Mayr lab

3'-seq protocol, October 2013

Time line:

Day -1: DNase treat your RNA samples (optional)
Day 1: Prepare beads, anneal oligo to beads, 1st, 2nd strand synthesis
Day 2: Introduce nick (Rnase HII), nick translation, T7 exo, mung bean nuclease, blunt-end, ligate adapter o/n (over night)
Day 3: Test-PCR, ethanol precipitate, run gel, cut out bands, elute o/n
Day 4: Process all libraries as above
Day 5: Resuspend library

Or:

Day1: Day1+Day2
Day2: Day3+Day4
Day3: Resuspend library

PROTOCOL

DNase treatment of RNA samples:

DNase treatment of 10 ug total RNA with Ambion (DNA-free kit) without using the Inactivation reagent (white solution), instead, after incubation with the DNAse enzyme for 30 min at 37ºC (volume 50 ul)

Next:

- add 50 ul H2O, now total volume is 100 ul
- add 100 uL Phenol/Chloroform/Isoamylalcohol (25:24:1), pH=4.5 to each sample
- vortex 30s
- on ice, 10 min
- spin 15 min, 14K, 4ºC
- transfer upper phase to new eppi
- add same volume of chloroform to it
- vortex 30s
- on ice, 10 min
- spin 14K, 15 min, 4ºC
- take again the upper phase and put it into a new eppi
- add 1/10 of the volume 3M NaOAc and 2.5x volume of 100% EtOH
- put at -20ºC, 2 hours
- spin, 20 min, 14K, 4ºC
- wash pellet with 300ul 70% EtOH
- spin, 14K, 5 min
- resuspend in 10 ul water
- spec with Nanodrop

Attach oligo(dT) primer to beads:

Prepare beads:
Use a magnetic stand. After the RNA was incubated with the beads we **DO NOT** vortex samples during each wash just wash off side of the tube while pipetting in the wash solution or other solutions; use magnet for 1 min for each step, unless indicated otherwise.

Beads: Dynabeads M280 streptavidin (Invitrogen). 10 mg/ml
Use 50 ul beads/sample

1. resuspend beads by vortexing.
2. transfer 50 ul of beads to siliconized tube
3. magnet 1 min, wash with 500 ul 1x B&W buffer
4. repeat 2x
5. wash 2x in Solution A (200 ul), 2 min
6. wash 2x in solution B.
8. wash 2x in solution 1x BB
9. resuspend in 2x BB buffer (100 ul)

Anneal biotinylated oligo:
Amount of oligo:
Stock: 10 uM of PS193 (/5Biosg/CAGACGTGTGCTCTTCCGATCTTTTTTTTrUTTTTTTTTVN)
Dilute 5 ul Oligo + 95 ul H2O (per sample)

1. add 100 ul above to 100 ul beads in 2x BB (this dilutes BB to 1x)
2. rotate 15 minutes at room temp
3. magnet 2 min, remove SN
4. wash 8x with 1x B&W buffer (600 ul) (extensive washing to prevent oligo carryover)
5. wash 2x in 1x BB
6. resuspend in 75 ul 2xBB buffer

Bind RNA to the beads:

Set Eppendorf Thermomixer to 65^{o}C
use 2 ug DNase treated total RNA/library
dilute RNA in 75 ul H2O

1. heat RNA at 65^{o}C, 5 min in Thermomixer
2. warm beads for 1-2 min in Thermomixer
3. mix RNA and beads
4. incubate at 45^{o}C in water bath for 10 min
5. magnet 1 min, remove SN
6. wash 3x with 1x BB buffer (750 ul)
7. wash with 100 uL 1x First strand buffer
8. repeat, have beads in 100 uL 1x First strand buffer

9. change Thermomixer to 50ºC

First strand synthesis:

1. prepare First strand mix:

	1x	4.5x	9x	18x	6.5x	5.5x
Water	69					
10X RT buffer	10					
DTT (0.1M)	10					
dNTP mix (10 mM)	5					
RNAse OUT	2					
Total	100					

2. magnet 1 min, discard SN
3. resuspend beads in 96 uL of mix
4. incubate beads with mix at 50ºC, 5 min
5. add 4 ul Superscript III, pipette up and down
6. incubate in Thermomixer at 50ºC, interval shaking,1300 rpm x 15 sec, stand x 2 min, 1 hr
7. prepare 2nd Thermomixer at 70ºC
8. 70ºC, 1300 rpm x 15 s, stand x 2 min, 15 min
9. set first Thermomixer at 16ºC
10. put samples on ice, 5 min

Second Strand Synthesis:

1. prepare Second strand mix:

	1x	4.5x	9x
5 X Second strand buffer	27.5		
dNTP mix (10 mM)	3	13.5	27
total	30.5		

2. add Sec. strand mix to RT on ice
3. mix, 5 min on ice
4. prepare enzyme mix

	1x	4.5x	9x
DNA pol I (NEB)	5	22.5	45
RNase H	1	4.5	9
E. coli ligase	1	4.5	9
total	7		
Total SS synthesis	137.5		

5. add enzyme mix to sample
6. spin, mix well
7. 16ºC, 1400 rpm x 15 s, stand x 2 min, 2.5 hr

Prepare cleaning solution:
You will need it for 6 steps

	1x	9x	10x	5x
Cleaning sol.	98.6	887.4	986	493
Pronase	1.4	12.6	14	7

Buffer exchange:

1. magnet, discard SN
2. 750 ul buffer C
3. magnet, discard SN
4. 100 ul cleaning solution, spin
5. 37C, 1300 rpm x 15 s, stand x 2 min, 15 min
6. magnet, discard SN
7. 750 ul buffer D wash
8. repeat wash 3x with buffer D
9. have 750 ul buffer D + beads. can stop O/N

From this point on steps 1-8 will be summarized as:
buffer C, cleaning solution, buffer D

Introduce Nick:

1. magnet, discard SN
2. resuspend in 100 ul 1x ThermoPol buffer
3. magnet, discard SN
4. resuspend in 100 ul 1x ThermoPol buffer, spin
5. prepare mix:

	1x	4.5x	9x
10x ThermoPol buffer	10	45	90
RNase HII	(4)		
Water	86	387	774
total	96		

1. magnet, discard SN
2. add 96 ul mix to beads
3. add 4 ul RNaseHII
4. mix by pipetting up and down
5. 37C, 1200 rpm x 15 s, stand x 2 min, 1 hr
   NO ICE here….

-buffer C, cleaning solution, buffer D

Nick Translation:

1. magnet, discard SN
2. wash 2x 100 ul 1x NEB buffer 2 (cold)
3. spin
4. make mix:

	1x	4.5x	9x
dNTP (10 mM)	5	22.5	45
NEB2 (10x)	10	45	90
Water	77	346.5	693
total	92		

5. resuspend beads in mix
6. transfer beads into PCR tubes at 8ºC in the PCR machine
7. place new PCR tubes also at 8ºC into the PCR machine
8. add 8 ul of E.coli DNA pol I (NEB) into empty PCR tubes and incubate for 2 min
9. add beads to enzyme, mix well
10. incubate exactly 8 min at 8ºC
11. prepare EDTA stop solution (100 mM)
12. add 100 ul EDTA stop solution (final conc, 50 mM)
13. magnet, discard SN
14. transfer to new siliconized tube

-buffer C, cleaning solution, buffer D
-change Thermomixer to 25ºC

-wash 2x 100 ul 1x NEB Buffer 4

T7 exonuclease digestion:

1. prepare mix:

	1x	4.5x	9x
10x NEB 4	10	45	90
T7 exonuclease	(5)		
Water	85	382.5	765
total	95		

2. magnet, discard SN
3. add 95 ul of mix
4. add 5 ul T7 exonuclease
5. mix well by pipetting up and down
6. 25ºC, 1400 rpm x 15 s, stand x 2 min, 45 min
7. change Thermomixer to 37ºC

-buffer C, cleaning solution, buffer D

-wash 2x 100 ul 1x mung bean nuclease buffer

Mung Bean Nuclease digestion:

1. prepare mix:

	1x	4.5x	9x
10x mung bean nucl buffer	10	45	90
Mung bean nuclease	0.1	0.45	0.9
Water	90	405	810
total	100		

2. magnet, discard SN
3. add 100 ul of the mix to the beads
4. 15ºC, 1400 rpm x 15 s, stand x 2 min, 30 min

-buffer C, cleaning solution, set mixer to 25ºC, buffer D

-wash 2x 100 ul 1x NEB buffer 2

Klenow Blunt End reaction:

1. Make 1x NEB buffer 2 with 33 uM of each dNTP, you will need 100 ul of this mix/sample:

	1x	10x
NEB buffer 2	10	100
dNTP (10mM)	0.33	3.3
Water	89.7	896.7
total	100	1000

2. magnet, discard SN
3. add 99 ul of the mix to the beads
4. add 1 ul Klenow enzyme
5. 25ºC, 1400 rpm x 15 s, stand x 2 min, 30 min

-buffer C, cleaning solution, set mixer to 16ºC, buffer D

-wash 2x 100 ul 1x T4 ligase buffer
-transfer to new siliconized tube

Ligate adapter:

1. prepare mix:

	1x	9x
10x T4 buffer	10	90
T4 ligase	(5)	
50 uM PS185+PS186*	4.15	37.35
Water	80.85	727.65
total	95	

(*) PS185: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PS186: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTAT
PS185+PS186 are pre-annealed, gel-purified Illumina Truseq universal Ad1 and Ad2

2. magnet, discard SN
3. add 95 ul of the mix to the beads
4. add 5 ul T4 ligase
5. mix by pipetting up and down
5. 16ºC, 1400 rpm x 15 s, stand x 2 min, o/n
6. 25ºC, 1400 rpm x 15 s, stand x 2 min, 1 hr

-buffer C, cleaning solution, buffer D

-8x wash in buffer D (this is to ensure removal of free adapter)
-wash 2x 100 ul 1x Phusion buffer, spin
-transfer to new siliconized tube

-magnet, discard SN
-resuspend in 39 ul of 1x Phusion buffer
(if storing beads at 4ºC, spin beads down and make sure cap is tight so beads don't dry out)

Phusion PCR:

1. 98C x 2 min
2. 98C x 20 s
3. 63C x 30 s
4. 72C x 15 s

cycle to step 2 - 14x cycles

5. 72C x 10 min
6. hold at 4ºC

To check the library: total volume: 100 ul, use 5 ul beads

	1x	4.5x	9x	12.5x
Beads	(5)			
Water	68	306	612	850
5x Phusion buffer	20	90	190	250
PS192*, 10 uM	2.5	11.25	22.5	31.25
Barcode prim., 10 uM	(2.5)			
25 mM dNTP	1	4.5	9	12.5
HotStar Phusion	1	4.5	9	12.5
total	92.5			

(*): PS192: AATGATACGGCGACCACCGAGATC
PS192 is three nucleotides longer than Truseq universal PCR primer R1

EtOH precipitation:

1. Use 100 ul SN
2. add 250 ul 100% EtOH
3. vortex
4. -20ºC, 2 h
5. spin 14K, 15 min, 4ºC
6. air dry the pellet, 6-8 min
7. resuspend in 8 ul water
8. let dissolve for 10 min, then resuspend, keep on ice
9. add 3 ul of 5x Ficoll dye, mix

Run Gel:

Novex 8% precast TBE PAGE gels
Use 1x TBE running buffer
Ladder: Low range Fermentas ladder (1 ul ladder, 3 ul 5x Ficoll dye, 7 ul water)
Load 11 ul per lane

1. run gel 200V x 35 minutes
2. stain for 15 minutes in 50 ml TBE + 10 ul Ethidium Bromide (can use top of 1 mL pipette tip box- do not re-use). Light shaking
3. destain 2 x 10 minutes in H2O. Light shaking
4. put gel on saran wrap.
5. take picture with UV imager

Phusion PCR – large scale:

1. As above, 5 ul of beads, 100 ul total volume, x6 for each library
2. Ethanol Precipitation, combine 3x 100 ul + 30 ul 3M NaOAc + 840 ul 100% EtOH (2 eppis/sample)
3. incubate at -20ºC o/n
4. spin 14K, 20 min, 4ºC
5. discard SN, wash 1x with 70% EtOH
6. spin, 14K, 10 min, 4ºC
7. air dry pellet, 6-8 min
8. add 24 ul H2O to pellet, let stand for 1 hour, add 9 ul Ficoll-dye
9. load 11 ul per lane (empty lane, Marker, 6x sample, marker, empty lane)
10. Run the gel, 200 V, 45-50 min
11. Stain, 15 min, destain 2x 10 min
12. Put the Gel in saran wrap
13. Take picture and save it
14. Mark the smear: between 150 – 210 bp
15. Cut out bands (cut 2 gel slabs containing 3 lanes each and place into 1.5 ml eppi) (Careful not to touch ladder lane with blade. Use new blade if it touches ladder.)
16. add 400 uL Lonza elution buffer

17. rotate o/n at 4ºC
18. remove SN from gel, transfer to new eppi
19. add 1000 ul (2.5x volume) of 100% EtOH, add 40 ul 3M NaOAc, 1ul glycogen
20. -20ºC, 2 hr
21. spin, 14K, 20 min, 4ºC
22. discard SN, wash with 700 ul 70% EtOH
23. spin, 14K, 10 min, 4ºC
24. air dry pellet 5-8 min
25. resuspend in 8 ul of water
26. let dissolve on ice for 15 min, then pipette up and down
27. spec 1.5 ul
28. bring to Genomics facility

Appendix

Oligos used:

PS193_Hiseq_17dT_HPLC
/5Biosg/CAGACGTGTGCTCTTCCGATCTTTTTTTTTrUTTTTTTTTVN

For Adapter:
PS185 + PS186

For PCR:
Use PS192 as reverse primer and any of the barcode forward primers (PS187-PS190, PS197-198)
PS197: BC1; CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
PS187: BC2; CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
PS198: BC3; CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
PS188: BC6: CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
PS189: BC7: CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
PS190: BC9: CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

The adapter oligos and the forward PCR primers are PAGE purified.

Anneal adapters:

10X annealing buffer

Reagent	Final conc	Stock	10 mL
Tris-HCL pH 7.5	100 mM	1M Tris pH 7.5	1 mL
NaCl	500 mM	2M NaCl	2.5 mL
EDTA	10 mM	0.5 M EDTA pH 8	0.2 mL
Water			6.3 mL

Stock: 50 uM of each oligo in water
-Combine 45 ul Oligo 1 + 45 ul Oligo 2 + 10 ul 10x annealing buffer
-Heat in heat block 95C, 5 min
-remove block from heater, put another hot block on top, let warm to room temp

Bind oligo(dT) oligo to beads:

Binding capacity for ss oligos: 200 pmol (=0.2 nmol) for 1 mg of beads
We have 10 mg/ml beads
If we use 50 ul of beads = 0.5 mg beads/sample
Binding capacity/sample: 100 pmol
Oligo: have 10 uM solution = 10 pmol/ul, therefore use 10 ul oligo / sample

Solutions:

Use 0.025% Tween 20 in all wash solutions
0.1% Tw20: 500 ul in 500 ml
0.01% Tw20: 50 ul in 500 ml
0.025% Tw20: 125 ul in 500 ml

2x Binding & Washing (B&W) buffer

FINAL	STOCK	50 ml
10 mM Tris-HCl pH 7.5	1 M	0.5 ml
1 mM EDTA pH 8	0.5 M	100 ul
2M NaCl	2 M	49.6 ml
Tween 20 (0.05%)		25 ul

Solution A

Final	Stock	50 ml
0.1 M NaOH	3 M	1.67 ml
0.05 M NaCl	5 M	0.5 ml
Water		47.9 ml

Solution B

Final	Stock	50 ml
0.1 M NaCl	5 M	1 ml
Water		49 ml

2x Binding buffer (BB):

FINAL	STOCK	50 ml
20 mM Tris-HCl ph 7.5	1 M	1 ml
1 M LiCl	1 M	48.8 ml
2 mM EDTA pH 8.0	0.5 M	0.2 ml

Buffer C

Final	STOCK	50 ml		
1x PBS	1x PBS	50 ml		
0.025% Tween 20		12.5 ul		

1X TE

10 mM Tris-HCL pH 8.0
2 mM EDTA

Buffer D

FINAL	STOCK	500 ml
10 mM Tris-HCl pH 8	1 M Tris	5
2 mM EDTA	0.5 M EDTA	2
0.025% Tween		125 ul
water		493

Cleaning Solution (without pronase)

FINAL	STOCK	50 ml		
1x PBS		50 ml		
1 mM $CaCl_2$	1 M	50 ul		

Pronase- stable at 4ºC for 6 months when resuspended (resuspend in water to 10 mg/ml)

25 mM each dNTPs
Have 100 mM dATP, dGTP, TTP, dCTP
Mix 50 ul + 50 ul + 50 ul + 50 ul

10 mM each dNTPs
Have 100 mM dATP, dGTP, TTP, dCTP
Mix 50 ul + 50 ul + 50 ul + 50 ul + 300 ul H2O

NEB buffer 2 plus 33 uM each dNTPs

Use 10 mM dNTP
Add 3.3 ul dNTPs + 996.7 ul H2O

5X Ficoll loading dye (high density TBE sample buffer)

Final
18 mM Tris base
18 mM Boric Acid
0.4 mM EDTA
3% Ficoll type 400
0.02% Bromo Blue
0.02% Xylene Cyanol
water

Reagent	**10 mL**
10X TBE	1 mL
Ficoll Type 400	1.5 g
Bromo Blue-0.25%	4 mL
Xylene Cyanol-1%	1 mL
water	4 mL

-store at 4ºC for up to 6 months

10X TBE

Reagent	**500 mL**
Tris Base	54 g
Boric Acid	27.5 g
EDTA 0.5 M pH 8	20 mL
water	

-stable at room temp up to 6 months.

Lonza Elution Buffer

Ammonium acetate 3.85 g
Magnesium acetate 0.215 g
0.5 M EDTA 0.2 mL
10% SDS 1mL
Up to 100 mL with H_2O

Reagents

Company	Reagent	Cat #
Invitrogen	Dynabeads M-280 streptavidin	112-05D
Fermentas	GeneRuler Low Range DNA Ladder, 25-700 bp	SM1191
Invitrogen	Superscript III RT	18080-044
Invitrogen	dNTPs, 10mM	AM8200
Roche	Pronase 10 mg/ml	10165921001
NEB	Phusion high-fidelity DNA Polymerase	F-540L
Invitrogen	8% TBE Gels 1.0 mm, 10 well	EC6215BOX
NEB	T4 DNA ligase	M0202M
NEB	Rnase H	M0297L
NEB	T7 exonuclease	M0263L
NEB	DNA Pol I (E.coli)	M0209L
NEB	Klenow, DNA Pol I, Lg (Klenow) fragment	M0210L
NEB	Rnase HII	M0288L
NEB	Mung bean nuclease	M0250S
NEB	Buffer 2	
NEB	Buffer 4	
Eppendorf	Thermomixer	
Bioplas/Applied biosystems	1.5ml Flat Top MCT, Natural, 500 Tubes/Pack (siliconized)	4165SL or 4030
Millipore	PureProteome Magnetic Stand	