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MPE-seq

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Protocol status: In development
We are still developing and optimizing this protocol

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Materials

MATERIALS

- Klenow Fragment (3'-5' exo-) (50,000 u/ml) - 1,000 units **New England Biolabs** **Catalog #**M0212M
- Phusion DNA polymerase **New England Biolabs**
- Dynabeads MyOne Streptavidin T1 **Invitrogen - Thermo Fisher**
- Superscript IV **Thermo Fisher Scientific** **Catalog #**18090050
- Zymo-Spin I Columns **Zymo Research** **Catalog #**C1003-250
- Biotin-11-dUTP Solution (1 mM) **Thermo Fisher Scientific** **Catalog #**R0081
- NEBNext® High-Fidelity 2X PCR Master Mix **New England Biolabs** **Catalog #**M0541L

RNA fragmentation

1 *Fragmentation may eliminate size biases against large primer extensions which are inefficiently PCR amplified and sequenced, but may be unnecessary depending on the application. I usually prefer not to fragment...

If you want to do fragmentation, I recommend fragmenting 15 to 40ug RNA by assembling the following reaction in a PCR tube for each RNA sample in PCR tubes. Fragmentation buffer should be added last, and reactions kept on ice to prevent excess fragmentation during reaction set up.

	Reagent	Volume (uL)
	15-40ug RNA	
	Fragmentation buffer	2
	DEPC Water	Up to 20
	total	20

2 Induce fragmentation by heating in thermocycler at 65°C for 15 minutes.

*(Different RNA samples may need optimization). In past, I have ran a time-course I and decided to use a fragmentation time about half-way between when the rRNA bands start to become indescernable (under fragmented) and saturating (over-fragmented).

3 Stop fragmentation by adding 2uL fragmentation STOP buffer and placing samples on ice

4 Exchange buffer by RNA by ethanol precipitation:Bring sample volume to 50uL by adding 26uL water and transfer to 1.5mL Eppendorf tube. Add 1/10 sample volumes (5uL) 3M sodium acetate, followed by 2.5 sample volumes (137.5uL) of 100% ethanol. Vortex and cool samples to encourage precipitation (2+ hours at -20°C, or 15min at -80°C).

5 Centrifuge max-speed in cold centrifuge for 10min to pellet. Pellet should be visible. Aspirate liquid.

6 Wash pellet with full sample volume (200uL) of 80% ethanol. Aspirate liquid. Repeat for a total of 2 washes

7 Dry pellet as much as possible, by aspirating and air-drying on benchtop before resuspending in water. Aim for a concentration of ~2ug/uL

8 Quantify by NanoDrop. Store at -20°C or continue.

Reverse transcription

9 For each sample, make a primer/template mix for each sample in a PCR tube. Keep on ice.
*Use mastermixes when applicable

	Reagent	Volume (uL)
	5X RT buffer	4
	Primer mix (10nM of each gene-specific primer)	2
	Fragmented RNA	10ug
	Water	Up to 20
	total	20

10 Prepare an enzyme mastermix. Make 21uL per sample in a single PCR tube. If volume exceeds 100uL, split into multiple PCR tubes.

	Reagent	**Volume (uL)**
	5X RT buffer	4
	0.1M DTT	4
	10mM dNTP mix	2
	water	6
	1mM Biotin dUTP	4
	SSIV	1
	Total	21

11 Program the following thermocylcer program, preferably on a cycler that can hold temperature during a user-pause

70°C 1min
65°C 5min
55°C 5min
(Optional user pause to hold at 55°C)
55°C 30min
80°C 10min
25°C hold

12 Add primer mix/template to thermocycler to denature and anneal primers to template. (70°C to 65°C to 55°C)

13 Once reaction hits 55°C or during user pause, preheat the enzyme mix by placing on thermocycler for a few minutes. Once reaction is preheated, can mix 20uL enzyme mix directly to primer/template mix.

*Not preheating enzyme leads to substantial increase of off-target primer-extensions.

14 Release the user pause and allow the reaction to proceed at 55°C for 30min followed by 80°C inactivation of enzyme.

15 Degrade the RNA by base hydrolysis:
Add 20uL RNA hydrolysis solution. Incubate at 65°C for 15min.

16 Add 20uL neautralization solution. Sample volume is now 80uL

17 Ampure cleanup:
1. Add 1.8 sample volumes AmpureXP beads. Capture beads on magnetic stand for 2min. Aspirate liquid
2. While on magnetic stand, add 200uL 70% ethanol wash and incubate for 30 seconds. Aspirate.
3. Repeat for a total of 2 washes
4. Remove from stand and pipet thouroughly with 50uL water to elute.
5. Place on magnetic stand for 2minutes and transfer supernate to labelled 1.5mL centrifuge tube.

First streptavadin bead purification of primer extensions

18 Bead preparation:
1. Vortex beads vigorously to resuspend
2. Transfer 20uL of beads per sample to be purified into a new 1.5mL Eppendorf tube (do not add to sample).
3. Place tube on magnetic stand and incubate for 1 min
4. While leaving tube on magnetic stand, aspirate buffer
5. Remove tube from magnetic stand
6. Wash beads by adding 500uL of 1x bind and wash buffer to tube containing the beads and mix well with a pipette
7. Place tube on magnetic stand and incubate for 1 min
8. Aspirate buffer
9. Repeat wash for a total of 2 washes
10. Resuspend beads in 50uL of 2x bind and wash buffer per sample to be purified
11. Mix beads well with a pipette
12. For each sample, aliquot 50uL of beads into a new PCR tube or tube for that can fit on magnetic stand.

19 Bead binding:
1. Add each 50uL prepared beads to each 50uL sample.
2. Place on rotator to gently agitate at RT for 30min
3. Place tubes on magnetic stand and incubate for 1 min

4. Aspirate buffer
5. Remove samples from magnetic stand

20 Bead wash:

1. Add 150uL of 1x bind and wash buffer and mix well with pipette
2. Place on magnetic stand and incubate for 1 min
3. aspirate buffer
4. repeat washing for a total of 3 washes

*I think it is acceptable here and in other steps to spray wash volume over beads with the same pipet tip and tap tube to mix if it breaks up the beads.

21 SSC wash:

1. Wash each sample with 100uL 1x SSC, mixing well by pipetting
2. Place on magnetic stand for 1 min
3. Aspirate buffer
4. Remove samples from magnetic stand

22 Denature/wash off unlabelled strand:

1. Add 100uL of denaturing solution to each sample
2. Incubate each sample for 10minat RT
3. Place samples on magnetic stand for 1 minand aspirate buffer
4. Wash by adding 100uL of denaturation solution to each sample. Mix well by pipetting
5. Place samples on magnetic stand for 1 minand aspirate buffer

23 TE wash:

1. Add 100uL of TE buffer and mix well with pipette
2. Place on magnetic stand and incubate for 1 min
3. aspirate buffer
4. Repeat TE buffer wash 2 times for a total of 3 washes

24 Elution:

1. Add 50uL of bead elution buffer to each sample
2. Incubate samples at 90°Cfor 2 min
3. Immediately place samples on magnetic stand and incubate for 1 min
4. Aspirate each sample and transfer to a new tube

25 Ampure cleanup:

1. Add 1.8 sample volumes AmpureXP beads. Capture beads on magnetic stand for 2min. Aspirate liquid
2. While on magnetic stand, add 200uL 70% ethanol wash and incubate for 30 seconds. Aspirate.

3. Repeat for a total of 2 washes
4. Remove from stand and pipet thouroughly with 40uL water to elute.
5. Place on magnetic stand for 2minutes and transfer supernate to labelled 1.5mL centrifuge tube.

Store at -20°C or continue.

First strand extension

26 1. Prepare the following reaction mix for each sample:

	Reagent	Volume (uL)
	10X NEB Buffer 2	5
	10mM dNTP Mix	1
	cDNA from previous	40
	1^{st}Strand Extension Primer	1
	Total	47

27 Incubate each sample at 65° Cfor 2 min.

28 Cool samples to RT by placing on bench top for~5min.

29 Add 3 uL of Klenow exo- enzyme to each sample.

30 Incubate samples at RT for 5 min

31 Heat samples to 37°C and incubate for 30 min

Second streptavidin bead purification

32 Bead preparation:

1. Vortex beads vigorously to resuspend
2. Transfer 20uL of beads per sample to be purified into a new 1.5mL Eppendorf tube (do not add to sample).
3. Place tube on magnetic stand and incubate for 1 min
4. While leaving tube on magnetic stand, aspirate buffer
5. Remove tube from magnetic stand
6. Wash beads by adding 500uL of 1x bind and wash buffer to tube containing the beads and mix well with a pipette
7. Place tube on magnetic stand and incubate for 1 min
8. Aspirate buffer
9. Repeat wash for a total of 2 washes
10. Resuspend beads in 50uL of 2x bind and wash buffer per sample to be purified
11. Mix beads well with a pipette
12. For each sample, aliquot 50uL of beads into a new PCR tube or tube for that can fit on magnetic stand.

33 Bead binding:

1. Add each 50uL prepared beads to each 50uL sample.
2. Place on rotator to gently agitate at RT for 30min
3. Place tubes on magnetic stand and incubate for 1 min
4. Aspirate buffer
5. Remove samples from magnetic stand

34 Bead wash:

1. Add 150uL of 1x bind and wash buffer and mix well with pipette
2. Place on magnetic stand and incubate for 1 min
3. aspirate buffer
4. repeat washing for a total of 3 washes

*I think it is acceptable here and in other steps to spray wash volume over beads with the same pipet tip and tap tube to mix if it breaks up the beads.

35 SSC wash:

1. Wash each sample with 100uL 1x SSC, mixing well by pipetting
2. Place on magnetic stand for 1 min
3. Aspirate buffer
4. Remove samples from magnetic stand

36 Denature/wash off unlabelled strand:

1. Add 100uL of denaturing solution to each sample
2. Incubate each sample for 10minat RT
3. Place samples on magnetic stand for 1 minand aspirate buffer
4. Wash by adding 100uL of denaturation solution to each sample. Mix well by pipetting
5. Place samples on magnetic stand for 1 minand aspirate buffer

37 TE wash:

1. Add 100uL of TE buffer and mix well with pipette
2. Place on magnetic stand and incubate for 1 min
3. aspirate buffer
4. Repeat TE buffer wash 2 times for a total of 3 washes

38 Elution:

1. Add 50uL of bead elution buffer to each sample
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3. Immediately place samples on magnetic stand and incubate for 1 min
4. Aspirate each sample and transfer to a new tube

39 Ampure cleanup:

1. Add 1.8 sample volumes AmpureXP beads. Capture beads on magnetic stand for 2min. Aspirate liquid
2. While on magnetic stand, add 200uL 70% ethanol wash and incubate for 30 seconds. Aspirate.
3. Repeat for a total of 2 washes
4. Remove from stand and pipet thouroughly with 20uL water to elute.
5. Place on magnetic stand for 2minutes and transfer supernate to labelled 1.5mL centrifuge tube.

Store at -20°C or continue.

PCR amplification and sequencing

40 Test PCR:

Best to do a test PCR with a small amount of template to verify that it works before using up all of the template!

Use NEB 2X HiFi MM with 2uL template

	Reagent	Volume
	Water	6
	template (from previous step)	2
	10uM 7XX Nextera Fwd primer	1
	10uM 5XX Nextera Rev primer	1
	100X SYBR green (dissolved in DMSO)	0.2
	2X Mastermix	10
	Total	20

Perform the following qPCR cycling:
Denaturation:
95 °C 3 min
Amplification (40x):
98 °C 10 sec
62 °C 20 sec
72 °C 30 sec
Final elongation:
72 °C 5 min

I typically get Ct values around ~20.
The best way to be sure the protocol worked is to look at the qPCR product on a gel, compared to a no template control.

41 Real PCR:

Do the same PCR as the test but using all the remaining template in a 40uL reaction and only do as many PCR cycles as necessary to get amplification. Clean with Ampure beads.

42 Sequence. MPE-seq libraries from human samples are not very complex in my experience. Perhaps on the order of a million or couple million unique molecules (UMIs) so there is diminishing returns with more sequencing. I think 1-5 million reads is usually sufficient. I usually observe 20-200 fold enrichment of targets compared to RNA-seq, though it varies quite a bit from target to target.

Unlike the MPE-seq publication in yeast, for whatever reason, we have not reads that appear derived from lariat intermediates, though we can measure plenty of unspliced message. In yeast, we reported ~ a 1:10 ratio of lariat derived reads to fully unspliced reads. That ratio is likely much less for humans.