User Manual

Version 1.1 – UMMT4160

Evercode™ MAX TCR v4 Library Prep

16 Reactions

For use with

ECMT4160

ECMT4161

Support Suite

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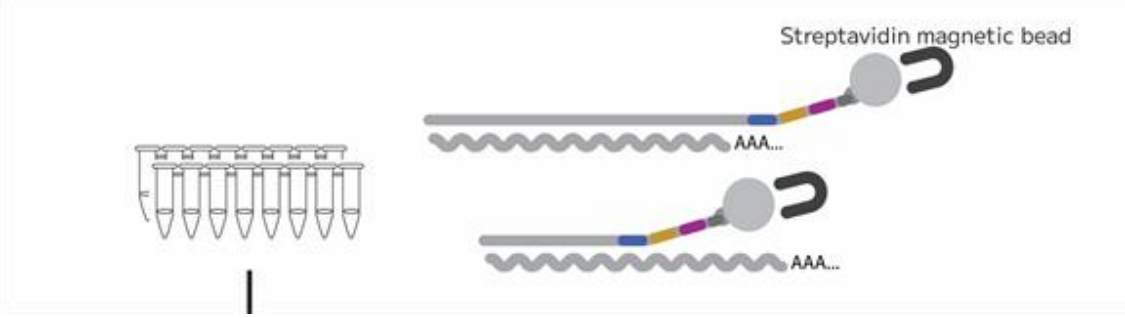
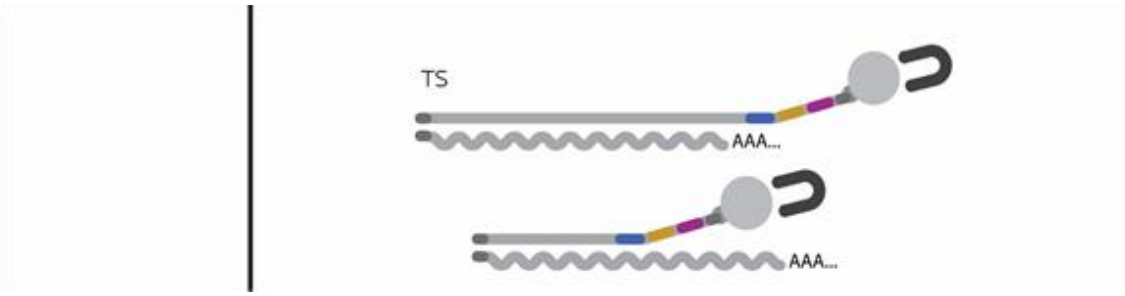
Overview

Workflow

This manual picks up where Evercode MAX TCR v4 Cell Barcoding user guides left off. In Cell Barcoding guide's Section 1, samples underwent three rounds of barcoding before being divided into sublibraries and lysed. Sections 2, 3, and 4 of this manual complete the workflow by amplifying the cDNA preparing the sequencing libraries followed by CDR3 amplification. This guide therefore should be completed after the Evercode MAX TCR v4 Cell Barcoding workflow.

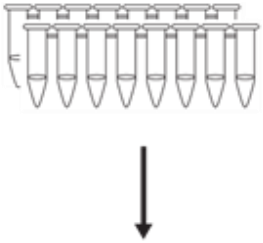
This user guide can be used to prepare between up to 16 sublibraries concurrently for sequencing.

This workflow supports the preparation of up to 32 sublibraries in a single run. For 32 reactions, two Evercode MAX TCR v4 Library Prep 16 Reactions are needed.

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

Section 2: cDNA Capture and Amplification	
3	 **cDNA Amplification:** The cDNA is amplified with primers specific to the template switch adapter and Illumina TruSeq Read 2.

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

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.

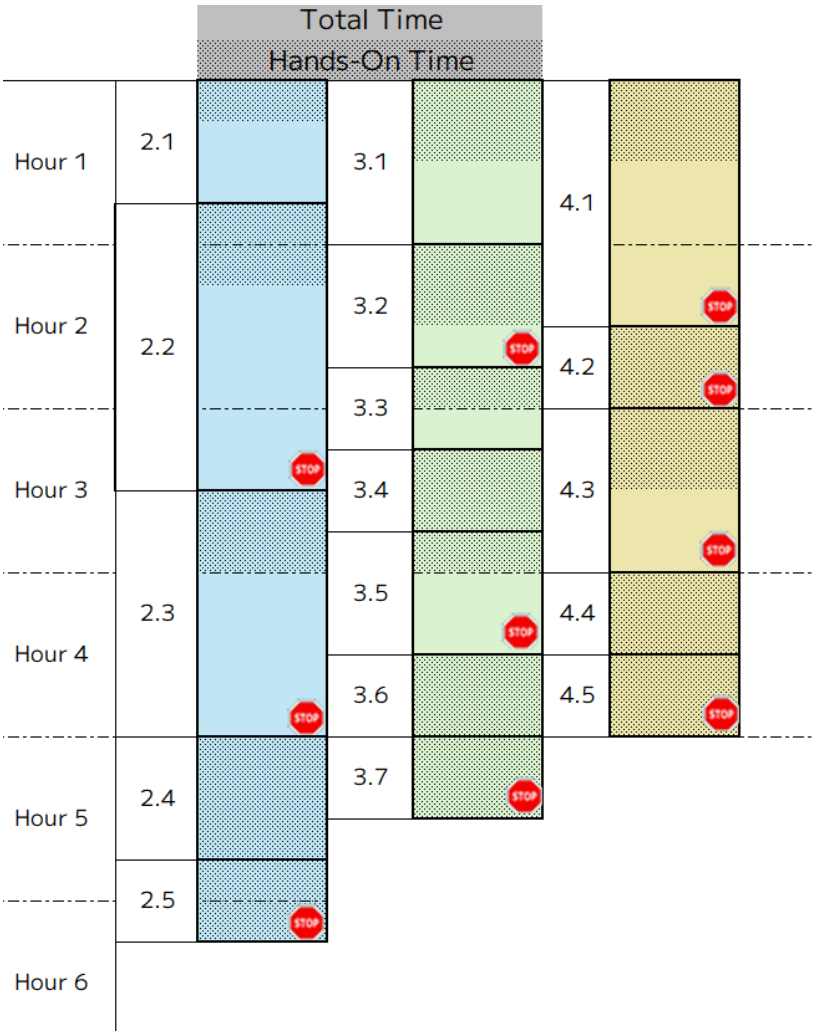
Protocol Timing

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

Preparing more than 16 sublibraries using plate magnets will add about 30 minutes to the workflow.

DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	45 min	10 min	
2.2 cDNA Template Switch	105 min	15 min	4°C ≤ 18 hrs
2.3 cDNA Amplification	90 min	10 min	4°C ≤ 18 hrs in the thermocycler
2.4 Post-Amplification Purification	30 min	30 min	
2.5 cDNA Quantification	30 min	30 min	4°C ≤ 48 hrs or -20°C ≤ 3 months
Section 3: Sequencing Library Preparation			
3.1 Fragmentation and End Prep	60 min	30 min	
3.2 Fragmentation and End Prep Size Selection	30 min	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
3.3 Adapter Ligation	30 min	15 min	
3.4 Post-Ligation Purification	30 min	30 min	
3.5 Barcoding Round 4	45 min	15 min	4°C ≤ 18 hrs in the thermocycler
3.6 Post-Barcoding Round 4 Size Selection	30 min	30 min	
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

DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
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/.

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. All user supplied consumables and reagents should be RNase free.
- 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.

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 on the benchtop and off ice will minimize this occurrence. PCR plate seals may be difficult to remove. Carefully peel the PCR plate seal while applying downward pressure on the plate to keep it in the PCR tube rack.

Magnetic Racks and Bead Cleanups

- The Evercode MAX TCR v4 Library Prep workflow uses magnetic beads to clean up libraries. A magnetic rack capable of holding 1.5 mL tubes and the Parse Biosciences Magnetic rack for 0.2 mL tubes are required for this protocol.
- When aspirating supernatants in the magnetic rack, angle pipette tips away from beads. Ensure tips are at the bottom of each tube to ensure all supernatant is removed.
- If beads are disturbed, dispense solution again and wait for 1 minute before trying to aspirate again.

- The Parse Biosciences Magnetic Rack 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

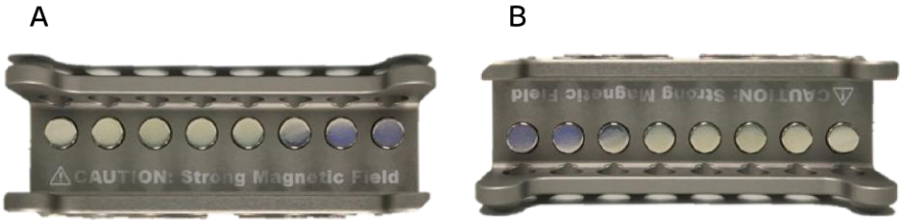


Figure 1: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- 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 2: Clear supernatant with compact bead pellets.

The Evercode MAX TCR v4 workflow supports preparation of up to 32 sublibraries using two Evercode MAX TCR v4 Library Prep 16-Reaction kits. When planning to barcode samples for more than 16 sublibraries, we recommend using a PCR plate magnet for capturing lysed cells and library preparation steps. Dispense lysates into low-bind 96-well plates if using a 96-well plate magnetic rack.

Sublibrary Processing

- 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.

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 kits.

- Depending on your sequencing instrument and number of sublibraries, consider base diversity when selecting UDI combinations for sequence information. See Appendix A for index sequences.

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

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

- Record which UDI is added to each sublibrary. This information will be critical for the sequencing provider.

Thermocycling Programs

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

Part List

The Evercode Max TCR v4 Library Prep 16 Reactions kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request

-20°C Reagents Store -20°C, XHT160 or XMT160

LABEL	ITEM	PN	FORMAT	QTY
	Wash Buffer A	MG143	5 mL tube	1
	Bead Wash Buffer	MG114	5 mL tube	1
	Binding Buffer	MG118	1.5 mL tube	1
	Wash Buffer B	MG144	5 mL tube	1
	Template Switch Buffer	WT148	1.5 mL tube	4
	Template Switch Enzyme	MG121	1.5 mL tube	1
	Template Switch Primer	MG122	1.5 mL tube	1
	PCR Additive	MG150	1.5 mL tube	1
	cDNA Amp Mix	MG123	1.5 mL tube	1
	cDNA Amp Primers	HTG124 or MTG124	1.5 mL tube	1
	Fragm/End Prep Buffer	MG125	1.5 mL tube	1
	Fragm/End Prep Enzymes	MG126	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Ligation Adapter	MG127	1.5 mL tube	1
	Adapter Ligation Buffer	MG128	1.5 mL tube	1
	Adapter Ligation Enzyme	MG129	1.5 mL tube	1
	Library Amp Mix	MG130	1.5 mL tube	1
	Receptor PCR Mix 1	HTG131or MTG131	1.5 mL tube	1
	Receptor PCR Mix 2	HTG132 or MTG132	1.5 mL tube	1
 	HT Primer or MT Primer	HTG133 or MTG133	1.5 mL tube	1

4°C Reagents Store 4°C, PN XHT170 or XMT170

LABEL	ITEM	PN	FORMAT	QTY
	Streptavidin Beads	PT201	2 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.

Equipment

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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.	☐
Permagen Bar Magnet	Permagen®	MSPU650	Optional: if preparing >16 sublibraries. We do not recommend alternative magnets, as they may result in lower transcript and gene detection.	☐
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.	☐
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.	☐
PCR tube rack	Various Suppliers	Varies	Capable of holding semi-skirted 96 well PCR plates.	☐
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.	☐
Single Channel Pipettes: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.	☐
T100 Thermal Cycler	Bio-Rad Laboratories®	1861096	Or an equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.	☐
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
6-inch Platform	Scientific Industries	146-6005-00	thermomixer or alternative shaker that can be set to 800-1000 RPM.	
Microplate Foam Insert	Scientific Industries	504-0235-00		
Qubit™ Flex Fluorometer	Thermo Fisher Scientific®	Q33327		☐
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.	☐
4200 TapeStation System	Agilent	G2991BA		

Consumables

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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.	☐
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.	☐
twin.tec™ PCR Plates LoBind™	Eppendorf	0030129504	Optional if preparing more than 16 sublibraries: Or equivalent DNA low-binding, nuclease-free 96-well plate.	☐
SealPlate®	Excel Scientific	100-SEAL-PLT	Optional: Or equivalent PCR plate seals.	☐

Reagents

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

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
UDI Plate-EC	Parse Biosciences	UDI1002	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode Library Prep (48 sublibraries). UDI Plate-EC can be purchased separately or bundled with Evercode kits.	☐
SPRIselect Reagent	Beckman Coulter®	B23317 (5mL) B23318 (60mL)	Choose one. We do not recommend substituting other magnetic beads.	☐
AMPure® XP Reagent	Beckman Coulter	A63880 (5 mL) A63881 (60mL)		
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)		
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780		
Ethyl alcohol, Pure	Sigma-Aldrich	459844	Or equivalent RNase decontamination solution.	☐
TE Buffer pH 8.0	Various Suppliers	Varies	Or equivalent ethanol.	☐
Nuclease-Free Water	Sigma-Aldrich	W4502	Optional. Choose one that is RNase-free.	☐
Qubit dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific	Q33230 (100 assays) Q33231 (500 assays)	Or equivalent nuclease-free water.	☐
High Sensitivity DNA Kit	Agilent	5067-4626	Or equivalent DNA quantifier.	☐
High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	Choose one that corresponds to the chosen Bioanalyzer or Tapestation.	☐
High Sensitivity D1000 ScreenTape and Reagents	Agilent	5067-5584 (screen tape) 5067-5585 (sample buffer and ladder)		

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 prepare up to 32 sublibraries at once, use two Evercode MAX WT Library Prep 16 Reactions kits.



Note: Optional: if preparing more than 16 sublibraries, we recommend using a plate magnet for beads clean up throughout Section 2. See "User Supplied Equipment and Reagents" for recommended plate magnetic racks.



CRITICAL! Always use new tips for each sublibrary.

To capture the cDNA:

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

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

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

Lysate Being Processed	4	8	12	16	32
● Streptavidin Beads	264 µL	528 µL	792 µL	1056 µL	2112 µL

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

6. Remove and discard the supernatant.

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

Lysate Being Processed	4	8	12	16	32
○ Bead Wash Buffer	200 µL	400 µL	600 µL	800 µL	1600 µL

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

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

9. Remove and discard the supernatant.

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

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

Lysate Being Processed	4	8	12	16	32
● Binding Buffer	220 µL	440 µL	660 µL	880 µL	1760 µL

12. Remove the desired tube(s) of lysate from the thermocycler (if continuing directly from Evercode MAX TCR v4 Cell Barcoding) or from storage at -80°C.

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

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



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

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

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

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

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

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



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

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

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

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



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

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

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



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

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

25. Proceed immediately to Section 2.2.

2.2. cDNA Template Switch

After an additional wash, the Template Switch Master Mix is added to the captured cDNA. The template switch reaction adds a 3′ adapter to the cDNA.

CRITICAL! Always use new tips for each sublibrary.

To perform template switch:

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

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

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

2. Prepare the Template Switch Master Mix in a new tube as follows, depending on the number of sublibraries being processed. Mix by pipetting 10x and store on ice. You will need 2 tubes if processing 32 samples.

TEMPLATE SWITCH MASTER MIX					
Number of Sublibraries	4	8	12	16	32
Template Switch Buffer	348 µL	696 µL	1044 µL	1392 µL	2784 µL
Template Switch Primer	12 µL	24 µL	36 µL	48 µL	96 µL
Template Switch Enzyme	24 µL	48 µL	72 µL	96 µL	192 µL
Total	384 µL	768 µL	1152 µL	1536 µL	3072 µL

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

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

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



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

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

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

8. Remove the tube(s) from the magnetic rack. Add **20 µL** nuclease-free water to each bead pellet and then fully resuspend by vortexing for **10 seconds**, followed by a brief centrifugation.

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



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

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

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

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

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

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

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

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

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

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

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

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

2.3. cDNA Amplification

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

CRITICAL! Always use new tips for each sublibrary.

To amplify the cDNA:

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

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

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

3. Prepare the cDNA Amplification Master Mix in a new tube as follows. Mix by pipetting 10x and store on ice. You will need 2 tubes if processing 32 samples.

cDNA AMPLIFICATION MIX					
Number of Libraries	4	8	12	16	32
cDNA Amp Mix	240 µL	480 µL	720 µL	960 µL	1920 µL
cDNA Amp Primers	230 µL	460 µL	690 µL	920 µL	1840 µL
PCR Additive	10 µL	20 µL	30 µL	40 µL	80 µL
Total	480 µL	960 µL	1440 µL	1920 µL	3840 µL

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

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

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

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

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

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

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

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

NUMBER OF PCR CYCLES			
Cells in Sublibrary	Primary T Cells	*In Vitro* Expanded T Cells	PBMC
200-1,000	14	13	13
1,000-2,000	12	11	11
2,000-4,000	10	9	9
4,000-8,000	10	9	8
8,000-16,000	9	8	7
16,000-32,000	7	6	6
32,000-64,000	6	5	5

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

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

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

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

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

2.4. Post-Amplification Purification

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

CRITICAL! Always use new tips for each sublibrary.

To purify the cDNA:

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

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

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

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

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

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

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



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

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

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

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



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

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



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

2.5. cDNA Quantification

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

To quantify the cDNA:

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

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

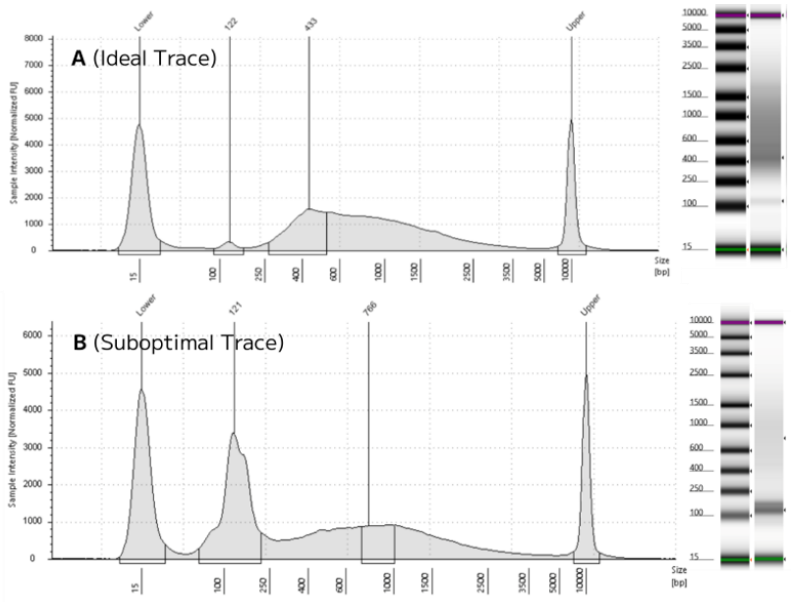


Figure 3: Expected post-amplification sublibrary cDNA size distribution on a Tapestation. A (Ideal Trace) shows a trace with ideal shape and peak sizes. B (Suboptimal trace) shows a trace with prominent primer-dimer peaks around 100-250bp in length.

CRITICAL! If a noticeable primer-dimer peak is present in the trace, repeat the SPRI clean-up step as these small fragments will impact the Section 4 outcome. Primer-dimer peaks do not affect Section 3 outcome, and the user can proceed with Section 3 without an additional clean up. Before proceeding to Section 4, repeat the SPRI clean-up only if there is ample amount of library material, 4 ng/µL or greater. Transfer 10 µL from each sublibrary to a separate new 0.2 mL tube (1 tube per sublibrary) and add

nuclease-free water to bring up the total volume of each sublibrary to 90 µL. Repeat the clean up steps starting at Step 2.4.5. If the trace exhibits minimal primer-dimer peaks after the additional clean up, proceed to Section 4.

Note: Samples may need to be diluted to be within the manufacturer's recommended concentration range. Typically, between a 1:3 to 1:10 dilution is appropriate.



Note: The traces above are representative of typical TapeStation cDNA traces. The shape and prominence of the trace is dependent on cell type, sublibrary size, and amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high quality data.

Section 3: Sequencing Library Preparation

3.1. Fragmentation and End Prep

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



Note: Optional: if preparing more than 16 sublibraries, we recommend using a plate magnet for beads clean up throughout Section 3. See "User Supplied Equipment and Reagents" for recommended plate magnetic racks.



CRITICAL! Always use new tips for each sublibrary.

To prepare for fragmentation and end prep:

1. For each sublibrary, prepare **1.2 mL** of fresh 85% ethanol with nuclease-free water. This will be used in Section 3.2.
2. Equilibrate **180 µL** of SPRI beads per sublibrary to room temperature. This will be used in Section 3.2.
3. Fill an ice bucket.
4. Take out the Parse Biosciences magnetic rack for 0.2 mL PCR tubes.
5. Obtain recorded cDNA concentrations from Section 2.5.
6. Gather the following items and handle as indicated below.

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

7. Vortex the tube(s) of cDNA for **5 seconds**. Briefly centrifuge.

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



Note: A recommended input of 5 ng of cDNA is needed for TCR library preparation in Section 4. If you have less than 105 ng of cDNA for a given sublibrary, less cDNA can be aliquoted towards the Whole Transcriptome library to ensure sufficient cDNA material for Section 4. Successful Whole Transcriptome libraries can be prepared from as little as 10 ng of cDNA. Record the amount added to each tube, as subsequent PCR cycles should be adjusted based on cDNA concentration.

DILUTED cDNA		
Amount of cDNA	≥10 ng/µL	<10 ng/µL
Purified cDNA	10 µL	100 ng
Nuclease-free water	25 µL	Variable
Total Volume	35 µL	35 µL

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

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



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

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



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

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

FRAGMENTATION AND END PREP MASTER MIX					
Number of Libraries	4	8	12	16	32
● Fragm/End Prep Buffer	24 µL	48 µL	72 µL	96 µL	192 µL
● Fragm/End Prep Enzymes	48 µL	96 µL	144 µL	192 µL	384 µL
Total	72 µL	144 µL	216 µL	288 µL	576 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.

CRITICAL! Always use new tips for each sublibrary.

To size select the fragmented and end prepped DNA:

1. Gather freshly prepared 85% ethanol prepared in Section 3.1.
2. Gather room temperature SPRI beads prepared in Section 3.1.

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 **20 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).



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

3.3. Adapter Ligation

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

To ligate adapters:

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

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

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

ADAPTER LIGATION MASTER MIX					
Number of Libraries	4	8	12	16	32
Nuclease-free water	77 µL	154 µL	231 µL	308 µL	616 µL
Adapter Ligation Buffer	88 µL	176 µL	264 µL	352 µL	704 µL
Adapter Ligation Enzyme	44 µL	88 µL	132 µL	176 µL	352 µL
Ligation Adapter	11 µL	22 µL	33 µL	44 µL	88 µL
Total	220 µL	440 µL	660 µL	880 µL	1760 µL

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

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

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

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

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

3.4. Post-Ligation Purification

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



CRITICAL! Always use new tips for each sublibrary.

To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (**~100 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

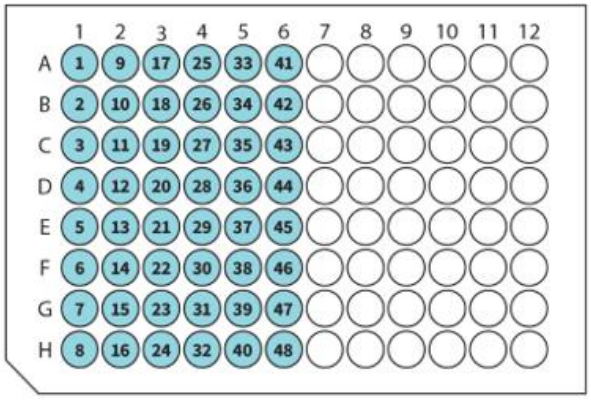
Purified adapter ligated DNA is PCR amplified with Illumina TruSeq Read 1 and Read 2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.

Note: Depending on your sequencing instrument and number of sublibraries, consider base diversity when selecting UDI combinations (see Appendix A) for sequence information. If sequencing two libraries, select index adapters with diverse sequences or contact Parse Support at support@parsebiosciences.com.

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

To add round 4 barcodes:

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



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

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

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

7. Add **25 µL** of ● Library Amp Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 25 µL. Briefly centrifuge.

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

Note: cDNA Input (ng) = cDNA concentration (ng/µL) x volume added (µL). Example: 10 µL at 10 ng/µL = 100 ng total input.

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

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

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

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

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

3.6. Post-Barcoding Round 4 Size Selection

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

CRITICAL! Always use new tips for each sublibrary.

To size select the sequencing libraries:

1. Gather freshly prepared 85% ethanol.
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 a 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 or TE Buffer (pH 8.0).

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 a new 0.2 mL tube(s). Store on ice.

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

3.7. Sequencing Library Quantification

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

To quantify the sequencing libraries:

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

2. Assess the size distribution of each purified sequencing library with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D5000 or D1000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

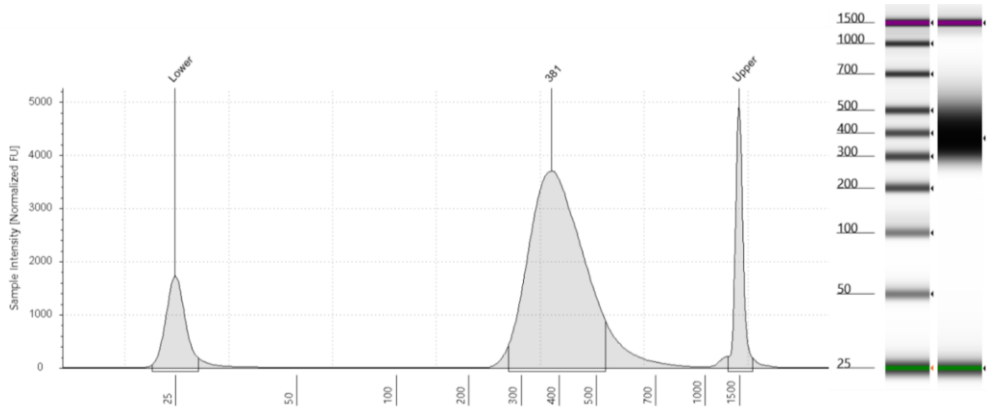


Figure 4: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

Note: Samples may need to be diluted to be within the manufacturer's recommended concentration range. Typically, between a 1:3 to 1:10 dilution is appropriate.

Note: The traces above are representative of typical TapeStation of DNA from indexed sublibraries. There should be a peak between 300-450 bp. The prominence of the trace is dependent on the amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high quality data.

Note: If using a Bioanalyzer, there may be an additional peak present. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 300-450 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, a HT Primer is used in the Human Evercode TCR, and a MT Primer is used in the Mouse Evercode TCR kit.

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	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
HT Primer/MT Primer	-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. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on cDNA input (See Appendix C for more details).

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

TCR AMPLIFICATION 1 MASTERMIX					
Number of Sublibraries	4	8	12	16	32
Receptor PCR Mix 1	220 µL	440 µL	660 µL	880 µL	1760 µL
HT Primer/MT Primer*	61.6 µL	123.2 µL	184.8 µL	246.4 µL	492.8 µL
Total	281.6 µL	563.2 µL	844.8 µL	1126.4 µL	2252.8 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	~50 minutes		
Lid Temperature	105°C		
Sample Volume	100 µL		
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	20
3	20 s	65°	
4	20 s	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

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. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on TCR cDNA concentration (See Appendix C for more details).

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

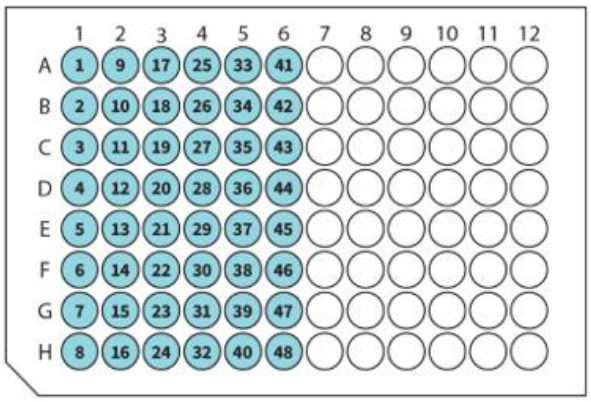
ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Receptor PCR Mix 2	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
UDI Plate - EC	UDI Plate - EC (-20°C)	1 sealed well per sublibrary	Thaw at room temperature then place on ice.

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	~30 minutes		
Lid Temperature	105°C		
Sample Volume	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.

4.5. TCR Sequencing Library Quantification

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

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.

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

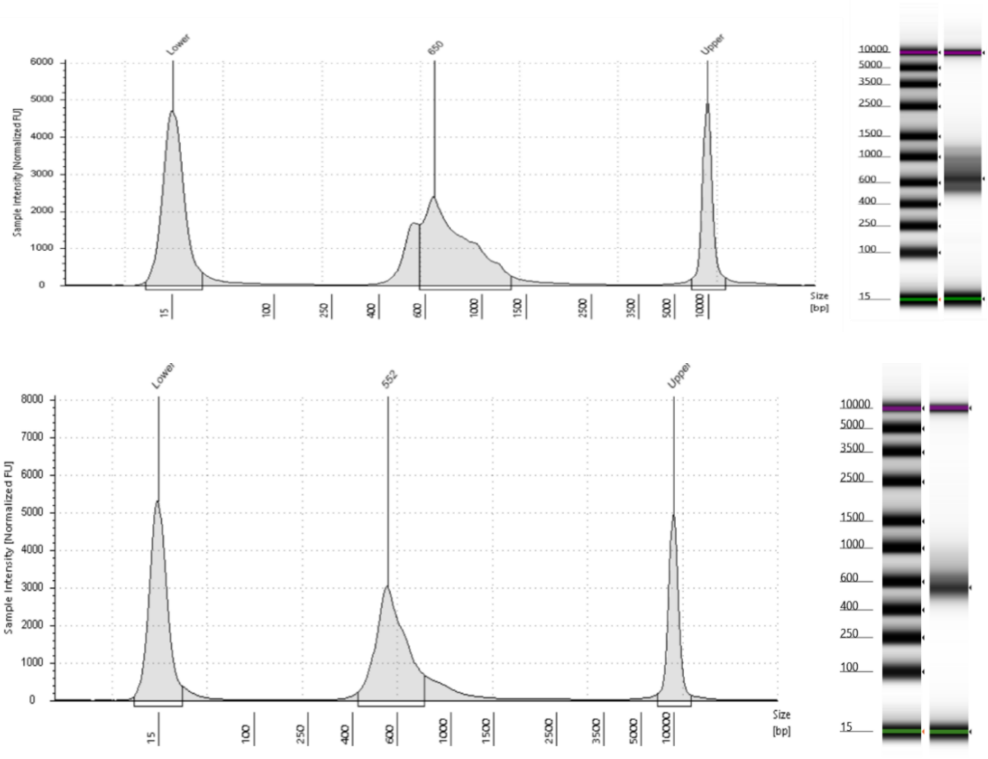


Figure 5: Expected TapeStation size distribution of a Human (Top) and Mouse (Bottom) TCR Sublibrary made with both Primary and Activated T cells.

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: Sequencing Information

We recommend a minimum sequencing depth of 10,000 reads per cell for whole transcriptome libraries and 5,000 reads per cell for the TCR 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, when analyzing a homogenous population, more shallow sequencing may be appropriate.

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

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

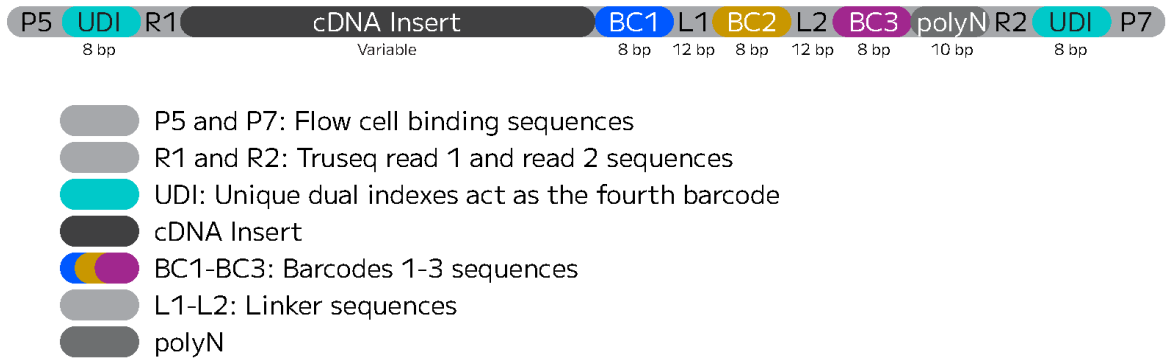


Figure 6: Whole transcriptome sequencing library structure.

Details of the final TCR sequencing library structure are below.

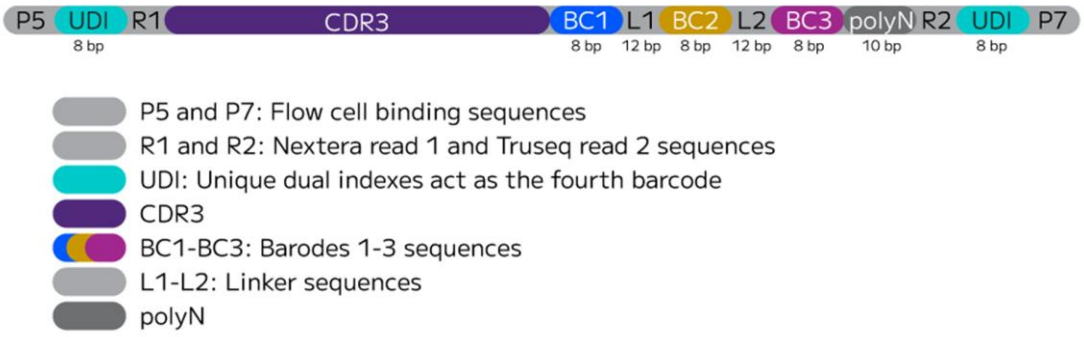


Figure 7: TCR sequencing library structure.

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

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 A1: Unique Dual Index (UDI) Plate - WT Sequences

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

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

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

Appendix A2: Unique Dual Index (UDI) Plate - WT 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 (for sample sheet)	i5 Reverse Complementary 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
UDI_Plate_EC_18	B3	TCGTAACT	TGTTGCTA	TAGCAACA
UDI_Plate_EC_19	C3	TCCGTGGT	TACTGTCA	TGACAGTA
UDI_Plate_EC_20	D3	AAGACAAC	AAGCCATA	TATGGCTT

Sublibrary Index ID	Well Position	i7 Forward Sequence (for sample sheet)	i5 Reverse Complementary Sequence	i5 Forward Sequence
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
UDI_Plate_EC_41	A6	TGACACGC	CGTGTGAA	TTCACACG
UDI_Plate_EC_42	B6	AAGCGCCT	TGTCACGG	CCGTGACA
UDI_Plate_EC_43	C6	TGATAACC	CGTAATCT	AGATTACG

Sublibrary Index ID	Well Position	i7 Forward Sequence (for sample sheet)	i5 Reverse Complementary Sequence	i5 Forward Sequence
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 B: Thermocycling Programs

Section 2: cDNA Capture and Amplification

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

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

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

Section 3: Sequencing Library Preparation

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

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

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

Section 4: TCR Sequencing Library Preparation

TCR AMPLIFICATION 1			
Run Time		~50 minutes	
Lid Temperature		105°C	
Sample Volume		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		~30 minutes	
Lid Temperature		105°C	
Sample Volume		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 C: 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 D: Revision History

Version	Description	Date
1.0	Initial release	October 2026
1.1	Section 2.1: Revised Streptavidin beads volumes	October 2026

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