Working Station Setup

Two working areas are needed for the following steps. PCR hoods are considered cleaner than clean stations described below. The clean station can be a bench-top in the general lab that is clean enough but far away from your PCR hoods. ***NO reagents/tubes/plates are allowed to go back and forth between your PCR hood and clean station to avoid any contamination. NO PCR amplification products are allowed in the PCR hood.***

Reagent Setup

If reagents are ordered in a large volume, make aliquots, avoid using stocks directly, and no used tips are allowed to go back into reagent tubes. If you have a large volume to pipet, it is always better to pipet multiple times with p200 than p1000 to have more accuracy.

1| RT Primer (QZ53): dissolve primers in EB buffer to a final concentration of 100μM, aliquot to 10μl/tube, and store at –20° up to 6 months. Add 90μl water to make final concentration of 10μM when use. – Enough for 100rxn.

RT Primer (QZ53): (PAGE purified, Invitrogen)
AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN (V: A/C/G, N: A/T/C/G)

2| TSO (QZ49): dissolve primers in EB buffer to a final concentration of 100μM, aliquot to 10μl/tube, and store at –80° up to 6 months. – Enough for 100rxn. ***Avoid repeated freeze-thaw cycles.***

TSO (QZ49): (RNase-free HPLC purified, Exiqon)
AAGCAGTGGTATCAACGCAGAGTACrGrG+G

3| Amp Primer 1 (QZ50): dissolve primers in EB buffer to a final concentration of 100μM, aliquot to 10μl/tube, and store at –20° up to 6 months. Add 90μl water to make final concentration of 10μM when use. – Enough for 400rxn.

Amp Primer 1 (QZ50): (PAGE purified, Invitrogen) AAGCAGTGGTATCAACGCAGAGT

4| i7 Barcoding Primers (QZ54-77): dissolve primers in EB buffer to a final concentration of 100μM, aliquot to 10μl/tube, and store at –20° up to 6 months. Add 190μl water to make final concentration of 5μM when use. – Enough for 40rxn.

i7 Barcoding Primers: (PAGE purified, Invitrogen)
CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG (X: barcodes)

Please note that ***N705 cannot be used alone on NextSeq***.

QZ54 (N701): TCGCCTTA (entry on sample sheet TAAGGCGA)

QZ55 (N702): CTAGTACG (entry on sample sheet CGTACTAG)

QZ56 (N703): TTCTGCCT (entry on sample sheet AGGCAGAA)

QZ57 (N704): GCTCAGGA (entry on sample sheet TCCTGAGC)
QZ58 (N705): AGGAGTCC (entry on sample sheet GGACTCCT)
QZ59 (N706): CATGCCTA (entry on sample sheet TAGGCATG)
QZ60 (N707): GTAGAGAG (entry on sample sheet CTCTCTAC)
QZ61 (N710): CAGCCTCG (entry on sample sheet CGAGGCTG)
QZ62 (N711): TGCCTCTT (entry on sample sheet AAGAGGCA)
QZ63 (N712): TCCTCTAC (entry on sample sheet GTAGAGGA)
QZ64 (N714): TCATGAGC (entry on sample sheet GCTCATGA)
QZ65 (N715): CCTGAGAT (entry on sample sheet ATCTCAGG)
QZ66 (N716): TAGCGAGT (entry on sample sheet ACTCGCTA)
QZ67 (N718): GTAGCTCC (entry on sample sheet GGAGCTAC)
QZ68 (N719): TACTACGC (entry on sample sheet GCGTAGTA)
QZ69 (N720): AGGCTCCG (entry on sample sheet CGGAGCCT)
QZ70 (N721): GCAGCGTA (entry on sample sheet TACGCTGC)
QZ71 (N722): CTGCGCAT (entry on sample sheet ATGCGCAG)
QZ72 (N723): GAGCGCTA (entry on sample sheet TAGCGCTC)
QZ73 (N724): CGCTCAGT (entry on sample sheet ACTGAGCG)
QZ74 (N726): GTCTTAGG (entry on sample sheet CCTAAGAC)
QZ75 (N727): ACTGATCG (entry on sample sheet CGATCAGT)
QZ76 (N728): TAGCTGCA (entry on sample sheet TGCAGCTA)
QZ77 (N729): GACGTCGA (entry on sample sheet TCGACGTC)

5| i5 Barcoding Primers (QZ78-93): dissolve primers in EB buffer to a final concentration of 100μM, aliquot to 10μl/tube, and store at –20° up to 6 months. Add 190μl water to make final concentration of 5μM when use. – Enough for 40rxn.

i5 Barcoding Primers: (PAGE purified, Invitrogen)
AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC (X: barcodes)

QZ78 (S502): CTCTCTAT (entry on sample sheet ATAGAGAG)
QZ79 (S503): TATCCTCT (entry on sample sheet AGAGGATA)
QZ80 (S505): GTAAGGAG (entry on sample sheet CTCCTTAC)
QZ81 (S506): ACTGCATA (entry on sample sheet TATGCAGT)
QZ82 (S507): AAGGAGTA (entry on sample sheet TACTCCTT)
QZ83 (S508): CTAAGCCT (entry on sample sheet AGGCTTAG)
QZ84 (S510): CGTCTAAT (entry on sample sheet ATTAGACG)

QZ85 (S511): TCTCTCCG (entry on sample sheet CGGAGAGA)
QZ86 (S513): TCGACTAG (entry on sample sheet CTAGTCGA)
QZ87 (S515): TTCTAGCT (entry on sample sheet AGCTAGAA)
QZ88 (S516): CCTAGAGT (entry on sample sheet ACTCTAGG)
QZ89 (S517): GCGTAAGA (entry on sample sheet TCTTACGC)
QZ90 (S518): CTATTAAG (entry on sample sheet CTTAATAG)
QZ91 (S520): AAGGCTAT (entry on sample sheet ATAGCCTT)
QZ92 (S521): GAGCCTTA (entry on sample sheet TAAGGCTC)
QZ93 (S522): TTATGCGA (entry on sample sheet TCGCATAA)

6| Lysis buffer: dilute Triton X-100 to 2% (vol) in water, and then dilute to 0.2% (vol) right before use.

7| Reagent ordering:

Triton X-100 (molecular grade)	Sigma T8787-50ML
Betaine solution (molecular grade)	Sigma B0300-5VL (3,750 rxn)
$MgCl_2$ solution (molecular grade)	Sigma M1028-100ML
RNase Inhibitor	Clontech 2313B (2,500 rxn)
dNTP mix	Thermo R0193 (5,000 rxn)
SuperScript II RT	Thermo 18064071 (400 rxn)
UltraPure Water	Thermo 10977-015
Hifi HotStart ReadyMix	KAPA KK2602 (500 rxn)
Buffer EB	Qiagen 19086
Agencourt AMPure XP	Beckman Coulter A63881
Hardshell PCR plate	Bio-rad HSP9601
Micro-seal A	Bio-rad MSA5001
Micro-seal B	Bio-rad MSB1001
Nextera XT DNA Library Prep kit	Illumina FC-131-1096 (96 rxn)
Tris-HCl w/ Tween-20	TEKnova T7724
Library Quantification Kit	KAPA KK4854 (1000 rxn)
Library Quantification Standards	KAPA KK4903 (120 rxn)
NextSeq 500/550 High Output v2 (75x)	Illumina FC-404-2005

Cell Sorting and Lysis

— PCR hood

1| Prepare fresh lysis buffer by adding 1μl of RNase inhibitor (40U/μl) to 19ul of 0.2% Triton X-100 solution, and aliquot 2μl/well into 96-well PCR plate for cell sorting. Seal with microseal B before and after sorting to avoid evaporation and contamination. – Enough for 10rxn.

2| Cell populations-of-interest will be sorted into eppendorf tubes containing 300μl of sorting buffer first. **Double-sort 500 cells** directly into each well of 96-well plate containing the lysis buffer, gently vortex and spin down the plate at 2,000g for 2min. Additional 500 cells will be sorted into eppendorf tubes for purity check. Any purity <99.5% should be taken notes.

3| Neg Ctrl: add nothing; Pos Ctrl: add 0.3μl of 1ng/μl total RNA.

4| Pre-mix 10μM RT Primers (QZ53) and 10mM dNTPs at 1:1 ratio (vol), and add 2μl of the mixture to each PCR well. – Total volume: 4μl.

5| Seal with microseal B, and spin down the mixture at 2,000g for 2min. ***ALWAYS spin first before you put the plate into a PCR cycler to make sure all reagents are down to the bottom.***

6| Incubate at 72° for 3min, and immediately put the plate on ice. Ideally, the next step should be processed ASAP. **PROGRAM: bprna1**

Reverse Transcription

— PCR hood

1| Prepare RT mix as below while performing denaturation. ***ALWAYS spin down all the regents down to the bottom of tubes as the concentration may be changed due to the evaporation onto the lid. Due to the low volume for pipetting, pre-mix more rxns of water and $MgCl_2$ first, and then pipet out the total volume you need for the rxns you have planned and mix with other reagents.***

Total volume (μl): 6μl	volume	final
Water	0.56	–
$MgCl_2$ (1M)	0.09	9mM
SuperScript II first-strand buffer (5x)	2	1x
DTT (100mM)	0.5	5mM
Betaine (5M)	2	1M
RNase inhibitors (40U/μl)	0.25	10U
TSO (100μM, QZ49)	0.1	1μM
SuperScript II reverse transcriptase (200U/μl)	0.5	100U

2| Spin down the incubated sample lysates at 2,000g for 2min before opening the seal. ***ALWAYS spin first before you open the seal to avoid any cross-contaminations between samples.***

3| Add 6μl of RT mix to 4μl of sample lysates to make up 10μl in total.

4| Seal with microseal A, and spin down the mixture at 2,000g for 2min.

5| Perform first-strand PCR reactions as below: ***The 10 cycles 50°/42° helps to open up some secondary structure and carry out further reverse transcription.***

PROGRAM: bprna2

1x	42°	90min
10x	50°	2min
	42°	2min
1x	70°	15min
1x	4°	hold

cDNA Amplification

— PCR hood

1| Prepare PCR amplification mix as below.

Total volume (μl): 15μl	volume	final
KAPA HiFi HotStart ReadyMix (2x)	12.5	1x
Water	2.25	–
Amp Primer 1 (10μM, QZ50)	0.25	0.1μM

2| Spin down first-strand product at 2,000g for 2min at room temperature before opening the seal. ***ALWAYS spin at room temperature if the last step has a long or high temperature incubation to avoid condensation of the seal pressure at low temperature.***

3| Add 15μl of PCR amplification mix to 10μl of first-strand product to make 25μl in total.

4| Seal with microseal A, and spin down the mixture at 2,000g for 2min.

5| Perform amplification PCR as below: **PROGRAM: bprna3**

1x	98°	3min
15x	98°	20s
	67°	30s
	72°	6min
1x	72°	5min
1x	4°	hold

PCR Purification

— Clean station ***NO PCR amplification products are allowed in PCR hood. All the following steps will be carried out in the clean station.***

1| Equilibrate Ampure XP beads at RT for at least 30min minimize the binding of pollens to beads.

2| Spin down amplified products at 2,000g for 2min at room temperature before opening the seal.

3| Add 15μl of Ampure XP beads (**0.6:1 ratio**) to amplification product, and pipet up and down ten times.

4| Incubate the mixture at room temperature for 5min.

5| Place the plate on magnetic stand for 5min, and carefully remove the clear supernatant.

6| Wash with 200μl of 80% (vol) ethanol solution twice, and pipet out the left ethanol. (ALWAYS make fresh 80% ethanol.)

7| Let the beads dry at room temperature for 5min or until some cracks are seen.

8| Add 17.5μl of EB buffer, and mix ten times to resuspend the beads.

9| Incubate the plate off the magnet for 5min.

10| Place the plate on the magnet for 2min.

11| Collect 15μl of supernatant to a new PCR plate without disturbing the beads. Store cDNA at –80° or –20°.

12| Check the fragment size >500bp with a peak at 1.5-2kb by TapeStation 5000, and quantify by Qubit.

cDNA Tagmentation

1| Thaw ATM (Amplicon Tagment Mix), TD (Tagment DNA Buffer), and cDNA on ice, and keep NT (Neutralize Tagment Buffer) at room temperature.

2| Dilute cDNA to 0.2ng/μl in water, and add 5μl to each well of 96-well plate (**1ng total input**).

3| Add 10μl TD to each well.

4| Add 5μl ATM to each well, and pipette up and down five times to mix.

5| Seal with microseal B, and spin down the mixture at 2,000g for 2min.

6| Incubate at 55° for 5min, hold at 10°, and immediately proceed to neutralization step. – Total volume: 20μl. **PROGRAM: bplib1**

7| Spin down at 2,000g for 2min at room temperature before opening the seal.

8| Add 5μl NT to the bottom of each well, and pipette up and down five times to mix.

9| Incubate at room temperature for 5min. – Total volume: 25μl.

Library Amplification

1| Thaw NPM (Nextera PCR Master Mix), and i7/i5 Index primers on ice.

2| Add 15μl NPM to each well.

3| Add 5μl i5 primers to each well.

4| Add 5μl i7 primers to each well.

5| Pipette up and down five times to mix. – Total volume: 50μl.

6| Seal with microseal A, and spin down the mixture at 2,000g for 2min.

7| Perform amplification PCR as below: **PROGRAM: bplib2**

1x	72°	3min
1x	95°	30s
12x	95°	10s
	55°	30s
	72°	30s
1x	72°	5min
1x	10°	hold

PCR Purification

1| Equilibrate Ampure XP beads at RT for at least 30min.

2| Spin down amplified product at 2,000g for 2min at room temperature before opening the seal.

3| Add 40μl of Ampure XP beads (**0.8:1 ratio**) to amplification product, and pipet up and down ten times.

4| Incubate the mixture at RT for 5min.

5| Place the plate on magnetic stand for 5min, and carefully remove the clear supernatant.

6| Wash with 200μl of 80% (vol) ethanol solution twice, and pipet out the left ethanol.

7| Let the beads dry at RT for 5min.

8| Add 30μl of EB buffer, and mix ten times to resuspend the beads.

9| Incubate the plate off the magnet for 5min.

10| Place the plate on the magnet for 2min.

11| Collect supernatant to a new PCR plate without disturbing the beads. Store libraries at –80° or –20°.

Sample Pooling and Sequencing

1| Use KAPA library quantification kit to quantify library concentrations.

A small aliquot of libraries are **diluted 10,000** times (2μl of library products into 198μl H_2O sequentially twice) before setting up the qPCR rxns.

qPCR system: 10μl in total (recommend setting up duplicates)

6μl KAPA buffer + 4μl of diluted libraries/standards/H_2O

qPCR program: **PROGRAM: Vignali Lab quant template**

1x	95°	5min
35x	95°	30s
	60°	45s
1x	65°	melting

Standards Conc.: Std1 (20pM), Std2 (2pM), Std3 (0.2pM), Std4 (0.02pM), Std5 (0.002pM), Std6 (0.0002pM). Std DNAs are 452bp, and have Tm around 86°-87°.

Calculation for library Conc.: **y** x 10,000pM = **y** x 10nM. (**y** is the mean conc. on the qPCR analyzer)

Run the qPCR products on a 1.5% gel, and the size should be 300bp-800bp with a peak at 450bp. Library Tm is around 81°-82°.

2| Dilute libraries to **2nM** in 10mM Tris-HCl w/ 0.1% Tween-20 (pH 8.5).

3| Pool 10μl of each diluted library together.

4| Submit the pooled libraries to Genomic Cores for sequencing on Illumina NextSeq platform. ***Refer to Page 1-3 to input the i5/i7 sequences (S5xx/N7xx entry on sample sheet) on the sample submission form.***

5| Around 10 libraries will be sequenced on the NextSeq 500/550 High Output v2 kit (75 cycles), so one will get about 40M reads per library.

Reference

This method is based on Smart-seq2, and can also be used for **plate-based single cell RNAseq with deep sequencing reads**.

Please cite the following reference when writing up your paper (Picelli et al., 2013).

Picelli, S., Bjorklund, A.K., Faridani, O.R., Sagasser, S., Winberg, G., and Sandberg, R. (2013). Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods *10*, 1096-1098.