Single-cell mRNA Seq with Integrated Barcoding
Off chip protocol for Illumina Sequencing:

Required primers:

Upper Transposon Oligonucleotide:
5’-AGA TGT GTA TAA GAG ACA G-3’

Lower Transposon Oligonucleotide:
5’-PhosCTG TCT CTT ATA CAC ATC T-3’

Forward Library Primer:
5’-AATGATACGGCGACCACCGAGAT-3’

Reverse Library Primer:
5’-CAAGCAGAAGACGGCATACGAGAT-3’

Extended Forward Library Primer:
5’-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACC ACA GTT GAG TGC GGT ATC TAA AGC GGT GAG-3’

Optional: Extended Forward Library Primer w/ Optional plate index

- replaces Extended Forward Illumina Library Primer if multiple C1 IFC are sequenced on one lane
- **for HiSeq sequencers only**, Nextseq uses different P5 index priming strategy.
- uses standard Illumina P5 index sequencing priming, no custom P5 sequencing primer required
- <P5-index> : either Illumina P5 index sequences or any custom designed index set.
- Suggested design, not tested

AAT GAT ACG GCG ACC ACC GAG ATC TAC AC <P5index> CCG AGA TCT ACA CCA CAG TTG AGT GCG GTA TCT AAA GCG GTG AG

Extended Reverse Library Primer:
5’-CAA GCA GAA GAC GGC ATA CGA GAT GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3’

Custom Illumina read 1 sequencing primer
5’-ACA CTC TTT CCG CAA TGA AGT CGC AGG GTT G-3’

Custom read 2 sequencing primer (Optional for paired end sequencing)
5’-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3’

Custom index 1 sequencing Primer
5’-CCA CAG TTG AGT GCG GTA TCT AAA GCG GTG AG-3’

Required reagents:

Ez-Tn5 Transposase (Lucigen PN TNP92110)
Taps buffer (50 mM TAPS-NaOH, 25 mM $MgCl_2$, pH 8.5 @ RT)
Glycerol
Dimethylformamide (Sigma PN D4551)
Dynabeads MyOne Streptavidin C1 (Thermo Fisher PN 65001)
Dynabead wash buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl)
SPRIselect beads (Beckman Coulter PN B23317)
85% Ethanol

Protocol

Harvest and pool samples for library prep:

1. Combine all material from the C1 harvest (~3.5 µl/cell) in a single 1.5 ml tube. The total volume should be ~336 µl.
2. Add one volume of SPRIselect beads to the pooled harvest material. Wash with 200 µl of 85% Ethanol. Retain beads.
3. Elute bound cDNA with 20 µl water.
4. Quantify cDNA concentration. Final concentration should be at least 1.1 ng/µl.

Transposon Assembly:
Note: For sequencing on Illumina instruments, a commercial Nextera tagmentation kit can also be used. (Nextera transposons have the Tn5 mosaic end sequences on which our PCR primers prime.)

1. Combine Upper and Lower transposon oligonucleotides at a final concentration of 50 µM each in TE.
2. In a thermal cycler run the following program to anneal the oligonucleotides:
    - 3 minutes at 95°C
    - 3 minutes at 70°C
    - Cool at 2°C/minute from 70°C to 26°C
3. Dilute 5x with H_2O
4. Add one vol. Glycerol
5. Mix diluted oligonucleotides and Ez-Tn5 transposase (1 U/µl) at a 4:1 (oligo:transposase) ratio and incubate for 30 minutes at room temperature to allow transposon formation
6. Use immediately or store at -20°C.

Tagmentation:

1. Dilute 10 ng of pooled amplified cDNA in water to a final volume of 9 µl.
2. To the 9 µl diluted cDNA add:
    - 4 µl TAPS buffer (50 mM TAPS-NaOH, 25 mM $MgCl_2$, pH 8.5 @ RT)
    - 2µl dimethylformamide
    - 5µl assembled transposon
3. Mix well and incubate for 5 minutes at 55°C
4. Cool sample to 4 -10°C or put on ice.
5. Add 4 µl of 0.1% SDS and incubate at 65°C for 10 minutes
6. Cool to 4°C

Capture and Library Amplification

1. Wash 24ul of MyOne Streptavidin C1 Dynabeads 3 times with 180ul 1X Dynabead wash buffer
2. Suspended beads in 24ul 2X Dynabead wash buffer .
3. Add the tagmented cDNA (24ul).
4. Shake or rotate for 15 min at room temperature.
5. Place on magnetic stand, discard supernatant.
6. Wash beads 3x with 180ul 1X Dynabead wash buffer
7. Resuspend washed beads with bound biotinylated fragments in 10.5 µl water
8. To the resuspended beads add:
    - 12.5 µl KAPA HiFi HotStart ReadyMix (2x)
    - 1ul mix of 10uM forward Library Primer and 0.5uM Extended Forward Library Primer
    - 1ul mix of 10uM reverse Library Primer and 0.5uM Extended reverse Library Primer
9. Amplify biotinylated fragments with the following conditions:

Temp and Time	Cycles
3 minutes at 72°C	1
30 seconds at 98°C	1
10 seconds at 98°C	14
30 seconds at 55°C	
30 second at 72°C	
2 minutes at 72°C	1
Hold at 4°C	1

Size Selection (> 200 bp)

1. Add 75 µl TE to 25 µl PCR reaction for a total volume of 100 µl.
2. Add 90 µl SPRIselect beads to the diluted PCR reaction to select fragments > 200 bp. Wash with 85% EtOH. Elute bound cDNA with 10 µl water.
3. Measure library quantity and size using an Agilent Bioanalyzer.

Sequence on Illumina instruments using customer primers following the manufacturer's instructions for the use of custom sequencing primers for the desired instrument. See required reagents for the sequences of the custom sequencing primers. Instructions for the use of custom sequencing primers are in the "NextSeq® System Custom Primers Guide" (Illumina Part # 15057456) or in the "HiSeq System Custom Primers Guide" (Illumina part # 15061846).