**PLATE-Seq**

NOTES: Assumes starting with 96-well plate of ~30ul lysate (TCL buffer w/ β-mercaptoethanol). Assumes enough starting material to use only half of the lysate. If all 30ul of lysate is needed, scale all reaction volumes up accordingly.

Reagents Needed:

TurboCapture 96 mRNA Kit (5) (Qiagen, 72251)

SUPERaseIN (ThermoFisher, AM2696)

Nuclease-free water (ThermoFisher, AM9939)

Adapter-linked oligo(dT) primers (IDT)\*

ProtoScript® II Reverse Transcriptase (NEB, M0368L)

DTT (1M)

dNTPs (ThermoFisher, R0182)

ExoI (NEB, M0293S)

NaOH

EDTA

HCl

pH strips

MinElute PCR Purification Kit (50) (Qiagen, 28004)

pH indicator (Qiagen)

sodium acetate (3M)

adapter-linked random hexamer primer (LowPassSS) (IDT)\*

Klenow Large Fragment (NEB, M0210L)

AMPureXP beads (Beckman Coulter, A63880)

Ethanol

Phusion® High-Fidelity DNA Polymerase (NEB, M0530S)

RP1 primer (IDT)\*

RPX (1-8) (IDT)\*

Custom R1/R2 sequencing primers for library (IDT)\*

Custom R1/R2 sequencing primers for PhiX (IDT)\*

Tris-HCl pH 7

PhiX (Illumina, FC-110-3001)

NextSeq High Output 75 cycles (Illumina, FC-404-2005)

\*primer sequences were published in citation

**mRNA Isolation**

1. Transfer 15ul lysate to each well of the mRNA capture plate using a multichannel pipette.
2. Add ERCC spike-in to half of the rows (12-channel pipette) or half the columns (8-channel pipette). The High-Throughput Screening facility uses 1ul of a 1:5000 dilution for a starting density of ~10K cells.
3. Incubate at room temperature for 90 minutes. If there’s access to a plate shaker, shake at 100rpm for the incubation.
4. Remove lysate and wash 3X with 20ul TCW. Spin and remove excess buffer after final wash.
5. Add 15ul water containing 0.15U/ul SUPERaseIN to each well. (Make stock of at least 1500ul)
6. Incubate at 65°C for 5 minutes.
7. Incubate at room temperature for 5 minutes.
8. Quick spin down, move 15ul supernatant to new plate.

**Reverse Transcription**

1. Add 3ul 100uM adapter-linked oligo(dT) primer to each well. Be sure to note plate orientation and keep wells matched between RNA plate and adapter plate.
2. Add 5ul 5X Protoscript RT Buffer to each well (assumes 25ul RT reaction). Mix well and spin down.
3. Incubate at 94°C for 2 minutes.
4. Immediately move plate to ice for 5 minutes.
5. Make the rest of the RT reaction for 2ul x 110 wells (FINAL CONCENTRATIONS):

1.8mM each dNTP

16mM DTT

0.35U/ul SUPERaseIN

3.6U/ul ProtoScript RT enzyme

Water to bring up to volume

1. Add 2ul RT mix to each well. Mix well and spin down briefly.
2. Incubate at 42°C for 2 hours.

The plate can be frozen here as an optional stopping point.

**Primer Removal and RNA Hydrolysis**

1. Remove 12.5ul RT reaction to new PCR plate. The rest can be saved and frozen.
2. Dilute ExoI 1:4 in water and add 1ul to each well. Mix well and spin down.
3. Incubate 1 hour at 25°C on thermocycler.
4. Add 5ul of a 1:1 mixture of 1M NaOH and 0.5M EDTA to each well. Mix well and spin down.
5. Incubate at 65°C for 15 minutes.
6. Spin down and pool total volume into a 15ml conical.
7. Neutralize with 10ul 12M HCl (**BE VERY CAREFUL AS HCl IS VOLATILE. DO UNDER A HOOD**).
8. Check on pH strip to see if pH is ~7.
9. If still basic, add 3ul HCl. Check pH strip again. Continue until pH is 7.

The tube can be frozen here as an optional stopping point.

**Clean-up and Concentration**

1. Add pH indicator to Buffer PB according to manufacturer’s instructions.
2. Add 5 volumes of Buffer PB + pH indicator to thawed pooled ss cDNA tube.
3. Check color of the mixture. It should be yellow. If it’s orange or purple, add 10ul 3M sodium acetate and mix well. It should turn the correct color.
4. If using a spin column, add 750ul to MinElute column and centrifuge max speed for 1 min.
5. Discard flow-through. Repeat step 4 until all volume has been passed through the column.
6. If using a vacuum manifold, add 750ul to MinElute column and follow manufacturer’s instructions for using the manifold with the column.
7. Keep adding 750ul to column until all volume has been passed through.
8. Wash with 750ul PE buffer. Spin 1 min or use vacuum to pull PE buffer through.
9. Spin column empty once for 1 min.
10. Move column to clean 1.5ml tube and add 15ul nuclease-free water. Incubate for 1 minute at room temperature.
11. Spin for 1 minute, discard MinElute tube.

**Second Strand Synthesis**

1. To 15ul ss cDNA from step above, add 1ul 100uM LowPassSS and 1ul 10mM dNTPs.
2. Incubate at 70°C for 2 minutes.
3. Immediately move to ice for 5 minutes.
4. Add 2ul NEB2 and 1ul Klenow Large Fragment.
5. Incubate at room temperature for 30 minutes.
6. Neutralize by adding EDTA to a final concentration of 50mM (2ul of 500mM). Mix well and spin down.

**Double AMPureXP Clean-up and Quantify**

1. Slowly add 22ul AMPure XP beads for a 1:1 bead:sample ratio and mix well.
2. Incubate for 5 minutes at room temperature.
3. Place tube on magnet and wait ~30 seconds for beads to separate from the solution.
4. Remove supernatant carefully, do not disturb beads. Leave ~3ul solution behind.
5. Add 200ul 80% ethanol, wait 30 seconds. Remove ethanol.
6. Repeat step 5 for a total of 2 ethanol washes.
7. Air dry beads 5-10 minutes. Slight cracking is okay.
8. Resuspend beads in 22ul nuclease-free water.
9. Incubate at room temperature for 3 minutes.
10. Place tube on magnet and wait ~30 seconds for beads to separate from the solution.
11. Remove 20ul to clean 1.5ml tube.
12. Slowly add 20ul AMPure XP beads for a 1:1 bead:sample ratio and mix well.
13. Repeat steps 2-11.
14. Qubit using 3ul cDNA. Range should be >0.1-0.2ng/ul.

**PCR Amplification**

1. Make PCR Master Mix:

1X

5X High Fidelity buffer 5ul

10mM dNTPs 1.25ul

25uM RP1 0.5ul

25uM RPIX 0.5ul

Template cDNA 5ul

Phusion DNA polymerase 0.25ul

Nuclease-free water 12.5ul

1. Cycling conditions:

98°C 30sec

10-12 cycles:

98°C 15sec

62°C 15sec

72°C 60sec

72°C 7min

4°C hold

1. Bioanalyze and Qubit to check quality and quantity.

**Sequencing**

1. Sequence on the NextSeq using the High Output 75 cycle kit. Read 1 = 26bp, read 2 = 60bp or 66bp depending on whether indexing is used (only use indexing if sequencing more than one plate together).
2. PhiX should be 30%. Loading concentration should be 1.6pM. This is lower than the NextSeq guide recommends due to short fragments in the library. Custom primers are used for read 1 and read 2. Read 1 primer mix contains 300nM each of the PhiX read 1 primer and the library read 1 primer in 2ml. Read 2 primer mix contains 300nM each of the PhiX read 2 primer and library read 2 primer in 2ml.