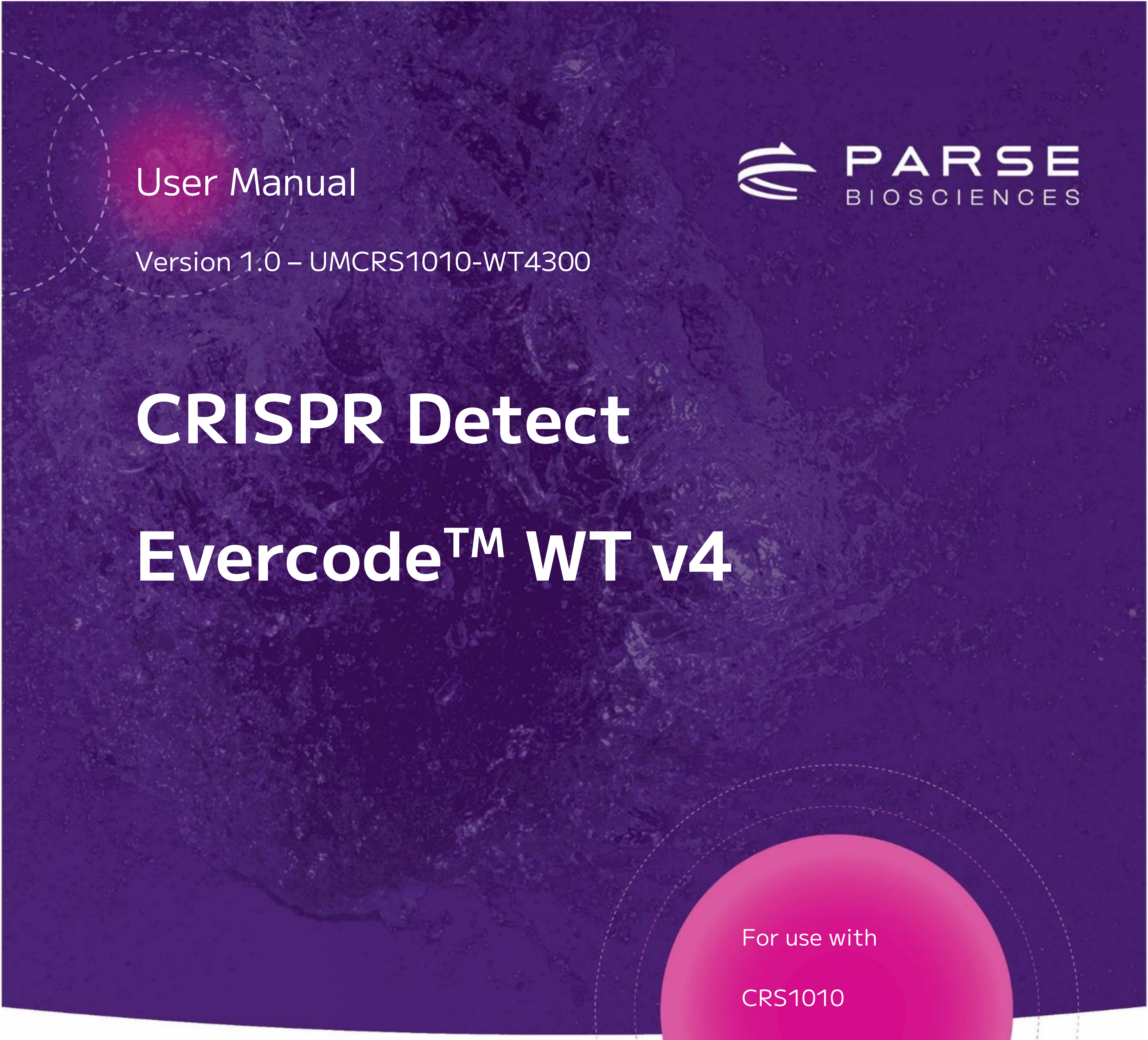

User Manual

Version 1.0 – UMCRS1010-WT4300

# CRISPR Detect

# Evercode™ WT v4

For use with

CRS1010

ECWt4300

Support Suite

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# Table of Contents

# Overview

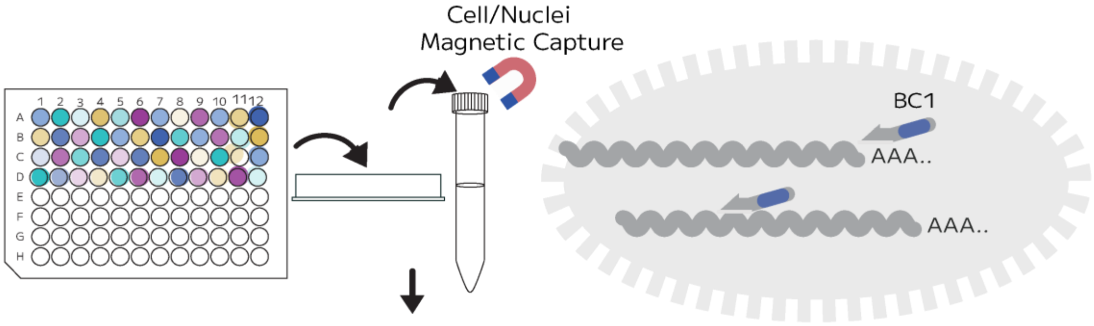
## Workflow

CRISPR Detect enables analysis of single guide RNAs (sgRNAs) in studies using CROP-seq or similar methods. Compatible methods generate a polyadenylated transcript containing the sgRNA sequence downstream of a human U6 promoter. When CRISPR Detect is combined with Evercode WT v4, paired sgRNA detection and whole transcriptome expression can be analyzed in up to 100,000 cells across up to 48 different biological samples or experimental conditions.

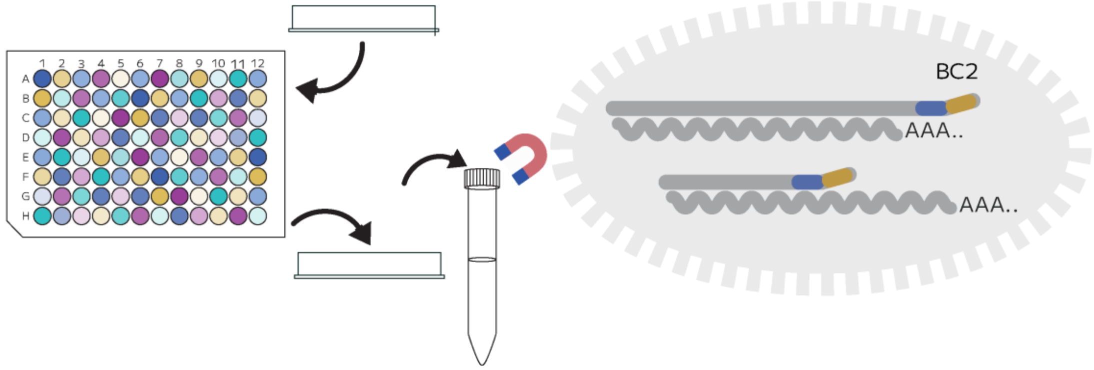
Evercode Cell Fixation kits convert the cells into individualized reaction compartments, thus avoiding the requirement for dedicated microfluidics hardware. Through three rounds of barcoding, the transcriptome of each fixed cell is uniquely labeled. In each round, pooled cells are randomly distributed into different wells, and transcripts are labeled with well-specific barcodes. Barcoded transcripts are amplified during cDNA amplification, and sgRNA containing polyadenylated transcripts are enriched with human U6 specific primers.

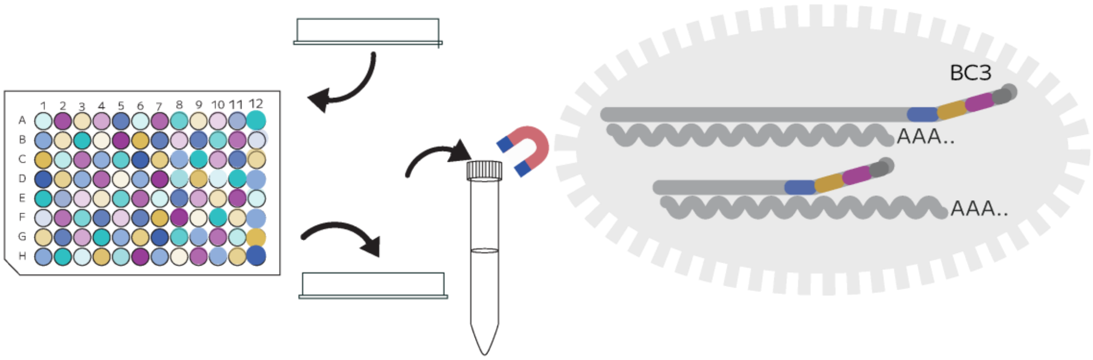
Amplified cDNA is split to create Whole Transcriptome and CRISPR sequencing libraries. After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same four barcodes combination to a single cell and associates sgRNAs to the appropriate cell.

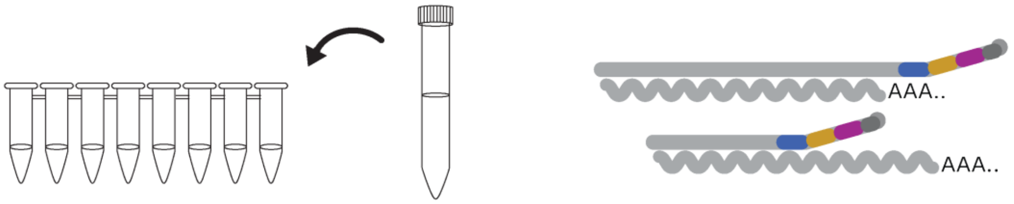
The tables below provide a high-level overview of the workflow.

<table>
<tr><th colspan="2">Section 1: In situ Sample Barcoding</th></tr>
<tr><td>1</td><td>


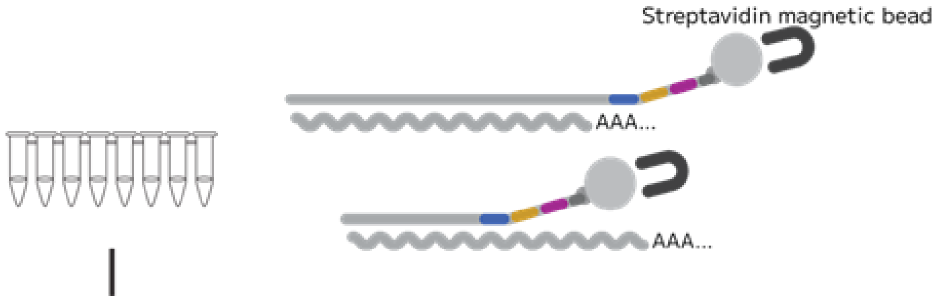
**Round 1 Barcoding:** Fixed and permeabilized cells are loaded into 48 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with well-specific barcodes that are associated with specific samples. Barcoded samples are pooled and captured via magnetic beads.
</td>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

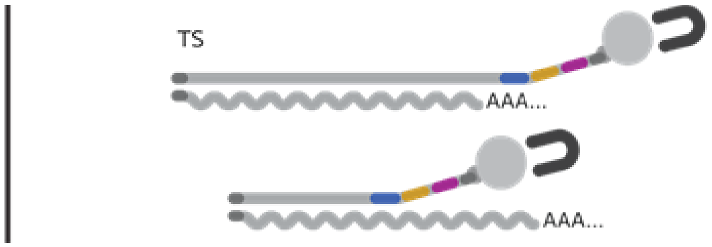
<table>
<tr><th colspan="2">Section 1: In situ Sample Barcoding</th></tr>
<tr><td>2</td><td>

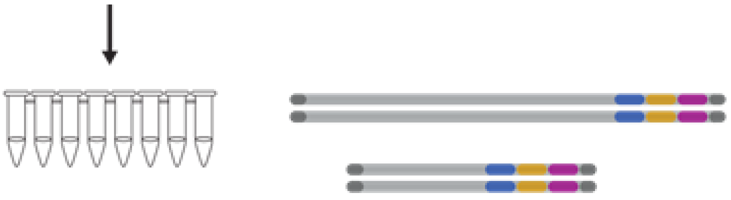
**Round 2 Barcoding:** The pooled cells pooled and loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode. Cells are pooled and then captured via magnet.</td></tr>
<tr><td>3</td><td>

**Round 3 Barcoding:** The cells are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina Truseq Read 2 sequence, and biotin. Cells are pooled and then captured via magnet.</td></tr>
<tr><td>4</td><td>

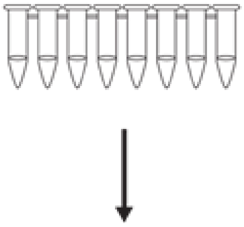
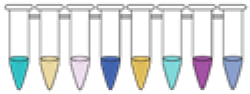
**Lysis and Sublibrary Generation:** Cells are split across 8 sublibraries and lysed.</td></tr>
</table>

<table>
<tr><th colspan="2">Section 2: cDNA Capture and Amplification</th></tr>
<tr><td>1</td><td>

**cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads.</td></tr>
<tr><td>2</td><td>

**cDNA Template Switch:** A template switch reaction adds an adapter to the 3′ end of the cDNA.</td></tr>
<tr><td>3</td><td>
**cDNA Amplification:** The cDNA is amplified with primers specific to the template switch adapter and Illumina Truseq Read 2.</td></tr>
</table>

| | 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: CRISPR Sequencing Library Preparation | |
|---|---|
| 1 | <br><br> <br>**CRISPR PCR:** A PCR reaction enriches the sgRNA containing cDNA with a second hU6 specific primer. This reaction also adds an Illumina adapter. |
| 2 | <br><br> <br>**CRISPR Index PCR:** The CRISPR Sequencing Library is amplified, adding P5/P7 adapters and a fourth barcode via the Illumina indexes in the UDI Plate - EC. |

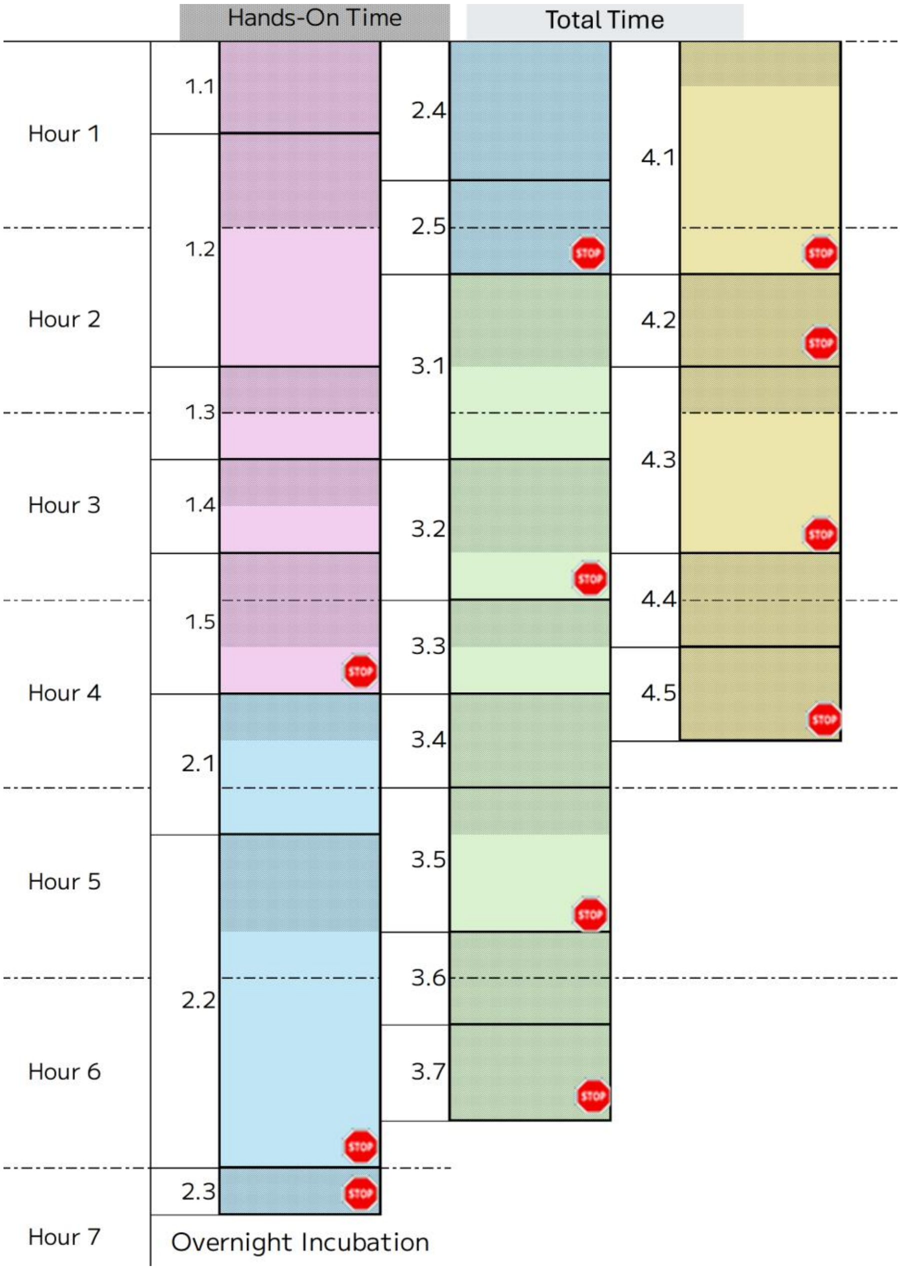
## Protocol Timing

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

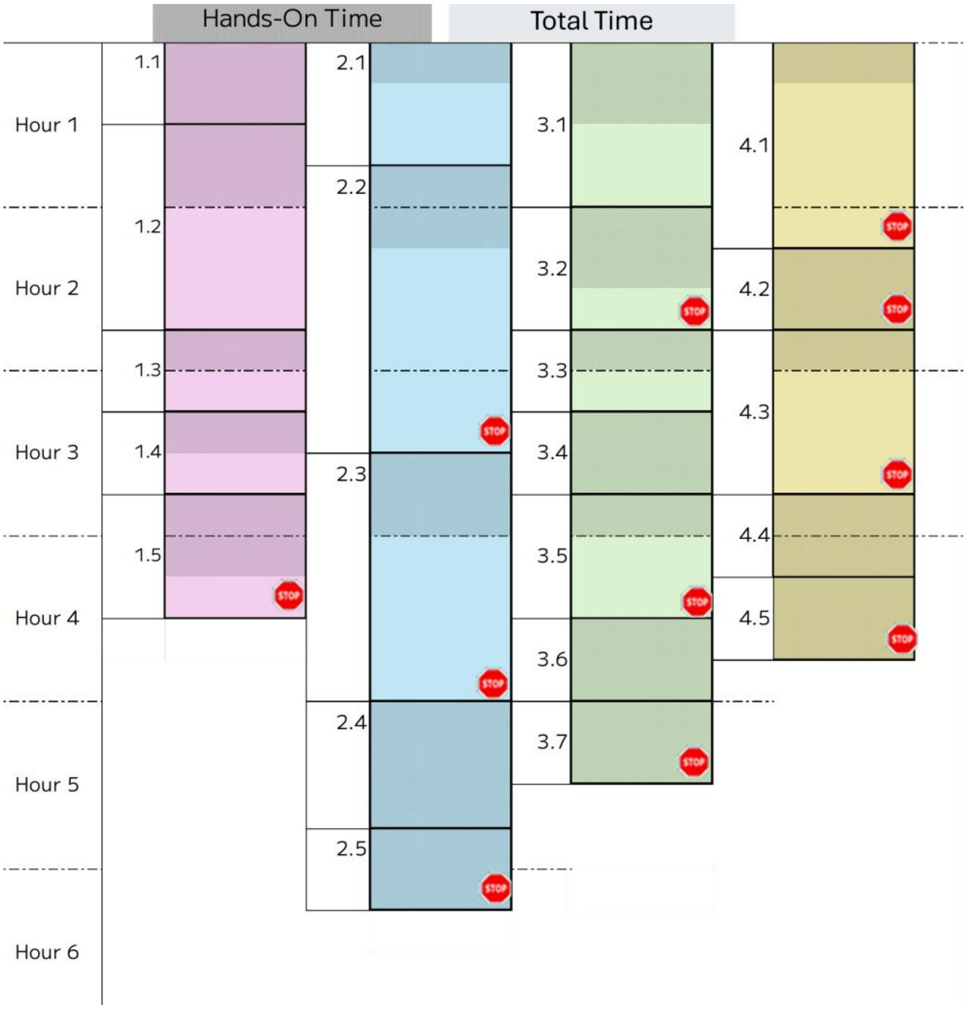
| DESCRIPTION | TIME | HANDS-ON-TIME | SAFE STOPPING POINTS |
|---|---|---|---|
| Section 1: In Situ Cell Barcoding | | | |
| 1.1 Set up and Sample Counting | Variable (30-90 min) | Variable (30-90 min) | |
| 1.2 Barcoding Round 1 | 75 min | 45 min | |
| 1.3 Barcoding Round 2 | 30 min | 15 min | |
| 1.4 Barcoding Round 3 | 30 min | 15 min | |
| 1.5 Lysis and Sublibrary Generation | 40 min | 25 min | -80°C ≤ 6 months |
| Section 2: cDNA Capture and Amplification | | | |
| 2.1 cDNA Capture | 45 min | 10 min | |
| 2.2 cDNA Template Switch | 105 min | 15 min | 4°C ≤ 18 hrs |

| DESCRIPTION | TIME | HANDS-ON-TIME | SAFE STOPPING POINTS |
|---|---|---|---|
| 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: WT 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: CRISPR Sequencing Library Preparation | | | |
| 4.1 CRISPR PCR | 90 min | 15 min | 4°C ≤ 18 hrs |
| 4.2 Post CRISPR PCR Size Selection | 30 min | 30 min | 4°C ≤ 2 days or -20°C ≤ 3 months |
| 4.3 CRISPR Index PCR | 60 min | 15 min | 4°C ≤ 18 hrs |
| 4.4 Post CRISPR Index PCR Size Selection | 30 min | 30 min | |
| 4.5 CRISPR Sequencing Library Quantification | 30 min | 30 min | -20°C ≤ 3 months |

3-days workflow



4-days workflow



# Important Guidelines

The following guidelines provide additional information to obtain optimal performance. For additional questions not discussed below, please contact us at support@parsebiosciences.com. We also have a library of additional resources and videos on our support site at https://support.parsebiosciences.com/.

## Sample Input

- This protocol begins with cells fixed with the Evercode Cell Fixation v3 and v4 kits.
- Even if samples were counted before freezing, we strongly recommend counting cells again after thawing to account for any changes during storage and freeze thaw. Typically, a 5-15% decrease after thawing should be expected. These counts are used to determine how cells are loaded in the Round 1 Plate, and their accuracy is critical to recover the desired number of cells.
- When processing many fixed samples, we recommend aliquoting samples after fixation and counting the aliquots the day before using an Evercode Whole Transcriptome kit. The Evercode Fixation User Manuals outline recommendations for generating aliquots. Because aliquots have undergone a similar storage time and a freeze thaw, cell counts from these aliquots will be more representative than using counts obtained immediately after fixation. Aliquots should be thawed in a water bath set to 37°C in sets of 2-4 and counted with a hemocytometer or alternative counting device. Counts should be recorded in the Sample Loading Table, and any remaining counting aliquot should be discarded.
- Once fixed samples have been thawed, they should not be refrozen.

## Cell Counting and Quality Assessment

- For initial cell counting a hemocytometer can be used. For pre-lysis sample counting, however, a fluorescent counting device is needed. If possible, cell validate counts from alternative devices to a hemocytometer when using Evercode Whole Transcriptome kits for the first time.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- When first using Evercode Whole Transcriptome kits, we suggest saving images at each counting step.
- Examples of AO/DAPI and trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells with <5% cell aggregation and no debris. Higher

levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed samples, it is critical to avoid counting cell debris to avoid overestimating the number of cells.

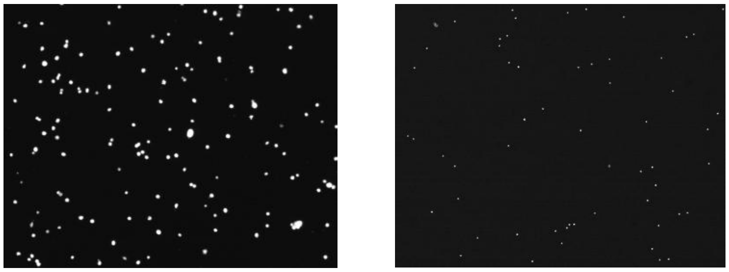
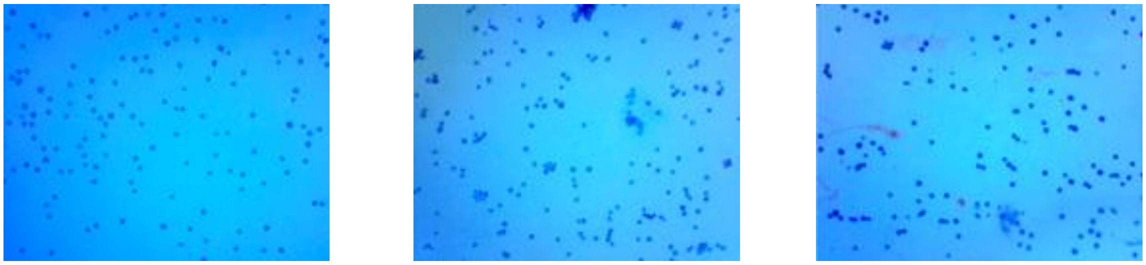


Figure 1: example of AO/DAPI stained HEK cells (left) and PBMCs (right).



High Quality Sample

Aggregation

Debris

Figure 2: Example of trypan blue stained fixed cells.

## Avoiding RNase Contamination

- Take standard precautions to avoid introducing RNases into samples or reagents throughout the workflow. Always wear proper laboratory gloves and use aseptic technique. 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.

## Optimizing Cell Recovery

- It is critical to thoroughly resuspend the cells after binding throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to cell adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell loss.

- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.

- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.

- The first time using an Evercode Whole Transcriptome kit, we recommend retaining supernatants after each barcoding step. In the unlikely event of unexpectedly high sample loss, these supernatants can be analyzed to identify points for optimization.

### Sample Loading Table

- The "Evercode WT v4 Sample Loading Table RevA" should be completed before starting the experiment

- If the table isn't working as expected, ensure that Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in the table to avoid disturbing any calculations.

- In the unlikely event that samples are not concentrated enough according to the Sample Loading Table (Excel spreadsheet), choose to either:

    - Decrease the "Max number barcoded cells" until the Sample Loading Table no longer gives an error, which will maintain the desired proportion between samples but barcode fewer cells.

    - Add 14 µL of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded cells but change the desired proportion between samples.

### Cell Strainers

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 µm is appropriate for many cell types, the mesh size should be chosen based on the sample type.

- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. An example video can be found in our support site.

### Vortexing

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

## Plate Sealing

- While sealing or unsealing 96 well plates, do not splash liquid onto the PCR plate seal or between wells. Securing plates in PCR tube racks 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 WT v4 workflow uses magnetic beads to clean up cells after each round of barcoding. A magnetic rack capable of holding 15 mL conical tubes and one capable of holding 1.5 mL tubes are needed throughout Section 1 of this protocol.

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

- When aspirating supernatants in the magnetic rack, angle pipette tips away from beads. Ensure tips are at the bottom of each tube to ensure all supernatant is removed.

- 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

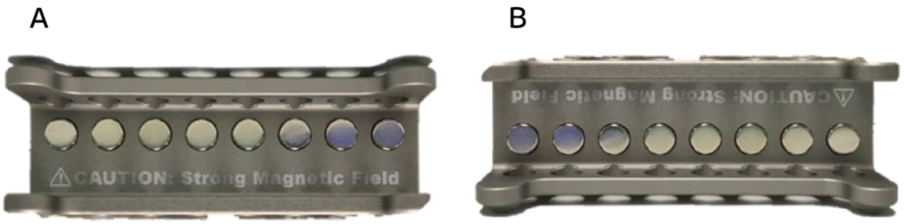


Figure 3: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- 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 4: Clear supernatant with compact bead pellets.

## Sublibrary Loading

- This Evercode WT kit generates 8 sublibraries with distinct Illumina indexing barcodes that can be processed and sequenced independently or concurrently after Section 1 of the protocol.

- The number of cells per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix A.

- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.

- Sublibraries can be loaded with different numbers of cells, and the maximum number of cells that can be analyzed is the sum of cells across all sublibraries.

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

## Indexing Primers

- UDI Plate-WT and UDI Plate-EC are 96-well plates containing 48 unique dual indexing (UDI) primers. Each well is a single-use reaction sufficient for one indexing PCR reaction for a single sublibrary. UDI Plate-WT should be used for Section 3 and UDI Plate-EC should be used for Section 4. Each UDI plate can be used for multiple CRISPR Detect kits.

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

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

- 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 C.

## Part List

Safety Data Sheets for these reagents can be provided upon request. This manual requires the use of Evercode WT v4 barcoding kit. Part list for the appropriate WT kit are in manual Evercode WT v4, UMWT4300. Note that the Evercode WT reagent "PCR Additive" will not be used in the CRISPR workflow.

**CRISPR Detect** Store at -20°C, PN CRS1010

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
| hU6 Primer 1 | Human U6 Primer 1 | CS101 | 1.5 mL tube | 1 |
| hU6 Primer 2 | Human U6 Primer 2 | CS102 | 1.5 mL tube | 1 |
| Univ Primer | Universal Primer | CS103 | 1.5 mL tube | 1 |
| CRISPR Amp | CRISPR Amplification Mix | CS104 | 1.5 mL tube | 1 |

## User Supplied Material and Equipment

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 |
|---|---|---|---|
| Parse Biosciences Magnetic Rack | Parse Biosciences | SB1004 | We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection. |
| 6-Tube Magnetic Separation Rack | New England Biolabs® | S1506S | Or an equivalent magnetic rack for 1.5 mL tubes. |
| 15 mL Conical Tube Magnet (choose one) | Permagen | MSR6X15 | 6 X 15 mL Centrifuge Magnetic Separation Rack. |
| | Millipore | LSKMAGS15 | PureProteome Magnetic Stand. |
| | STEMCELL Technologies | 18103 | EasyEights™ EasySep™ Magnet. |
| Centrifuge with Swinging Bucket Rotors | Various Suppliers | Varies | Compatible with 96 well plates and capable of reaching 4°C. |
| Microcentrifuge | Various Suppliers | Varies | Compatible with 1.5 mL and 0.2 mL tubes. |
| Water bath | Various Suppliers | Varies | Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C. |
| Hemocytometer | Sigma-Aldrich® | Z359629 | Or other cell counting device. We recommend validating alternatives relative to a hemocytometer. |
| Fluorescence Cell Counter | Various Suppliers | Varies | |
| PCR tube rack | Various Suppliers | Varies | Capable of holding semi-skirted 96 well PCR plates and a tight fitting lid. |
| Plate Seal Applicator | Various Suppliers | Varies | Capable of adhering plate sealing films to 96 well plates. |

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| Single Channel Pipettes: P20, P200, P1000<br>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 | |
| Microplate Foam Insert | Scientific Industries | 504-0235-00 | |
| Qubit™ Flex Fluorometer | Thermo Fisher Scientific® | Q33327 | |
| 2100 Bioanalyzer | Agilent® | G2939BA | Choose one. |
| 4200 TapeStation System | Agilent | G2991BA | |
| Serological Pipette Controller | Various Suppliers | Varies | Optional. |

### Consumables

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| Evercode WT v4 | Parse Biosciences | ECWT4300 | |
| UDI Plate - WT | Parse Biosciences | UDI1001 | Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode Whole Transcriptome kits (48 sublibraries). UDI Plate - WT can be purchased separately or bundled with Evercode Whole Transcriptome kits. |
| UDI Plate - EC | Parse Biosciences | UDI1002 | Each 96 well plate contains 48 unique single-use reactions sufficient for multiple CRISPR sequencing libraries (48 sublibraries). |

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| Reagent basins | Various Suppliers | Varies | Sterile, nuclease-free, 10 mL or 25 mL polypropylene reagent basins. |
| Falcon® High Clarity PP Centrifuge Tubes, 15 mL | Corning® | 352097 | Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial cell loss. |
| SWiSH™ Mini Cell Strainer | Stellar Scientific® | TC70-SWM-20<br>TC70-SWM-40<br>TC70-SWM-70<br>TC70-SWM-100 | Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm, 70 µm, 100 µm).<br>We do not recommend FlowMi Cell Strainers (SP Bel-Art). |
| pluriStrainer® Mini | pluriSelect® | 43-10020-40<br>43-10040-40<br>43-10070-40<br>43-10100-40 | |
| Falcon®Cell Strainer | Corning® | 431750<br>431751<br>431752 | |
| EASYstrainer™, small | Greiner Bio-One™ | 542120<br>542140<br>542170<br>542100 | |
| DNA LoBind® Tubes, 1.5 mL, Snap Cap | Eppendorf® | 022431021 | Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes. |
| DNA LoBind® Tubes, 2 mL, Snap Cap | Eppendorf | 022431048 | Or equivalent DNA low-binding, nuclease-free 2 mL tubes. |
| DNA LoBind® Tubes, 5 mL, Snap Cap | Eppendorf | 030108310 | Or equivalent DNA low-binding, low retention, nuclease-free 5 mL tubes. |
| TempAssure® PCR 8-Tube Strips, 0.2 mL | USA Scientific® | 1402-4700 | Or equivalent nuclease-free 0.2 mL PCR tubes. |
| SealPlate® | Excel Scientific | 100-SEAL-PLT | Or equivalent PCR plate seals. |
| Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL | Rainin® | 17014961<br>17014963<br>17014967 | Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips. |
| 5 mL serological pipettes | Various Suppliers | Varies | Optional. Use DNase/RNase-free pipettes |

### Reagents

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| SPRIselect Reagent | Beckman Coulter® | B23317 (5mL)<br>B23318 (60mL) | Choose one. We do not recommend substituting other magnetic beads. |
| AMPure® XP Reagent | Beckman Coulter | A63880 (5 mL)<br>A63881 (60mL) | |
| KAPA® Pure Beads | Roche® | KK8000 (5 mL)<br>KK8001 (30mL) | |
| MAGFLO™ NGS Size Selection | INTEGRA | 7000 (1 mL)<br>7002 (5 mL) | |
| RNaseZap™ RNase Decontamination Solution | Thermo Fisher Scientific | AM9780 | Or equivalent RNase decontamination solution. |
| Ethyl alcohol, Pure | Sigma-Aldrich | 459844 | Or equivalent ethanol. |
| TE Buffer pH 8.0 | Various Suppliers | Varies | Optional. Choose one that is RNase-free. |
| Nuclease-Free Water | Sigma-Aldrich | W4502 | Or equivalent nuclease-free water. |
| AO/PI | Various Suppliers | Varies | Or alternative viability dyes. |
| Qubit dsDNA HS (High Sensitivity) Assay Kit | Thermo Fisher Scientific | Q33230 (100 assays)<br>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)<br>5067-5593 (sample buffer and ladder) | |
| High Sensitivity D1000 ScreenTape and Reagents | Agilent | 5067-5584 (screen tape)<br>5067-5585 (sample buffer and ladder) | |

# Section 1: In Situ Cell Barcoding

## 1.1. Set up and Sample Counting

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

**To set up for barcoding:**

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

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

7. If cells show acceptable integrity and minimal debris, start setting up for the barcoding and cool a centrifuge with swinging bucket rotors to 4°C.
8. Gather the following items and handle as indicated below.

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ▭ Round 1 Plate | -20°C Reagents | 1 | Place directly on ice. |
| 🟢 Sample Dilution Buffer | -20°C Reagents | 1 | *Used for standard fixed samples only*.<br>Thaw at room temperature then store on ice. Mix by inverting 3x. |
| 🔵 Bead Prep Buffer* | -20°C Reagents | 1 | |
| 🔴 Round 1 Stop Mix | -20°C Reagents | 1 | |
| ○ Ligation Buffer | -20°C Reagents | 1 | |

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ● Ligation Enzyme | -20°C Reagents | 1 | Place directly on ice. Briefly centrifuge before use. |
| ○ Barcoding Buffer | -20°C Reagents | 1 | Thaw in 37°C water bath, then store on ice. |
| ● Sample Binding Beads* | 4°C Reagents | 1 | *Used for standard fixed samples only*. Keep at room temperature. Ensure beads are not settled before use. |

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

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

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

13. Proceed immediately to Section 1.2.

## 1.2. Barcoding Round 1

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

**To add round 1 barcodes:**

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

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

**Note:** PCR plate seals may be difficult to remove. Carefully peel the seal while applying downward pressure to keep the plate from moving to minimize cross-contamination.

3. With the Round 1 Plate on ice, add **14 µL** of each diluted sample to the appropriate wells of Round 1 Plate as defined in the Sample Loading Table. Mix immediately after dispensing each sample by pipetting 3x.

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

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

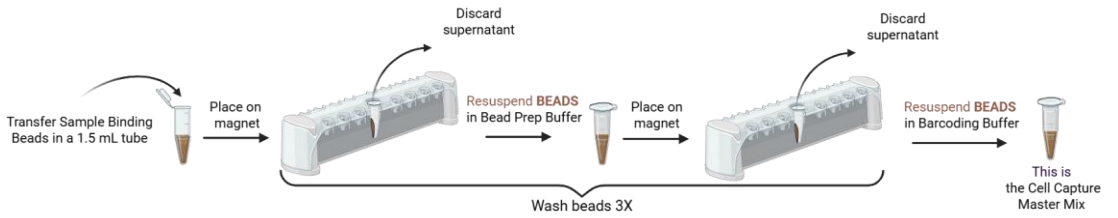
4. While secured in a PCR tube rack on a flat surface, add a new plate seal.

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

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

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

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



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

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

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

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

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

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

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

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

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

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

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

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

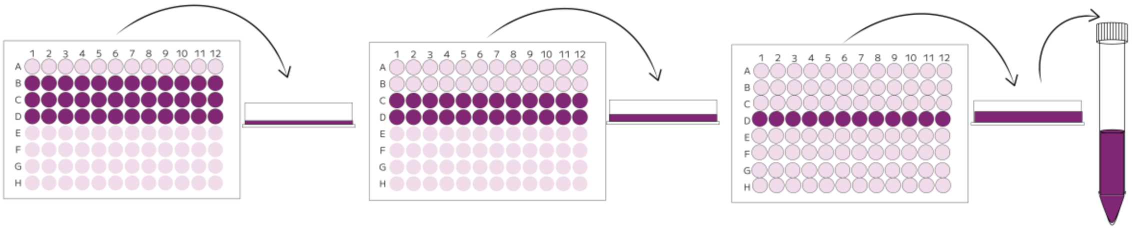
17. Mix the Ligation Master Mix by inverting the tube 10x. Store on ice.

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

18. Remove the Round 1 Plate from the thermocycler, place it in a PCR tube rack on the benchtop. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 1 Plate on ice.

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

20. With a 12–channel multi-pipette transfer **10 µL** of ● Round 1 Stop Mix into Row A of the Round 1 Plate, still keeping the plate on ice.
    a. Repeat step 20, **3 more times** using the same pipette tips for Rows B-D to dispense the Round 1 Stop Mix into all wells of the Round 1 Plate.
    b. With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.
    c. Transfer **50 µL** from **row A** into a clean basin (bubbles will be generated, this will not impact experimental results).
    d. Repeat b-c for **rows B-D**.
    e. Once cells from all wells are in the basin, using a P1000 transfer the mix into a 15 mL conical tube. Keep on ice.



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

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

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

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

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

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

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

27. Remove or loosen the cap, then place the tube on the 15 mL conical tube magnet to allow magnetic beads to bind to the magnet. If the cap is adjusted afterward, the rotational motion may cause the pellet to shift. Keep magnet on ice the entire time,

covering the tube in ice up to the liquid level to keep the tube cold. Allow beads to bind to the magnet for **10 minutes**.

28. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids. Holding the tube in the magnet will ensure the tube is kept from moving.

29. With the tube still on the magnet, add **5 mL** of ○ **Barcoding Buffer**. Do not resuspend. Incubate on ice for **1 minute** before removing and discarding the supernatant with a P1000 or serological pipette.

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

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

## 1.3. Barcoding Round 2

The pooled beads-bound cells are loaded into the Round 2 Plate. An in situ ligation reaction adds a well-specific barcode to the 5′ end of the cDNA. The ligation reaction is quenched with Round 2 Stop Mix, and the cells are pooled and strained.

**To add Round 2 barcodes:**

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ▭ Round 2 Plate v4 | -20°C Reagents | 1 | Place directly on ice. |
| ● Round 2 Stop Mix | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by vortexing. |

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

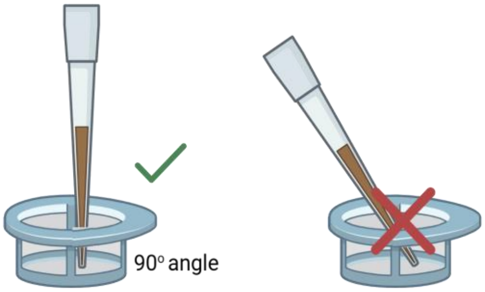
| THAW ROUND 2 PLATE | | |
|---|---|---|
| Run Time | 3 minutes | |
| Lid Temperature | 70°C | |
| Sample Volume | 10 µL | |
| Step | Time | Temperature |
| 1 | 3 min | 25°C |
| 2 | Hold | 4°C |

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

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

5. Pipette all of the sample in Ligation Master Mix (**4.2 mL)** through a cell strainer into a new basin with a P1000.

**CRITICAL!** To ensure that all of the liquid passes through the strainer, keep the tip of the pipette straight up and against the mesh to create a tight seal and press the pipette plunger down steadily. All the liquid should pass through the strainer in ~1 second.



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

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

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

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

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

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

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

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

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

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

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

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

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

    b. Transfer the entire content (**50 µL)** from row A to the basin containing ● Round 2 Stop Mix and mix 2x.

    c. Repeat steps a-b for **rows B-H** to mix the sample then transfer to the basin. The same pipette tips may be used while pooling.

12. Transfer the Round 2 pool into a 15 mL conical tube and apply to the magnet. Loosen or remove the cap to avoid a rotational motion may cause the pellet to shift. Keep the magnet on ice. Allow beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear.

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

14. Keep the 15 mL conical tube on the magnet on ice and with a P1000 set to 1000 µL or a 5 mL serological pipette, add **5 mL** of the ○ Barcoding Buffer to rinse the pellet. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound.

15. Remove and discard all the supernatant with a P1000 set to 1000 µL or a 5 mL serological pipette.

16. Remove tube from magnet and resuspend the beads with **4.2 mL** of the Ligation Master Mix. Store the sample on ice and proceed to section 1.4.

## 1.4. Barcoding Round 3

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

**To add round 3 barcodes:**

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

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

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

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

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

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

5. Pipette all of the sample in Ligation Master Mix (**4.2 mL**) through a cell strainer into a new basin with a P1000.

**CRITICAL!** To ensure that all of the liquid passes through the strainer, keep the tip of the pipette straight up and against the mesh to create a tight seal and press the pipette plunger down steadily. All the liquid should pass through the strainer in ~1 second.

6. With the Round 3 Plate on ice and the basin on the bench, transfer the cells in Ligation Master Mix to each well in the Round 3 Plate as follows:
   a. Mix the sample in the basin by pipetting 2x with a multichannel P200 set to 40 µL.
   b. Transfer **40 µL** of the mix to row **A** of the Round 3 Plate and mix by pipetting 2x.
   c. Changing tips for each row, repeat steps a-b to mix the sample in the basin then transfer to rows **B-H**.

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

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

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

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

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

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

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

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

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

    b. Transfer **50 µL** from row **A** to the basin and mix 2x.

    c. Repeat steps a-b for rows **B-H** to mix the sample then transfer to the basin.

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

13. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind to the magnet for **5 minutes**, or until supernatant is clear.

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

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

16. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind for **5 minutes**, until supernatant is clear.

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

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

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

19. Proceed to Lysis and Sublibrary Generation Step 1.5.

## 1.5. Lysis and Sublibrary Generation

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

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

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

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

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

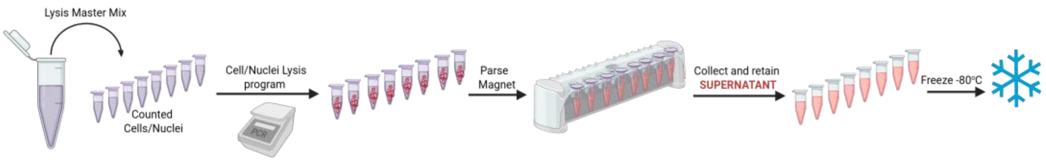
3. Ensure the cells are in suspension by pipetting 5x with a P200 set to 200 µL prior to each transfer. Add the appropriate volume of sample to 8 different 0.2 mL PCR tubes.
4. Keeping the samples on ice, add the appropriate volume of ●Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of **25 µL**.
5. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P200 set to 200 µL. Store at room temperature.

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

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

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

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



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

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

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

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

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

**CRITICAL!** Do NOT discard the supernatant as it contains the lysed cells.

11. Freeze the lysate(s) at -80°C or proceed to Section 2.

Safe stopping point: Sublibrary lysates can be stored at -80°C for up to 6 months.

# Section 2: cDNA Capture and Amplification

## 2.1. cDNA Capture

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

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

**To capture the cDNA:**

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

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

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

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

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

6. Remove and discard the supernatant.

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

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

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

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

9. Remove and discard the supernatant.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

25. Proceed immediately to Section 2.2.

## 2.2. cDNA Template Switch

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

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

**To perform template switch:**

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

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

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

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

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

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

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

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

**CRITICAL!** Do not discard the ○ 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 ○ **Wash Buffer B**.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## 2.3. cDNA Amplification

The captured cDNA is washed and amplified with Template Switch Primer and Illumina Truseq R2 specific primers. The sgRNA containing polyadenylated transcripts are also enriched with Human U6 Primer 1. Note that the cDNA Amplification Master Mix does not use the PCR Additive reagent included in the Evercode WT v4 kits.

**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 | |
| ● Human U6 Primer 1 | CRISPR Detect (-20°C) | 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 for later use.

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

| cDNA AMPLIFICATION MASTER MIX | | |
|---|---|---|
| Number of Sublibraries | 1 | 8 |
| ● cDNA Amp Mix | 60.5 µL | 484 µL |
| ● cDNA Amp Primers | 60.5 µL | 484 µL |
| ● Human U6 Primer 1 | 2.4 µL | 19.2 µL |
| Total | 123.4 µL | 987.2 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.

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

6. While still on the magnetic rack, add **125 µL** of ○Wash Buffer B to each tube.

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

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 cDNA 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 | High RNA content such as cell lines | Low RNA content such as PBMCs |
| 200-1,000 | 11 | 13 |
| 1,000-2,000 | 9 | 11 |
| 2,000-5,000 | 7 | 9 |
| 5,000-12,500 | 6 | 8 |

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

| cDNA AMPLIFICATION | | | |
|---|---|---|---|
| Run Time | | 60-80 minutes | |
| Lid Temperature | | 105°C | |
| Sample Volume | | 100 µL | |
| Step | Time | Temperature | Cycles |
| 1 | 3 min | 95°C | 1 |
| 2 | 20 sec | 98°C | 5 |
| 3* | 45 sec | 65°C* | |
| 4 | 3 min | 72°C | |
| 5 | 20 sec | 98°C | Variable, see above |
| 6* | 20 sec | 67°C* | |
| 7 | 3 min | 72°C | |
| 8 | 5 min | 72°C | 1 |
| 9 | Hold | 4°C | 1 |

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

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

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

## 2.4. Post-Amplification Purification

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

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

**To purify the cDNA:**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## 2.5. cDNA Quantification

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

**To quantify the cDNA:**

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

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

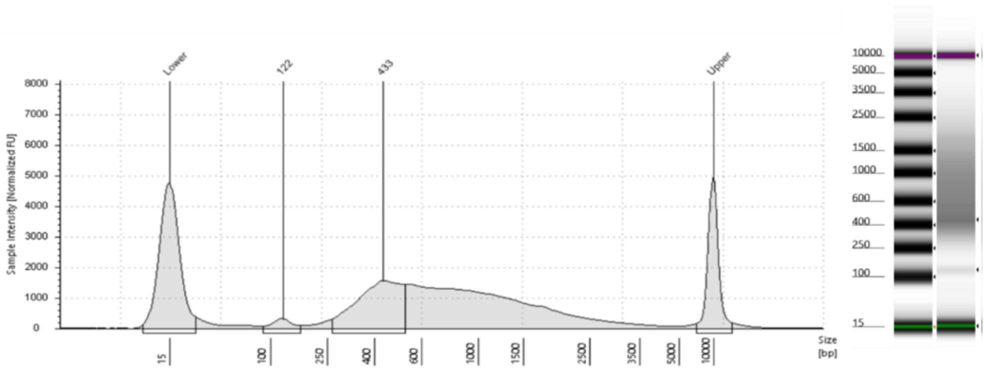


Figure 5: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a Tapestation.

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

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

# Section 3: WT Sequencing Library Preparation

## 3.1. Fragmentation and End Prep

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

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

**To prepare for fragmentation and end prep:**

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

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

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

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

**Note:** A recommended input of 5 ng of cDNA is needed for CRISPR 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 be adjusted based on cDNA concentration.

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 Sublibraries | 1 | 8 |
| 🟠 Fragm/End Prep Buffer | 6 µL | 48 µL |
| 🔴 Fragm/End Prep Enzymes | 12 µL | 96 µL |
| Total | 18 µL | 144 µL |

12. Add **15 µL** of Fragmentation and End Prep master mix to each tube of diluted cDNA. Mix by pipetting 10x with a P200 multichannel pipette set to 40 µL. Briefly centrifuge.
13. Place the tube(s) into the cooled thermocycler. Skip the 4°C hold in Step 1 so the thermocycler proceeds to Step 2 of the Fragmentation and end prep program.

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

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

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

The fragmented and end prepped DNA are size selected with a 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.
2. Gather room temperature SPRI beads.

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

3. Vortex the SPRI beads until fully mixed. Add **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.

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

**To ligate adapters:**

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

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

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

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

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

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

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

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

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

## 3.4. Post-Ligation Purification

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

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

**To purify the ligated DNA:**

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

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

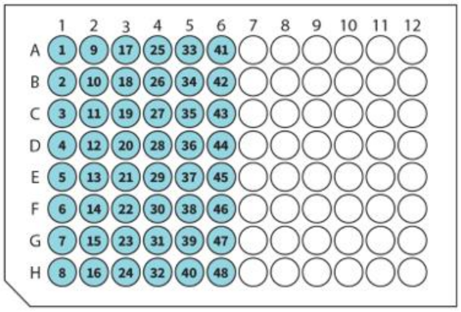
**To add round 4 barcodes:**

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.

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



| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | 9 | 17 | 25 | 33 | 41 | | | | | | |
| B | 2 | 10 | 18 | 26 | 34 | 42 | | | | | | |
| C | 3 | 11 | 19 | 27 | 35 | 43 | | | | | | |
| D | 4 | 12 | 20 | 28 | 36 | 44 | | | | | | |
| E | 5 | 13 | 21 | 29 | 37 | 45 | | | | | | |
| F | 6 | 14 | 22 | 30 | 38 | 46 | | | | | | |
| G | 7 | 15 | 23 | 31 | 39 | 47 | | | | | | |
| H | 8 | 16 | 24 | 32 | 40 | 48 | | | | | | |

4. With a multichannel P20, pierce the seal of the chosen wells of the UDI Plate - WT.

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

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

6. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C. Do not reuse wells.
7. Add **25 µL** of ● Library Amp Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 25 µL. Briefly centrifuge.
8. Determine the number of PCR cycles required for the Indexing PCR based on the amount of cDNA added to the fragmentation and end prep reaction as recorded in Section 2.5.

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

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

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

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

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

## 3.6. Post-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.

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.

STOP 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 D1000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

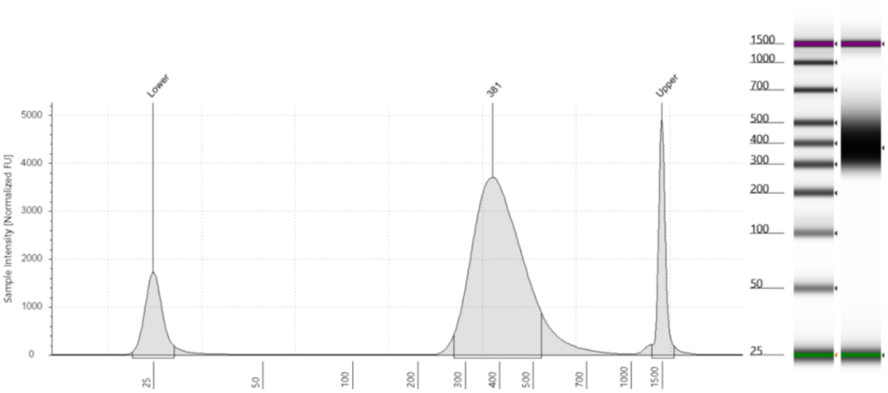


Figure 6: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

**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: CRISPR Sequencing Library Preparation

## 4.1 CRISPR PCR

A PCR reaction further enriches sgRNA containing transcripts.

**To amplify the sgRNA transcripts:**

1. Prepare 1.2 mL 85% ethanol per sublibrary with nuclease-free water.
2. Equilibrate approximately 90 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 the end of Section 2.
6. Gather the following items and handle as indicated below.

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| 🔴 Human U6 Primer 2 | CRISPR Detect (-20°C) | 1 | Thaw at room temperature then place on ice. Mix by inverting 3x. |
| 🟢 Universal Primer | CRISPR Detect (-20°C) | 1 | |
| 🔵 CRISPR Amplification Mix | CRISPR Detect (-20°C) | 1 | |
| UDI Plate - EC | UDI Plate - EC (-20°C) | 1 sealed well per sublibrary | Thaw at room temperature then place on ice. |

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 21 µL using nuclease-free water. Store any remaining cDNA at -20°C.

| DILUTED cDNA | |
|---|---|
| Sublibrary cDNA | 50 ng |
| Nuclease-free water | Variable |
| Total Volume | 21 µL |

**Note:** If the concentration of cDNA is higher than 25 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 50 ng of cDNA after Whole Transcriptome Library Preparation for a given sublibrary, use all remaining cDNA at this step. We recommend a minimum amount of 5 ng/µL. If the input is greater than 5 ng/µL, the quality of the library is unlikely to be affected. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on cDNA concentration (See Appendix D for more details).

9. Prepare the CRISPR PCR Reaction Solution in the order shown below. Mix by pipetting 10x and store on ice.

| CRISPR PCR REACTION SOLUTION | | |
|---|---|---|
| Number of Sublibraries | 1 | 8 |
| ● CRISPR Amplification Mix | 27.5 µL | 220 µL |
| ● Universal Primer | 2.2 µL | 17.6 µL |
| ● Human U6 Primer 2 | 2.2 µL | 17.6 µL |
| Total Volume | 31.9 µL | 255.2 µL |

10. Add **29 µL** of the CRISPR PCR Reaction Solution to each sublibrary. Mix sublibraries 10x with a P200 pipette set to 40 µL. Briefly centrifuge.

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

**Note:** For primer annealing, steps 3 and 6 below (*) have different temperature settings. Double check the settings inputted into the thermocycler before starting the amplification protocol.

| CRISPR PCR | | | |
|---|---|---|---|
| Run Time | | ~50 minutes | |
| Lid Temperature | | 105°C | |
| Sample Volume | | 50 µL | |
| Step | Time | Temperature | Cycle |
| 1 | 3 min | 95°C | 1 |
| 2 | 20 sec | 98°C | 5 |
| 3* | 20 sec | 65°C* | |
| 4 | 1 min | 72°C | |
| 5 | 20 sec | 98°C | 13 |
| 6* | 20 sec | 72°C* | |
| 7 | 1 min | 72°C | |
| 8 | 5 min | 72°C | 1 |
| 9 | Hold | 4°C | 1 |

**Note:** If you added less than 50 ng of input for any sublibrary, see Appendix D for PCR cycling recommendations.

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

## 4.2. Post CRISPR PCR Size Selection

sgRNA enriched cDNA sublibraries are size selected with a double sided SPRI cleanup.

**To size select the sublibraries:**

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~45 µL per sublibrary).

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

3. Vortex the SPRI beads until fully mixed. Add **30 µL** of SPRI beads to each tube of CRISPR enriched 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 **75 µL** of the supernatant containing the CRISPR enriched cDNA into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **10 µL** of SPRI beads to each tube.
9. Vortex the tube(s) for **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 **20 µL** of nuclease-free water.

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

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

21. While still on the magnetic rack, transfer **20 µL** of the supernatant into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).

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

STOP Safe stopping point: The size-selected CRISPR enriched cDNA can be stored at 4°C for up to 2 days or -20°C for up to 3 months.

## 4.3. CRISPR Index PCR

A final PCR adds i5/i7 UDIs that act as a fourth cell barcode.

**To prepare the final CRISPR sequencing libraries:**

1. Obtain amplified and purified CRISPR cDNA from Section 4.2.
2. Vortex the tube(s) for **2-3 seconds**. Briefly centrifuge.
3. Prepare diluted CRISPR cDNA in new 0.2 mL tube(s) as follows and store on ice. Bring the total volume up to **21 µL** using nuclease-free water. Store any remaining product from Section 4.2 at -20°C.

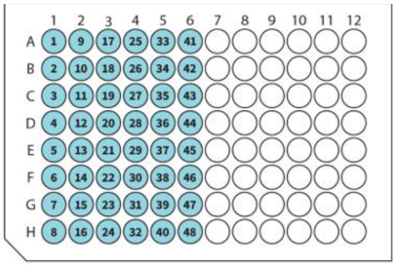
| DILUTED CRISPR cDNA | |
|---|---|
| CRISPR cDNA | 10 ng |
| Nuclease-free water | Variable |
| Total Volume | 21 µL |

**Note:** If you have less than 10 ng of input for a given sublibrary, add the entire amount of cDNA at this step. This is unlikely to affect the library quality. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on cDNA concentration (See Appendix D for more details).

4. 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.

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



| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | 9 | 17 | 25 | 33 | 41 | | | | | | |
| B | 2 | 10 | 18 | 26 | 34 | 42 | | | | | | |
| C | 3 | 11 | 19 | 27 | 35 | 43 | | | | | | |
| D | 4 | 12 | 20 | 28 | 36 | 44 | | | | | | |
| E | 5 | 13 | 21 | 29 | 37 | 45 | | | | | | |
| F | 6 | 14 | 22 | 30 | 38 | 46 | | | | | | |
| G | 7 | 15 | 23 | 31 | 39 | 47 | | | | | | |
| H | 8 | 16 | 24 | 32 | 40 | 48 | | | | | | |

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

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

9. If any unused wells remain in the UDI Plate - EC, store the plate at -20°C.
10. Add **25 µL** of CRISPR Amplification Master Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 40 µL. Briefly centrifuge.
11. Place the tube(s) into a thermocycler and run the following program.

| SUBLIBRARY INDEX AMPLIFICATION | | | |
|---|---|---|---|
| Run Time | | ~30 min | |
| Lid Temperature | | 105°C | |
| Sample Volume | | 50 µL | |
| Step | Time | Temperature | Cycle |
| 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 |

**Note:** If you added less than 10 ng of input for any sublibrary, see Appendix D for PCR cycling recommendations.

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

## 4.4. Post CRISPR Index PCR Size Selection

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

**To size select the sublibraries:**

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~45 µL per sublibrary).

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

3. Vortex the SPRI beads until fully mixed. Add **30 µL** of SPRI beads to each tube of CRISPR 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 **75 µL** of the supernatant containing the CRISPR sequencing library into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **10 µL** of SPRI beads to each tube.
9. Vortex the tube(s) for **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 **20 µL** of nuclease-free water.
19. Incubate for **5 minutes** at room temperature.
20. Place the tube(s) on the low position of the magnetic rack. Incubate until the solution clears (~2 minutes).
21. While still on the magnetic rack, transfer **20 µL** of the supernatant into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s). Product is ready for quantification.

## 4.5. CRISPR Sequencing Library Quantification

The concentration and size distribution of CRISPR 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 CRISPR sequencing libraries:**

1. Measure the concentration of each purified CRISPR 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 CRISPR 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. See below for an expected Tapestation trace when amplifying CRISPR sequencing libraries. There should be an expected peak around 530 bp.

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

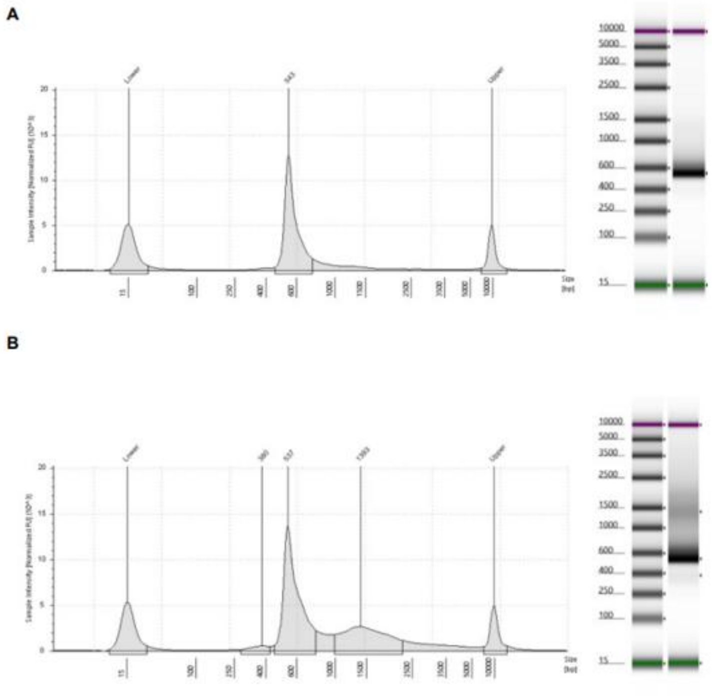


Figure 7: Expected Size Distribution of CRISPR Libraries before Illumina Sequencing. (A) Example trace of a CRISPR sequencing library with a minimal higher molecular weight product.

(B) Example trace of a CRISPR sequencing library with more prominent higher molecular weight product.

**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:** There might be high molecular weight product above 530 bp. This is due to linear amplification of the cDNA template that gets carried through the index PCR reaction. This is not a concern, as sublibraries with higher molecular weight still produce high quality data.

**Note:** Do not consider the high molecular weight products when estimating amplicon size to ensure that CRISPR libraries do not under-cluster on the sequencing instrument.

# Appendices

## Appendix A: Sublibrary Generation Table

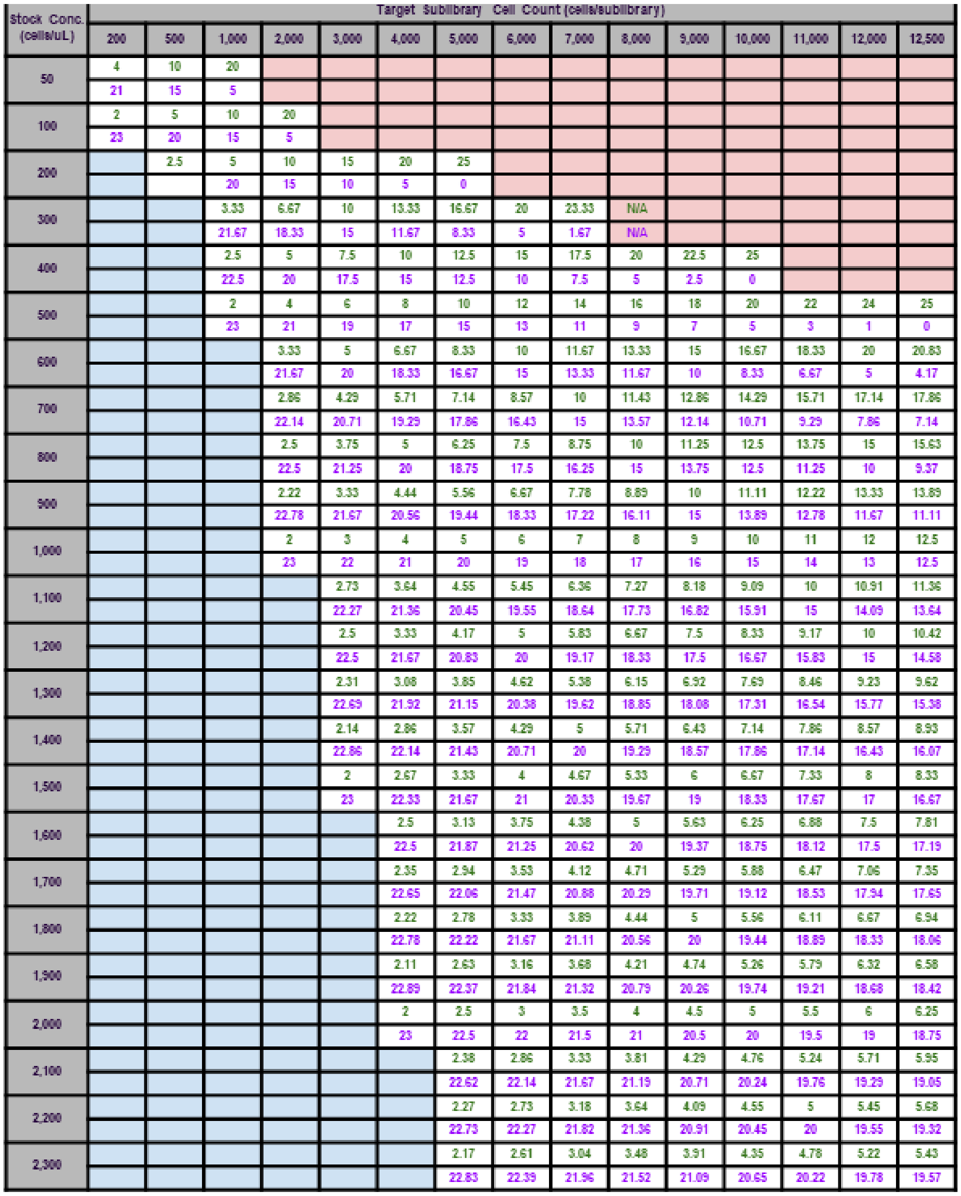
The Sublibrary Generation Table provides guidance for sample dilution in Section 1.5 to achieve a target sublibrary concentration.

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

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

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

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



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

# Appendix B: Sequencing Information

We recommend a minimum sequencing depth of 10,000 reads per cell for whole transcriptome libraries. However, ideal sequencing depth is dependent on the sample type and experimental goals. For example, if trying to capture rare cell types in a heterogenous population, deeper sequencing is required. Alternatively, analyzing a homogenous population, more shallow sequencing may be appropriate.

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

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

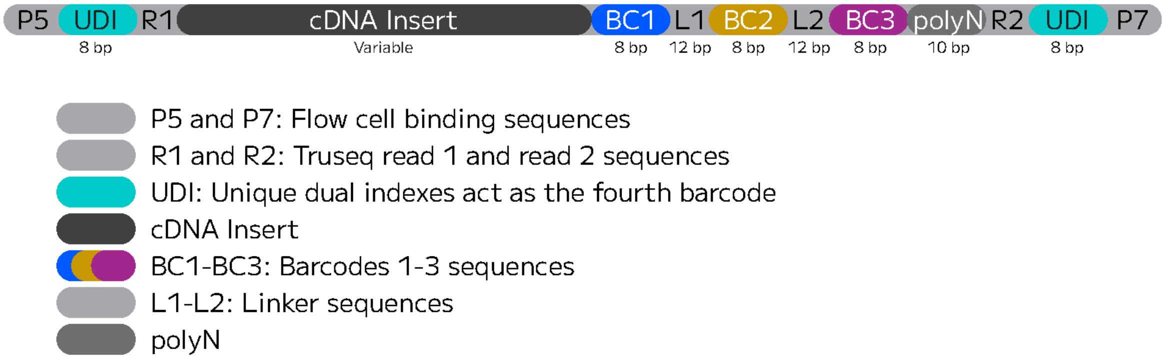


Figure 8: Whole transcriptome sequencing library structure.

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

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

The fourth barcode that tags each sublibrary act as a standard Illumina i7 and i5 indexes.

CRISPR sequencing libraries can be sequenced together or separately from Whole Transcriptome libraries. We recommend sequencing CRISPR libraries at 2,000-5,000 reads per cell, so the ratio between the libraries may need to be adjusted if they are sequenced together. For CRISPR libraries sequenced alone, we suggest using a minimum of 10% PhiX due to their limited complexity.

| READ | CYCLE |
|---|---|
| Read 1 | 64 |
| i7 Index (Index 1) | 8 |
| i5 Index (Index 2) | 8 |
| Read 2 | 58 |

Please refer to table in Appendices B1 and B2 to demultiplex the whole transcriptome libraries and the CRISPR libraries that have been sequenced together in the same run.

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

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

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

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---|---|---|---|---|
| UDI_Plate_WT_22 | F3 | CTATACTC | TGTTCGAG | CTCGAACA |
| UDI_Plate_WT_23 | G3 | CACTCACA | AAGGCTGA | TCAGCCTT |
| UDI_Plate_WT_24 | H3 | CTCGAACA | CTCGAGTG | CACTCGAG |
| UDI_Plate_WT_25 | A4 | CTCTATCG | ATCGGTGG | CCACCGAT |
| UDI_Plate_WT_26 | B4 | TCCTCATG | AGGTCTTG | CAAGACCT |
| UDI_Plate_WT_27 | C4 | AACAACCG | AGGAAGCG | CGCTTCCT |
| UDI_Plate_WT_28 | D4 | GCCAATGT | ACATGTGT | ACACATGT |
| UDI_Plate_WT_29 | E4 | TGGTTGTT | ATACAGTT | AACTGTAT |
| UDI_Plate_WT_30 | F4 | TCTGCTGT | ATCGCCTT | AAGGCGAT |
| UDI_Plate_WT_31 | G4 | TTGGAGGT | TTCGACGC | GCGTCGAA |
| UDI_Plate_WT_32 | H4 | TCGAGCGT | TGTCGTTC | GAACGACA |
| UDI_Plate_WT_33 | A5 | TGCGATCT | TCCATAGC | GCTATGGA |
| UDI_Plate_WT_34 | B5 | TTCCTGCT | TAAGTGTC | GACACTTA |
| UDI_Plate_WT_35 | C5 | TTCCATTG | CTGGCATA | TATGCCAG |
| UDI_Plate_WT_36 | D5 | TAACGCTG | CTGAGCCA | TGGCTCAG |
| UDI_Plate_WT_37 | E5 | TTGGTATG | CTCAATGA | TCATTGAG |
| UDI_Plate_WT_38 | F5 | TGAACTGG | CGCATACA | TGTATGCG |
| UDI_Plate_WT_39 | G5 | TCCAGTCG | CCGAAGTA | TACTTCGG |
| UDI_Plate_WT_40 | H5 | TGTATGCG | CCAGTTCA | TGAACTGG |
| UDI_Plate_WT_41 | A6 | TGGCTCAG | CAGCGTTA | TAACGCTG |
| UDI_Plate_WT_42 | B6 | TATGCCAG | CAATGGAA | TTCCATTG |
| UDI_Plate_WT_43 | C6 | GGTTGGAC | ATCCTGTA | TACAGGAT |
| UDI_Plate_WT_44 | D6 | GACACTTA | AGCAGGAA | TTCCTGCT |

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---|---|---|---|---|
| UDI_Plate_WT_45 | E6 | GAACGACA | ACGCTCGA | TCGAGCGT |
| UDI_Plate_WT_46 | F6 | AAGGCGAT | ACAGCAGA | TCTGCTGT |
| UDI_Plate_WT_47 | G6 | ATGCTTGA | ACAAGCTA | TAGCTTGT |
| UDI_Plate_WT_48 | H6 | AGTATCTG | CATCAAGT | ACTTGATG |

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

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

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---|---|---|---|---|
| UDI_Plate_EC_1 | A1 | AACAGATC | TGCCATGA | TCATGGCA |
| UDI_Plate_EC_2 | B1 | CCTGTCTT | CTAACGAT | ATCGTTAG |
| UDI_Plate_EC_3 | C1 | ATATCGAG | TTCCGGAA | TTCCGGAA |
| UDI_Plate_EC_4 | D1 | TAGTCCGC | TCTCCATA | TATGGAGA |
| UDI_Plate_EC_5 | E1 | TGCTGTTA | AGTGACGT | ACGTCACT |
| UDI_Plate_EC_6 | F1 | TAGCGAAT | CTCTGGCA | TGCCAGAG |
| UDI_Plate_EC_7 | G1 | AGGACCGT | AGAAGATG | CATCTTCT |
| UDI_Plate_EC_8 | H1 | TTATCAGG | TCTAACAG | CTGTTAGA |
| UDI_Plate_EC_9 | A2 | CCTCGGAA | TACGTAGA | TCTACGTA |
| UDI_Plate_EC_10 | B2 | TTGATCGA | TAAGCTCT | AGAGCTTA |
| UDI_Plate_EC_11 | C2 | TCAACCTC | TTGGTCGA | TCGACCAA |
| UDI_Plate_EC_12 | D2 | CGGAATAA | CGGTAATA | TATTACCG |
| UDI_Plate_EC_13 | E2 | TATGAGAC | CACAGTGG | CCACTGTG |
| UDI_Plate_EC_14 | F2 | CCTACCAT | AGACGCGA | TCGCGTCT |
| UDI_Plate_EC_15 | G2 | CATAGGCC | AGCAATGG | CCATTGCT |
| UDI_Plate_EC_16 | H2 | CACGATAA | CCTCTTGA | TCAAGAGG |
| UDI_Plate_EC_17 | A3 | TTCTGGCG | TTGTCTAG | CTAGACAA |
| 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 Complement 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 |
| UDI_Plate_EC_44 | D6 | AAGAGTGT | CCTTCTGG | CCAGAAGG |

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---|---|---|---|---|
| UDI_Plate_EC_45 | E6 | TGCTACCG | AATCGCTA | TAGCGATT |
| UDI_Plate_EC_46 | F6 | CCATCGTC | TGGACCAA | TTGGTCCA |
| UDI_Plate_EC_47 | G6 | CCACGAGA | TGTGCACT | AGTGCACA |
| UDI_Plate_EC_48 | H6 | ACGGTCAT | CTTATGGA | TCCATAAG |

# Appendix C: Thermocycling Programs

### Section 1: In situ Cell Barcoding

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

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

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

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

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

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

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

### Section 2: cDNA Capture and Amplification

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

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

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

| 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 CRISPR PCR

| CRISPR PCR | | | |
|---|---|---|---|
| Run Time | | ~50 minutes | |
| Lid Temperature | | 105°C | |
| Sample Volume | | 50 µL | |
| Step | Time | Temperature | Cycle |
| 1 | 3 min | 95°C | 1 |
| 2 | 20 sec | 98°C | 5 |
| 3* | 20 sec | 65°C* | |
| 4 | 1 min | 72°C | |
| 5 | 20 sec | 98°C | 13 |
| 6* | 20 sec | 72°C* | |
| 7 | 1 min | 72°C | |
| 8 | 5 min | 72°C | 1 |
| 9 | Hold | 4°C | 1 |

| SUBLIBRARY INDEX AMPLIFICATION | | | |
|---|---|---|---|
| Run Time | | ~30 min | |
| Lid Temperature | | 105°C | |
| Sample Volume | | 50 µL | |
| Step | Time | Temperature | Cycle |
| 1 | 3 min | 95°C | 1 |
| 2 | 20 s | 98°C | 9 |
| 3 | 20 s | 67°C | |
| 4 | 1 min | 72°C | |
| 5 | 5 min | 72°C | 1 |
| 6 | Hold | 4°C | 1 |

## Appendix D: CRISPR-Specific Troubleshooting

**Adjusting PCR Cycles:**

Section 4.1: CRISPR PCR requires 50 ng of input for optimal amplification. Section 4.3: CRISPR Sublibrary Index PCR requires 10 ng of input. However, PCR cycles can be adjusted to better suit different input quality. See tables below for recommended PCR cycles based on input.

| CRISPR PCR (Step 4.1.12) | |
|---|---|
| Input (ng) | Total number of cycles |
| 40-50 | 18 |
| 20-49 | 19 |
| 5-19 | 20 |

**Note:** For CRISPR PCR (Step 4.1.12), keep total cycling for steps 2-4 at 5 cycles. Adjust the cycling for steps 5-7 to achieve the total cycling in the table above.

| CRISPR SUBLIBRARY INDEX PCR (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 CRISPR PCR or CRISPR Sublibrary Index PCR may result in suboptimal sequencing results.

## Appendix E: Revision History

| Version | Description | Date |
|---|---|---|
| 1.0 | Initial Release: updated for Evercode WT v4 chemistry | March 2026 |

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