User Manual

Version 1.0 – UMWT3730

Evercode™ WT Penta 384 v3

For use with

ECWT3730

Legal Notices

Table of Contents

Overview

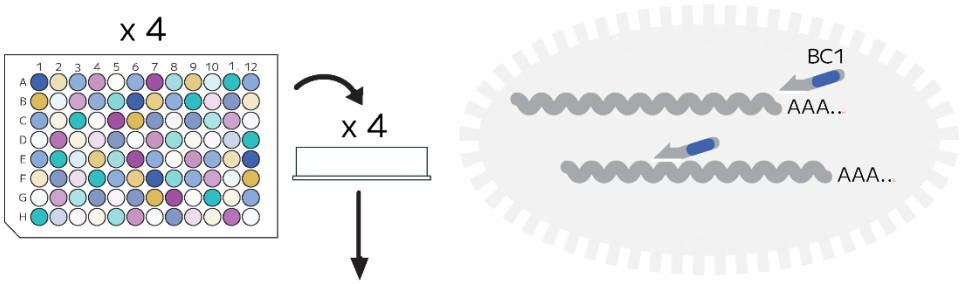
Workflow

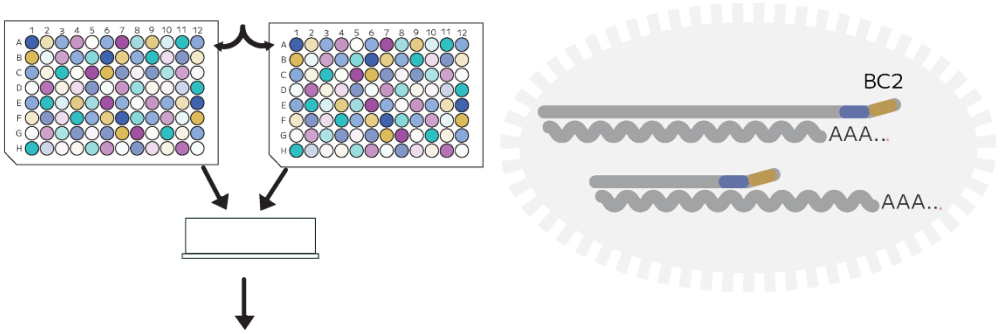
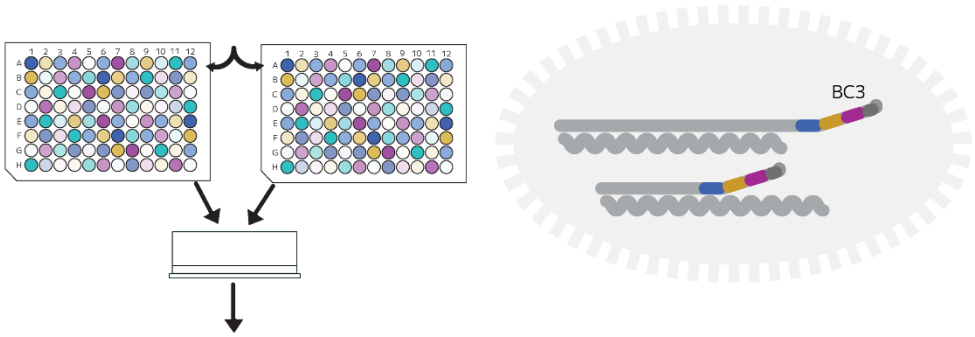
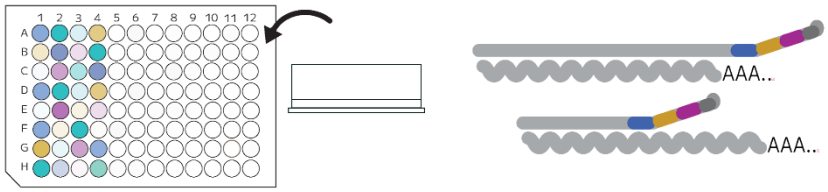
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT Penta 384 v3 kit can profile up to 5,000,000 cells/nuclei across 384 different biological samples or experimental conditions.

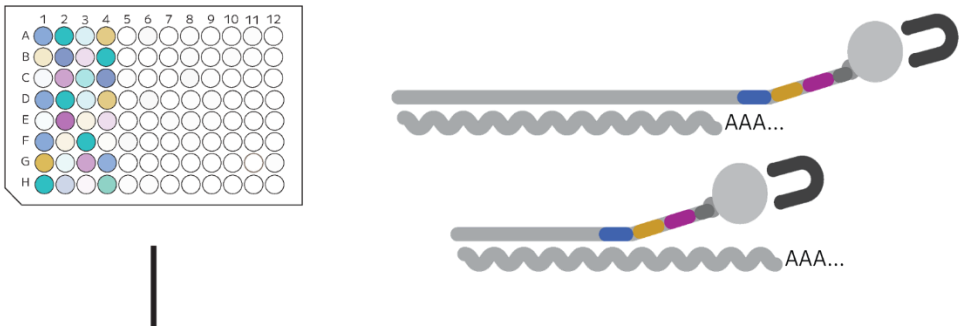
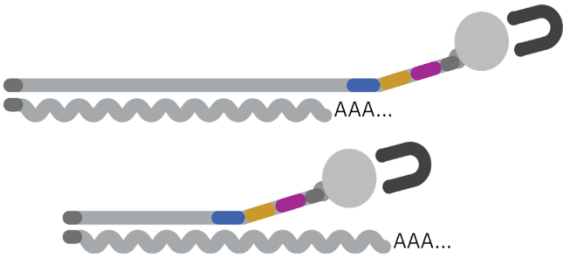
Evercode Fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding can yield a very large number of possible barcode combinations which is more than sufficient to uniquely label up to 5,000,000 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 two Round 2 Plates. 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 two Round 3 Plates. 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 32 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.

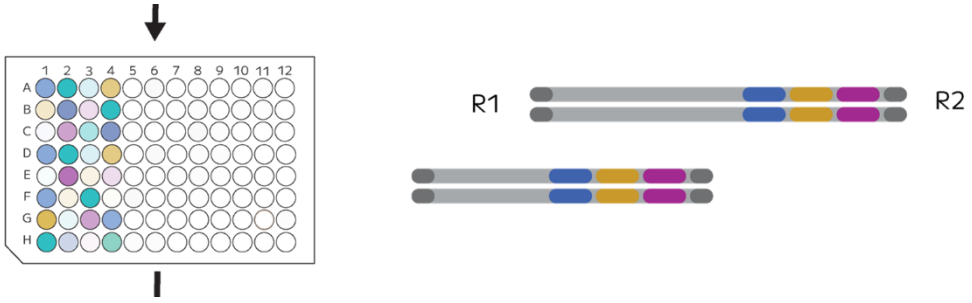
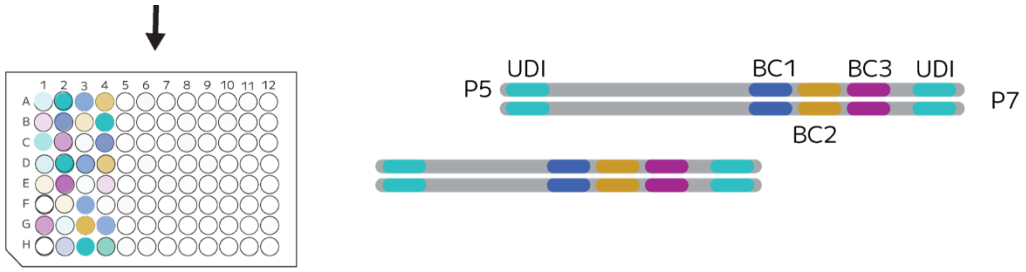
	Section 3: Sequencing Library Preparation
1	 **Fragmentation and End Repair:** DNA is fragmented, ends are repaired, and A-tailed.
2	 **Adapter Ligation:** An Illumina Truseq R1 Adapter is ligated to the 5’ end of the DNA.
3	 **Round 4 Barcoding:** The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.

Figure 1 shows the WT Penta 384 workflow. This kit can barcode up to 5 million cells/nuclei across 384 samples in a single reaction. The Penta 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 two Round 2 Plates. The barcodes from Round 1 and Round 2 serve as sample identifiers.

Samples from Round 2 are pooled and distributed into two Round 3 plates. Cells/nuclei are then pooled and subsequently split to generate 32 sublibraries. Sample plates are easily identified by labeling color-coded stickers (Figure 2).

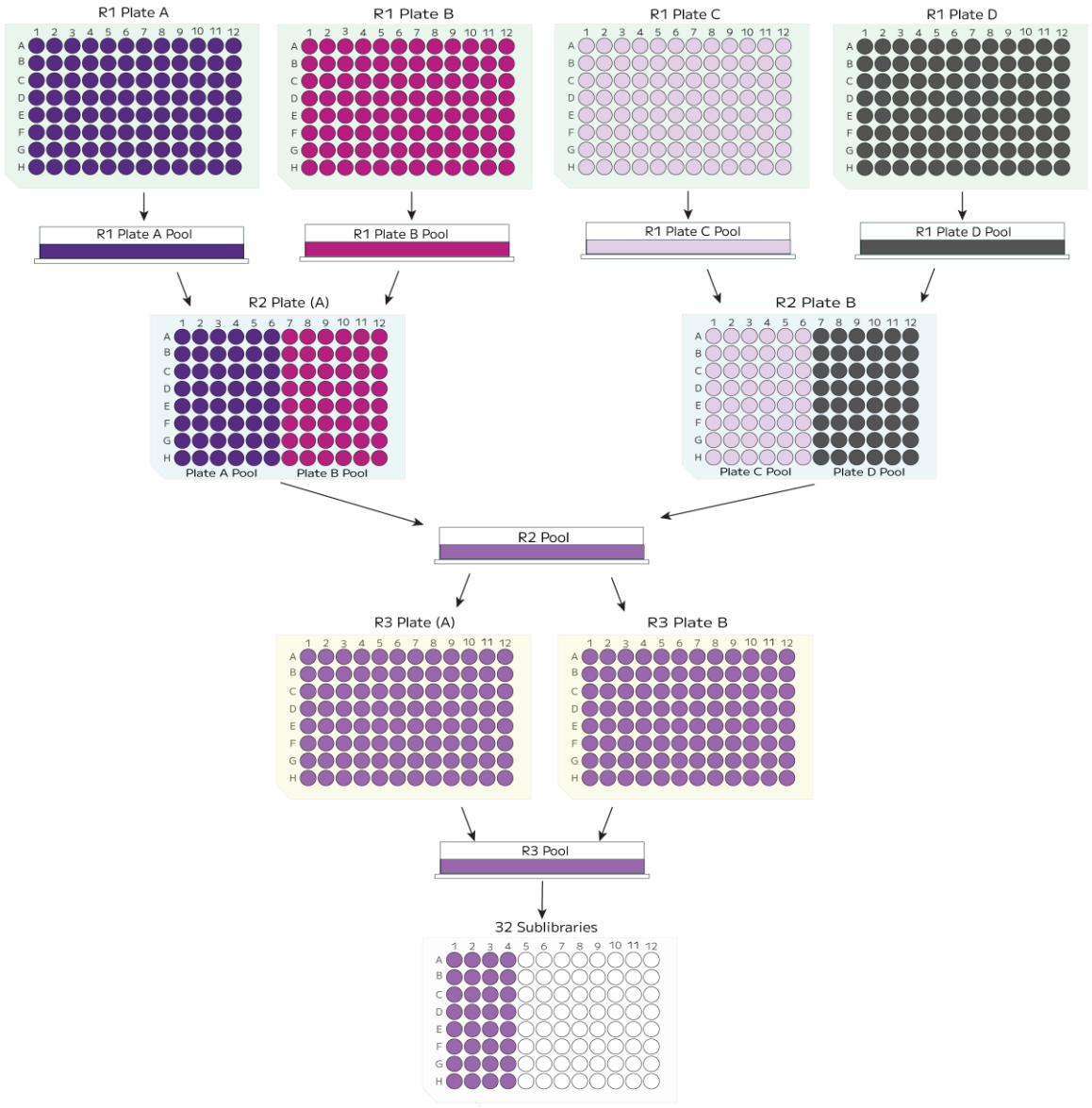


Figure 1: Evercode WT Penta 384 workflow pools 384 samples from four identical Round 1 Barcoding Plates into two distinct Round 2 and Round 3 Barcoding Plates respectively, generating 32 Sequencing Libraries.

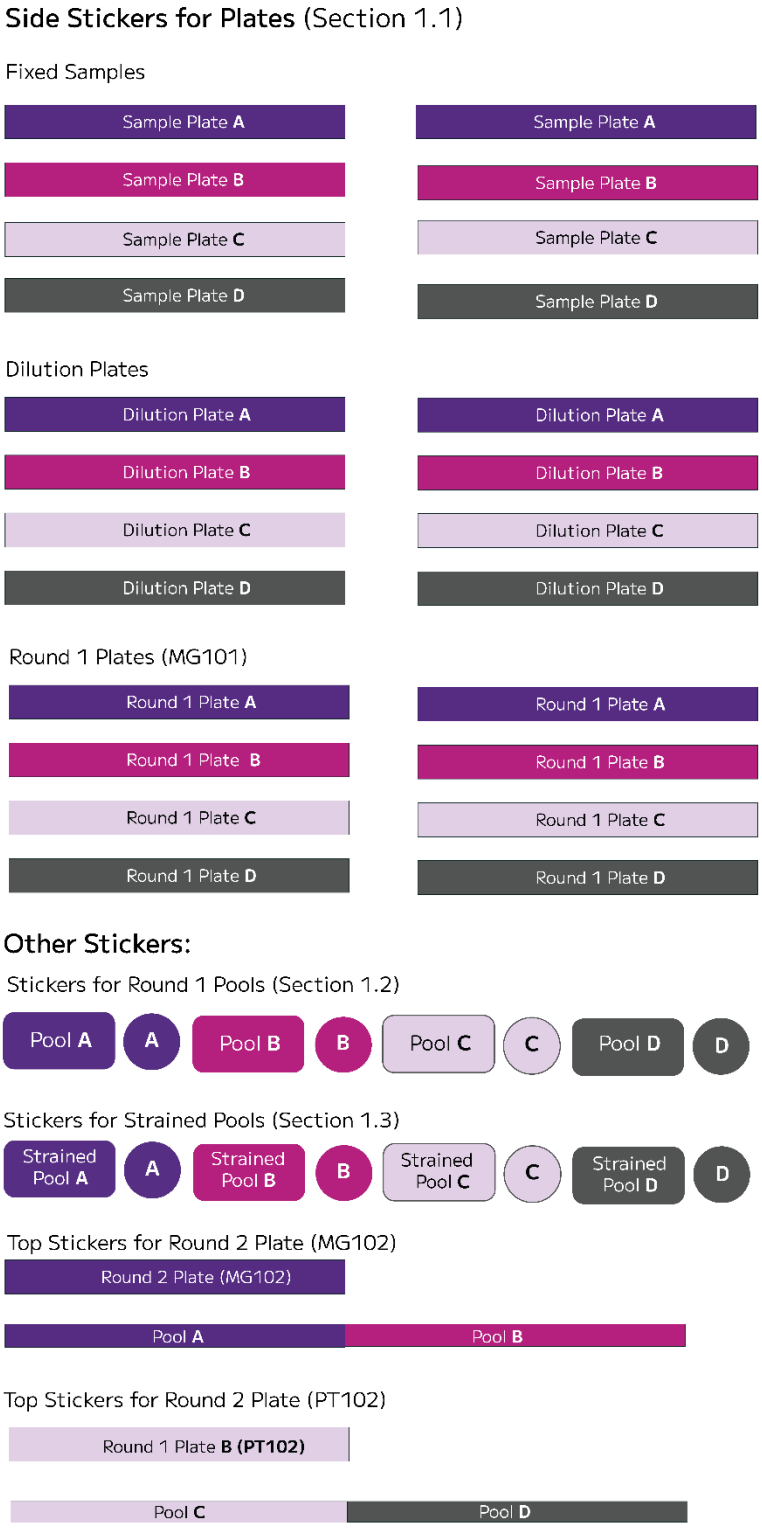


Figure 2: Plates are easy to handle with labeled color-coded stickers throughout the Round 1 Barcoding section.

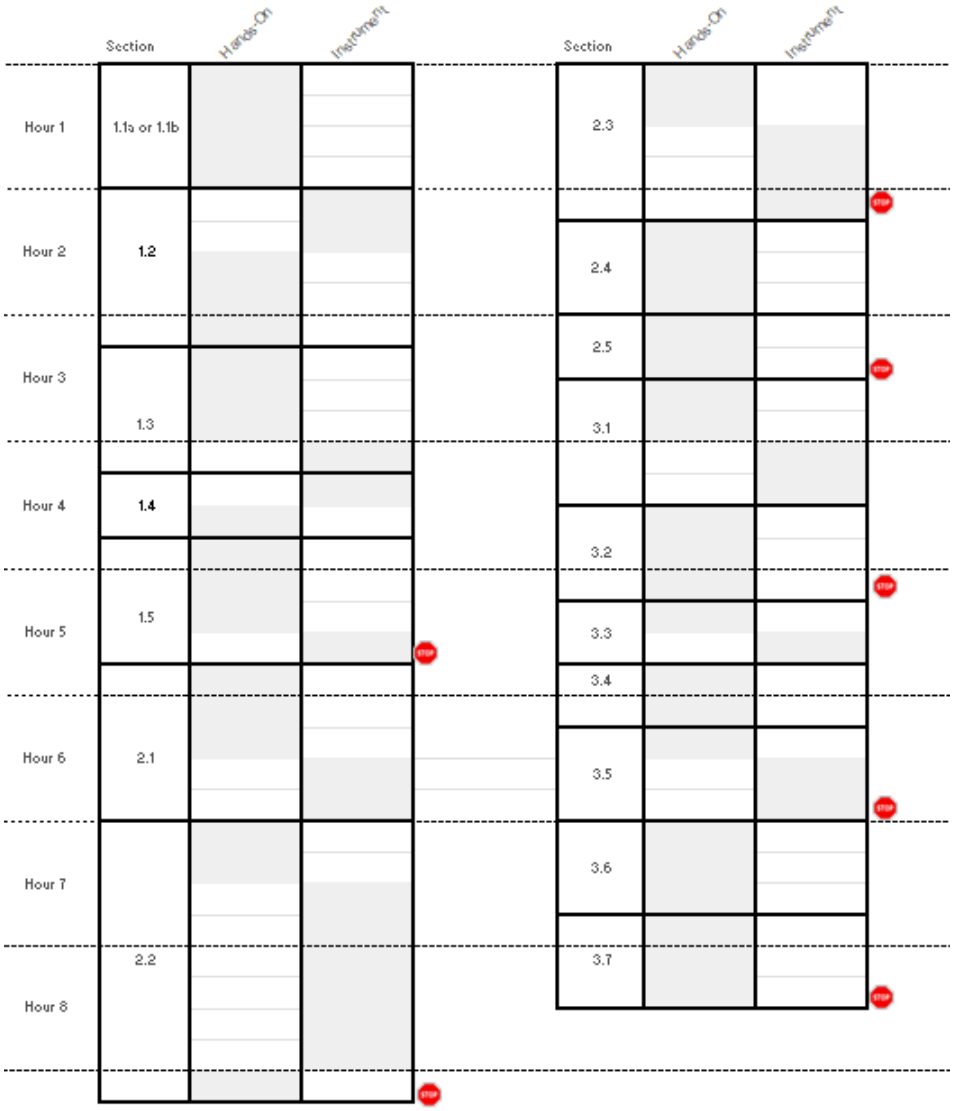
Protocol Timing

The table below provides details of the total and hands-on time required for the whole transcriptome workflow. Visual representations of a 2-day and a 3-day workflow are shown below the table.

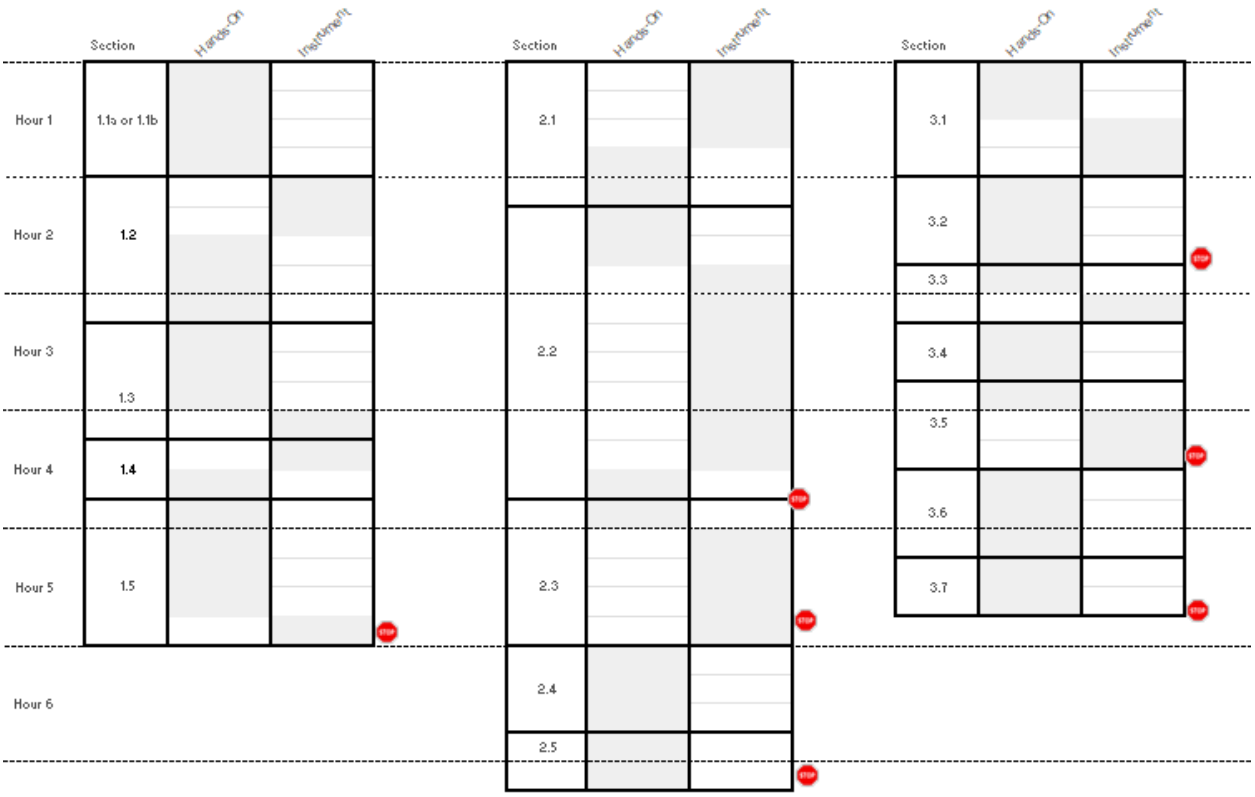
DESCRIPTION	TIME	HANDS-ON-TIME	SAFE STOPPING POINTS
Section 1: *In Situ* Cell/Nuclei Barcoding			
1.1a Set up for Low Input Fixation Samples OR 1.1b Set up for Standard Fixation	60-90 min	60-90 min	
1.2 Pooling Round 1	75 min	45 min	
1.3 Barcoding Round 2	60 min	45 min	
1.4 Barcoding Round 3	30 min	15 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	75 min	45 min	
2.2 cDNA Template Switch	135 min	45 min	4°C ≤ 18 hrs
2.3 cDNA Amplification	80 min	30 min	4°C ≤ 18 hrs in the thermocycler
2.4 Post-Amplification Purification	45 min	45 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	45 min	45 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	45 min	45 min	
3.7 Sequencing Library Quantification	45 min	45 min	-20°C ≤ 3 months

2-day Workflow



3-day Workflow



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 or Evercode Nuclei Fixation v3 kit.
- Even if samples were counted before freezing, and if you have a set aside a counting aliquot, we strongly recommend counting cells/nuclei again after thawing to account for any changes during storage and freeze thaw. Typically, a 5-15% decrease after thawing should be expected. These counts are used to determine how cells/nuclei are loaded in the Round 1 Plate, and their accuracy is critical to recover the desired number of cells/nuclei.
- When processing many fixed samples, we recommend aliquoting samples after fixation and counting the aliquots the day before using an Evercode Whole Transcriptome kit. The Evercode Fixation User Manuals outline recommendations 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 obtained immediately after fixation. Aliquot plates should be thawed at 37°C and counted with an automated cell counter or alternative 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 and Penta 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).
- Once fixed samples have been thawed, they should not be refrozen.

Cell/Nuclei Counting and Quality Assessment

- Due to the scale of high-throughput experiments, we recommend an automated cell counter for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices to a hemocytometer when using Evercode Whole Transcriptome kits for the first time.
- 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.

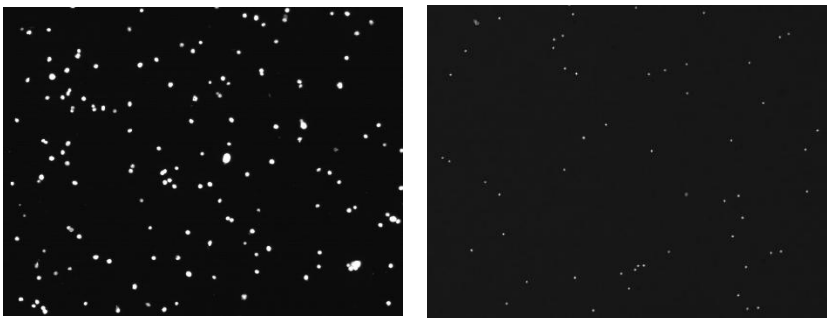


Figure 3: example of AO/DAPI stained HEK cells (left) and PBMCs (right).

High Quality Sample Aggregation Debris

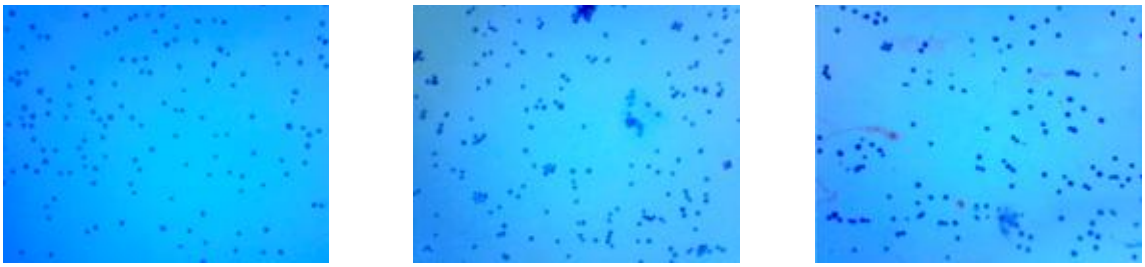


Figure 4: Standard Fixation: example of trypan blue stained fixed cells.

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.

- Use polypropylene 15 mL centrifuge tubes and basins, as using polystyrene 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.

Sample Loading Table

- The Parse Biosciences Evercode Sample Loading Table (Excel spreadsheet) should be completed before starting the experiment. Based on the selected fixation method, fill out either the Low Input Fixation Sample Loading Table or the Standard Fixation Sample Loading Table accordingly.

- 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 Plates, which will result in more overall barcoded cells but change the desired proportion between samples.

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

- A ring plate magnet is needed for capturing low input cell lysates only. A bar magnet is recommended for the remaining library preparation steps for its ease of use.

- When aspirating supernatants in the magnetic rack, angle pipette tips away from beads. Ensure tips are at the bottom of each well to ensure all wells will get all supernatants removed.

- If the beads are uptaken, dispense solution again and wait for 1 min before trying to aspirate again.

- To ensure material is not lost during bead purifications, ensure supernatants are completely clear before moving to the next step. The incubation times at each step are recommendations, but visual confirmation of clearing should be used to make the final determination. Discarding any beads in supernatants will result in a reduction of transcripts and genes detected per cell.

Sublibrary Loading

- The Evercode WT Penta 384 kit generates 32 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.
- 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.
- This manual is designed for the preparation of 32 sublibraries. However, to prepare 16 sublibraries at a time, use two columns in a plate or a set of 16 PCR tubes. Simply adjust the volumes by either multiplying the amount per sublibrary by 16 or halving the volumes designated for 32 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.
- The UDI Plates are 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 A.
- We recommend recording which UDI is added to each sublibrary. This information will be critical for the sequencing provider.

Thermocycling Programs

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

Part List

The Evercode WT Penta 384 v3 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents Store at -20°C, PTT100

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 2 Plate B	PT102	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Round 3 Plate B	PT103	Yellow semi-skirted 96 well plate	1
Resuspen Buf	Resuspension Buffer	MG131	5 mL tube	1
Sample Dil	Sample Dilution Buffer	MG132	5 mL tube	4
R2 Lig Buf	Round 2 Ligation Buffer	MG133	5 mL tube	1
R2 Lig Enzy	Round 2 Ligation Enzyme	PT104	1.5 mL tube	1
R2 Stop	Round 2 Stop Buffer	PT105	5 mL tube	1
R3 Lig Enzy	Round 3 Ligation Enzyme	PT106	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Final Stop Buffer	PT107	5 mL tube	1
	Pre-Lysis Wash Buffer	MG110	5 mL tube	2
	Pre-Lysis Dilution Buffer	PT108	5 mL tube	1
	Lysis Enzyme	PT109	1.5 mL tube	1
	Penta Lysis Buffer	PT126	2 mL tube	1
	Capture Enhancer	PT110	1.5 mL tube	1
	Bead Wash Buffer	PT111	5 mL tube	2
	Binding Buffer*	PT112	5 mL tube	1
	Wash Buffer 1	PT113	5 mL tube	3
	Wash Buffer 2	PT114	5 mL tube	3
	Wash Buffer 3	PT115	5 mL tube	3
	Template Switch Buffer	PT116	5 mL tube	1
	Template Switch Enzyme	PT117	1.5 mL tube	1
	Template Switch Primer	PT118	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
cDNA Amp Mix	cDNA Amp Mix	PT119	5 mL tube	1
cDNA Amp Primers	cDNA Amp Primers	PT120	5 mL tube	1
Fragm/End Prep Buf	Fragm/End Prep Buffer	PT121	1.5 mL tube	1
Fragm/End Prep Enzy	Fragm/End Prep Enzymes	PT122	1.5 mL tube	1
Lig Adapter	Ligation Adapter	MG127	1.5 mL tube	1
Adap Lig Buffer	Adapter Ligation Buffer	PT123	1.5 mL tube	1
Adap Lig Enzy	Adapter Ligation Enzyme	PT124	1.5 mL tube	1
Library Amp Mix	Library Amp Mix	PT125	1.5 mL tube	1
N/A	Sticker Labels	N/A	Sticker Sheet	N/A

Note: * Check before using the Pre-Lysis Wash Buffer and the Pre-Lysis Dilution Buffer. The two tube caps resemble each other and could be mistaken.

4°C Reagents Store at 4°C, PTT200

LABEL	ITEM	PN	FORMAT	QTY
Spin Add	Spin Additive	MG204	1.5 mL tube	1
Strep Beads	Streptavidin Beads	PT201	2 mL tube	2

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
Permagen Bar Magnet	Permagen®	MSPU650	We do not recommend alternative magnets, as they may result in lower transcript and gene detection.
Permagen Ring Magnet	Permagen®	S500	Needed only for processing low input samples
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.
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.
Single Channel Pipettes: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.

ITEM	SUPPLIER	PN	NOTES
2 or 4 T100 Thermal Cyclers	Bio-Rad Laboratories®	1861096	Or equivalent thermocyclers 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	
QubitTM 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. Use only polypropylene basins as polystyrene may lead to some cell loss.

ITEM	SUPPLIER	PN	NOTES
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.
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	
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.
PCR Plate 96 LoBind®	Eppendorf	0030129504	
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.
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)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	

ITEM	SUPPLIER	PN	NOTES
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.
TempPlate® EXT Sealing Foil	USA Scientific®	2998-0100	
5 mL tubes	Eppendorf	30108310	
Strip of 8 Domed Caps	Fisherbrand	14230231	
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 and Penta 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 and Penta 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 and Penta 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 and Penta 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	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	-20°C Reagents	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: For every 96 samples being processed, a 1.5 mL tube is needed to resuspend and wash the beads. Therefore, up to 4 tubes would be needed to process all 384 samples.

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 each 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 each 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 each well of **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. If four thermocyclers are available, they can be operated simultaneously.

20. Remove **two** of the four plates of fixed cells/nuclei from -80°C storage. Set the 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 starting with "Sample Plate A". See Evercode WT Mega 384 and Penta 384 Low Input Fixation 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, switching tips. 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 and switching tips between columns.

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

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

29. Add **20 mL** of 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. Switch tips between columns. Refer to the Sample Loading Table to determine O Storage Buffer volumes (ranges from 20-46 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.

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.

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

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 33-35 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 20-37 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 Section 1.2, individually pooling Round 1 Plate A into Pool A and Round 1 Plate B into Pool B.

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 and Penta 384 Standard Fixation Sample Loading Table. Dilution plates may be made up to 48 hours in advance of barcoding.

Dilution plate set up:

1. Prepare an automated counter, 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 and Penta 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	-20°C Reagents	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 -20°C Reagents and Stickers box.

7. Create the corresponding dilution plates of O Sample Dilution Buffer using the Evercode WT Mega 384 and Penta 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 and Penta 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
O Resuspension Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
O Round 2 Ligation Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.

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 the 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 at 4°C.

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. Example of the workflow is shown in the diagram below. See Sample Loading Table plate configuration tab for sample location.

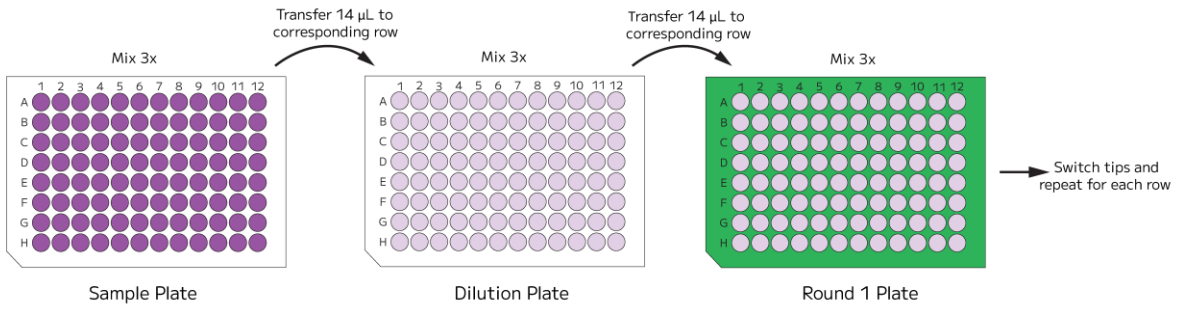


Figure 5: Samples in the sample plate are diluted and then transferred to the Round 1 Plate.

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, switching tips after every transfer.

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.

Note: If only 2 thermocyclers are available, store the completed Plates A and B on ice, while Plates C and D are barcoded.

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.
Round 2 Plate B	-20°C Reagents	1	
● Round 2 Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
○ Round 2 Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.

* The Round 2 Plate will be referred to as "Round 2 Plate (A)" to maintain consistency with Round 2 Plate B.

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

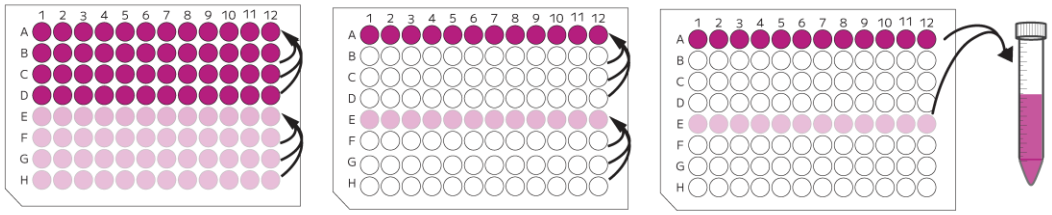
3. Place the two Round 2 Plates into two thermocyclers and run the following program. Proceed immediately to the next step while the program is still running.

THAW ROUND 2 PLATE (2)		
Run Time	Lid Temperature	Sample Volume
3 min	70°C	10 µL
Step	Time	Temperature
1	3 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 (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.

CRITICAL! Do not mix pools together. Separate pools from each Round 1 Plate are needed for demultiplexing barcode information.

7. Add **19.2 µL** of ● Spin Additive to the 15 mL tube (Pool A) 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 1-8 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	5 mL
○ Round 2 Ligation Buffer	4.88 mL
● Round 2 Ligation Enzyme	40 µL
Total Volume	9.92 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, using new pipette tips for each tube.

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 are 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. Working with one sample pool at the time, resuspend the pellets from each pool (A-D) in **2.3 mL** of the Round 2 Ligation Master Mix to make a Ligation Cell Mix. Store on ice.

CRITICAL! Do not reuse any tips between resuspension steps.

2. Label four new basins with the provided stickers.

3. Pipette Pool A through a cell strainer into the basin labeled Pool A with a P1000.

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. Do not directly touch the mesh of the cell strainer with a gloved hand.

4. Repeat the previous step for the remaining three resuspended pools (B-D).

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

6. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate (A), and apply the provide adhesive labels to assist with loading. We suggest not removing the seal from Round 2 Plate B yet to avoid confusion.

7. With Round 2 Plates on ice, transfer the Ligation Cell Mix to each well in the Round 2 Plate (A) as follows. See figure 6 for an overview.

    i. Mix **Pool A** in the basin 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 (A) and mix by pipetting 2x.

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.

iii Repeat i-ii to mix Pool A in the basin then transfer to each well of columns 2-6 of the Round 2 Plate (A).

iv Mix **Pool B** in the basin by pipetting 2x with a multichannel P200 set to 40 µL.

v Transfer **40 µL** of the mix to column 7 of the Round 2 Plate (A) and mix by pipetting 2x.

vi Repeat iv-v to mix Pool B in the basin then transfer to each well of columns 8-12 of the Round 2 Plate (A).

vii While secured in a PCR tube rack on a flat surface, add a plastic PCR seal to Round 2 Plate (A) and place back on ice.

vii Remove the seal from the Round 2 Plate B and apply appropriate label.

viii Repeat i-vii to add Pool C and Pool D to Round 2 Plate B: load **Pool C** in each well of columns 1-6, and **Pool D** in each well of columns 7-12 in Round 2 Plate B, respectively.

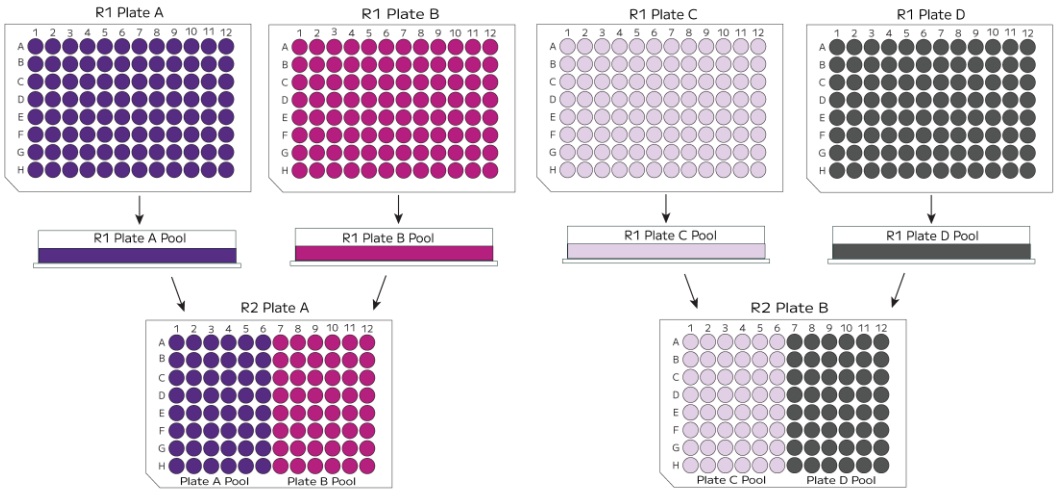


Figure 6: After straining Round 1 Pools into four basins, each Pool is loaded column-wise into six columns each of the Round 2 Plates (A) and B.

Note: If when loading column 6 and 12 in each round 2 plate (last column loaded for each pool), the volume remaining is less than 40 µL per well, the wells can be partially loaded without impacting experimental results.

8. Place the Round 2 Plates into two thermocyclers and run the following program. Upon completion, proceed immediately to the next step.

BARCODING ROUND 2 (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

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

10. Remove the Round 2 Plates from the thermocyclers, place in PCR tube racks, remove the plate seals, and store on ice.

11. With the Round 2 Plates on ice and the basin on the bench, transfer **10 µL** of the O Round 2 Stop Buffer to each well in the Round 2 Plates 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 O Round 2 Stop Buffer into each well of the plates. Never place a tip that has entered one of the wells back into the basin or a different well.

12. While secured in PCR tube racks on a flat surface, add new plate seals to Round 2 Plates.

13. Place the Round 2 Plates into two thermocyclers and run the following program. Proceed to the next step while the program is still running.

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

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

ITEM	SOURCE	QTY	HANDING AND STORAGE
Round 3 Plate*	-20°C Reagents	1	Place directly on ice.
Round 3 Plate B	-20°C Reagents	1	

* The Round 3 Plate will be referred to as "Round 3 Plate (A)" to maintain consistency with Round 3 Plate B.

15. Place the Round 3 Plates (A) and B into a third and fourth thermocycler and run the program below. Proceed to the next step while the program is still running.

Note: If a third and fourth thermocyclers are not available, this step can be performed later, after the Round 2 Stop program is completed.

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

16. Immediately upon completion of the Round 2 Stop program, transfer the Round 2 Plates from the thermocyclers to PCR tube racks, remove the plate seals, and store on ice.

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

    i With a multichannel P200 set to 50 µL, mix the sample in Round 2 Plate (A) 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 (A) to the basin with a multichannel P20 set to 10 µL.

    v Repeat steps i-iv to pool Round 2 Plate B into the **same** basin.

18. 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.

19. Proceed immediately to section 1.4.

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 Plates. 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
○ Final 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.
● Penta Lysis Buffer	-20°C Reagents	1	Thaw at room temperature.

2. Add **40 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 Plates from the thermocyclers, place in PCR tube racks, and centrifuge for **1 minute** at 100 x g at 4°C.

4. While secured in PCR tube racks on a flat surface, remove the plate seal from the Round 3 Plate (A). We suggest not removing the seal from Round 3 Plate B yet to avoid confusion.

5. With the Round 3 Plates on ice and the basin on the bench, transfer **50 µL** from the basin to each well in the Round 3 Plate (A) 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 (A) and mix by pipetting 2x.

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

    iv While secured in a PCR tube rack on a flat surface, add a plastic PCR seal to Round 3 Plate (A).

    v Remove the seal from the Round 3 Plate B.

    vi Repeat steps i-iv to add the sample to Round 3 Plate B.

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. Place the Round 3 Plates into two thermocyclers 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

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

8. Remove the Round 3 Plates from the thermocyclers, place in PCR tube racks, remove the plate seals, and store on ice.

9. With the Round 3 Plates on ice and the basin on the bench, transfer **20 µL** of the O Final Stop Buffer from the basin to each well in the Round 3 Plates 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 O Final 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.

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

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

    i. With a multichannel P200 set to 70 µL, mix the sample in Round 3 Plate (A) 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 (A) to the basin with a multichannel P20 pipette set to 10 µL.

    v Repeat steps i-iv to pool Round 3 Plate B into the **same** basin.

12. Pipette 7.5 mL of 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.

13. Repeat step 12 for a second 15 mL tube.

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 to create 32 sublibraries of 156,250 cells/nuclei. These sublibraries are lysed and stored at -80°C.

To generate and lyse sublibraries:

1. Add **70 µL** of ● Spin Additive to each of the 15 mL tubes with the sample. Gently invert once to mix.

2. Centrifuge the 15 mL tubes 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 from each tube until about ~10 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 each pellet in **1 mL** of ○ Pre-Lysis Wash Buffer.

5. Add an additional **3 mL** of ○ Pre-Lysis Wash Buffer to each tube for a total addition of 4 mL each.

6. Transfer the full volume in one 15 mL tube to the other 15 mL tube to have a total of **8 mL** of sample resuspended in ○ Pre-Lysis Wash Buffer.

7. 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.

8. Remove the supernatant until about ~100 µL of liquid remains above the pellet. Use a P1000 for the first 7 mL and then a P200 for the remaining volume.

9. Fully but gently resuspend the pellet in **650 µL** of ○ Pre-Lysis Dilution Buffer for a final total volume of 750 µL. Store on ice.

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

10. While minimizing time on ice, count the number of cells/nuclei in the sample with an automated cell counter or alternative cell counting device. Record the cell/nuclei count.

CRITICAL! An accurate cell count is critical for correct sublibraries loading. We strongly recommend using a fluorescent automated cell counter and carefully mixing the sample before collecting an aliquot for counting to ensure accurate sublibrary loading.

11. Dilute cells/nuclei further using the ○ Pre-Lysis Dilution Buffer to reach a concentration of 6,250 cells/nuclei per µL and to reach a final total volume of at least 880 µL.

Note: Even if the concentration is below 6,250 cells/nuclei per µL, we still recommend maintaining the volume of at least 880 µL so all 32 sublibraries can be created.

12. While minimizing time on ice, recount the number of cells/nuclei in the sample. Record the cell/nuclei count for the final library size.

CRITICAL! Do not add more than 156,250 cells/nuclei to a sublibrary. Adding additional cells/nuclei will result in an increased multiplet rate.

13. Ensure the cells/nuclei are in suspension by pipetting 5x with a P1000 set to 500 µL prior to each transfer.

14. Aliquot out **110 µL** of the diluted cells with a P200 single channel into each well of an 8-tube PCR strip for multichanneling. Mix 2x between each aliquot. Store on ice.

Note: Keep any remaining volume on ice until all sublibraries are filled.

15. With a new semi-skirted low bind plate on ice, and the 8-tube PCR strip containing cells on ice, transfer **25 µL** from the strip into the semi-skirted plate as follows:

    i. With a multichannel P200 set to 25 µL, mix the sample in the 8-tube strip by pipetting 3x.

    ii. Transfer **25 µL** in the 8-tube strip to column from the strip to the first column in the semi-skirted plate.

    iii. Repeat for columns 2-4.

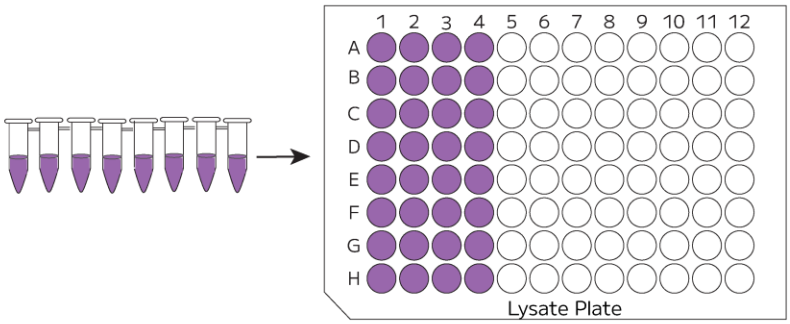


Figure 7: Each tube in the 8-tube PCR strip holds 110 µL of sample. Aliquots of 25 µL are dispensed into four columns of the semi-skirted plate, generating a total of 32 sublibraries.

16. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P1000 set to 800 µL. Store at room temperature.

LYSIS MASTER MIX	
● Lysis Buffer	1000 µL
● Lysis Enzyme	200 µL
Total Volume	1200 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.

17. Aliquot out **145 µL** of Lysis Master Mix into each well of an 8-strip PCR tube. Centrifuge briefly.

18. Add **30 µL** of Lysis Master Mix to each well with diluted cells/nuclei using a P200 multichannel pipette. Store at room temperature.

CRITICAL! Change tips between each column after dispensing the Lysis Master Mix.

19. While secured in a PCR tube rack on a flat surface, seal the plate with a foil seal and layer with a plastic seal to prevent punctures.

20. While secured in the PCR tube rack, vortex the PCR plate for **1 minute**.

21. Centrifuge the lysate plate for **1 minute** at 100 x g at 4°C.

22. Place the plate into a thermocycler and run the following program.

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

23. For Standard Fixation samples, freeze the lysates at -80°C or proceed to Section 2. For Low Input Fixation samples, proceed to step 24.

24. Remove samples from the thermocycler, remove the seal, and place the plate on the ring plate magnet. Incubate until the solution clears (~2 minutes).

25. While still on the magnet, transfer **55 µL** of the supernatant containing lysed cells into a new PCR plate, switching tips between transfers.

26. If not immediately proceeding to Section 2, seal the plate with a foil seal, and freeze the lysates at -80°C.

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. Gather the following equipment:
    i. Magnetic rack for 1.5 mL tubes
    ii. Magnetic plate for semi-skirted PCR plates
    iii. Vortex with an adapter for 96 well plates
    iv. 96 well PCR tube rack (for stabilizing plate)
    v. 8-cap strips
    vi 25 mL basins
3. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Streptavidin Beads	4°C Reagents	2	Keep at room temperature.
○ Bead Wash Buffer	-20°C Reagents	2	Thaw at room temperature then store on ice. Mix by inverting 3x
○ Wash Buffer 1	-20°C Reagents	2	
○ Wash Buffer 2	-20°C Reagents	2	
○ Wash Buffer 3	-20°C Reagents	2	
● 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
○ Template Switch Buffer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 10x.

4. Remove the plate of lysates from the thermocycler (if continuing directly from Section 1) or from storage at -80°C.

5. If previously frozen, incubate the plate in a thermocycler at 37°C for **5 minutes**.

THAW LYSATE PLATE		
Run Time	Lid Temperature	Sample Volume
5 min	40°C	55 µL
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

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

6. Briefly centrifuge in a swinging bucket centrifuge at 100 x g for **30 seconds**. Store at room temperature.

7. Briefly centrifuge ● Capture Enhancer and gently mix by pipetting 2x with a P200 set to 150 µL.

8. Aliquot **20 µL** of ● Capture Enhancer into each tube of an 8-tube PCR strip to facilitate the use of a multichannel pipette.

9. Using a P20 multichannel, add **2.5 µL** of ● Capture Enhancer to each well of the lysate plate, column-wise, switching tips between columns.

10. Place the plate into a 96 well PCR tube rack, press to secure, and seal with 8-cap strips.

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

12. Remove the plate from the vortex mixer. Briefly centrifuge in a swinging bucket centrifuge at 100 x g for **20 seconds**.

13. 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.

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

Lysates being processed	1	16	32*
OStreptavidin Beads	66 µL	1056 µL	2x1056 µL

***** For processing 32 lysates, process two tubes of 16 reactions (i.e. two 1.5 mL tubes with 1056 µL of Streptavidin Beads)

15. Place the tube(s) on the magnetic rack for 1.5 mL tubes until the solution clears (1 minute).

16. Remove and discard the supernatant.

17. Add the appropriate volume of O Bead Wash Buffer as follows:

Lysates being processed	1	16	32*
O Bead Wash Buffer	50 µL	800 µL	2x800 µL

*For processing 32 lysates, process two tubes of 16 reactions (i.e. two 1.5 mL tubes with 800 µL of Bead Wash Buffer)

18. Remove the tube(s) from the magnetic rack and vortex for **10 seconds** to fully resuspend the bead pellet(s). Briefly centrifuge.

19. Place the tube(s) on the magnetic rack for 1.5 mL tubes until the solution clears (1 minute).

20. Remove and discard the supernatant.

21. Repeat steps 17-20 twice for a total of 3 washes.

Note: Avoid over-centrifuging the streptavidin beads, as this can make them difficult to resuspend.

22. Remove the 1.5 mL tube(s) from the magnetic rack. Fully resuspend the pellet(s) in the appropriate volume of OBinding Buffer as follows and store at room temperature.

Lysates being processed	1	16	32*
O Binding Buffer	55 µL	880 µL	2x880 µL

*For processing 32 lysates, process two tubes of 16 reactions (i.e. two 1.5 mL tubes with 880 µL of Binding Buffer)

23. Aliquot **220 µL** of O Streptavidin Beads in O Binding Buffer into each tube of an 8-tube PCR strip, mixing well between each dispense.

24. Using a P200 multichannel pipette, add **50 µL** of O Streptavidin Beads in O Binding Buffer to each well of the lysate plate, switching tips between columns.

25. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

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

27. Briefly centrifuge for **10 seconds** at 100 x g without letting beads collect at the bottom of the wells.

28. 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 5-10 minutes into the incubation. If settled, increase the vortex mixing speed to keep the beads in solution.

29. Remove the plate from the vortex mixer and briefly vortex the plate on a standard vortex adapter.

30. Briefly centrifuge at 100 x g for **10 seconds.**

31. Secure the plate of lysates on a 96 tube rack, and remove the caps.

32. Place the plate on the PCR plate magnet. 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.

33. Prepare and label 3 empty basins to add wash buffers at appropriate steps and an additional basin for waste.

Note: The 4 basins should be labeled for “Wash Buffer 1”, “Wash Buffer 2”, “Wash Buffer 3”, and “Waste”.

34. Add **10 mL** of O Wash Buffer 1 to a labeled and empty basin using a P1000 pipette or a serological pipette.

35. Add **5 mL** of O Wash Buffer 2 to a labeled and empty basin using a P1000 pipette or a serological pipette. Cover basin with a plastic PCR seal.

36. Add **5 mL** of O Wash Buffer 3 to a labeled and empty basin using a P1000 pipette or a serological pipette. Cover basin with a plastic PCR seal.

37. While still on the plate magnet, remove and discard the supernatant from all 4 columns of the Lysate plate into the waste basin. Change tips between each column of lysate.

Note: Angle pipette tips away from the bead pellet to prevent bead loss.

38. While still on the magnet, carefully add **125 µL** O Wash Buffer 1 to all four lysate columns, using a P200 multichannel pipette, switching tips between columns. Be careful to prevent spillage.

39. Secure the plate onto a 96 tube rack, and seal with 8-cap strips, ensuring the caps are tightly secured.

40. Vortex the plate for **15 seconds**.

41. Centrifuge the plate for **10 seconds** at 100 x g in a swinging bucket centrifuge.

42. Secure the plate onto a 96 well PCR tube rack, and remove the caps.

43. Return the plate to the plate magnet. Incubate until the solution clears (~2 minute).

44. While still on the plate magnet, remove and discard the supernatant from all 4 columns, changing tips between each column.

Note: Angle pipette tips away from the bead pellet to prevent bead loss.

45. Repeat steps 38-44 once for a total of 2 washes with O Wash Buffer 1.

46. While still on the magnet, carefully add **125 µL** O Wash Buffer 2 to all four columns, using a P200 multichannel pipette, switching tips between columns. Be careful to prevent spillage.

Note: The remaining 5 mL tube of OWash Buffer 2 can be used for optional storage before cDNA amplification.

47. Secure the plate onto a 96 tube rack, and seal with 8-cap strips, ensuring the caps are tightly secured.

48. Vortex the plate for **15 seconds**.

49. Centrifuge the plate for **10 seconds** at 100 x g in a swinging bucket centrifuge.

50. Remove from the centrifuge and store at room temperature.

51. 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
● Template Switch Primer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
● Template Switch Enzyme	-20°C Reagents	1	Keep on ice. Briefly centrifuge before use.
○ cDNA Amp Mix*	-20°C Reagents	1	Thaw at room temperature and store on ice. Mix by inverting 10x.
○ cDNA Amp Primers*	-20°C Reagents	1	

Note: * If planning to store the cDNA at the end of this section and prior to the cDNA Amplification steps, do not thaw the ○ cDNA Amp Mix and the ○ cDNA Amp Primers as they are for cDNA amplification.

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

2. Gently mix the ○ Template Switch Buffer by pipetting 10x with a P1000 set to 800 µL.

3. Prepare the Template Switch Master Mix in a new 5 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	32
○ Template Switch Buffer	111 µL	3552 µL
● Template Switch Primer	3 µL	96 µL
● Template Switch Enzyme	6 µL	192 µL
Total	120 µL	3840 µL

4. Aliquot **220 µL** of Template Switch Master Mix into 16 PCR tubes to facilitate the use of a multichannel pipette, and store on ice.

5. Secure the plate of captured cDNA from Section 2.1 onto a 96 well PCR tube rack, and remove the caps.

6. Place the plate onto a plate magnet. Incubate until the solution clears (~2 minutes).

7. While still on the magnet, remove and discard the supernatant from all 4 columns, switching pipette tips between each column.

8. While still on the magnet, pour the second tube into the basin and add **125 µL** of ○ Wash Buffer 3 to each column, switching tips between columns.

Note: No vortexing of the plate is required at this step.

CRITICAL! Do not discard the other 5 mL tube of ○ Wash Buffer 3 as it will be used in another step.

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

10. While still on the magnet, remove and discard the ○ Wash Buffer 3 from all 4 columns, switching pipette tips between each column. With a P20 remove any leftover liquid.

11. Remove the plate from the magnet and secure the plate onto a 96 well PCR tube rack.

12. Add **100 µL** of the Template Switch Master Mix with a multichannel P200 to all 4 columns, switching pipette tips between each column.

CRITICAL! We recommend adding the Template Switch Master Mix before pipette mixing to avoid drying the beads.

13. Seal the plate with a plastic seal.

14. Centrifuge the plate for **1 minute** at 100 x g in a swinging bucket centrifuge to collect the beads at the bottom of each well.

15. Secure the plate onto a 96 well PCR tube rack, remove the seal, and mix each column 20x vigorously using a P200 multichannel pipette set to 75 µL, switching pipette tips between each column. Ensure the beads are fully resuspended by checking all angles of the plate.

Note: Because the Template Switch Master Mix is viscous, you may need to perform additional pipette mixes to make sure the beads are fully resuspended. Perform a visual check to make sure no beads are stuck to the side of the wells before proceeding to the next step.

16. Seal the plate with a new plastic PCR seal and briefly centrifuge without letting beads collect at the bottom of the wells (**10 seconds** at 100 x g).

17. Incubate for **30 minutes** at room temperature.

18. Fully resuspend each bead pellet by mixing 5x with a P200 set to 75 µL, switching pipette tips between each column.

19. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

20. Place the plate 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

21. Proceed immediately to Section 2.3. Alternatively, proceed to the following steps (step 22-30) to store samples prior to cDNA amplification.

22. Place the plate on the plate magnet and incubate for **3 minutes**.

23. Mix each column 5x using a P200 multichannel set to 75 µL. Change tips between each column.

Note: Pipette mixing is necessary to resuspend settled beads.

24. Incubate for an additional **2 minutes**.

25. Add **5 mL** of O Wash Buffer 2 to a labeled and new basin using a P1000 pipette, switching tips between each column.

26. While still on the magnet, remove and discard the supernatant from all 4 columns into the waste basin, switching tips between columns.

27. While still on the magnet, add **125 µL** O Wash Buffer 2 using a P200 multichannel pipette, switching pipette tips between each column.

28. Remove the plate from the plate magnet and secure it in a 96 PCR tube rack.

29. Fully resuspend each bead pellet in O Wash Buffer 2 using a P200 multichannel set to 100 µL, switching pipette tips between each column.

30. Seal with a foil seal.

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. Prepare the cDNA Amplification Master Mix in a new 5 mL tube as follows. Mix by pipetting 10x and store on ice.

cDNA AMPLIFICATION MASTER MIX		
Number of Sublibraries	1	32
O cDNA Amp Mix	60.5 µL	1936 µL
O cDNA Amp Primers	60.5 µL	1936 µL
Total	121 µL	3872 µL

2. Aliquot **220 µL** of cDNA Amplification Master Mix into 16 PCR tubes to facilitate the use of the multichannel pipette. Store on ice.

3. Secure the plate of template switched cDNA from Section 2.2 onto a 96 well PCR tube rack, and remove the caps.

4. Place the plate on the plate magnet. Incubate for **3 minutes**.

5. Mix each column 5x with a P200 multichannel set to 75uL. Change tips between each column.

Note: Pipette mixing is necessary to resuspend settled beads.

6. Incubate for an additional **2 minutes**.

7. While still on the magnet, remove and discard the supernatant from all 4 columns, switching tips between each column.

8. While still on the magnetic rack, add **125 µL** of OWash Buffer 3 to each well, switching tips between each column.

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

Note: No vortexing of the plate is required at this step.

10. While still on the magnet, remove and discard the ○ Wash Buffer 3, switching pipette tips between each column.

11. Remove the plate from the magnet and secure it onto a 96 PCR tube rack.

12. With a multichannel P220, add **100 µL** of the Amplification Master Mix to each column, switching pipette tips between each column.

13. Seal the plate with a plastic PCR seal.

14. Centrifuge the plate for **1 minute** at 100 x g in a swinging bucket centrifuge to collect the beads at the bottom of each well.

15. With the plate secured onto a 96 PCR tube rack, mix each column 10x using a P200 multichannel pipette set to 75 µL. Change tips between each column.

16. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

17. Centrifuge for **10 seconds** at 100 x g. Store on ice.

18. 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
12,500-25,000	4	6	5
25,000-62,500	3	5	4
62,500-125,000	2	4	3
125,000-156,250	2	3	2

19. Place the plate into a thermocycler and run the following program.

cDNA AMPLIFICATION			
Run Time	Lid Temperature	Sample Volume	
40-60 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

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 well of amplified cDNA, gather **400 µL** of freshly prepared 85% ethanol (prepare **15 mL** total for 32 sublibraries).
2. Gather room temperature SPRI beads **(85 µL** per well of amplified cDNA, or **2.72 mL** for 32 sublibraries).
3. Vortex the SPRI beads until fully mixed. Aliquot **170 µL** of SPRI beads into each tube of **two** 8-tube PCR strips to facilitate the use of a multichannel pipette. Centrifuge briefly.
4. Preload a new low-bind semi-skirted plate with **72 µL** of SPRI beads in columns 1-4.

Note: Prior to use, ensure the SPRI beads have been equilibrated to room temperature for at least **30 minutes**.

5. Centrifuge the plate of amplified cDNA from Section 2.3 for **10 seconds** at 100 x g.
6. Secure the plate onto a 96 well PCR tube rack, and remove the caps.
7. Place the plate of amplified cDNA from Section 2.3 on the plate magnet. Incubate for **3 minutes**.
8. Mix each column 5x using a P200 multichannel set to 75uL. Change pipette tips between each column.

Note: Mixing is necessary to resuspend the settled beads.

9. Incubate for an additional **2 minutes**.
10. While still on the magnet, transfer **90 µL** of the supernatant containing the amplified cDNA into the PCR plate preloaded with **72 µL** of SPRI Beads, switching tips between each column. Store at room temperature.
11. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips or plastic PCR seal.
12. Vortex the plate for **5 seconds**.

13. Centrifuge for **10 seconds** at 100 x g.

14. Incubate for **3 minutes** at room temperature.

15. With the plate secured on a 96 PCR tube rack, remove the caps.

16. Place the plate on the plate magnet. Incubate until the solution clears (~2 minutes).

17. Add **13 mL** of 85% Ethanol into a new and labeled basin.

18. Add **4 mL** of nuclease-free water into a new and labeled basin. Seal basin with plastic seal.

19. While still on the magnet, remove and discard the supernatant from all 4 columns into a waste basin, switching tips between each column.

20. While still on the magnet, add **180 µL** of 85% ethanol to each well, switching tips between each column.

21. Incubate for **30 seconds** at room temperature after adding to the last column.

22. While still on the magnet, remove and discard the supernatant from all 4 columns, switching tips between each column.

23. Repeat steps 20-22 once for a total of 2 washes. Remove any residual ethanol with a P20.

24. While still on the magnet, air dry the SPRI beads (~1 minute).

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.

25. Remove the plate from the magnet and secure onto a 96 PCR tube rack.

26. Add **75 µL** of nuclease-free water to each well using a P200 multichannel, switching tips between each column.

27. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

28. Vortex for **10 seconds** to resuspend the beads.

29. Centrifuge for **10 seconds** at 100 x g.

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

31. Place the plate on the plate magnet. Incubate until the solution clears (~1 minute).

32. While still on the magnet, transfer **75 µL** of the supernatant containing the purified cDNA into corresponding columns of a new low-bind semi-skirted PCR plate, switching tips between each column.

33. If proceeding immediately with cDNA quantification (Section 2.5) and Section 3, store the plate 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. Before storing, secure the plate in a 96 PCR tube rack on a flat surface, seal with a foil seal and layer with a plastic seal to prevent punctures.

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 well 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.

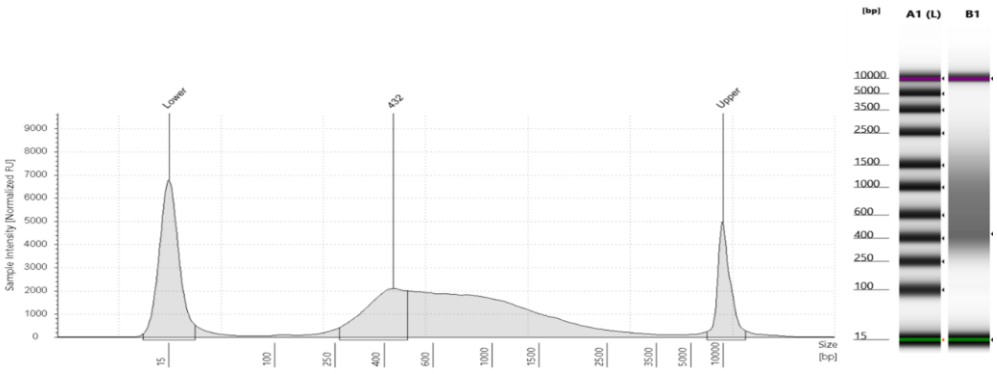


Figure 8: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a Tapestation.

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 TapeStation cDNA traces. The shape and prominence of the trace is dependent on cell type, sublibrary size, and amount of DNA loaded into the 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. Fill an ice bucket.
2. For each sublibrary, prepare 1.2 mL of 85% ethanol with nuclease-free water (total of **50 mL** for 32 sublibraries). The 85% ethanol will be used in Section 3.2.
3. Equilibrate 200 µL of SPRI beads per sublibrary to room temperature (**6.4 mL** for 32 sublibraries). The equilibrated SPRI beads will be used in Section 3.2.
4. Gather the following equipment:
    i. Magnetic plate for semi-skirted PCR plates
    ii. 96 tube rack (for stabilizing plate)
    iii. 8-cap strips
    iv. 25 mL basins
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. If the amplified cDNA was stored at -20°C, start the following program to thaw the plate.

THAW AMPLIFIED cDNA PLATE		
Run Time	Lid Temperature	Sample Volume
5 min	40°C	75 µL
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

8. Vortex the plate of cDNA for **5 seconds**. Briefly centrifuge.

9. Transfer **35 µL** of cDNA into a new low-bind, semi-skirted PCR plate and store on ice. Store any remaining purified cDNA at -20°C.

10. 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 18.

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

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

12. 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	32
Fragm/End Prep Buffer	6 µL	192 µL
Fragm/End Prep Enzymes	12 µL	384µL
Total	18 µL	576 µL

13. Aliquot **70 µL** of Fragmentation and End Prep Master Mix into each well of an 8-tube PCR strip to facilitate the use of multichannel pipette. Store on ice.

14. Add **15 µL** of Fragmentation and End Prep Master Mix to each well tube of the cDNA plate using a P20 multichannel and switching tips between each column.

15. Mix by pipetting 10x with a P200 multichannel pipette set to 40 µL, switching tips between each column.

16. While the plate is secured on a PCR tube rack, seal the plate with caps.

17. Briefly centrifuge for **10 seconds** at 100 x g.

18. Place the plate 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 plate.

19. As soon as the program reaches step 4 of the thermocycling program (4°C), store the plate 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 the 85% ethanol prepared in Section 3.1.
2. Prepare and label the basins as follows: "ethanol", "nuclease-free water" and "waste" for later use.
3. Gather room temperature SPRI beads equilibrated in Section 3.1.

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

4. Vortex the SPRI beads until fully mixed. Aliquot **140 µL** of SPRI Beads into each tube of an 8-tube PCR strip to facilitate the use of a multichannel pipette. Centrifuge briefly.
5. With the plate containing fragmented DNA secured on a 96 PCR tube rack, carefully remove the caps.
6. Add **30 µL** of SPRI beads to each well of fragmented and end prepped DNA using a P200 multichannel, switching tips between each column.
7. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.
8. Vortex the plate for **5 seconds**.
9. Centrifuge for **10 seconds** at 100 x g.
10. Incubate for **3 minutes** at room temperature.
11. With the plate secured on a 96 PCR tube rack, remove the caps.
12. Place the plate on the plate magnet. Incubate until the solution clears (~3 minutes)
13. During the incubation, vortex again the prepared SPRI beads until fully mixed. Aliquot **100 µL** of SPRI beads into each tube of a new 8-tube PCR strip to facilitate the use of a multichannel pipette. Centrifuge briefly.
14. Preload a new low-bind semi-skirted plate with **20 µL** of SPRI beads in columns 1-4. Secure on 96 PCR tube rack.

15. While still on the magnetic rack, transfer **75 µL** of the supernatant containing the fragmented and end prepped DNA to the PCR plate preloaded with **20 µL** of SPRI Beads, switching tips between each column. Set the plate with original bead pellets aside.

16. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

17. Vortex the plate for **5 seconds**.

18. Centrifuge for **10 seconds** at 100 x g.

19. Incubate for **3 minutes** at room temperature.

20. With the plate secured on a 96 PCR tube holder, remove the caps.

21. Place the plate on the plate magnet. Incubate until the solution clears (~3 minutes).

CRITICAL! Ensure the solution is completely clear before proceeding.

22. Add **13 mL** of 85% Ethanol into the previously labeled basin.

23. While still on the magnetic rack, remove and discard the supernatant from all 4 columns into a waste basin, switching tips between each column.

24. While still on the magnetic rack, add **180 µL** of 85% ethanol to each well, switching tips between each column.

25. After adding ethanol to the last column, start the timer to incubate for **30 seconds** at room temperature.

26. While still on the magnetic rack, remove and discard the supernatant from all 4 columns, switching tips between each column.

27. Repeat steps 24-26 once for a total of 2 washes. Remove any residual ethanol with a P20 multichannel.

28. 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.

29. Remove the plate from the magnet and secure onto a 96 PCR tube rack.

30. Using a P200 multichannel, transfer **50 µL** of nuclease-free water into each well from the pre-prepared water basin, switching pipette tips between each column.

31. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

32. Vortex for **10 seconds** to resuspend the beads.

33. Centrifuge for **10 seconds** at 100 x g.

34. Incubate for **3 minutes** at room temperature.

35. While secured in a 96 PCR tube rack, remove the caps.

36. Place the plate on the magnet. Incubate until the solution clears (~1 minute).

37. While still on the magnet rack, transfer **50 µL** of the supernatant into corresponding columns in a new low-bind semi-skirted PCR plate, switching tips between each column. Seal with a plastic seal and store on ice.

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	
Library Amp Mix	-20°C Reagents	1	
UDI Plate - WT	-20°C Reagents	1 sealed well per sublibrary	Thaw at room temperature then place on ice.
Adapter Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

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

ADAPTER LIGATION MASTER MIX		
Number of Sublibraries	1	32
Nuclease-free water	21 µL	672 µL
Adapter Ligation Buffer	24 µL	768 µL
Adapter Ligation Enzyme	12 µL	384 µL
Ligation Adapter	3 µL	96 µL
Total	60 µL	1920 µL

3. Aliquot **220 µL** of Adaptor Ligation Master Mix into each tube of an 8-tube PCR strip to facilitate the use of multichannel pipette. Store on ice.

4. Add **50 µL** of Adapter Ligation Master Mix to each well of purified fragmented and end prepped DNA from Section 3.2 using a P200 multichannel pipette. Switch tips between columns.

5. Mix by pipetting 10x with a P200 multichannel pipette set to 80 µL, switching tips between each column.

6. Seal the plate with a plastic PCR seal.

7. Briefly centrifuge for **10 seconds** at 100 x g.

8. Place the plate 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.

9. 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 (prepare **15 mL** for 32 sublibraries) and 1-3 mL nuclease-free water.
2. Prepare and label the basins as follows: "ethanol", "nuclease-free water" and "waste" for later use.
3. Gather room temperature SPRI beads (~90 µL per sublibrary, ~3 mL for 32 sublibraries).

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

4. Vortex the SPRI beads until fully mixed. Aliquot **180 µL** of SPRI beads into each of two 8-tube PCR tubes (total of 16 tubes) to facilitate the use of multichannel pipette. Centrifuge briefly.
5. Secure the plate of adapter ligated DNA from Section 3.3 onto a 96 tube PCR rack. Remove the plastic PCR seal.
6. Add **80 µL** of SPRI beads to each well of adapter ligated DNA plate using a P200 multichannel, switching tips between each column.
7. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.
8. Vortex the plate for **5 seconds**.
9. Centrifuge for **10 seconds** at 100 x g.
10. Incubate for **3 minutes** at room temperature.
11. With the plate secured on a 96 PCR tube rack, remove the caps.
12. Place the plate on the plate magnet. Incubate until the solution clears (~2 minutes).
13. Add **15 mL** of 85% Ethanol into the previously labeled basin.
14. While still on the magnetic rack, remove and discard the supernatant from all 4 columns into a waste basin, switching pipette tips between columns.

15. While still on the magnetic rack, add **180 µL** of 85% ethanol to each well, switching tips between each column.

16. Incubate for **30 seconds** at room temperature after adding to the last column.

17. While still on the magnet, remove and discard the supernatant from all 4 columns, switching pipette tips between columns.

18. Repeat steps 15-17 once for a total of 2 washes. Remove any residual ethanol with a P20.

19. While still on the magnetic rack, air dry the SPRI beads (~1 minute).

20. Remove the plate from the magnetic rack and secure onto a 96 PCR tube rack.

21. Pour 1 mL of nuclease-free water into the basin labeled "water" and add **23 µL** of nuclease-free water to each well using a P200 multichannel, switching tips between each column.

22. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secure

23. Vortex for **10 seconds** to resuspend the beads.

24. Centrifuge for **10 seconds** at 100 x g.

25. Incubate for **3 minutes** at room temperature.

26. Place the plate on the plate magnet. Incubate until the solution clears (~1 minute).

27. While still on the magnetic rack, transfer exactly **21 µL** of the supernatant containing the purified adapter ligated DNA into a new low-bind semi-skirted PCR plate, switching tips between each column. Store on ice.

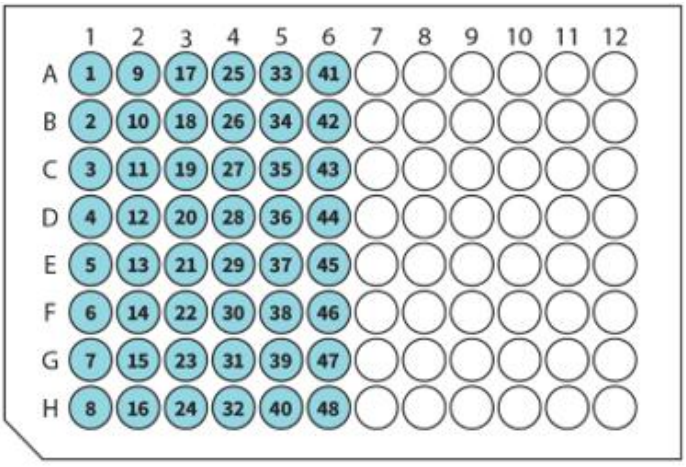
28. Proceed immediately to Section 3.5.

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 column, choose one unused column of the UDI Plate - WT to use and record the well position and number for each sublibrary.



4. With a multichannel P20, pierce the seal of the chosen columns 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 column of the UDI Plate - WT to its corresponding column of purified adapter ligated DNA from Section 3.4.

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

6. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C. Unused wells can be used for future sublibraries. Do not reuse wells once they have been pierced.
7. Mix ● Library Amp Mix 10x with a P1000 pipette set to 800 µL.

8. Aliquot **120 µL** of ● Library Amp Mix into each well of an 8-tube PCR strip to facilitate the use of a multichannel pipette. Briefly centrifuge, and store on ice.

9. Add **25 µL** of ● Library Amp Mix to each well and mix by pipetting 10x with a P200 multichannel pipette set to 25 µL, switching tips between columns.

10. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

11. Centrifuge for **10 seconds** at 100 x g.

12. Determine the number of PCR cycles required for the Indexing PCR based on the **average** amount of cDNA inputted into the fragmentation and end prep reaction as recorded in Section 2.5.

NUMBER OF PCR CYCLES		
cDNA concentration (End of Section 2.5)	cDNA Input (ng)	PCR Cycles
0.35 - 0.7 ng/µL	10-24	13
0.71 - 1.4 ng/µL	25-49	12
1.41 - 2.84 ng/µL	50-99	11
2.85 - 8.56 ng/µL	100-299	10
8.57- 28.4 ng/µL	300-999	8
28.5+ ng/µL	1,000 or more	7

13. 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

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 (prepare **15 mL** for 32 sublibraries).
2. Prepare and label three basins for later use.

Note: Label the basins as follows: "ethanol", "nuclease-free water" and "waste".

3. Gather room temperature SPRI beads (~50 µL per sublibrary, ~1.8mL for 32 sublibraries).

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

4. Vortex the SPRI beads until fully mixed. Aliquot **140 µL** of SPRI Beads into an 8-tube PCR strip to facilitate the use of a multichannel pipette. Centrifuge briefly.
5. With the plate containing DNA secured on a 96 PCR tube rack, carefully remove the caps.
6. Add **30 µL** of SPRI beads to each well containing a sequencing library using a P200 multichannel pipette, switching tips between each column.
7. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.
8. Vortex the plate for **5 seconds**.
9. Centrifuge for **10 seconds** at 100 x g.
10. Incubate for **3 minutes** at room temperature.
11. With the plate secured on a 96 PCR tube rack, remove the caps.
12. Place the plate on the plate magnet. Incubate until the solution clears (~3 minutes)
13. While incubating, vortex the SPRI beads until fully mixed. Aliquot **50 µL** of SPRI beads into each tube of an 8-tube PCR strip to facilitate the use of a multichannel pipette. Centrifuge briefly.

14. Preload a new low-bind semi-skirted plate with **10 µL** of SPRI beads in columns 1-4. Secure on 96 PCR tube rack.

15. While still on the magnet, transfer **75 µL** of the supernatant containing the DNA to the PCR plate preloaded with 10 µL of SPRI Beads, switching tips between each column. Discard the plate with bead pellets.

16. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

17. Vortex the plate for **5 seconds**.

18. Centrifuge for **10 seconds** at 100 x g.

19. Incubate for **3 minutes** at room temperature.

20. With the plate secured on a 96 PCR tube holder, remove the caps.

21. Place the plate on the plate magnet. 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.

22. Add **13 mL** of 85% Ethanol into previously prepared labeled basin.

23. Add **3 mL** of nuclease-free water into the previously prepared basin. Cover with plastic PCR seal.

24. While still on the magnetic rack, remove and discard the supernatant from all 4 columns into the waste basin, switching tips between each column.

25. While still on the magnetic rack, add **180 µL** of 85% ethanol to each well, switching tips between each column.

26. Starting the timer after adding ethanol to the last column, incubate for **30 seconds** at room temperature.

27. While still on the magnetic rack, remove and discard the supernatant from all 4 columns.

28. Repeat steps 25-27 once for a total of 2 washes. Remove any residual ethanol with a P20 multichannel set to 20 µL.

29. Remove the plate from the magnet and secure onto a 96 PCR tube rack.

30. Add **20 µL** of nuclease-free water to each well using a P200 multichannel pipette. Change tips between each column.

31. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

32. Vortex for **10 seconds** to resuspend the beads.

33. Centrifuge for **10 seconds** at 100 x g.

34. Incubate for **3 minutes** at room temperature.

35. While secured in a 96 PCR tube rack, remove the caps.

36. Place the plate on the magnet. Incubate until the solution clears (~1 minute).

37. While still on the magnetic rack, transfer **20 µL** of the supernatant containing the final library DNA into corresponding columns in a new low-bind semi-skirted PCR plate, switching tips between each column.

38. If proceeding immediately with library quantification, store the plate on ice.

Safe stopping point: Sequencing libraries can be stored at -20°C for up to 3 months. Before storing, secure the plate in a 96 PCR tube rack on a flat surface, seal with a foil seal and layer with a plastic seal to prevent punctures.

3.7. Sequencing Library Quantification

The concentration and size distribution of the sequencing libraries are measured with fluorescent dyes and capillary electrophoresis. Sequencing libraries can be stored at -20°C for up to 3 months.

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.

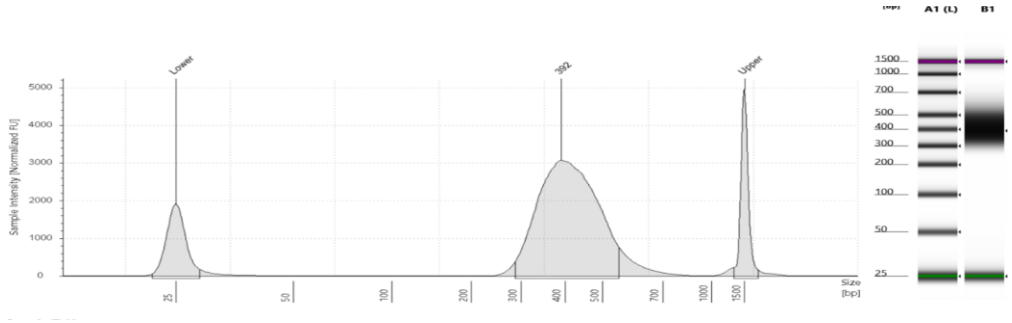


Figure 9: Expected Size Distribution before Illumina Sequencing. Example trace of DNA from indexed sublibraries run on a TapeStation.

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 TapeStation of DNA from indexed sublibraries. There should be a peak between 350-450 bp. The prominence of the trace is dependent on the amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high quality data.

Note: If using a Bioanalyzer, there may be an additional peak present. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 350-450 bp). Do not use this additional peak when estimating amplicon size.

Appendices

Appendix A: 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.

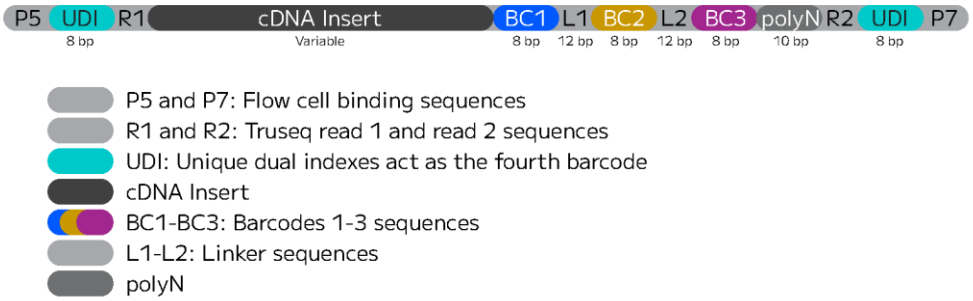


Figure 10: Whole transcriptome sequencing library structure.

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 acts 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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
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
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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
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 B: Thermocycling Programs

Section 1*: In Situ* Cell/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

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	Hold

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

THAW LYSATE PLATE		
Run Time	Lid Temperature	Sample Volume
5 min	40°C	55 µL
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

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	
40-60 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
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

THAW AMPLIFIED cDNA PLATE		
Run Time	Lid Temperature	Sample Volume
5 min	40°C	75 µL
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

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 C: Revision History

Version	Description	Date
1.0	Initial Release	February 2025

PARSE BIOSCIENCES

parsebiosciences.com

support@parsebiosciences.com