mRNA prep with magnetic beads

Original: Brad Townsley and Kristina Zumstein http://journal.frontiersin.org/article/10.3389/fpls.2015.00366/full.
Updated by Kaisa Kajala (latest: 11/19/15).

Purpose and Background

Extract polyadenylated mRNAs from plant tissue. This protocol has been optimized for tomato and successfully carried out with M82, pennellii and hairy root cultures. Great for multiplexing!

Materials

- Root material (1-8 root tips, 1cm long)
- If using a bead beater to disrupt the material:
    - RNase free 2ml tubes with 4-6 large (2.3mm) ceramic beads and 9-11 small (1.0mm) ceramic beads
- Liquid nitrogen
- Biotin-linker-polyT oligo (12.5 µM, store at -20 C. Return to -20 C promptly after each use. Oligo sequence: Biotin-ACAGGACATTCGTCGCTTCCTTTTTTTTTTTTTTTTTTT (linker region can also be replaced with TTTTT…). Prepare as needed by adding 12.5 µl from the 100 µm stock to 87.5 µl RNAse-free H_20. Vortex thoroughly.)
- RNase-free PCR strips and filter tips

Item	**Cat number**	**Provider**
		(VMCS = UCD stores)
Consumables		
NEB Streptavadin Magnetic Beads, 5ml	S1420S	NEB / VMCS
For buffers & washes		
1 M Tris-HCl pH 8	E76004	VMCS
Lithium chloride solution (8 M)	L7026-500ml	Sigma
500 mM EDTA pH 8	E76000	VMCS
SDS	ABI01266	VMCS
DTT	D0632-1G	Sigma
Antifoam A	A5633-100G	Sigma
5 M NaCl	E76025	VMCS
2-Mercaptoethanol	WIMM2993	VMCS
Rnase-free water	WI717861LT	VMCS
Devices		
Magwell 96 well magnetic separator	#57624	EdgeBio
MicroPlate Genie™ multiple well plate mixer	SI-0400	Scientific Industries
8-multichannel pipettes (2-20ul, 20-200ul)		

Lysis/binding buffer (**LBB**)
(Store at 4 C and warm up to RT before use by placing at 37 C for several minutes)

Solution components	**Stock solutions**	**volumes for 50 ml**
100 mM Tris-HCl	1 M pH 8	5ml
1000 mM LiCl	8M	6.25 ml
10 mM EDTA	500mM pH 8	1ml
1% SDS (or LiDS)	10% w/v	5 ml
5 mM DTT	.5 M	500 µl
Antifoam A	(full strength)	750 ul
		RNAse-free H2O to 50 ml

Immediately before adding to ground tissue add 5 µl/ml 2-Mercaptoethanol.
Ensure salt crystals are fully dissolved and Antifoam A is fully homogenized in solution prior to each use.

Washing Buffer A (**WBA**)
(Store at 4 C and keep on ice)

Solution components	**stock solutions**	**volumes for 50 ml**
10 mM Tris-HCl	1 M pH 8	500 µl
150 mM LiCl	8M	940 µl
1 mM EDTA	500mM pH8	100 µl
0.1% SDS	10% w/v	500 µl
		RNAse-free H2O to 50 ml

Washing Buffer B (**WBB**)
(Store at 4 C and keep on ice prior to use)

Solution components	**stock solutions**	**volumes for 50 ml**
10 mM Tris-HCl	1 M pH 8	500 µl
150 mM LiCl	8 M	940 µl
1mM EDTA	500mM pH 8	100 µl
		RNAse-free H2O to 50 ml

Low-salt Buffer (**LSB**)
(Store at 4 C and keep on ice prior to use)

Solution components	**stock solutions**	**volumes for 50 ml**
20 mM Tris-HCl	1M pH8	1 ml
150 mM NaCl	5M	1.5 ml
1 mM EDTA	500mM pH8	100 µl
		RNAse-free H2O to 50 ml

10 mM Tris-HCl pH 8
(Store at room temperature)

Solution components	**stock solutions**	**volumes for 50 ml**
10 mM Tris-HCl	1 M pH 8	500 µl
		RNAse-free H2O to 50 ml

1 M (1000 mM) 2-Mercaptoethanol
(freeze immediately at -20C between uses and re-make frequently)

Solution components	**stock solutions**	**volumes for 100 µl**
1 M 2-Mercaptoethanol	14.3 M	7 µl
RNAse-free H2O		93 µl

RNA elution buffer 10 mM Tris-HCL, 1mM 2-Mercaptoethanol
(make fresh each time)

Solution components	**stock solutions**	**volumes for 1 ml**
10 mM Tris-HCl	10 mM	999 µl
1 M 2-Mercaptoethanol	1 M	1 µl

General tip:

Use multichannel pipettes and nuclease-free reservoirs to make the work faster. Filter tips can be used to ensure contamination-free samples.

Procedure

Lysate preparation (Ravi's RNA library protocol)

From frozen tissue:

1. Harvest the roots into 2ml tubes (RNase-free) with 4-6 large beads and 9-11 small beads. Flash freeze in liquid nitrogen. Store in -80C.
2. Prepare 750ul of LBB with 5ul/ml 2-Mercaptoethanol per sample in 50ml falcon tube. Make sure LBB is warmed up and has no precipitate in it.
3. Bead beat up to 27 tubes at a time. Keep tubes and cool beater inserts on liquid nitrogen. 2x1min.
4. Add 400ul of LBB (w/SDS) quickly, mix by turning. (Use spatula to get tubes out of the insert.)
5. Bead beat for another 2x1min.
6. Let sit at RT for 10 min and spin down at 13,000rpm for 10min.
7. Transfer cleared lysate into PCR strips (200ul to storage at -80C + 200ul into mRNA isolation).

From TRAP samples:

1. Prepare 800ul of LBB with 5ul/ml 2-Mercaptoethanol per sample. Make sure LBB is warmed up and has no precipitate in it.
2. Add 105ul of LBB onto the anti-FLAG beads. (For TOTAL samples – add 400ul LBB)
3. Vortex 5min.
4. Let sit at RT for 10 min and spin down at 13,000rpm for 10min.
5. Transfer the lysate into PCR strips. (If more than 200ul, it is safe to store extra at -80C)
6. For TRAP, clear the lysate x2 by placing on magnet & moving the supernatant into a fresh tube.
7. Store at -80C or proceed to mRNA isolation.

mRNA isolation (Brad's DGE protocol)

Add 1uL of 12.5uM biotin-20nt-dT oligos to PCR tubes.
Add 200 uL of lysate to each PCR tube and mix well by pipetting.
Incubate at RT for 10 min.

Meanwhile, in a new set of pcr tubes, add 20ul streptavidin beads per reaction. Resuspend beads carefully with vortexing before pipetting them. (10 ul for small samples like TRAP)
Place on magnet and remove supernatant.
Resuspend in 200uL LBB, place on magnet, and remove supernatant.

Add lysate to beads and mix on agitator for 10 min at RT.
Briefly spin down to get lysate off of the lids.
Place on magnet and remove supernatant.
Wash with 200ul WBA. (Remove from magnet, mix well by pipetting, place back onto magnet, remove supernatant)
Wash with 200ul WBB. (Remove from magnet, mix well by pipetting, place back onto magnet, remove supernatant)
Wash with 200ul LSB. (Remove from magnet, mix well by pipetting, place back onto magnet, remove supernatant FULLY)
Resuspend pellet in 16uL (10mM Tris + 1mM b-Me). (10 uL for small samples like TRAP)
Heat at 80C for 2 min.
Place on magnet immediately and transfer supernatant into new tubes as quickly as possible (cooling down will re-anneal the mRNA onto the oligo-dT). If your samples cool down before transfer, heat them back to 80C.

Secondary wash (needs to be done for random primer-primed libraries):
Add 200 µl of LBB to your mRNA sample (10-16ul) and repeat from the beginning of the mRNA isolation:
(add 1ul of biotin-20nt-dT oligos; etc…).
The streptavidin beads can be re-used: wash them first with 200ul of 10mM Tris-HCl and then with 200ul LBB.

Store at -80C, proceed to cDNA synthesis OR to library prep (Brad-seq step 4 – for TRAP).

cDNA synthesis

Virtually no genomic contamination so no need to do DNase treatment. Remember to set up no RT controls.

	(per rxn)
RNA	5uL
oligo-dT	0.5uL
10mM dNTP	0.5uL

Denature RNA by heating to 65C for 5min.

5X First Strand buffer	2uL
DTT	1uL
SuperScript III	0.5uL
RNase OUT	0.5uL

50C	50min
85C	5min

Dilute the cDNA 5-fold before using as template in the qRT-PCR (by addition of 40ul nuclease-free water).