

User Manual

Version 1.0 – UMWT4730



Evercode™ WT Penta 384

v4

For use with

ECWT 4730



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U.S. Pat. No. 10,900,065

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U.S. Pat. No. 11,639,519

U.S. Pat. No. 11,680,283

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Overview

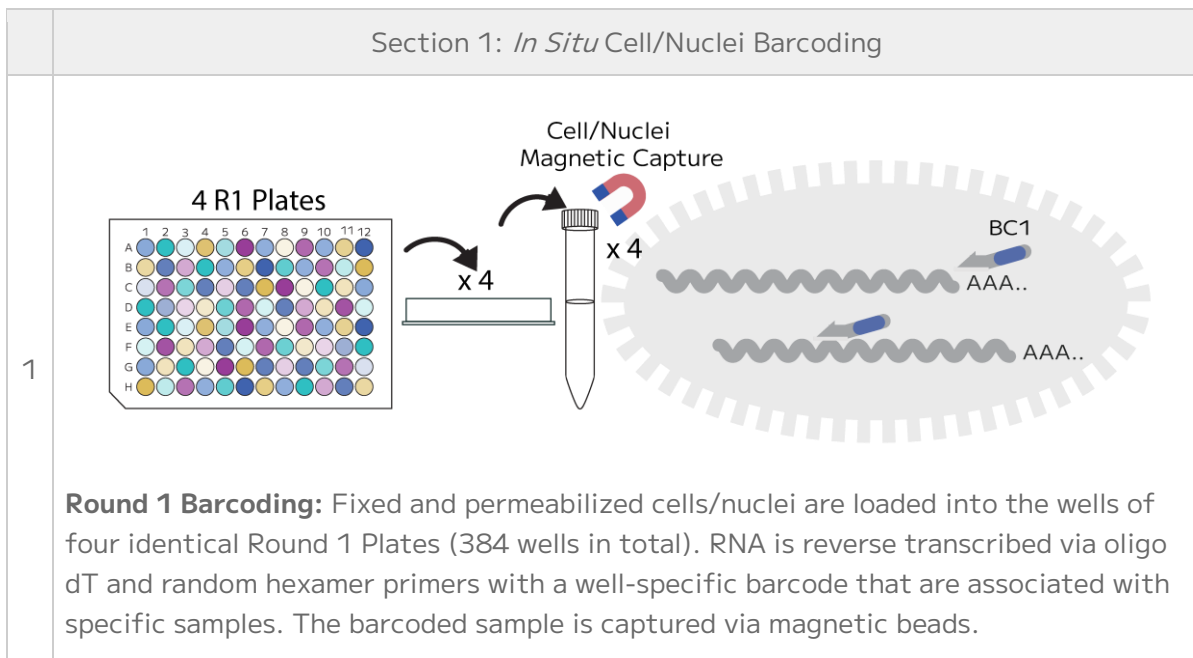
Workflow

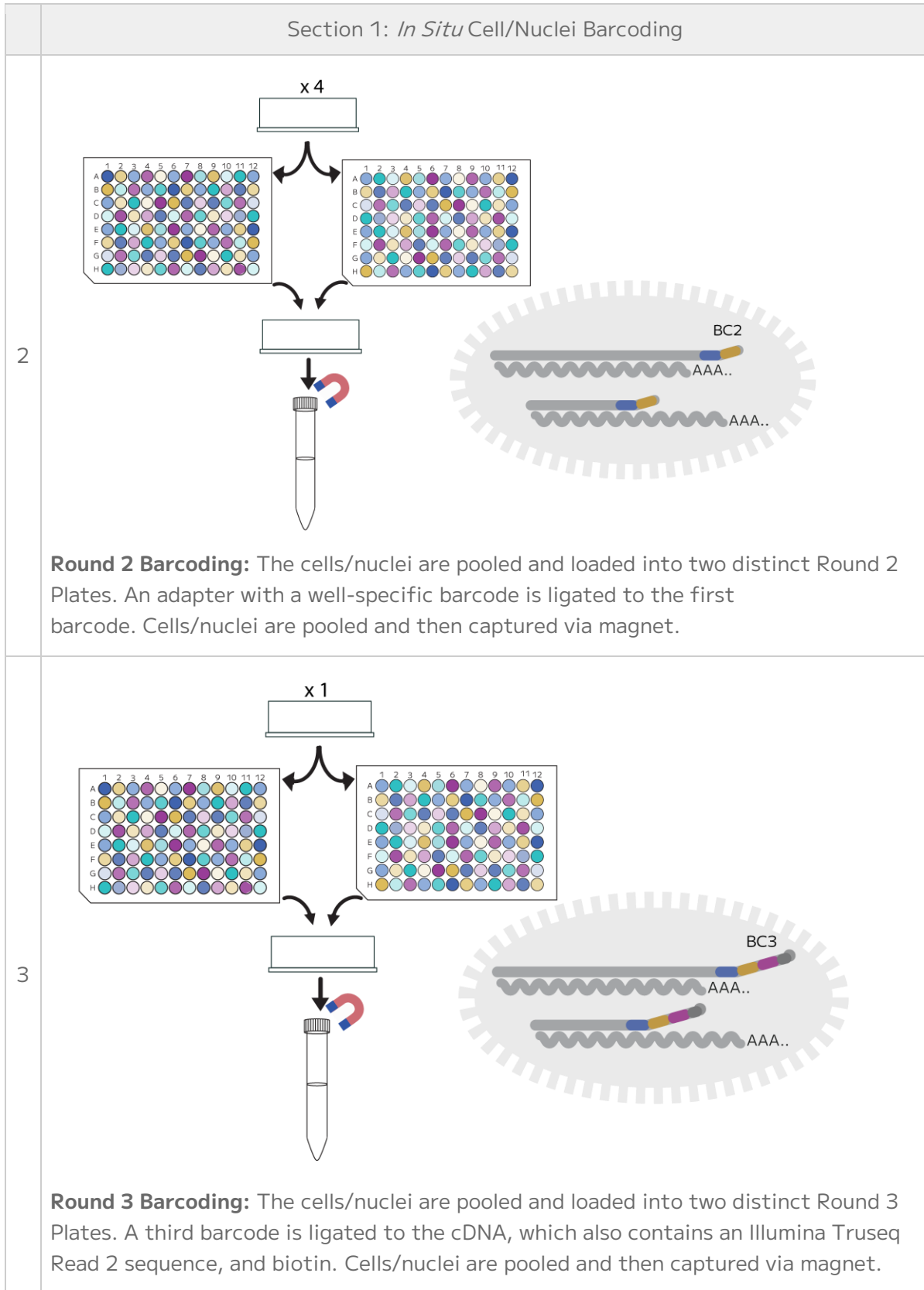
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT Penta 384 v4 kit can profile up to 5,000,000 cells across up to 384 different biological samples or experimental conditions.

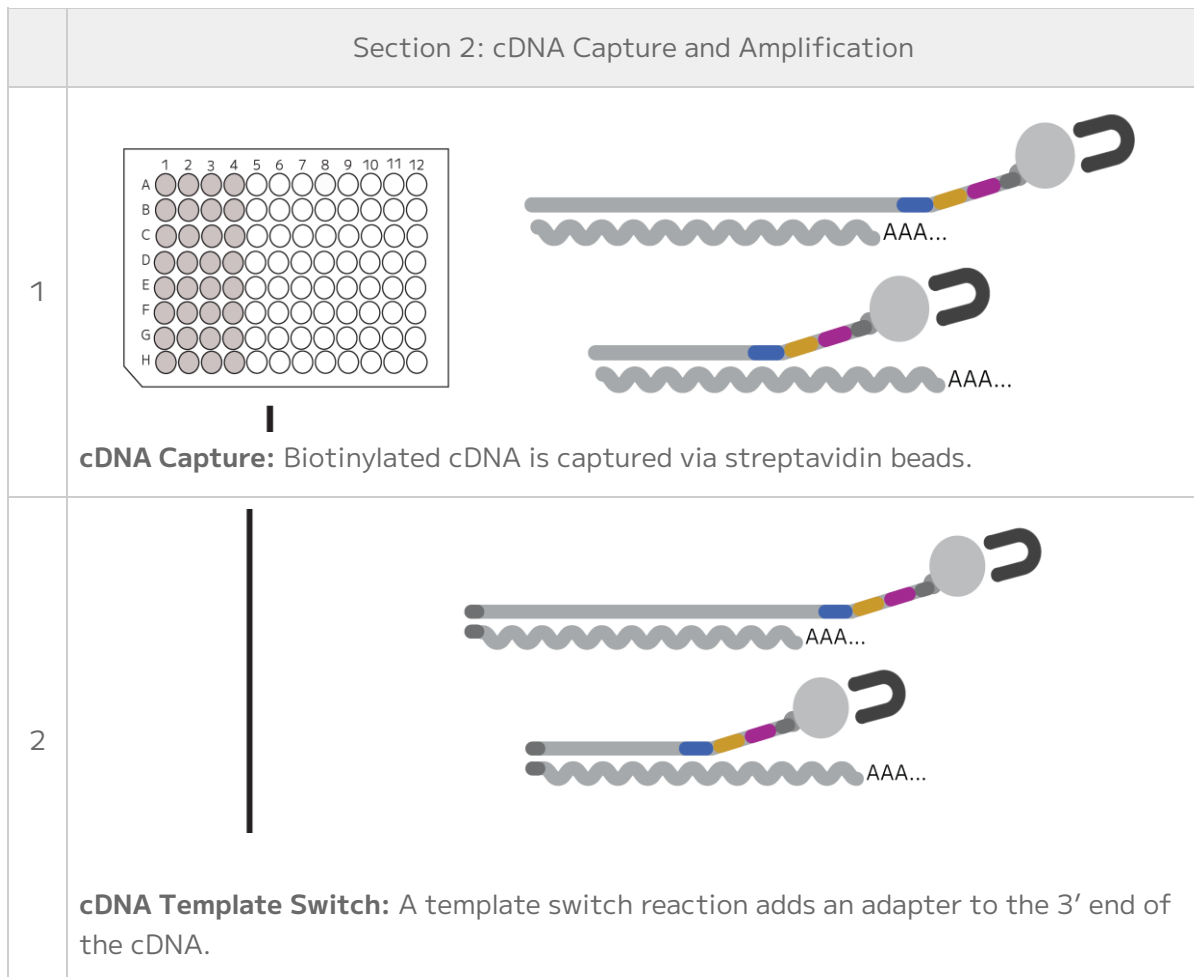
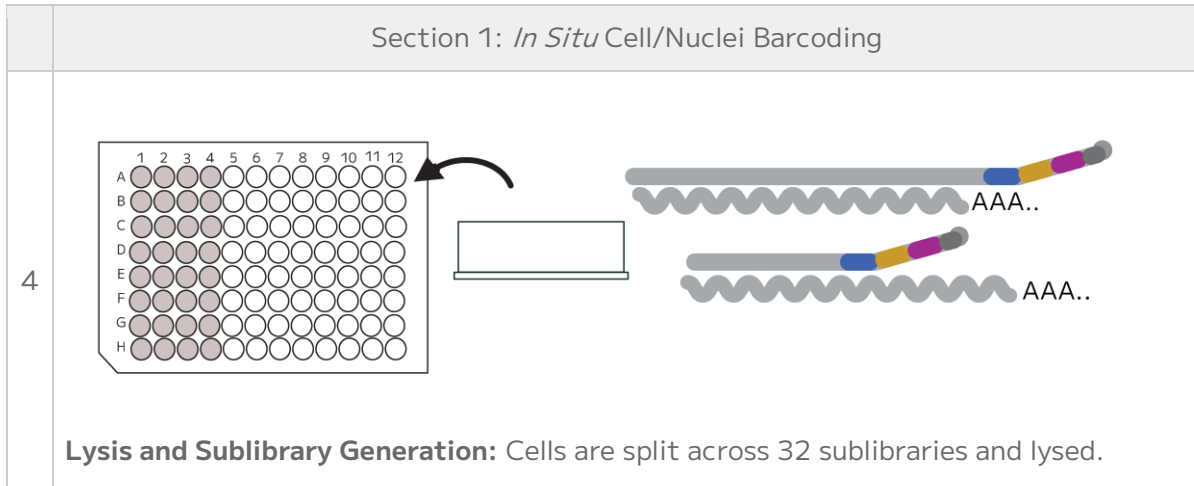
Evercode Fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding 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.

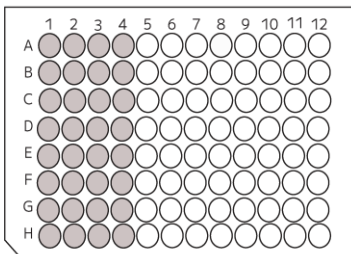


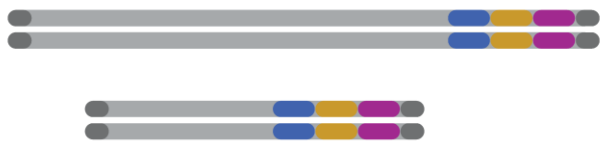




Section 2: cDNA Capture and Amplification

3

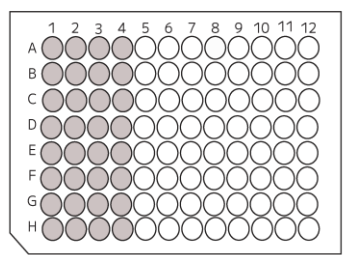


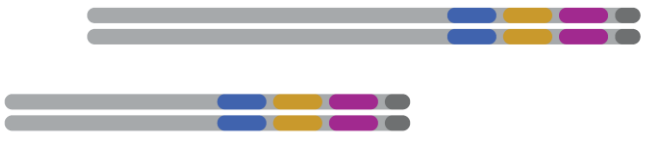


cDNA Amplification: The cDNA is amplified with primers specific to the template switch adapter and Illumina Truseq Read 2.

Section 3: Sequencing Library Preparation

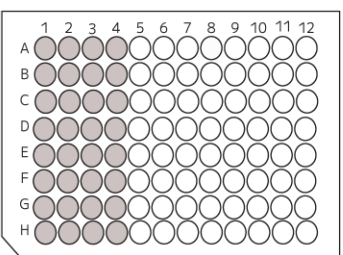
1

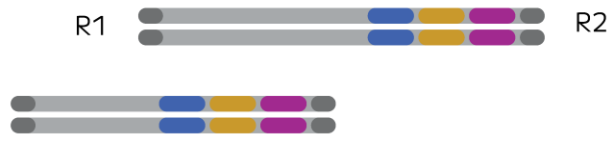




Fragmentation and End Repair: DNA is fragmented, ends are repaired, and A-tailed.

2





Adapter Ligation: An Illumina Truseq R1 Adapter is ligated to the 5' end of the DNA.

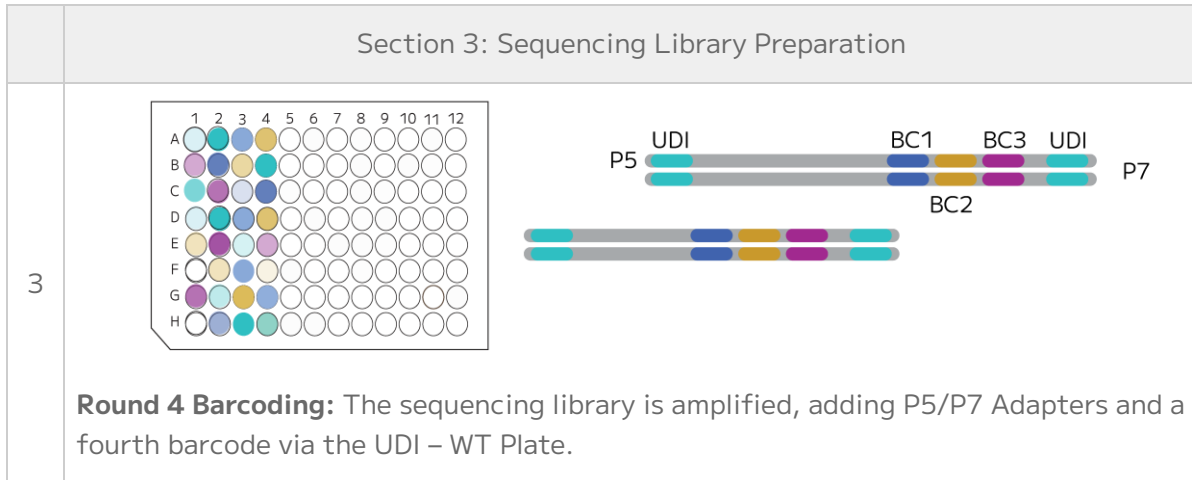


Figure 1 shows the WT Penta 384 workflow. This kit can barcode up to 5 million cells/nuclei across 384 samples in a single reaction. The Penta 384 utilizes four identical Round 1 Plates, each holding a unique set of 96 samples, totaling 384 samples. These samples are pooled from the four Round 1 Plates 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).

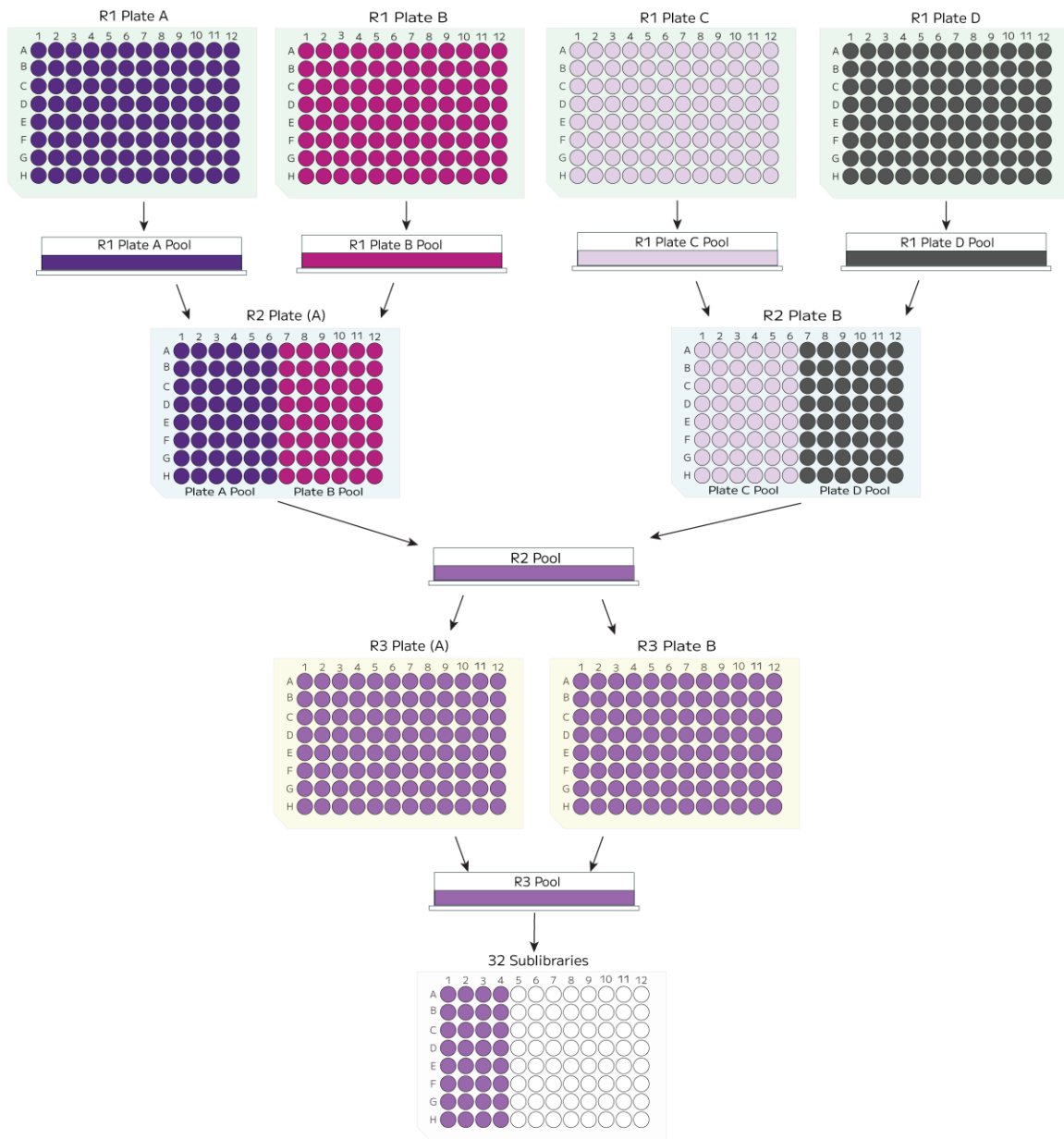


Figure 1: Evercode™ WT Penta 384 workflow pools 384 samples from four identical Round 1 Barcoding Plates into two distinct Round 2 and Round 3 Barcoding Plates respectively, generating 32 Sequencing Libraries.

Penta 384 Stickers v4:

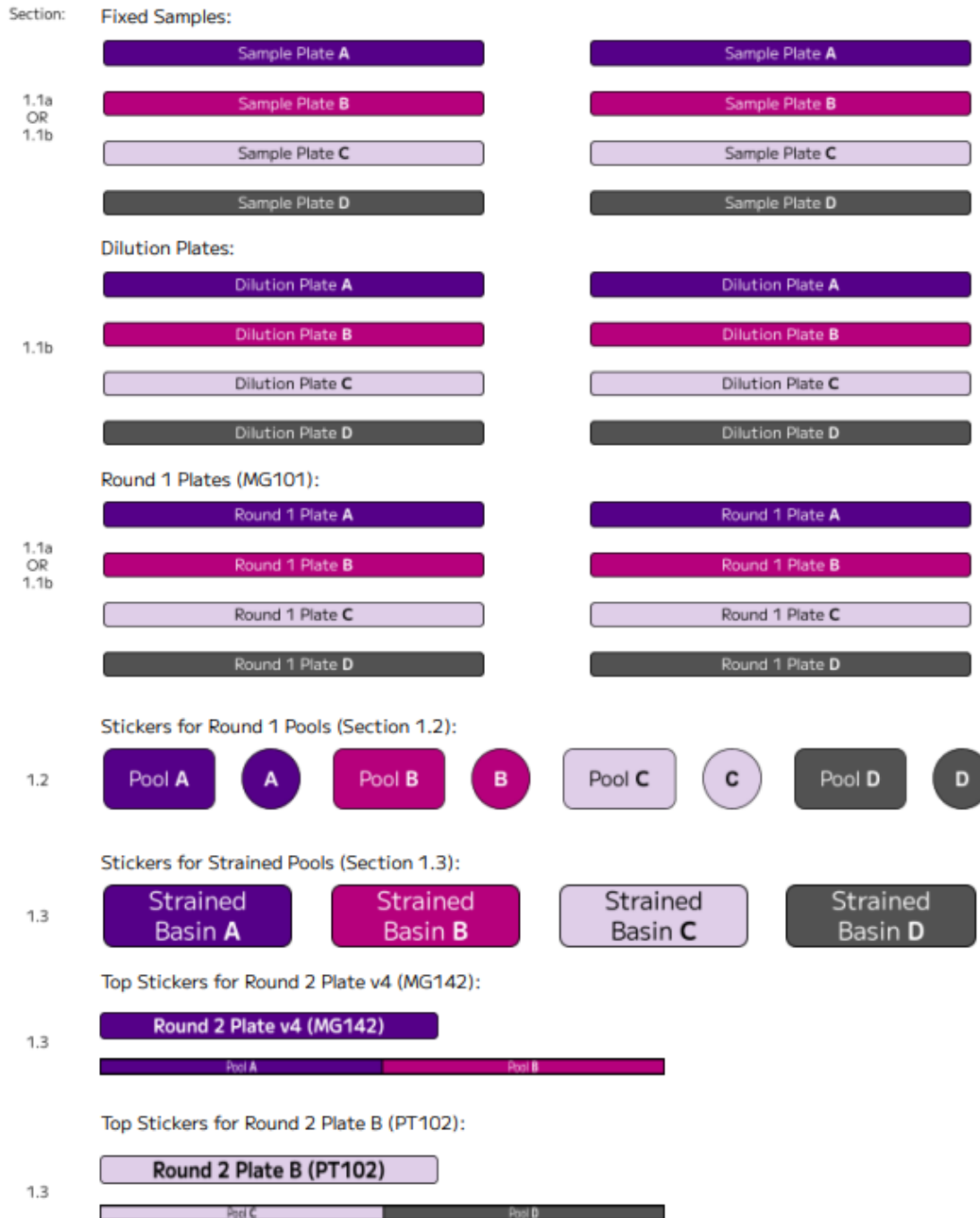


Figure 2: Plates are easy to handle with labeled color-coded stickers throughout the Round 1 Barcoding section.

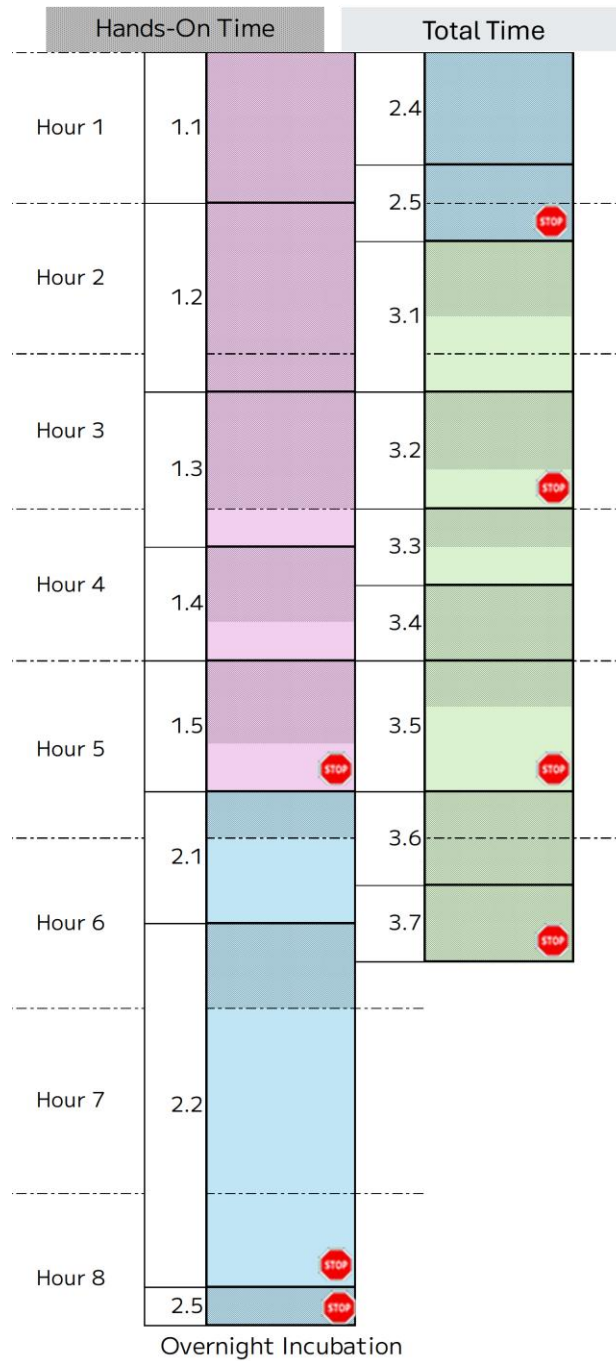
Protocol Timing

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.

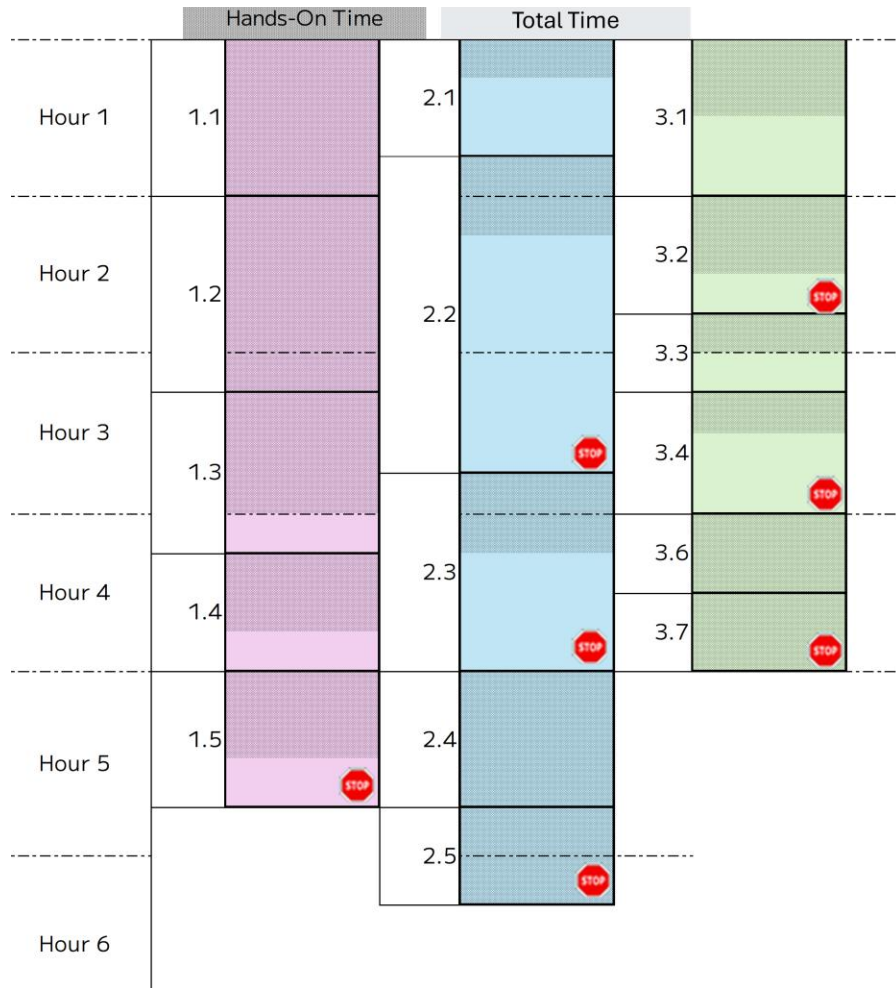
DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
Section 1: <i>In Situ</i> Cell/Nuclei Barcoding			
1.1a Set up for Low Input Fixation Samples OR 1.1b Set up for Standard Fixation	Variable 60-90 min	Variable 60-90 min	
1.2 Pooling Round 1	75 min	75 min	
1.3 Barcoding Round 2	60 min	45 min	
1.4 Barcoding Round 3	45 min	30 min	
1.5 Lysis and Sublibrary Generation	45 min	30 min	-80°C ≤ 6 months
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	45 min	15 min	
2.2 cDNA Template Switch	120 min	30 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	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
3.3 Adapter Ligation	30 min	15 min	
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

DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
3.6 Post-Barcoding Round 4 Size Selection	30 min	30 min	
3.7 Sequencing Library Quantification	30 min	30 min	-20°C ≤ 3 months

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 the Evercode Cell Fixation v3 and v4 kits, or Evercode Nuclei Fixation v3 and v4 kits, the Evercode Low Input Cell and Nuclei Fixation v3 and v4 kits, or the Evercode Whole Blood Fixation kit, high throughput workflow.
- 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 Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA”, create Sample Dilution Plates as shown on the “Dilution and R1 Plate Config.” tab (see Section 1.1b for more information).
- For low input fixed samples, bead and storage buffer volumes vary based on cell/nuclei input. With 384 samples, loading can become complex and time-consuming. To streamline bead capture and keep it within a manageable timeframe, we strongly recommend normalizing cell/nuclei counts across sets of 12 or 8 prior to fixation.
- Once fixed samples have been thawed, they should not be refrozen.

Cell/Nuclei Counting and Quality Assessment

- For initial cell/nuclei counting a hemocytometer can be used. For pre-lysis sample counting, however, a fluorescent counting device is needed. Given the scale of high-throughput experiments, we recommend using an automated fluorescence cell counter. 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.

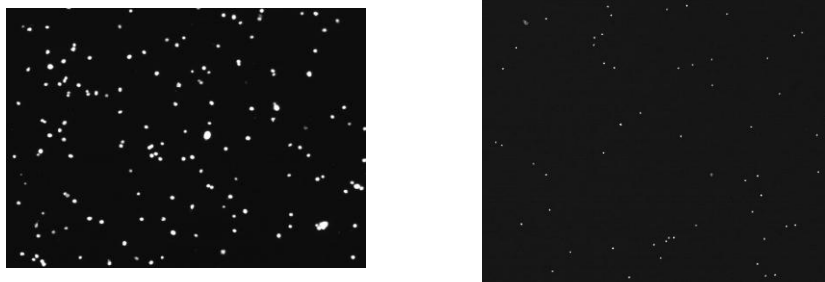


Figure 3: example of AO/DAPI stained HEK cells (left) and PBMCs (right).

High Quality Sample

Aggregation

Debris

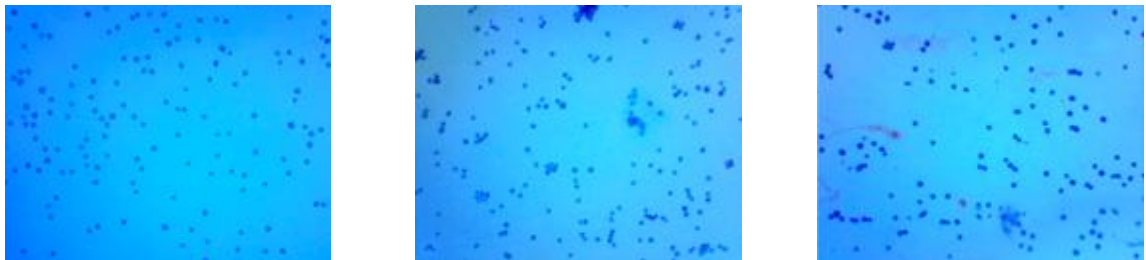


Figure 4: Example of trypan blue stained fixed cells.

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.

Optimizing Cell/Nuclei Recovery

- 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 sample loading table should be completed before starting the experiment. Based on the selected fixation method, fill out either the Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA or the Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA.
- 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 μ L 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 μ m is appropriate for many cell types, the mesh size should be chosen based on the sample type.
- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. An example video can be found in our support site.

Vortexing

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

Plate Sealing

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

Magnetic Racks and Bead Cleanups

- The Evercode WT Penta 384 v4 workflow uses magnetic beads to clean up cells/nuclei pools after each round of barcoding. A magnetic rack capable of holding 15 mL conical tubes and one capable of holding 1.5 mL tubes are needed throughout Section 1 of this protocol.
- Three validated 15 mL conical tube magnets are described in "User Supplied Equipment and Reagents" section of this manual to choose from.
- A PCR plate magnet is needed for capturing lysed cells and library preparation steps.
- 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 tube to ensure all supernatant is removed.
- If beads are disturbed, dispense solution again and wait for 1 minute 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 2 sets of 8 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. If using different UDI plates, use unique wells to avoid duplicate barcodes.
- We recommend selecting UDIs column-wise from left to right (starting with indices 1-8). UDI sequences can be found in Appendix A.
- Record 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 v4 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents Store at -20°C, PTT400

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	MG101	Green semi-skirted 96 well plate	4
	Round 2 Plate v4	MG142	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
	Sample Dilution Buffer	MG132	5 mL tube	1
	Bead Prep Buffer	PT136	5 mL tube	1
	Barcoding Buffer	PT137	15 mL tube	3
	Ligation Buffer	PT134	15 mL tube	2
	Round 1 Stop Mix	PTT138	5 mL tube	1
	Ligation Enzyme	PT140	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Round 2 Stop Mix	PT139	5 mL tube	1
	Final Stop Mix	PT141	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
	Lysis Solution	PT146	2 mL tube	1
	Bead Wash Buffer	PT111	5 mL tube	2
	Binding Buffer	PT112	5 mL tube	1
	Wash Buffer A	PT143	5 mL tube	3
	Wash Buffer B	PT144	5 mL tube	3
	Template Switch Buffer	PT148	5 mL tube	1
	Template Switch Enzyme	PT117	1.5 mL tube	1
	Template Switch Primer	PT118	1.5 mL tube	1
	cDNA Amp Mix	PT119	5 mL tube	1
	cDNA Amp Primers	PT120	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	PCR Additive	PT150	1.5 mL tube	1
	Fragm/End Prep Buffer	PT121	1.5 mL tube	1
	Fragm/End Prep Enzymes	PT122	1.5 mL tube	1
	Ligation Adapter	MG127	1.5 mL tube	1
	Adapter Ligation Buffer	PT123	1.5 mL tube	1
	Adapter Ligation Enzyme	PT124	1.5 mL tube	1
	Library Amp Mix	PT125	1.5 mL tube	1
N/A	Sticker Labels	N/A	Sticker Sheet	N/A

4°C Reagents Store at 4°C, PTT500

LABEL	ITEM	PN	FORMAT	QTY
	Sample Binding Beads	PT135	2 mL tube	1
	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
Parse Biosciences Magnetic Rack	Parse Biosciences	SB1004	Optional: use when processing 16 libraries at the time. We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection.
Permagen Bar Magnet	Permagen®	MSPU650	To use in Section 2 and 3 only. We do not recommend alternative magnets, as they may result in lower transcript and gene detection.
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.
15 mL Conical Tube Magnet (choose one)	Permagen	MSR6X15	6 X 15 mL Centrifuge Magnetic Separation Rack.
	Millipore	LSKMAGS15	PureProteome Magnetic Stand.
	STEMCELL Technologies	18103	EasyEights™ EasySep™ Magnet.
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.
Fluorescence Cell Counter	Various Suppliers	Varies	

ITEM	SUPPLIER	PN	NOTES
PCR tube rack	Various Suppliers	Varies	Capable of holding semi-skirted 96 well PCR plates.
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.
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	
Qubit™ Flex Fluorometer	Thermo Fisher Scientific®	Q33327	Or an equivalent fluorometer.
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.
4200 TapeStation System	Agilent	G2991BA	

Consumables

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

ITEM	SUPPLIER	PN	NOTES
			basins as polystyrene may lead to some cell loss.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial cell loss.
Falcon® High Clarity PP Centrifuge Tubes, 50 mL	Corning®	352098	
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	
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.
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.
TempPlate® EXT Sealing Foil	USA Scientific®	2998-0100	

ITEM	SUPPLIER	PN	NOTES
5 mL tubes	Eppendorf	30108310	
Strips 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.

Reagents

ITEM	SUPPLIER	PN	NOTES
SPRIselect Reagent	Beckman Coulter	B23317 (5mL) B23318 (60mL)	Choose one. We do not recommend substituting other magnetic beads.
AMPure® XP Reagent	Beckman Coulter®	A63880 (5 mL) A63881 (60mL)	
MAGFLO™ NGS Size Select	INTEGRA	7000 (1 mL) 7002 (5 mL)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	
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 Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA. 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 Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA", 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 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.
 Ligation 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 or pipette mix until completely 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.
 Storage Buffer	Low Input Fixation Reagents (-20°C)	8**	Thaw at room temperature then immediately store on ice. Mix by inverting. Do not vortex.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Sticker Sheet	4°C Reagents and Stickers	1	Remove sheet and follow instructions for plate and tube labeling.

*The high throughput 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.

- Gently pulse-vortex (1-2 seconds per pulse) or pipette mix) ● Cell/● Nuclei Binding Beads until completely resuspended.
- 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

- Place the four tubes on the 1.5 mL tube magnetic rack until the solution clears (~ 2 minutes).
- With the tubes still on the magnetic rack, remove and discard the supernatant.
- Remove the tubes from the magnetic rack and fully resuspend each bead pellet.
- 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 **○ Storage Buffer** following the table below.

STORAGE BUFFER (4)		
Number of Samples	1	96
○ Storage Buffer	15 μ L	1.44 mL



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

16. Using a P200 pipette set to 110 μ L, aliquot **110 μ L** of beads resuspended in **○ 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	3 minutes	
Lid Temperature	70°C	
Sample Volume	26 μ L	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

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

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



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

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

THAW CELLS/NUCLEI (2)		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	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 the Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA 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. During the bind step, add **20 mL** of O Storage Buffer to a clean basin (refilling with more volume as needed). The Storage Buffer will be used in a later step.

28. 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).

29. Remove the plate from the magnetic rack and place it on ice.

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



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	3 minutes	
Lid Temperature	40°C	
Sample Volume	200 μ L*	
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 μ L** 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 Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA" 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	40 minutes		
Lid Temperature	70°C		
Sample Volume	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 ([Section 1.2](#)).

1.1b Set Up for Standard Fixation Samples

Prior to barcoding, users are expected to count cells or nuclei using a counting plate in order to ascertain the concentration of all 384 samples. This will allow for using variable Sample Dilution Buffer volumes to dilute with a constant volume of fixed samples. This will streamline the loading process into the Round 1 Plate and minimize dedicated processing time. In the following protocol, we will designate the four plates of fixed samples as A, B, C and D. Appropriate dilutions, loading concentrations, and loading positions are determined by the Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA. 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 Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA", 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
○ Sample Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Sticker Sheet	4°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 4°C Reagents and Stickers box.

7. Create the corresponding dilution plates of Sample Dilution Buffer using the Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA.



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 Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA", which will guide sample dilutions and plate loading in later steps.
9. Fill a bucket with ice.
10. Have two or four thermocyclers on standby.



Note: If only 2 thermocyclers are available, process Plates A and B first, keeping Plates C and D on ice.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
<input type="checkbox"/> Round 1 Plate	-20°C Reagents	4	Place directly on ice.
<input type="radio"/> Round 1 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
<input type="radio"/> Ligation Buffer	-20°C Reagents	1	
<input checked="" type="radio"/> Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

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	3 minutes	
Lid Temperature	40°C	
Sample Volume	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	3 minutes	
Lid Temperature	70°C	
Sample Volume	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	3 minutes	
Lid Temperature	40°C	
Sample Volume	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.

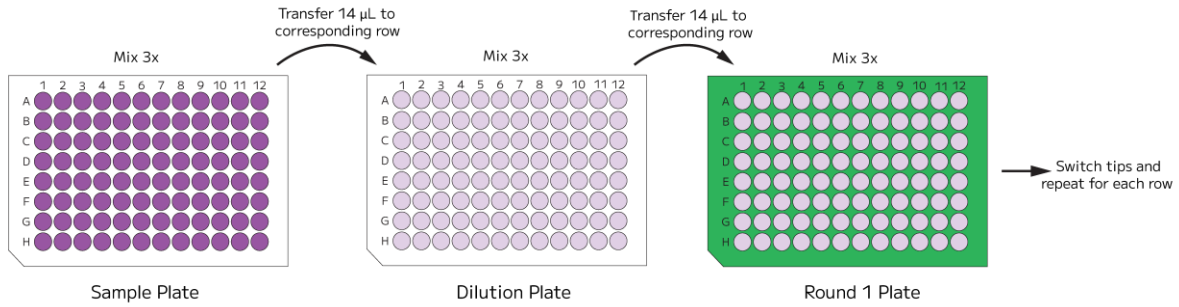


Figure 5: Samples in the sample plate are diluted and then transferred to the Round 1 Plate.

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	40 minutes		
Lid Temperature	70°C		
Sample Volume	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	1
8	5 min	50°C	
9	Hold	4°C	1

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

25. 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 (Section 1.2).



Note: If only 2 thermocyclers are available, store the completed Plates A and B on ice, while Plates C and D undergo Barcoding Round 1.

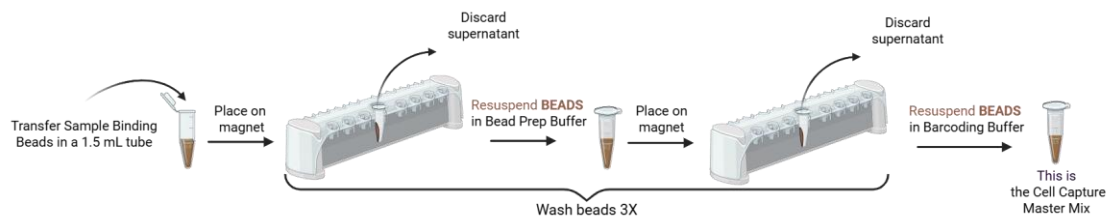
1.2 Pooling Round 1 Plates

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
○ Bead Prep Buffer*	-20°C Reagents	1	*Used for standard fixed samples only*. Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Round 1 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
○ Barcoding Buffer	-20°C Reagents	1	Thaw in 37°C water bath, then store on ice.
○ Ligation Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Sample Binding Beads*	4°C Reagents	1	*Used for standard fixed samples only*. Keep at room temperature. Mix before use to ensure beads are not settled.

2. **If using low input samples**, the cells/nuclei are already attached to the binding beads and no new beads need to be prepared. **Proceed directly to step 12.**
3. **If using standard fixation samples**, proceed to the next step to prepare the ● Sample Binding Beads to capture the cells/nuclei. See workflow below.



Cell Capture Master Mix: Sample Binding Beads are washed three times with Bead Prep Buffer, then resuspended in Barcoding Buffer.

4. While the thermocycling program is still running, gently pulse-vortex ● Sample Binding Beads to resuspend and store at room temperature. Do not let them settle for >3 minutes before pipetting. Transfer **800 µL** of ● Sample Binding Beads into two 1.5 mL Eppendorf tubes (for a total of **1,600 µL** of ● Sample Binding Beads used).
5. Place the tubes on the magnetic rack compatible with 1.5 mL tubes and wait for the solution to clear (~2 minutes).
6. While still on the magnet, remove and discard the supernatants.
7. Remove the tubes from the magnetic rack and fully resuspend each bead pellet in **800 µL** of ○ Bead Prep Buffer.
8. Place the tube on the magnetic rack until the solution clears (~2 minutes).
9. While still on the magnet, remove and discard the supernatant.
10. Repeat steps 7-9 twice for a total of 3 washes.
11. Remove the tubes from the magnetic rack. Fully resuspend each pellet in **800 µL** of ○ Barcoding Buffer and store on ice. This is your Cell Capture Master Mix (for a total of **1,600 µL** of resuspended beads).



Note: Save the Barcoding Buffer as it will be used later in this section.

12. Proceed to make the Ligation Master Mix for both Round 2 and Round 3 Ligation:

LIGATION MASTER MIX	
○ Ligation Buffer	17.6 mL
● Ligation Enzyme	88 µL

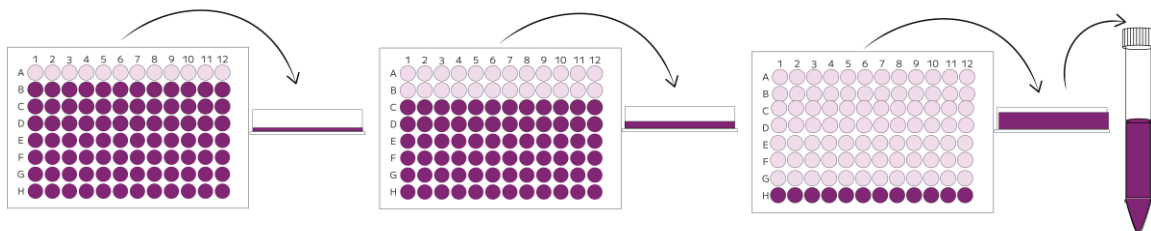
13. Mix the Ligation Master Mix by inverting the tube 10x. Store on ice.



CRITICAL! **8.8 mL** will be used for the Round 2 ligation barcoding step, and later **8.4 mL** will be used for the Round 3 ligation barcoding step.

14. Remove the Round 1 Plate A from the thermocycler, place it in a PCR tube rack on the benchtop. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 1 Plate A on ice.
15. Transfer all the ○ Round 1 Stop Mix into a new basin with a P1000.

16. With a 12-channel pipette P20 transfer **10 μ L** of **○ Round 1 Stop Mix** into Row A of the Round 1 Plate A, still keeping the plate on ice.
17. Switching tips between each row, repeat step 16, **7 more times** for Rows B-H to dispense the **○ Round 1 Stop Mix** into all wells of the Round 1 Plate A.
18. Change tips between plates, and repeat steps 16-17 with Plates B, C, and D to add the **○ Round 1 Stop Mix** to all samples. Keep plates on ice.
19. Label four 15 mL conical tubes with the provided stickers as Pool A, Pool B, Pool C, and Pool D.
20. Start pooling the samples from Round 1 Plate A into Pool A tube as follows:
 - a. With a multichannel P200 set to 50 μ L, mix the sample in row A by pipetting 3x.
 - b. Transfer **50 μ L** from row A into a clean basin (bubbles will be generated, this will not impact experimental results).
 - c. Repeat steps b-c for rows **B-H**.
 - d. Once cells from all wells are in the basin, using a P1000 transfer the mix into a 15 mL conical tube. Keep on ice.
 - e. Change pipette tips after each plate and repeat steps a-d 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.



21. **If using low input fixation samples**, cells/nuclei have been already captured. Place the 15 mL conical tube magnet on ice and wait 1 minute to let it pre-cool. **Proceed directly to step 27.**
22. **If using standard fixation samples**, proceed to the next step to capture the cells/nuclei.
23. Resuspend any settled beads in the Cell Capture Master Mix from step 12 by pipette mixing.

24. Using a P1000, transfer **384 µL** of the Cell Capture Master Mix into each Pool [A-D] tube containing the pooled Round 1 cells/nuclei. Mix each pool 10x with the P1000 set to 900 µL. Switch pipette tips between each pool.
25. Incubate the cells/nuclei on ice for **5 minutes** to allow beads to bind.



CRITICAL! Let beads incubate for **the entire 5 minutes**.

26. While cells are incubating, place the magnet on ice to pre-cool.



Note: In the next step, if the magnet cannot accommodate all four Pools simultaneously, **process one or two Pools at a time keeping the other on ice**.



27. Remove or loosen the cap, then place the Pool tubes on the 15 mL conical tube magnet to allow magnetic beads to bind to the magnet. If the cap is adjusted afterward, the rotational motion may cause the pellet to shift. Keep magnet on ice the entire time, covering the tube in ice up to the liquid level to keep the tube cold. Allow beads to bind to the magnet for **10 minutes and ensure the supernatant is clear before proceeding**.
28. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, hold the tube steady and remove and discard the supernatant while the 15 mL Pool tube is still on the magnet. Use a P1000 to remove any excess liquids. Holding the tube in the magnet will ensure the tube is kept from moving.
29. With the tube still on the magnet, add **5 mL** of **O** Barcoding Buffer. Do not resuspend. Incubate on ice for **1 minute**. Then remove and discard the supernatant with a P1000 or serological pipette.
30. Remove tube from magnet and resuspend the beads-bound cells/nuclei in **2.2 mL** of Ligation Master Mix. Using a P1000 set to 1000 µL, pipette mix 10x to fully resuspend the beads into the Ligation Master Mix. Store on ice.
31. If processing one or two Pools at a time, repeat steps 27-30 with the remaining Pools. Then proceed 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 5' end of the cDNA. The ligation reaction is quenched with ○ Round 2 Stop Buffer, and the cells/nuclei are pooled and strained.

To add round 2 barcodes:

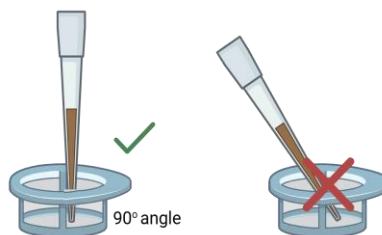
1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 2 Plate v4 (A)	-20°C Reagents	1	Place directly on ice. For clarity, we will refer to Round 2 Plate v4 as "Round 2 Plate (A)".
 Round 2 Plate B	-20°C Reagents	1	
○ Round 2 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.

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. Switching tips between each pool, repeat the previous step for the remaining three resuspended pools (B-D).

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

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

6. 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.
7. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate (A), and apply the provided adhesive labels to assist with loading. We suggest not removing the seal from Round 2 Plate B yet to avoid confusion.
8. With both Round 2 Plates on ice, transfer the Ligation Cell Mix to each well in the Round 2 Plate (A) as follows. See figure below for an overview.
 - a. Mix **Pool A** in the basin by pipetting 2x with a multichannel P200 set to 40 µL.
 - b. 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.

- c. Repeat steps a-b to mix Pool A in the basin then transfer to each well of columns 2-6 of the Round 2 Plate (A).
- d. Mix **Pool B** in the basin by pipetting 2x with a multichannel P200 set to 40 µL.
- e. Switching tips between each column, transfer **40 µL** of the mix to column 7 of the Round 2 Plate v4 and mix by pipetting 2x.
- f. Repeat steps c-d to mix Pool B in the basin then transfer to each well of columns 8-12 of the Round 2 Plate (A).

- g. 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.
- h. Remove the seal from the Round 2 Plate B and repeat a-g 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.

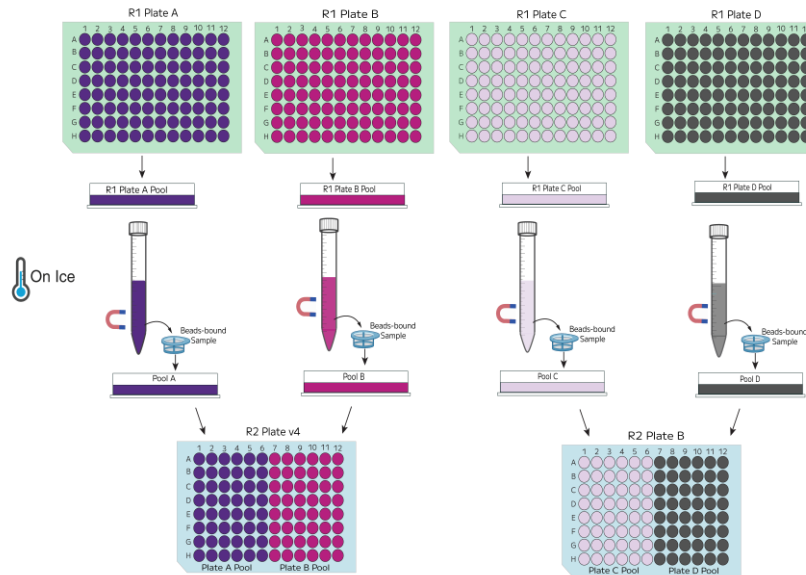


Figure 6: After pooling Round 1 samples into four basins, Pools are transferred into tubes and beads are added. Beads-bound cells/nuclei are strained into a new basin, and loaded column-wise in six columns each of the Round 2 Plates (A) and B.



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.

9. 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	15 minutes	
Lid Temperature	50°C	
Sample Volume	50 µL	
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C








10. Briefly vortex the **○** Round 2 Stop Mix. Transfer **2 mL** of this tube to a new basin with a P1000.
11. Remove the Round 2 Plates from the thermocyclers, place in PCR tube racks, remove the plate seals, and store on ice.
12. With the Round 2 Plates on ice and the basin on the bench, transfer the contents from each of the Round 2 Barcoding plate into the basins containing the **○** Round 2 Stop Mix as follows:
 - a. Starting with Round 2 Plate **(A)**, with a multichannel P200 set to 50 μ L, mix the sample in row A by pipetting 3x.
 - b. Transfer the entire content (**50 μ L**) from row A to the basin containing **○** Round 2 Stop Mix and mix 2x.
 - c. Repeat steps a-b for **rows B-H** to mix the sample then transfer to the basin. The same pipette tips may be used while pooling.
 - d. Repeat steps a-c with Round 2 Plate B, transferring the content into the same basin.
13. Transfer the Round 2 pool into a 15 mL conical tube and apply to the magnet. Loosen or remove the cap to avoid a rotational motion may cause the pellet to shift. Keep the magnet on ice. Allow beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear.
14. With a P1000 set to 1000 μ L or a 5 mL serological pipette and without touching the pellet, remove the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.
15. Keep the 15 mL conical tube on the magnet on ice and with a P1000 set to 1000 μ L or a 5 mL serological pipette, add **10 mL** of the **○** Barcoding Buffer to rinse the pellet. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound.
16. Remove and discard the supernatant with a P1000 set to 1000 μ L or a 5 mL serological pipette.
17. Remove tube from magnet and resuspend the beads with **8.4 mL** of the Ligation Master Mix. Store the sample on ice and proceed to section 1.4.

1.4 Barcoding Round 3

The cells are strained and 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 ligation reaction is stopped, and the sample is then pooled and strained.

To add round 3 barcodes:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 3 Plate (A)	-20°C Reagents	1	Place directly on ice. For clarity, we will refer to Round 3 Plate as Round 3 Plate (A).
 Round 3 Plate B	-20°C Reagents	1	
 Final Stop Mix	-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.
 Pre-Lysis Dilution Buffer	-20°C Reagents	1	
 Lysis Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
 Lysis Solution	-20°C Reagents	1	Thaw and store at room temperature.

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

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

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

4. While secured in a PCR tube rack on a flat surface, remove the plate seals from the Round 3 Plates.
5. Pipette all of the sample in Ligation Master Mix (**8.4 mL**) through a cell strainer into a new basin with a P1000.



CRITICAL! To ensure that all of the liquid passes through the strainer, keep the tip of the pipette straight up and 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.

6. With the Round 3 Plates on ice and the basin on the bench, transfer **40 µL** of sample in the basin to each well in the Round 3 Plates as follows:
 - a. Starting with Round 3 Plate (**A**), mix the sample in the basin by pipetting 2x with a multichannel P200 set to 40 µL.
 - b. Transfer **40 µL** of the mix to **row A** of the Round 3 Plate (A) and mix by pipetting 2x.
 - c. Changing tips between rows, repeat steps a-b to mix the sample in the basin then transfer to **rows B-H**.
 - d. While secured in a PCR tube rack on a flat surface, add a new plate seal to the fully loaded Round 3 Plate (A).
 - e. Repeat steps a-d to add the samples to rows **B-H** of 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.

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

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

8. Briefly vortex the **OFinal Stop Mix** and ensure there is no precipitate. Transfer **3.4 mL** to a new basin with a P1000.
9. Remove the Round 3 Plates from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.
10. With the Round 3 Plates on ice and the basin on the bench, transfer the content from each of the Round 3 Plates into the basin containing the **O Final Stop Mix** as follows:
 - a. Starting with Round 3 plate **(A)**, with a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.
 - b. Transfer **50 µL** from **row A** of the Round 3 Plate (A) to the basin.
 - c. Repeat steps a-b for **rows B-H** to mix the sample then transfer to the basin and mix 2x.
 - d. Repeat steps a-c for Round 3 Plate B, transferring the content into the same basin.
11. Pipette the pooled sample from the basin 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.
12. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear.
13. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 µL or a serological pipette.

14. Remove the 15 mL tube from the magnet and fully resuspend the pellet in **8 mL** of **○ Pre-Lysis Wash Buffer**. Do not invert.
15. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind for **5 minutes**. Ensure the supernatant is clear.
16. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 μ L or a serological pipette.
17. Remove the tube from the magnet and fully resuspend the cells in **700 μ L** of **○ Pre-Lysis Dilution Buffer**.



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

18. Proceed to Lysis and Sublibrary Generation Step 1.5.

1.5. Lysis and Sublibrary Generation

The cell/nuclei pools resuspended in Pre-Lysis Dilution Buffer are counted and divided into sublibraries based on the Sublibrary Generation Table in Appendix A. These sublibraries are lysed, transferred into a new plate, and stored at -80°C .

1. While minimizing time on ice, count the number of cells/nuclei in the sample with a fluorescent cell counting device. Record the cell/nuclei count.



CRITICAL! We strongly recommend carefully mixing the sample before collecting an aliquot for counting to ensure accurate sublibrary loading.

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



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

CRITICAL! 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

3. While minimizing time on ice, recount the number of cells/nuclei in the sample. Record the cell/nuclei count for the final library size.
4. Ensure the cells/nuclei are in suspension by pipetting 3x with a P1000 set to 500 μL .
5. Aliquot out at least **110 μL** of the diluted cells with a P200 single channel into each well of an 8-tube PCR strip to allow multichanneling, mixing between each aliquot. Store on ice.
6. See the image below to visualize the next steps. With a new low-bind semi-skirted plate on ice, and the 8-tube PCR strip containing cells on ice, transfer **25 μL** from the strip into the low-bind semi-skirted plate as follows:
 - a. With a multichannel P200 set to 25 μL , mix the sample in the 8-tube strip by pipetting 3x.
 - b. Transfer 25 μL in the 8-tube strip to column from the strip to the first column in the semi-skirted plate.
 - c. Repeat for columns 2-4.

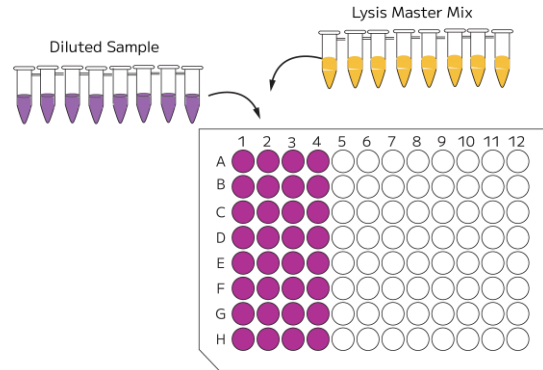


Figure 7: Each tube in the 8-tube PCR strip contains Diluted Sample. Aliquots are pipetted into four columns of the semi-skirted plate, resulting in 4 columns for 32 sublibraries, followed by Lysis Master Mix addition.

7. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 10x with a P1000 set to 800 μ L. Store at room temperature.

LYSIS MASTER MIX	
● Lysis Solution	1,000 μ L
● Lysis Enzyme	200 μ L
Total	1,200 μ L



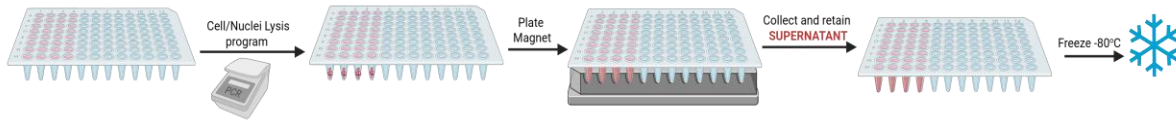
Note: Ensure that there is no precipitate before using the Lysis Solution.



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

8. Aliquot out **145 μ L** of Lysis Master Mix into each well of an 8-strip PCR tube. Centrifuge briefly
9. With a multichannel and switching tips between each column, add **30 μ L** of Lysis Master Mix to each column of the plate containing the diluted cells/nuclei. Store at room temperature.
10. 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.

11. While secured on a PCR tube rack, vortex for **1 minute**. Centrifuge the plate for **1 minute** at 100 x g at 4°C. For a visual representation of the next steps, see the workflow below.



12. Place the lysate plate into a thermocycler and run the following program.

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



Note: Handle the plate with caution as the plastic PCR seal may be hot.

13. Remove samples from the thermocycler and place the plate on the plate magnet. Incubate until the solution clears (~2 minutes).

14. While still on the magnetic rack and switching tips between each column, using a multichannel P200 transfer **55 µL** of the supernatant **containing lysed cells into a new low-bind plate in the same format**. If tips have volume/bubbles formed, results will not be impacted.



CRITICAL! Do **NOT** discard the supernatant as it contains the lysed cells/nuclei.

15. 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.

16. Freeze the lysate(s) at -80°C or proceed to Section 2.



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

Section 2: cDNA Capture and Amplification

2.1. cDNA Capture

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



CRITICAL! Always use new tips for each sublibrary.

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 A	-20°C Reagents	2	
○ Wash Buffer B	-20°C Reagents	2	
○ Binding Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Template Switch Buffer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 10x.
● Template Switch Primer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.

- If not continuing directly from Section 1, remove the lysate plate from storage at -80°C.
- If previously frozen, incubate the plate in a thermocycler at 37°C for **5 minutes**.

THAW LYSATE PLATE		
Run Time	5 minutes	
Lid Temperature	40°C	
Sample Volume	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.

- Briefly centrifuge in a swinging bucket centrifuge at 100 x g for **1 minute**. Store at room temperature.
- Vortex ● Streptavidin Beads until fully mixed. Add the appropriate volume of ● 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*
● Streptavidin 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)

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

9. Remove and discard the supernatant.
10. Add the appropriate volume of **O** Bead Wash Buffer as follows:

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

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

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



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

12. Place the tube(s) on the magnetic rack for 1.5 mL tubes until the solution clears (1 minute).
13. Remove and discard the supernatant.
14. Repeat steps 10-13 twice for a total of 3 washes.
15. Remove the 1.5 mL tube(s) from the magnetic rack. Fully resuspend the pellet(s) in the appropriate volume of **O**Binding Buffer as follows and store at room temperature.

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

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

16. Aliquot **220 μ L** of ● Streptavidin Beads in **O**Binding Buffer into each tube of an 8-tube PCR strip, mixing well between each dispense.
17. Using a P200 multichannel pipette, add **50 μ L** of ● Streptavidin Beads in **O**Binding Buffer **to each well of the Lysate Plate**, switching tips between columns.
18. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.

19. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex plastic holder on 100% power for **1 minute**.
20. Briefly centrifuge for **10 seconds** at 100 x g without letting beads collect at the bottom of the wells.
21. 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.

22. Remove the plate from the vortex and briefly vortex the plate on a standard vortex adapter.
23. Briefly centrifuge at 100 x g for **10 seconds**.
24. Secure the plate of lysates on a 96 tube rack, and remove the caps.
25. 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.

26. Prepare and label 2 empty basins to add wash buffers at appropriate steps and two additional basins (one for water, one for waste).



Note: The 4 basins should be labeled for "Wash Buffer A", "Wash Buffer B", "nuclease-free water", and "Waste".

27. Add **5 mL** of **O** Wash Buffer A to a labeled and empty basin using a P1000 pipette or a serological pipette.
28. Add **5 mL** of **O** Wash Buffer B to a labeled and empty basin using a P1000 pipette or a serological pipette. Cover basin with a plastic PCR seal.
29. Add **3 mL** of nuclease-free water to a labeled and empty basin using a P1000 pipette or a serological pipette. Cover basin with a plastic PCR seal.
30. 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.

31. While still on the magnet, carefully add **125 μ L** **O** Wash Buffer A to all four lysate columns, using a P200 multichannel pipette, switching tips between columns. Be careful to prevent spillage.
32. Secure the plate onto a 96 tube rack, and seal with 8-cap strips, ensuring the caps are tightly secured.
33. Vortex the plate for **15 seconds**.
34. Centrifuge the plate for **10 seconds** at 100 x g in a swinging bucket centrifuge.
35. Remove from the centrifuge and store at room temperature.
36. 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.



CRITICAL! Always use new tips for each sublibrary.

To perform template switch:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● 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	87 µL	2784 µL
● Template Switch Primer	3 µL	96 µL
● Template Switch Enzyme	6 µL	192 µL
Total	96 µL	3072 µL

4. Aliquot **185 μ L** 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, with a multichannel P200 remove and discard the supernatant from all 4 columns, switching pipette tips between each column.



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

8. While still on the magnet, add **125 μ L** of **O**Wash Buffer B to each column, switching tips between columns.



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

9. Secure the plate onto a 96 tube rack, and seal with 8-cap strips, ensuring the caps are tightly secured.
10. Vortex the plate for **15 seconds**.
11. Centrifuge the plate for **10 seconds** at 100 x g in a swinging bucket centrifuge.
12. Secure the plate onto a 96 well PCR tube rack, and remove the caps.
13. Return the plate to the plate magnet. Incubate until the solution clears (~2 minute).
14. While still on the magnet, with a multichannel P200 pipette remove and discard the **O** Wash Buffer B from all 4 columns, switching pipette tips between each column. With a P20 remove any leftover liquid.
15. Remove the plate from the magnet and secure the plate onto a 96 well PCR tube rack.
16. Switching tips between each column, add **20 μ L** of nuclease-free water from the pre-prepared basin into each column.
17. Secure the plate onto a 96 tube rack, and seal with 8-cap strips, ensuring the caps are tightly secured.
18. Vortex the plate for **10 seconds**.

19. Centrifuge the plate for **10 seconds** at 100 x g in a swinging bucket centrifuge.
20. Secure the plate onto a 96 well PCR tube rack, remove the seal.
21. Add **80 µL** of the Template Switch Master Mix with a multichannel P200 to column 1 (for a total volume of 100 µL per well). Mix 10x with the P200 multichannel set to 80 µL.
22. Repeat step 21 for the remaining 3 columns, switching pipette tips between each column.
23. 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).
24. Incubate for **30 minutes** at room temperature.
25. Fully resuspend each bead pellet by mixing 5x with a multichannel P200 set to 75 µL, switching pipette tips between each column.
26. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.
27. Place the plate into a thermocycler and run the following program.

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

28. Proceed immediately to Section 2.3. Alternatively, proceed to the following steps (step 29-37) to store samples prior to cDNA amplification.
29. Place the plate on the plate magnet and incubate for **3 minutes**.
30. 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.

31. Incubate for an additional **2 minutes**.
32. Add **5 mL** of **O Wash Buffer A** to a labeled basin using a P1000 pipette.
33. While still on the magnet, remove and discard the supernatant from all 4 columns into the waste basin, switching tips between columns.
34. While still on the magnet, add **125 µL** **O Wash Buffer A** using a P200 multichannel pipette, switching tips between each column.
35. Remove the plate from the plate magnet and secure it in a 96 PCR tube rack.
36. Fully resuspend each bead pellet in **O Wash Buffer A** using a P200 multichannel set to 100 µL, switching pipette tips between each column.
37. 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.



CRITICAL! Always use new tips for each sublibrary.

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
○ cDNA Amp Mix	60 μ L	1,920 μ L
○ cDNA Amp Primers	57.5 μ L	1,840 μ L
● PCR Additive	2.5 μ L	80 μ L
Total	120 μ L	3,840 μ L

2. Aliquot **220 μ L** 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 seal/caps.
4. Place the plate on the plate magnet. Incubate for **3 minutes**.
5. Mix each column 5x with a P200 multichannel set to 75 μ L. 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, using a multichannel P200 remove and discard the supernatant from all 4 columns, switching tips between each column.

8. While still on the magnetic rack, transfer the second tube of **O Wash Buffer B** into the previously labeled basin (if continuing directly from Section 2.2) or into a new labeled basin (if resuming after an overnight stop).
9. With a multichannel P200, add **125 µL** of **O**Wash Buffer B to each well, switching tips between each column.
10. Secure the plate onto a 96 tube rack, and seal with 8-cap strips, ensuring the caps are tightly secured.
11. Vortex the plate for **15 seconds**.
12. Centrifuge the plate for **10 seconds** at 100 x g in a swinging bucket centrifuge.
13. While still on the magnet, with a multichannel P200 remove and discard the **O** Wash Buffer B, switching pipette tips between each column.
14. Remove the plate from the magnet and secure it onto a 96 PCR tube rack.
15. With a multichannel P200, add **100 µL** of the cDNA Amplification Master Mix to each column, switching pipette tips between each column.
16. Seal the plate with a plastic PCR seal.
17. 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.
18. 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.
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. Centrifuge for **10 seconds** at 100 x g. Store on ice.
21. 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

NUMBER OF PCR CYCLES			
25,000-62,500	3	5	4
62,500-125,000	2	4	3
125,000-156,250	2	3	2

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

cDNA AMPLIFICATION			
Run Time		40-60 min	
Lid Temperature		105°C	
Sample Volume		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	
6*	20 sec	67°C*	Variable, see above
7	3 min	72°C	
8	5 min	72°C	
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.



CRITICAL! Always use new tips for each sublibrary.

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).



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

3. Vortex the SPRI beads until fully mixed. Aliquot **170 μL** of SPRI beads into each of 16 PCR tubes 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.
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 75 μL . 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.


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 using a P200 multichannel, switching tips between each column.
20. While still on the magnet, add **180 µL** of 85% ethanol to each well using a P200 multichannel, 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. The sample is in the beads. Do not disturb it.
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 **5 minutes** at room temperature.
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.

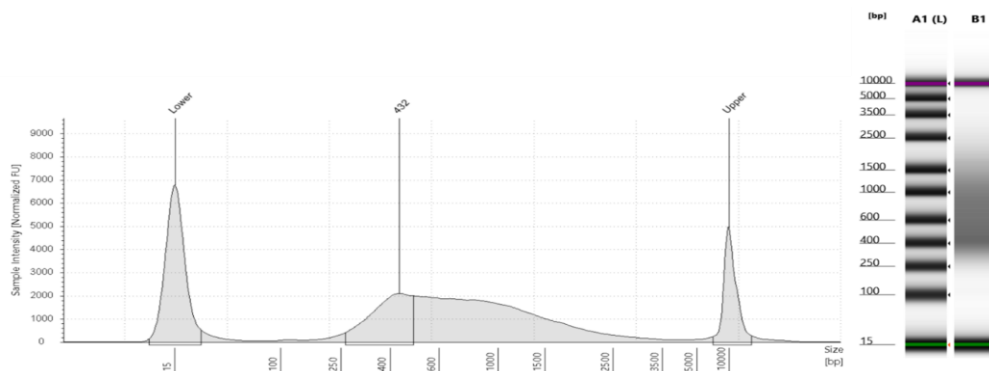


Figure 8: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a TapeStation.

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.



CRITICAL! Always use new tips for each sublibrary.

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 μ L 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	5 min	
Lid Temperature	40°C	
Sample Volume	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 in 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	40 minutes	
Lid Temperature	70°C	
Sample Volume	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 μ L	192 μ L
● Fragm/End Prep Enzymes	12 μ L	384 μ L
Total	18 μ L	576 μ L

13. Aliquot **70 μ L** 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 μ L** 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 μ L, 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.



CRITICAL! Always use new tips for each sublibrary.

To size select the fragmented and end prepped DNA:

1. Gather the 85% ethanol prepared in Section 3.1.
2. Prepare and label three basins as follows: "ethanol", "nuclease-free water" and "waste" for later use. Add **5 mL** nuclease-free water into the corresponding labeled basin.
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 μ L** of SPRI Beads into each tube of an 8-tube PCR strip to facilitate the use of a multichannel pipette (in step 6). Centrifuge briefly.
5. With the plate containing fragmented DNA secured on a 96 PCR tube rack, carefully remove the caps.
6. Add **30 μ L** 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 stock 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 (in step 14). 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, with a multichannel 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, using a multichannel remove and discard the supernatant from all 4 columns into a waste basin, switching pipette tips between each column.
24. While still on the magnetic rack, **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, with a multichannel P200 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 if sealed with a foil seal.

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.



CRITICAL! Always use new tips for each sublibrary.

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	15 min	
Lid Temperature	30°C*	
Sample Volume	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.



CRITICAL! Always use new tips for each sublibrary.

To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol (prepare **15 mL** for 32 sublibraries) and 3 mL nuclease-free water.
2. Prepare and label three 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 tips between columns.
15. While still on the magnetic rack, add **180 μ L** 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. Add **2 mL** of nuclease-free water into the basin labeled as "water" and add **23 μ L** 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 μ L** 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

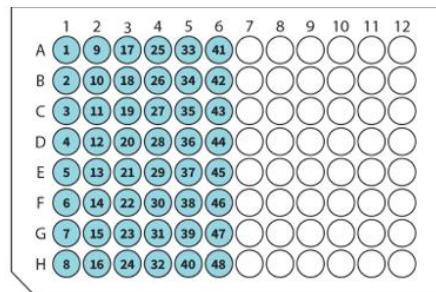
Purified adapter ligated DNA is PCR amplified with Illumina Truseq Read 1 and Read 2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.



CRITICAL! Always use new tips for each UDI-Plate's well and corresponding sublibrary.

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
0.28 - 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- 28.5 ng/µL	300-999	8
28.6+ ng/µL	1,000 or more	7

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

INDEXING PCR			
Run Time		~30 minutes	
Lid Temperature		105°C	
Sample Volume		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.



CRITICAL! Always use new tips for each sublibrary.

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 μ L** 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 μ L** of SPRI beads in columns 1-4. Secure on 96 PCR tube rack.
15. While still on the magnet, transfer **75 μ L** of the supernatant containing the DNA to the PCR plate preloaded with 10 μ L 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 the 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 μ L** 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.

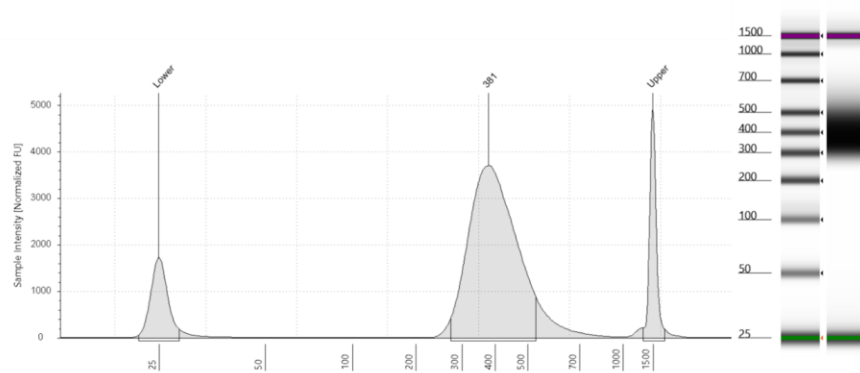


Figure 9: Expected Size Distribution before Illumina Sequencing. Example trace of DNA from indexed sublibraries run on a TapeStation.

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 10,000 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.

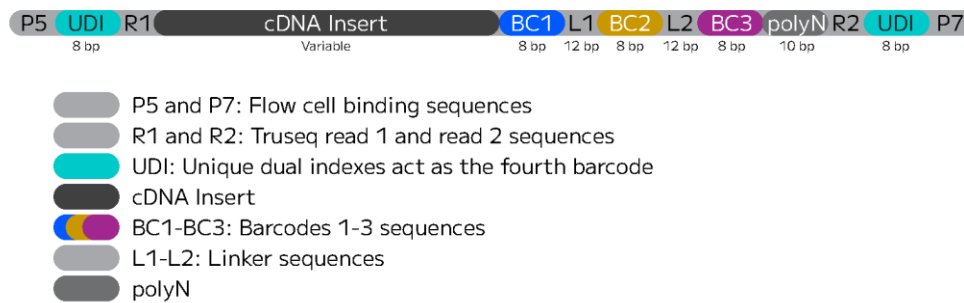


Figure 10: Whole transcriptome sequencing library structure.

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	CAAAGTC	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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_22	F3	CTATACTC	TGTTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
UDI_Plate_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
UDI_Plate_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
UDI_Plate_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
UDI_Plate_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
UDI_Plate_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT
UDI_Plate_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
UDI_Plate_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
UDI_Plate_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA
UDI_Plate_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA
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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix B: Thermocycling Programs

Section 1: *In Situ* Cell/Nuclei Barcoding

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

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

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

BARCODING ROUND 1			
Run Time	40 minutes		
Lid Temperature	70°C		
Sample Volume	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	3 minutes	
Lid Temperature	70°C	
Sample Volume	10 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

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

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

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

CELL/NUCLEI LYSIS		
Run Time	15 minutes	
Lid Temperature	80°C	
Sample Volume	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	5 minutes	
Lid Temperature	40°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

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

cDNA AMPLIFICATION			
Run Time	40-60 minutes		
Lid Temperature	105°C		
Sample Volume	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	5 minutes	
Lid Temperature	40°C	
Sample Volume	75 µL	
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

FRAGMENTATION AND END PREP		
Run Time	40 minutes	
Lid Temperature	70°C	
Sample Volume	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	15 minutes	
Lid Temperature	30°C	
Sample Volume	100 µL	
Step	Time	Temperature
1	15 min	20°C
2	Hold	4°C

INDEXING PCR			
Run Time		~30 minutes	
Lid Temperature		105°C	
Sample Volume		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	March 2026



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