

# TRAP purification of polysomes

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Bench copy for Kaisa Kajala – updated 12-2016 (after ATLAS TRAP)

## Purpose and Background

Polyribosomes (polysomes) are multiple ribosomes engaged in translation on a single mRNA. To evaluate the translation state of an mRNA, ribosomal subunits, ribosomes, and polysomes can be isolated from detergent-treated cell extracts. Traditionally this is done by high-speed differential centrifugation to obtain a ribosome pellet. The ribonucleoprotein particles of the pellet can be further purified by centrifugation through a sucrose density gradient. By fractionation of the gradient the amount of an individual mRNA in a sub-population of polysomes can be quantitatively determined. In this lab, we will perform polysome isolation using transgenic *Arabidopsis thaliana* that express an epitope tagged version of ribosomal protein L18 (RPL18). The addition of a FLAG epitope to the amino terminus of RPL18 allows for the rapid immunoprecipitation of ribosomes from crude cell extracts (Zanetti et al., 2005).

## Safety note

This protocol uses toxic chemicals. Read the SOPs for Cycloheximide and PMSF before starting. All buffer waste is collected to a waste container located by the fume hood and disposed by EH&S.

## Materials

- All solutions and equipment used in this protocol need to be free of RNases. Glassware, pipette tips, tubes and solutions must be sterilized by autoclaving for 15 min.

Homogenizers & glass rods

Mortars & pestles

- 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 (1003D Life technologies)

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	EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), adjust to pH 8.0 with 10 M NaOH. Note: EGTA only dissolves after the pH has been adjusted.
1 M	MgCl <sub>2</sub>

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

20 % (v/v) Polyoxyethylene 10 tridecyl ether (PTE). Note, shake before use.

20 %	Detergent mix (Dissolve while heating to about 60 °C)	
	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	Cycloheximide, dissolved in ethanol
50 mg/mL	Chloramphenicol, dissolved in ethanol
0.25 M	Phenylmethylsulfonyl fluoride (PMSF), dissolved in isopropanol

## Materials and equipment

- This technique is based on the usage of transgenic Arabidopsis thaliana 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) Oakridge Polycarbonate tubes are great (e.g. Thermo 3118-0050)
- Rocking shaker, capable of shaking at about 60 rpm/min

## Procedure

1. **Prepare buffers** as needed. For eight samples prepare

2x50ml PEB (**Polysome Extraction Buffer**)

3x50ml WB (**Wash Buffer**)

1x10ml BB (**Bead wash and binding Buffer**)

All of them are made on the day of each experiment and kept on ice.

Handy chart for prepping all of the buffers:

Final concentration	Component	V of stock to add for 50ml of PEB	V of stock to add for 50ml of WB	V of stock to add for 10ml of BB
0.2M	Tris pH 9.0	5ml	5ml	1ml
0.2M	KCl	5ml	5ml	1ml
0.025M	EGTA	2.5ml	2.5ml	0.5ml
0.035M	MgCl <sub>2</sub>	1.75ml	1.75ml	0.35ml
1%	Detergent mix	2.5ml	-	-
0.02%	Tween-20	-	-	2ul
		Bring volume up to 40ml with nuclease-free water	Bring volume up to 40ml with nuclease-free water	Bring volume to 10ml with water
		Heat up to 42C, mix (do not vortex), cool down on ice	Cool down on ice	Keep at RT

1%	PTE	2.5ml	-	-
5mM	DTT	0.5ml	0.5ml	-
1mM	PMSF (heat to 42C & vortex)	200ul	200ul	-
50 ug/ml	Cycloheximide	50ul	50ul	-
50 ug/ml	Chloramphenicol	50ul	50ul	-
		Up to 50ul with nuclease-free water, keep on ice	Up to 50ul with nuclease-free water, keep on ice	

## 2. Bead Preparation

1. Aliquot 50 ul of resuspended Dynabeads Protein G in 1.7 ml microfuge tube.
2. Separate the beads by placing to a magnet for 3 min.
3. Discard supernatant by pipetting.
4. Wash beads one time with 400 ul of BB by pipetting and repeat steps 2 and 3.
5. Prepare a anti-FLAG master mix:  
5 ul (1 ug/ul) of AntiFLAG monoclonal antibody per sample  
400 ul of BB per sample
6. Add the diluted antibody to the washed beads and resuspend by pipetting.
7. Incubate with gentle agitation for 1 h at room temperature. (Move to Step 3a)
8. Magnetically separate the beads and discard carefully the supernatant by pipetting.
9. Wash the beads with 400 ul of BB by pipetting as in steps 2 and 3.
10. Precool the beads on ice.

## 3. Tissue extraction

- a. **Grind** frozen tissue in liquid nitrogen with ceramic mortars and pestles until very fine. 50 root tips of 1-cm length are about 0.5ml of tissue, which seems to work with libraries.
- b. Add 10ml freshly prepared **PEB** with a serological pipette onto the mortar and let the mixture thaw on ice. Mix with pestle, transfer as soon as thawed.
- c. Transfer tissue to glass homogenizer. **Homogenize** the mixture with five strokes of pressing the plunger down. Note: pull the plunger up carefully not to lose your sample.
- d. Let the mixture stand on ice for 10min or until all samples are processed. Wait for the samples to unfoam (KK does at least 10min).
- e. Pour into 15ml Oak Ridge centrifuge tube. **Balance** tubes to within 0.5g (with additional PEB).
- f. **Centrifuge** samples at 4C with 16,000 g for 15 min.
- g. Prepare fresh 15ml Falcon tubes on ice with folded sterile Miracloth in the mouth of the tube. Using a 1ml pipette, **filter** the supernatant through the Miracloth into the tube. (If pellet gets disturbed, repeat the 16,000g centrifugation). This is the **clarified extract**.
- h. **Set aside** samples for total protein (0.1ml) for Westerns and **TOTAL RNA (0.2ml)**. The total RNA sample can be taken from the unclarified part too. Use the total RNA for as a control for qPCR/libraries.

## Side adventure into TOTAL RNA

Do these steps after you've got the immunoprecipitation (step 4b) started.

- i. Add 400ul LBB buffer with 2-mercaptoethanol (see RNA protocol for library preps) onto the TOTAL RNA samples. Vortex tubes at Room Temperature for 5min.
- j. Let samples sit at RT for 10 min. If you used unclarified part, spin down at 13,000rpm for 10min and transfer the supernatant to a fresh tube.
- k. Store at -80C for RNA pull-down at a later date.

#### 4. Immunoprecipitation of polysomes

- a. **Add the clarified extract** to the beads (after reserving samples for RNA and protein).
- b. **Incubate for 2 hrs at 4C** with gentle back-and-forth shaking on a rocking platform.
- c. After the 2hr incubation, put the tubes on **magnetic rack** and let stand for 5min (at 4C).
- d. ~~Set aside 200ul of the supernatant ("Unbound") if you want to do a Western to check where your FLAG tag is.~~
- e. Remove the supernatant using disposable pipettes (or pouring). Add 6ml of Wash Buffer. Place tubes on **nutator** for 5min (at 4C).
- f. Repeat steps (c) and (e) so that the beads have been **washed 3 times** (with large volume).
- g. Resuspend the beads in 1ml and transfer to 1.5ml tube. Place on nutator for 5min, then on a small magnet for 5 min.
- h. Repeat step (h) so that the beads have been **washed 3 times** (with small volume).
- i. ~~Set aside 10ul of the supernatant ("Bound") if you want to do a Western to check where your FLAG tag is.~~
- j. Remove the supernatant fully.
- k. Add 105ul LBB buffer with 2-mercaptoethanol (see RNA protocol for library preps) onto the beads. Vortex tubes at Room Temperature for 5min.
- l. Let samples sit at RT for 10 min. Spin down at 13,000rpm for 5min.
- m. Place tubes on a magnet. Transfer supernatant to fresh tubes (or PCR strip). Continue with the RNA pull-downs or store at -80C.

## 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. Ron, M.R., Kajala K., et al., (2014) Hairy root transformation using Agrobacterium rhizogenes as a tool for exploring cell type-specific gene expression and function using tomato as a model. Plant Physiology.