User Manual

Version 1.0 - UMWT3530

Evercode™ WT Mega 384 v3

For use with

ECWT3530

Legal Notices

Table of Contents

Overview

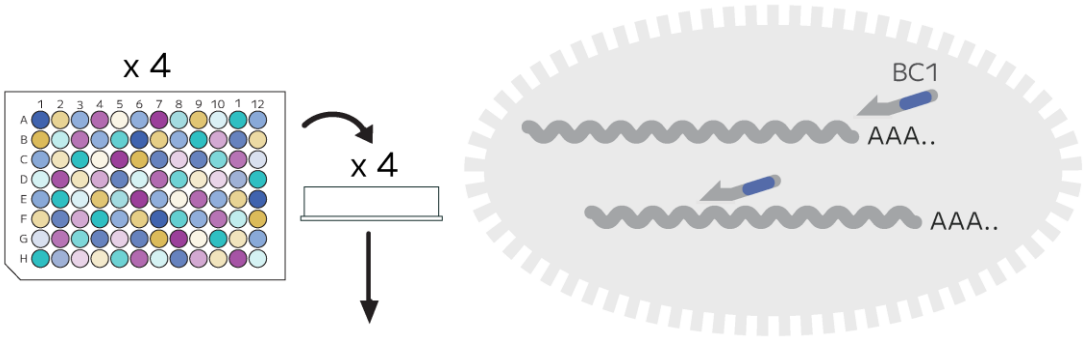
Workflow

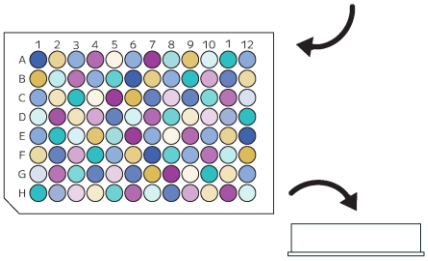
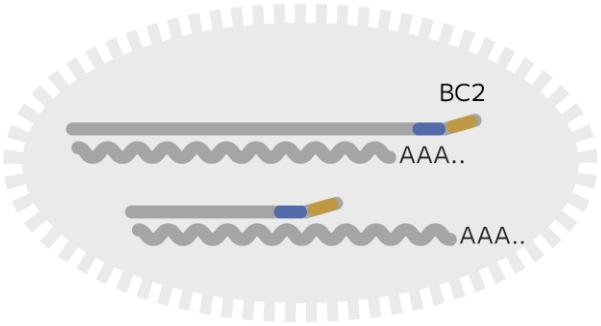
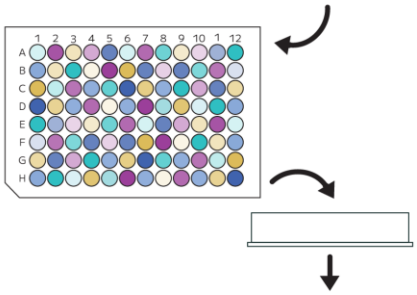
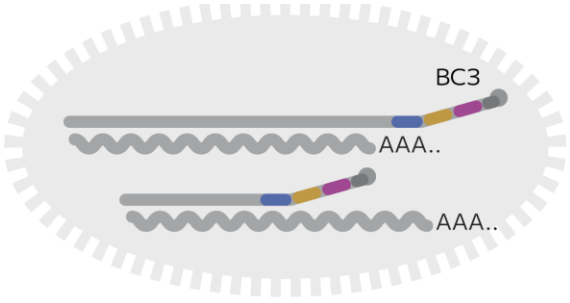
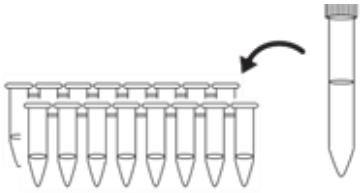
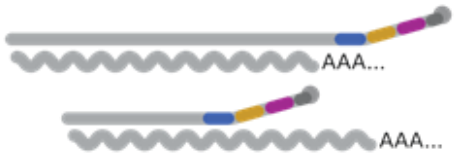
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT Mega 384 v3 kit can profile up to 1,000,000 cells/nuclei across 384 different biological samples or experimental conditions.

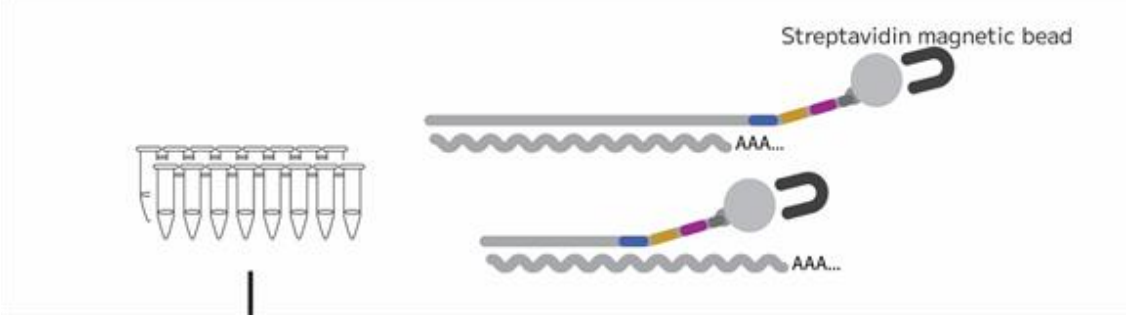
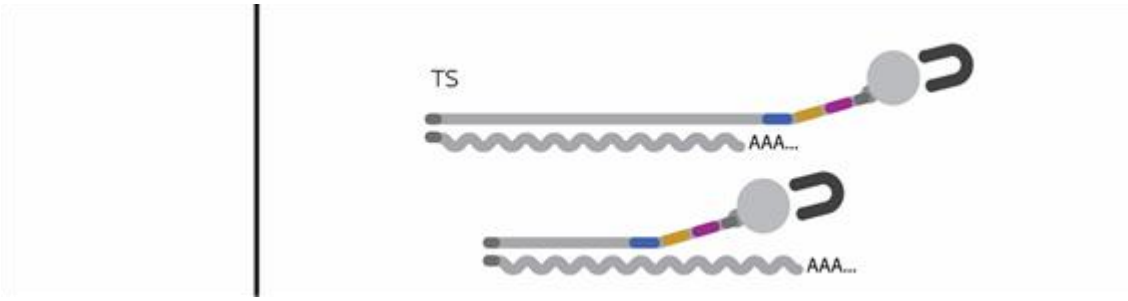
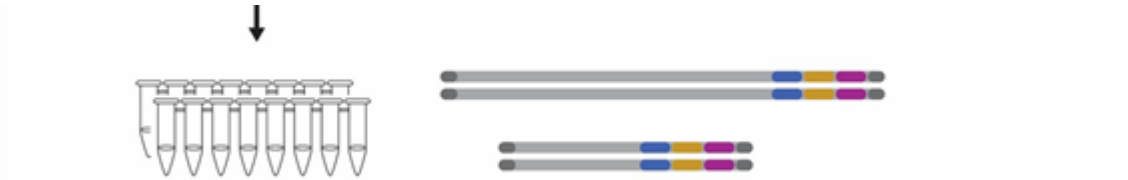
Evercode Fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding can yield a very large number of possible barcode combinations which is more than sufficient to uniquely label up to 1,000,000 cells/nuclei while avoiding doublets.

After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same 4 barcode combination to a single cell/nuclei.

The tables below provide a high-level overview of the whole transcriptome workflow.

Section 1: *In situ* Cell/Nuclei Barcoding	
1	 **Round 1 Barcoding:** Fixed and permeabilized cells/nuclei are loaded into the wells of four identical Round 1 Plates (384 wells in total). RNA is reverse transcribed via oligo dT and random hexamer primers with a well-specific barcode that are associated with specific samples.

Section 1: *In situ* Cell/Nuclei Barcoding	
2	  **Round 2 Barcoding:** The cells/nuclei are pooled and loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode.
3	  **Round 3 Barcoding:** The cells/nuclei are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina Read 2 sequence, and biotin.
4	  **Lysis and Sublibrary Generation:** Cells/nuclei are split across 16 sublibraries and lysed.

Section 2: cDNA Capture and Amplification	
1	 **cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads.
2	 **cDNA Template Switch:** A template switch reaction adds an adapter to the 3′ end of the cDNA.
3	 **cDNA Amplification:** The cDNA is amplified with primers specific to the template switch adapter and Illumina Truseq Read 2.

Section 3: Sequencing Library Preparation	
1	 **Fragmentation and End Repair:** DNA is fragmented, ends are repaired, and A-tailed.
2	 **Adapter Ligation:** An Illumina Truseq Read 1 Adapter is ligated to the 5′ end of the DNA.
3	 **Round 4 Barcoding:** The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.

Figure 1 shows the Mega 384 workflow. This kit can barcode up to 1 million cells/nuclei across 384 samples in a single reaction. The Mega 384 utilizes four identical Round 1 Plates, each holding a unique set of 96 samples, totaling 384 samples. These samples are pooled from the four Round 1 Plates into a single Round 2 Plate. The barcodes from Round 1 and Round 2 serve as sample identifiers.

Samples from Round 2 are pooled and distributed into the Round 3 plate. Cells/nuclei are then pooled and subsequently split to generate 16 sublibraries (Figure 1). Sample plates are easily identified by labeling color-coded stickers (Figure 2).

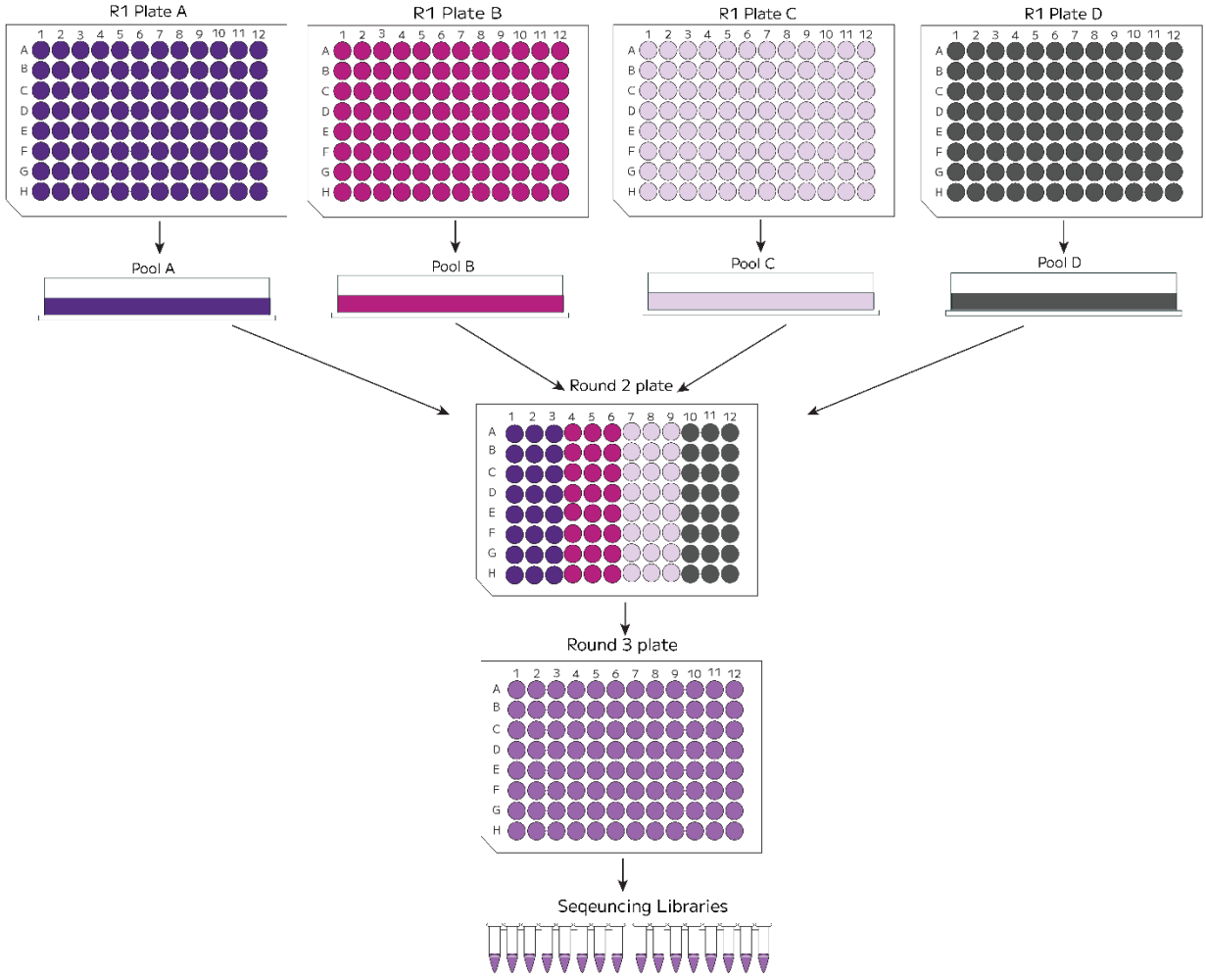


Figure 1: Evercode WT Mega 384 workflow pools 384 samples from four Round 1 Barcoding Plates into one Round 2 and Round 3 Barcoding Plates respectively, generating 16 Sequencing Libraries

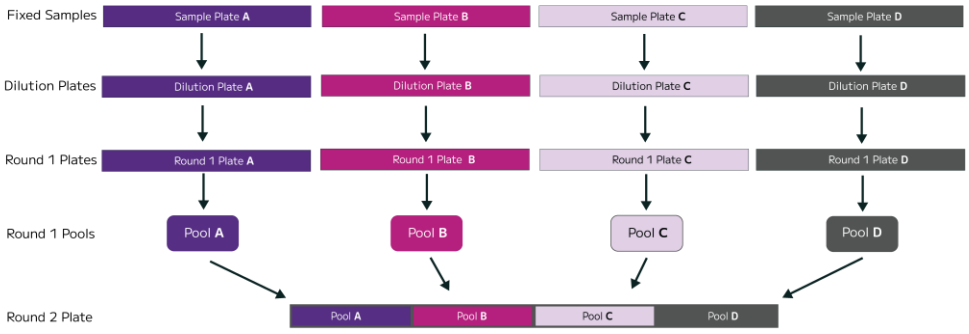


Figure 2: Plates are easy to handle with labeled color-coded stickers throughout the Round 1 Barcoding section.

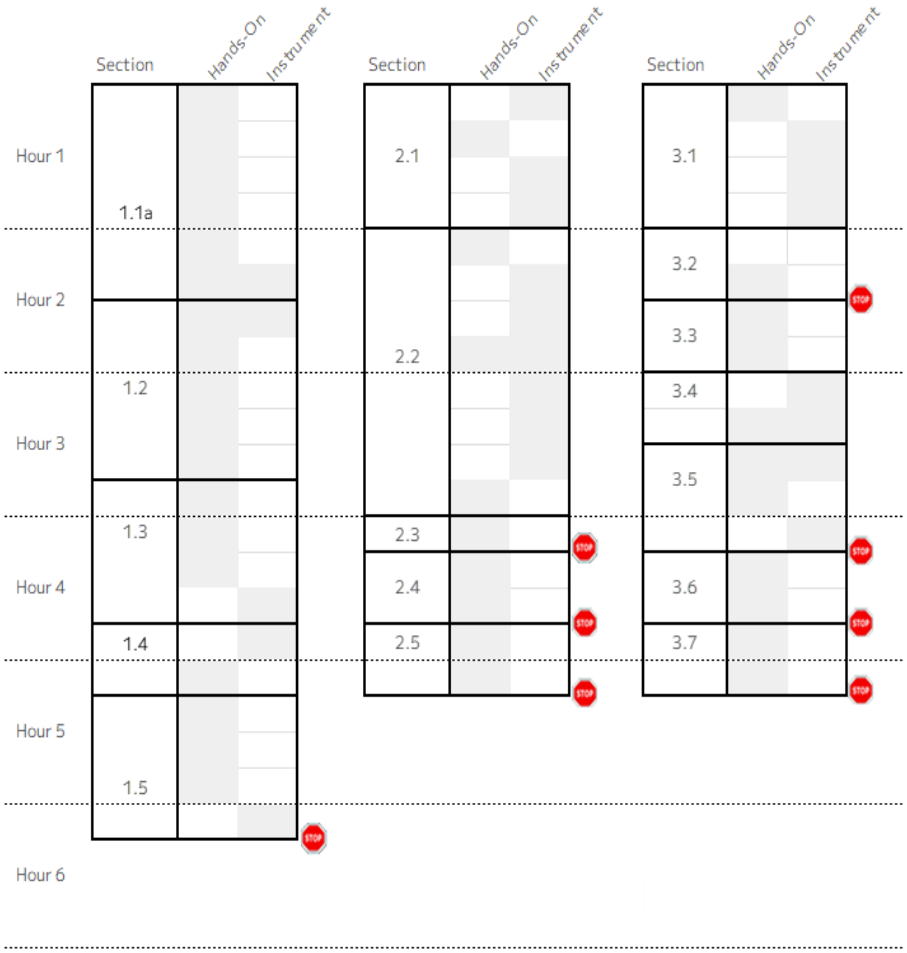
Protocol Timing

The table below provides details of the total and hands-on time required for the whole transcriptome workflow. A visual representation of the workflow timing is shown below the table. The first visual representation details the workflow for Low-Input Fixation samples. The second details the workflow for Standard Fixation samples.

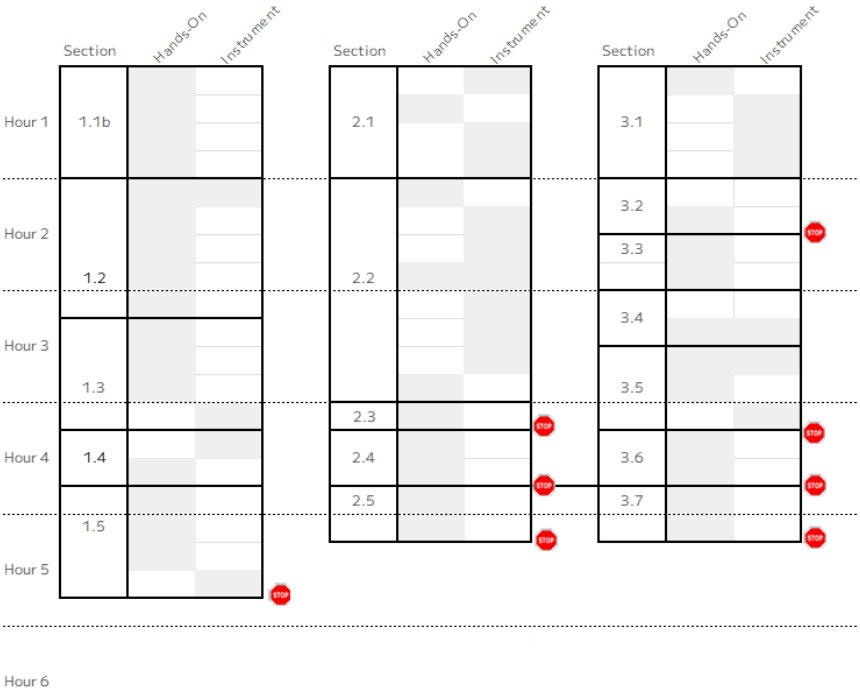
DESCRIPTION	TIME	HANDS-ON-TIME	SAFE STOPPING POINTS
Section 1: *In Situ* Cell/Nuclei Barcoding			
1.1a Set up for Low Input Fixation Samples OR 1.1b Set up for Standard Fixation Samples	60-90 min	60-90 min	
1.2 Pooling Round 1 Plates	75 min	45 min	
1.3 Barcoding Round 2	60 min	45 min	
1.4 Barcoding Round 3	30 min	15 min	
1.5 Lysis and Sublibrary Generation	60 min	45 min	-80°C ≤ 6 months
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	60 min	30 min	
2.2 cDNA Template Switch	120 min	30 min	4°C ≤ 18 hrs
2.3 cDNA Amplification	90 min	15 min	4°C ≤ 18 hrs in the thermocycler
2.4 Post-Amplification Purification	30 min	30 min	
2.5 cDNA Quantification	30 min	30 min	4°C ≤ 48 hrs or -20°C ≤ 3 months
Section 3: Sequencing Library Preparation			
3.1 Fragmentation and End Prep	60 min	30 min	
3.2 Fragmentation and End Prep Size Selection	30 min	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
3.3 Adapter Ligation	30 min	15 min	

DESCRIPTION	TIME	HANDS-ON-TIME	SAFE STOPPING POINTS
3.4 Post-Ligation Purification.	30 min	30 min	
3.5 Barcoding Round 4	45 min	15 min	4°C ≤ 18 hrs in the thermocycler
3.6 Post-Barcoding Round 4 Size Selection	30 min	30 min	
3.7 Sequencing Library Quantification	30 min	30 min	-20°C ≤ 3 months

Protocol timing when using Low Input Fixation samples



Protocol timing when using Standard Fixation samples



Important Guidelines

The following guidelines provide additional information to obtain optimal performance. For additional questions not discussed below, please contact us at support@parsebiosciences.com. We also have a library of additional resources and videos on our support site at https://support.parsebiosciences.com/.

Sample Input

- This protocol begins with cells or nuclei fixed with an Evercode Cell Fixation v3 Kit, Evercode Nuclei Fixation v3 kit, Evercode Low-Input Cell Fixation Kit or Evercode Low-Input Nuclei Fixation Kit. It is also compatible with samples fixed with Evercode Cell Fixation v2 or Evercode Nuclei Fixation v2 kits.

- For the Standard Fixation kit, aliquot the samples after fixation and count the aliquots the day before using an Evercode kit. Refer to the guidelines in the Evercode Fixation User Manuals for generating aliquots. Because aliquots have undergone a similar storage time and a freeze thaw, cell/nuclei counts from these aliquots will be more representative than using counts from immediately after fixation. Aliquots should be thawed in a thermocycler set to 37°C and counted with an automated counting device. Counts should be recorded in the Sample Loading Table, and any remaining counting aliquot should be discarded.

- For the Standard Fixation kit, after recording the concentrations of the counting aliquots in the Evercode WT Mega 384 Standard Fixation Sample Loading Table, create Sample Dilution Plates as shown on the "Dilution and R1 Plate Config." tab (see Section 1.1b for more information).

- For Low Input Fixation samples, use recommended volumes of sample input and dilution buffer from Evercode WT Mega 384 Low Input Fixation Sample Loading Table based on prefixation cell input. We do not recommend counting the samples directly post cell capture due to time constraints.

- Once fixed samples have been thawed, they should not be refrozen.

Cell/Nuclei Counting and Quality Assessment

- For the Standard Fixation kit, we recommend using a plate based automated cell counter to count cells/nuclei from the counting plates of Standard Fixation samples due to the high-throughput nature of the workflow.

- For Low Input samples, we recommend counting prefixation and optionally spot-counting post fixation.

- Fluorescent sample counting is recommended for low input samples after beads have been added for ease of counting.
- When first using Evercode Whole Transcriptome kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- Examples of AO/DAPI and trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells/nuclei with <5% cell aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed samples, it is critical to avoid counting cell debris to avoid overestimating the number of cells/nuclei.

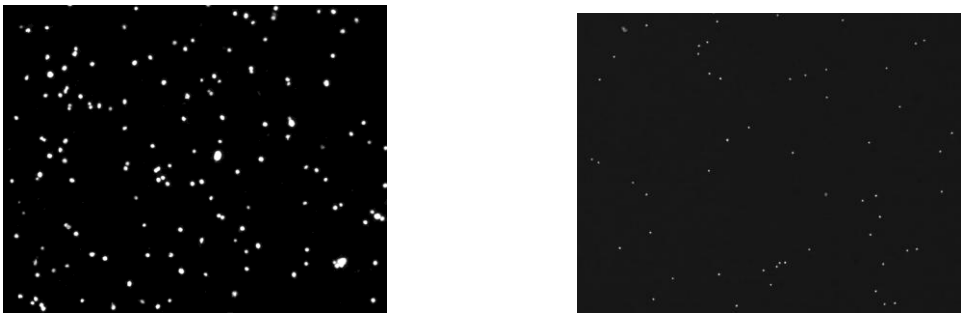


Figure 3: Low Input Fixation: example of post-bind AO/DAPI stained HEK cells (left) and PBMCs (right).

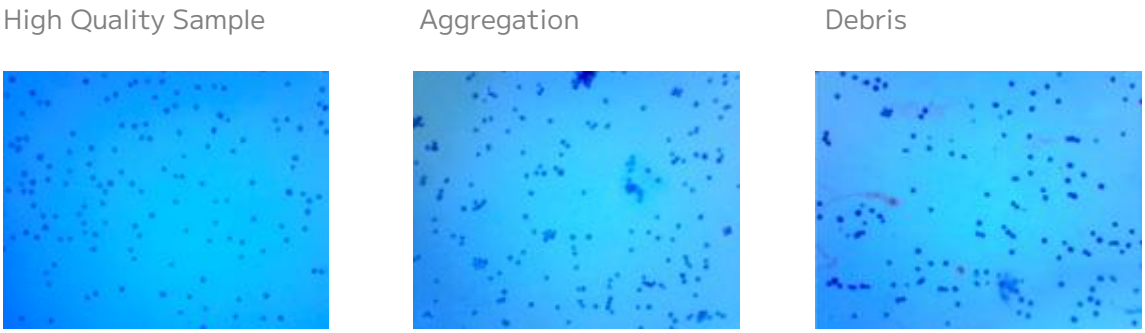


Figure 4: Standard Fixation: example of trypan blue stained fixed cells.

Avoiding RNase Contamination

- Take standard precautions to avoid introducing RNases into samples or reagents throughout the workflow. Always wear proper laboratory gloves and use aseptic technique.

- Although RNases are not inactivated by ethanol or isopropanol, they are inactivated by products such as RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific). These can be sprayed on benchtops and pipettes.

- Filtered pipette tips should be used to reduce RNase contamination from pipettes.

Centrifugation

- A range of centrifugation speeds and durations are provided in this protocol. Optimize centrifugation conditions for each sample type to balance retention and resuspension efficiencies.

- Use a swinging bucket rotor for all high-speed centrifugation steps in this protocol. A fixed-angle rotor will lead to substantial cell/nuclei loss.

Optimizing Cell/Nuclei Recovery

- It is critical to thoroughly resuspend the cells/nuclei after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to cell/nuclei adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell/nuclei loss.

- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell/nuclei pellets adequately.

- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.

- The first time using an Evercode Whole Transcriptome kit, we recommend retaining supernatants after each barcoding step. In the unlikely event of unexpectedly high sample loss, these supernatants can be analyzed to identify points for optimization.

- For Low Input Fixation samples only: if the bead/cell pellet is disturbed during the bead binding aspiration step, dispense all liquid back into the wells and wait an additional 2 minutes for the bead-bound cells to bind to the magnet again before re-aspirating.

Sample Loading Table

- The Parse Biosciences Evercode WT Mega 384 Sample Loading Table (Excel spreadsheet) should be completed before starting the experiment. Be sure to use either the Evercode WT Mega 384 Low Input Fixation Sample Loading Table or the Evercode WT Mega 384 Standard Fixation, since workflow requirements are different.

- If the table isn't working as expected, ensure that Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in the table to avoid disturbing any calculations.

- In the unlikely event that samples are not concentrated enough according to the Sample Loading Table (Excel spreadsheet), choose to either:

    - Decrease the "Max number barcoded cells" until the Sample Loading Table no longer gives an error, which will maintain the desired proportion between samples but barcode fewer cells.

    - Add 14 µL of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded cells but change the desired proportion between samples.

- In the unlikely event that samples are too highly concentrated according to the Sample Loading Table (Excel spreadsheet), choose to either:

    - Standard Fixation Samples: dilute samples upstream of adding them into the Sample Dilution Plates. This can be done using the left over Cell/Nuclei Storage Buffer from Fixation.

    - For Low Input fixation, do not input more than 100,000 cells/nuclei per fixation sample because it will negatively impact the subsequent accuracy of the Evercode WT Mega 384 Low Input Fixation Sample Loading Table.

- Evercode WT Mega 384 Low Input Fixation Sample Loading Table only: sample concentrations used to input into the Sample Loading Table should be obtained from spot counts during fixation while samples are in Prefixation Buffer. Averaged spot count can be used to estimate the number of cells per well throughout the plate. Account for up to 10% cell/nuclei loss that can happen during freeze-thaw.

Cell Strainers

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 µm is appropriate for many cell types, the mesh size should be chosen based on the sample type.

- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. An example video can be found in our support site.

Vortexing

- Unless specified in an individual step, we strongly discourage vortexing samples or enzymes throughout the protocol.

Plate Sealing

- While sealing or unsealing 96 well plates, do not splash liquid onto the PCR plate seal or between wells. Securing plates in PCR tube racks will minimize this occurrence. PCR plate seals may be difficult to remove. Carefully peel the PCR plate seal while applying downward pressure on the plate to keep it in the PCR tube rack.

Magnetic Racks and Bead Cleanups

- The Parse Biosciences Magnetic Rack uses powerful rare earth magnets for rapid and efficient magnetic bead purification for 0.2 mL tubes. The rack has high and low magnet positions important for optimal yield at key steps. We do not recommend substituting alternative racks.

- To alternate between the positions, the rack can be flipped upside down so the magnet is closer to the top (high) or bottom (low) of the 0.2 mL tubes. See the figures below for clarification.

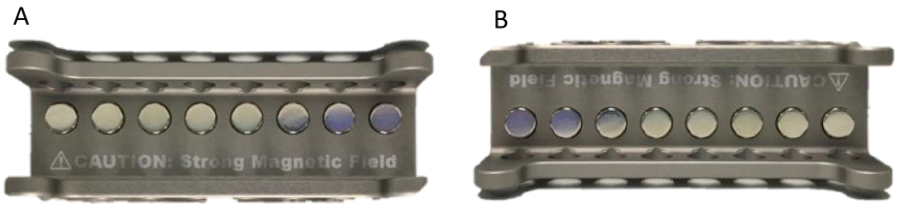


Figure 5: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.

Figure 6: Clear supernatant with compact bead pellets.

- The Low Input Fixation kit uses a plate magnetic rack. We validated the plate magnetic racks listed in the "User Supplied Equipment and Consumables". We do not recommend substituting alternative racks.

Sublibrary Loading

- The Evercode WT Mega 384 kit generates 16 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.
- The number of cells or nuclei per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix A.
- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.
- Sublibraries can be loaded with different numbers of cells, and the maximum number of cells that can be analyzed is the sum of cells/nuclei across all sublibraries.
- Asymmetric sublibrary loading can enable cost-effective sequencing quality control. One sublibrary can be loaded with a few hundred cells/nuclei and sequenced very deeply. This data can be used to choose an appropriate sequence depth for the remaining sublibraries.

Indexing Primers

- The UDI Plate - WT is a 96-well plate containing 48 unique dual indexing (UDI) primers. Each well is a single-use reaction sufficient for one Barcoding Round 4 PCR reaction for a single sublibrary. Thus, the UDI Plate - WT can be used for multiple Evercode Whole Transcriptome kits.
- The UDI Plate - WT is sealed with a pierceable foil to minimize cross-contamination. The plate seal should be wiped with 70% ethanol and pierced with a new pipette tip immediately prior to use. Avoid splashing or mixing the liquid between individual wells. Once a well has been used, it should not be resealed or reused.
- We recommend selecting UDIs column-wise from left to right (starting with indices 1-8). UDI sequences can be found in Appendix B.

Thermocycling Programs

- We recommend pre-programming the thermocycling programs required for the entire workflow. A summary of these can be found in Appendix C.

Part List

The Evercode WT Mega 384 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

WT -20°C Reagents. Store -20°C, MGT100

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	MG101	Green semi-skirted 96 well plate	4
	Round 2 Plate	MG102	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Resuspension Buffer	MG131	5 mL tube	1
	Sample Dilution Buffer	MG132	5 mL tube	4
	Round 2 Ligation Buffer	MG133	5 mL tube	1
	Round 2 Ligation Enzyme	MG107	1.5 mL tube	1
	Round 2 Stop Buffer	MG108	2 mL tube	1
	Round 3 Stop Buffer	MG109	5 mL tube	1
	Pre-Lysis Wash Buffer	MG110	5 mL tube	1
	Round 3 Ligation Enzyme	MG111	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Pre-Lysis Dilution Buffer	MG112	2 mL tube	1
	Lysis Enzyme	MG113	1.5 mL tube	1
	Bead Wash Buffer	MG114	5 mL tube	1
	Wash Buffer 1	MG115	5 mL tube	1
	Wash Buffer 2	MG116	5 mL tube	1
	Capture Enhancer	MG117	1.5 mL tube	1
	Binding Buffer	MG118	1.5 mL tube	1
	Wash Buffer 3	MG119	5 mL tube	1
	Template Switch Buffer	MG120	2 mL tube	1
	Template Switch Enzyme	MG121	1.5 mL tube	1
	Template Switch Primer	MG122	1.5 mL tube	1
	cDNA Amp Mix	MG123	1.5 mL tube	1
	cDNA Amp Primers	MG124	1.5 mL tube	1
	Fragm/End Prep Buffer	MG125	1.5.mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Fragm/End Prep Enzymes	MG126	1.5 mL tube	1
	Ligation Adapter	MG127	1.5 mL tube	1
	Adapter Ligation Buffer	MG128	1.5 mL tube	1
	Adapter Ligation Enzyme	MG129	1.5 mL tube	1
	Library Amp Mix	MG130	1.5 mL tube	1

4°C Reagents and Stickers. Store 4°C, MGT200

LABEL	ITEM	PN	FORMAT	QTY
	Spin Additive	MG204	1.5 mL tube	1
	Lysis Buffer	MG202	1.5 mL tube	1
	Streptavidin Beads	MG203	1.5 mL tube	1

4°C Reagents and Stickers. Store at room temperature, MGT200

LABEL	ITEM	PN	FORMAT	QTY
N/A	Sticker Labels	N/A	Sticker Sheet	1

To avoid confusion while handling the plates, specifically labeled color-coded stickers are provided with the kit. When working with four fixed sample plates, we recommend labeling each as Sample Plate A, Sample Plate B, Sample Plate C, and Sample Plate D respectively.

For subsequent plates, use the corresponding stickers (e.g., Dilution Plate A, Round 1 Plate A, Pool A). Below is a visual representation of the stickers.

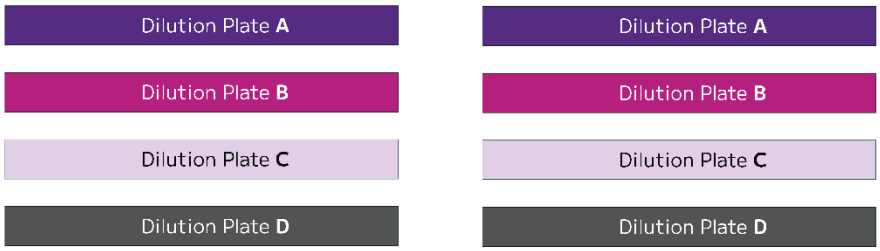
Side Stickers for Plates (Section 1.1)

Fixed Samples

1.1a, step 23 or 1.1b, step 19

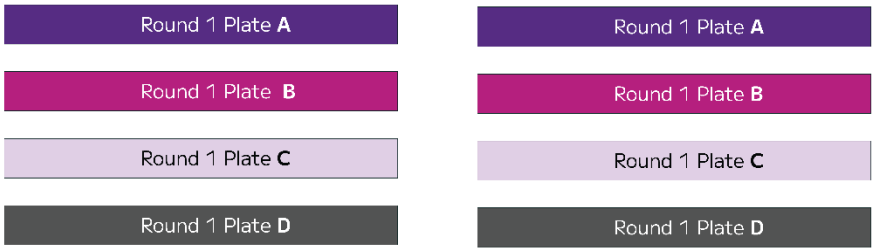
Dilution Plates

1.1b, step 6



Round 1 Plates (MG101)

1.1a, step 19 or 1.1b, step 17



Stickers for Tubes

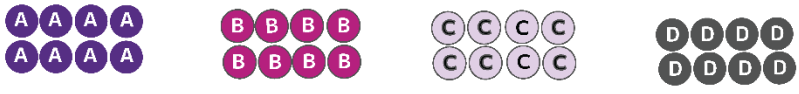
Stickers for Round 1 Pools (Section 1.2)

1.2, step 5

Stickers for Strained Pools (Section 1.3)

1.3, step 3

1.3, step 4



Top Stickers for Round 2 Plate (MG102)

1.3, step 8

User Supplied Equipment and Consumables

The following materials and equipment are required to perform the protocol but are not provided within the kit. Note that this list does not include standard laboratory equipment, such as freezers.

Equipment

ITEM	SUPPLIER	PN	NOTES
Parse Biosciences Magnetic Rack	Parse Biosciences	SB1004	We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection.
Plate Magnet	Permagen® or Alpaqua®	s500 or A000405	96-well Ring Plate Magnet or Magnum FLX®
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 15 mL centrifuge tubes and 96 well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL and 0.2 mL tubes.
Water bath	Various Suppliers	Varies	Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.
Heat Block	Various Suppliers	Varies	Or equivalent water bath, bead bath, or thermomixer capable of holding temperatures from 48°C to 68°C. Compatible with 1.5 mL, 2 mL, and 5 mL tubes.
Hemocytometer or a plate-based automated cell counter.	Various Suppliers	Varies	We recommend validating alternatives relative to a hemocytometer.
PCR tube rack	Various Suppliers	Varies	Capable of holding semi-skirted 96 well PCR plates and a tight fitting lid.
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.

ITEM	SUPPLIER	PN	NOTES
Single Channel Pipettes: P20, P200, P1000. 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.
T100 Thermal Cycler (Quantity: 2-4)	Bio-Rad Laboratories®	1861096	Or equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM.
6-inch Platform	Scientific Industries	146-6005-00	
Microplate Foam Insert	Scientific Industries	504-0235-00	
Qubit™ Flex Fluorometer	Thermo Fisher Scientific®	Q33327	Or an equivalent fluorometer.
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.
4200 TapeStation System	Agilent	G2991BA	

Consumables

ITEM	SUPPLIER	PN	NOTES
UDI Plate - WT	Parse Biosciences	UDI1001	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode kits (48 sublibraries). UDI Plate - WT can be purchased separately or bundled with Evercode kits.
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free, 10 mL or 25 mL reagent basins.

ITEM	SUPPLIER	PN	NOTES
Eppendorf twin.tec® PCR Plate 96 LoBind®	Eppendorf	0030129504	Or equivalent DNA low-binding, nuclease-free PCR plate or 0.2 mL tube strips.
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-20 TC70-SWM-40 TC70-SWM-70 TC70-SWM-100	Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm, 70 µm, 100 µm). We do not recommend FlowMi Cell Strainers (SP Bel-Art).
pluriStrainer® Mini	pluriSelect®	43-10020-40 43-10040-40 43-10070-40 43-10100-40	
Falcon®Cell Strainer	Corning®	431750 431751 431752	
EASYstrainer™, small	Greiner Bio-One™	542120 542140 542170 542100	
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial cell loss.
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.
DNA LoBind® Tubes, 2 mL, Snap Cap	Eppendorf®	022431048	Or equivalent DNA low-binding, nuclease-free 2 mL tubes.
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes with attached caps.
AMPure® XP Reagent	Beckman Coulter®	A63880 (5 mL) A63881 (60mL)	Choose one. We do not recommend substituting other magnetic beads.
SPRIselect Reagent	Beckman Coulter	B23317 (5mL) B23318 (60mL)	

ITEM	SUPPLIER	PN	NOTES
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Ethyl alcohol, Pure	Sigma-Aldrich	459844	Or equivalent 100% non-denatured ethanol.
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
Trypan Blue	Various Suppliers	Varies	Or alternative dyes to assess cell viability, such as AO/PI.
Qubit dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific	Q33230 (100 assays) Q33231 (500 assays)	Or equivalent DNA quantifier.
High Sensitivity DNA Kit	Agilent	5067-4626	Choose one that corresponds to the chosen Bioanalyzer or Tapestation.
High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	

Section 1: In Situ Cell/Nuclei Barcoding

1.1 Sample Set Up and Barcoding Round 1

Prior to starting barcoding, fixed samples are thawed and prepared.

If the Low-Input Fixation workflow was performed, set up and prepare samples following Section 1.1a and using the “Evercode WT Mega 384 Low Input Fixation Sample Loading Table”.

If the Standard Fixation workflow was performed, set up and prepare samples following Section 1.1b and using the “Evercode WT Mega 384 Standard Fixation Sample Loading Table".

1.1a Set Up for Low Input Fixation Samples

Capture and barcoding of Low-Input samples requires planning to minimize sample processing time. Prior to barcoding, cells or nuclei are thawed and captured using magnetic beads. Due to the precise nature of the bead capture, sample input into Round 1 barcoding can be calculated based on known sample input prior to fixation. This is determined by the Evercode WT Mega 384 Low Input Fixation Sample Loading Table. In the following protocol, the four plates with fixed samples will be labeled as A, B, C, and D. To minimize the processing time, we recommend capturing, loading and initiating Round 1 barcoding reactions for plates A and B first, then repeating the process for plates C and D.

To set up for barcoding:

1. Open the "Evercode WT Mega 384 Low Input Fixation Sample Loading Table" (Excel spreadsheet), which will guide sample dilutions and plate loading in later steps.
2. Cool a centrifuge with plate swinging bucket rotors to 4°C.
3. Fill a bucket with ice.
4. Set the 4 thermocyclers at the temperatures needed.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 1 Plate	-20°C Reagents	4	Place directly on ice.
● Spin Additive	4°C Reagents and Stickers	1	Keep at room temperature
○ Resuspension Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Cell/● Nuclei Binding Beads	Low Input Prefixation and Binding Reagents (4°C)	4*	Gently pulse-vortex until resuspended and store at room temperature. Do not let it settle for >3 minutes before pipetting.
○ Cell/Nuclei Binding Bead Wash Buffer	Low Input Fixation Reagents (-20°C)	4*	Thaw at room temperature then store on ice. Mix by inverting 3x.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Storage Buffer	Low Input Fixation Reagents (-20°C)	8**	Thaw at room temperature then immediately store on ice. Mix by inverting. Do not vortex.
Sticker Sheet	4°C Reagents and Stickers	1	Remove sheet and follow instructions for plate and tube labeling.

*The Low Input Fixation kit can prepare up to 96 samples. Reagents from four Low Input Fixation kits are needed to process 384 samples.

** The eight tubes come from four different Low Input Fixation kits.

Note: If processing more than 96 samples at once (up to 384), up to four 1.5 mL tubes are needed to resuspend and wash the beads.

6. Gently pulse-vortex ● Cell/● Nuclei Binding Beads until resuspended.
7. Add **1.44 mL** of ● Cell/● Nuclei Binding Beads to **four** new 1.5 mL tubes.

BINDING BEADS VOLUMES (4)		
Number of Samples	1	96
● Cell/● Nuclei Binding Beads	15 µL	1.44 mL

8. Place the four tubes on the 1.5 mL tube magnetic rack until the solution clears (~ 2 minutes).
9. With the tubes still on the magnetic rack, remove and discard the supernatant.
10. Remove the tubes from the magnetic rack and fully resuspend the bead pellet.
11. Add **1.44 mL** of ○ Cell/Nuclei Binding Bead Wash Buffer to each tube.

CELL/NUCLEI BINDING BEAD WASH BUFFER (4)		
Number of Samples	1	96
○ Cell/Nuclei Binding Bead Wash Buffer	15 µL	1.44 mL

Note: Ensure no beads are stuck to the sides of the 1.5 mL tube(s).

12. Place the tubes on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).

13. With the tubes still on the magnetic rack, remove and discard the supernatant.

14. Repeat steps 11-13 twice for a total of 3 washes.

15. Remove the tubes from the magnetic rack. Fully resuspend the pellet in the appropriate volume of O Storage Buffer following the table below.

STORAGE BUFFER (4)		
Number of Samples	1	96
O Storage Buffer	15 µL	1.44 mL

CRITICAL! Do not discard the O Storage Buffer after this step as it is used again in the following steps.

16. Using a P200 pipette set to 120 µL, aliquot **120 µL** of beads resuspended in O Storage Buffer into **four** sets of 12 PCR tubes.

CRITICAL! Ensure beads are thoroughly mixed several times before and throughout the aliquoting process.

17. Thaw the four Round 1 Plates in the thermocyclers according to the following protocol:

THAW ROUND 1 PLATE (4)		
Run Time	Lid Temperature	Sample Volume
3 min	70°C	26 µL
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

18. Gently remove the Round 1 Plates from the thermocyclers, place in PCR plate holder, and centrifuge for **1 minute** at 100 x g at 4°C.

19. Remove the Round 1 Plates from the centrifuge, store on ice, and label the four Round 1 Plates uniquely with "Round 1 - Plate [A-D]" using the stickers provided.

Note: To minimize the processing time, we recommend capturing, loading and initiating Round 1 barcoding reactions for plates A and B first, then repeating the process for plates C and D.

20. Remove **two** of the four plates of fixed cells/nuclei from -80°C storage. Set thermocycler to the following protocol and thaw cells/nuclei:

THAW CELLS/NUCLEI (2)		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	100 µL
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

21. To thaw, place the plate of frozen cells/nuclei in the thermocycler and start the program.

22. Once the thaw protocol has finished, check that all wells are fully thawed. If ice remains, place the plate back into the thermocycler for another minute at 37°C. Once fully thawed, place the plate into a PCR plate holder, and store on ice.

23. Label the plates with the corresponding adhesive labels provided in the kit, following designations as filled out in the Sample Loading Table (i.e. “Sample Plate - [A-D]”).

24. Use the table below as guidance for loading the binding beads into each sample. See the Low Input Fixation Mega 384 Sample Loading Table for a visual diagram of loading the appropriate amount of beads to each sample.

CELL/NUCLEI BINDING BEADS ADDITION	
Cell/Nuclei Input Number	Cell/Nuclei Binding beads (µL)
10,000-49,999	4
50,000-74,999	6
75,000-89,999	9
90,000-100,000	12

Note: Be sure to mix beads by pipetting thoroughly to fully resuspend before using them for cell/nuclei capture.

25. With a P200 multichannel pipette set to 120 µL, pipette 3x to ensure beads are fully suspended in cell/nuclei samples. The beads will begin binding to the cells/nuclei almost immediately.

26. Place the plate on a plate magnet and bind at room temperature for **5 minutes**.

27. With the plate still on the magnet, remove the supernatant from the wells, without disturbing the pellet with a multichannel P200 set to 200 µL.

CRITICAL! Move quickly to resuspend beads at this step to minimize time beads are out of liquid (<5 minutes).

28. Remove plate from the magnet rack and place it on ice.

29. Add **42 mL** of O Storage Buffer to a clean basin.

30. Keep plate on ice while vigorously resuspending cells/nuclei in the O Storage Buffer with a P200 multichannel pipette to ensure no pellets remain. Refer to the Sample Loading Table to determine O Storage Buffer volumes (ranges from 20-100 µL).

CRITICAL! Resuspending the beads in less than the volume required for resuspension will harm the barcoding chemistry and result in a loss of transcript detection.

31. Repeat steps 24-30 for the second sample plate ("Sample - Plate B").

32. Before loading the first and second sample plates (A and B) into the corresponding Round 1 Plate, remove the third and fourth sample plates (C and D) from the -80°C storage and thaw according to the following protocol. Once the program has completed, store plates on ice and label plates with corresponding adhesive labels.

THAW CELLS/NUCLEI (2)		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	200 µL*
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

* Or maximum volume allowed by the thermocycler.

33. Remove the plastic seal from a thawed Round 1 Plate (labeled "Round 1 - Plate A").

Note: PCR plate seals may be difficult to remove. Carefully peel the seal while applying downward pressure to keep the plate from moving to minimize cross-contamination.

34. With the Round 1 Plate on ice, add **14 µL** of “Sample Plate - A” to the appropriate wells of “Round 1 Plate - A” as defined in the Sample Loading Table. Mix immediately after dispensing each sample by pipetting 3x.

Note: Refer to the "Evercode WT Mega 384 Low Input Fixation Sample Loading Table" Plate Configuration tab for visual reference on how to load samples into Round 1 plates.

CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting cells into each well of the plate. Never place a tip that has entered one of the wells into a sample or different well.

35. While secured in a PCR plate holder on a flat surface, add a new plate seal and place back on ice.

36. Repeat steps 32-34 for "Sample Plate - B" with "Round 1 Plate - B".

37. Place both Round 1 Plates (A and B) into thermocyclers and run the program below for both plates using two separate thermocyclers. Once the program has been started, proceed immediately to the next step.

BARCODING ROUND 1 (2)			
Run Time	Lid Temperature	Sample Volume	
40 min	70°C	40 µL	
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 s	8°C	3
3	45 s	15°C	
4	45 s	20°C	
5	30 s	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	1

38. Repeat steps 22-36 to load third and fourth sample plates (C and D) with the corresponding Round 1 Plates (C and D).

39. Once the Round 1 plates A and B have finished the “Barcoding Round 1” program, place them on ice.

40. When there are **10 minutes** remaining in "Barcoding Round 1" for Round 1 Plates C and D, proceed with individually pooling Round 1 Plate A into Pool A and Round 1 Plate B into Pool B (Section 1.2).

1.1b Set Up for Standard Fixation Samples

Prior to barcoding, users are expected to count cells or nuclei using a counting plate in order to ascertain the concentration of all 384 samples. This will allow for using variable Sample Dilution Buffer volumes to dilute with a constant volume of fixed samples. This will streamline the loading process into the Round 1 plate and minimize dedicated processing time. In the following protocol, we will designate the four plates of fixed samples as A, B, C and D. Appropriate dilutions, loading concentrations, and loading positions are determined by the Evercode WT Mega 384 Standard Fixation Sample Loading Table.

Dilution plate set up:

1. Prepare a hemocytometer, flow cytometer, or other plate-based counting device.
2. Using a counting plate aliquot of fixed samples, count the number of cells/nuclei in the sample with an automated cell counter or alternative cell counting device. Record the cell/nuclei count.
3. Fill out the “Evercode WT Mega 384 Standard Fixation Sample Loading Table" (Excel spreadsheet), which will guide sample dilutions and plate loading in later steps.
4. Fill a bucket with ice and cool a centrifuge with plate swinging bucket rotors to 4°C.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
O Sample Dilution Buffer	-20°C Reagents	4	Thaw at room temperature then store on ice. Mix by inverting 3x.
Sticker Sheet	4°C Reagents and Stickers	1	Remove sheet and follow instructions for plate and tube labeling.

6. Label four (user-provided) PCR Plates uniquely with stickers “Dilution Plate - [A-D]” provided in the 4°C Reagents and Stickers box.
7. Create the corresponding dilution plates of O Sample Dilution buffer using the Evercode WT Mega 384 Standard Fixation Sample Loading Table.

Note: If there are errors in the Sample Loading Table, see the Important Guidelines section for suggestions.

Safe Stopping Point: Dilution plates can be made in advance, sealed, and frozen at -20°C for 48 hours prior to barcoding.

Barcoding plate set up:

8. Open the "Evercode WT Mega 384 Standard Fixation Sample Loading Table" (Excel spreadsheet), which will guide sample dilutions and plate loading in later steps.
9. Fill a bucket with ice.
10. Have four thermocyclers on standby.
11. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 1 Plate	-20°C Reagents	4	Place directly on ice.
Spin Additive	4°C Reagents and Stickers	1	Keep at room temperature
Resuspension Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Round 2 Ligation Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Round 2 Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Sticker Sheet	4°C Reagents and Stickers	1	Remove sheet and follow instructions for plate and tube labeling.

12. If the Dilution Plates are stored at -20°C, thaw the four pre-prepared Dilution Plates in the thermocyclers according to the following protocol. Then gently remove the Dilution Plates from the thermocyclers, place in 0.2 mL tube racks, and centrifuge for **1 minute** at 100 x g at 4°C.

THAW DILUTION PLATES (4)		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	100 µL
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

13. Place the Dilution Plates on ice, and label with corresponding adhesive labels, if not done previously.

14. Thaw the four Round 1 Plates in the thermocyclers according to the following protocol:

THAW ROUND 1 PLATES (4)		
Run Time	Lid Temperature	Sample Volume
3 min	70°C	26 µL
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

15. Gently remove the Round 1 Plates from the thermocyclers, place in 0.2 mL tube racks, and centrifuge for **1 minute** at 100 x g at 4°C.

16. Remove the Round 1 Plates from the centrifuge, and store on ice.

17. Label Round 1 Plates with a sticker "Round 1 - Plate [A-D]" sticker provided, taking care that each plate is associated with a unique letter from A-D.

Note: Each Round 1 Plate is identical until samples are loaded. Ensure traceability by using the provided adhesive labels.

18. From the -80°C storage remove the sample plate that is associated with Plate A loading in Sample Loading Table and thaw it according to the following protocol.

THAW CELLS/NUCLEI		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	100 µL
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

19. Gently remove the sample plate from the thermocycler and place on ice. Label sample plate with "Sample - Plate A" sticker provided.

Note: At this point, there will be 9 plates on ice: four Dilution Plates, four Round 1 Plates, and the Sample Plate A. Be sure to have each plate labeled for traceability using the provided sticker sheet. To avoid confusion, when not in use, Dilution Plates and Round 1 Plates can be stored in the fridge.

20. Using a multichannel P200 set to 40 µL, mix cells/nuclei 3x in the Sample Plate A, Row A. Then, using a multichannel P20 set to 14 µL, transfer **14 µL** from Sample Plate A, Row A into Dilution Plate A, Row A.

21. Mix 3x with the same pipette tips to ensure even mixing, immediately transfer **14 µL** of the diluted cells/nuclei into the corresponding wells of the Round 1 Plate A, Row A and mix again 3x. An example of the workflow is shown in the diagram below. See sample loading table plate configuration tab for sample location.

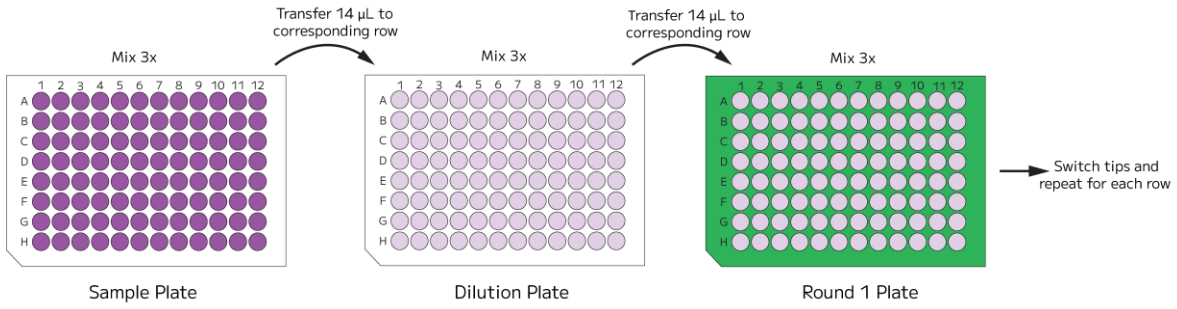


Figure 7: Samples in the sample plate are diluted and then transferred to the Round 1 Plate.

22. Repeat the process for the rest of rows B-H until all samples from sample Plate A are diluted and transferred into Round 1 Plate A.

CRITICAL! Do not reuse any tips that have entered the Round 1 Plate wells. Do not reuse tips across non-correlating rows.

23. Place the Round 1 Plate A into the thermocycler and run the program below.

BARCODING ROUND 1			
Run Time	Lid Temperature	Sample Volume	
40 min	70°C	40 µL	
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 s	8°C	3
3	45 s	15°C	
4	45 s	20°C	
5	30 s	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	1

24. Repeat steps 18-23 for Plates B, C, and D labeling the plates accordingly.

25. Once Round 1 barcoding is completed for all four Plates A, B, C, and D, proceed immediately to Section 1.2.

1.2 Pooling Round 1 Plates

After the Round 1 reverse transcription reaction, the cells/nuclei are pooled, centrifuged, and resuspended.

1. Gather the following item and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 2 Plate	-20°C Reagents	1	Place directly on ice.

2. Remove the Round 1 Plates from the thermocyclers, and store on ice.

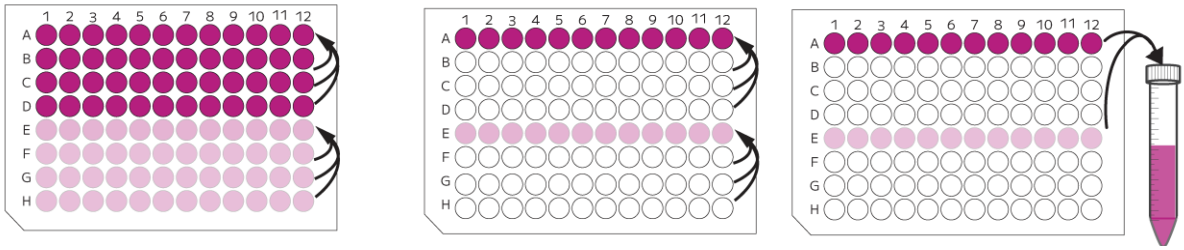
3. Place the Round 2 Plate into a thermocycler and run the following program. Proceed immediately to the next step while the program is still running.

THAW ROUND 2 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	10 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

4. While secured in PCR tube racks on a flat surface, remove the plate seal from the Round 1 Plate A.

5. Label four 15 mL centrifuge tubes with the [A-D] stickers provided.

6. With the plate and 15 mL centrifuge tube on ice, pool all wells from the Round 1 plate A into a labeled 15 mL centrifuge tube. Pipette as follows:



i. With a multichannel P200 set to 30 µL, mix the sample in row B by pipetting 3x.

ii. Transfer **30 µL** from row B to row A.

iii. Repeat i-ii for rows C-D to mix the sample then transfer to row A.

iv. Transfer any residual liquid in rows B-D to row A with a multichannel P20 set to 10 µL.

v. Ensure the cells/nuclei in row A are in suspension as described in i. Then, transfer the total volume of each well in row A into the same 15 mL tube A (Pool A) with a single channel P200 set to 200 µL.

vi. Repeat i-ii for rows F-H to mix the sample then transfer to row E.

vii. Transfer any residual liquid in rows F-H to row E with a multichannel P20 set to 10 µL.

viii. Ensure the cells/nuclei in row E are in suspension as described in i. Then, transfer the total volume of each well in row E into the same 15 mL tube A (Pool A) with a single channel P200 set to 200 µL.

7. Add **19.2 µL** of ● Spin Additive to the 15 mL tube with pooled cells/nuclei. Do not discard the Spin Additive as it will be needed throughout Section 1.

8. Mix by gently inverting the tube just once.

9. Repeat steps 1-7 with the remaining three Round 1 Plates B-D to generate four separate pools of cells/nuclei: Pool A, Pool B, Pool C, and Pool D.

10. Centrifuge the four separate pools in 15 mL tubes using a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Immediately move to the next step after centrifugation.

CRITICAL! Ideal centrifugation speed and duration should be determined empirically for each sample type to optimize retention and resuspension efficiencies. Overly aggressive centrifugation can damage cell integrity and decrease data quality. If the centrifugation speeds used during fixation gave satisfactory retention, they should be used throughout this protocol.

CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will have a significant impact on data quality.

11. While samples are spinning, make the Round 2 Ligation Master Mix in a 15 mL tube on ice as per the table below. Mix thoroughly by pipetting 10x with a P1000 set to 1000 µL. Store on ice.

ROUND 2 LIGATION MASTER MIX	
Resuspension Buffer	2.5 mL
O Round 2 Ligation Buffer	2.44 mL
● Round 2 Ligation Enzyme	20 µL
Total Volume	4.96 mL

12. Remove the supernatant from all four tubes until about ~40 µL of liquid remains above the pellet. Use a P1000 for the first 3 mL and then a P200 for the remaining volume.

CRITICAL! Do not reuse pipette tips between tubes.

CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will have a significant impact on data quality.

Note: Depending on the number of cells/nuclei and cell types, a pellet may or may not be visible.

13. Proceed immediately to Section 1.3.

1.3 Barcoding Round 2

The Ligation Master Mix is added to the separately pooled cells/nuclei, which is loaded **column-wise** into the Round 2 Plate. An *in situ* ligation reaction adds a well-specific barcode to the 3' end of the cDNA. The ligation reaction is quenched with Round 2 Stop Buffer, and the cells/nuclei are pooled and strained.

To add round 2 barcodes:

1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDING AND STORAGE
● Round 2 Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Round 3 Plate	-20°C Reagents	1	Place directly on ice.

2. Resuspend the pellets from each pool in 1200 µL of the Round 2 Ligation Master Mix to make a Ligation Cell Mix, one at a time.

CRITICAL! Do not reuse any tips between resuspension steps.

3. Strain each Ligation Cell Mix into four new labeled 1.5 mL tubes (stickers provided).

CRITICAL! To ensure that all of the liquid passes through the strainer, press the tip of the pipette against the mesh to create a tight seal and press the pipette plunger down steadily. All the liquid should pass through the strainer in ~1 second.

4. Label **four** 8-tubes PCR strips with the provided stickers. You can either label only the first tube of each strip or all 8 tubes in the strip.

5. Using a P200 single-channel, aliquot **139 µL** of the resuspended pool (from Pool A) into 8 labeled PCR tubes (strip format), mixing between each transfer step.

6. Repeat previous step for the remaining three resuspended pools corresponding to Pools B-D.

7. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.

8. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate, and apply the provided appropriate adhesive labels to assist with loading. Store on ice.

9. With the Round 2 Plate and PCR strip tubes on ice, transfer the Ligation Cell Mix to each well in the Round 2 Plate as follows. See figure 8 for an overview.

    i. Mix Pool A in the PCR strip tubes by pipetting 2x with a multichannel P200 set to 40 µL.

    ii Transfer **40 µL** of the mix to column 1 of the Round 2 Plate and mix by pipetting 2x.

    iii Repeat i-ii to mix Pool A in the tubes then transfer to columns 2-3.

    iv Repeat i-iii to add the remaining 3 samples to columns 4-6, 7-9, and 10-12, respectively.

CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting cells into each well of the plate. Never place a tip that has entered one of the wells back into the PCR strip or a different well.

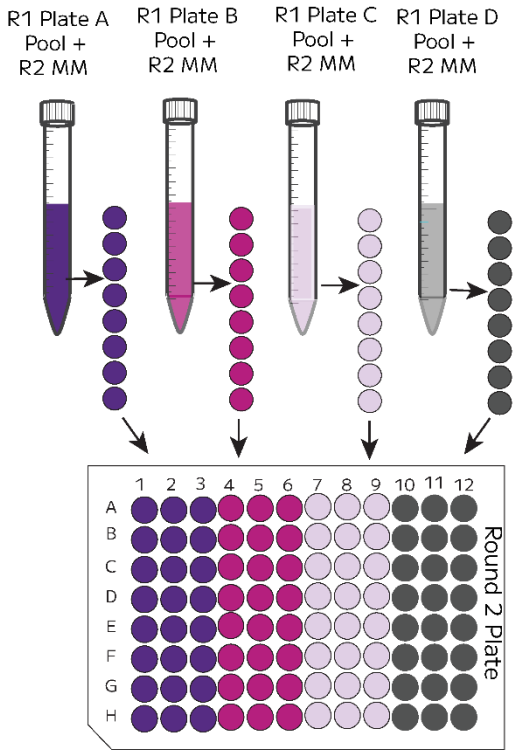


Figure 8: After resuspending the four Round 1 pools with Round 2 Ligation Master Mix, each Ligation Cell Mix is strained and aliquoted across **four** 8-tubes PCR strips respectively. The Ligation Cell Mix is loaded into three columns each of the Round 2 Plate.

Note: If when loading column 3,6, 9, and 12 (last column loaded for each pool), the volume remaining is less than 40 µL, the wells can be partially loaded without impacting experimental results.

10. While secured in a PCR tube rack on a flat surface, add a new plate seal to Round 2 Plate.

11. Place the Round 2 Plate into a thermocycler and run the following program. Upon completion, proceed immediately to the next step.

BARCODING ROUND 2		
Run Time	Lid Temperature	Sample Volume
15 min	50°C	50 µL
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

12. Briefly vortex the ● Round 2 Stop Buffer. Transfer the entire volume of this tube to a new basin with a P1000.

13. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.

14. With the Round 2 Plate on ice and the basin on the bench, transfer **10 µL** of the ● Round 2 Stop Buffer to each well in the Round 2 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.

CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting Round 2 Stop Buffer into each well of the plate. Never place a tip that has entered one of the wells back into the basin or a different well.

15. While secured in a PCR tube rack on a flat surface, add a new plate seal to Round 2 Plate.

16. Place the Round 2 Plate into a thermocycler and run the following program. Proceed to the next step while the program is still running.

ROUND 2 STOP		
Run Time	Lid Temperature	Sample Volume
5 min	50°C	60 µL
Step	Time	Temperature
1	5 min	16°C
2	Hold	4°C

17. Place the Round 3 Plate into a second thermocycler and run the program below. Proceed to the next step while the program is still running.

THAW ROUND 3 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	10 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

18. Immediately upon completion of the Round 2 Stop program, transfer the Round 2 Plate from the thermocycler to a PCR tube rack, remove the plate seal, and store on ice.

19. With the Round 2 Plate on ice and the basin on the bench, transfer all the liquid in the Round 2 Plate into a new basin as follows:

    i With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.

    ii Transfer **50 µL** from row A to the basin.

    iii Repeat i-ii for rows B-H to mix the sample then transfer to the basin.

    iv Transfer any residual liquid in the Round 2 Plate to the basin with a multichannel P20 set to 10 µL.

20. Pipette the sample through a cell strainer into a new basin with a P1000. Before each transfer, gently mix the cells in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.

Note: Bubbles may form while pooling or straining. They will not affect the quality of the experiment.

1.4 Barcoding Round 3

The Round 3 Ligation Enzyme is added to the pooled cells/nuclei, which are then loaded into the Round 3 Plate. A second *in situ* ligation reaction adds a third well-specific barcode, the Illumina Truseq Read 2 sequence, and a biotin. The sample is then pooled and strained.

To add round 3 barcodes:

1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 3 Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
Pre-Lysis Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Round 3 Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Pre-Lysis Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Lysis Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Lysis Buffer	4°C Reagents and Stickers	1	Place in a 37°C water bath until use.

2. Add **20 µL** of Round 3 Ligation Enzyme to the basin containing the strained sample. Mix by gently pipetting 20x with a P1000 set to 1000 µL.

3. Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.

4. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 3 Plate.

5. With the Round 3 Plate on ice and the basin on the bench, transfer **50 µL** from the basin to each well in the Round 3 Plate as follows:

    i Mix the sample in the basin by pipetting 2x with a multichannel P200 set to 50 µL.

    ii Transfer **50 µL** of the mix to row A of the Round 3 Plate and mix by pipetting 2x.

    iii Repeat i-ii to mix the sample in the basin then transfer to rows B-H.

CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting cells into each well of the plate. Never place a tip that has entered one of the wells back into the basin or a different well.

Note: If the volume is insufficient to transfer the last row with a multichannel, tilt the basin and transfer the remaining volume with a single channel pipette. If the volume is still insufficient to fill every well, a few can be left empty without impacting experimental results.

6. While secured in a PCR tube rack on a flat surface, add a new plate seal to Round 3 Plate.

7. Place the Round 3 Plate into a thermocycler and run the following program.

BARCODING ROUND 3		
Run Time	Lid Temperature	Sample Volume
15 min	50°C	60 µL
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

8. Briefly vortex the O Round 3 Stop Buffer and ensure there is no precipitate. Transfer the entire volume to a new basin with a P1000.

9. Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.

10. With the Round 3 Plate on ice and the basin on the bench, transfer **20 µL** of the O Round 3 Stop Buffer from the basin to each well in the Round 3 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.

CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting ORound 3 Stop Buffer into each well of the plate. Never place a tip that has entered one of the wells back into the basin or a different well.

11. Without incubation, proceed immediately to the next step.

12. With the Round 3 Plate on ice and a new basin on the bench, transfer all the liquid in the Round 3 Plate into the new basin as follows:

    i. With a multichannel P200 set to 70 µL, mix the sample in row A by pipetting 3x.

    ii. Transfer **70 µL** from row A to the basin.

    iii. Repeat i-ii for rows B-H to mix the sample then transfer to the basin.

    iv. Transfer any residual liquid in the Round 3 Plate to the basin with a multichannel P20 pipette set to 10 µL.

13. Pipette the sample through a cell strainer into a new 15 mL tube with a P1000. Before each transfer, gently mix the cells in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.

Note: Bubbles may form while pooling or straining. They will not affect the quality of the experiment.

14. Proceed immediately to Section 1.5.

1.5 Lysis and Sublibrary Generation

The cell/nuclei pool is centrifuged, washed, and resuspended in ● Pre-Lysis Dilution Buffer. The cells/nuclei are counted and divided into sublibraries based on the Sublibrary Generation Table in Appendix A. These sublibraries are lysed and stored at -80°C.

To generate and lyse sublibraries:

1. Add **70 µL** of ● Spin Additive to the 15 mL tube with the sample. Gently invert once to mix.
2. Centrifuge the 15 mL tube in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Immediately move to the next step after centrifugation.

CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

3. Remove the supernatant until about ~40 µL of liquid remains above the pellet. Use a P1000 for the first 6 mL and then a P200 for the remaining volume.

Note: Depending on the number of cells/nuclei and sample type, the pellet may or may not be visible.

4. Fully but gently resuspend the pellet in **1 mL** of ○ Pre-Lysis Wash Buffer.
5. Add an additional **3 mL** of ○ Pre-Lysis Wash Buffer for a total addition of 4 mL.
6. Centrifuge the 15 mL tube in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Immediately move to the next step after centrifugation.

CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

7. Remove the supernatant until about ~40 µL of liquid remains above the pellet. Use a P1000 for the first 3 mL and then a P200 for the remaining volume.
8. Fully but gently resuspend the pellet in **200 µL** of ● Pre-Lysis Dilution Buffer for a final total volume of 240 µL. Store on ice.

Note: Do not discard ● Pre-Lysis Dilution Buffer as it will be used in another step.

9. While minimizing time on ice, count the number of cells/nuclei in the sample with a hemocytometer or alternative cell counting device. Record the cell/nuclei count.

CRITICAL! We strongly recommend using a fluorescent automated cell counter or hemocytometer and carefully mixing the sample before collecting an aliquot for counting to ensure accurate sublibrary loading.

10. Decide how to divide cells/nuclei across the sublibraries. See the "Sublibrary Loading" in the Important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix A to determine the volume of sample and ● Pre-Lysis Dilution Buffer that should be added to each sublibrary.

CRITICAL! Do not add more than 62,500 cells/nuclei to a sublibrary. Adding additional cells/nuclei will result in an increased multiplet rate.

11. Ensure the cells/nuclei are in suspension by pipetting 5x with a P200 set to 200 µL prior to each transfer. Add the appropriate volume of sample to 16 different 0.2 mL PCR tubes.

12. Add the appropriate volume of ● Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of 25 µL.

13. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P1000 set to 425 µL. Store at room temperature.

LYSIS MASTER MIX	
● Lysis Buffer	440 µL
● Lysis Enzyme	88 µL
Total Volume	528 µL

Note: Ensure that there is no precipitate before using the ● Lysis Buffer.

CRITICAL! Do not place Lysis Master Mix on ice, as a precipitate will form.

14. Add **30 µL** of Lysis Master Mix to each 0.2 mL tube with diluted cells/nuclei. Store at room temperature.

15. Vortex the 0.2 mL tubes for **1 minute**. Briefly centrifuge.

16. Place the tubes into a thermocycler and run the following program. If continuing to Section 2 without freezing the sample, proceed to Section 2 while the program is still running.

CELL/NUCLEI LYSIS		
Run Time	Lid Temperature	Sample Volume
15 min	80°C	55 µL
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C

17. For Standard Fixation samples, freeze the lysates at -80°C or proceed to Section 2. For Low Input Fixation samples, proceed to step 18.

18. Remove samples from the thermocycler and place the tubes on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

19. While still on the magnetic rack, transfer **55 µL** of the supernatant containing lysed cells into a new 0.2 mL tubes.

20. Freeze the lysates at -80°C or proceed to Section 2.

Safe stopping point: Sublibrary lysates can be stored at -80°C for up to 6 months.

Section 2: cDNA Capture and Amplification

2.1 cDNA Capture

The barcoded cDNA is captured with streptavidin-coated magnetic beads and washed to remove cellular debris.

To capture the cDNA:

1. Fill an ice bucket.
2. For each lysate, prepare 400 µL of 85% ethanol with nuclease-free water.
3. Equilibrate 80 µL of SPRI beads per lysate to room temperature.
4. Gather the following equipment:
    i. Magnetic rack for 1.5 mL tubes
    ii. Parse Biosciences magnetic rack for 0.2 mL PCR tubes
    iii. Vortex with an adapter for 96 well plates
5. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Streptavidin Beads	4°C Reagents and Stickers	1	Keep at room temperature.
○ Bead Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x
○ Wash Buffer 1	-20°C Reagents	1	
○ Wash Buffer 2	-20°C Reagents	1	
● Capture Enhancer	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
● Binding Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x

6. Remove the desired tubes of lysate from the thermocycler (if continuing directly from Section 1) or from storage at -80°C.

7. If previously frozen, incubate the tube(s) in water bath or thermocycler at 37°C for **5 minutes**.

Note: Ensure there is no precipitation before proceeding. Otherwise, an additional 5 minutes at 37°C should be sufficient.

8. Briefly centrifuge and store at room temperature.

9. Briefly centrifuge ● Capture Enhancer and gently mix by pipetting 2x with a P20 set to 15 µL.

10. Add **2.5 µL** of ● Capture Enhancer to each tube of lysate.

11. Place the tube(s) into a 96 well PCR tube rack, press to secure, and ensure the caps are secured tightly. Place the lid on the rack.

12. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex the plastic holder on 100% power for **1 minute**.

13. Remove the tube(s) from the vortex mixer. Briefly centrifuge.

14. Incubate for **10 minutes** at room temperature. Proceed immediately to the next step during the incubation.

Note: This incubation can be extended by 5 additional minutes (up to a total of 15 minutes) without negatively impacting performance.

15. Vortex ● Streptavidin Beads until fully mixed. Add the appropriate volume of ● Streptavidin Beads to a new 1.5 mL tube as follows, depending on the number of lysates being processed:

Lysates being processed	1	16
● Streptavidin Beads	44 µL	704 µL

16. Place the tube on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).

17. Remove and discard the supernatant.

18. Remove the tube from the magnetic rack and fully resuspend the bead pellet in the appropriate volume of ○ Bead Wash Buffer as follows:

Lysates being processed	1	16
○ Bead Wash Buffer	50 µL	800 µL

Note: Ensure no beads are stuck to the sides of the 1.5 mL tube.

19. Place the tube on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).

20. Remove and discard the supernatant.

21. Repeat steps 18-20 twice for a total of 3 washes.

22. Remove the tube from the magnetic rack. Fully resuspend the pellet in the appropriate volume of ● Binding Buffer as follows and store at room temperature.

Lysates being processed	1	16
● Binding Buffer	55 µL	880 µL

23. Add **50 µL** of ● Streptavidin Beads in ● Binding Buffer to each tube of lysate.

24. Place the tube(s) into a 96 well PCR tube rack, press to secure, and ensure the caps are secured tightly. Place the lid on the rack.

25. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex plastic holder on 100% power for **1 minute**.

26. Vortex on 20% power (~800-1000 RPM) for **30 minutes** at room temperature.

Note: To ensure the beads are being mixed sufficiently, check that the beads are in solution 10 minutes into the incubation. If settled, increase the vortex mixing speed to keep the beads in solution.

27. Remove the tube(s) from the vortex mixer.

28. Briefly vortex the tube(s) on a standard vortex adapter. Briefly centrifuge without letting beads collect at the bottom of the tube(s).

29. Place the tube(s) on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

CRITICAL! Ensure the supernatant is completely clear before proceeding. Discarding any beads in the supernatant will result in a reduction of transcripts and genes detected per cell. See "Magnetic Racks and Bead Cleanups" in the Important Guidelines section for representative images.

30. While still on the magnetic rack, remove and discard the supernatant.

31. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** O Wash Buffer 1.

32. Incubate for **1 minute** at room temperature.

33. Return the tube(s) to the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).

34. While still on the magnetic rack, remove and discard the supernatant.

35. Repeat steps 31-34 once for a total of 2 washes with O Wash Buffer 1.

36. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** O Wash Buffer 2.

Note: Save O Wash Buffer 2 to use for optional storage before cDNA amplification.

37. Incubate for **1 minute** at room temperature.

38. Proceed immediately to Section 2.2.

2.2 cDNA Template Switch

After an additional wash, the Template Switch Master Mix is added to the captured cDNA. The template switch reaction adds a 5′ adapter to the cDNA.

To perform template switch:

1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Wash Buffer 3	-20°C Reagents	1	Thaw and store at room temperature. Mix by inverting 3x.
● Template Switch Buffer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
● Template Switch Primer	-20°C Reagents	1	
● Template Switch Enzyme	-20°C Reagents	1	Keep on ice. Briefly centrifuge before use.

Note: Ensure that there is no precipitate in the ● Template Switch Buffer before proceeding.

2. Prepare the Template Switch Master Mix in a new 2 mL tube as follows, depending on the number of sublibraries being processed. Mix by pipetting 10x and store on ice.

TEMPLATE SWITCH MASTER MIX		
Number of Samples	1	16
● Template Switch Buffer	101.75 µL	1628 µL
● Template Switch Primer	2.75 µL	44 µL
● Template Switch Enzyme	5.5 µL	88 µL
Total	110 µL	1760 µL

3. Place each tube of captured cDNA from Section 2.1 on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).

4. While still on the magnetic rack, remove and discard the supernatant.

5. While still on the magnetic rack, add **125 µL** of Wash Buffer 3 to each tube.

CRITICAL! Do not discard the Wash Buffer 3 as it will be used in another step.

6. Incubate for **1 minute** at room temperature.
7. While still on the magnetic rack, remove and discard the Wash Buffer 3.
8. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 µL** of the Template Switch Master Mix.

Note: Because the Template Switch Master Mix is viscous, it may take time to fully resuspend the beads.

9. Briefly centrifuge without letting beads collect at the bottom of the tube(s).
10. Incubate for **30 minutes** at room temperature.
11. Fully resuspend each bead pellet by mixing 5x with a P200 set to 75 µL.
12. Place the tube(s) into a thermocycler and run the following program.

TEMPLATE SWITCH		
Run Time	Lid Temperature	Sample Volume
60 min	70°C	100 µL
Step	Time	Temperature
1	60 min	42°C
2	Hold	4°C

13. Proceed immediately to Section 2.3. Alternatively, proceed to step 14 to store samples prior to cDNA amplification.
14. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).

Note: Beads may need to be resuspended if they have settled.

15. While still on the magnetic rack, remove and discard the supernatant.

16. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ○ Wash Buffer 2.

Safe stopping point: Template switched cDNA can be stored at 4°C for up to 18 hours. Do not freeze.

2.3. cDNA Amplification

The captured cDNA is washed and amplified with Template Switch Primer and Illumina Truseq Read 2 specific primers.

To amplify the cDNA:

1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● cDNA Amp Mix	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
● cDNA Amp Primers	-20°C Reagents	1	

2. Prepare the cDNA Amplification Master Mix in a new 2 mL tube as follows. Mix by pipetting 10x and store on ice.

cDNA AMPLIFICATION MASTER MIX		
Number of Sublibraries	1	16
● cDNA Amp Mix	60.5 µL	968 µL
● cDNA Amp Primers	60.5 µL	968 µL
Total	121 µL	1936 µL

3. Place each tube of template switched cDNA from Section 2.2 on the high position of the Parse Biosciences Magnetic Rack. Incubate until the solution clears (~2 minutes).

Note: You may need to pipette mix to resuspend settled beads so they separate appropriately.

4. While still on the magnetic rack, remove and discard the supernatant.
5. While still on the magnetic rack, add **125 µL** of O Wash Buffer 3 to each tube.
6. Incubate for **1 minute** at room temperature.
7. While still on the magnetic rack, remove and discard the O Wash Buffer 3.
8. Remove tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 µL** of the Amplification Master Mix. Store on ice.
9. Determine the number of PCR cycles required for cDNA amplification based on the table below. Although these recommendations are appropriate for many cell types, the number of cycles may need to be optimized for each sample type.

NUMBER OF PCR CYCLES			
Cells/Nuclei in Sublibrary	High RNA content such as cell lines	Low RNA content such as PBMCs	Nuclei
200-999	11	13	12
1,000-1,999	9	11	10
2,000-5,999	7	9	8
6,000-12,499	6	8	7
12,500-24,999	4	6	5
25,000-62,500	3	5	4

10. Place the tube(s) into a thermocycler and run the following program.

cDNA AMPLIFICATION			
Run Time	Lid Temperature	Sample Volume	
50-70 min	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 sec	98°C	5
3*	45 sec	65°C*	
4	3 min	72°C	
5	20 sec	98°C	Variable, see above
6*	20 sec	67°C*	
7	3 min	72°C	
8	5 min	72°C	1
9	Hold	4°C	1

CRITICAL! If processing sublibraries with different numbers of cells/nuclei, they should be amplified in separate thermocyclers according to the recommendations above.

Note: Annealing steps 3* and 6* have different time and temperature settings. Ensure these are correct before starting the program

Safe stopping point: Amplified cDNA can be stored at 4°C for up to 18 hours.

2.4 Post-Amplification Purification

Amplified cDNA is purified with a 0.8x SPRI bead cleanup.

To purify the cDNA:

1. For each tube of amplified cDNA, gather 400 µL of freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (80 µL per tube of amplified cDNA).

Note: Ensure the SPRI beads have been equilibrated to room temperature for at least 30 minutes.

3. Place each tube of amplified cDNA from Section 2.3 on the high position of the Parse Biosciences Magnetic Rack. Incubate until the solution clears (~2 minutes).

Note: If beads remain in solution after 2-3 minutes, pipette 3x in the bottom of the PCR tube with a P200 set to 40 µL. Then return to the magnet and incubate until the solution clears.

4. While still on the magnetic rack, transfer **90 µL** of the supernatant containing the cDNA into a new 0.2 mL tube(s). Store at room temperature.
5. Vortex the SPRI beads until fully mixed. Add **72 µL** of SPRI beads to each tube with amplified cDNA.
6. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
7. Incubate for **5 minutes** at room temperature.
8. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
9. While still on the magnetic rack, remove and discard the supernatant.
10. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
11. Incubate for **1 minute** at room temperature.
12. While still on the magnetic rack, remove and discard the supernatant.
13. Repeat steps 10-12 once for a total of 2 washes. Remove any residual ethanol with a P20.

14. While still on the magnetic rack, air dry the SPRI beads (~2 minutes).

CRITICAL! Do not over-dry the beads, which can lead to a substantial loss in yield. "Cracking" of the beads is a sign of over-drying.

15. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **25 µL** of nuclease-free water.

16. Incubate for **10 minutes** at 37°C in a thermocycler.

17. Place the tube(s) on the low magnet position of the magnetic rack, so the magnet is closer to the bottom of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

Note: See 'Magnetic Racks and Bead Cleanups' in the Important Guidelines section for an image of the low position.

18. While still on the magnetic rack, transfer **25 µL** of the supernatant containing the purified cDNA into new 0.2 mL tube(s). Store on ice.

Safe stopping point: Amplified cDNA can be stored at 4°C for up to 48 hours or at -20°C for up to 3 months.

2.5 cDNA Quantification

The concentration and size distribution of the cDNA are measured with fluorescent dyes and capillary electrophoresis. The cDNA is then stored at 4°C for up to 48 hours or at -20°C for up to 3 months.

To quantify the cDNA:

1. Measure the concentration of each tube of purified cDNA from Section 2.4 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions. Record the concentration(s), which will be used in Section 3.

2. Assess the size distribution of each tube of purified cDNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D5000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

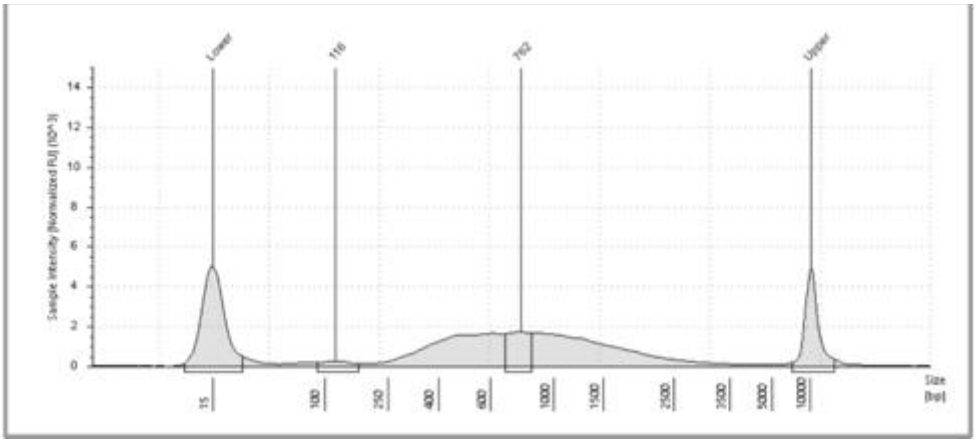


Figure 9: Expected post-amplification sublibrary cDNA size distribution. Example trace of cDNA run on a TapeStation.

Note: Samples may need to be diluted to be within the manufacturer's recommended concentration range. Typically, between a 1:3 to 1:10 dilution is appropriate.

Note: The traces above are representative of typical Bioanalyzer and TapeStation cDNA traces. The shape and prominence of the trace is dependent on cell type, sublibrary size, and amount of DNA loaded into the Bioanalyzer or TapeStation. Sublibraries with minor deviations can still produce high quality data.

Section 3: Sequencing Library Preparation

3.1 Fragmentation and End Prep

Barcoded and amplified cDNA is fragmented, end repaired and A-tailed in a single reaction.

To prepare for fragmentation and end prep:

1. For each sublibrary, prepare 1.2 mL of 85% ethanol with nuclease-free water.
2. Equilibrate 180 µL of SPRI beads per sublibrary to room temperature.
3. Fill an ice bucket.
4. Take out the Parse Biosciences magnetic rack for 0.2 mL PCR tubes.
5. Obtain recorded cDNA concentrations from Section 2.5.
6. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Fragm/End Prep Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice.
Fragm/End Prep Enzymes	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

7. Vortex the tube(s) of cDNA for **5 seconds**. Briefly centrifuge.
8. Prepare Diluted cDNA in new 0.2 mL tube(s) as follows to a final volume of 35 µL and store on ice. Store any remaining purified cDNA at -20°C.

DILUTED cDNA	
Purified cDNA	10 µL
Nuclease-free water	25 µL
Total Volume	35 µL

9. Start the following program to ensure the thermocycler is cool prior to use.

FRAGMENTATION AND END PREP		
Run Time	Lid Temperature	Sample Volume
40 min	70°C	50 µL
Step	Time	Temperature
1	Hold*	4°C
2	10 min	32°C
3	30 min	65°C
4	Hold	4°C

Note: * This hold ensures that the thermocycler is cooled and ready for step 13.

10. Vortex the ● Fragm/End Prep Buffer for **5 seconds**. Briefly centrifuge.

Note: Confirm the ● Fragm/End Prep Buffer is fully thawed without precipitation.

11. Prepare the Fragmentation and End Prep Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 10x and store on ice.

FRAGMENTATION AND END PREP MASTER MIX		
Number of Sublibraries	1	16
● Fragm/End Prep Buffer	5.5 µL	88 µL
●Fragm/End Prep Enzymes	11 µL	176 µL
Total	16.5 µL	264 µL

12. Add **15 µL** of Fragmentation and End Prep Master Mix to each tube of Diluted cDNA. Mix by pipetting 10x with a P200 multichannel pipette set to 40 µL. Briefly centrifuge.

13. Place the tube(s) into the cooled thermocycler. Skip the 4°C hold in Step 1 so the thermocycler proceeds to Step 2 of the Fragmentation and End Prep program.

Note: Ensure the thermocycler has cooled to 4°C prior to adding the tubes.

14. As soon as the program reaches step 4 of the thermocycling program (4°C), store the tube(s) on ice and proceed immediately to Section 3.2.

3.2. Post-Fragmentation and End Prep Size Selection

The fragmented and end prepped DNA are size selected with a double sided SPRI cleanup.

To size select the fragmented and end prepped DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~50 µL per sublibrary).

Note: Ensure the SPRI beads have been equilibrated to room temperature for at least 30 minutes.

3. Vortex the SPRI beads until fully mixed. Add **30 µL** of SPRI beads to each tube of fragmented and end prepped DNA.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, transfer **75 µL** of the supernatant containing the fragmented and end prepped DNA into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **10 µL** of SPRI beads to each tube.
9. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.

11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~3 minutes).

CRITICAL! Ensure the solution is completely clear before proceeding.

12. While still on the magnetic rack, remove and discard the supernatant.
13. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
14. Incubate for **1 minute** at room temperature.
15. While still on the magnetic rack, remove and discard the supernatant.
16. Repeat steps 13-15 once for a total of 2 washes. Remove any residual ethanol with a P20.
17. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).

CRITICAL! Do not over-dry the beads, which can lead to substantial loss in yield. "Cracking" of the beads is a sign of over-drying.

18. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **50 µL** of nuclease-free water.
19. Incubate for **5 minutes** at room temperature.
20. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
21. While still on the magnetic rack, transfer **50 µL** of the supernatant into new 0.2 mL tube(s).

STOP Safe stopping point: The size-selected fragmented and end prepped DNA can be stored at 4°C for up to 18 hours or at -20°C for up to 2 weeks.

3.3 Adapter Ligation

Adapters with an Illumina Truseq Read 1 sequence are ligated to the 5' end of the fragmented and end prepped DNA.

To ligate adapters:

1. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Ligation Adapter	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
Adapter Ligation Buffer	-20°C Reagents	1	
Adapter Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Library Amp Mix	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
UDI Plate - WT	-20°C Reagents	1 sealed well per sublibrary	Thaw at room temperature then place on ice.

2. Prepare the Adapter Ligation Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 10x and store on ice.

ADAPTER LIGATION MASTER MIX		
Number of Sublibraries	1	16
Nuclease-free water	19.25 µL	308 µL
Adapter Ligation Buffer	22 µL	352 µL
Adapter Ligation Enzyme	11 µL	176 µL
Ligation Adapter	2.75 µL	44 µL
Total	55 µL	880 µL

3. Add **50 µL** of Adapter Ligation Master Mix to each tube of purified fragmented and end prepped DNA from Section 3.2. Mix by pipetting 10x with a P200 multichannel pipette set to 80 µL. Briefly centrifuge.

4. Place the tube(s) into a thermocycler and run the program below.

ADAPTER LIGATION		
Run Time	Lid Temperature	Sample Volume
15 min	30°C*	100 µL
Step	Time	Temperature
1	15 min	20°C
2	Hold	4°C

Note: * If the thermocycler's lid can't reach the recommended temperature of 30°C, turn the lid heating off.

5. As soon as the program reaches 4°C, store the adapter ligated DNA on ice and proceed immediately to Section 3.4.

3.4 Post-Ligation Purification

Adapter ligated DNA is purified with a 0.8x SPRI bead cleanup.

To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~90 µL per sublibrary)

Note: Ensure the SPRI beads have been equilibrated to room temperature for at least 30 minutes.

3. Vortex the SPRI beads until fully mixed. Add **80 µL** of SPRI beads to each tube of adapter ligated DNA from Section 3.3.
4. Vortex the tube(s) for **5 seconds.** Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~2 minutes).
13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **23 µL** of nuclease-free water.
14. Incubate for **5 minutes** at room temperature.
15. Place the tube(s) on the low magnet position of the magnetic rack, so the magnet is closer to the bottom of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

16. While still on the magnetic rack, transfer exactly **21 µL** of the supernatant containing the purified adapter ligated DNA into new 0.2 mL tube(s). Store on ice.

17. Proceed immediately to Section 3.5.

3.5 Barcoding Round 4

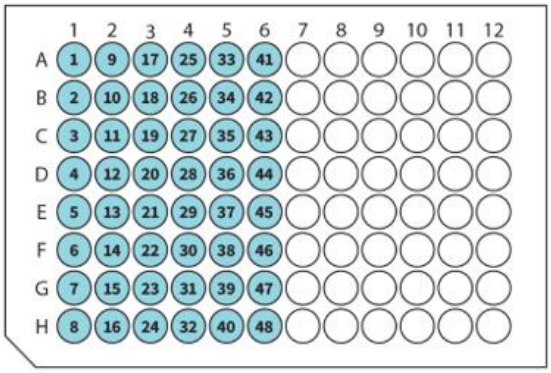
Purified adapter ligated DNA is PCR amplified with Illumina Truseq Read 1 and Read 2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.

To add round 4 barcodes:

1. Centrifuge the UDI Plate - WT at 100 x g for **1 minute**.

2. Wipe the surface of the plate with 70% ethanol and allow it to dry.

3. Orient the UDI Plate - WT with the notch on the bottom left as pictured below. For each sublibrary being processed, choose one unused well of the UDI Plate - WT and record the well position and number for each sublibrary.



4. With a multichannel P20, pierce the seal of the chosen wells of the UDI Plate - WT.

5. With a multichannel P20 and new tips, mix by pipetting 5x then immediately transfer **4 µL** from a chosen unused well of the UDI Plate - WT to its corresponding tube of purified adapter ligated DNA from Section 3.4.

CRITICAL! Only transfer primers from 1 well of the UDI Plate - WT to 1 tube of adapter ligated DNA.

6. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C. Do not reuse wells.

7. Add **25 µL** of ● Library Amp Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 25 µL. Briefly centrifuge.

8. Determine the number of PCR cycles required for the Indexing PCR based on the amount of cDNA added to the fragmentation and end prep reaction as recorded in Section 2.5.

NUMBER OF PCR CYCLES	
cDNA Input (ng)	PCR Cycles
10-24	13
25-49	12
50-99	11
100-299	10
300-999	8
1,000 or more	7

9. Place the tube(s) into a thermocycler and run the following program.

INDEXING PCR			
Run Time	Lid Temperature	Sample Volume	
~30 min	105°C	50 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	Varies, see table above
3	20 s	67°C	
4	1 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

CRITICAL! If processing sublibraries with different cDNA input, they should be amplified in separate thermocyclers according to the recommendations above.

Safe stopping point: Prior to size selection, sequencing libraries can be stored at 4°C for up to 18 hours.

3.6 Post-Barcoding Round 4 Size Selection

The sequencing libraries are size selected with a double sided SPRI cleanup.

To size select the sequencing libraries:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~50 µL per sublibrary).

Note: Ensure the SPRI beads have been equilibrated to room temperature for at least 30 minutes.

3. Vortex the SPRI beads until fully mixed. Add **30 µL** of SPRI beads to each sequencing library tube.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, transfer **75 µL** of the supernatant containing the DNA into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **10 µL** of SPRI beads to each tube.
9. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~3 minutes).

CRITICAL! This may take longer due to the low volume of beads. Ensure the solution is completely clear before proceeding.

12. While still on the magnetic rack, remove and discard the supernatant.
13. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
14. Incubate for **1 minute** at room temperature.

15. While still on the magnetic rack, remove and discard the supernatant.

16. Repeat steps 13-15 once for a total of 2 washes. Remove any residual ethanol with a P20.

17. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).

18. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **20 µL** of nuclease-free water.

19. Incubate for **5 minutes** at room temperature.

20. Place the tube(s) on the low position of the magnetic rack. Incubate until the solution clears (~2 minutes).

21. While still on the magnetic rack, transfer the supernatant into new 0.2 mL tube(s). Store on ice.

Safe stopping point: Sequencing libraries can be stored at -20°C for up to 3 months.

3.7 Sequencing Library Quantification

The concentration and size distribution of the sequencing libraries are measured with fluorescent dyes and capillary electrophoresis.

To quantify the sequencing libraries:

1. Measure the concentration of each purified sequencing library from Section 3.6 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

2. Assess the size distribution of each purified sequencing library with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D1000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

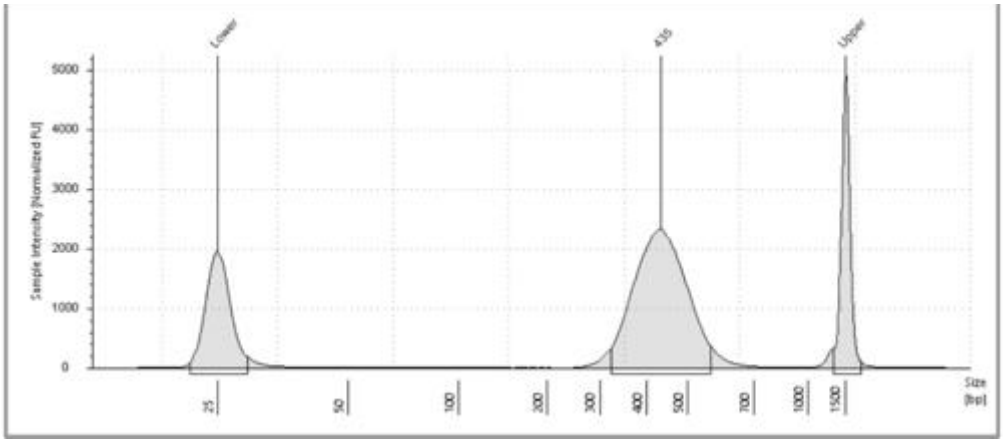


Figure 10: Expected Size Distribution before Illumina Sequencing. Example trace of DNA from indexed sublibraries run on a TapeStation.

Note: Samples may need to be diluted to be within the manufacturer's recommended concentration range. Typically, between a 1:3 to 1:10 dilution is appropriate.

Note: The traces above are representative of typical Bioanalyzer and TapeStation of DNA from indexed sublibraries. There should be a peak between 400-500 bp. The prominence of the trace is dependent on amount of DNA loaded into the Bioanalyzer or TapeStation. Sublibraries with minor deviations can still produce high quality data.

Note: There may be an additional peak present on the Bioanalyzer. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 400-500 bp). Do not use this additional peak when estimating amplicon size.

Appendices

Appendix A: Sublibrary Generation Table

The Sublibrary Generation Table provides guidance for sample dilution in Section 1.5 to achieve a target sublibrary concentration.

Green text (top): Volume of cell suspension in µL (from step 1.5.10) to add to each sublibrary.

Purple text (bottom): Volume of Dilution Buffer in µL to add to each sublibrary.

Blue Shading: Serial dilution of cell stock is required to improve sublibrary cell count.

Red Shading: Insufficient cell stock concentration for target sublibrary cell count.

Stock con (cells/uL)	Target Sublibrary Cell Count (cells/sublibrary)													
	200	500	1,000	2,000	5,000	10,000	12,000	12,500	15,000	20,000	25,000	30,000	31,250	62,500
50	4 21	10 15	20 5											
100	2 23	5 20	10 15	20 5										
200		2.5 22.5	5 20	10 15	25 0									
400			2.5 22.5	5 20	12.5 12.5	25 0								
600				3.33 21.67	8.33 16.67	16.67 8.33	20 5	20.83 4.17	25 0					
800				2.5 22.5	6.25 18.75	12.5 12.5	15 10	15.63 9.37	18.75 6.25	25 0				
1,000				2 23	5 20	10 15	12 13	12.5 12.5	15 10	20 5	25 0			
1,200					4.17 20.83	8.33 16.67	10 15	10.42 14.58	12.5 12.5	16.67 8.33	20.83 4.17	25 0		
1,400					3.57 21.43	7.14 17.86	8.57 16.43	8.93 16.07	10.71 14.29	14.29 10.71	17.86 7.14	21.43 3.57	22.32 2.68	
1,600					3.13 21.87	6.25 18.75	7.5 17.5	7.81 17.19	9.38 15.63	12.5 12.5	15.63 9.38	18.75 6.25	19.53 5.47	
1,800					2.78 22.22	5.56 19.44	6.67 18.33	6.94 18.06	8.33 16.67	11.11 13.89	13.89 11.11	16.67 8.33	17.36 7.64	
2,000					2.5 22.5	5 20	6 19	6.25 18.75	7.5 17.5	10 15	12.5 12.5	15 10	15.63 9.38	
2,500					2 23	4 21	4.8 20.2	5 20	6 19	8 17	10 15	12 13	12.5 12.5	25 0
3,000						3.33 21.67	4 21	4.17 20.83	5 20	6.67 18.33	8.33 16.67	10 15	10.42 14.58	20.83 4.17
3,500						2.86 22.14	3.43 21.57	3.57 21.43	4.29 20.71	5.71 19.29	7.14 17.86	8.57 16.43	8.93 16.07	17.86 7.14
4,000						2.5 22.5	3 22	3.13 21.88	3.75 21.25	5 20	6.25 18.75	7.5 17.5	7.81 17.19	15.63 9.38
4,500						2.22 22.78	2.67 22.33	2.78 22.22	3.33 21.67	4.44 20.56	5.56 19.44	6.67 18.33	6.94 18.06	13.89 11.11
5,000						2 23	2.4 22.6	2.5 22.5	3 22	4 21	5 20	6 19	6.25 18.75	12.5 12.5
5,500							2.18 22.82	2.27 22.73	2.73 22.27	3.64 21.36	4.55 20.45	5.45 19.55	5.68 19.32	11.36 13.64
6,000							2 23	2.08 22.92	2.5 22.5	3.33 21.67	4.17 20.83	5 20	5.21 19.79	10.42 14.58
7,000									2.14 22.86	2.86 22.14	3.57 21.43	4.29 20.71	4.46 20.54	8.93 16.07
8,000										2.5 22.5	3.13 21.88	3.75 21.25	3.91 21.09	7.81 17.19
9,000										2.22 22.78	2.78 22.22	3.33 21.67	3.47 21.53	6.94 18.06
10,000										2 23	2.5 22.5	3 22	3.13 21.88	6.25 18.75

Appendix B: Sequencing Information

We recommend a minimum sequencing depth of 20,000 reads per cell for whole transcriptome libraries. However, ideal sequencing depth is dependent on the sample type and experimental goals. For example, if trying to capture rare cell types in a heterogenous population, deeper sequencing is required. Alternatively, analyzing a homogenous population, more shallow sequencing may be appropriate.

Whole transcriptome sequencing libraries should be diluted and denatured according to the manufacturer's instructions for the relevant sequencing instrument. If using the Illumina platform, we strongly recommend adding 5% PhiX for optimal sequencing quality.

Details of the final whole transcriptome sequencing library structure are below.

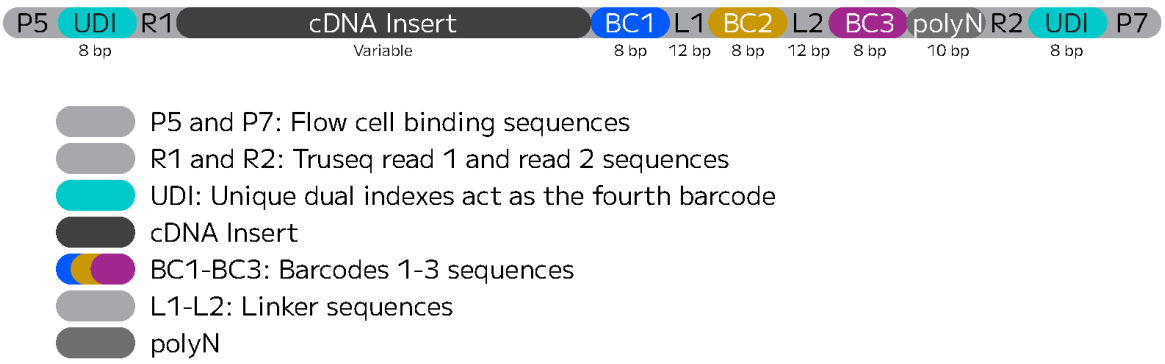


Figure 11: Whole transcriptome sequencing library structure.

Libraries should be sequenced with paired reads using the read structure in the table below. Read lengths longer than those recommended below are acceptable, but additional bases in read 2 are trimmed by the Parse Analysis Pipeline.

READ	FUNCTION	INSERT
Read 1	cDNA Insert	64
i7 Index (Index 1)	Barcode 4 / UDI	8
i5 Index (Index 2)	Barcode 4 / UDI	8
Read 2	Barcodes 1-3	58

The fourth barcode that tags each sublibrary act as a standard Illumina i7 and i5 indexes. Please refer to the following table to demultiplex the whole transcriptome libraries.

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_1	A1	CAGATCAC	ATGTGAAG	CTTCACAT
UDI_Plate_WT_2	B1	ACTGATAG	GTCCAACC	GGTTGGAC
UDI_Plate_WT_3	C1	GATCAGTC	AGAGTCAA	TTGACTCT
UDI_Plate_WT_4	D1	CTTGTAAT	AGTTGGCT	AGCCAACT
UDI_Plate_WT_5	E1	AGTCAAGA	ATAAGGCG	CGCCTTAT
UDI_Plate_WT_6	F1	CCGTCCTA	CCGTACAG	CTGTACGG
UDI_Plate_WT_7	G1	GTAGAGTA	CATTCATG	CATGAATG
UDI_Plate_WT_8	H1	GTCCGCCT	AGATACGG	CCGTATCT
UDI_Plate_WT_9	A2	GTGAAACT	TACAGACT	AGTCTGTA
UDI_Plate_WT_10	B2	TCATTCCT	AATGCCTG	CAGGCATT
UDI_Plate_WT_11	C2	GGTAGCAT	TGCTTGCC	GGCAAGCA
UDI_Plate_WT_12	D2	ACTTGATC	TTTGGGTG	CACCCAAA
UDI_Plate_WT_13	E2	ATGAGCAT	GAATCTGA	TCAGATTC
UDI_Plate_WT_14	F2	GCGCTATC	CGACTGGA	TCCAGTCG
UDI_Plate_WT_15	G2	TGACCAGT	ACATTGGC	GCCAATGT
UDI_Plate_WT_16	H2	TATAATCA	ACCACTGT	ACAGTGGT
UDI_Plate_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
UDI_Plate_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
UDI_Plate_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
UDI_Plate_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
UDI_Plate_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
UDI_Plate_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
UDI_Plate_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
UDI_Plate_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
UDI_Plate_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
UDI_Plate_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
UDI_Plate_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT
UDI_Plate_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
UDI_Plate_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
UDI_Plate_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA
UDI_Plate_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_35	C5	TTCCATTG	CTGGCATA	TATGCCAG
UDI_Plate_WT_36	D5	TAACGCTG	CTGAGCCA	TGGCTCAG
UDI_Plate_WT_37	E5	TTGGTATG	CTCAATGA	TCATTGAG
UDI_Plate_WT_38	F5	TGAACTGG	CGCATACA	TGTATGCG
UDI_Plate_WT_39	G5	TCCAGTCG	CCGAAGTA	TACTTCGG
UDI_Plate_WT_40	H5	TGTATGCG	CCAGTTCA	TGAACTGG
UDI_Plate_WT_41	A6	TGGCTCAG	CAGCGTTA	TAACGCTG
UDI_Plate_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
UDI_Plate_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix C: Thermocycling Programs

Section 1: *In Situ* cells/nuclei Barcoding

THAW ROUND 1 PLATE		
Run Time	Lid Temperature	Sample Volume
3 min	70°C	26 µL
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

THAW CELLS/NUCLEI		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	100 µL
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

THAW DILUTION PLATES		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	100 µL
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

BARCODING ROUND 1			
Run Time	Lid Temperature	Sample Volume	
40 min	70°C	40 µL	
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 s	8°C	3
3	45 s	15°C	
4	45 s	20°C	
5	30 s	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	1

THAW ROUND 2 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	10 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

BARCODING ROUND 2		
Run Time	Lid Temperature	Sample Volume
15 min	50°C	50 µL
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

ROUND 2 STOP		
Run Time	Lid Temperature	Sample Volume
5 min	50°C	60 µL
Step	Time	Temperature
1	5 min	16°C
2	Hold	4°C

THAW ROUND 3 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	10 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

BARCODING ROUND 3		
Run Time	Lid Temperature	Sample Volume
15 min	50°C	60 µL
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

CELL/NUCLEI LYSIS		
Run Time	Lid Temperature	Sample Volume
15 min	80°C	55 µL
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C

Section 2: cDNA Capture and Amplification

TEMPLATE SWITCH		
Run Time	Lid Temperature	Sample Volume
60 min	70°C	100 µL
Step	Time	Temperature
1	60 min	42°C
2	Hold	4°C

cDNA AMPLIFICATION			
Run Time	Lid Temperature	Sample Volume	
50-70 min	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	5
3	45 s	65°C	
4	3 min	72°C	
5	20 s	98°C	Variable, see section 2.3
6	20 s	67°C	
7	3 min	72°C	
8	5 min	72°C	1
9	Hold	4°C	1

Section 3: Sequencing Library Preparation

FRAGMENTATION AND END PREP		
Run Time	Lid Temperature	Sample Volume
40 min	70°C	50 µL
Step	Time	Temperature
1	Hold	4°C
2	10 min	32°C
3	30 min	65°C
4	Hold	4°C

ADAPTER LIGATION		
Run Time	Lid Temperature	Sample Volume
15 min	30°C	100 µL
Step	Time	Temperature
1	15 min	20°C
2	Hold	4°C

INDEXING PCR			
Run Time	Lid Temperature	Sample Volume	
~30 min	105°C	50 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	Variable, see table in Section 3.5
3	20 s	67°C	
4	1 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Appendix D: Revision History

Version	Description	Date
1.0	Initial Release	Jan 2025

PARSE BIOSCIENCES

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