InDrop Library Preparation

Materials provided by 1CellBio

- ☐ 100 µM PE2-N6 primer
- ☐ 5 µM PE1/PE2 primer mixture with indices (24 sets provided in plate)
- ☐ 100 µM Custom read 1, read2, and index primers

Materials not provided by 1CellBio

- ☐ Nuclease-free water
- ☐ Agencourt AMPure XP magnetic beads (Beckman Coulter, cat. no. A63881)
- ☐ Exonuclease I (ExoI; 20,000 U/mL; NEB, cat. no. M0293L)
- ☐ NEBNext® mRNA Second Strand Synthesis Module (NEB, cat. no. E6111S)
- ☐ HiScribe™ T7 High Yield RNA Synthesis Kit (NEB, cat. no. E2040S)
- ☐ RNA Fragmentation Reagents (Ambion/Life technologies, cat. no. AM8740)
- ☐ PrimeScript™ Reverse Transcriptase (Takara Clontec, cat. no. 2680A)
- ☐ RNaseOUT™ Recombinant Ribonuclease Inhibitor (ThermoFisher Scientific, cat. no. 10777-019)
- ☐ Deoxynucleotide (dNTP) Solution Mix (10 mM each; NEB, cat. no. N0447L)
- ☐ Kapa 2x HiFi Hot Start PCR mix (Kapa Biosystems, cat. no. KK2601)
- ☐ Eva Green Dye (20x in water; Biotium, cat. no. 31000-T)
- ☐ Qiagen MinElute Reaction Cleanup Kit (Qiagen, cat. no. 28204 or 28206)
- ☐ BioAnalyzer RNA Pico chip and reagents (Agilent, cat. no. 5067-1513)
- ☐ BioAnalyzer High Sensitivity DNA chip and reagents (Agilent, cat. no. 5067-4626)
- ☐ Low adhesions gel pipette tip (optional)
- ☐ Magnetic racks for microfuge tubes
- ☐ Centrifuge (e.g., Eppendorf AG, Cat. No. 5424R)
- ☐ Thermal cycler
- ☐ qPCR machine
- ☐ Heat block for 1.5 ml tubes
- ☐ BioAnalyzer (Agilent)

Reagent setup

Reagents should be prepared in a sterile environment using RNAse-free techniques to avoid introducing unwanted nucleic acids and nucleases. Reagents may also be sterile filtered using either syringe filters or fast flow filters with 0.2 µm pores. These reagents can be made in advance and stored long term under the conditions indicated.

RNA Elution Buffer (REB)

10mM Tris-HCl, pH 7.5
0.1mM EDTA

For 5 mL:

1M Tris-HCl, pH 7.5	50 µL
0.5M EDTA	1 µL
RNAse-free water	4940 µL
TOTAL	5 mL

Aliquot into 1.5 mL nuclease-free microfuge tubes. REB can be stored at room temperature.

DNA Suspension Buffer (DSB)

10mM Tris-HCl, pH 8.0
0.1mM EDTA

For 5 mL:

1M Tris-HCl, pH 8.0	50 µl
0.5M EDTA	1 µl
RNAse-free water	4940 µl
TOTAL	5 mL

Aliquot into 1.5 mL nuclease-free microfuge tubes. DSB can be stored at room temperature.

80% Ethanol (v/v)

Combine 8 volumes of ethanol with 2 volumes of nuclease-free water, respectively. Always measure the volumes of both the ethanol and water rather than pouring the reagents into a graduated vessel. ***This reagent should be made fresh daily***.

AMPure Clean up Protocol

***CRITICAL**: Prepare 80% ethanol fresh daily. Bring Ampure beads to room temperature before using. Always mix beads thoroughly by vortexing. Pipette volumes carefully*.

1) Measuring carefully add the appropriate amount of AMPure beads, given the reaction volume and bead ratio indicated, to a sterile 1.5 mL microfuge tube (0.2 mL PCR tubes can also be used). (i.e., to perform a 0.8X AMPure cleanup of a 100 µL reaction use 80 µL of AMPure beads.)
2) Add the sample to the tube of AMPure beads.
3) Gently but thoroughly vortex the sample and bead mixture. If beads stick to the sides of the tube quick spin the contents and vortex gently again keeping the mixture in the bottom of the tube.
4) Incubate the sample/bead mixture at room temperature for 5 min, gently vortexing occasionally.
5) Quick spin the tube and place it in a magnetic rack until the beads have pelleted against the wall of the tube and the supernatent is clear of floating magnetic beads.
6) Carefully aspirate the supernatant without removing any beads.*
7) Keeping the tube on the magnet gently wash the magnetic beads with an ample amount of 80% ethanol (~0.5 mL). It is best not to overly disturb the bead pellet but rather to let the ethanol wash over the pellet.
8) Allow loose beads to re-pellet on the magnet and remove the ethanol wash. Repeat one time.
9) Before removing the second ethanol wash quick spin the tube and place it back on the magnet allowing any loose beads to pellet.
10) Collect all remaining ethanol from the bottom and walls of the tube by using a fine pipette tip. Use care not to disturb the beads on the wall of the tube.
11) Leave the tube on the magnetic rack with the cap open for 5 minutes at room temperature. All ethanol must be allowed to dissipate but avoid over-drying the beads. **Note** – When beads are very dry, you may see cracks forming on the bead pellet.
12) Add the indicated amount of eluent to the beads. Pipette up and down to ensure beads are thoroughly resuspended. Gently vortex. Incubate at room temperature for 5 minutes gently vortexing occasionally.
13) Re-pellet the beads through a quick spin to pull-down beads stuck to side of tube. Return the tubes to the magnet and allow 1 min or more for the magnetic beads to re-pellet against the magnet. When the solution is clear carefully transfer the eluate to a clean, labeled microfuge tube.

TIP – Traces of magnetic beads will interfere with the BioAnalyzer. If you are instructed to remove an aliquot of the sample for a QC step, place the tube of eluate on the magnet and then remove the aliquot for QC to a fresh tube.

*Up to 100 µL of the supernatant removed from the first step in an AMPure clean up reaction can be retained in a clean 1.5 mL centrifuge tube for QC. After the supernatant is cleaned with a Qiagen MinElute Reaction Cleanup column it can be viewed on the BioAnalyzer as an additional QC measure. Do not attempt to run the supernatant material on a BioAnalyzer without cleaning the reaction with a Qiagen column as this will cause the BioAnalyzer assay to fail.

Protocol Summary

First Day

- Oligo Removal
- Second Strand Synthesis
- In vitro Transcription

Quantify yield using BioAnalyzer

Second Day

- Reverse Transcription
- qPCR to estimate PCR cycles
- Quantitative PCR
- Final Library Amplification

Quantify yield using BioAnalyzer

Library Preparation Procedure

RT Reaction Clean-up (30 min)

[note – perform this clean-up only if it was not performed at the end of the Emulsion/RT reaction protocol]

1) If necessary, thaw post-RT libraries on ice.

2) Centrifuge libraries for 2 minutes at full speed to pellet cell debris and to fully separate the layers in the sample.

3) Carefully remove and discard any remaining mineral oil from above the aqueous phase containing the single cell library and as much of the HFE-7500 oil layer below the library phase as possible. This process can be made easier with sterile gel loading pipette tips.

4) Following manufacturer's instructions, clean each single cell library aliquot using a Qiagen MinElute Reaction Cleanup column. Elute each sample in 12 µL of Qiagen buffer EB or in sterile water. [**Note** – If the encapsulation was less than 4 minutes add 20 µL of sterile water to the sample to bring the volume to the appropriate amount and proceed to Qiagen clean up]

Oligo Removal (1 h)

1) Set up the following reaction to remove excess oligos from the RT reaction.

Sample/library	12 µL
10X NEB ExoI buffer	2 µL
NEB ExoI	1 µL
ddH2O	5 µL
TOTAL:	20 µL

2) Incubate at 37°C, 30 minutes.

3) Add 30 µL of sterile water to sample for a total volume of 50 µL.

4) AMPure clean the sample with 0.9X ratio [45 µL AMPure beads]. Resuspend in 22 µL of sterile water [**Note** – See AMPure Cleanup Instructions].

5) Remove 1.5 µL of the AMPure clean post-Exo sample to a separate tube for BioAnalyzer analysis [**Note** – See TIP at the end of the AMPure Cleanup instructions].

Second Strand Synthesis (1.5 h)

1) On ice set up the following reaction for second strand DNA synthesis using the NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Kit.

Sample/library	20 µL
Sterile H2O	48 µL
10X SSS Reaction Buffer	8 µL
SSS Enzyme Mix	4 µL
TOTAL:	80 µL

2) Incubate at 16°C for 60 minutes. If using a thermal cycler do not use a heated lid.

3) Clean reaction with a Qiagen MinElute column following the manufacturer's protocol and using RNAse-free precautions. Elute the sample in 12 µL of sterile water. If not already in a 1.5 mL tube transfer SSS product into a clean 1.5 mL tube.

4) Remove 1.5 µL of cleaned second strand synthesis reaction to a fresh tube for analysis on the BioAnalyzer.

In Vitro-Transcription (IVT) (4.5 h)

1) Set up IVT as follows using the NEB HiScribe T7 High Yield RNA Synthesis Kit and careful RNAse free technique.

Sample/library	10.0 µL
10X T7 rxn buffer	2.2 µL
ATP	2.0 µL
CTP	2.0 µL
UTP	2.0 µL
GTP	2.0 µL
T7 RNA Pol	2.0 µL
TOTAL	22.2 µL

2) Mix gently by pipetting up and down or by 'flicking' the tube. Quick spin the tube to bring the contents to the bottom.

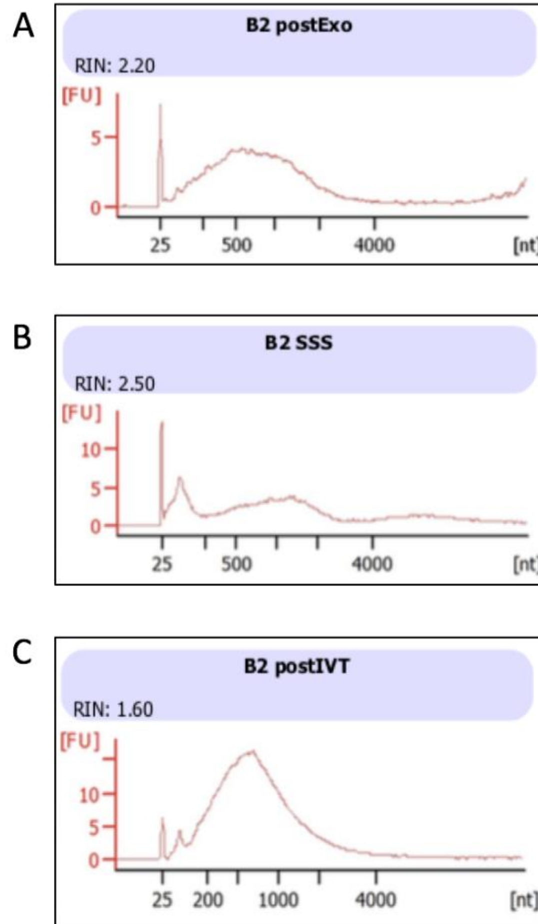
3) Incubate the IVT reaction at 37°C (lid at 50°C) for 4 h.

4) Add 28 µL of sterile water to bring the reaction volume to 50.2 µL.

5) Using RNAse-free, sterile technique, AMPure clean the IVT reaction with 0.9X ratio (45 µL AMPure beads). Resuspend in 22 µL of REB.

6) Remove 1.5 µL of the post-IVT reaction for evaluation using a BioAnalyzer RNA Pico Chip Assay.

BioAnalyzer Traces from quality checkpoints



BioAnalyzer traces from designated quality checkpoints. A) The sample post Exo1 treatment. The amount of material at this and all steps can vary depending on cells and experimental conditions. B) The products of the second strand synthesis that will serve as template for the subsequent IVT reaction. It is not uncommon to see a concentration of small products at this stage. C) The library after amplification by IVT. Small products have been largely removed by the post-IVT AMPure bead reaction clean up.

Fragmentation of Amplified RNA (20 min)

Provided the library (post IVT) has an average fragment size (<800 bp) we do not recommend fragmenting the library, and you can proceed to Reverse Transcription using Random Hexamers. If you do plan on fragmenting the Amplified RNA.

1) Pre-heat a thermal cycler block to 70°C (lid at 100-110°C).
2) On ice, add 1 µL 10X RNA Fragmentation reagent to 9 µL of purified IVT product.
3) Flick, quick spin, and immediately incubate at 70°C for about 1.5 min.
4) Cool the fragmentation reaction on ice and immediately add 1 µL of Fragmentation Stop solution. Mix well and spin very briefly (~1 s) to collect any drops from walls of the tube.
5) Add 10 µl of RNA elution buffer to the sample. Conduct a 0.8X AMPure clean up reaction (16 µl of room temperature AMPure beads). Elute the fragmented material in 10 µl of RNA Elution Buffer. Retain 1.5 µl for using a BioAnalyzer RNA Pico Chip Assay.

Reverse Transcription using Random Hexamers (2.5 h)

1) On ice, combine:

Purified Fragmented RNA	8 µL
dNTP (10 mM)	1 µL
PE2-N6 primer (100 µM)	2 µL
TOTAL	11 µL

2) Vortex and spin down. Incubate at 65°C (lid at 105°C) for exactly 3 min and cool immediately on ice.
3) On ice, add the following components to a total volume of 20 µL:

Nuclease-free water	3 µL
5x PrimerScript Buffer	4 µL
RNaseOUT (40U/µL)	2 µL
PrimerScript RT (200U/µL)	1 µL
TOTAL	9 µL

4) Mix gently and incubate the reaction mixture at 30°C for 10 min followed by 42°C for 1 h.
5) Heat inactivate the enzyme at 70°C for 15 min.
6) Add 30 µL nuclease-free water to the reaction for a total volume of 50 µL. Purify the reverse transcription product with **0.7X** (35 µL) AMPure beads as described above and re-suspend in 20 µL of DNA elution buffer.

Diagnostic qPCR (2 h)

Perform a quantitative PCR (qPCR) to determine the correct number of cycles for the next PCR amplification of sequence-ready libraries. Combine the following components on ice [**Note** - the PE1 and PE2 primers can be found in the plate of primers provided by 1CellBio]:

Sample/library	0.5 µL
Nuclease-free water	6.5 µL
2x Kapa HiFi Hot Start PCR Mix	10 µL
20x Eva Green Dye	1 µL
PE1/PE2 primer mix (5 µM)	2 µL
TOTAL	20 µL

Cycle the qPCR reaction using the following qPCR thermalcycling program:

Temperature	Duration	Cycles
98°C	2 min	
98°C	20 s	2
55°C	30 s	
72°C	40 s	
98°C	20 s	24
65°C	30 s	
72°C	40 s (with fluorescent read)	

Note – Eva Green uses the same excitation/emission settings as for SYBR Green.

Set the qPCR threshold within the exponential phase of amplification, but closer to where the signal starts to emerge from the noise to avoid the possibility of over-amplification.

Correct the resulting C_t value to account for differences in the amount of input material between the qPCR and library PCR. The library input is 19 times higher and, assuming a 100% PCR efficiency, the difference corresponds to $\log_2 19 = 4.25$ cycles. For example, if the number of cycles determined by qPCR is 2 + 12.67, the required number of PCR cycles for library is 2 + (12.67-4.25) = 2 + 8.

Library Amplification by PCR (2 h)

1) Combine the following components on ice:

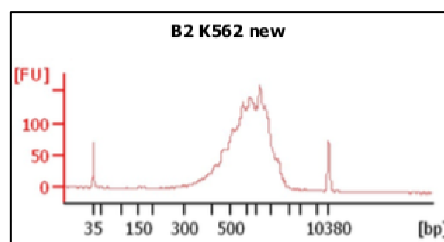
Nuclease-free water	0.5 µL
Eluate (from 2nd RT)	9.5 µL
2x Kapa HiFi Hot Start PCR Mix	12.5 µL
PE1/PE2 primer mix (5 µM)	2.5 µL
TOTAL	25 µL

2) If multiple libraries are to be multiplexed in one sequencing run, use different PE1/PE2 primer mix variants to introduce standard Illumina library indices.

***CRITICAL**: Whenever multiplexing libraries, two of the indices must be index 6 and index 12. This ensures proper signal registration during index read using Illumina sequencing platforms It is best to avoid multiplexing indices with common bases at any one position.*

3) Perform the thermal cycling protocol shown above. If working with multiple libraries that require different numbers of cycles, set the thermal cycler to perform the highest number of cycles required, and manually remove individual libraries from the thermal cycler, when they reach the desired cycle number. For example, if library X requires 8 cycles, at the end of the 72°C step of cycle 8 pause the cycler and transfer library X to an ice bucket. Then, resume the PCR program. Once all the samples have undergone the required number of PCR cycles, transfer all tubes back to the thermal cycler for the final step, a 5 min incubation at 72°C.
4) Add 25 µL of Elution buffer to the PCR product.
5) Purify using **0.7X** (35 µL) AMPure beads, as described above and elute in 20 µL of Elution buffer. The eluate is the final sequencing-ready library.
6) Test library quality by running 1 µL of the library on a BioAnalyzer DNA HS Chip. Libraries should look like a smooth hump starting at 200 bp and ending at 1.5 kb, and have an average size between 400 and 600 bp.

***PAUSE POINT**: Store the libraries at -20°C or -80°C, they should be stable for at least one year.*



Bioanalyzer trace showing a sequence-ready library made from K562 cells.

Sequencing Considerations

1) Sequence libraries on an Illumina sequencer using custom sequencing primers. Read 2, which is

the cell barcode and UMI read, should be at least 50 bp.

2) Distribute the rest of cycles between the index read, if multiplexing, and read 1, the transcript read, e.g., for NextSeq 75 cycle kit, allow 51 cycles to read the barcode, 6 cycles to read the index, and 37 cycles for the transcript.

Sequencing Barcodes

V2 Index sequences

The following sequences are written as read by the sequencer and are the sequences to reference when submitting your libraries for sequencing.

```
idx1:     ATCACG
idx2:     CGATGT
idx3:     TTAGGC
idx4:     TGACCA
idx5:     ACAGTG
idx6:     GCCAAT
idx7:     CAGATC
idx8:     ACTTGA
idx9:     GATCAG
idx10:    TAGCTT
idx11:    GGCTAC
idx12:    CTTGTA
idx13:    AGTCAA
idx14:    AGTTCC
idx15:    ATGTCA
idx16:    CCGTCC
idx17:    GTAGAG
idx18:    GTCCGC
idx19:    GTGAAA
idx20:    GTGGCC
idx21:    GTTTCG
idx22:    CGTACG
idx23:    GAGTGG
idx24:    GGTAGC
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