Polysomal RNA isolation and purification
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This protocol is optimized for isolation of polysomal RNA from leaves of mature *Arabidopsis thaliana*. If you isolate polysomes from different types of tissue, you may need to optimize the condition. This protocol has been used for maize and tobacco tissues. Polysome isolation from seedlings, and other tissues with low cytoplasmic density, may require concentration of polysomes by centrifugation through a sucrose cushion. The RNA isolated by this protocol can be used for DNA microarray hybridizations, RT-PCR or RNA blot hybridizations.

Information regarding the ISCO sucrose gradient absorbance profile analyzer and fractionator is available at: http://cepceb.ucr.edu/resources/protocol/htm

Day 1

Before you start:

1. Cool rotor (Beckman SW55Ti) at 4°C at least 1 hour prior to use.
2. Warm 20% detergent mix at 42-45°C
3. Thaw gradients (20-60% sucrose*) at 37°C for 1 h in a rotor buckets, then cool at 4°C for 1h (see page 3 for how to make gradients).
4. Label collection tubes (1.7 ml Eppendorf tubes) for each gradient.
5. Wash Corex tubes (30 ml) and rinse with 0.1% DEPC, then autoclave at 121°C for 30 min.

Polysome Extraction:

1. Prepare polysome extraction buffer.

1	2M Tris (pH 9.0)**	1 mL
2	2M KCl**	1 mL
3	0.5M EGTA (pH 8.3)**	0.5 mL
4	1M $MgCl_2$**	0.36 mL
5	ddH_2O**	To 8ml
6	Mix well	
7	β-mercaptoethanol	80 uL
8	50 mg/mL cycloheximide in EtOH	10 uL
9	50 mg/mL chroramphenicol in EtOH	10 uL
10	20% Detergent MIX*** (see recipe)	0.5 mL
11	2% PTE, 10% DOC****	1 mL
12	Heparin	10 mg
13	ddH_2O	To 10 mL

** Need to prepare stock solutions and autoclave prior to use. Store at -20°C in aliquots.

2. Chill microtubes (2.0mL) and spatulas in liquid nitrogen.
3. Place 750 uL of packed tissue volume of frozen Arabidopsis leaf (ground) into a microtube (**do not thaw**), then **immediately** add 1250 uL extraction buffer.

4. Mix the extract with spatula well and place on ice for 10 min. occasionally mix by inverting tubes (**do not vortex**).
5. Spin samples down for 2 min at 14 Krpm (4°C).
6. If there is debris that remains in the supernatant, it can be removed by the following optional step. Place the supernatant onto a QIA shredder (QIAGEN) (700 uL/ column). Spin the column for 1 min at 14 Krpm (4°C). Combine the flow through (usually from 2 columns) into a new 1.7 mL microtube. Briefly mix.
7. Carefully layer 700 uL of sample onto a sucrose gradient. Balance tubes and buckets to 0.03g by adding either sample or extraction buffer.
8. Centrifuge for 90 min at 50,000 rpm (275,000g) in Beckman LM-80 centrifuge.
9. Turn on ISCO system at least 30 min prior to polysome analysis.
10. Run blanks and samples on ISCO with sensitivity=1.0, flow rate=1.5 mL/min.
11. Fractionate polysomes into14 microtubes (16 drops/tube) (ISCO fraction collector Model 180).
12. Combine fractions 1 to 7 (non-polysomal) and fractions 8 to 13 (polysomal) in separate 30 mL Corex tubes. [If you wish to perform analyses on individual fractions you will not want to combine samples at this step.]
13. Add 7 mL (non-polysomal) or 5 mL(polysomal) 8 M guanidine HCl (0.1% DEPC, filtered. Not autoclaved). Vortex for 3 min (seal the tube with parafilm to avoid spilling). [Note: If samples were not combined in Step 12, then an equal volume of 8 M guanidine HCl is added to each fraction. RNA precipitation can be performed in 2 mL Eppendorf tubes; add as close to 2 volumes of EtOH, spin at ≥14K rpm to pellet sample. Resuspend each pellet in 20 µL DEPC-treated water. This RNA obtained by this method should be pure enough for RT-PCR]
14. Add 10.5 mL (to non-polysomal sample) or 7.5 mL (to polysomal sample) 100% EtOH. Vortex for 1 min.
15. Precipitate RNA at -20°C overnight.

Day 2

Before you start:

1. Cool Beckman JA-20 rotor at 4°C
2. Wash a corex tube insert for centrifuge rotor with 0.1% DEPC water and autoclave for 30 min.

RNA purification:

1. Centrifuge samples at 10,000 rpm for 45 min.
2. Remove supernatant **carefully**. Remove the residual solution by pipette.
3. Invert the corex tube and let the pellet dry for 20 min.
4. Prepare extraction buffer (EB) by adding β-mercaptoethanol to RLT (QIAGEN) (10ul/1mL RLT).
5. Add 450 uL EB to the sample and vortex vigorously.
6. Add 225 uL 100% EtOH to the sample. Mix well by pipetting (**do not vortex**).
7. Apply sample (700 uL) to an RNeasy mini spin column (pink). Let it sit for 3 min.
8. Spin the column for 15 sec at 14 Krpm.
9. Take the flow through and put it back to the column again (double loading). Let it sit for 3 min.

10. Spin the column for 15 sec at 14 K rpm.
11. Add 700 uL RW1 and spin for 15 sec at 14 K rpm. Discard the flow through.
12. Transfer the column into a new 2 mL microtube (without a cap). Add 500 uL RPE (Four volumes of EtOH is already added. See QIAGEN protocol.) onto the column and spin for 15 sec at 14 Krpm.
13. Add 500 RPE to the column and spin for 2 min at 14 Krpm.
14. Place the column in a new 2mL microtube and spin for 1 min at 14 Krpm.
15. Transfer the column in a new 1.7 mL microtube and add 50 uL RNase free water. Let it sit for 5 min.
16. Elute RNA by centrifuge for 1 min at 14 K rpm (repeat 15-16).
17. Take 5 uL RNA solution into a new tube and add 95 uL TE (pH 8.0). Take a spectrophotomer reading at A260 and A280 and calculate RNA conc.
18. Add 9.5 uL 3 M Sodium acetate (pH 5.3) and 190 uL 100% EtOH to the remaining RNA solution (95 uL). Mix briefly and precipitate at -20°C overnight.

Day 3

RNA wash and final conc determination:

1. Centrifuge microtubes for 30 min at 14 Krpm at 4°C.
2. Wash the pellet with 0.8 mL 75% EtOH
3. Centrifuge the tube for 10 min at 14 Krpm at 4°C (repeat 2-3 one more time).
4. Quick spin and remove residual EtOH by pipette.
5. Invert tubes and let the pellet dry for 20 min.
6. Add appropriate quantity of RNase free water (assuming 90% recovery from Day2) to get approximately final conc. of 1.0 ug/uL
7. Mix well by pipetting. Let it sit for 20 min at 4°C and vortex 1 min.
8. Take spec (1 ul RNA solution + 99 uL TE8.0) in triplicate and determine the final concentration.
9. Store samples at –80°C until use.

*** 20-60% Sucrose Gradients** (5 mL gradients)

Prepare stock solutioins:

10x Sucrose Salts:

Trizma base	2.43 g
KCl	0.75 g
$MgCl_2$	1.02 g
ddWater	to 50 ml
Adjust pH with HCl	to 8.4
Autoclave	10 min

2M (68.5% Sucrose)

Sucrose	171.2 g
ddWater	to 250 ml (approx. for 70 gradients)

Make up gradient layers as proportioned below. Then make up gradients in 5 mL polycarbonate SW55.1 centrifuge tubes. This is best done by freezing each layer at -80°C before adding the next layer.

Add **1ul per 10 ml volume of both Cyclohexamide** and **Chloramphenicol** to each layer.

Layer	2M (68.5%) Sucrose	10x Sucrose Salts	dd Water	Vol per gradient	% Sucrose
1 (Bottom)	0.88 (44) ml	0.1 (5) ml	0.02 (1) ml	0.75 ml	60
2	1.32 (66)ml	0.2 (10) ml	0.48 (24)ml	1.50 ml	45
3	0.88 (44)ml	0.2 (10)ml	0.92 (46) ml	1.50 ml	30
4 (Top)	0.29 (14.5) ml	0.1 (5) ml	0.61 (30.5) ml	0.75 ml	20

The 1st value is for 1 5 mL gradient. The value in parentheses is for 50 gradients.

*****20% detergent mix**

Triton X-100	10 mL
Brij 35	10g
Tween-40	10mL
NP-40	10mL
ddH_2O	to 50mL

******20% PTE and 10% DOC**

4 ml of poly
2 g Deoxycholine
ddH_2O to 20 ml
No need to autoclave