Modified Weissman protocol for 3' end tag sequencing
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Overview
1. poly(A) selection, followed by precipitation
2. RNase T1 digest, precipitate
3. Reverse transcription, gel purify, precipitate
4. Circularize and PCR

Step 1: poly(A) selection
1. Magnetize **160 µl Dynal oligo dT_{25}** magnetic beads for 30~60 seconds and discard supernatant by pipetting
2. Wash beads by resuspending in **100 µl binding buffer** by pipetting or vortexing, magnetize and discard supernatant as before
3. Resuspend in **100 µl binding buffer**
4. Dilute 60 µg of total RNA to a final volume of **100 µl in wate**r
5. Add **100 µl binding buffer** to diluted RNA and heat at 65°C for 2 minutes, then place on ice for 2 minutes to denature
6. Add 200 µl denatured RNA to washed beads (in 100µl binding buffer), and rotate tubes at room temperature for 5 minutes to anneal
7. Magnetize beads and save the supernatant (just in case). Resuspend in **200 µl buffer B** for 1 minute rotating at room temperature.
8. Repeat wash in **buffer B**
9. Resuspend beads in **25 µl 1X Sequence Buffer** (from RNase T1 biochemistry grade, Ambion)
10. Incubate at 75° C for 2 minutes, then magnetize. The supernatant contains the $polyA^+$ RNA

Step 2: RNase T1 digestion
1. Add:
   - 1 µl glycoblue
   - optional: 0.75 µl (low molarity) radiolabeled rna
2. Heat to 50° C in pcr machine with heated lid for 5 minutes
3. Reduce temperature to 22°C
4. Add .5 µl RNase T1 (Ambion RNase T1, biochemistry grade)
5. Incubate at 22° C for 20 minutes
6. Add 54 µl stop solution, precipitate at –20° C for at least 30min

Step 3: Reverse transcription
1. resuspend RNA samples in 12µl water; keep remainder for no RT control
2. Prepare template mixes:
   - __ 9µl tailed RNA
   - __ 1µl dNTPs 10mM each
   - __ 1µl IW-RT1p reverse transcription oligo 5µM
   - __ 3.2µl water
3. denature 5min at 65°C, then put on ice for 1min
4. Add:
   - __ 4µl 5x FSB
   - __ 0.2µl SUPERase-In
   - __ 1µl 0.1M DTT
   - __ 1µl SuperScript III
5. Incubate 30min at 48°C
6. Add 2.3µl 1M NaOH to each RT reaction
7. Incubate 15min at 98°C
8. Add 22.5 µl 2x denaturing loading dye to each reaction
9. Run a 6% TBE-Urea gel:
   - Make a size standard sample with 0.5µl RT primer, 9.5µl water and 10µl 2x denaturing loading dye [this could be optional, as the primer shows up strongly even after reverse transcription]
   - Run the RT product, the RT primer size standard and a single-stranded ladder with bands around 200–400bp (can use ssRNA ladder)
10. stain 5min in SYBR Gold 1:10,000 in 1x TBE
11. photograph gel
12. excise a ~200bp smear above the size standard
13. crush the gel and elute in 0.3M NaCl at 65°C for 2h
14. precipitate with glycoblue

Step 4: Circularization
1. Resuspend gel-purified RT products in 10µl water
2. Add:
   - 2µl water
   - 2µl 10x CircLigase II buffer
   - 4µl 5M betaine
   - 1µl 50mM $MnCl_2$
   - 1µl CircLigase II
3. Incubate for 2hr at 60°C (longer incubations may increase yield)
4. Heat inactivate 10min at 80°C

Step 5: PCR
5. PCR volumes are optimized so the entire PCR reaction can be loaded directly onto an agarose gel
6. Prepare 4.5x PCR reactions per sample:
7. Add 70.6µl PCR mix to 4.5µl ssDNA template
8. Divide the resulting 75.1µl into four samples, taking 16.7µl of each sample
9. Perform PCR (*recipe below*)
   - initial denaturation, 30s at 98°C
   - 12 cycles of:
     - 10s denaturation at 98°C
     - 10s annealing at 60°C
     - 15s extension at 72°C
10. Take out an aliquot from each sample at 9, 12 and 15 cycles and leave the last sample in for the full 18 cycles
11. Add 3.3µl 6x gel loading dye to each reaction
12. Run no RT control here
13. Perform an agarose gel extraction
    - select ~200–400bp region

1x	10 reactions		
1x Phusion buffer	33.4 µl 5x HF buffer		
100 µM each dNTPs	3.34 µl dNTPs (10mM each)		
500 nM reverse PCR primer	0.84 µl IW-PCR-RPI*x*, 100µM		
500 nM forward PCR primer	0.84 µl IW-PCR-F.*y*, 100µM		
0.02 U/µl Phusion	1.67µl Phusion 2U/µl		
	117 µl Water		

Recipes
Oligo dT Binding buffer [20 mM Tris-Cl pH 7.5, 1 M LiCl, 2 mM EDTA]
Oligo dT Buffer B [10 mM Tris-Cl pH 7.5, .15 M LiCl, 1 mM EDTA]
RNase T1 Sequencing buffer [20 mM Sodium Citrate, pH 5.0, 1 mM EDTA (pH 8.0), 7 M Urea]

IW-RT1p : /5phos/TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC/iSp18/CACTCA/iSp18/CTTTCCCTACACGACGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTVN

IW-PCR-F.1: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

IW-PCR-RPI1: CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA (reverse PCR primer, index1)
IW-PCR-RPI2: CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA (index2)

IW-Seq-PE1.1: ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTTTTTTTTTTTTTTT
IW-Seq-PE2.1: GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
IW-Seq-Barcode: TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC