Derti et al. 2011

polyA-seq

The following is a method for amplification of 3' ends of transcripts anchored at the poly-A tail. It is a modified form of NSR where 1st strand is done using a T10VN primer, and 2nd strand using the standard NSR-AS primer pool. Sequencing is carried out using a primer ending in 10 Ts to initiate read directly at 3'end.

Primers

1st strand cDNA synthesis: NSR with short 5' PCR annealing site (100 nM)

TB1003 ACACGTCGCGTTTTTTTTTTVN

2nd strand synthesis: N7 with short 5' PCR annealing site (10 uM):

TB-rev (GA) TCCGATCTGANNNNNNN (ends in 7 Ns)

PCR: annealing sites with 5' extensions for sequencing the antisense strand (10 uM):

TB1007 AATGATACGGCGACCACCGAGCACGCTGTCCCGACACGTCGCG
TB1002 CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTGA

Sequencing primer (100 uM):

TB1005 GCACGCTGTCCCGACACGTCGCGTTTTTTTTTT

1st strand Synthesis Reaction (Invitrogen)

Mix the following in PCR strip cap tube:

x ul	polyA+ RNA (100 ng: can go down to 50 if mRNA is limiting, 100 is best)
2.0 ul	**TB1003** primer (**0.1 uM**)
To 11.4 ul	water

Heat to 65°C for 10 min and snap-chill at 4 C (use separate cycler @ 4°C).

Add 8.4 ul of RT cocktail and mix:

4.0 ul	5X buffer (Invitrogen)
1.6 ul	dNTPs (25 mM)
1.0 ul	DTT
1.0 ul	RNAse OUT (Invitrogen)
1.0 ul	Superscript III Reverse Transcriptase (Invitrogen)

Incubate at 40°C for 90 min, 70°C for 15 min and then cool to 4°C.

Add 1 ul RNase H (Invitrogen) and mix

Incubate at 37°C for 20 min, 75°C for 15min and then cool to 4°C.

1st Qiagen purification – all spins @ 13k

Add 79 uL water and mix. Add 500 uL buffer PB and pass over qiaquick (13k – 1'). Wash with 750 uL wash buffer. Dry (spin 2'). Elute twice with 55°C, 30 uL water (let sit for 1 minute prior to each spin).

2nd Strand Synthesis Reaction (New England Biolabs)

Add 40 uL of 2nd strand mix (below) to the 1st strand product (60 uL from above):

11.7 ul	water
10.0 ul	10X NEBuffer 2
10.0 ul	**TB-rev (GA) – 10 uM**
5.0 ul	10 mM dNTPs
3.3 ul	Klenow enzyme (5U/ul of NEB exo$^-$ Klenow #M0212L)

Incubate at 37°C for 30 min.

Ampure XP-Purification

Add 1.8 volumes of Agencourt AMPure XP beads (Beckman Coulter) for 5 minutes, washing twice with 70% EtOH and eluting with 50 µL of elution buffer.

PCR (Roche – 50 uL)

Add 31 ul of purified 2nd Strand Synthesis product to 18 uL of PCR mix:

10 uL	10X Buffer (Roche reagent #2)
2.5 uL	99.9% DMSO (for 5% final concentration)
1 uL	10 mM dNTP
2 uL	10 uM **TB1007** primer
2 uL	10 uM **TB1002** primer
1 uL	25 mM $MgCl_2$ (Roche reagent #4)

To each tube separately (@ 4°C), add and mix by pipetting up-down):

0.5 uL	Roche HF Enzyme Mix

Amplify using the following cycling routine in an ABI 2700:

94 C for 2 min

2 cycles of:
- 94 C for 10 sec
- 40 C for 2 min
- 72 C for 1 min

8 cycles of:
94 C for 10 sec
60 C for 30 sec
72 C for 1 min

20 cycles of:
94C for 15 sec
60 C for 30 sec
72 C for 1 min + 10 sec ↺ (use AutoX to add 10 sec/cycle)

72 C - 5 min to polish ends
4 C hold

Post PCR clean-up -> Perform second Ampure purification as described above

Post PCR Analysis:

1. run PCR reaction out on gel, should see band ranging from ~80-250 nt
2. Nanodrop spec.

Sequencing:

Standard Illumina setup, but use **TB1005** as sequencing primer.