User Manual

Version 1.3 – UMIT2300

Evercode™ TCR

For use with

ECIT2300 (Human)

ECIT2301 (Human)

ECIT1310 (Mouse)

ECIT1311 (Mouse)

Legal Notices

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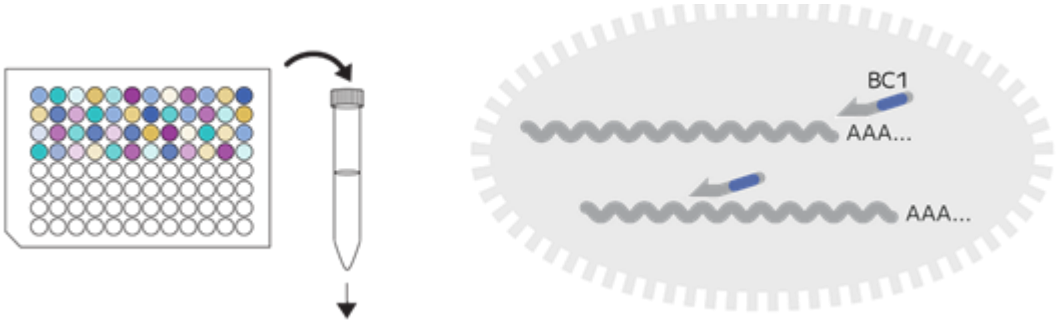
Overview

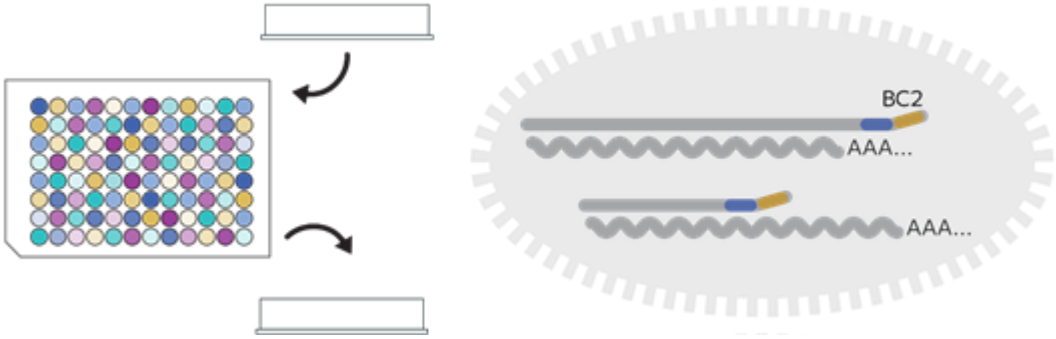
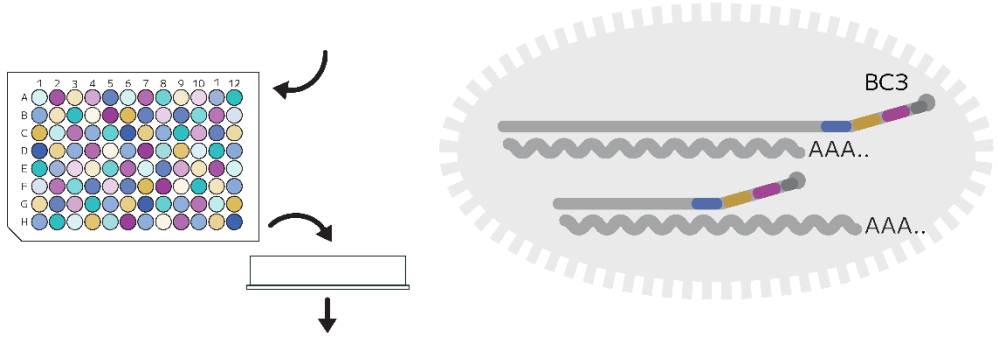
Workflow

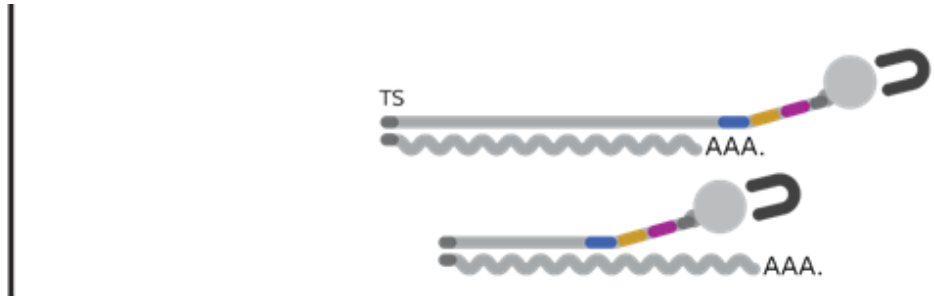
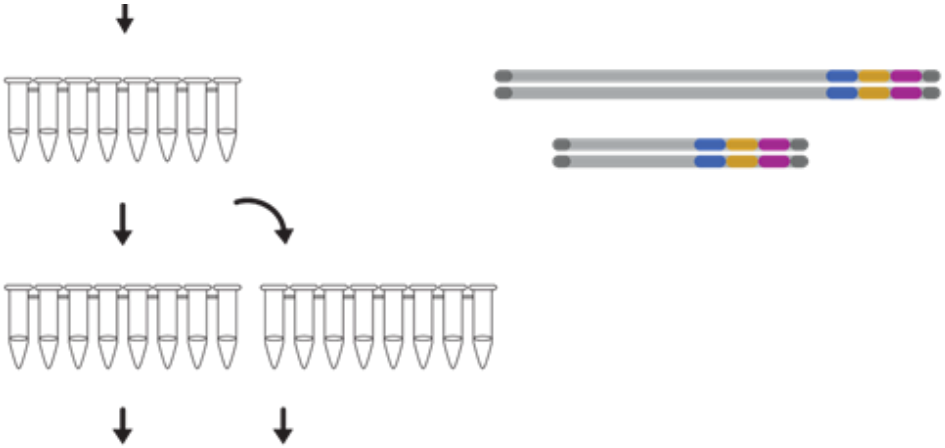
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode TCR kit can profile up to 100,000 cells across up to 48 different biological samples or experimental conditions. Evercode fixation kits fix and permeabilize cells so they 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.

Evercode TCR expands capabilities of scRNA-seq by simultaneously capturing T-cell receptor (TCR) information. A sufficient sample size is crucial to detecting and tracking rare clones. Discover up to 100,000 T cells, identify phenotypes and their paired TCR sequences.

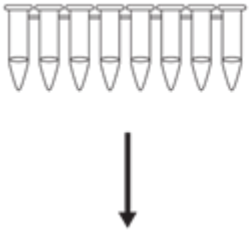
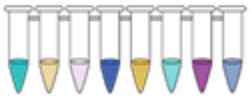
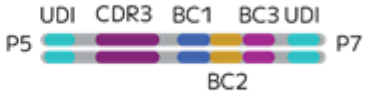
The tables below provide a high-level overview of the workflow.

Section 1: *In situ* Cell 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 a well-specific barcode that are associated with specific samples.

Section 1: *In situ* Cell Barcoding	
2	 **Round 2 Barcoding:** The cells are pooled and loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode.
3	 **Round 3 Barcoding:** The cells 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.
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 template switch adapter and Illumina Truseq Read 2 specific primers. Amplified cDNA is split to create Whole Transcriptome and TCR sequencing libraries in Section 3 and Section 4.

Section 3: WT 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.

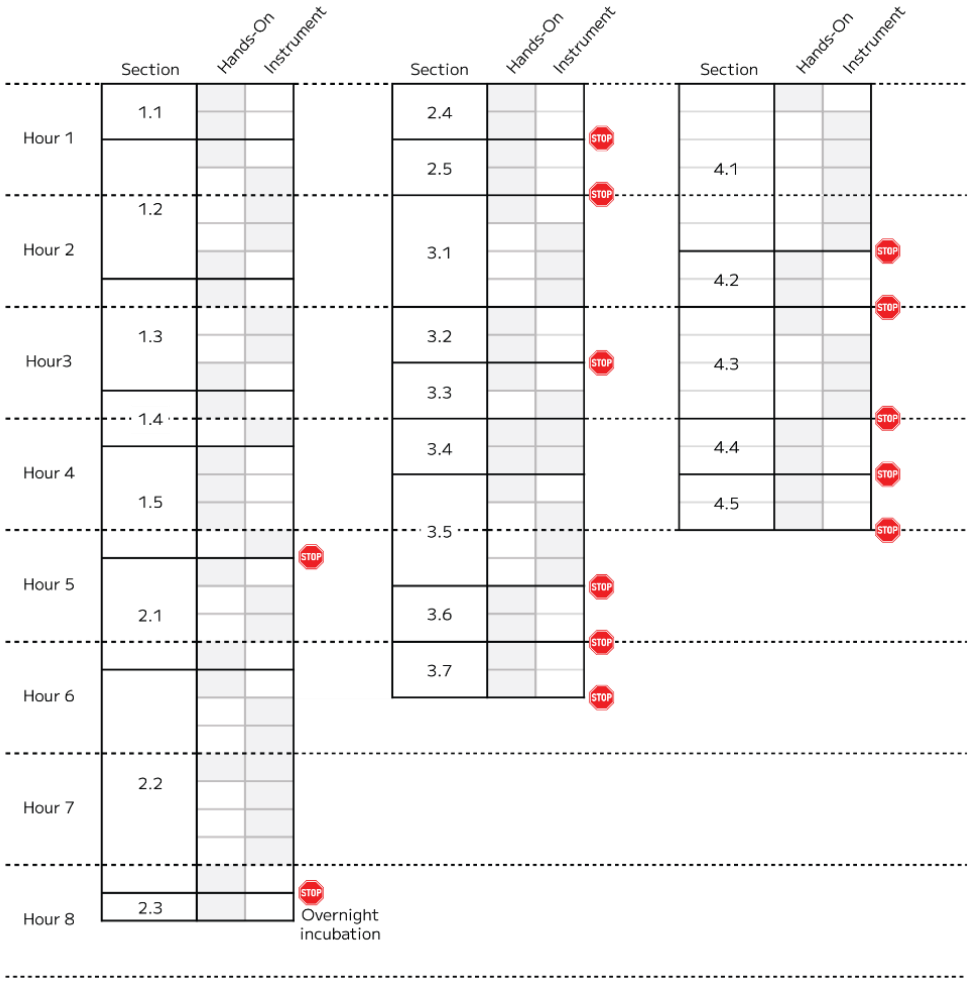
Section 4: TCR Sequencing Library Preparation	
1	  **TCR Amplification 1:** A PCR reaction amplifies a subset of the cDNA from the whole transcriptome that contains V(D)J segments in the CDR3 repertoire of the T cell. This reaction also adds an Illumina adapter.
2	  **TCR Amplification 2:** A final PCR amplifies the TCR Amplification 1 product and appends the fourth DNA barcodes, UDIs from the UDI - EC Plate, as well as the P5 and P7 adapters.

Protocol Timing

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

DESCRIPTION	TIME	HANDS-ON-TIME	SAFE 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	45 min	
1.3 Barcoding Round 2	60 min	45 min	
1.4 Barcoding Round 3	30 min	15 min	
1.5 Lysis and Sublibrary Generation	60 min	45 min	-80°C ≤ 6 months
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	60 min	30 min	
2.2 cDNA Template Switch	120 min	30 min	4°C ≤ 18 hrs
2.3 cDNA Amplification	90 min	15 min	4°C ≤ 18 hrs in the thermocycler
2.4 Post-Amplification Purification	30 min	30 min	
2.5 cDNA Quantification	30 min	30 min	4°C ≤ 48 hrs or -20°C ≤ 3 months
Section 3: Sequencing Library Preparation			
3.1 Fragmentation and End Prep	60 min	30 min	
3.2 Fragmentation and End Prep Size Selection	30 min	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
3.3 Adapter Ligation	30 min	15 min	
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	SAFE STOPPING POINTS
3.7 Sequencing Library Quantification	30 min	30 min	-20°C ≤ 3 months
Section 4: TCR Sequencing Library Preparation			
4.1 TCR Amplification 1	90 min	30 min	4°C ≤ 18 hrs
4.2 Post TCR Amplification 1 Size Selection	30 min	30 min	-20°C ≤ 3 months
4.3 TCR Amplification 2	60 min	30 min	4°C ≤ 18 hrs
4.4 Post TCR Amplification 2 Size Selection	30 min	30 min	
4.5 TCR Sequencing Library Quantification	30 min	30 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

- This protocol begins with cells fixed with an Evercode Cell Fixation v3 kit. It is also compatible with samples fixed with Evercode Cell Fixation v2 kit.
- When working with mouse T cells, use the Evercode Cell Fixation (Mouse TCR) kit, 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 TCR kits. Because aliquots have experienced comparable storage durations and freeze-thaw cycle, cell counts derived from these aliquots will provide a more accurate representation compared to counts obtained immediately following 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 counting aliquot should be discarded.
- Once fixed samples have been thawed, they should not be refrozen.

Cell Counting and Quality Assessment

- We recommend a hemocytometer for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices to a hemocytometer when using Evercode TCR kits for the first time.
- When first using Evercode TCR kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).

- Examples of 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.

High Quality Sample | Aggregation | Debris

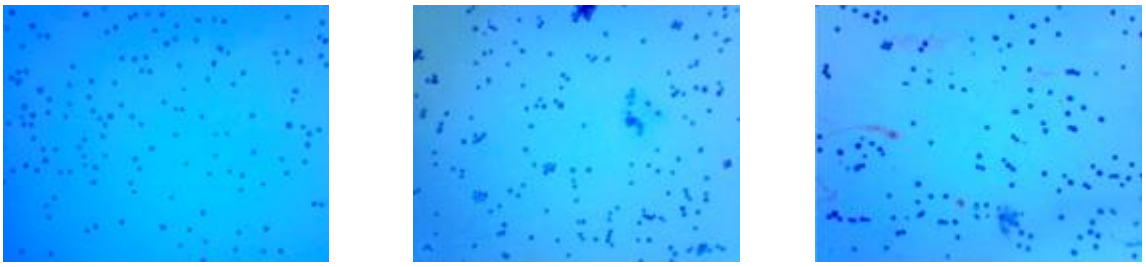


Figure 1: Example trypan blue stained fixed cells.

Avoiding RNase Contamination

- Take standard precautions to avoid introducing RNases into samples or reagents throughout the workflow. Always wear proper laboratory gloves and use aseptic technique.
- Although RNases are not inactivated by ethanol or isopropanol, they are inactivated by products such as RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific). These can be sprayed on benchtops and pipettes.
- Filtered pipette tips should be used to reduce RNase contamination from pipettes.

Centrifugation

- A range of centrifugation speeds and durations are provided in this protocol. Optimize centrifugation conditions for each sample type to balance retention and resuspension efficiencies.
- Use a swinging bucket rotor for all high-speed centrifugation steps in this protocol. A fixed-angle rotor will lead to substantial cell loss.

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 TCR kit, we recommend retaining supernatants after each barcoding step. In the unlikely event of unexpectedly high sample loss, these supernatants can be analyzed to identify points for optimization.

Sample Loading Table

- The Parse Biosciences Evercode WT Sample Loading Table v2 (Excel spreadsheet) 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 T cell types, the mesh size should be chosen based on the sample type.
- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. An example video can be found in our support site.

Vortexing

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

Plate Sealing

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

Magnetic Racks and Bead Cleanups

- The Parse Biosciences Magnetic Rack (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.

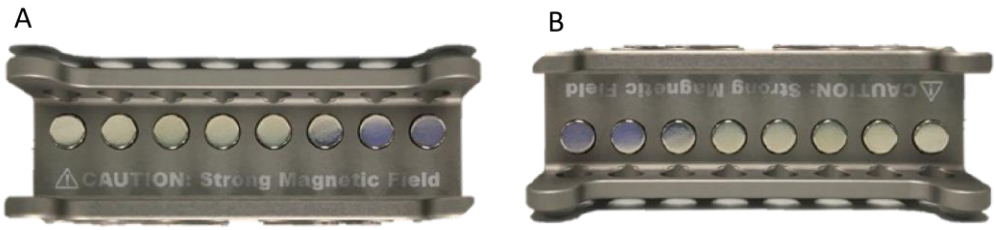


Figure 2: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.

- 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 3: Clear supernatant with compact bead pellets.

Sublibrary Loading

- The Evercode TCR 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.
- Asymmetric sublibrary loading can enable cost-effective sequencing quality control. One sublibrary can be loaded with a few hundred cells and sequenced very deeply. This data can be used to choose an appropriate sequence depth for the remaining sublibraries.

Indexing Primers

- UDI Plate-WT and UDI Plate-EC are 96-well plates containing 48 unique dual indexing (UDI) primers. Each well is a single-use reaction sufficient for one indexing PCR reaction for a single sublibrary. UDI Plate-WT should be used for Section 3 and UDI Plate-EC should be used for Section 4. Each UDI plate can be used for multiple TCR kits.
- The UDI Plates are sealed with a pierceable foil to minimize cross-contamination. The plate seal should be wiped with 70% ethanol and pierced with a new pipette tip immediately prior to use. Avoid splashing or mixing the liquid between individual wells. Once a well has been used, it should not be resealed or reused.
- We recommend selecting UDIs column-wise from left to right (starting with indices 1-8). UDI sequences can be found in Appendix B.

Thermocycling Programs

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

Part List

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

-20°C Reagents. Store -20°C, PN HTT100 or MTT100

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	HTT101 or MTT101	Green semi-skirted 96 well plate	1
	Round 2 Plate	WT102	Blue semi-skirted 96 well plate	1
	Round 3 Plate	WT103	Yellow semi-skirted 96 well plate	1
	Resuspension Buffer	WT104	5 mL tube	1
	Sample Dilution Buffer	WT105	2 mL tube	1
	Round 2 Ligation Buffer	WT106	5 mL tube	1
	Round 2 Ligation Enzyme	WT107	1.5 mL tube	1
	Round 2 Stop Buffer	WT108	2 mL tube	1
	Round 3 Stop Buffer	WT109	5 mL tube	1
	Pre-Lysis Wash Buffer	WT110	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Round 3 Ligation Enzyme	WT111	1.5 mL tube	1
	Pre-Lysis Dilution Buffer	WT112	2 mL tube	1
	Lysis Enzyme	WT113	1.5 mL tube	1
	Bead Wash Buffer	WT114	5 mL tube	1
	Wash Buffer 1	WT115	5 mL tube	1
	Wash Buffer 2	WT116	5 mL tube	1
	Capture Enhancer	WT117	1.5 mL tube	1
	Binding Buffer	WT118	1.5 mL tube	1
	Wash Buffer 3	WT119	5 mL tube	1
	Template Switch Buffer	WT120	1.5 mL tube	1
	Template Switch Enzyme	WT121	1.5 mL tube	1
	Template Switch Primer	WT122	1.5 mL tube	1
	cDNA Amp Mix	WT123	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	cDNA Amp Primers	HTT124 or MTT124	1.5 mL tube	1
	Fragm/End Prep Buffer	WT125	1.5.mL tube	1
	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
	Receptor PCR Mix 1	HTT131 or MTT131	1.5 mL tube	1
	Receptor PCR Mix 2	HTT132 or MTT132	1.5 mL tube	1
 or 	HT Primer or MT Primer	HTT133 or MTT133	1.5 mL tube	1

4°C Reagents. Store 4°C, PN HTT200 or MTT200

LABEL	ITEM	PN	FORMAT	QTY
	Spin Additive	WT201	1.5 mL tube	1
	Lysis Buffer	WT202	1.5 mL tube	1
	Streptavidin Beads	WT203	1.5 mL tube	1

User Supplied Equipment and Reagents

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.

Note: UDI Plates are included in ECIT2301 or ECIT1311, but not in ECIT2300 or ECIT1310. If using ECIT2300 or ECIT1310, UDI Plates must be purchased separately.

Equipment

ITEM	SUPPLIER	PN	NOTES
Parse Biosciences Magnetic Rack	Parse Biosciences	SB1004	We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection.
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 15 mL centrifuge tubes and 96 well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL and 0.2 mL tubes.
Water bath	Various Suppliers	Varies	Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. We recommend validating alternatives relative to a hemocytometer.
PCR tube rack	Various Suppliers	Varies	Capable of holding semi-skirted 96 well PCR plates and a tight fitting lid.
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.
Single Channel Pipettes: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.

ITEM	SUPPLIER	PN	NOTES
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.
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM.
6-inch Platform	Scientific Industries	146-6005-00	
Microplate Foam Insert	Scientific Industries	504-0235-00	
Qubit™ Flex Fluorometer	Thermo Fisher Scientific®	Q33327	Or an equivalent fluorometer.
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.
4200 TapeStation System	Agilent	G2991BA	

Consumables

ITEM	SUPPLIER	PN	NOTES
UDI Plate - WT	Parse Biosciences	UDI1001	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode Whole Transcriptome kits (48 sublibraries). UDI Plate - WT can be purchased separately or bundled with Evercode Whole Transcriptome kits.

ITEM	SUPPLIER	PN	NOTES
UDI Plate - EC	Parse Biosciences	UDI1002	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple TCR sequencing libraries (48 sublibraries). UDI Plate - EC can be purchased separately or bundled with the kit.
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free reagent basins.
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-20 TC70-SWM-40 TC70-SWM-70 TC70-SWM-100	Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm, 70 µm, 100 µm). We do not recommend FlowMi Cell Strainers (SP Bel-Art).
pluriStrainer® Mini	pluriSelect®	43-10020-40 43-10040-40 43-10070-40 43-10100-40	
Falcon®Cell Strainer	Corning®	431750 431751 431752	
EASYstrainer™, small	Greiner Bio-One™	542120 542140 542170 542100	
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial cell loss.
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.

ITEM	SUPPLIER	PN	NOTES
AMPure® XP Reagent	Beckman Coulter®	A63880 (5 mL) A63881 (60 mL)	Choose one. We do not recommend substituting other magnetic beads.
SPRIselect Reagent	Beckman Coulter	B23317 (5mL) B23318 (60mL)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30 mL)	
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Ethyl alcohol, Pure	Sigma-Aldrich	459844	Or equivalent 100% non-denatured ethanol.
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
Trypan Blue	Various Suppliers	Varies	Or alternative dyes to assess cell viability, such as AO/PI.
Qubit dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific	Q33230 (100 assays) Q33231 (500 assays)	Or equivalent DNA quantifier
High Sensitivity DNA Kit	Agilent	5067-4626	Choose one that corresponds to the chosen Bioanalyzer or Tapestation.
High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	

Section 1: In Situ Cell 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. Open the "Evercode WT Sample Loading Table v2" (Excel spreadsheet), which will guide sample dilutions and plate loading in later steps.
2. Cool a centrifuge with swinging bucket rotors to 4°C.
3. Set a water bath to 37°C.
4. Fill a bucket with ice.
5. Prepare a hemocytometer, flow cytometer, or other cell counting device.
6. 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.
Round 2 Plate	-20°C Reagents	1	
○ Resuspension Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x
● Sample Dilution Buffer	-20°C Reagents	1	
○ Round 2 Ligation Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice until use in section 1.3. Mix by inverting 3x before use.
● Spin Additive	4°C Reagents	1	Keep at room temperature.

7. 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.

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

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

9. 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.

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

THAW ROUND 1 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	26 µL
Step	Time	Temperature
1	10 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 Round 1 Plate. An *in situ* reverse transcription reaction adds well-specific barcodes that also act as sample barcodes. Cells are then pooled, centrifuged, and 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, 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. Upon completion, proceed immediately to the next step.

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

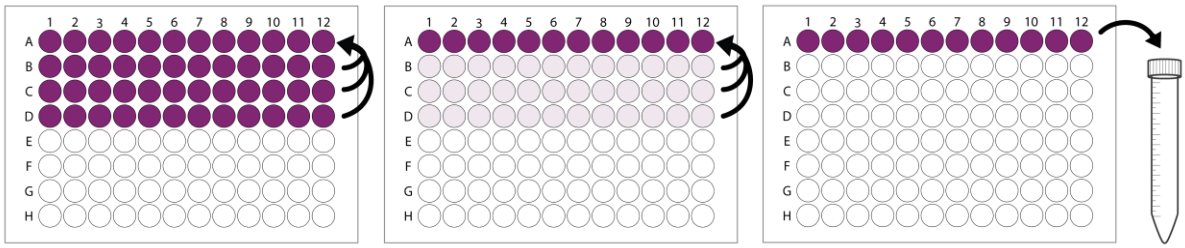
6. Remove the Round 1 Plate from the thermocycler, place it in a PCR tube rack, and store it on ice.

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

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

8. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 1 Plate.

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



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

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

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

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

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

10. Add **9.6 µL** of ● Spin Additive to the 15 mL tube with pooled cells. Do not discard the ● Spin Additive as it will be needed in another step.

11. Mix by gently inverting the tube just once.

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

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

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

13. Remove the supernatant until about ~40 µL of liquid remains above the pellet. Use a P1000 for the first 1 mL and then a P200 for the remaining volume.

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

14. Fully but gently resuspend the pellet in **1 mL** of O Resuspension Buffer.

15. Add an additional **1 mL** of O Resuspension Buffer for a total addition of 2 mL. Store on ice.

16. Proceed immediately to Section 1.3.

Note: If the low input fixation workflow was performed, execute the following step before proceeding to Section 1.3.

17. Pipette the sample through a cell strainer into a new 15 mL tube with a P1000. Before transfer, gently mix the cells by pipetting 2x.

1.3. Barcoding Round 2

The pooled cells are added to the Ligation Master Mix, which is loaded into the Round 2 Plate. An *in situ* ligation reaction adds a well-specific barcode to the 3′ end of the cDNA. The ligation reaction is quenched with Round 2 Stop Buffer, and the cells 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 Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
● Round 2 Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
Round 3 Plate	-20°C Reagents	1	Place directly on ice.

2. On ice, prepare the Round 2 Ligation Master Mix by adding the following to the samples in ○ Resuspension Buffer prepared in Section 1.2. Mix thoroughly by pipetting 10x with a P1000 set to 1000 µL. Store on ice.

Note: Prior to use, mix by inverting 3x the ○ Round 2 Ligation Buffer that was gathered and stored on ice in Section 1.1.

ROUND 2 LIGATION MASTER MIX	
Sample in Resuspension Buffer	2 mL
○ Round 2 Ligation Buffer	1.95 mL
● Round 2 Ligation Enzyme	20 µL
Total Volume	3.97 mL

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. Transfer the Round 2 Ligation Master Mix to a basin with a P1000.

6. With the Round 2 Plate on ice and the basin on the bench, transfer Round 2 Ligation Master Mix to each well in the Round 2 Plate as follows:

    i. Mix the sample in the basin by pipetting 2x with a multichannel P200 set to 40 µL.

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

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

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

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

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

9. Briefly vortex the ● Round 2 Stop Buffer and ensure there is no precipitate. Transfer the entire volume of 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 **10 µL** of the ● Round 2 Stop Buffer to each well in the Round 2 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.

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

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

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

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

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

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

Note: If a second thermocycler is not available, the same thermocycler used in step 14 can subsequently be used in step 15. However, the Round 2 Plate should be stored on ice until the Thaw Round 3 Plate program is complete.

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

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

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

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

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

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

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

CRITICAL! Do not directly touch the mesh of the cell strainer with gloved hands. To ensure that all of the liquid passes through the strainer, press the tip of the pipette against the mesh to create a tight seal and press the pipette plunger down steadily. All the liquid should pass through the strainer in ~1 second.

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

18. Proceed immediately to Section 1.4.

1.4. Barcoding Round 3

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

To add round 3 barcodes:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

14. Proceed immediately to Section 1.5.

1.5. Lysis and Sublibrary Generation

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

To generate and lyse sublibraries:

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

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

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

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

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

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

7. Remove the supernatant until about ~40 µL of liquid remains above the pellet. Use a P1000 for the first 3 mL and then a P200 for remaining volume.
8. Fully but gently resuspend the pellet in **60 µL** of ● Pre-Lysis Dilution Buffer for a final total volume of **100 µL**. Store on ice.

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

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

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

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

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

11. Ensure the cells are in suspension by pipetting 5x with a P200 set to 75 µL prior to each transfer. Add the appropriate volume of sample to 8 different 0.2 mL PCR tubes.

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

11. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P1000 set to 220 µL. Store at room temperature.

LYSIS MASTER MIX	
● Lysis Buffer	220 µL
● Lysis Enzyme	44 µL
Total Volume	264 µL

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

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

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

15. Vortex the 0.2 mL tube(s) for **10 seconds**. Briefly centrifuge.

16. 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	Lid Temperature	Sample Volume
15 min	80°C	55 µL
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C

17. 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.

To capture the cDNA:

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

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

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

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

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

8. Briefly centrifuge and store at room temperature.

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

10. Add **2.5 µL** of ● Capture Enhancer to each tube of lysate and mix by pipetting 5x with a P200 set to 40 µL. Briefly centrifuge.

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

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

12. 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

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

14. Remove and discard the supernatant.

15. 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.

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

17. Remove and discard the supernatant.

18. Repeat steps 15-17 twice for a total of 3 washes.

19. 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

20. Add **50 µL** of ● Streptavidin Beads in ● Binding Buffer to each tube of lysate and fully mix by pipetting 2x with a P200 set to 90 µL.

21. 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.

22. Place the rack onto a vortex mixer with a plate adapter. Push to secure. 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.

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

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

25. 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.

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

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

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

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

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

31. Repeat steps 27-30 once for a total of 2 washes with O Wash Buffer 1.

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

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

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

34. Proceed immediately to Section 2.2.

2.2. cDNA Template Switch

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

To perform template switch:

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

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

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

2. Prepare the Template Switch Master Mix in a new 1.5 mL tube as follows, depending on the number of sublibraries being processed. Mix by pipetting 10x and store on ice.

TEMPLATE SWITCH MASTER MIX		
Number of Samples	1	8
● Template Switch Buffer	101.75 µL	814 µL
● Template Switch Primer	2.75 µL	22 µL
● Template Switch Enzyme	5.5 µL	44 µL
Total	110 µL	880 µL

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

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

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

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

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

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

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

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

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

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

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

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

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

2.3. cDNA Amplification

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

To amplify the cDNA:

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

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

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

cDNA AMPLIFICATION MASTER MIX		
Number of Sublibraries	1	8
● cDNA Amp Mix	60.5 µL	484 µL
● cDNA Amp Primers	60.5 µL	484 µL
Total	121 µL	968 µL

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

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

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

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

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

7. While still on the magnetic rack, remove and discard the OWash Buffer 3.

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

9. Determine the number of PCR cycles required for cDNA amplification based on the table below. Although these recommendations are appropriate for many cell types, the number of cycles may need to be optimized for each sample type.

NUMBER OF PCR CYCLES		
Cells in Sublibrary	Primary T cells	Activated T cells
200-1,000	14	13
1,000-2,000	12	11
2,000-6,000	10	9
6,000-12,500	8	7

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

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

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

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

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

2.4. Post-Amplification Purification

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

To purify the cDNA:

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

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

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

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

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

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

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

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

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

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

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

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

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

2.5 cDNA Quantification

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

To quantify the cDNA:

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

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

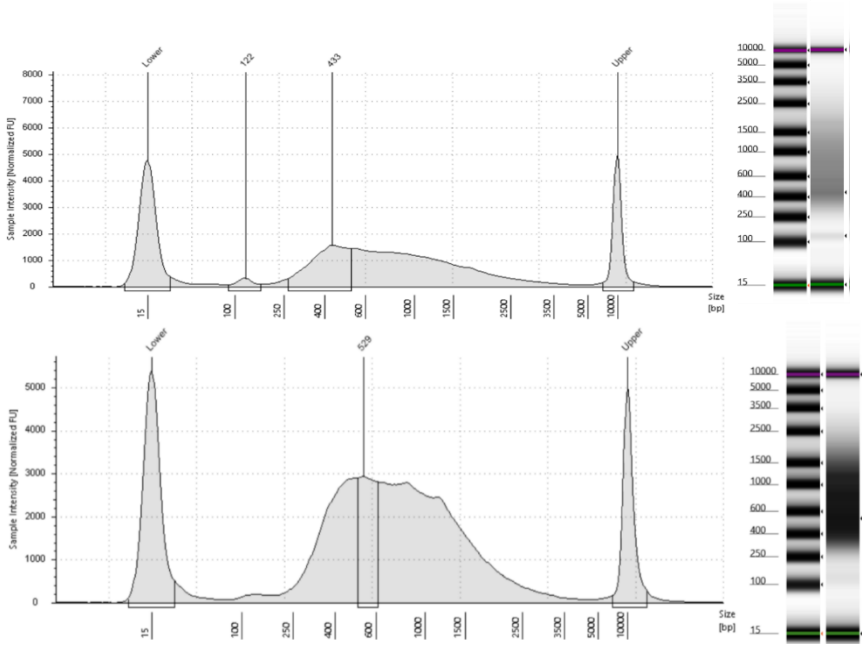


Figure 4: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a Tapestation (Top) and Mouse cDNA (Bottom).

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: WT Sequencing Library Preparation

3.1. Fragmentation and End Prep

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

To prepare for fragmentation and end prep:

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

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

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

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

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

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

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

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

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

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

FRAGMENTATION AND END PREP MASTER MIX		
Number of Sublibraries	1	8
● Fragm/End Prep Buffer	5.5 µL	44 µL
● Fragm/End Prep Enzymes	11 µL	88 µL
Total	16.5 µL	132 µL

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

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

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

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

3.2. Post-Fragmentation and End Prep Size Selection

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

To size select the fragmented and end prepped DNA:

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

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

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

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

CRITICAL! Ensure the solution is completely clear before proceeding.

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

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

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

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

3.3. Adapter Ligation

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

To ligate adapters:

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

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

2. Prepare the Adapter Ligation Master Mix in a new 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	Lid Temperature	Sample Volume
15 min	30°C*	100 µL
Step	Time	Temperature
1	15 min	20°C
2	Hold	4°C

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

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

3.4. Post-Ligation Purification

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

To purify the ligated DNA:

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

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

3. Vortex the SPRI beads until fully mixed. Add **80 µL** of SPRI beads to each tube of adapter ligated DNA from Section 3.3.
4. Vortex the tube(s) for **5 seconds.** Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

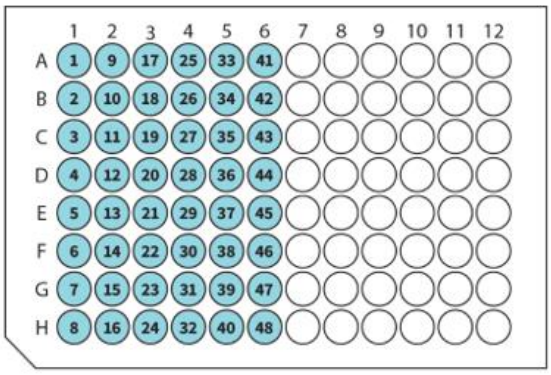
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~2 minutes).
13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **23 µL** of nuclease-free water.
14. Incubate for **5 minutes** at room temperature.
15. Place the tube(s) on the low magnet position of the magnetic rack, so the magnet is closer to the bottom of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
16. While still on the magnetic rack, transfer exactly **21 µL** of the supernatant containing the purified adapter ligated DNA into new 0.2 mL tube(s). Store on ice.
17. Proceed immediately to Section 3.5.

3.5. Barcoding Round 4

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

To add round 4 barcodes:

1. Centrifuge the UDI Plate - WT at 100 x g for **1 minute**.
2. Wipe the surface of the plate with 70% ethanol and allow it to dry.
3. Orient the UDI Plate - WT with the notch on the bottom left as pictured below. For each sublibrary being processed, choose one unused well of the UDI Plate - WT and record the well position and number for each sublibrary.



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

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

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

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

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

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

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

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

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

3.6. Post-Barcoding Round 4 Size Selection

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

To size select the sequencing libraries:

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

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

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

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

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

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

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

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

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

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

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

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

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

3.7. Sequencing Library Quantification

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

To quantify the sequencing libraries:

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

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

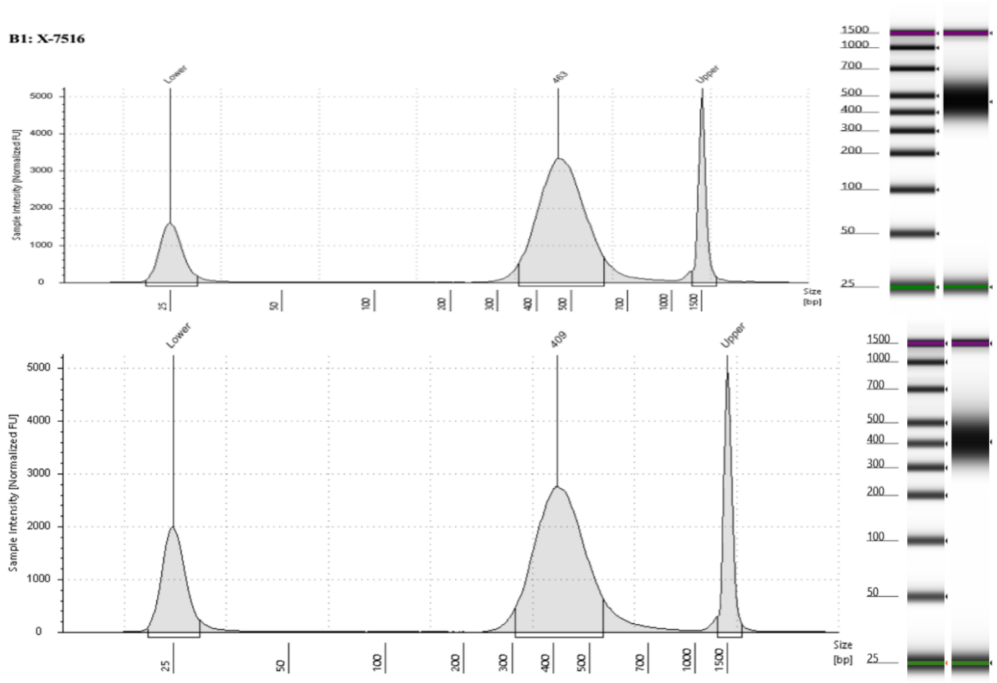


Figure 5: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA (Top) and Mouse DNA (Bottom) from indexed sublibraries run on a TapeStation.

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 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: TCR Sequencing Library Preparation

4.1. TCR Amplification 1

A PCR reaction amplifies a subset of the cDNA containing V(D)J segments in the CDR3 repertoire of the T cells. For the first TCR Amplification Reaction, an HT Primer is used in the Human Evercode TCR, and a MT Primer is used in the Mouse Evercode.

To amplify the V(D)J segments:

1. Prepare **1.2 mL** 85% ethanol per sublibrary with nuclease-free water.
2. Equilibrate approximately **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 amplified cDNA from Section 2.4.
6. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Receptor PCR Mix 1	TCR -20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
HT Primer/MT Primer	TCR -20°C Reagents	1	

7. Vortex the tube(s) of amplified cDNA for **2-3 seconds**. Briefly centrifuge.
8. Prepare diluted cDNA in a new 0.2 mL tube(s) as follows and store on ice. Bring the total volume up to **36 µL** using nuclease-free water. Store any remaining cDNA at -20°C.

DILUTED cDNA	
Sublibrary cDNA	10 ng
Nuclease-free water	Variable
Total volume	36 µL

Note: Accuracy of input is important at this step. If the concentration of cDNA is higher than 5 ng/µL, a dilution is recommended to ensure sufficient volume is used.

Note: If you have less than 10 ng of cDNA for a given sublibrary, add the entire amount of cDNA at this step. This will not affect the quality of your libraries. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on cDNA concentration (See Appendix D for more details).

9. Prepare the TCR Amplification Reaction Solution in a new 1.5 mL tube as follows. Mix by pipetting 10x and store on ice.

TCR AMPLIFICATION 1 MASTERMIX		
Number of Sublibraries	1	8
Receptor PCR Mix 1	55 µL	440 µL
HT Primer/MT Primer*	15.4 µL	123.2 µL
Total	70.4 µL	563.2 µL

Note: *The initial TCR Amplification Reaction uses an HT Primer for human TCR and an MT Primer for mouse TCR.

10. Add **64 µL** of the TCR Amplification Reaction Solution to each sublibrary, bringing the total volume to **100 µL**. Mix sublibraries 10x with a P200 pipette set to 90 µL. Briefly centrifuge.

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

TCR AMPLIFICATION 1			
Run Time	Lid Temperature	Sample Volume	
~50 minutes	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	20
3	20 s	65°C	
4	20 s	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

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

4.2. Post TCR Amplification 1 Size Selection

TCR enriched cDNA sublibraries are size selected with a double sided 0.5X and 0.8X SPRI cleanup.

To size select the sublibraries:

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 **50 µL** of SPRI beads to each tube of TCR-amplified cDNA.
4. Vortex the tube(s) for **2-3 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, transfer **145 µL** of the supernatant containing the TCR-amplified cDNA into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **30 µL** of SPRI beads to each tube.
9. Vortex the tube(s) for **2-3 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

CRITICAL! This may take longer due to the low volume of beads. Ensure all the solutions are completely clear before proceeding.

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

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

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

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

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

18. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **16 µL** of nuclease-free water.

19. Incubate for 5 minutes at room temperature.

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

21. While still on the magnetic rack, transfer **15 µL** of the supernatant into new 0.2 mL tube(s) and store on ice. Discard the tube(s) with bead pellet(s).

22. Measure the concentration of each tube of size-selected TCR amplified cDNA with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

Safe stopping point: The size-selected TCR amplified cDNA can be stored at -20°C for up to 3 months.

4.3. TCR Amplification 2

A final PCR further amplifies the TCR cDNA and adds i5/i7 UDIs that act as a fourth cell barcode.

To prepare the TCR sequencing libraries:

1. Obtain amplified and purified TCR cDNA from Section 4.2.
2. Vortex the tube(s) for **2-3 seconds**. Briefly centrifuge.
3. Prepare diluted TCR cDNA in new 0.2 mL tube(s) as follows and store on ice. Bring the total volume up to 46 µL using nuclease-free water. Store any remaining product from Section 4.2 at -20°C.

DILUTED TCR cDNA	
TCR cDNA	10 ng
Nuclease-free water	Variable
Total volume	46 µL

Note: Accuracy of input is important at this step. If the concentration of TCR cDNA is higher than 5 ng/µL, a dilution is recommended to ensure sufficient volume is used while pipetting (at least 2 µL used).

Note: If you have less than 10 ng of TCR cDNA for a given sublibrary, add the entire amount of TCR cDNA at this step. This will not affect the quality of your libraries. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on TCR cDNA concentration (See Appendix D for more details).

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

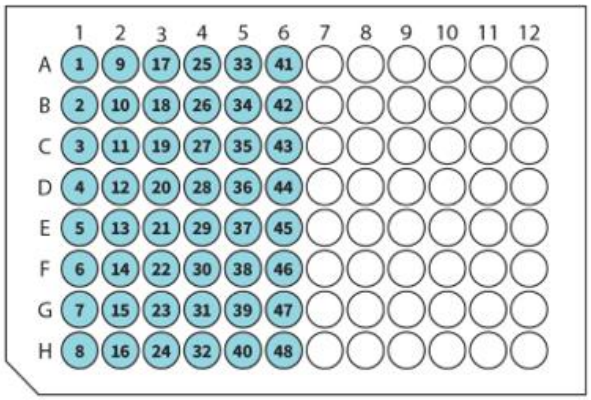
ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Receptor PCR Mix 2	TCR -20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
UDI Plate - EC	UDI Plate - EC (-20°C)	1 sealed well per sublibrary	Thaw at room temperature then place on ice.

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

CRITICAL! Ensure not to confuse UDI plates. Double-check the label on the plate as specific plates are used in different sections.

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

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



8. With a multichannel P20, pierce the seal of the chosen wells of the UDI Plate - EC.

9. 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 - EC to its corresponding tube of diluted TCR DNA.

CRITICAL! Only transfer primers from 1 well of the UDI Plate - EC to 1 tube of diluted TCR cDNA.

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

11. Add **50 µL** of ● Receptor PCR Mix 2 to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 90 µL.

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

TCR AMPLIFICATION 2			
Run Time	Lid Temperature	Sample Volume	
~30 minutes	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	9
3	20 s	67°C	
4	1 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Safe stopping point: TCR sequencing libraries can be stored at 4°C for up to 18 hours.

4.4. Post TCR Amplification 2 Size Selection

TCR sequencing libraries are size selected with a double sided 0.5X and 0.65X SPRI cleanup.

To size select the sublibraries:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~80 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 **50 µL** of SPRI beads to each tube of TCR sequencing library.
4. Vortex the tube(s) for **2-3 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, transfer **145 µL** of the supernatant containing the TCR sequencing library into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **15 µL** of SPRI beads to each tube.
9. Vortex the tube(s) for **2-3 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

CRITICAL! This may take longer due to the low volume of beads. Ensure all the solutions are completely clear before proceeding.

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

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

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

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

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

18. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **21 µL** of nuclease-free water.

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

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

21. While still on the magnetic rack, transfer **20 µL** of the supernatant into new 0.2 mL tube(s) and store on ice. Discard the tube(s) with bead pellet(s). Product is now ready for quantification.

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

4.5. TCR Sequencing Library Quantification

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

To quantify the TCR sequencing libraries:

1. Measure the concentration of each purified TCR sequencing library from Section 4.4 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

2. Assess the size distribution of each purified TCR 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. Samples may need to be diluted to be within the manufacturer's recommended concentration range.

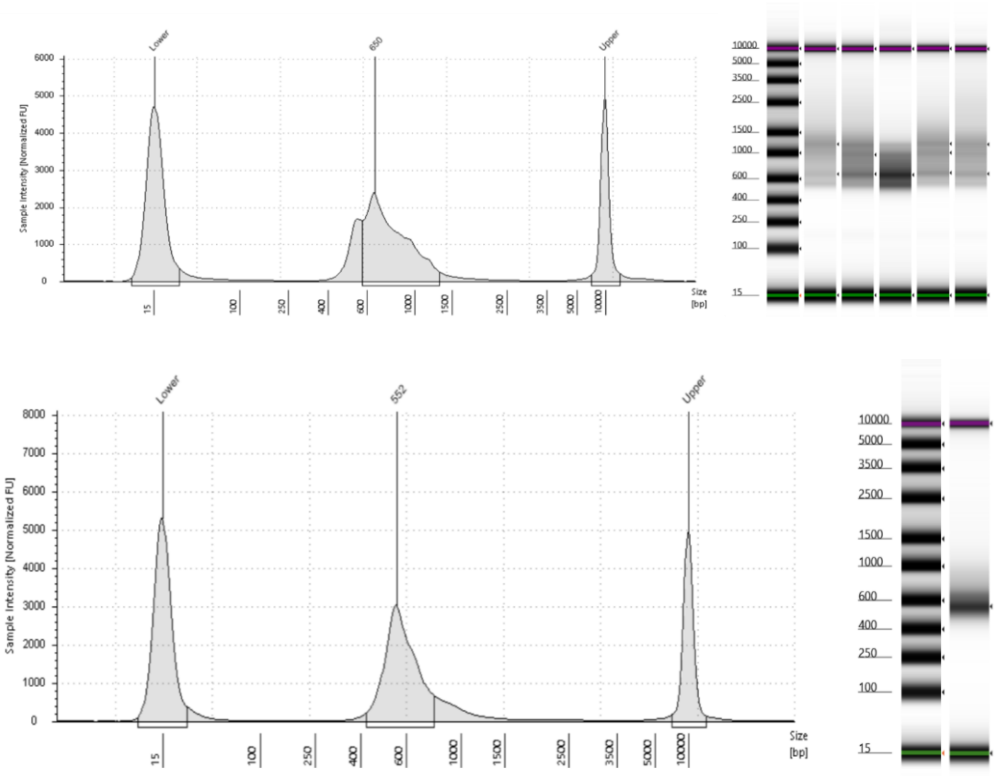


Figure 6: Expected TapeStation size distribution of a Human (Top) and Mouse (Bottom) TCR Sublibrary made with both Primary and Activated T cells.

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 trace above is representative of a typical TapeStation trace of DNA from an indexed TCR sublibrary. Amplicon sizes should be within 450-1000 bp range. The shape and prominence of the trace is dependent on cell type, sublibrary size, and amount of DNA loaded into the TapeStation. Experimental conditions can also contribute to variability in the trace. Sublibrary with minor deviations can still produce high quality data.

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 con (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 25	10 25	20 23												
100	2 23	5 20	10 15	20 5											
200		2.5 22.5	5 20	10 15	15 10	20 5	25 0								
300			3.33 21.67	6.67 18.33	10 15	13.33 11.67	16.67 8.33	20 5	23.33 1.67						
400			2.5 22.5	5 20	7.5 17.5	10 15	12.5 12.5	15 10	17.5 7.5	20 5	22.5 2.5	25 0			
500			2 23	4 21	6 19	8 17	10 15	12 13	14 11	16 9	18 7	20 5	22 3	24 1	25 0
600				3.33 21.67	5 20	6.67 18.33	8.33 16.67	10 15	11.67 13.33	13.33 11.67	15 10	16.67 8.33	18.33 6.67	20 5	20.83 4.17
700				2.86 22.14	4.29 20.71	5.71 19.29	7.14 17.86	8.57 16.43	10 15	11.43 13.57	12.86 12.14	14.29 10.71	15.71 9.29	17.14 7.86	17.86 7.14
800				2.5 22.5	3.75 21.25	5 20	6.25 18.75	7.5 17.5	8.75 16.25	10 15	11.25 13.75	12.5 12.5	13.75 11.25	15 10	15.625 9.375
900				2.22 22.78	3.33 21.67	4.44 20.56	5.56 19.44	6.67 18.33	7.78 17.22	8.89 16.11	10 15	11.11 13.89	12.22 12.78	13.33 11.67	13.89 11.11
1,000				2 23	3 22	4 21	5 20	6 19	7 18	8 17	9 16	10 15	11 14	12 13	12.5 12.5
1,100					2.73 22.27	3.64 21.36	4.55 20.45	5.45 19.55	6.36 18.64	7.27 17.73	8.18 16.82	9.09 15.91	10 15	10.91 14.09	11.36 13.64
1,200					2.50 22.50	3.33 21.67	4.17 20.83	5 20	5.83 19.17	6.67 18.33	7.5 17.5	8.33 16.67	9.17 15.83	10 15	10.42 14.58
1,300					2.31 22.69	3.08 21.92	3.85 21.15	4.62 20.38	5.38 19.62	6.15 18.85	6.92 18.08	7.69 17.31	8.46 16.54	9.23 15.77	9.62 15.38
1,400					2.14 22.86	2.86 22.14	3.57 21.43	4.29 20.71	5 20	5.71 19.29	6.43 18.57	7.14 17.86	7.86 17.14	8.57 16.43	8.93 16.07
1,500					2.00 23.00	2.67 22.33	3.33 21.67	4.00 21.00	4.67 20.33	5.33 19.67	6.00 19.00	6.67 18.33	7.33 17.67	8.00 17.00	8.33 16.67
1,600						2.5 22.5	3.125 21.875	3.75 21.25	4.375 20.625	5 20	5.625 19.375	6.25 18.75	6.88 18.13	7.50 17.50	7.81 17.19
1,700						2.35 22.65	2.94 22.06	3.53 21.47	4.12 20.88	4.71 20.29	5.29 19.71	5.88 19.12	6.47 18.53	7.06 17.94	7.35 17.65
1,800						2.22 22.78	2.78 22.22	3.33 21.67	3.89 21.11	4.44 20.56	5 20	5.56 19.44	6.11 18.89	6.67 18.33	6.94 18.06
1,900						2.11 22.89	2.63 22.37	3.16 21.84	3.68 21.32	4.21 20.79	4.74 20.26	5.26 19.74	5.79 19.21	6.32 18.68	6.58 18.42
2,000						2 23	2.5 22.5	3.00 22.00	3.5 21.5	4.00 21.00	4.50 20.50	5 20	5.50 19.50	6.00 19.00	6.25 18.75
2,100							2.38 22.62	2.86 22.14	3.33 21.67	3.81 21.19	4.29 20.71	4.76 20.24	5.24 19.76	5.71 19.29	5.95 19.05
2,200							2.27 22.73	2.73 22.27	3.18 21.82	3.64 21.36	4.09 20.91	4.55 20.45	5.00 20.00	5.45 19.55	5.68 19.32
2,300							2.17 22.83	2.61 22.39	3.04 21.96	3.48 21.52	3.91 21.09	4.35 20.65	4.78 20.22	5.22 19.78	5.43 19.57

Appendix B: Sequencing Information

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

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

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

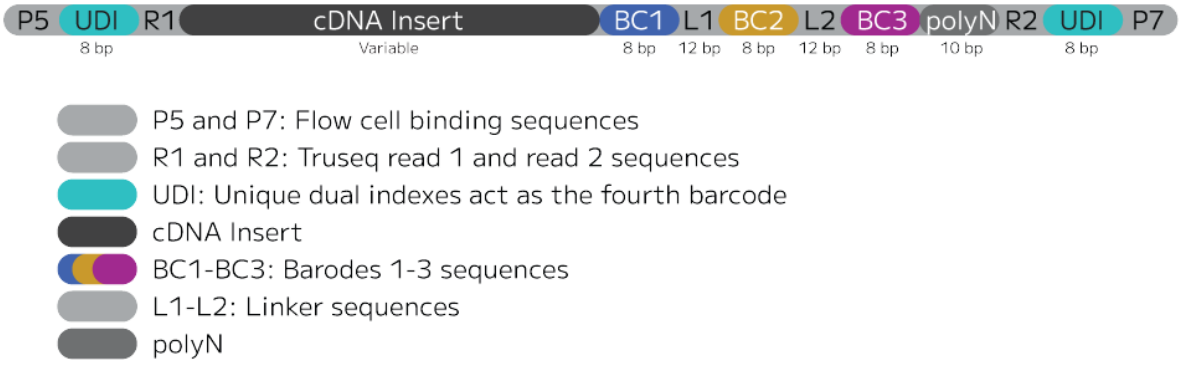


Figure 7: Whole transcriptome sequencing library structure.

Evercode sequencing libraries should be diluted and denatured according to the instruction for the relevant sequencing instrument. We strongly recommended adding 5% PhiX for optimal sequencing quality.

Details of the final TCR sequencing library structure are below.

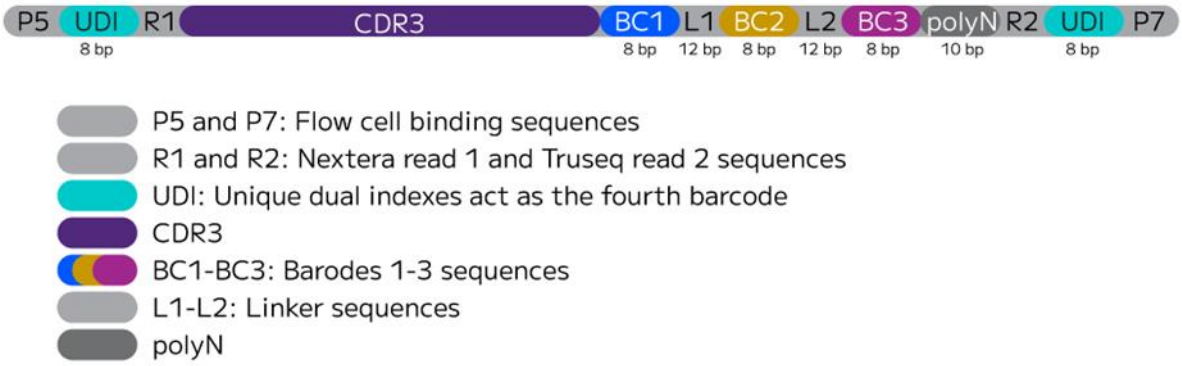


Figure 8: TCR sequencing library structure.

Paired end sequencing is used for both Whole Transcriptome and TCR Library. Whole Transcriptome and TCR Libraries have different sequencing configurations as shown below.

WHOLE TRANSCRIPTOME ILLUMINA RUN CONFIGURATION			
Run	**Function**	**WT Library Cycles**	**Notes**
Read 1	cDNA Insert	64	The DNA product used for this sequencing run should have come from Section 3.
i7 Index (Index 1)	Barcode 4 / UDI	8	
i5 Index (Index 2)	Barcode 4 / UDI	8	
Read 2	Barcodes 1-3	58	
TCR ILLUMINA RUN CONFIGURATION			
Run	**Function**	**TCR Library Cycles**	**Notes**
Read 1	CDR3 Insert	242	The DNA product used for this sequencing run should have come from Section 4.
i7 Index (Index 1)	Barcode 4 / UDI	8	
i5 Index (Index 2)	Barcode 4 / UDI	8	
Read 2	Barcodes 1-3	58	

Appendix B1: Unique Dual Index (UDI) Plate - WT Sequences

Please refer to the following table to demultiplex the whole transcriptome libraries.

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

UDI_Plate_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
UDI_Plate_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
UDI_Plate_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
UDI_Plate_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
UDI_Plate_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
UDI_Plate_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT
UDI_Plate_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
UDI_Plate_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
UDI_Plate_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA
UDI_Plate_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA
UDI_Plate_WT_35	C5	TTCCATTG	CTGGCATA	TATGCCAG
UDI_Plate_WT_36	D5	TAACGCTG	CTGAGCCA	TGGCTCAG
UDI_Plate_WT_37	E5	TTGGTATG	CTCAATGA	TCATTGAG
UDI_Plate_WT_38	F5	TGAACTGG	CGCATACA	TGTATGCG
UDI_Plate_WT_39	G5	TCCAGTCG	CCGAAGTA	TACTTCGG
UDI_Plate_WT_40	H5	TGTATGCG	CCAGTTCA	TGAACTGG
UDI_Plate_WT_41	A6	TGGCTCAG	CAGCGTTA	TAACGCTG
UDI_Plate_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
UDI_Plate_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT

UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix B2: Unique Dual Index (UDI) Plate- EC Sequences

Please refer to the following table to demultiplex TCR sequencing libraries that have been sequenced together in the same run.

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_EC_1	A1	AACAGATC	TGCCATGA	TCATGGCA
UDI_Plate_EC_2	B1	CCTGTCTT	CTAACGAT	ATCGTTAG
UDI_Plate_EC_3	C1	ATATCGAG	TTCCGGAA	TTCCGGAA
UDI_Plate_EC_4	D1	TAGTCCGC	TCTCCATA	TATGGAGA
UDI_Plate_EC_5	E1	TGCTGTTA	AGTGACGT	ACGTCACT
UDI_Plate_EC_6	F1	TAGCGAAT	CTCTGGCA	TGCCAGAG
UDI_Plate_EC_7	G1	AGGACCGT	AGAAGATG	CATCTTCT
UDI_Plate_EC_8	H1	TTATCAGG	TCTAACAG	CTGTTAGA
UDI_Plate_EC_9	A2	CCTCGGAA	TACGTAGA	TCTACGTA
UDI_Plate_EC_10	B2	TTGATCGA	TAAGCTCT	AGAGCTTA
UDI_Plate_EC_11	C2	TCAACCTC	TTGGTCGA	TCGACCAA
UDI_Plate_EC_12	D2	CGGAATAA	CGGTAATA	TATTACCG
UDI_Plate_EC_13	E2	TATGAGAC	CACAGTGG	CCACTGTG
UDI_Plate_EC_14	F2	CCTACCAT	AGACGCGA	TCGCGTCT
UDI_Plate_EC_15	G2	CATAGGCC	AGCAATGG	CCATTGCT
UDI_Plate_EC_16	H2	CACGATAA	CCTCTTGA	TCAAGAGG
UDI_Plate_EC_17	A3	TTCTGGCG	TTGTCTAG	CTAGACAA

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_EC_18	B3	TCGTAACT	TGTTGCTA	TAGCAACA
UDI_Plate_EC_19	C3	TCCGTGGT	TACTGTCA	TGACAGTA
UDI_Plate_EC_20	D3	AAGACAAC	AAGCCATA	TATGGCTT
UDI_Plate_EC_21	E3	ATTGCTTC	CGGCACTT	AAGTGCCG
UDI_Plate_EC_22	F3	AGAGAAGT	TCATGGAT	ATCCATGA
UDI_Plate_EC_23	G3	TACGTCTT	TAATCAGG	CCTGATTA
UDI_Plate_EC_24	H3	TCCACGTT	CATCTGAG	CTCAGATG
UDI_Plate_EC_25	A4	CAATGAGT	CAGAATCG	CGATTCTG
UDI_Plate_EC_26	B4	CGTCAGTT	TTGATGCA	TGCATCAA
UDI_Plate_EC_27	C4	TTCACTCC	ACGTATGG	CCATACGT
UDI_Plate_EC_28	D4	CTACCTGA	TGCGGATA	TATCCGCA
UDI_Plate_EC_29	E4	AGGTGATT	TAACAGCA	TGCTGTTA
UDI_Plate_EC_30	F4	TCTCACAT	AATGTTCG	CGAACATT
UDI_Plate_EC_31	G4	TTAGTGAG	TTACGTGT	ACACGTAA
UDI_Plate_EC_32	H4	TGTTCACT	ATCTACTG	CAGTAGAT
UDI_Plate_EC_33	A5	CGAAGCCT	ATACCTCT	AGAGGTAT
UDI_Plate_EC_34	B5	TGGCCGTA	TACATCTG	CAGATGTA
UDI_Plate_EC_35	C5	CGCTTCAC	AAGGAGCA	TGCTCCTT
UDI_Plate_EC_36	D5	CGGAGAAC	CTCCTAGA	TCTAGGAG
UDI_Plate_EC_37	E5	AAGTTCAG	TCAGCCTA	TAGGCTGA
UDI_Plate_EC_38	F5	AAGCCTTC	TTCGCTCA	TGAGCGAA
UDI_Plate_EC_39	G5	ACACCTCA	CGGATTAA	TTAATCCG
UDI_Plate_EC_40	H5	TTCTCCTA	ATTAGAGG	CCTCTAAT

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_EC_41	A6	TGACACGC	CGTGTGAA	TTCACACG
UDI_Plate_EC_42	B6	AAGCGCCT	TGTCACGG	CCGTGACA
UDI_Plate_EC_43	C6	TGATAACC	CGTAATCT	AGATTACG
UDI_Plate_EC_44	D6	AAGAGTGT	CCTTCTGG	CCAGAAGG
UDI_Plate_EC_45	E6	TGCTACCG	AATCGCTA	TAGCGATT
UDI_Plate_EC_46	F6	CCATCGTC	TGGACCAA	TTGGTCCA
UDI_Plate_EC_47	G6	CCACGAGA	TGTGCACT	AGTGCACA
UDI_Plate_EC_48	H6	ACGGTCAT	CTTATGGA	TCCATAAG

Appendix C: Thermocycling Programs

Section 1: *In Situ* Cell Barcoding

THAW ROUND 1 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	26 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

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

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

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

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

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

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

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

Section 2: cDNA Capture and Amplification

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

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

Section 3: Sequencing Library Preparation

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

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

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

Section 4: TCR Sequencing Library Preparation

TCR AMPLIFICATION 1			
Run Time	Lid Temperature	Sample Volume	
~50 minutes	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	20
3	20 s	65°C	
4	20 s	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

TCR AMPLIFICATION 2			
Run Time	Lid Temperature	Sample Volume	
~30 minutes	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	9
3	20 s	67°C	
4	1 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Appendix D: TCR-Specific Troubleshooting

Adjusting TCR Amplification Cycles

Section 4.1: TCR Amplification 1 and Section 4.3: TCR Amplification 2 requires 10 ng of input for optimal TCR amplification. However, PCR cycles can be adjusted to better suit different input quantities. See the tables below for recommended PCR cycles based on input.

TCR AMPLIFICATION 1 (STEP 4.1.11)	
Input (ng)	Total Number of Cycles
10	20
5-9	21

TCR AMPLIFICATION 2 (STEP 4.3.12)	
Input (ng)	Total Number of Cycles
10	9
5-9	10

Note: Having less than 5 nanograms of input material for TCR Amplification 1 or 2 might indicate poor quality data.

Appendix E: Revision History

Version	Description	Date
1.0	Updated for Evercode WT v3 chemistry	May 2024
1.1	Appendix B: Updated sequencing information	June 2024
1.2	Updated box configurations	August 2024
1.3	Section 1.2.17: added a step to accommodate low Input fixation samples	November 2024

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