Gan-Or Lab PD MIPs Protocol

Attached files:

- List of all PD MIP probes
- Benchtop worksheet in excel format; useful for calculating reagent quantities and having a quick reference sheet, please read written protocol before using.
- List of barcode sequences

General notes:

- As is standard, add 10% to reagent calculations to account for pipetting error
- Keep enzymes on ice while working with them
- Buffers and MIP pools do not need to be kept on ice
- Centrifuge all plates before opening
- Store AMPure beads according to manufacturer's instructions

Prepare MIP pools:

Order all PD MIPs library MIP probes from IDT (see supplementary material for list of MIPS; to order, you will need the MIP name and MIP Sequence) as 25 nmol oligos in plates. To make separating the poor performers easier, you can put them together on the last plates. Once they arrive, thaw and prepare pools by adding 5 uL of each probe to one 5 mL tube for each pool, as follows:

Pool 1: Plates 1-8 of MIP probes (768 probes total)

Pool 2: Plates 2-16 of MIP probes (768 probes total)

Pool 3: Plates 17-24 of MIP probes (768 probes total)

Pool 4: Plates 25-31 of MIP probes (627 probes total)

Poor Performers Pool: pool according to poor performers list. Note that the poor performers will be included in the regular pools as well as the poor performers pool; there is no need to exclude them from the regular pools. (108 probes total)

Order Primers:

MIP Forward primer: `CATACGAGATCCGTAATCGGGAAGCTGAAG`

MIP barcodes: see attached file

Step-by-step MIPs protocol

Step 1: Phosphorylation of MIPs

Reagents

	MIP pool				
	1	**2**	**3**	**4**	**Poor Performers**
vol uL MIP Pool	38.4	38.4	38.4	31.35	5.4
T4 Ligase Buffer (10X)	5	5	5	5	5
T4 PNK	3.5	3.5	3.5	3.5	3.5
water	3.1	3.1	3.1	10.15	36.1
final volume	50	50	50	50	50

1. Prepare the reactions in a strip of PCR tubes, preparing one tube for each pool of MIP probes (5 total). Vortex MIP pools and T4 ligase buffer before adding.
2. Run the MIP phosphorylation program in a PCR machine.

MIP Phosphorylation PCR program:

37 °C 45 min
65 °C 20 min

Note: phosphorylated MIPs will last up to 3 weeks, stored at 4 °C (do not freeze!)

3. After the phosphorylation program has run, combine the phosphorylated MIP pools with serial dilutions:

Dilution 1 (1:10)

1:10 dilution (5uL in 50uL final H2O)	picomoles/uL	uL
pool 1 @0.1 (Phosphorylated)	0.01	5
pool 2 @0.1 (Phosphorylated)	0.01	5
pool 3 @0.1 (Phosphorylated)	0.01	5
pool 4 @0.1 (Phosphorylated)	0.01	5
H2O		30
Final volume dil. 1:10		50

Dilution 2 (1:50 for final dilution 1:500)

1:50 dilution (4 uL in 200 uL final H20)	picomoles/uL	uL
Dilution 1, all pools @ 0.01	0.0002	4
Poor performers @0.1 (Phosphorylated)	0.02	40
H2O		156
Final volume dil. 1:500		200

As above, this final pool can be stored for 3 weeks at 4 °C.

Step 2: MIP Capture

Reagents, per 96-well plate

DNA, 10 µL @ 10 ng/µL

Mastermix:

	X 1	X 96
Ampligase 10X Buffer	2.5	240
MIP Probes	2.19E-01	21.02
dNTPs 0.1mM each	0.080	7.68
Hemo Kleen Taq	0.32	30.72
Ampligase (100 units/ul)	0.01	0.96
Ultrapure water	11.87	1139.6
Total	**15**	1440.00

1. Prepare your DNA, plating onto 96-well PCR plate: 10 µL DNA @ 10 ng/µL.
2. Prepare the MIP capture mastermix, combining all reagents listed above. Flick enzyme tubes and vortex buffers before adding. Vortex mastermix.
3. Distribute 15 µL of mastermix to each well.
4. Seal plate well, centrifuge, and run the MIP capture program in a PCR machine.

MIP Capture PCR program:

95°C 10min
60°C 24-48 hrs

After capture, cool plate on ice and proceed IMMEDIATELY to exo treatment.

Step 3: Exo Treatment

Reagents, per 96-well plate

	X1	X96
Exo I	0.5	48
Exo III	0.5	48
Ampligase buffer 10X	0.2	19.2
H2O	0.8	76.8
Total	**2ul**	192

1. Prepare exo treatment mastermix with reagents above. Before adding, flick the tubes of Exos to mix, and vortex the ampligase buffer. Briefly vortex your master mix before distributing.

2. Run MIPs exo program in a PCR machine.

MIP Exo PCR program:

37°C 45 min
95°C 2 min to inactivate the exo

When finished store at -20 ° indefinitely.

Step 4: MIP Real-Time PCR

Reagents, per 96-well plate

Barcoded R primers @10 uM (1.25 uL)

MIP capture reaction (5 uL)

Mastermix:

	X1	X96
2X iProof	12.5	1200
F primer (100uM)	0.125	12
Sybr Green invitrogen 100X (0.5X final)	0.125	12
H2O	6	576
Total	**18.75**	1800

1. Prepare your reactions in a 0.1 mL PCR plate. First add 1.25 uL of the barcoded R primer, and 5 uL of the MIP capture reaction (with exo treatment completed) to each well. Then distribute your mastermix. Total volume should now be 25 uL.
2. Seal with an optical plate cover and run the QPCR program, setting SYBR as the reporter.

QPCR program:

98C 30s	
98C 10s 60C 30s 72C 30s	22 cycles
72C 2min	
4C forever	

When finished store at -20 ° indefinitely.

Step 5: Library preparation

Reagents

0.9V of AMPure purification beads; 432 uL for a 96-well plate.
70% ethanol
2.5% Agarose gel
Elution buffer

1. First, prepare a 2.5% agarose gel and check a few samples to make sure that the QPCR was successful. Two bands should be visible; the lower band will be cleaned up with purification beads.
2. Pool your capture plate (5 µL of each well into a labelled 1.5 mL tube)
3. Reserve 5 µL of the pool to run on a gel.
4. Add purification beads at 0.9x volume of your total pool.
5. Pipette mix and let incubate 5 minutes at room temperature.
6. Place tube in the magnetic rack until beads are separated.
7. Remove supernatant and place in a new (labelled) tube.
8. Wash the bead pellet twice with 200 µL of 70% ethanol without removing the tube from the magnet. Leave EtOH for 30 seconds, then remove with pipette.
9. Remove remaining EtOH and dry beads for 10 minutes.
10. Remove from magnet and add elution buffer, pipette mix and let incubate 5-10 minutes.
11. Place tube on rack until beads have separated.
12. Remove the eluant to a new labelled 1.5 mL tube. THIS IS YOUR FINAL PRODUCT.
13. Run 5 µL each of the pre-purification pool, the supernatant, and the eluant on the gel and see if you have cleaned up the lower band.
14. When you look at the gel, what you will ideally see is a band at approx. 280 bp for your pre-purification DNA, along with (possibly) a band at 150 bp. The supernatant should show the 150 bp band, if there was one to begin with, and the eluant should show only a clear 280 bp band, though it may be very faint.
15. Measure concentration and dilute as needed before sequencing.

Sequencing:

To achieve a good depth of coverage, we sequence 3 plates (288 samples) per lane of Illumina HiSeq 100 bp PE. Note that you must therefore use 288 different barcodes!