User Manual

Version 1.2 – UMWT4700

Evercode™ WT Penta v4

For use with

ECWT4700

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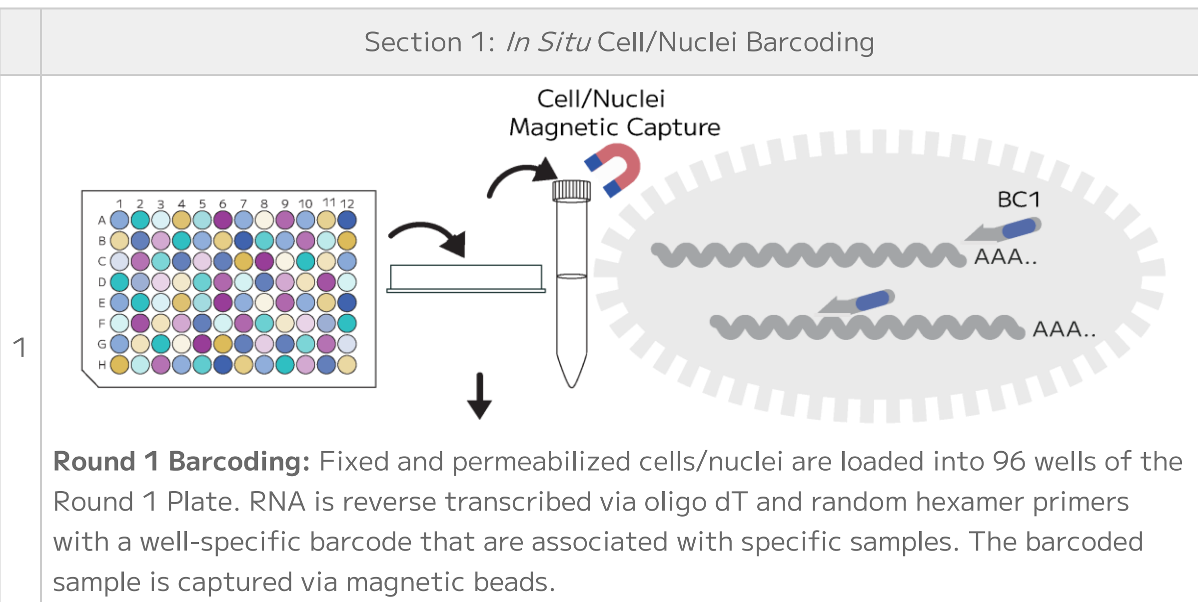
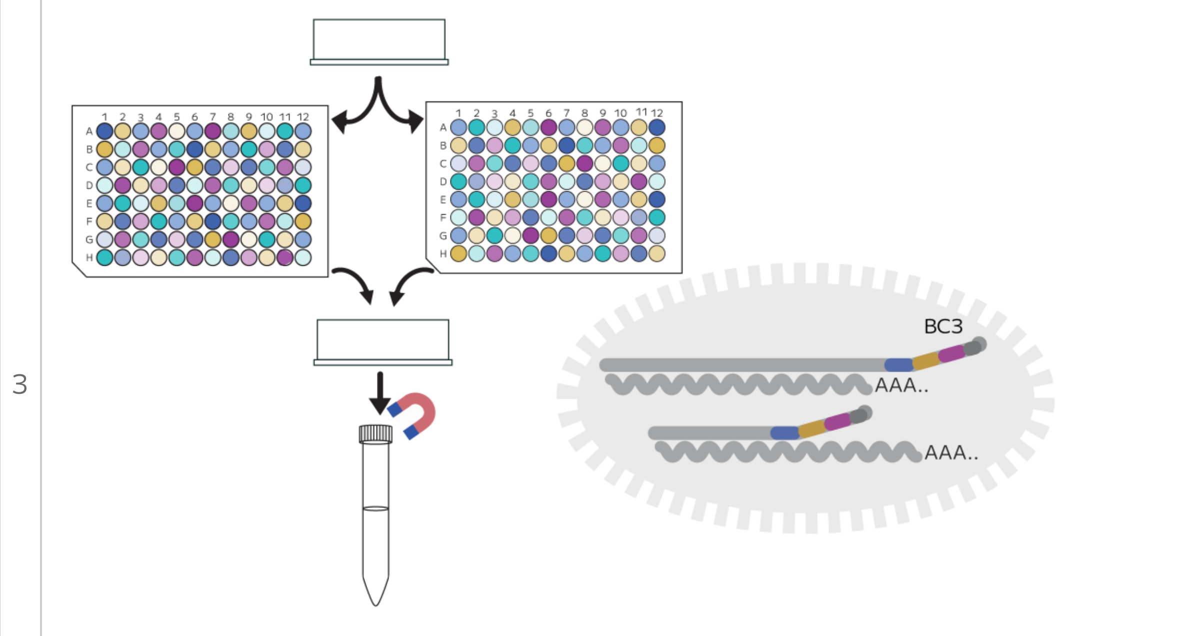
Overview

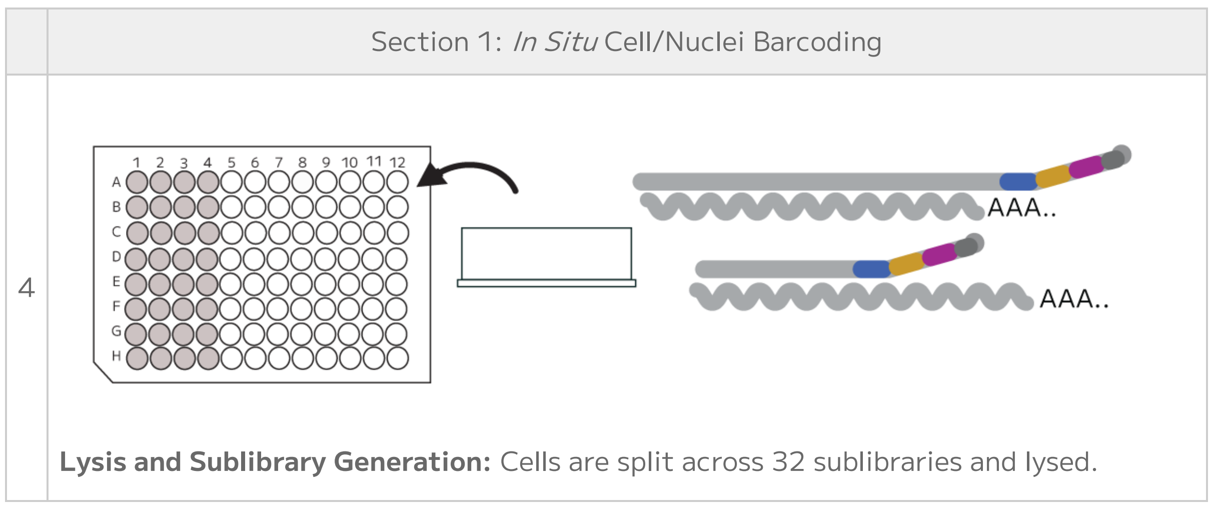
Workflow

Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT Penta v4 kit can profile up to 5,000,000 cells across up to 96 different biological samples or experimental conditions. Evercode Fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding can yield a very large number of possible barcode combinations which is more than sufficient to uniquely label up to 5,000,000 cells/nuclei while avoiding doublets. After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same 4 barcode combination to a single cell/nuclei.

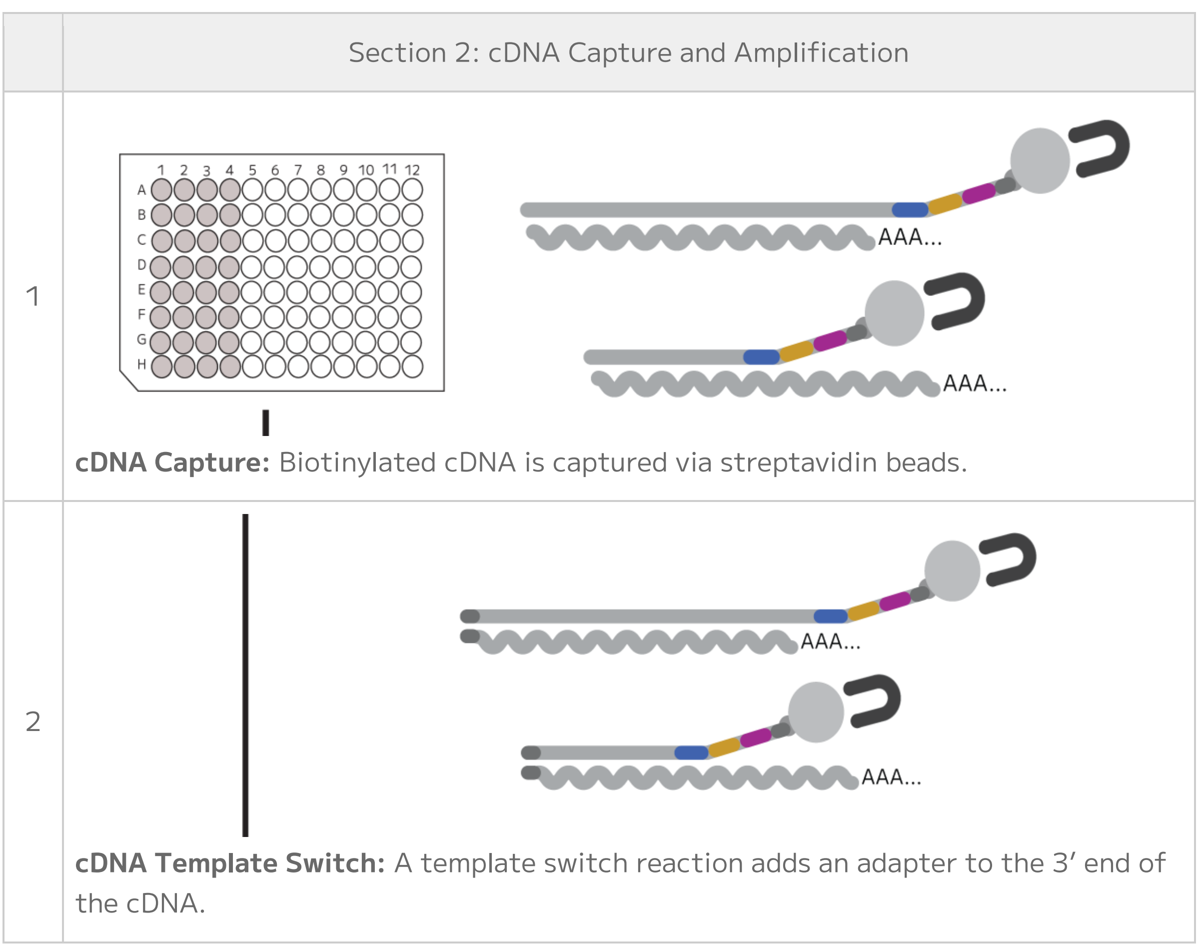
The tables below provide a high-level overview of the whole transcriptome workflow.

	Section 1: *In Situ* Cell/Nuclei Barcoding
1	 **Round 1 Barcoding:** Fixed and permeabilized cells/nuclei are loaded into 96 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with a well-specific barcode that are associated with specific samples. The barcoded sample is captured via magnetic beads.

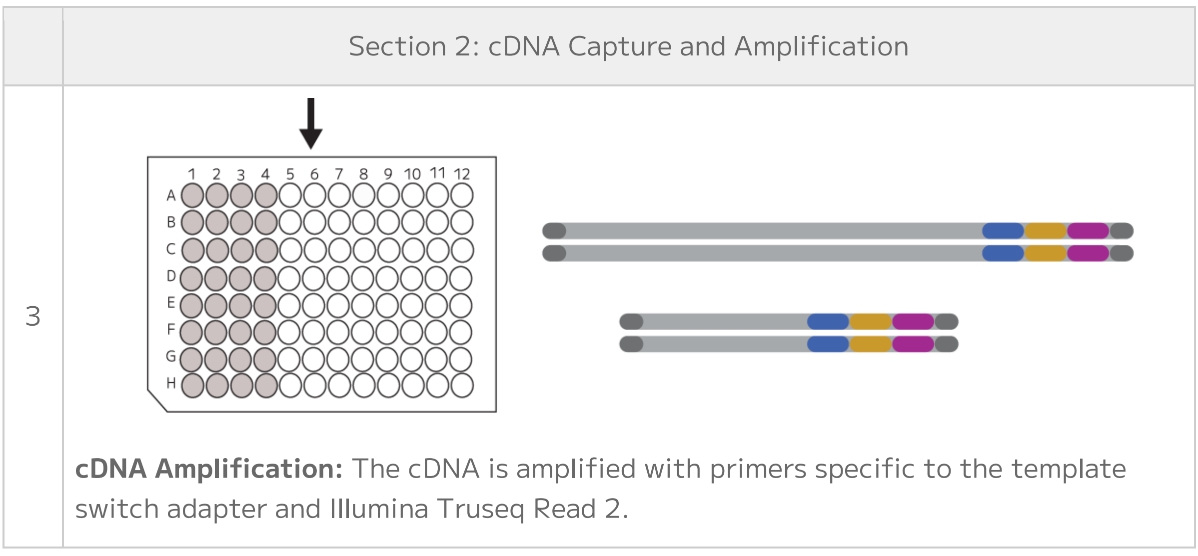
	Section 1: *In Situ* Cell/Nuclei Barcoding
2	**Round 2 Barcoding:** The cells/nuclei are pooled and loaded into two distinct Round 2 Plates. An adapter with a well-specific barcode is ligated to the first barcode. Cells/nuclei are pooled and then captured via magnet.
3	 **Round 3 Barcoding:** The cells/nuclei are pooled and loaded into two distinct Round 3 Plates. A third barcode is ligated to the cDNA, which also contains an Illumina Truseq Read 2 sequence, and biotin. Cells/nuclei are pooled and then captured via magnet.

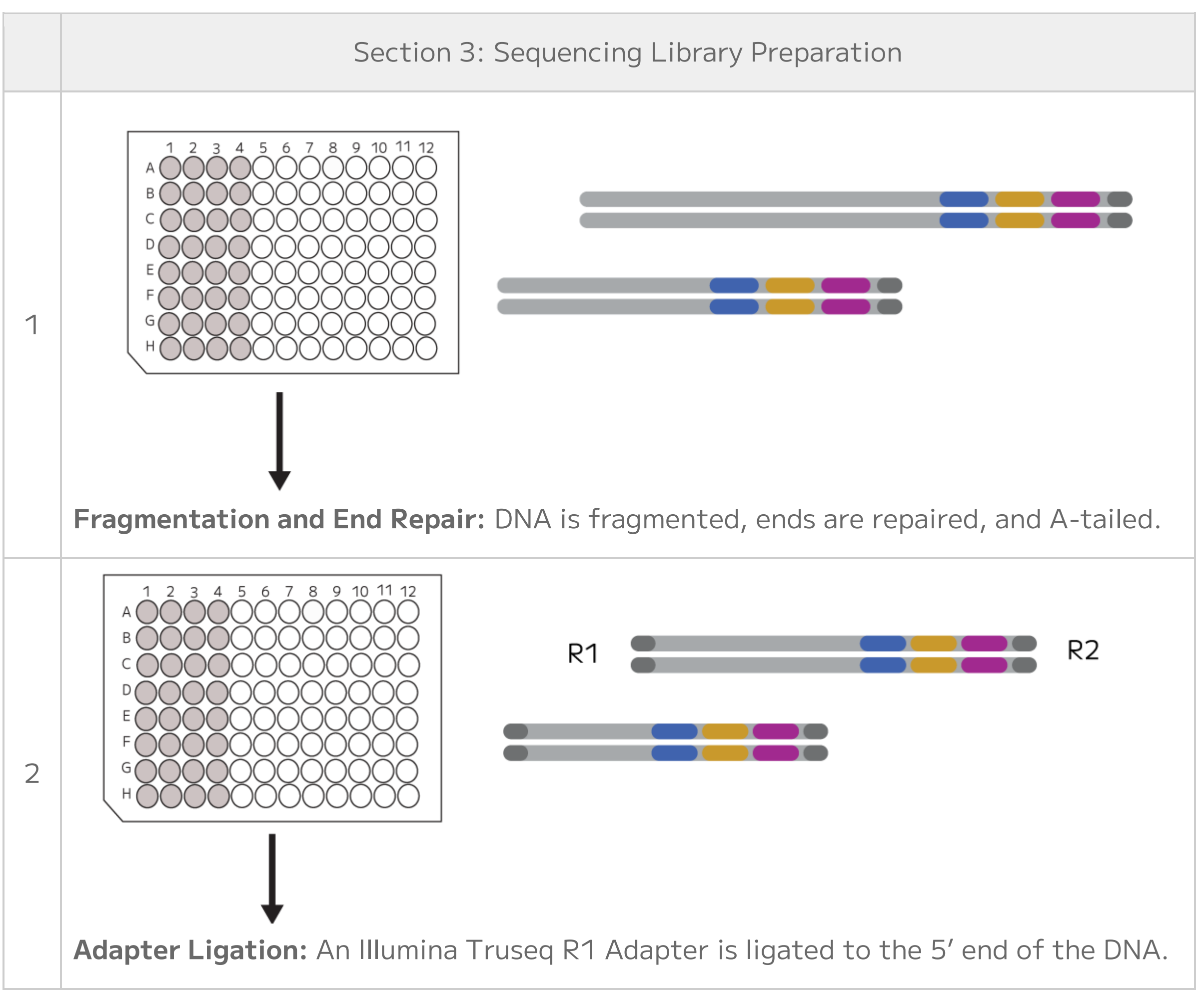


	Section 1: *In Situ* Cell/Nuclei Barcoding
4	**Lysis and Sublibrary Generation:** Cells are split across 32 sublibraries and lysed.



	Section 2: cDNA Capture and Amplification
1	**cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads.
2	**cDNA Template Switch:** A template switch reaction adds an adapter to the 3′ end of the cDNA.


Section 2: cDNA Capture and Amplification
3
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
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
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Fragmentation and End Repair: DNA is fragmented, ends are repaired, and A-tailed.
2
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
R1
R2
Adapter Ligation: An Illumina Truseq R1 Adapter is ligated to the 5′ end of the DNA.

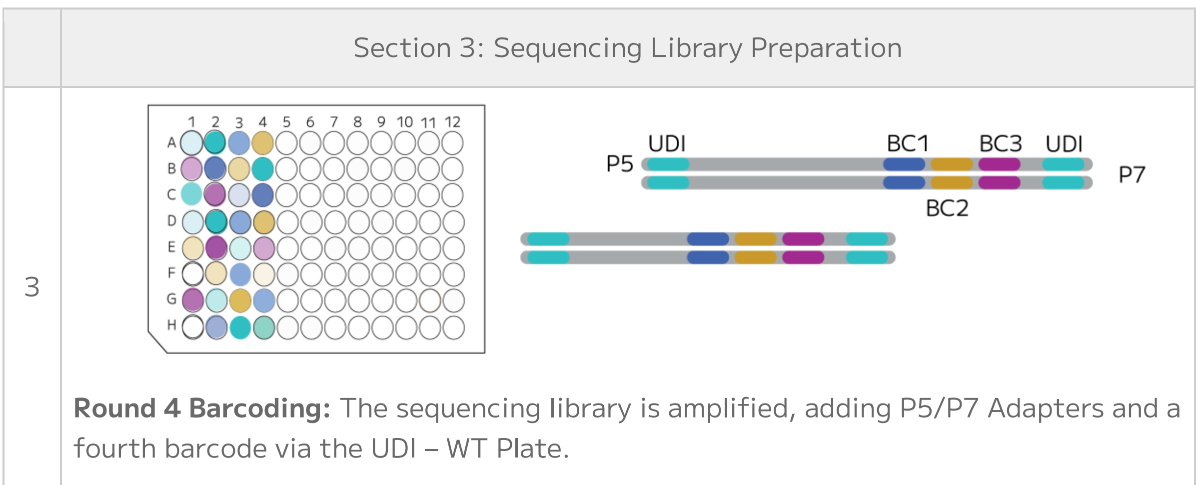
	Section 3: Sequencing Library Preparation
3	 **Round 4 Barcoding:** The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.

Figure 1 shows the Evercode WT Penta workflow. This kit can barcode up to 5 million cells/nuclei across 96 samples in a single reaction. The WT Penta kit utilizes one Round 1 Plate holding 96 samples. Samples are pooled from the Round 1 Plate and randomly distributed across two Round 2 Plates, each carrying different barcode sets. Cells are pooled from both Round 2 Plates, and randomly split again across two Round 3 Plates, also each carrying different sets of barcodes.

Cells/nuclei are then pooled and subsequently split to generate 32 sublibraries.

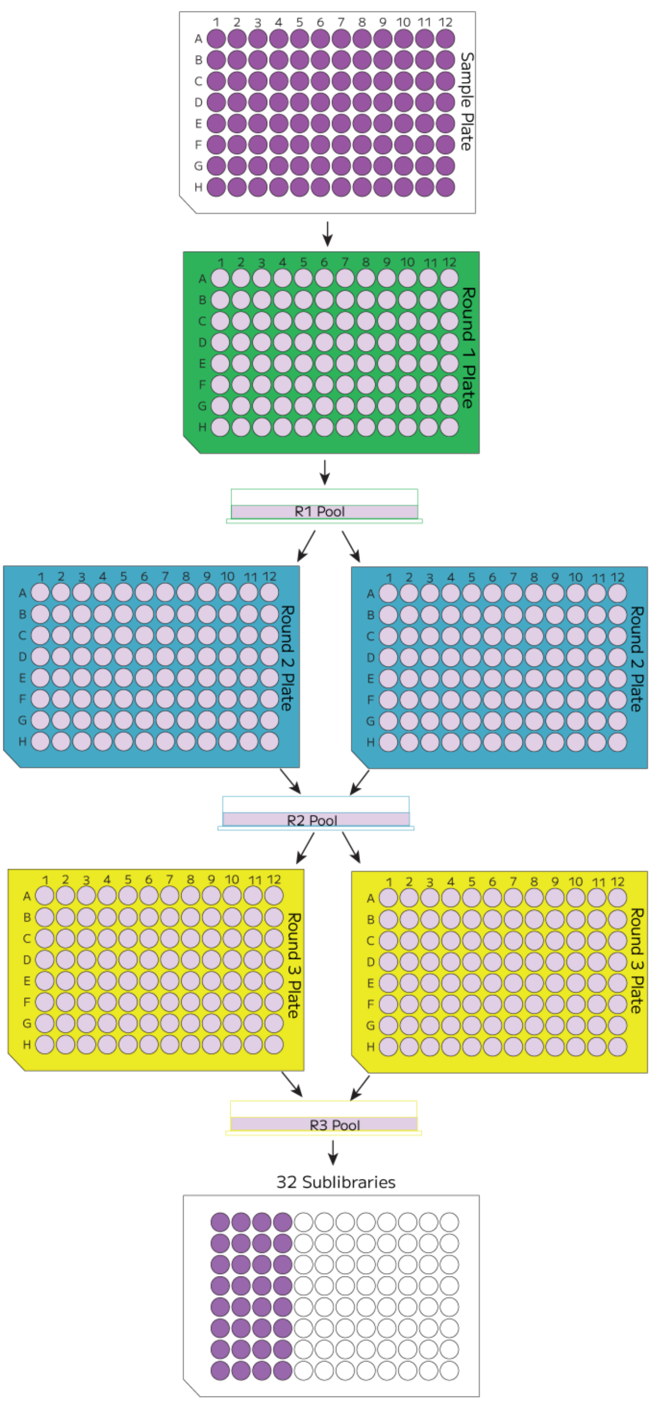


Figure 1: Evercode WT Penta workflow pools 96 samples from a single Round 1 Barcoding Plate into two Round two and Round 3 Barcoding Plates respectively, generating 32 Sequencing Libraries.

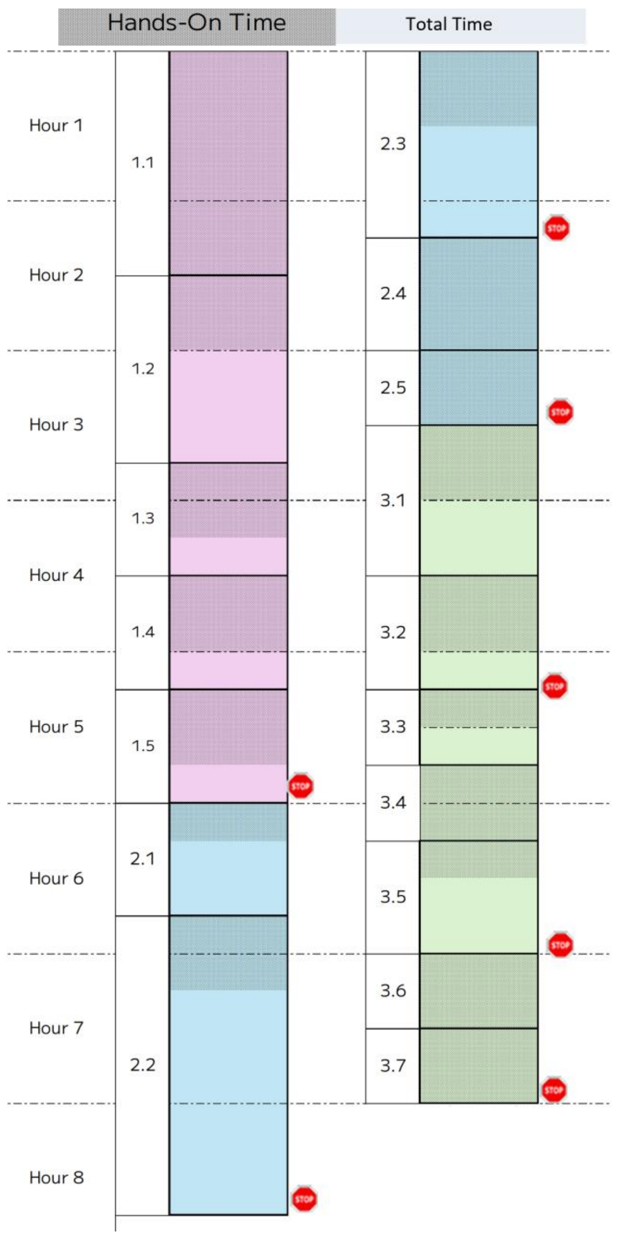
Protocol Timing

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.

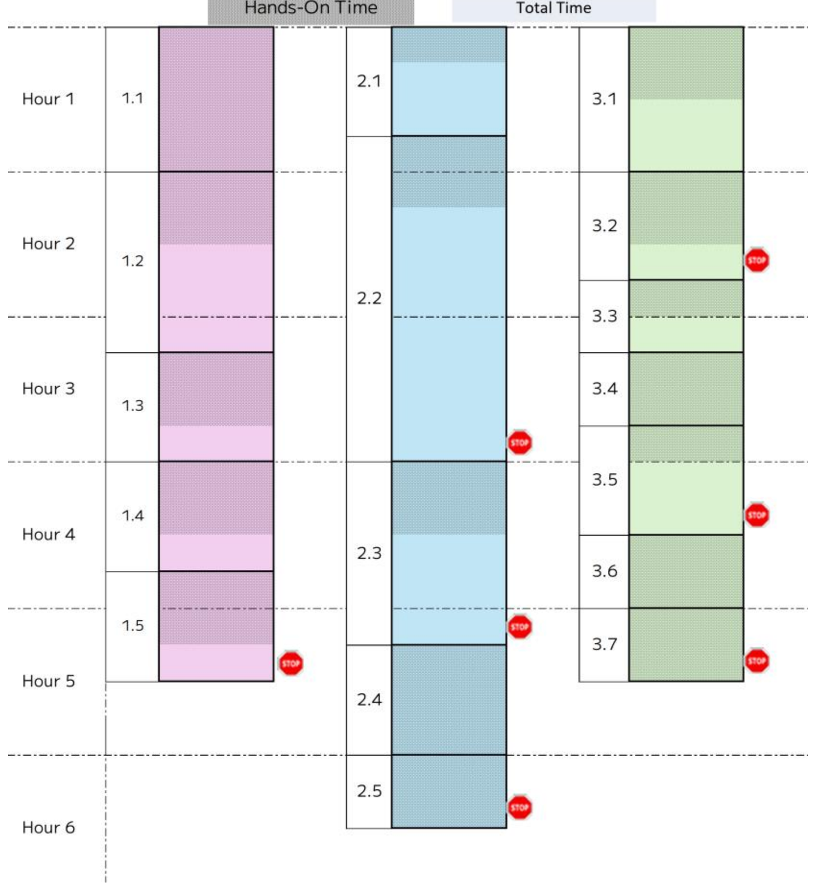
DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
Section 1: In Situ Cell/Nuclei Barcoding (~4:00 to 5:00 hours)			
1.1 Set up and Sample Prep	Variable 30-90 min	Variable 60-90 min	
1.2 Pooling Round 1	75 min	35 min	
1.3 Barcoding Round 2	45 min	30 min	
1.4 Barcoding Round 3	45 min	30 min	
1.5 Lysis and Sublibrary Generation	45 min	30 min	-80°C ≤ 6 months
Section 2: cDNA Capture and Amplification (~5:20 hours)			
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 (~4:30 hours)			
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	

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

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, 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.
- 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.

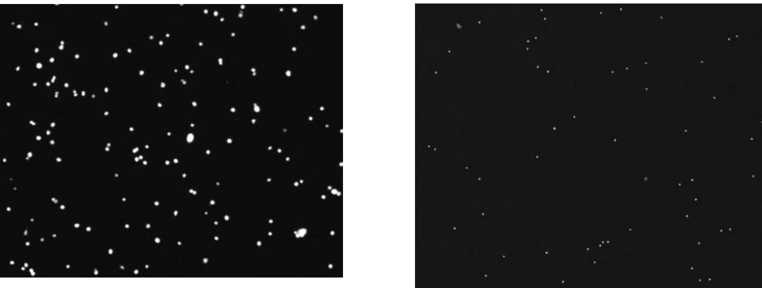


Figure 2: example of AO/DAPI stained HEK cells (left) and PBMCs (right).

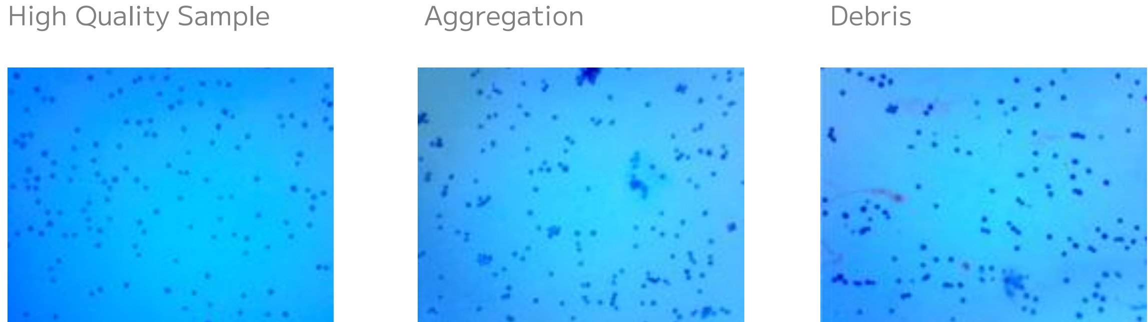


Figure 3: Example of trypan blue stained fixed cells.

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 Evercode WT Penta v4 Sample Loading Table RevA should be completed before starting the experiment.
- 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 35 µL of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded cells but change the desired proportion between samples.

Cell Strainers

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 µm is appropriate for many cell types, the mesh size should be chosen based on the sample type.
- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. An example video can be found in our support site.

Vortexing

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

Plate Sealing

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

Magnetic Racks and Bead Cleanups

- The Evercode WT Penta 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.
- 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 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 v4 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents Store at -20°C, PT400

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate P	PT101	Green semi-skirted 96 well plate	1
	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 Dil	Sample Dilution Buffer	MG132	5 mL tube	1
Bead Prep	Bead Prep Buffer	PT136	5 mL tube	1
Barcode Buffer	Barcoding Buffer	MG137	15 mL tube	2
Ligation Buf	Ligation Buffer	PT134	15 mL tube	2
R1 Stop Mix	Round 1 Stop Mix	PT138	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
Ligation Enzy	Ligation Enzyme	PT140	1.5 mL tube	1
R2 Stop Mix	Round 2 Stop Mix	PT139	5 mL tube	1
Final Stop	Final Stop Mix	PT141	5 mL tube	1
Pre-Lysis Wash	Pre-Lysis Wash Buffer	MG110	5 mL tube	2
Pre-Lysis Dil	Pre-Lysis Dilution Buffer	PT108	5 mL tube	1
Lysis Enzy	Lysis Enzyme	PT109	1.5 mL tube	1
Lysis Solution	Lysis Solution	PT146	2 mL tube	1
Bead Wash	Bead Wash Buffer	PT111	5 mL tube	2
Binding Buf	Binding Buffer	PT112	5 mL tube	1
Wash A	Wash Buffer A	PT143	5 mL tube	3
Wash B	Wash Buffer B	PT144	5 mL tube	3
TS Buffer	Template Switch Buffer	PT148	5 mL tube	1
TS Enzyme	Template Switch Enzyme	PT117	1.5 mL tube	1
TS Primer	Template Switch Primer	PT118	1.5 mL tube	1
cDNA Amp Mix	cDNA Amp Mix	PT119	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
cDNA Amp Primers	cDNA Amp Primers	PT120	5 mL tube	1
PCR Additive	PCR Additive	PT150	1.5 mL tube	1
Fragm/End Prep Buf	Fragm/End Prep Buffer	PT121	1.5 mL tube	1
Fragm/End Prep Enzy	Fragm/End Prep Enzymes	PT122	1.5 mL tube	1
Lig Adapter	Ligation Adapter	MG127	1.5 mL tube	1
Adap Lig Buffer	Adapter Ligation Buffer	PT123	1.5 mL tube	1
Adap Lig Enzy	Adapter Ligation Enzyme	PT124	1.5 mL tube	1
Library Amp Mix	Library Amp Mix	PT125	1.5 mL tube	1

4°C Reagents Store at 4°C, PT500

LABEL	ITEM	PN	FORMAT	QTY
Sample Beads	Sample Binding Beads	PT149	2 mL tube	1
Strep Beads	Streptavidin Beads	PT201	2 mL tube	2

User Supplied Equipment and Consumables

The following materials and equipment are required to perform the protocol, but are not provided within the kit.

Note that this list does not include standard laboratory equipment, such as freezers.

Equipment

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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.	
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.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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		☐
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.	☐
2 T100 Thermal Cyclers	Bio-Rad Laboratories®	1861096	Or equivalent thermocyclers compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 μL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.	☐
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.	☐
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM.	☐
6-inch Platform	Scientific Industries	146-6005-00		
Microplate Foam Insert	Scientific Industries	504-0235-00		
QubitTM Flex Fluorometer	Thermo Fisher Scientific®	Q33327	Or an equivalent fluorometer.	☐
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.	☐
4200 TapeStation System	Agilent	G2991BA		

Consumables

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
UDI Plate - WT	Parse Biosciences	UDI1001	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode kits (48 sublibraries). UDI Plate - WT can be purchased separately or bundled with Evercode kits.	☐
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free, 10 mL or 25 mL reagent basins. Use only polypropylene basins as polystyrene may lead to some cell loss.	☐
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	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.	☐
DNA LoBind® Tubes, 2 mL, Snap Cap	Eppendorf®	022431048	Or equivalent DNA low-binding, nuclease-free 2 mL tubes.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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		☐
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	CHECK LIST
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)		
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)		
MAGFLO NGS Size Selection	INTEGRA	7000 (1 mL) 7002 (5 mL)		
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.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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. Set up and Sample Counting

Prior to barcoding, cells/nuclei are thawed and counted. Appropriate dilutions, loading concentrations, and loading positions are determined by the Evercode WT Penta v4 Sample Loading Table RevA.

To load the barcoding plate:

1. If working with standard fixation samples, remove the ● Sample Binding Beads from the 4°C Reagents box. Gently pulse-vortex (1-2 seconds per pulse) or pipette mix the beads until completely resuspended. Observe the beads to confirm they stay in solution for **at least 2-3 minutes** before beginning to settle. Store the ● Sample Binding Beads at room temperature until use.

CRITICAL! If you observe that the Sample Binding Beads are frozen or dried, and settled at the bottom the tube within ~10 seconds after pulse-vortexing, do not proceed. Contact your FAS or support@parsebiosciences.com for assistance.

2. Open the completed "Evercode WT Penta v4 Sample Loading Table RevA", which will guide sample dilutions and plate loading in later steps.
3. Cool a centrifuge with swinging bucket rotors and spinner plate capabilities to 4°C.
4. Fill a bucket with ice.
5. Prepare a hemocytometer, flow cytometer, or other cell counting device.
6. Thaw the previously fixed cell/nuclei samples in a water bath set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.
7. While minimizing time on ice, count the cells/nuclei in the sample with a hemocytometer or alternative cell counting device.
8. Record the sample names and cell/nuclei count in the Sample Loading Table.

Note: When processing many fixed samples, we recommend aliquoting samples after fixation and counting the aliquots the day before using an Evercode Whole Transcriptome kit. See the Important Guidelines section for details.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 1 Plate P	-20°C Reagents	1	Place directly on ice.
Sample Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x
Bead Prep Buffer	-20°C Reagents	1	
Round 1 Stop Mix	-20°C Reagents	1	
Ligation Buffer	-20°C Reagents	2	
Barcoding Buffer	-20°C Reagents	2	Thaw in a 37°C water bath, then store on ice.
Ligation Enzyme	-20°C Reagents	1	Place directly on ice
Sample Binding Beads	4°C Reagents	1	Keep at room temperature. Ensure beads are not settled before use.

10. Record the sample names and cell/nuclei count in the Evercode WT Penta Standard Fixation Sample Loading Table.

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

11. Place the Round 1 Plate P into a thermocycler and run the following program.

THAW ROUND 1 PLATE P		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	65 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

12. Based on the values defined in the Sample Loading Table, dilute each sample with ○ Sample Dilution Buffer and store on ice.

13. Proceed immediately to Section 1.2.

1.2. Barcoding Round 1

Samples are loaded into the Round 1 Plate. An in situ reverse transcription reaction adds well-specific barcodes that also act as sample barcodes. The reverse transcription reaction is stopped, cells/nuclei are pooled, and then cell/nuclei are resuspended.

To add round 1 barcodes:

1. Gently remove the Round 1 Plate P from the thermocycler, place in a 0.2 mL tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.

2. Remove the Round 1 Plate P from the centrifuge, place in a PCR tube rack, remove the plate seal, and store on ice.

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.

3. With the Round 1 Plate P on ice, add **35 µL** of each diluted sample to the appropriate wells of Round 1 Plate P as defined in the Evercode WT Penta v4 Sample Loading Table RevA. 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: When pipetting the same sample into many wells, the sample must be mixed by gentle pipetting prior to each transfer to avoid cells or nuclei settling. Do not vortex the samples.

4. While secured in a PCR tube rack on a flat surface, add a new plate seal.

5. Place the Round 1 Plate P into a thermocycler and run the program below. While running, proceed immediately to the next step.

BARCODING ROUND 1			
Run Time		40 minutes	
Lid Temperature		70°C	
Sample Volume		100 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

6. Prepare the Sample Binding Beads to capture the cells/nuclei. See workflow below.

7. While the thermocycling program is still running, gently pulse-vortex the ● Sample Binding Beads to resuspend and store at room temperature. Do not let them settle for >3 minutes before pipetting. Transfer **1 mL** of ● **Sample Binding Beads** to a 1.5 mL Eppendorf tube.

8. Place the tube on the magnetic rack and wait for the solution to clear (~2 minutes).

9. Remove and discard the supernatant.

10. Remove the tube from the magnetic rack and fully resuspend the bead pellet in **1 mL** of ○ **Bead Prep Buffer**.

11. Place the tube on the magnetic rack until the solution clears (~2 minutes).

12. Remove and discard the supernatant.

13. Repeat steps 10-12 twice for a total of 3 washes.

14. Remove the tube from the magnetic rack. Fully resuspend the pellet in **1 mL** of ○ **Barcoding Buffer**. This is your Cell Capture Master Mix. Store on ice.

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

15. After the Cell Capture Master Mix is prepared, proceed to making the Ligation Master Mix for both Round 2 and Round 3 Ligation:

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

16. Mix by inverting the tube 10x, and store Ligation Master Mix on ice.

CRITICAL! 8.4 mL of Ligation Master Mix 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.

17. Remove the Round 1 Plate P 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 P. Place the Round 1 Plate P on ice.

18. Transfer all the Round 1 Stop Mix in a new basin.

19. With a 12–channel multi-pipette transfer **25 µL** of Round 1 Stop Mix into Row A of the Round 1 Plate P, still keeping the plate on ice.

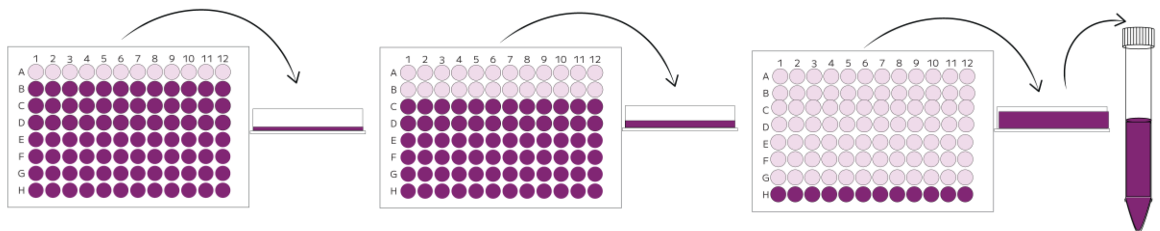
    a. Repeat step 19 **7 more times** using the same pipette tips for Rows B-H to dispense the Round 1 Stop Mix into all wells of the Round 1 Plate P.

    b. With a multichannel P200 set to 90 µL, mix the sample in row A by pipetting 3x.

c. Transfer **125 µL** from row A into a clean basin (bubbles will be generated, this will not impact experimental results).

d. Repeat steps b-c for rows **B-H.**

e. Once cells from all wells are in the basin, transfer the mix into a 15 mL conical tube. Keep on ice.



20. To capture the cells/nuclei resuspend any settled beads in the Cell Capture Master Mix from step 14 by pipette mixing

21. Using a P1000, transfer **960 µL** of the Cell Capture Master Mix to the pooled Round 1 cells/nuclei. Pipette mix 10x with the P1000, set to 1000 µL.

22. Incubate the cells on ice for **5 minutes** to allow beads to bind.

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

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

24. Remove or loosen the cap, then place the tube with the pooled cells/nuclei 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 the 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**.

Note: At this point your cells/nuclei are bound to beads. Preserve the beads in the subsequent rounds of barcoding, as loss of any beads from this point forward will result in loss of cells/nuclei.

25. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL 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.

26. With the tube still on the magnet, add **10 mL** of ○ **Barcoding Buffer**. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound to the magnet.

27. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.

28. Remove the tube from the magnet and resuspend the pellet in **8.4 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. Keep the sample on ice before proceeding to the next step.

CRITICAL! Do not throw away Ligation Master Mix after use: the remainder will be used in Section 1.3.

1.3. Barcoding Round 2

The pooled cells/nuclei are resuspended in the Ligation Master Mix, which is loaded into the Round 2 Plates. 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 Mix, 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. Place the two Round 2 Plates into two thermocyclers and run the following program. Proceed immediately to the next step.

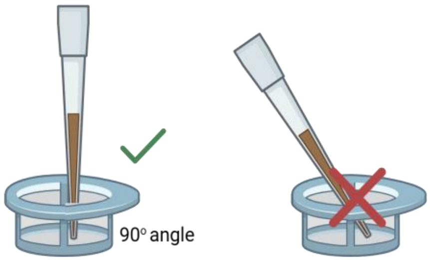
THAW ROUND 2 PLATE (2)		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	10 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

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

5. Pipette the **8.4 mL** of sample in Round 2 Ligation Master Mix 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 2 Plates on ice and the basin on the bench, transfer the cells/nuclei in Round 2 Ligation Master Mix to each well in the Round 2 Plates as follows:

   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 2 Plate **v4 (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** of Round 2 Plate v4 (A).

   d. While secured in a PCR tube rack on a flat surface, add a new plate seal to the fully loaded Round 2 Plate v4 (A).

   e. Repeat steps a-d to load cells into rows **A-H** of Round 2 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 of Round 2 Plate B 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 2 Plates into 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

8. Briefly vortex the Round 2 Stop Mix and ensure there is no precipitate. Transfer **2 mL** from this tube to a new basin with a P1000.

9. Remove the Round 2 Plates from the thermocycler, place in PCR tube racks, remove the plate seal, and store on ice.

10. With the Round 2 Plates on ice and the basin on the bench, transfer the contents from each of the Round 2 Barcoding plates into a basin containing Round 2 Stop Mix as follows:

    a. Starting with Round 2 Plate **v4 (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 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.

11. Transfer the Round 2 pool into a new 15 mL conical tube and apply to the magnet. Loosen or remove the cap to avoid a rotational motion that may cause the pellet to shift. Keep the magnet on ice the entire time, covering the tube in ice up to the liquid level to keep the tube cold. Allow the beads to bind to the magnet for **5 minutes** before proceeding to the next step. If the supernatant is not clear, continue binding to the magnet for an additional 5 minutes.

12. With 5 mL serological pipette or a P1000, and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.

13. With the tube still on the magnet, add **10 mL** of the Barcoding Buffer to rinse the beads. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound to the magnet.

14. With a 5 mL serological pipette or a P1000, and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.

15. Remove tube from magnet and resuspend the beads-bound cells/nuclei in the remaining **8.4 mL** of the Ligation Master Mix. Using a P1000 set to 1000 µL, pipette mix 10x to fully resuspend the beads into 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 Plate. A second in situ ligation reaction adds a third well-specific barcode, the Illumina Truseq Read 2 sequence, and a biotin. The sample is then pooled and strained.

To add round 3 barcodes:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 3 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	2	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 Plates into a thermocycler and run the following program. Proceed immediately to the next step while the program is still running.

THAW ROUND 3 PLATES (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 seal from the Round 3 Plates. Place the Round 3 Plates on ice.

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** from the basin to each well in the Round 3 Plates as follows:

    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-c to add the sample to rows **A-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 thermocyclers and run the following program.

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

8. Briefly vortex the ○ Final 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 ○ 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 to the basin 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 for Round 3 Plate **B,** transferring the content into the same basin.

11. Pipette the pooled sample in 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 the beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear before proceeding to the next step. If the supernatant is not clear, continue binding to the magnet for an additional 5 minutes.

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**, until supernatant is clear.
16. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 µL or a serological pipette.
17. Remove the tube from the magnet and fully resuspend the cells/nuclei in **700 µL** of ○ **Pre-Lysis Dilution Buffer**.

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

18. Proceed immediately to Section 1.5.

1.5. Lysis and Sublibrary Generation

The cell/nuclei pool 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 ○ 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.

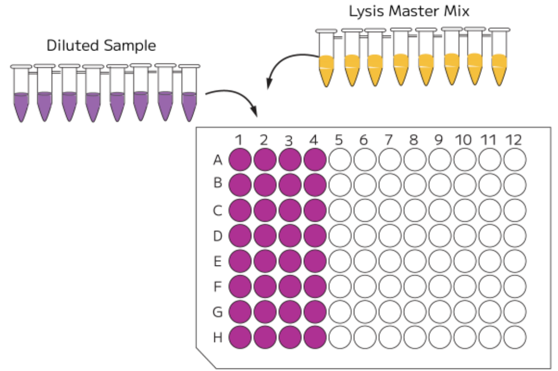


Figure 5: Each tube in the 8-tube PCR strip contains Diluted Sample. Aliquots are pipetted into four columns of the semi-skirted plate, resulting in 4 columns for 32 sublibraries, followed by Lysis Master Mix addition.

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 µL. Store at room temperature.

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

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

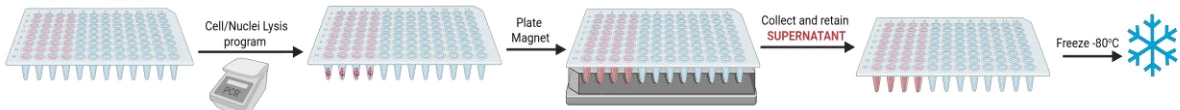
CRITICAL! Do not place Lysis Master Mix on ice, as a precipitate will form.

8. Aliquot out **145 µL** 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 µL** 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).

CRITICAL! Do **NOT** discard the supernatant. At this point cells/nuclei are lysed, so the supernatant contains the cDNA. The beads are no longer needed, and will be discarded.

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.

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.

4. If not continuing directly from Section 1, remove the lysate plate from storage at -80°C.

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

THAW LYSATE PLATE		
Run Time	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.

6. Briefly centrifuge in a swinging bucket centrifuge at 100 x g for **1 minute**. Store at room temperature.

7. 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)

8. 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 ○ Bead Wash Buffer as follows:

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

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

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 ○Binding Buffer as follows and store at room temperature.

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

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

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

17. Using a P200 multichannel pipette, add **50 µL** of ● Streptavidin Beads in ○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 ○ Wash Buffer A to a labeled and empty basin using a P1000 pipette or a serological pipette.

28. Add **5 mL** of ○ 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 µL** ○ 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 µL** of Template Switch Master Mix into 16 PCR tubes to facilitate the use of a multichannel pipette, and store on ice.

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

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

7. While still on the magnet, 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 µL** of ○Wash Buffer B to each column, switching tips between columns.

CRITICAL! Do not discard the other 5 mL tube of ○ 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 ○ 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 µL** 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 ○ **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** ○ 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 ○ **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 µL	1,920 µL
○ cDNA Amp Primers	57.5 µL	1,840 µL
● PCR Additive	2.5 µL	80 µL
Total	120 µL	3,840 µL

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

3. Secure the plate of template switched cDNA from Section 2.2 onto a 96 well PCR tube rack, and remove the 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 75uL. Change tips between each column.

Note: Pipette mixing is necessary to resuspend settled beads.

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

7. While still on the magnet, 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 ○ **Wash Buffer B i**nto 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 ○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 ○ 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
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	Variable, see above
6*	20 sec	67°C*	
7	3 min	72°C	
8	5 min	72°C	1
9	Hold	4°C	1

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

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

2.4. Post-Amplification Purification

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

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.

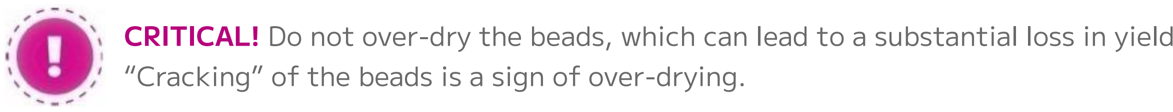
20. While still on the magnet, add **180 µL** of 85% ethanol to each well using a P200 multichannel, switching tips between each column.

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.

STOP 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.

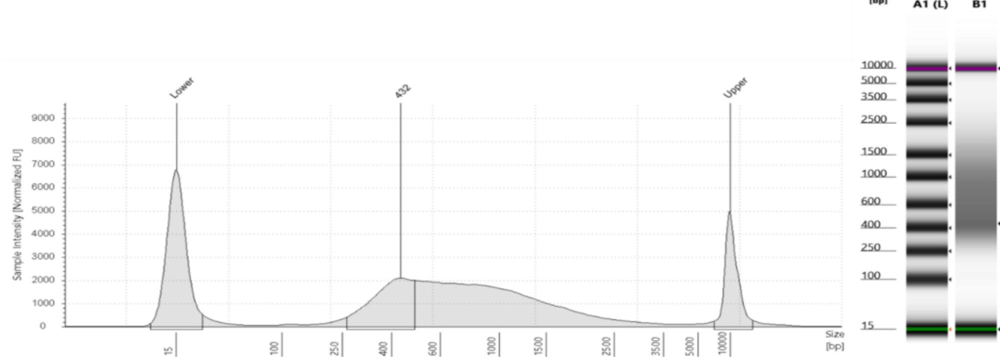


Figure 6: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a Tapestation.

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 µL of SPRI beads per sublibrary to room temperature (**6.4 mL** for 32 sublibraries). The equilibrated SPRI beads will be used in Section 3.2.
4. Gather the following equipment:

   i. Magnetic plate for semi-skirted PCR plates

   ii. 96 tube rack (for stabilizing plate)

   iii. 8-cap strips

   iv. 25 mL basins
5. Obtain recorded cDNA concentrations from Section 2.5.
6. Gather the following items and handle as indicated below.

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

7. If the amplified cDNA was stored at -20°C, start the following program to thaw the plate.

THAW AMPLIFIED cDNA PLATE		
Run Time	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 µL	192 µL
● Fragm/End Prep Enzymes	12 µL	384µL
Total	18 µL	576 µL

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

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

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

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

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

18. Place the plate into the cooled thermocycler. Skip the 4°C hold in Step 1 so the thermocycler proceeds to Step 2 of the Fragmentation and End Prep program.

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

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

3.2. Post-Fragmentation and End Prep Size Selection

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

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 µL** 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 µL** of SPRI beads to each well of fragmented and end prepped DNA using a P200 multichannel, switching tips between each column.
7. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secured.
8. Vortex the plate for **5 seconds**.
9. Centrifuge for **10 seconds** at 100 x g.
10. Incubate for **3 minutes** at room temperature.
11. With the plate secured on a 96 PCR tube rack, remove the caps.
12. Place the plate on the plate magnet. Incubate until the solution clears (~3 minutes)

13. During the incubation, vortex again the 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.

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

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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 µL** of 85% ethanol to each well, switching tips between each column.
16. Incubate for **30 seconds** at room temperature after adding to the last column.
17. While still on the magnet, remove and discard the supernatant from all 4 columns, switching pipette tips between columns.
18. Repeat steps 15-17 once for a total of 2 washes. Remove any residual ethanol with a P20.
19. While still on the magnetic rack, air dry the SPRI beads (~1 minute).
20. Remove the plate from the magnetic rack and secure onto a 96 PCR tube rack.
21. Add **2 mL** of nuclease-free water into the basin labeled as "water" and add **23 µL** of nuclease-free water to each well using a P200 multichannel, switching tips between each column.
22. With the plate secured on a 96 PCR tube rack, seal the plate with 8-cap strips, ensuring each cap is tightly secure
23. Vortex for **10 seconds** to resuspend the beads.
24. Centrifuge for **10 seconds** at 100 x g.
25. Incubate for **3 minutes** at room temperature.
26. Place the plate on the plate magnet. Incubate until the solution clears (~1 minute).
27. While still on the magnetic rack, transfer exactly **21 µL** of the supernatant containing the purified adapter ligated DNA into a new low-bind semi-skirted PCR plate, switching tips between each column. Store on ice.
28. Proceed immediately to Section 3.5.

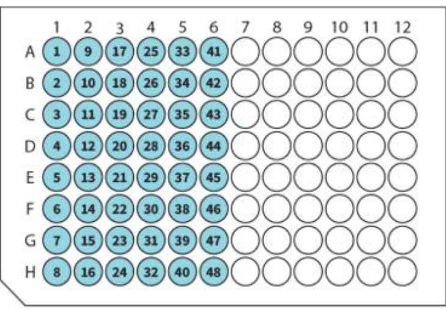
3.5. Barcoding Round 4

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

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 µL** of SPRI beads into each tube of an 8-tube PCR strip to facilitate the use of a multichannel pipette. Centrifuge briefly.

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

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

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

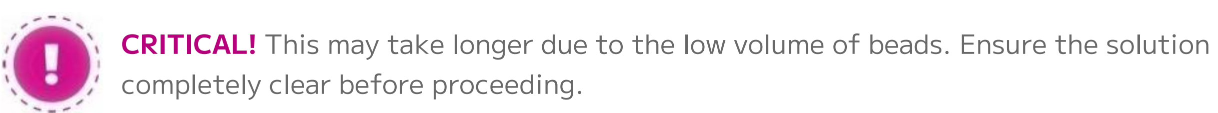
17. Vortex the plate for **5 seconds**.

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

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

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

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



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

22. Add **13 mL** of 85% Ethanol into 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 µL** of 85% ethanol to each well, switching tips between each column.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.7. Sequencing Library Quantification

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

To quantify the sequencing libraries:

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

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

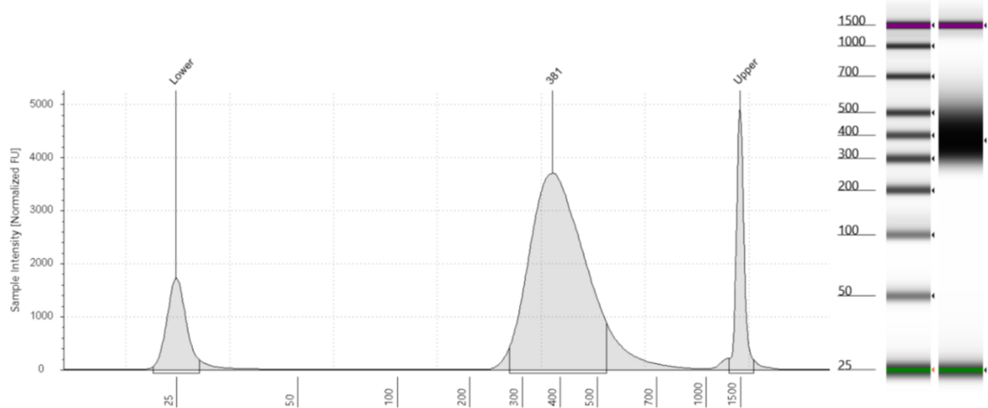


Figure 7: Expected Size Distribution before Illumina Sequencing. Example trace of DNA from indexed sublibraries run on a TapeStation.

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.

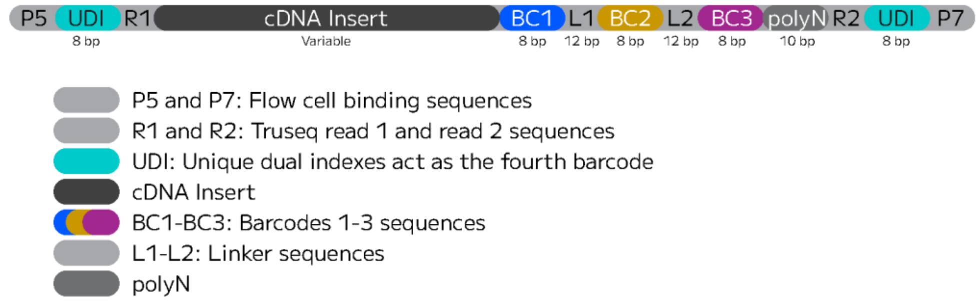


Figure 8: Whole transcriptome sequencing library structure.

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

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

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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_1	A1	CAGATCAC	ATGTGAAG	CTTCACAT
UDI_Plate_WT_2	B1	ACTGATAG	GTCCAACC	GGTTGGAC
UDI_Plate_WT_3	C1	GATCAGTC	AGAGTCAA	TTGACTCT
UDI_Plate_WT_4	D1	CTTGTAAT	AGTTGGCT	AGCCAACT
UDI_Plate_WT_5	E1	AGTCAAGA	ATAAGGCG	CGCCTTAT
UDI_Plate_WT_6	F1	CCGTCCTA	CCGTACAG	CTGTACGG
UDI_Plate_WT_7	G1	GTAGAGTA	CATTCATG	CATGAATG
UDI_Plate_WT_8	H1	GTCCGCCT	AGATACGG	CCGTATCT
UDI_Plate_WT_9	A2	GTGAAACT	TACAGACT	AGTCTGTA
UDI_Plate_WT_10	B2	TCATTCCT	AATGCCTG	CAGGCATT
UDI_Plate_WT_11	C2	GGTAGCAT	TGCTTGCC	GGCAAGCA
UDI_Plate_WT_12	D2	ACTTGATC	TTTGGGTG	CACCCAAA
UDI_Plate_WT_13	E2	ATGAGCAT	GAATCTGA	TCAGATTC
UDI_Plate_WT_14	F2	GCGCTATC	CGACTGGA	TCCAGTCG
UDI_Plate_WT_15	G2	TGACCAGT	ACATTGGC	GCCAATGT
UDI_Plate_WT_16	H2	TATAATCA	ACCACTGT	ACAGTGGT
UDI_Plate_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
UDI_Plate_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
UDI_Plate_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
UDI_Plate_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
UDI_Plate_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
UDI_Plate_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
UDI_Plate_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
UDI_Plate_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
UDI_Plate_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
UDI_Plate_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
UDI_Plate_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT
UDI_Plate_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
UDI_Plate_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
UDI_Plate_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA
UDI_Plate_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA
UDI_Plate_WT_35	C5	TTCCATTG	CTGGCATA	TATGCCAG
UDI_Plate_WT_36	D5	TAACGCTG	CTGAGCCA	TGGCTCAG
UDI_Plate_WT_37	E5	TTGGTATG	CTCAATGA	TCATTGAG
UDI_Plate_WT_38	F5	TGAACTGG	CGCATACA	TGTATGCG
UDI_Plate_WT_39	G5	TCCAGTCG	CCGAAGTA	TACTTCGG
UDI_Plate_WT_40	H5	TGTATGCG	CCAGTTCA	TGAACTGG
UDI_Plate_WT_41	A6	TGGCTCAG	CAGCGTTA	TAACGCTG
UDI_Plate_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
UDI_Plate_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix B: Thermocycling Programs

Section 1: ***In Situ*** **Cell/Nuclei Barcoding**

THAW ROUND 1 PLATE P		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	65 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

BARCODING ROUND 1			
Run Time	40 minutes		
Lid Temperature	70°C		
Sample Volume	100 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

<table>
<tr><th colspan="4">INDEXING PCR</th></tr>
<tr><td colspan="2">Run Time</td><td colspan="2">~30 minutes</td></tr>
<tr><td colspan="2">Lid Temperature</td><td colspan="2">105°C</td></tr>
<tr><td colspan="2">Sample Volume</td><td colspan="2">50 µL</td></tr>
<tr><td>Step</td><td>Time</td><td>Temperature</td><td>Cycles</td></tr>
<tr><td>1</td><td>3 min</td><td>95°C</td><td>1</td></tr>
<tr><td>2</td><td>20 s</td><td>98°C</td><td rowspan="3">Variable, see table in Section 3.5</td></tr>
<tr><td>3</td><td>20 s</td><td>67°C</td></tr>
<tr><td>4</td><td>1 min</td><td>72°C</td></tr>
<tr><td>5</td><td>5 min</td><td>72°C</td><td>1</td></tr>
<tr><td>6</td><td>Hold</td><td>4°C</td><td>1</td></tr>
</table>

Appendix C: Revision History

Version	Description	Date
1.0	Initial release	March 2026
1.1	Section 1: Clarified barcoding beads language: discard vs. retain	April 2026
1.2	Section 1.1: Added a critical recommendation on Sample Binding Beads handling.	May 2026

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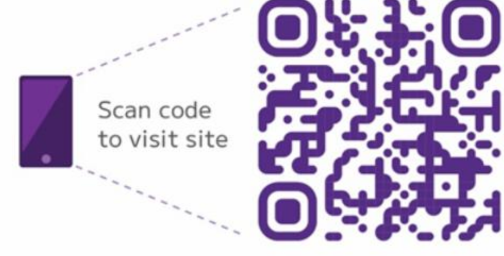

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