User Manual

Version 1.1 – UMMT4080

# Evercode™ MAX TCR v4 Library Prep

## 8 Reactions

For use with

ECMT4080

ECMT4081

Support Suite



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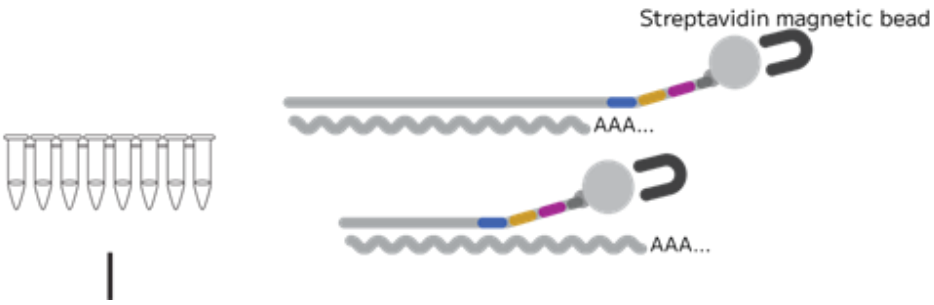
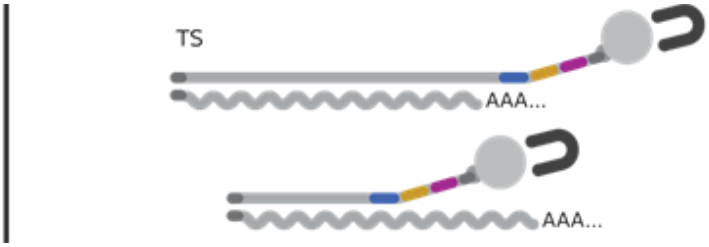
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# Overview

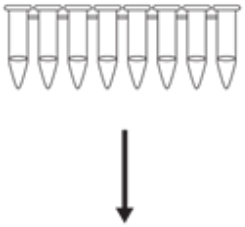
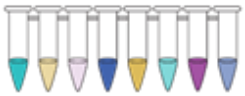
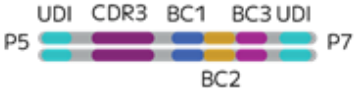
## Workflow

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 2-8 sublibraries concurrently for sequencing.

| Section 2: cDNA Capture and Amplification | |
|---|---|
| 1 | <br>**cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads. |
| 2 | <br>**cDNA Template Switch:** A template switch reaction adds an adapter to the 3' end of the cDNA. |
| 3 | <br>**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 | <br>**Fragmentation and End Repair:** DNA is fragmented, ends are repaired, and A-tailed. |
| 2 | <br>**Adapter Ligation:** An Illumina TruSeq Read 1 Adapter is ligated to the 5′ end of the DNA. |
| 3 | <br>**Round 4 Barcoding:** The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate. |

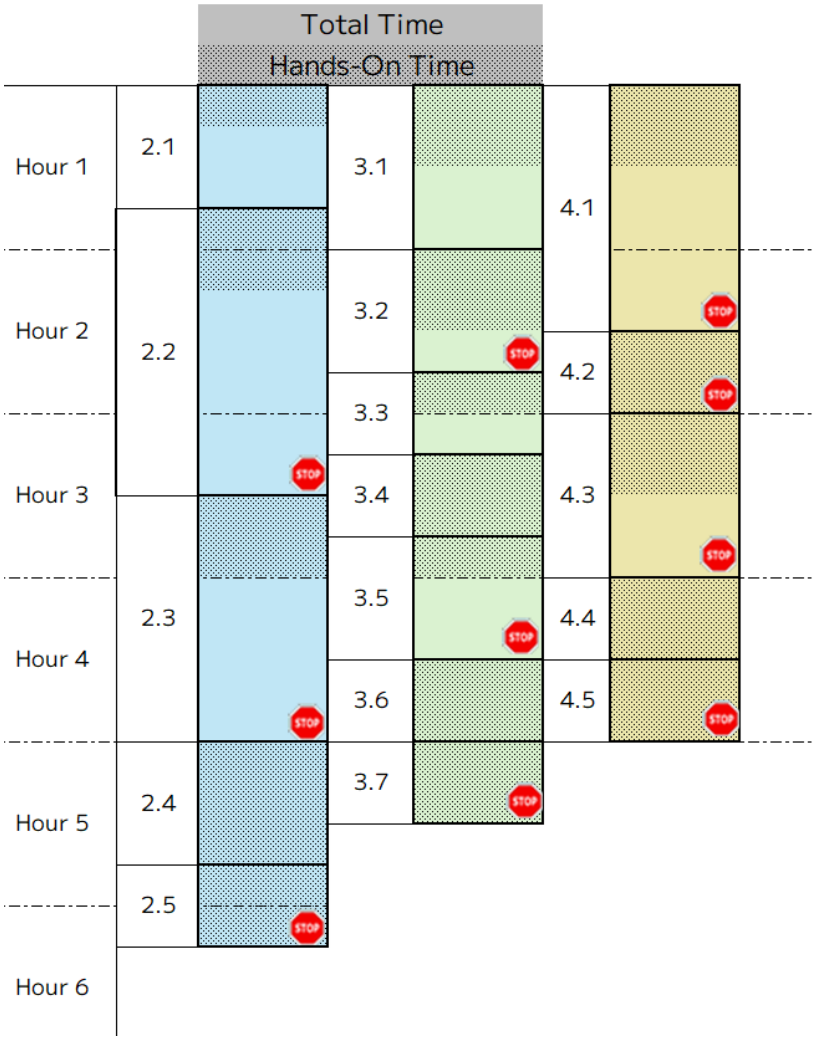
| Section 4: TCR Sequencing Library Preparation | |
|---|---|
| 1 |     **TCR Amplification 1:** A PCR reaction amplifies a subset of the cDNA from the whole transcriptome that contains V(D)J segments in the CDR3 repertoire of the T cell. This reaction also adds an Illumina adapter. |
| 2 |    **TCR 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 workflow. A visual representation of the workflow is shown below the table.

| 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 |
| 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 |

| DESCRIPTION | TIME | HANDS-ON-TIME | STOPPING POINTS |
|---|---|---|---|
| 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.

## 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

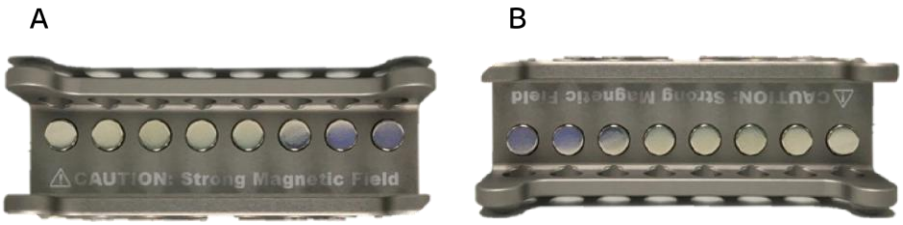



Figure 1: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- 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.

## 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 8 Reactions kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request

**-20°C Reagents** Store -20°C, XHT080 or XMT080

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|   | Wash Buffer A | WT143 | 5 mL tube | 1 |
|   | Bead Wash Buffer | WT114 | 5 mL tube | 1 |
|   | Binding Buffer | WT118 | 1.5 mL tube | 1 |
|   | Wash Buffer B | WT144 | 5 mL tube | 1 |
|   | Template Switch Buffer | WT148 | 1.5 mL tube | 2 |
|   | Template Switch Enzyme | WT121 | 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 | WT123 | 1.5 mL tube | 1 |
|   | cDNA Amp Primers | HTG124 or MTG124 | 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 |

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|   | 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 |
|     | HT Primer or MT Primer | HTT133 or MTT133 | 1.5 mL tube | 1 |

**4°C Reagents.** Store 4°C, XHT090 or XMT090

| 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. | ☐ |
| 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 thermomixer or alternative shaker that can be set to 800-1000 RPM. | ☐ |
| 6-inch Platform | Scientific Industries | 146-6005-00 | | |

| ITEM | SUPPLIER | PN | NOTES | CHECK LIST |
|---|---|---|---|---|
| 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<br>17014963<br>17014967 | Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips. | ☐ |

**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. | ☐ |
| 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. | ☐ |

| ITEM | SUPPLIER | PN | NOTES | CHECK LIST |
|---|---|---|---|---|
| SPRIselect Reagent | Beckman Coulter® | B23317 (5mL) B23318 (60mL) | Choose one. We do not recommend substituting other magnetic beads. | ☐ |
| AMPure® XP Reagent | Beckman Coulter | A63880 (5 mL) A63881 (60mL) | | |
| KAPA® Pure Beads | Roche® | KK8000 (5 mL) KK8001 (30mL) | | |
| RNaseZap™ RNase Decontamination Solution | Thermo Fisher Scientific | AM9780 | Or equivalent RNase decontamination solution. | ☐ |
| Ethyl alcohol, Pure | Sigma-Aldrich | 459844 | Or equivalent ethanol. | ☐ |
| TE Buffer pH 8.0 | Various Suppliers | Varies | Optional. Choose one that is RNase-free. | ☐ |
| Nuclease-Free Water | Sigma-Aldrich | W4502 | Or equivalent nuclease-free water. | ☐ |
| Qubit dsDNA HS (High Sensitivity) Assay Kit | Thermo Fisher Scientific | Q33230 (100 assays) Q33231 (500 assays) | Or equivalent DNA quantifier. | ☐ |
| High Sensitivity DNA Kit | Agilent | 5067-4626 | Choose one that corresponds to the chosen Bioanalyzer or Tapestation. | ☐ |
| High Sensitivity D5000 ScreenTape and Reagents | Agilent | 5067-5592 (screen tape) 5067-5593 (sample buffer and ladder) | | |
| High Sensitivity D1000 ScreenTape and Reagents | Agilent | 5067-5584 (screen tape) 5067-5585 (sample buffer and ladder) | | |

# Section 2: cDNA Capture and Amplification

## 2.1. cDNA Capture

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

**CRITICAL!** Always use new tips for each sublibrary.

**To capture the cDNA:**

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

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

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 | 2 | 4 | 6 | 8 |
|---|---|---|---|---|
| ● Streptavidin Beads | 132 µL | 264 µL | 396 µL | 528 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 | 2 | 4 | 6 | 8 |
|---|---|---|---|---|
| ○ Bead Wash Buffer | 100 µL | 200 µL | 300 µL | 400 µL |



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

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

9. Remove and discard the supernatant.

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

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

| Lysate Being Processed | 2 | 4 | 6 | 8 |
|---|---|---|---|---|
| ● Binding Buffer | 110 µL | 220 µL | 330 µL | 440 µL |

12. Remove the desired tube(s) of lysate from the thermocycler (if continuing directly from Evercode MAX WT Cell Barcoding v4) 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.

| TEMPLATE SWITCH MASTER MIX | | | | |
|---|---|---|---|---|
| Number of Sublibraries | 2 | 4 | 6 | 8 |
| Template Switch Buffer | 174 µL | 348 µL | 522 µL | 696 µL |
| Template Switch Primer | 6 µL | 12 µL | 18 µL | 24 µL |
| Template Switch Enzyme | 12 µL | 24 µL | 36 µL | 48 µL |
| Total | 192 µL | 384 µL | 576 µL | 768 µL |

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

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

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

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

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

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

8. Remove the tube(s) from the magnetic rack. 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** O Wash Buffer A by vortexing for **10 seconds** (100% power).



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

## 2.3. cDNA Amplification

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



**CRITICAL!** Always use new tips for each sublibrary.

**To amplify the cDNA:**

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

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

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

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

| cDNA AMPLIFICATION MIX | | | | |
|---|---|---|---|---|
| Number of Libraries | 2 | 4 | 6 | 8 |
| cDNA Amp Mix | 120 µL | 240 µL | 360 µL | 480 µL |
| cDNA Amp Primers | 115 µL | 230 µL | 345 µL | 460 µL |
| PCR Additive | 5 µL | 10 µL | 15 µL | 20 µL |
| Total | 240 µL | 480 µL | 720 µL | 960 µL |

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



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

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

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

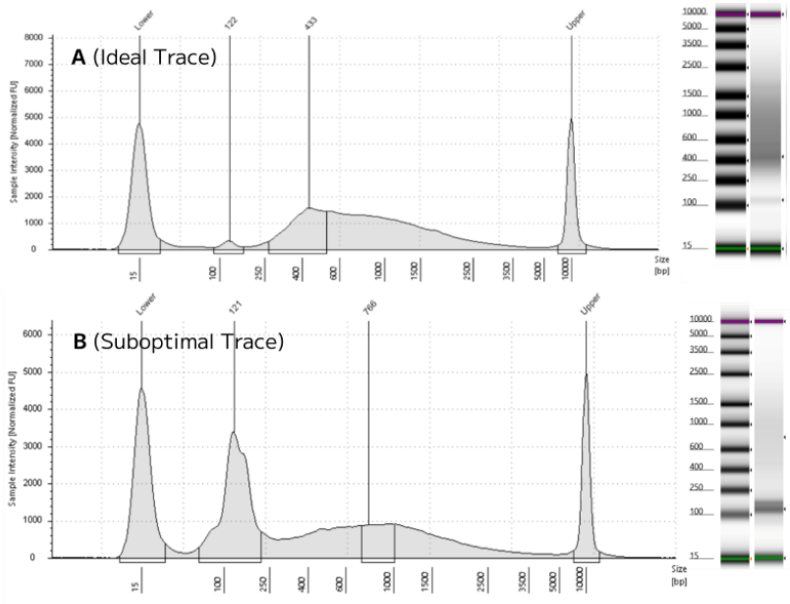



Figure 3: Expected post-amplification sublibrary cDNA size distribution on a Tapestation. A (Ideal Trace) shows a trace with ideal shape and peak sizes. B (Suboptimal trace) shows a trace with prominent primer-dimer peaks around 100-250bp in length.

**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.



**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 | 2 | 4 | 6 | 8 |
| ● Fragm/End Prep Buffer | 12 µL | 24 µL | 36 µL | 48 µL |
| ● Fragm/End Prep Enzymes | 24 µL | 48 µL | 72 µL | 96 µL |
| Total | 36 µL | 72 µL | 108 µL | 144 µL |

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

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



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

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

## 3.2. Post-Fragmentation and End Prep Size Selection

The fragmented and end prepped DNA are size selected with a 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 | 2 | 4 | 6 | 8 |
| Nuclease-free water | 38.5 µL | 77 µL | 115.5 µL | 154 µL |
| Adapter Ligation Buffer | 44 µL | 88 µL | 132 µL | 176 µL |
| Adapter Ligation Enzyme | 22 µL | 44 µL | 66 µL | 88 µL |
| Ligation Adapter | 5.5 µL | 11 µL | 16.5 µL | 22 µL |
| Total | 110 µL | 220 µL | 330 µL | 440 µL |

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

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

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

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

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

## 3.4. Post-Ligation Purification

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



**CRITICAL!** Always use new tips for each sublibrary.

**To purify the ligated DNA:**

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

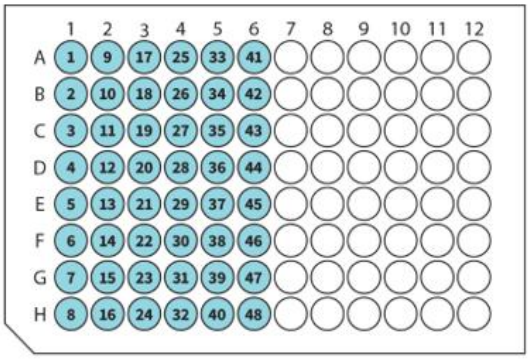
Purified adapter ligated DNA is PCR amplified with Illumina TruSeq Read 1 and Read 2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.

**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.

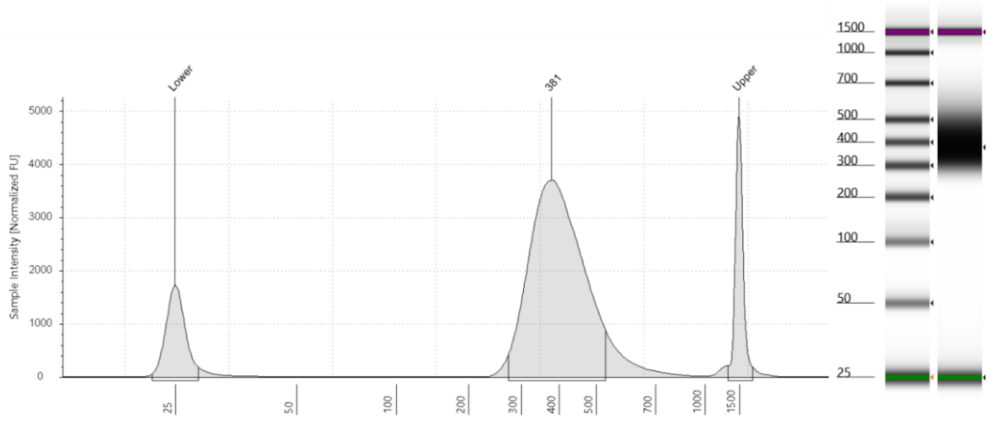



Figure 4: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

**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 | 2 | 4 | 6 | 8 |
| Receptor PCR Mix 1 | 110 µL | 220 µL | 330 µL | 440 µL |
| HT Primer/MT Primer* | 30.8 µL | 61.6 µL | 92.4 µL | 123.2 µL |
| Total | 140.8 µL | 281.6 µL | 422.4 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 | ~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.

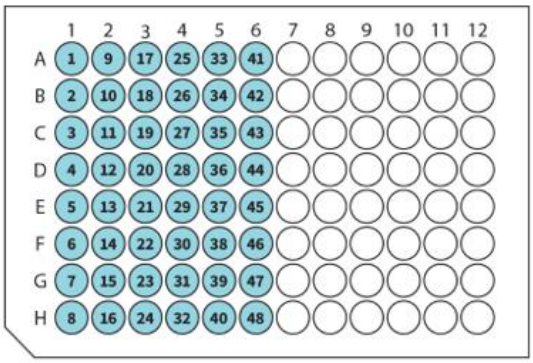
| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ● Receptor PCR Mix 2 | -20°C Reagents | 1 | Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use. |
| UDI Plate - EC | UDI Plate - EC (-20°C) | 1 sealed well per sublibrary | Thaw at room temperature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

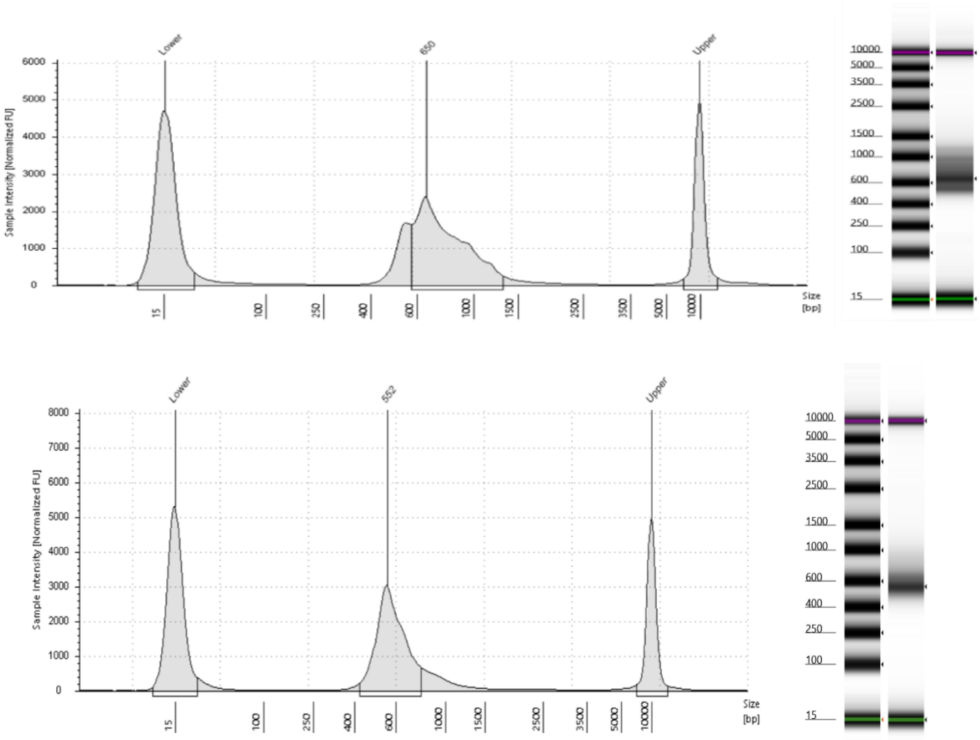



Figure 5: Expected TapeStation size distribution of a Human (Top) and Mouse (Bottom) TCR Sublibrary made with both Primary and Activated T cells.

**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.

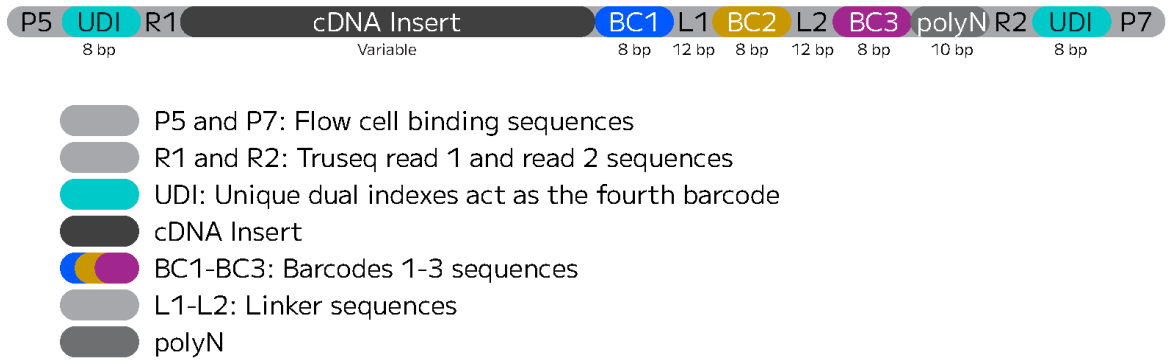



Figure 6: Whole transcriptome sequencing library structure.

Details of the final TCR sequencing library structure are below.

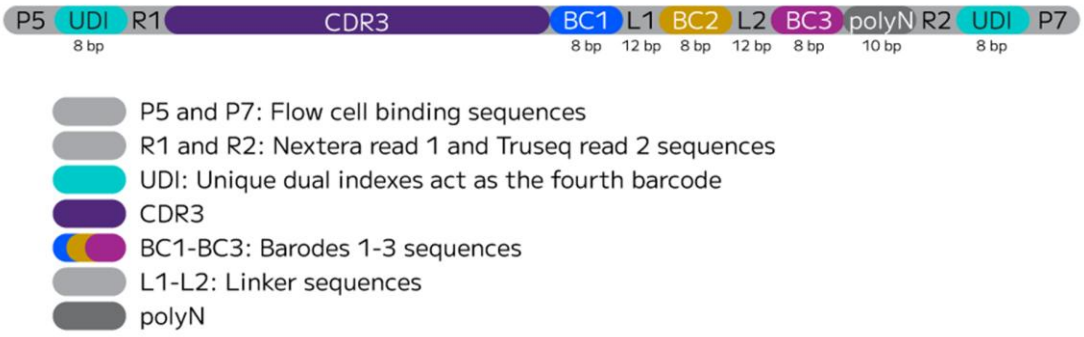



Figure 7: TCR sequencing library structure.

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 Complementary 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 Complementary 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 - 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 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 | 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 | 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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