters.

TRAP: Immunoprecipitation of polysomes (Estimated time 4 h, including 2 h pause)

- Add the rest of the **clarified extract** to each of the two tubes with washed a-FLAG M2 coupled Protein G Dynabeads
- To bind the epitope tagged ribosomes to the affinity matrix, incubate for **2 h** at 4 °C with gentle back-and-forth shaking on a rocking platform. If done at room temperature, tubes should be lying on their sides in an ice bucket with maximal movement of the liquid.

Bead Capture and Washing (estimated time 1h 10min)

- Collect the beads with the magnet for 4 minutes at 4 °C.
- Carefully, remove the supernatant from the beads (IP) using a 1 mL disposable pipette. This should be done without disturbing the bead pellet. If necessary, re-collect the beads to the magnet to get all but about 100 µl of the supernatant off. The beads should have absorbed the epitope tagged ribosomes from the solution. You can discard the supernatant.
- Add 6 mL of Wash Buffer to the beads, mix by gently inverting the tube, incubate at 4 °C for 2 min with gentle shaking on a rocking platform on ice; collect the beads to the magnet for 4 minutes at 4 ^{o}C (First wash).
- Remove the supernatant with a pipette, add 6 mL of Wash Buffer to the beads. Incubate at 4 °C for 2 min with gentle shaking (Second wash)
- Collect the beads to the magnet for 4 minutes at 4 ^{o}C. Remove the supernatant. Repeat for a third wash.
- Remove the supernatant, add 1 mL Wash Buffer. Carefully transfer the sample from the Falcon tube to a pre-labelled microfuge tube
- Collect the beads to the magnet for 4 minutes at 4 ^{o}C. Remove the supernatant. Repeat the washing steps two more times for a total of 6 washes.
- Collect the beads to the magnet for 4 min.
- Use a fine tipped pipette to remove as much of the supernatant as possible from the beads. Proceed directly to mRNA preparation.