User Manual

Version 1.1 – UMIB4300

Evercode™ BCR v4

For use with

ECIB4300

ECIB4310

ECIB4320

Support Suite

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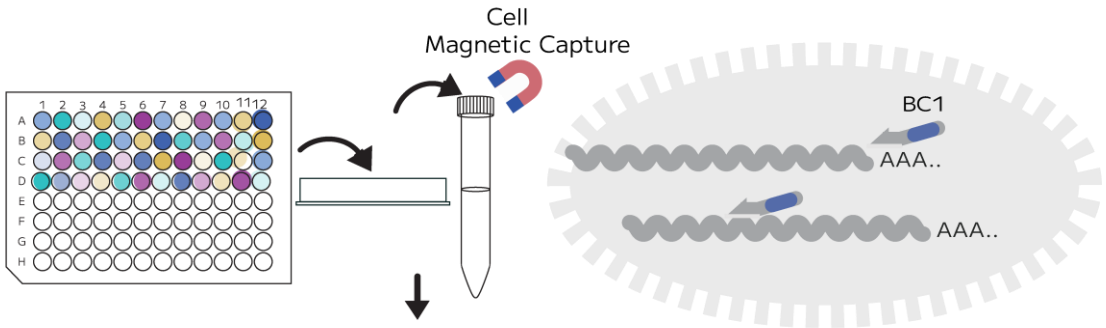
Overview

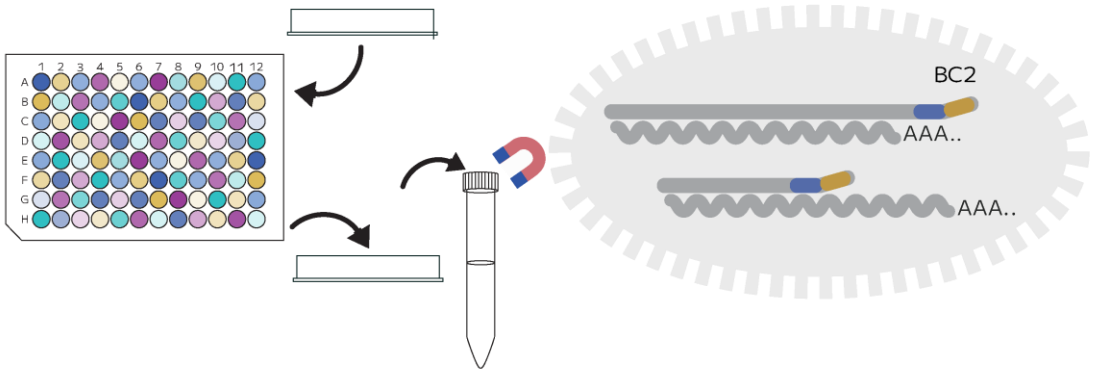
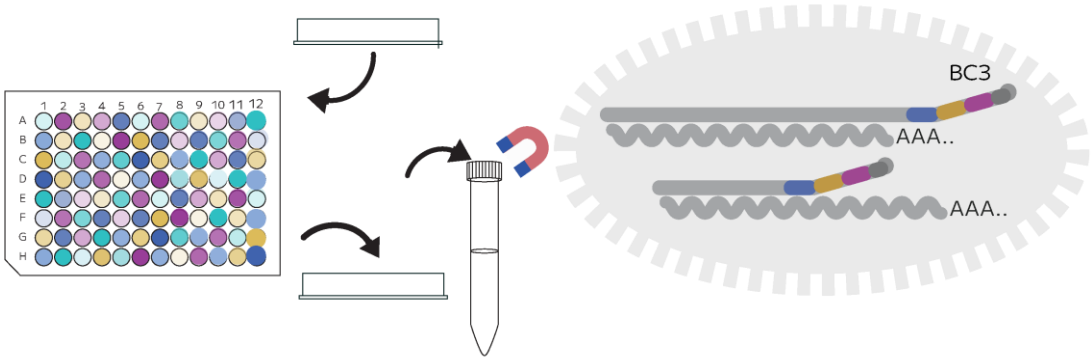
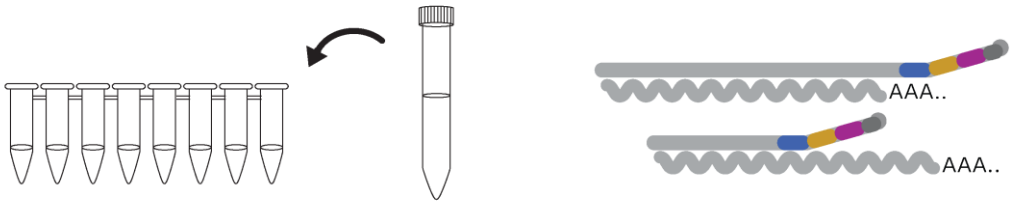
Workflow

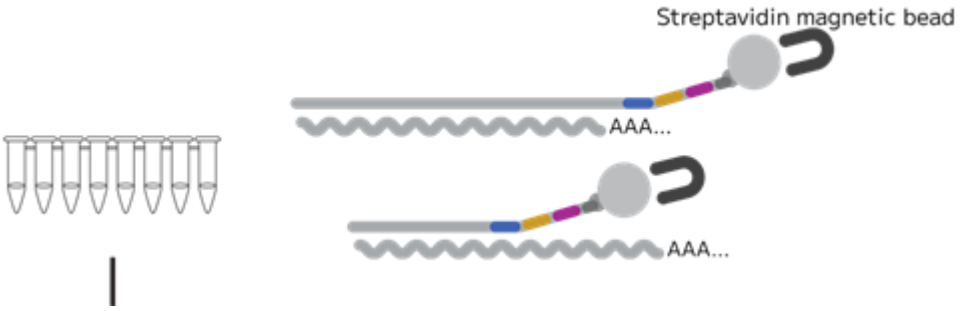
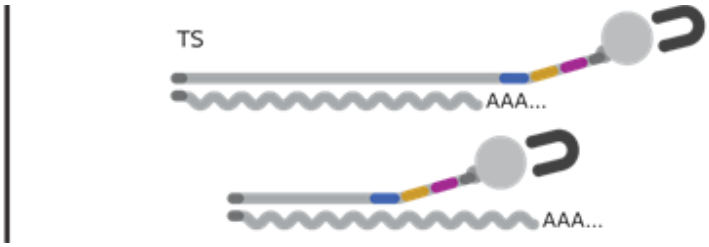
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode BCR kit can profile up to 100,000 cells across up to 48 different biological samples or experimental conditions. Evercode Fixation kits fix and permeabilize cells so they act as individual reaction compartments. This eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell is uniquely labeled. The four rounds of barcoding can yield a vast number of possible barcode combinations, which is more than sufficient to uniquely label up to 100,000 cells while avoiding doublets. After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same four barcode combinations to a single cell.

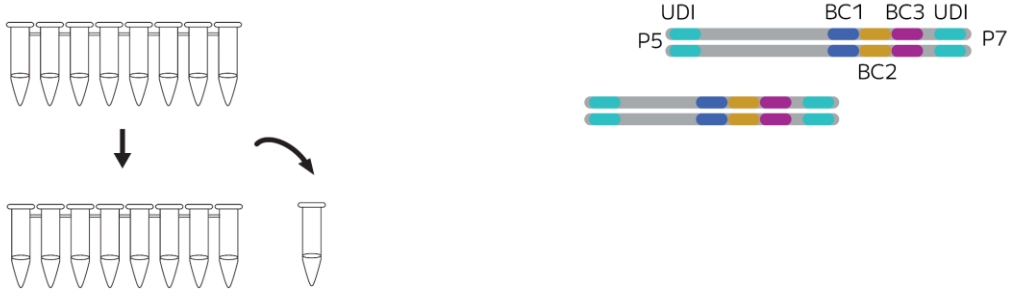
The Evercode BCR kit is a hybridization-based technology that captures B cells' full length V(D)J sequences.

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

Section 1: *In situ* Sample Barcoding	
1	 **Round 1 Barcoding:** Fixed and permeabilized cells are loaded into 48 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with well-specific barcodes that are associated with specific samples. Barcoded samples are pooled and captured via magnetic beads.

Section 1: *In situ* Sample Barcoding	
2	 **Round 2 Barcoding:** The pooled cells are loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode. Cells are pooled and then captured via magnet.
3	 **Round 3 Barcoding:** The cells are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina TruSeq Read 2 sequence, and biotin. Cells are pooled and then captured via magnet.
4	 **Lysis and Sublibrary Generation:** Cells are split across 8 sublibraries and lysed.

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

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

Section 4: BCR Library Preparation	
1	 **WT Libraries Pool and Blocker Hybridization:** Up to 8 WT sublibraries are pooled per BCR capture reaction. Evercode library-specific and repetitive sequences blockers are hybridized.
2	 **BCR Hybridization:** Biotinylated probes in the BCR Panel are hybridized to the target DNA.

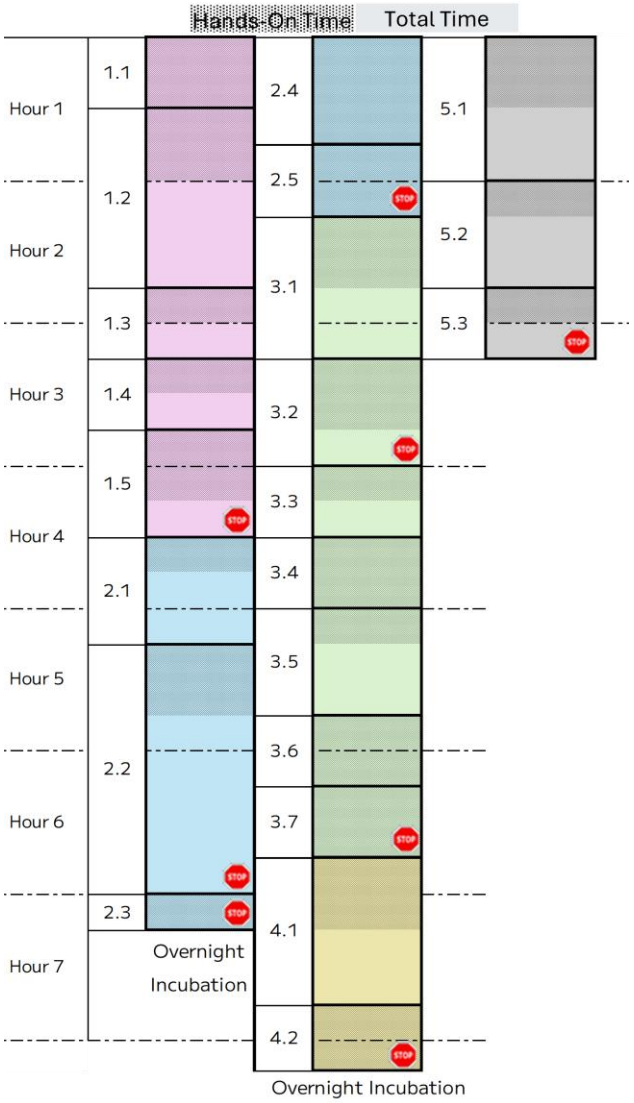
Section 5: BCR Amplification	
1	 **Bind and wash:** Target molecules are captured with Streptavidin Binder Beads and washed.
2	 PCR-Amplified BCR Libraries **BCR Enrichment:** Captured molecules are enriched via PCR. The amplified double stranded libraries are ready to sequence.

Protocol Timing

The table below provides details of the total and hands-on time required for the whole transcriptome and BCR workflow. A visual representation of the workflow is shown below the table.

DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
Section 1: In Situ Cell Barcoding			
1.1 Set up and Sample Counting	Variable (30-90 min)	Variable (30-90 min)	
1.2 Barcoding Round 1	75 min	35 min	
1.3 Barcoding Round 2	30 min	15 min	
1.4 Barcoding Round 3	30 min	15 min	
1.5 Lysis and Sublibrary Generation	40 min	25 min	-80°C ≤ 6 months
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	45 min	10 min	
2.2 cDNA Template Switch	105 min	15 min	4°C ≤ 18 hrs
2.3 cDNA Amplification	90 min	10 min	4°C ≤ 18 hrs in the thermocycler
2.4 Post-Amplification Purification	30 min	30 min	
2.5 cDNA Quantification	30 min	30 min	4°C ≤ 48 hrs or -20°C ≤ 3 months
Section 3: Sequencing Library Preparation			
3.1 Fragmentation and End Prep	60 min	30 min	
3.2 Fragmentation and End Prep Size Selection	30 min	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
3.3 Adapter Ligation	30 min	15 min	
3.4 Post-Ligation Purification.	30 min	30 min	
3.5 Barcoding Round 4	45 min	15 min	4°C ≤ 18 hrs in the thermocycler
3.6 Post-Barcoding Round 4 Size Selection	30 min	30 min	

DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
3.7 Sequencing Library Quantification	30 min	30 min	-20°C ≤ 3 months
Section 4: BCR Library Preparation			
4.1 Pool Libraries	60 min	30 min	
4.2 Hybridization	15-17 hrs	30 min	
Section 5: BCR Amplification			
5.1 Bind and Wash	60 min	30 min	
5.2 BCR Amplification	40 min	10 min	
5.3 BCR Library Quantification	20 min	10 min	-20°C ≤ 3 months



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

- When working with mouse B cells, use the Evercode Mouse TCR/BCR Cell Fixation workflow, which includes a murine RNAse Inhibitor.
- Even if samples were counted before freezing, we strongly recommend counting cells again after thawing to account for any changes during storage and freeze thaw. Typically, a 5-15% decrease after thawing should be expected. These counts are used to determine how cells are loaded in the Round 1 Plate, and their accuracy is critical to recover the desired number of cells.
- When processing many fixed samples, we recommend aliquoting samples after fixation and counting the aliquots the day before using an Evercode BCR kit. The Evercode Fixation User Manuals outline recommendations for generating aliquots. Because aliquots have undergone a similar storage time and a freeze thaw, cell counts from these aliquots will be more representative than using counts from immediately after fixation. Aliquots should be thawed in a water bath set to 37°C in sets of 2-4 and counted with a hemocytometer or alternative counting device. Counts should be recorded in the Sample Loading table, and any remaining sample material from the counting aliquot should be discarded.
- Once fixed samples have been thawed, they should not be refrozen.

Cell Counting and Quality Assessment

- For initial cell counting a hemocytometer can be used. For pre-lysis sample counting, however, a fluorescent counting device is needed. If possible, validate cell counts from alternative devices to a hemocytometer when using Evercode Whole Transcriptome kits for the first time.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- When first using Evercode Whole Transcriptome kits, we suggest saving images at each counting step.

- Examples of AO/DAPI and trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells 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.

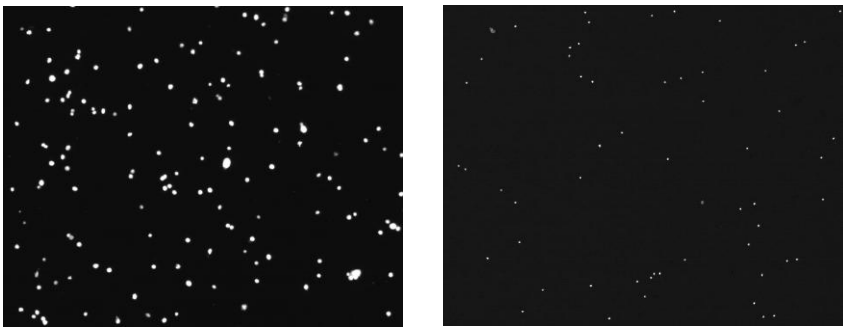


Figure 1: example of AO/DAPI stained HEK cells (left) and PBMCs (right).

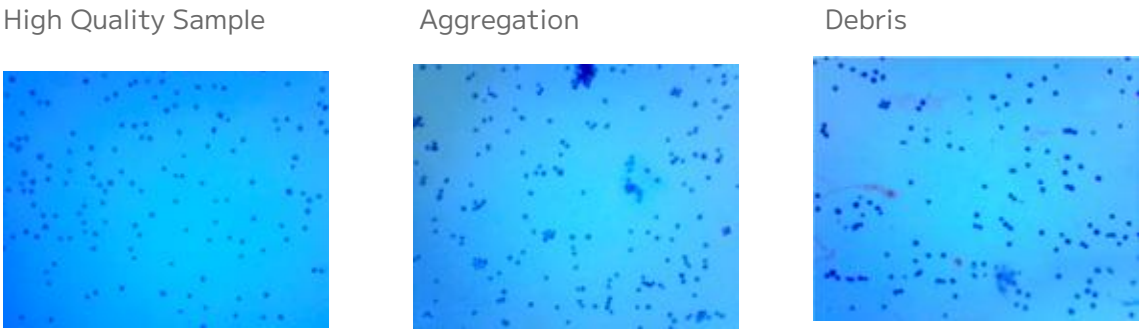


Figure 2: Example of trypan blue stained fixed cells.

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 Recovery

- It is critical to thoroughly resuspend the cells after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are

visible. Due to cell adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell loss.

- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.
- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- The first time using an Evercode 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

- Ensure that the Evercode WT v4 Sample Loading Table being used is the most up-to-date version. The Sample Loading Table can be downloaded from the Parse Biosciences Customer Support Suite. Customer log-in is required to access the Sample Loading Table.
- The Evercode WT v4 Sample Loading Table 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 14 µL of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded cells but change the desired proportion between samples.

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 on 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 the provided 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 BCR v4 workflow uses magnetic beads to pool cells 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.

- A list of three validated 15 mL conical tube magnets are described in "User Supplied Equipment and Reagents" section of this manual to choose from.

- 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.

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

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

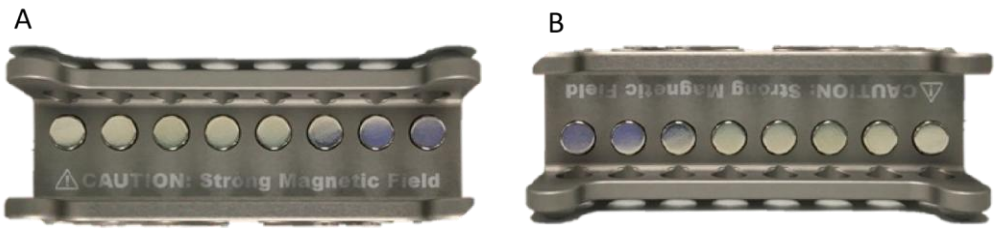


Figure 3: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.

- 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. See the figure below for an example of completely cleared supernatants. Discarding any beads in supernatants will result in a reduction of transcripts and genes detected per cell.



Figure 4: Clear supernatant with compact bead pellets.

Sublibrary Loading

- The Evercode BCR kit generates 8 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.
- The number of cells per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix A.
- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.
- Sublibraries can be loaded with different numbers of cells, and the maximum number of cells that can be analyzed is the sum of cells across all sublibraries.

Sublibrary Concentration

- When pooling sublibraries in Section 4.1, vacuum concentration can be used rather than SPRI bead concentration. See Appendix D for a protocol.

Indexing Primers

- The UDI Plate - WT is a 96-well plate containing 48 unique dual indexing (UDI) primers. Each well is a single-use reaction sufficient for one Barcoding Round 4 PCR reaction for a single sublibrary. Thus, the UDI Plate - WT can be used for multiple Evercode kits.

- The UDI Plate - WT is sealed with a pierceable foil to minimize cross-contamination. The plate seal should be wiped with 70% ethanol and pierced with a new pipette tip immediately prior to use. Avoid splashing or mixing the liquid between individual wells. Once a well has been used, it should not be resealed or reused.

- We recommend selecting UDIs column-wise from left to right (starting with indices 1-8). UDI sequences can be found in Appendix B.

BCR Library Generation

- The BCR library generation protocol begins with indexed sublibraries obtained with Evercode BCR kit. For each library pool, up to 8 indexed sublibraries can be used for BCR library preparation. Pooling sublibraries require at least a concentration of 5 ng/µL per sublibrary. We recommend adding a minimum of 100 ng of each sublibrary to the BCR library pool for a maximum of 800 ng of total input per reaction.

- To ensure even sequencing coverage, sublibraries with similar cell numbers should be mixed in equal proportion. However, if any sublibraries have less than 100 ng of input, 50 ng of each sublibrary can be pooled in equal proportion to a total of 400 ng. Adjust the number of PCR cycles based on the indexed sublibraries input amount to ensure optimal amplification.

Sublibraries Concentration

- SPRI beads are used to concentrate libraries during Section 4.1 of the Evercode BCR workflow and will remain in the solution through subsequent steps. These beads are not removed immediately but will be removed during the post-PCR library cleanup in Section 5.2. In Section 5.1, streptavidin beads are introduced to capture target molecules. As a result, during Section 5 both SPRI and streptavidin beads will be present in the sample.



Thermocycling Programs

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

Part List

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

WT -20°C Reagents Store -20°C, PN HBT500 or MBT600

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	HBT101 or MBT101	Green semi-skirted 96 well plate	1
	Round 2 Plate v4	WT142	Blue semi-skirted 96 well plate	1
	Round 3 Plate	WT103	Yellow semi-skirted 96 well plate	1
	Bead Prep Buffer	WT136	2 mL tube	1
	Barcoding Buffer	WT137	15 mL bottle	1
	Round 1 Stop Mix	WT138	2 mL tube	1
	Ligation Enzyme	WT140	1.5 mL tube	1
	Sample Dilution Buffer	WT105	2 mL tube	1
	Ligation Buffer	WT134	15 mL bottle	1
	Round 2 Stop Mix	WT139	2 mL tube	1
	Final Stop Mix	WT141	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Pre-Lysis Dilution Buffer	WT112	2 mL tube	1
	Pre-Lysis Wash Buffer	WT110	5 mL tube	1
	Lysis Solution	WT146	1.5 mL tube	1
	Lysis Enzyme	WT113	1.5 mL tube	1
	Wash Buffer A	WT143	5 mL tube	1
	Bead Wash Buffer	WT114	5 mL tube	1
	Binding Buffer	WT118	1.5 mL tube	1
	Wash Buffer B	WT144	5 mL tube	1
	Template Switch Buffer	WT148	1.5 mL tube	1
	Template Switch Enzyme	WT121	1.5 mL tube	1
	Template Switch Primer	WT122	1.5 mL tube	1
	PCR Additive	WT150	1.5 mL tube	1
	cDNA Amp Mix	WT123	1.5 mL tube	1
	cDNA Amp Primers	WT124	1.5 mL tube	1
	Fragm/End Prep Buffer	WT125	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Fragm/End Prep Enzymes	WT126	1.5 mL tube	1
	Ligation Adapter	WT127	1.5 mL tube	1
	Adapter Ligation Buffer	WT128	1.5 mL tube	1
	Adapter Ligation Enzyme	WT129	1.5 mL tube	1
	Library Amp Mix	WT130	1.5 mL tube	1

WT+BCR 4°C Reagents. Store 4°C, PN HBT600 or MBT700

LABEL	ITEM	PN	FORMAT	QTY
	Sample Binding Beads	WT135	0.5 mL tube	1
	Streptavidin Beads	WT203	1.5 mL tube	1
	Bead Wash Buffer A	GC301	1.5 mL tube	1
	Bead Wash Buffer B	GC302	2 mL tube	1
	Streptavidin Binding Buffer	GC305	8 mL tube	1
	Streptavidin Binder Beads	GC304	1.5 mL tube	1

BCR -20°C Reagents Store -20°C, PN HBT700 or MBT800 or MBT900

LABEL	ITEM	PN	FORMAT	QTY
	Hybridization Mix	GC101	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Hybridization Enhancer	GC102	1.5 mL tube	1
	Blocker Solution	GC103	1.5 mL tube	1
	Evercode Blocker Solution	GC108	0.5 mL tube	1
 or  or 	Human BCR Panel v4 or Mouse BCR Panel v4 or Transgenic Mouse BCR Panel v4	GC112 or GC113 or GC114	0.5 mL tube	1
	Enrichment Primer Mix	GC105	1.5 mL tube	1
	Enrichment Amplification Mix	GC106	0.5 mL tube	1

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
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.	☐
Parse Biosciences Magnetic Rack	Parse Biosciences	SB1004	We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection.	☐
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 96 well plates and capable of reaching 4°C.	☐
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL and 0.2 mL tubes.	☐
Water bath	Various Suppliers	Varies	Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.	☐
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. 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.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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.	☐
2-4 T100 Thermal Cycler	Bio-Rad Laboratories®	1861096	Or an equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.	☐
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM.	☐
6-inch Platform	Scientific Industries	146-6005-00		
Microplate Foam Insert	Scientific Industries	504-0235-00		
Qubit™ Flex Fluorometer	Thermo Fisher Scientific®	Q33327		☐
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.	☐
4200 TapeStation System	Agilent	G2991BA		
Serological Pipette Controller	Various Suppliers	Varies	Optional.	☐

Consumables

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free, 10 mL or 25 mL polypropylene reagent basins. Do not use polystyrene basins.	☐
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
			centrifuge tubes, as it will lead to substantial cell loss.	
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-20 TC70-SWM-40 TC70-SWM-70 TC70-SWM-100	Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm, 70 µm, 100 µm). We do not recommend FlowMi Cell Strainers (SP Bel-Art).	☐
pluriStrainer® Mini	pluriSelect®	43-10020-40 43-10040-40 43-10070-40 43-10100-40		
Falcon®Cell Strainer	Corning®	431750 431751 431752		
EASYstrainer™, small	Greiner Bio-One™	542120 542140 542170 542100		
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.	☐
DNA LoBind® Tubes, 2 mL, Snap Cap	Eppendorf	022431048	Or equivalent DNA low-binding, nuclease-free 2 mL tubes.	☐
DNA LoBind® Tubes, 5 mL, Snap Cap	Eppendorf	030108310	Or equivalent DNA low-binding, low retention, nuclease-free 5 mL tubes.	☐
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.	☐
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.	☐
5 mL serological pipettes	Various Suppliers	Varies	Optional. Use DNase/RNase-free pipettes.	☐

Reagents

ITEM	SUPPLIER	PN	NOTES	CHEK 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)		
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.	☐
Ethyl alcohol, Pure	Sigma-Aldrich	459844	Or equivalent ethanol.	☐
TE Buffer pH 8.0	Various Suppliers	Varies	Optional. Choose one that is RNase-free.	☐
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.	☐
AO/DAPI	Various Suppliers	Varies	Or alternative viability dyes.	☐
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)		
High Sensitivity D1000 ScreenTape and Reagents	Agilent	5067-5584 (screen tape) 5067-5585 (sample buffer and ladder)		

Section 1: In situ Cell Barcoding

1.1. Set up and Sample Counting

Prior to barcoding, cells are thawed and counted. Appropriate dilutions, loading concentrations, and loading positions are determined by the Sample Loading Table.

To set up for barcoding:

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 Evercode WT v4 Sample Loading Table, which will guide sample dilutions and plate loading in later steps. The Sample Loading Table can be downloaded from the Parse Biosciences Customer Support Suite. Customer log-in is required to access the Sample Loading Table.

3. Fill a bucket with ice.

4. Prepare a hemocytometer, flow cytometer, or other cell counting device.

5. Thaw the previously fixed cell samples in a water bath set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.

6. While minimizing time on ice, count the cells in the sample with a hemocytometer or alternative cell counting device.

7. Record the sample names and cell count in the Sample Loading Table.



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

8. If cells show acceptable integrity and minimal debris, start setting up for barcoding and cool a centrifuge with swinging bucket rotors to 4°C.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 1 Plate	-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. *Note: Bead prep buffer is only used for standard fixed samples.
Bead Prep Buffer*	-20°C Reagents	1	
Round 1 Stop Mix	-20°C Reagents	1	
Ligation Buffer	-20°C Reagents	1	
Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Barcoding Buffer	-20°C Reagents	1	Thaw in a 37°C water bath, then store on ice.
Sample Binding Beads*	4°C Reagents	1	*Used for standard fixed samples only*. Keep at room temperature. Ensure beads are not settled before use.

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

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

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

12. 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 are pooled, and then cell are resuspended.

To add round 1 barcodes:

1. Gently remove the Round 1 Plate 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 from the centrifuge, place in a PCR tube rack on a flat surface, 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 on ice, add **14 µL** of each diluted sample to the appropriate wells of Round 1 Plate as defined in the Sample Loading Table. Mix immediately after dispensing each sample by pipetting 3x.



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



Note: When pipetting the same sample into many wells, the sample must be mixed by gentle pipetting prior to each transfer to avoid cells settling. Do not vortex the samples.

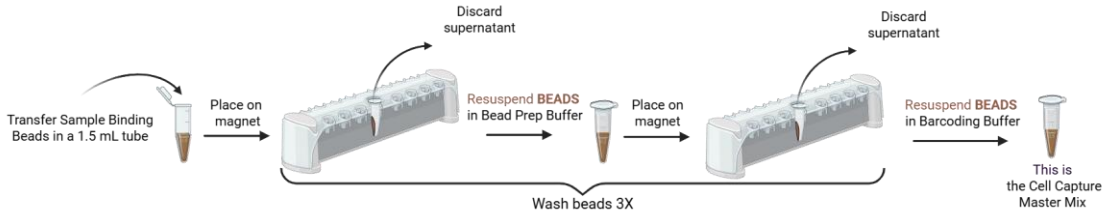
4. While secured in a PCR tube rack on a flat surface, add a new plate seal.

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

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

6. **If using low input samples**, the cells are already attached to the binding beads and don't need further washing. **Proceed directly to step 16**.

7. **If using standard fixation samples**, proceed to the next step to prepare the ● Sample Binding Beads to capture the cells. See workflow below.



Cell Capture Master Mix: Sample Binding Beads are washed three times, then Barcoding Buffer is added.

8. While the thermocycling program is still running, gently pulse-vortex ● Sample Binding Beads to resuspend and store at room temperature. Do not let them settle for >3 minutes before pipetting. Transfer **200 µL** of ● Sample Binding Beads to a 1.5 mL Eppendorf tube.

9. Place the tube on the magnetic rack compatible with 1.5 mL tubes and wait for the solution to clear (~2 minutes).

10. While still on the magnet, remove and discard the supernatant.

11. Remove the tube from the magnetic rack and fully resuspend the bead pellet in **200 µL** of ● **Bead Prep Buffer**.

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

13. While still on the magnet, remove and discard the supernatant.

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

15. Remove the tube from the magnetic rack. Fully resuspend the pellet in **200 µL** of ○ **Barcoding Buffer** and store on ice. This is your Cell Capture Master Mix.

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

16. Proceed to make the Ligation Master Mix for both Round 2 and Round 3 Ligation in a new tube:

LIGATION MASTER MIX	
○ Ligation Buffer	8.8 mL
● Ligation Enzyme	44 µL

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

CRITICAL! **4.2 mL** will be used for the Round 2 ligation barcoding step, and later **4.2 mL** will be used for the Round 3 ligation barcoding step.

18. Remove the Round 1 Plate 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. Place the Round 1 Plate on ice.

19. Transfer all the Round 1 Stop Mix in a new basin with a P1000.

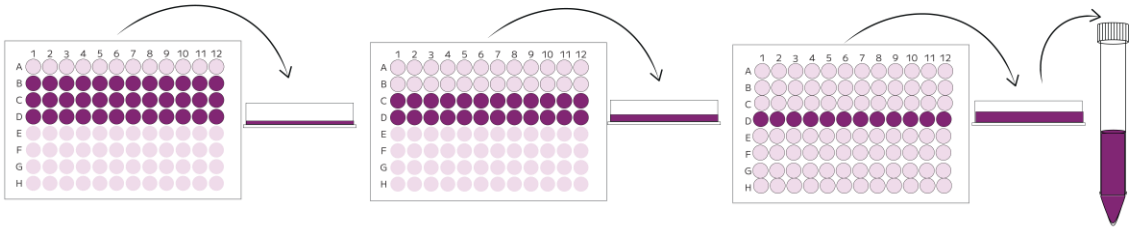
20. With a 12–channel multi-pipette transfer **10 µL** of ● Round 1 Stop Mix into Row A of the Round 1 Plate, still keeping the plate on ice.

    a. Repeat step 20, **3 more times** using the same pipette tips for Rows B-D to dispense the Round 1 Stop Mix into all wells of the Round 1 Plate. \With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.

    b. Transfer **50 µL** from **row A** into a clean basin (bubbles will be generated, this will not impact experimental results).

    c. Repeat b-c for **rows B-D**.

    d. Once cells from all wells are in the basin, using a P1000 transfer the mix into a 15 mL conical tube. Keep on ice.



21. **If using low input fixation samples**, cells have been already captured. Place the 15 mL conical tube **magnet on ice** and wait **1 minute** to let it pre-cool. **Then proceed directly to step 27**.

22. **If using standard fixation samples**, proceed to the next step to capture the cells.

23. Resuspend any settled beads in the Cell Capture Master Mix from step 15 by pipette mixing.

24. Using a P1000, transfer **192 µL** of the Cell Capture Master Mix to the pooled Round 1 cells. Pipette mix 10x with the P1000 set to 900 µL.

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

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

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

27. Remove or loosen the cap, then place the tube with the pooled cells in 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 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.

28. 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.

29. With the tube still on the magnet, add **5 mL** of **O Barcoding Buffer**. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound to the magnet.

30. 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.

31. Remove the tube from the magnet and resuspend the beads-bound cells in **4.2 mL** of Ligation Master Mix. Using a P1000 set to 1000 µL, pipette mix 10x to fully resuspend the beads into the Ligation Master Mix. Keep the sample on ice before proceeding to the next step.



CRITICAL! Do not throw away Ligation Master Mix after use, keep it on ice: the remainder will be used in Section 1.4.

1.3. Barcoding Round 2

The pooled beads-bound cells are loaded into the Round 2 Plate. An in situ ligation reaction adds a well-specific barcode to the 5′ end of the cDNA. The ligation reaction is quenched with Round 2 Stop Mix, and the cells 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	-20°C Reagents	1	Place directly on ice.
Round 2 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.

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

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

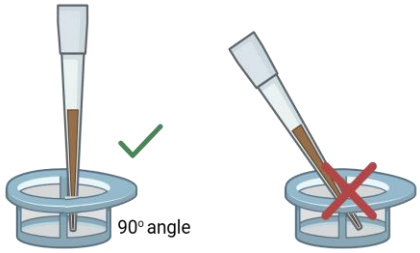
3. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.

4. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate and store on ice.

5. Pipette all of the sample in Ligation Master Mix (**4.2 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 2 Plate on ice and the basin on the bench, transfer the cells in Ligation Master Mix to each well in the Round 2 Plate 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 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**.

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

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

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

9. Briefly vortex the ● Round 2 Stop Mix and ensure there is no precipitate. Transfer **1 mL** from this tube to a new basin with a P1000.

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

11. With the Round 2 Plate on ice and the basin on the bench, transfer the contents of the Round 2 Barcoding plate into the ● Round 2 Stop Mix as follows:

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

    b. Transfer the entire content (**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.

12. Transfer the Round 2 pool into a 15 mL conical tube and apply to the magnet. Loosen or remove the cap to avoid a rotational motion that may cause the pellet to shift. Keep the magnet on ice. 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 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.

14. While the tube is still on the magnet, add **5 mL** of ○ Barcoding Buffer. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound to the magnet.

15. 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.

16. Remove the tube from magnet and resuspend beads-bound cells with **4.2 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 ligation reaction is stopped, and the sample is then pooled and strained.

To add round 3 barcodes:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 3 Plate	-20°C Reagents	1	Place directly on ice.
Final Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
Pre-Lysis Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Pre-Lysis Dilution Buffer	-20°C Reagents	1	
Lysis Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Lysis Solution	-20°C Reagents	1	Thaw and store at room temperature.

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

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

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

5. Pipette all of the sample in Ligation Master Mix (**4.2 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 Plate on ice and the basin on the bench, transfer the cells in Ligation Master Mix to each well in the Round 3 Plate 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 and mix by pipetting 2x.

    c. Changing tips for each row, repeat steps a-b to mix the sample in the basin then transfer to rows **B-H**.

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

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

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

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

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

9. Briefly vortex the Final Stop Mix and ensure there is no precipitate. Transfer **2 mL** to a new basin with a P1000.

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

11. With the Round 3 Plate on ice and the basin on the bench, transfer all the liquid in the Round 3 Plate into the basin with the Final Stop Mix as follows:

    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.

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

13. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear before proceeding to the next step. If the supernatant is not clear, continue binding to the magnet for an additional 5 minutes.

14. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 µL.

15. Remove the 15 mL tube from the magnet and fully resuspend the cells in **4 mL** of **Pre-Lysis Wash Buffer**. Do not invert.

16. 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.

17. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 µL or serological pipette.

18. Remove the tube from the magnet and resuspend the cells in **80 µL** of ● Pre-Lysis Dilution Buffer.



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

19. Proceed to Lysis and Sublibrary Generation Step 1.5.

1.5. Lysis and Sublibrary Generation

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

1. While minimizing time on ice, count the number of cells in the sample with a fluorescent (e.g. DAPI) cell counting device. Record the cell count.



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

2. Decide how to divide cells across the 8 sublibraries. See the "Sublibrary Loading" in the Important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix A to determine the volume of sample and ● Pre-Lysis Dilution Buffer that should be added to each sublibrary.



CRITICAL! Do not add more than 12,500 cells to a sublibrary. Adding additional cells will result in an increased multiplet rate.

3. Ensure the cells are in suspension by pipetting 5x with a P200 set to 200 µL prior to each transfer. Add the appropriate volume of sample to 8 different 0.2 mL PCR tubes.

4. Keeping the samples on ice, add the appropriate volume of ● Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of **25 µL**.

5. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P200 set to 200 µL. Store at room temperature.

LYSIS MASTER MIX	
● Lysis Solution	220 µL
● Lysis Enzyme	44 µL
Total	264 µL

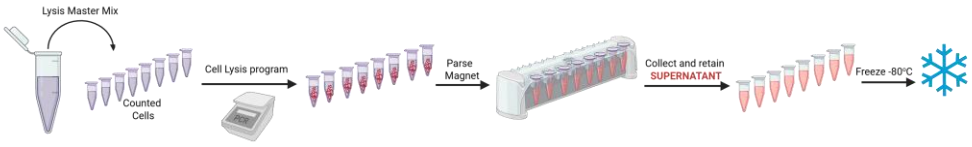


Note: Ensure that there is no precipitate before using the ● Lysis Solution.



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

6. For a visual representation of the next few steps see the workflow below. Add **30 µL** of Lysis Master Mix to each 0.2 mL tube with diluted cells. Store at room temperature.



7. Vortex the 0.2 mL tube(s) for **1 minute**. Briefly centrifuge.

8. Place the tube(s) into a thermocycler and run the following program. If continuing to Section 2 without freezing the sample, proceed to Section 2 while the program is still running.

CELL 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

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

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

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

11. 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.
    a. Magnetic rack for 1.5 mL tubes
    b. Parse Biosciences magnetic rack for 0.2 mL PCR tubes
    c. Vortex with an adapter for 96 well plates
3. Gather the following items and handle as indicated below:

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

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

Lysates being processed	1	8
● Streptavidin Beads	44 µL	352 µL

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

6. Remove and discard the supernatant.

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

Lysates being processed	1	8
○ Bead Wash Buffer	50 µL	400 µL



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

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

9. Remove and discard the supernatant.

10. Repeat steps 7-9 twice for a total of 3 washes.

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

Lysates being processed	1	8
● Binding Buffer	55 µL	440 µL

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

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

THAW LYSATES		
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 precipitation before proceeding. Otherwise, an additional 5 minutes at 37°C should be sufficient.

14. Briefly centrifuge the lysates and store at room temperature.

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

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

17. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex plastic holder on 100% power for **10 seconds**.

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



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

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

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

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



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

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

23. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ○ **Wash Buffer A** by vortexing for **10 seconds** (100% power).



Note: Save ○Wash Buffer A to use for optional storage before cDNA amplification.

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

25. 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 Buffer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by pipetting **5x**. Briefly centrifuge before use.
Template Switch Primer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by pipetting 3x. Briefly centrifuge before use.
Template Switch Enzyme	-20°C Reagents	1	Keep on ice. Briefly centrifuge before use.

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

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

TEMPLATE SWITCH MASTER MIX		
Number of Samples	1	8
Template Switch Buffer	87 µL	696 µL
Template Switch Primer	3 µL	24 µL
Template Switch Enzyme	6 µL	48 µL
Total	96 µL	768 µL

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

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

5. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** O**Wash Buffer B** by vortexing for **10 seconds** (100% power).

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

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

7. While still on the magnetic rack, remove and discard the O **Wash Buffer B**.

8. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **20 µL** Nuclease-Free water by vortexing for **10 seconds**, followed by a brief centrifugation.

9. Add **80 µL** of the Template Switch Master Mix to each resuspended pellet and mix 5x with a P200 set to 80 µL.

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

10. Briefly centrifuge without letting beads collect at the bottom of the tube(s).

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

12. Fully resuspend each bead pellet by mixing 5x with a P200 set to 75 µL.

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

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

14. Proceed immediately to Section 2.3. Alternatively, proceed to step 15 to store samples prior to cDNA amplification.

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



Note: Beads may need to be resuspended if they have settled prior to placing on the magnet.

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

17. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** Wash Buffer A by vortexing for **10 seconds** (100% power).



Safe stopping point: Template switched cDNA can be stored in Wash Buffer A 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. Gather the following items and handle as indicated below:

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

2. Equilibrate 80 µL of SPRI beads per lysate to room temperature. Immediately proceed to step 3 after placing SPRI beads at room temperature. These will be used later.

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

cDNA AMPLIFICATION MIX		
Number of Sublibraries	1	8
cDNA Amp Mix	60 µL	480 µL
cDNA Amp Primers	58.75 µL	470 µL
PCR Additive	1.25 µL	10 µL
Total	120 µL	960 µL

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



Note: You may need to pipette mix to resuspend settled beads so they separate appropriately prior to placing them on the magnet.

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

6. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ○ **Wash Buffer B** by vortexing for **10 seconds** (100% power).

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

8. While still on the magnetic rack, remove and discard the ○ Wash Buffer B.

9. Remove tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 µL** of the Amplification Master Mix. Store on ice.

10. 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 in Sublibrary	Cell lines	Human Primary B Cells	Human PBMCs	Mouse Primary B Cells
200-999	11	12	13	14
1,000-1,999	9	10	11	12
2,000-5,999	7	8	9	10
6,000-12,500	6	7	8	9

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

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

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

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

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

2.4. Post-Amplification Purification

Amplified cDNA is purified with a single sided SPRI bead cleanup.

CRITICAL! Always use new tips for each sublibrary.

To purify the cDNA:

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

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

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

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

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

13. Repeat steps 10-12 once for a total of 2 washes. Remove any residual ethanol with a P20.

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



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

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

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

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



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

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



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

2.5. cDNA Quantification

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

To quantify the cDNA:

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

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

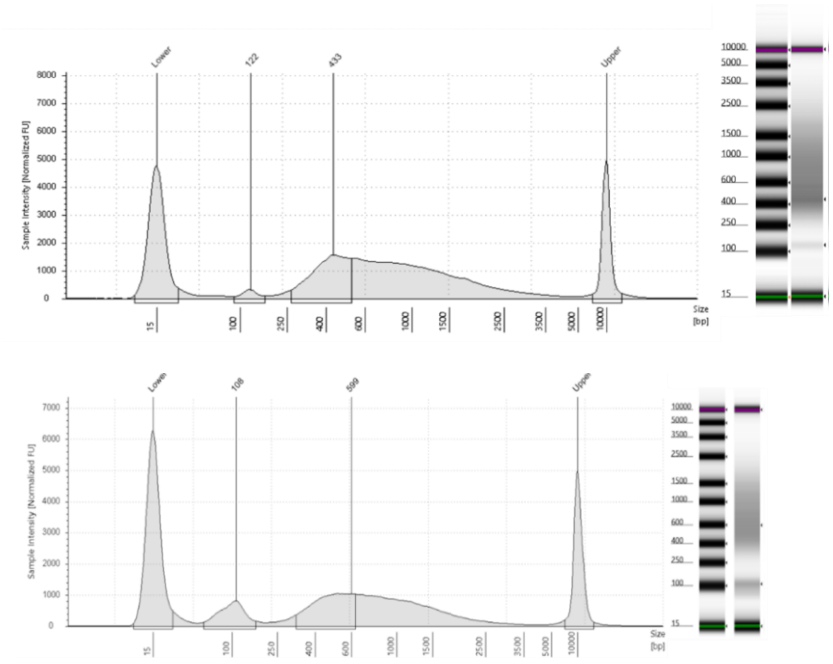
3. 

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

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

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

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

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

FRAGMENTATION and END PREP		
Run Time	35 minutes	
Lid Temperature	70°C	
Sample Volume	50 µL	
Step	Time	Temperature
1	Hold*	4°C
2	5 min	32°C
3	30 min	65°C
4	Hold	4°C



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

10 Mix ● Fragm/End Prep Buffer by vortexing for **5 seconds**. Briefly centrifuge and store on ice.



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

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

FRAGMENTATION AND END PREP MASTER MIX		
Number of Sublibraries	1	8
● Fragm/End Prep Buffer	6 µL	48 µL
● Fragm/End Prep Enzymes	12 µL	96 µL
Total	18 µL	144 µL

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

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

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

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

3.2. Post-Fragmentation and End Prep Size Selection

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

CRITICAL! Always use new tips for each sublibrary.

To size select the fragmented and end prepped DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads.

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

3 Vortex the SPRI beads until fully mixed. Add **40 µL** of SPRI beads to each tube of fragmented and end prepped DNA.

4 Vortex the tube(s) for **5 seconds**. Briefly centrifuge.

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

6 Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

CRITICAL! Ensure the solution is completely clear before proceeding.

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

8 While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.

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

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

11 Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.

12 While still on the magnetic rack, air dry the SPRI beads (~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.

13 Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **50 µL** of nuclease-free water.

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

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

16 While still on the magnetic rack, transfer **50 µL** of the supernatant into new 0.2 mL tube(s).



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

3.3. Adapter Ligation

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

To ligate adapters:

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

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

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

ADAPTER LIGATION MASTER MIX		
Number of Sublibraries	1	8
Nuclease-free water	19.25 µL	154 µL
Adapter Ligation Buffer	22 µL	176 µL
Adapter Ligation Enzyme	11 µL	88 µL
Ligation Adapter	2.75 µL	22 µL
Total	55 µL	440 µL

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

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

ADAPTER LIGATION		
Run Time	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.

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

3.4. Post-Ligation Purification

Adapter ligated DNA is size selected with a single sided SPRI bead cleanup.

CRITICAL! Always use new tips for each sublibrary.

To purify the ligated DNA:

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

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

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

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

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

17. Proceed immediately to Section 3.5.

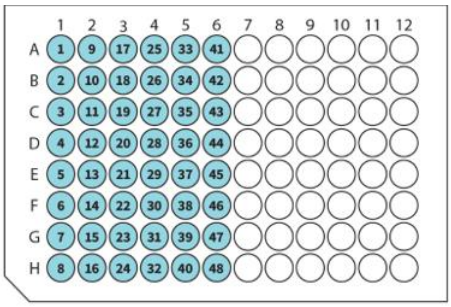
3.5. Barcoding Round 4

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

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 sublibrary being processed, choose one unused well of the UDI Plate - WT and record the well position and number for each sublibrary.



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

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

6. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C. Do not reuse wells.
7. Add **25 µL** of ● Library Amp Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 25 µL. Briefly centrifuge.

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

NUMBER OF PCR CYCLES	
cDNA Input (ng)	PCR Cycles
10-24	13
25-49	12
50-99	11
100-199	10
200-399	9
400-999	8
1,000 or more	7

9. 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 sec	98°C	Varies, see table above
3	20 sec	67°C	
4	1 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

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

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

3.6. Post-Round 4 Barcoding Size Selection

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

CRITICAL! Always use new tips for each sublibrary.

To size select the sequencing libraries:

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

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

3. Vortex the SPRI beads until fully mixed. Add **40 µL** of SPRI beads to each sequencing library tube.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes)

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

7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).
13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **20 µL** of nuclease-free water or TE Buffer.

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

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

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

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

3.7. Sequencing Library Quantification

The concentration and size distribution of the sequencing libraries are measured with fluorescent dyes and capillary electrophoresis. 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 D5000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

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

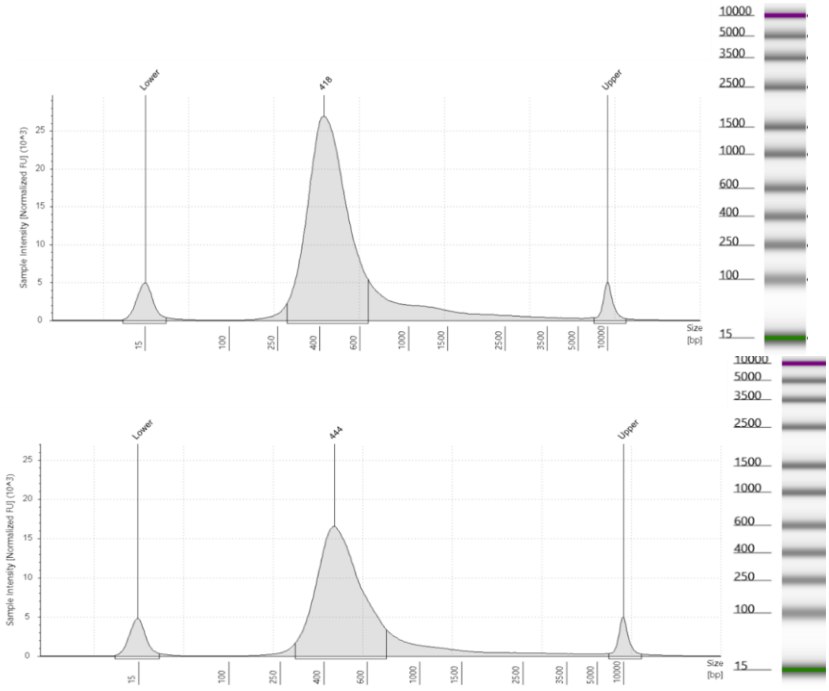


Figure 6: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA (Top) and Mouse DNA (Bottom) from indexed sublibraries run on a TapeStation.

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 400-500 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 400-500 bp). Do not use this additional peak when estimating amplicon size.

Section 4: BCR Library Preparation

4.1. Pool Libraries

Libraries are pooled and prepared for the hybridization reaction. Up to 8 indexed sublibraries can be used for BCR library preparation. No more than **12 µL** per sublibrary should be used to prepare the BCR library to ensure that there is sufficient input for WT sequencing. When pooling sublibraries in step 10, vacuum concentration can be used rather than SPRI bead concentration. See Appendix D for a protocol.

To prepare for BCR library preparation:

1. Fill a bucket with ice.
2. Set a heat block to 65°C.
3. Program a thermocycler to 95°C and set the heated lid to 105°C.
4. Equilibrate SPRI beads (Ampure XP or KAPA Pure Beads) to room temperature for at least 30 minutes.
5. Prepare at least 700 µL of 85% ethanol per reaction and the magnetic rack for 1.5 mL tubes.
6. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Evercode BCR Indexed Sublibraries	User Stored Location (-20°C)	Up to 8 per reaction	Thaw on ice.
● Hybridization Mix	BCR -20°C Reagents	1	Heat at 65°C for 10 minutes, then keep at room temperature
● Hybridization Enhancer	BCR -20°C Reagents	1	Thaw on ice. Briefly centrifuge before use.
● Blocker Solution	BCR -20°C Reagents	1	
● Evercode Blocker Solution	BCR -20°C Reagents	1	
● Human or Mouse or Transgenic Mouse BCR Panel v4	BCR -20°C Reagents	1	

7. Incubate the ● Hybridization Mix in the heat block at 65°C for **10 minutes** or until no precipitate is present before proceeding.

8. Incubate the ● Hybridization Mix at room temperature for **5 minutes** to equilibrate the solution.

9. Vortex sublibraries (2-3 seconds). Briefly centrifuge (~2 seconds).

10. Pool up to 8 indexed sublibraries into a 1.5 mL tube to make a Library Pool. We recommend adding **100 ng** of each sublibrary going into BCR library preparation for a maximum of 800 ng of input per tube.



Note: See "BCR Library Generation" in Important Guidelines if your sublibraries contain less than 100 ng of total mass.

11. If the total volume of the Library Pool is less than **100 µL**, bring the volume up to 100 µL with nuclease-free water.

12. Vortex the SPRI beads until fully mixed. Add **1.8x** of SPRI beads to the Library Pool.



Note: For example, if a Library Pool is 100 µL, add (1.8 x 100 µL) = 180 µL of SPRI beads.

13. Vortex the tube for **5 seconds**. Briefly centrifuge (~2 seconds).

14. Incubate tube at room temperature for **5 minutes**.

15. Place the tube in a 1.5 mL magnetic rack and wait for all the beads to bind to the magnet (~3 minutes: ensure the liquid is clear).

16. With the tube still on the magnetic rack, slowly remove and discard the clear supernatant.

17. Without resuspending beads, add **300 µL** of 85% ethanol to the tube using a P1000 and wait **1 minute**.

18. Using a pipette, aspirate and discard the ethanol from the tube.

19. Without resuspending beads, add another **300 µL** of 85% ethanol to the tube using a P1000 and wait **1 minute**.

20. Using a pipette, aspirate and discard all of the ethanol from the tube (it may be necessary to remove the final few drops with a P20 pipette). With the tube still on the rack, air dry the beads (~2 minutes).



CRITICAL! Do not over-dry the beads. Over-drying of beads can lead to substantial losses in yield. “Cracking” of the beads is a sign of over-drying.

21. Remove the tube from the magnetic rack. Proceed directly to Section 4.2.

4.2. Blocking & Hybridization

Blockers are added to the library pool to reduce off-target binding. Next, during hybridization, biotinylated probes bind to the BCR repertoire.

1. Add the following reagents individually to Library Pool from step 4.1.21.

ADD TO THE LIBRARY POOL	
ITEM	VOLUME (µL)
Evercode Blocker Solution	7
Blocker Solution	5
TOTAL	12

2. Resuspend the beads by gentle pipetting with a P20 set to 10 µL.

3. Briefly centrifuge and carefully transfer the entire volume (12 µL) into a **new 0.2 mL tube** using a P20 set to 15 µL.

4. Heat the Library Pool for **5 minutes** at 95°C in a thermocycler with the lid heated to 105°C, then immediately place at room temperature.

5. Prepare a Probe Solution in a **new 0.2 mL tube**. Mix by pipetting.

PROBE SOLUTION	
ITEM	Volume for 1 Library pool (µL)
Hybridization Mix	21
Human or Mouse or Transgenic Mouse BCR Panel v4	4.2
Nuclease-free Water	4.2
TOTAL	29.4

6. Heat the Probe Solution for **2 minutes** at 95°C in a thermocycler with the lid heated to 105°C, then immediately cool for **5 minutes** on ice.

7. Separately, incubate both the Probe Solution and Library Pool at room temperature for **5 minutes**. For timing, see the figure below.



Figure 7: Heating and denaturing steps for Probe Solution and Library Pool are efficiently performed together.

8. Set a thermocycler to 70°C with the lid at 85°C for infinite time.
9. Vortex the Probe Solution for **5 seconds**. Briefly centrifuge (~2 seconds).
10. Transfer **28 µL** of the Probe Solution into the Library Pool to create the Hybridization Reaction.
11. Vortex the tube for **5 seconds**. Briefly centrifuge (~2 seconds).
12. Without mixing, add **30 µL** of ● Hybridization Enhancer to the top of the meniscus of the Hybridization Reaction from step 11.

CRITICAL! Do not mix after adding the Hybridization Enhancer. It should form a distinct layer above the hybridization reaction.

13. Briefly centrifuge tube to ensure there are no bubbles present.
14. Ensure the tube is tightly capped to prevent excess evaporation.
15. Incubate the Hybridization Reaction (72 µL) at 70°C for **15-17 hours** in a thermocycler with the lid at 85°C.

Note: Hybridizing for less than 15 hours or more than 17 hours will impact hybridization efficiency.

Section 5: BCR Amplification

5.1. Bind and Wash

Streptavidin binder beads capture the hybridized BCR repertoire prior to amplification.

To prepare BCR capture pool for amplification:

1. Gather two heat blocks, set one to 68°C (for 1.5 mL tube) and the other to 48°C (for 2 mL and 5 mL tubes).

2. Equilibrate ● Streptavidin Binder Beads and the SPRI beads to room temperature for at least **30 minutes**.



Note: See Important Guidelines, "Sublibraries Concentration" for clarifications about the Streptavidin Beads and the SPRI beads in the sample.

3. Prepare at least 400 µL of 85% ethanol.

4. Prepare the magnetic rack for 1.5 mL tubes.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Bead Wash Buffer A	WT+ BCR 4°C Reagents	1	Heat tube at 68°C and keep at 68°C.
● Bead Wash Buffer B	WT+ BCR 4°C Reagents	1	Heat tube at 48°C and keep at 48°C.
○ Streptavidin Binding Buffer	WT+ BCR 4°C Reagents	1	Heat tube at 48°C for 5 minutes. Then keep at room temperature.
● Streptavidin Binder Beads	WT+ BCR 4°C Reagents	1	Equilibrate at room temperature for at least 30 minutes.
● Enrichment Primer Mix	BCR -20°C Reagents	1	Thaw at room temperature, then place on ice.
● Enrichment Amplification Mix	BCR -20°C Reagents	1	Thaw on ice. Briefly centrifuge before use.

6. Check if precipitate is dissolved, then equilibrate the ○Streptavidin Binding Buffer to room temperature. Keep the ● Bead Wash Buffer A and ● Bead Wash Buffer B in their respective heat blocks.

7. Ensure the ● Streptavidin Binder Beads have been equilibrated to room temperature for at least **30 minutes**. Vortex the ● Streptavidin Binder Beads until mixed.

8. Add **100 µL** of ● Streptavidin Binder Beads to a new 1.5 mL tube for the hybridization reaction.

9. Add **200 µL** of ○Streptavidin Binding Buffer to the tube with ● Streptavidin Binder Beads. Mix thoroughly by pipetting 10x with a P200 set to 200 µL.

10. Place the tube in a 1.5 mL magnetic rack and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).

11. Using a pipette, aspirate and discard the supernatant from the tube. Remove tube from the magnetic rack.

12. Repeat steps 9-11 twice for a total of three washes.

13. Add **200 µL** of ○Streptavidin Binding Buffer and resuspend the beads by vortexing until fully homogenized.

14. Heat the resuspended beads at 68°C for **10 minutes** before proceeding to the next step.

15. Set a P200 pipette to 80 µL. After the hybridization reaction from Section 4.2 is complete, open the thermocycler lid and immediately transfer the entire volume of the Hybridization Reaction into a corresponding tube of preheated ● Streptavidin Binder Beads from step 5.1.14. Mix by pipetting.



CRITICAL! The rapid transfer from the 70°C thermocycler in step 4.2.15 is a critical step for minimizing off-target binding. Do not remove the Hybridization Reaction tube from the thermocycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptavidin Binder Beads. Allowing the Hybridization Reaction to cool to room temperature for more than 5 minutes will result in as much as 10–20% increase in off-target binding.

16. Incubate the tube containing the Hybridization Reaction and ● Streptavidin Binder Beads mixture at 68°C for **5 minutes**.

17. Remove the tube from the heat block. Do not vortex, briefly centrifuge (~2 seconds).

18. Place the tube in a magnetic rack and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).

19. Using a pipette, aspirate and discard the clear supernatant from the tube.

Note: Some Hybridization Enhancer reagent residue may be visible after supernatant removal and throughout each wash step. This will not affect the final capture product.

20. Remove the tube from the magnetic rack, add **200 µL** of 68°C ● Bead Wash Buffer A. Mix by pipetting.

21. Incubate the tube at 68°C for **5 minutes**. Briefly centrifuge.

22. For the hybridization reaction, transfer the entire volume from step 21 (~200 µL) into a new 1.5 mL tube.

CRITICAL! This step reduces background from non-specific binding to the surface of the tube.

23. Place the tube in a 1.5 mL magnetic rack and wait for all the beads to bind to the magnet (~1 minute: liquid should be clear).

24. Using a pipette, aspirate and discard the clear supernatant from the tube.

25. Remove the tube from the magnetic rack and add **200 µL** of 48°C ● Bead Wash Buffer B. Mix by pipetting and briefly centrifuge.

26. Incubate the tube at 48°C for **5 minutes** and place ● Bead Wash Buffer B back into the 48°C heat block.

27. Place the tube in a magnetic rack and wait for all the beads to bind to the magnet (~1 minute: liquid should be clear).

28. Using a pipette, aspirate and discard the clear supernatant from the tube.

29. Repeat steps 25-28 twice for a total of three washes.

30. Remove any residual supernatant with a P20 pipette. Proceed immediately to the next step.

31. Remove the tube from the magnetic rack and resuspend in **45 µL** of nuclease-free water. Mix by pipetting until homogenized, then incubate on ice. This solution will be referred to as Streptavidin Binding Bead Slurry.

32. Proceed immediately to Section 5.2.

5.2. BCR Amplification

The BCR repertoire is amplified for sequencing.

1. Prepare 400 µL of 85% ethanol per reaction.
2. Prepare the magnetic rack for 1.5 mL tubes.
3. Mix the Streptavidin Binding Bead Slurry by pipetting.
4. For the hybridization reaction, prepare a PCR mixture in a new 0.2 mL PCR tube as follows. Then, mix by pipetting, centrifuge briefly, and store on ice.

PCR MIXTURE	
REAGENTS	VOLUME (µL)
Streptavidin Binding Bead Slurry	22.5*
Enrichment Primer Mix	2.5
Enrichment Amplification mix	25
TOTAL	50

Note: *When preparing the PCR mixture, only half the slurry is necessary for immediate use. Store the unused slurry at -20°C.

5. Determine the number of PCR cycles required for the total amount of input into the library pool, as shown in the table below.

NUMBER OF PCR CYCLES	
Library Pool (ng)	PCR Cycles
800	13
400	14
200	15
100	16

6. Place the tube into a thermocycler and run the following program

BCR AMPLIFICATION			
Run Time		~30 min	
Lid Temperature		105°C	
Sample Volume		50 µL	
Step	Time	Temperature	Cycles
1	45 sec	98°C	1
2	15 sec	98°C	Variable, see above
3	30 sec	60°C	
4	30 sec	72°C	
5	1 min	72°C	1
6	Hold	4°C	1

7. As soon as the program reaches 4°C, place the tube at room temperature and proceed immediately to the next step.

8. Ensure the SPRI beads have been equilibrated to room temperature for at least **30 minutes**. Vortex the SPRI beads until fully mixed. Add **90 µL** of SPRI beads (1.8x) to the tube. Vortex for **5 seconds**.

Note: It is not necessary to recover supernatant or remove Streptavidin Binder Beads from the amplified PCR product.

9. Incubate tube at room temperature for **5 minutes**.

10. Place the tube on the high position of the magnetic rack for 0.2 mL tubes and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).

11. With tube still on the magnetic rack, remove and discard the clear supernatant.

12. With tube still on the magnetic rack, add **180 µL** of 85% ethanol to the tube.

13. Incubate tube at room temperature for **1 minute**.



CRITICAL! Do not over-dry the beads. Over-drying of beads can lead to substantial losses in yield. “Cracking” of the beads is a sign of over-drying.

14. With tube still on the magnetic rack, remove and discard the supernatant.
15. Repeat steps 12-14 once more for a total of 2 washes. Remove any residual ethanol with a P20 pipette.
16. With tube still on the magnetic rack, air dry the beads (~**30 seconds**).
17. Remove the tube from the magnetic rack. Fully resuspend the bead pellet with **32 µL** of nuclease-free water.
18. Incubate the tube at room temperature for **5 minutes**.
19. Place the tube on the low magnet position of the magnetic rack for 0.2 mL tubes and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).
20. With tube still on the magnetic rack, transfer **30 µL** of the supernatant containing the purified BCR sublibraries into new 0.2 mL tube. Store on ice.
21. Proceed to Section 5.3.

5.3. BCR Library Quantification

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

1. Measure the concentration of the BCR library from Section 5.2 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

2. Assess the size distribution of the purified DNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System according to the manufacturer's instructions. See Figure 8 for the expected size distribution.

3. For sequencing, refer to run configuration details in Appendix B.

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

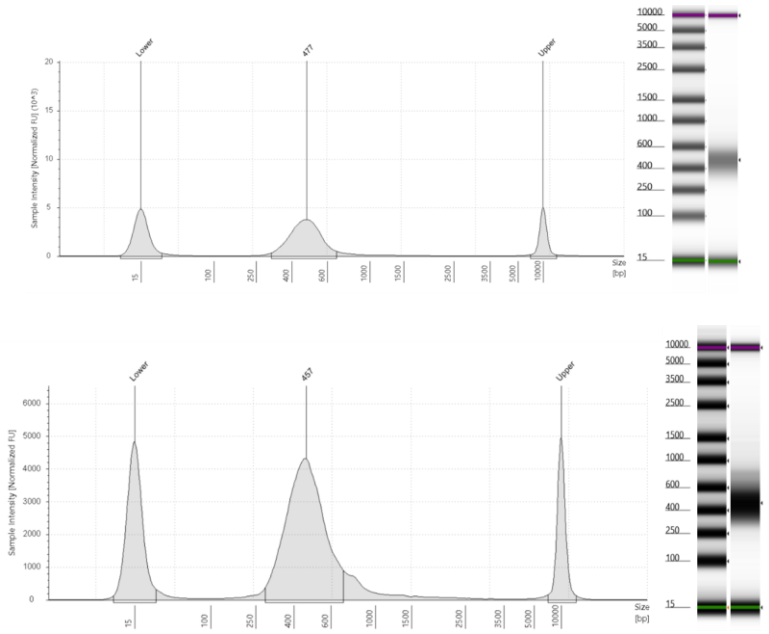


Figure 8: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA (top) and mouse DNA (bottom) from indexed sublibraries run on a TapeStation.

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

Note: If quantifying DNA with a Bioanalyzer, there may be an additional peak present. If there is a 400-500 bp peak, it should not impact sequencing or data quality, but care should be taken when calculating the library concentration before sequencing.

Appendices

Appendix A: Sublibrary Generation Table

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

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

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

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

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

Stock Conc. (cells/uL)	Target Sublibrary Cell Count (cells/sublibrary)														
	200	500	1,000	2,000	3,000	4,000	5,000	6,000	7,000	8,000	9,000	10,000	11,000	12,000	12,500
50	4	10	20												
	21	15	5												
100	2	5	10	20											
	23	20	15	5											
200		2.5	5	10	15	20	25								
			20	15	10	5	0								
300			3.33	6.67	10	13.33	16.67	20	23.33	N/A					
			21.67	18.33	15	11.67	8.33	5	1.67	N/A					
400			2.5	5	7.5	10	12.5	15	17.5	20	22.5	25			
			22.5	20	17.5	15	12.5	10	7.5	5	2.5	0			
500			2	4	6	8	10	12	14	16	18	20	22	24	25
			23	21	19	17	15	13	11	9	7	5	3	1	0
600				3.33	5	6.67	8.33	10	11.67	13.33	15	16.67	18.33	20	20.83
				21.67	20	18.33	16.67	15	13.33	11.67	10	8.33	6.67	5	4.17
700				2.86	4.29	5.71	7.14	8.57	10	11.43	12.86	14.29	15.71	17.14	17.86
				22.14	20.71	19.29	17.86	16.43	15	13.57	12.14	10.71	9.29	7.86	7.14
800				2.5	3.75	5	6.25	7.5	8.75	10	11.25	12.5	13.75	15	15.63
				22.5	21.25	20	18.75	17.5	16.25	15	13.75	12.5	11.25	10	9.37
900				2.22	3.33	4.44	5.56	6.67	7.78	8.89	10	11.11	12.22	13.33	13.89
				22.78	21.67	20.56	19.44	18.33	17.22	16.11	15	13.89	12.78	11.67	11.11
1,000				2	3	4	5	6	7	8	9	10	11	12	12.5
				23	22	21	20	19	18	17	16	15	14	13	12.5
1,100					2.73	3.64	4.55	5.45	6.36	7.27	8.18	9.09	10	10.91	11.36
					22.27	21.36	20.45	19.55	18.64	17.73	16.82	15.91	15	14.09	13.64
1,200					2.5	3.33	4.17	5	5.83	6.67	7.5	8.33	9.17	10	10.42
					22.5	21.67	20.83	20	19.17	18.33	17.5	16.67	15.83	15	14.58
1,300					2.31	3.08	3.85	4.62	5.38	6.15	6.92	7.69	8.46	9.23	9.62
					22.69	21.92	21.15	20.38	19.62	18.85	18.08	17.31	16.54	15.77	15.38
1,400					2.14	2.86	3.57	4.29	5	5.71	6.43	7.14	7.86	8.57	8.93
					22.86	22.14	21.43	20.71	20	19.29	18.57	17.86	17.14	16.43	16.07
1,500					2	2.67	3.33	4	4.67	5.33	6	6.67	7.33	8	8.33
					23	22.33	21.67	21	20.33	19.67	19	18.33	17.67	17	16.67
1,600						2.5	3.13	3.75	4.38	5	5.63	6.25	6.88	7.5	7.81
						22.5	21.87	21.25	20.62	20	19.37	18.75	18.12	17.5	17.19
1,700						2.35	2.94	3.53	4.12	4.71	5.29	5.88	6.47	7.06	7.35
						22.65	22.06	21.47	20.88	20.29	19.71	19.12	18.53	17.94	17.65
1,800						2.22	2.78	3.33	3.89	4.44	5	5.56	6.11	6.67	6.94
						22.78	22.22	21.67	21.11	20.56	20	19.44	18.89	18.33	18.06
1,900						2.11	2.63	3.16	3.68	4.21	4.74	5.26	5.79	6.32	6.58
						22.89	22.37	21.84	21.32	20.79	20.26	19.74	19.21	18.68	18.42
2,000						2	2.5	3	3.5	4	4.5	5	5.5	6	6.25
						23	22.5	22	21.5	21	20.5	20	19.5	19	18.75
2,100							2.38	2.86	3.33	3.81	4.29	4.76	5.24	5.71	5.95
							22.62	22.14	21.67	21.19	20.71	20.24	19.76	19.29	19.05
2,200							2.27	2.73	3.18	3.64	4.09	4.55	5	5.45	5.68
							22.73	22.27	21.82	21.36	20.91	20.45	20	19.55	19.32
2,300							2.17	2.61	3.04	3.48	3.91	4.35	4.78	5.22	5.43
							22.83	22.39	21.96	21.52	21.09	20.65	20.22	19.78	19.57

Appendix B: Sequencing Information

We recommend a minimum sequencing depth of 10,000 reads per cell for whole transcriptome libraries and 5,000 reads per cell for the BCR library. 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. We strongly recommend adding 5% PhiX for optimal sequencing quality.

The diagram below illustrates the composition of the sequencing libraries generated. This library structure applies to both the whole transcriptome library and the BCR-enriched library.

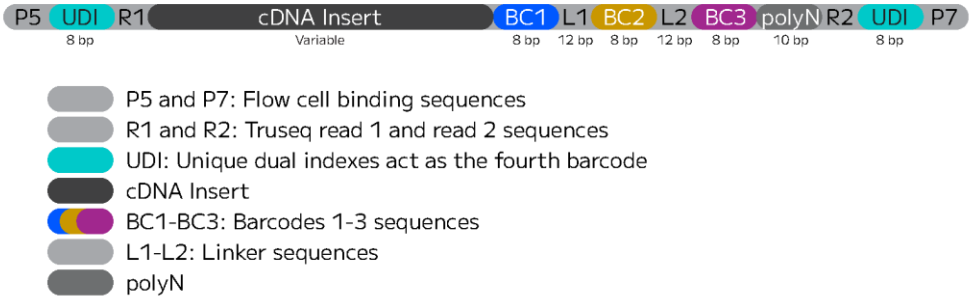


Figure 9: Whole transcriptome sequencing library structure.

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. Note that WT and BCR libraries have different sequencing requirements. The WT and BCR libraries should not be run together; however, they can be run in different lanes.

WHOLE TRANSCRIPTOME ILLUMINA RUN CONFIGURATION			
Run	**Function**	**WT Library Cycles**	**Note**
Read 1	cDNA Insert	64	The DNA product used for this sequencing run should have come from Section 3: Preparing WT Libraries for Sequencing.
i7 Index (Index 1)	Barcode 4/UDI	8	
i5 Index (Index 2)	Barcode 4/UDI	8	
Read 2	Barcodes 1-3	58	

Sequencing BCR libraries requires an Illumina 300 cycle kit. See the table below for details:

BCR ILLUMINA RUN CONFIGURATION			
Run	**Function**	**BCR Library Cycles**	**Note**
Read 1	cDNA Insert	242	The DNA product used for this sequencing run should have come from Section 5: BCR Libraries Preparation
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 UDI Whole Transcriptome and BCR sublibraries.

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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
UDI_Plate_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
UDI_Plate_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
UDI_Plate_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
UDI_Plate_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
UDI_Plate_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
UDI_Plate_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
UDI_Plate_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
UDI_Plate_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
UDI_Plate_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
UDI_Plate_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT
UDI_Plate_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
UDI_Plate_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
UDI_Plate_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA
UDI_Plate_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA
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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
UDI_Plate_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix C: Thermocycling Programs

Section 1: In situ Cell Barcoding

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

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

THAW ROUND 2 PLATE		
Run Time	3 min	
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 min	
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 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 LYSATES		
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		50-70 minutes	
Lid Temperature		105°C	
Sample Volume		100 µL	
Step	Time	Temperature	Cycle
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

Section 3: Sequencing Library Preparation

FRAGMENTATION and END PREP		
Run Time	35 minutes	
Lid Temperature	70°C	
Sample Volume	50 µL	
Step	Time	Temperature
1	Hold*	4°C
2	5 min	32°C
3	30 min	65°C
4	Hold	4°C

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

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

Section 5: BCR Amplification

BCR AMPLIFICATION			
Run Time		~30 min	
Lid Temperature		105°C	
Sample Volume		50 µL	
Step	Time	Temperature	Cycles
1	45 sec	98°C	1
2	15 sec	98°C	Variable, see Section 5.2.5
3	30 sec	60°C	
4	30 sec	72°C	
5	1 min	72°C	1
6	Hold	4°C	1

Appendix D: Vacuum Concentration

Pooled Sublibraries require at least a concentration of 5 ng/µL per sublibrary. We recommend adding a minimum of 100 ng of each sublibrary to the BCR library pool for a maximum of 800 ng of total input. However, if any sublibraries have less than 100 ng of input, sublibraries can be pooled in equal proportion to a total of 400 ng where the minimum sublibrary input is 50 ng.

When pooling sublibraries in Section 4.1, vacuum concentration can be used rather than SPRI bead concentration. See protocol below.

To vacuum concentrate the Pooled Sublibraries:

1. Pool 100 ng of each indexed library into a 0.2 mL PCR tube. Briefly centrifuge.
2. Dry the pool of sublibraries using a vacuum concentrator without heat or on the lowest heat setting.
3. The dried Library Pool can be stored at -20°C for 1 week. Otherwise, proceed directly to section 4.2 Hybridization, step 4.2.2 should be skipped.

Appendix E: Revision History

Version	Description	Date
1.0	Initial release	May 2026
1.1	Sample Loading Table update	June 2026

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