Brad's Rapid Ravi for low starting mRNA amounts

Original: Brad Townsley, annotated by Kaisa Kajala, latest update 12/8/16.

Purpose and Background

Preparing indexed non-strand specific RNA-seq libraries from low mRNA starting amounts. Great for multiplexing!
Note: this is **not** Brad-seq (which is strand-specific 3' sequencing) but Brad's rapid version of Ravi Kumar's RNA-seq prep.

References:
http://journal.frontiersin.org/article/10.3389/fpls.2015.00366/full - Brad's paper incl. the protocol for this prep and for Brad-seq
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3428589/ - Original Ravi prep

Materials

Item	**Cat number**	**Provider**
		(VMCS = UCD stores)
Consumables		
NEB Streptavadin Magnetic Beads, 5ml	S1420S	NEB / VMCS
RevertAid Reverse Transcriptase (200 U/µL), 10,000U	EP0441	Thermo / VMCS
DNA polymerase I (10U/ul), 500 units	EP0041	Fermentas / VMCS
Ampure XP beads	A63881	Beckman
Phusion high-fidelity polymerase	M0530S	NEB
dNTP mix, 25 mM each	R1121	Thermo / VMCS
Random Primers	48190-011	Invitrogen
RnaseH	M0297S	NEB / VMCS
NEBNext® End Repair Module	E6050L	NEB / VMCS
Taq	M0273L	NEB / VMCS
T4 DNA Ligase (Rapid) 600 units/µl	L603-HC-L	Enzymatics
Biotinylated oligos (Bio-linker-dT20)	Custom primer	
Adapters (annealed primers PE1-lig and ILL-lig)	Custom primer	
Primers (S1, S2, PE1, barcoded primers)	Custom primer	
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
ethanol		
PEG 8000		
Plastics		

RNase-free PCR strips		USA scientific
Filter tips, 1-10ul		Denville
Filter tips, 2-20ul		Denville
Filter tips, 20-200ul		Denville
Devices		
Magwell 96 well magnetic separator	#57624	EdgeBio
Beadbeater		
PCR machine		
MicroPlate Genie™ multiple well plate mixer	SI-0400	Scientific Industries
8-multichannel pipettes (2-20ul, 20-200ul)		
Chillette™ 20 Portable Tube Cooler	R6620	Denville

Preparations:

This protocol starts with mRNA extracted with streptavidin-coated magnetic beads (see separate protocol).

ABR buffer (Ampure XP Bead Resuspension)

15% PEG 8000
2.5M NaCL

Non-strand specific "Y" adapter annealing

Non-strand specific libraries use a "Y" shaped adapter which is ligated to both ends of a double stranded DNA molecule, The adapters should be prepared in advance and can be stored at -20 C indefinitely.

- Hydrate the main stocks of sense and antisense 5-prime adapter oligos when they arrive to a concentration of 100 µM.
- Prepare adapters by adding 8µl of 100µM PE1-lig oligo and 8µl of 100µM 5' phosphorylated ILL-lig oligo. Add 784 µl of H2O, vortex and spin down. Aliquot 100 µl in 8 strip flip-cap strip tubes and spin the strip so that all droplets are collected and run the following annealing program:

  94C 1min, (94C 10sec) X 60 cycles -1C/cycle, 20C 1min, 4C hold)

- The final concentration should be 1 µM of the Y shaped ligation adapter.
- It is a good idea to use different colors of flip-cap 8 strip tubes for the different types of adapters to avoid confusion.
- OLIGO SEQUENCES:

  PE1-lig CACTCTTTCCCTACACGACGCTCTTCCGATCT
  ILL-lig **P**-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC

General tips:

- Use multichannel pipettes, nuclease-free reservoirs and master mixes (always add +10%) to make the work faster. Filter tips can be used to ensure contamination-free samples. Master mixes can be first pipetted into one PCR strip and then into all the samples using a multi-channel pipette.
- Remember to resuspend all beads carefully before pipetting them. Ampure XP beads are expensive – pipette out the total amount you need for a day and then aliquot into individual tubes to prevent contaminating back-and-forth pipetting.

Protocol:

4. RNA fragmentation and cDNA priming

Here magnesium ions in the first strand buffer are used to fragment the mRNA at high temperature, followed by priming of the 1st strand cDNA by random primers.

- For each sample, add following into a fresh tube:
  - 1.5 µl 5X Thermo Scientific RT buffer
  - 0.5 µl Invitrogen random primers
  - 8 µl RNA
- (Place remaining RNA into -80C for troubleshooting or future library preps.)
- Spin down samples to ensure all of sample is at the bottom of the tube.
- Place in thermocycler for fragmentation and 1st strand priming.
  - 25°C 1 second,
  - 94°C 1.5 min,
  - 4°C 5 min,
  - 4°C hold

5. First strand cDNA synthesis

- Prepare 1st strand master mix.

Reagent	vol/rxn
5X Thermo Scientific RT buffer	1.5 µl
0.1M DTT	1.5 µl
H2O	1 µl
25mM dNTPs	.5 µl
RevertAid RT enzyme	.5 µl

  Add 5 µl of mix to each fragmented RNA sample and mix well.
  Total reaction volume 15 µl

- Incubate in thermocycler for reverse transcription step with the following program:
  - 25C 10min
  - 42C 50min
  - 50C 10min
  - 70C 10min
  - 4C hold

X2. Rapid Ravi steps: Second strand synthesis, end repair, A-tailing, adapter ligation

1: Second strand synthesis, end preparation and A-tailing

- Prepare master mix.

Reagent	vol/rxn
H2O	1.5 uL
25mM dNTPs	0.4 uL
PolI	1 uL
RNaseH	0.1 uL
T4 Pol+PNK mix (End Repair Module)	0.4 uL
Taq	0.2 uL
End Repair Buffer	1.4 uL
Total	5 uL

- Add 5 ul of mix to each sample on ice. Mix and spin down strip.

- Place in thermocycler and run following program:

  16C 20m,
  20C 20m,
  72C 20m,
  4C Hold

- Add 30 ul Ampure XP beads, mix and let stand at RT for 5 minutes.
- Magnetize and remove all but 5 uL of the supernatant.
- Wash 2x with 200 ul 80% EtOH – both times add the ethanol and leave it on for 30 sec. No need to resuspend beads. Remove all the supernatant.
- Leave strip on magnetic rack and allow beads to dry (until look dry & start to crack)

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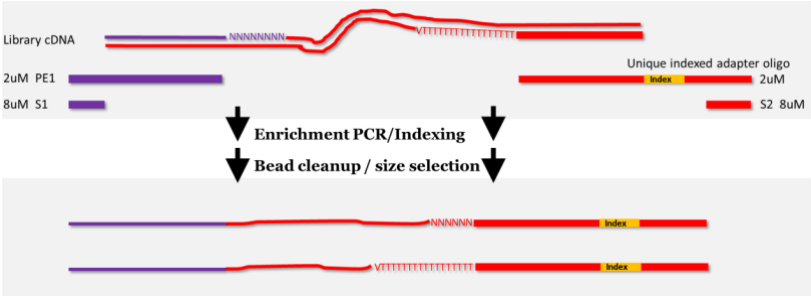
2: Adapter ligation

- Add 3 ul of annealed 1 uM universal adapters to each dry bead pellet. (It is possible that PEG with the DNA ligase affects elution, so you might pipette up and down to elute already here – very hard with such low volume though, and KK does not do it.)
- Prepare following master mix:

Reagent	volume per reaction	
H2O	1.75 ul	
2X Rapid T4 ligase buffer	5.0 ul	(note: if not rapid – will not work)
DNA ligase	0.25 ul	
Total	7 ul	

- Add 7 ul of the ligase master mix to each sample and mix by pipetting up and down making sure to resuspend Ampure beads.
- Place strip lid on to avoid evaporation and allow to stand at Room Temperature for 15 minutes.
- Add 10 ul of 50 mM EDTA to each sample.
- Add 25 ul of ABR to each sample and mix by pipetting up and down.
- Allow to stand 5 min at RT.
- Magnetize and remove all but 5 uL of the supernatant.
- Wash 2x with 200 ul 80% EtOH – both times add the ethanol and leave it on for 30 sec. No need to resuspend beads. Remove all the supernatant.
- Leave strip on magnetic rack and allow beads to dry (until look dry & start to crack).
- When beads are dry add 20-22 ul 10mM Tris to each sample and re-suspend beads by pipetting up and down 10 times. Incubate at RT for 2min.
- Magnetize and transfer the supernatant into fresh tubes.

10. Enrichment, adapter extension and final cleanup



10.1: Enrichment and adapter extension

The enrichment PCR uses four primers concurrently, two long primers to complete the adapter sequence at low concentration, and two short primers consisting of the terminal most sequence of the adapters at higher concentration. This is to selectively amplify molecules with complete adapter sequences.

- Make enrichment master mix (at RT, hot start is not necessary).

Reagent	volume per reaction
5X Phusion HF Buffer	4 µl
H2O	2.6 µl
2 µM PE1 primer	1 µl
8 µM each EnrichS1 + S2 primers	1 µl
25mM dNTPs	0.2 µl
Phusion Polymerase	0.2 µl

- Add **9 µl** of master mix to a set of new tubes.
- Transfer **1 µl** of appropriate unique indexed enrichment oligo to each well (2 µM ILL-BC primer).
- Transfer **10 µl** of adapterized cDNA (that has been eluted off the Ampure beads) to each well.

- Primer sequences:

  EnrichS1 AATGATACGGCGACCACCGA
  EnrichS2 CAAGCAGAAGACGGCATACGA
  PE1 AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
  Indexed enrichment oligo sequences at the end of this document

- Mix well by pipetting up and down. The total volume of the reaction is 20ul.

- [**Optional but HIGHLY recommended**, especially if doing libraries for these samples for the first time] Without changing tips from previous step, transfer 10 ul of enrichment PCR mix to second 8 strip PCR tubes

  ! Hedging your bets: Particularly with small samples or any time you don't have a reasonable idea how many amplification cycles will provide sufficient amplification without over-amplifying, transfer 10 µl of the pre-amplification reaction volume and freeze at -20 C, then amplify the remainder of the reaction volume and run 2 ul on an agarose gel. This will allow you to adjust the number of cycles for the remainder of the reaction volume. The remaining 8 ul of enriched libraries should provide enough sample for the final cleanup and pooling.

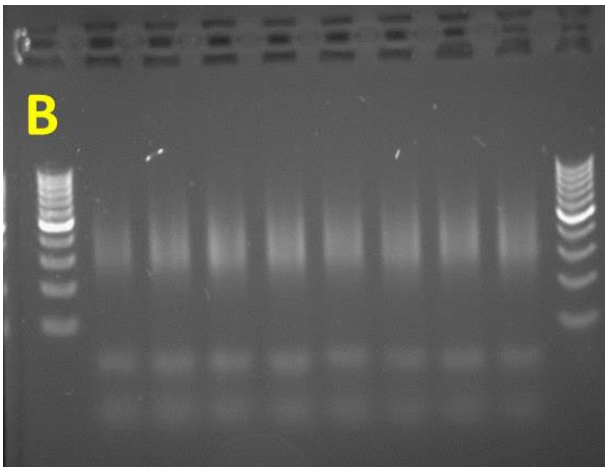
- Incubate in thermocycler with following program:

  98C 30 sec,
  (98 C 10 sec, 65 C 30 sec, 72 C 30 sec) *N cycles,
  72 C 5 min,
  10 C hold.

  N for our TRAP work has been 15 cycles.

- If you have not optimized your library cycle number for the sample type yet, check PCR results on a gel. **"If you can see it, you can sequence it".** If desired amplification is obtained proceed to cleanup.
  - Run 2 µl on 1% Agarose gel for 20 minutes at 100 Volts to check results (KK) **or**
  - **Or** run 2ul on 8% acrylamide gel and stain with SYBR Gold to increase the detection (JBS lab).
  - Sometimes KK runs up to 5ul of the first enrichment test to see the sample, enriches the rest (10-15ul), cleans it up, and does spot checks for libraries for adapter/primer contamination by running 1ul on gel.

- If desired amplification is not obtained for all samples, use remaining adapterized cDNA for enrichment with increased or decreased number of cycles.



Non-strand specific RNA-seq libraries run at 9 cycles.
2 ul of enrichment reaction loaded on to 1% SB gel run 20 minutes at 100 volts.
Ladder used is 1 ul of Thermo Scientific O'GeneRuler 100bp DNA ladder.

10.2: Final library cleanup

This step gets rid of primers and adapters and selects for specific size of library molecules. Use fresh Ampure beads. Two alternative approaches (depending how good your Ampure beads are).

Brady lab uses homemade beads and following cleanup:

- Add 1.5 volumes (i.e. 12 ul for 8 ul of enrichment product) resuspended Ampure beads to sample, mix well and let sit at room temperature for 5 minutes.
- Place on magnetic tray and remove supernatant
- Wash 2X with 200 ul of 80% EtOH. Allow pellet to dry.
- Re-suspend pellet in 10uL 10mM Tris pH 8.0 and re-suspend beads by pipetting up and down 10 times. Incubate at RT for 2min.
- Magnetize and transfer the supernatant into fresh tubes.
- **Repeat the wash.**

Bailey-Serres lab uses AmpureXP beads and following cleanup:

- Add 1.1 volumes (i.e. 8.8 ul for 8 ul of enrichment product) resuspended Ampure beads to sample, mix well and let sit at room temperature for 5 minutes.
- Place on magnetic tray and remove supernatant
- Wash 2X with 200 ul of 80% EtOH. Allow pellet to dry.
- Re-suspend pellet in 10uL 10mM Tris pH 8.0 and re-suspend beads by pipetting up and down 10 times. Incubate at RT for 2min.
- Magnetize and transfer the supernatant into fresh tubes.

- 1-2 ul can be run on a gel to make sure of (sufficient recovery and) complete lack of adapter contamination.
- Proceed to quantification (SYBR green with plate reader – see separate protocol) and pooling.
- Clean up pooled libraries with 0.8 volumes of Ampure beads to bring down the volume and to remove any residual adapters and empty library molecules.

Sequences of indexed adapter enrichment primers

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ILLSINHA01 CAAGCAGAAGACGGCATACGAGATTCAATCATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA02 CAAGCAGAAGACGGCATACGAGATAGGCAGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA03 CAAGCAGAAGACGGCATACGAGATAGAATAGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA04 CAAGCAGAAGACGGCATACGAGATGTAACGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA05 CAAGCAGAAGACGGCATACGAGATTGTAGCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA06 CAAGCAGAAGACGGCATACGAGATGCACCACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
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ILLSINHA07 CAAGCAGAAGACGGCATACGAGATGACTCGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
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ILLSINHA14 CAAGCAGAAGACGGCATACGAGATAGGTGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA15 CAAGCAGAAGACGGCATACGAGATCATCGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
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ILLSINHA19 CAAGCAGAAGACGGCATACGAGATCTTCCAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA20 CAAGCAGAAGACGGCATACGAGATCCTGATGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
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ILLSINHA22 CAAGCAGAAGACGGCATACGAGATGATATCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
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ILLSINHA27 CAAGCAGAAGACGGCATACGAGATTCAGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILLSINHA28 CAAGCAGAAGACGGCATACGAGATATAAGCAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
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