

User Manual

Version 1.1 – UM0024



CRISPR Detect for Evercode™ WT Mini v2

For use with

CRS1010

ECW02110

ECW02115

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U.S. Pat. No. 10,900,065

U.S. Pat. No. 11,168,355

U.S. Pat. No. 11,427,856

U.S. Pat. No. 11,634,751

U.S. Pat. No. 11,639,519

U.S. Pat. No. 11,680,283

Patents pending in the U.S. and other countries

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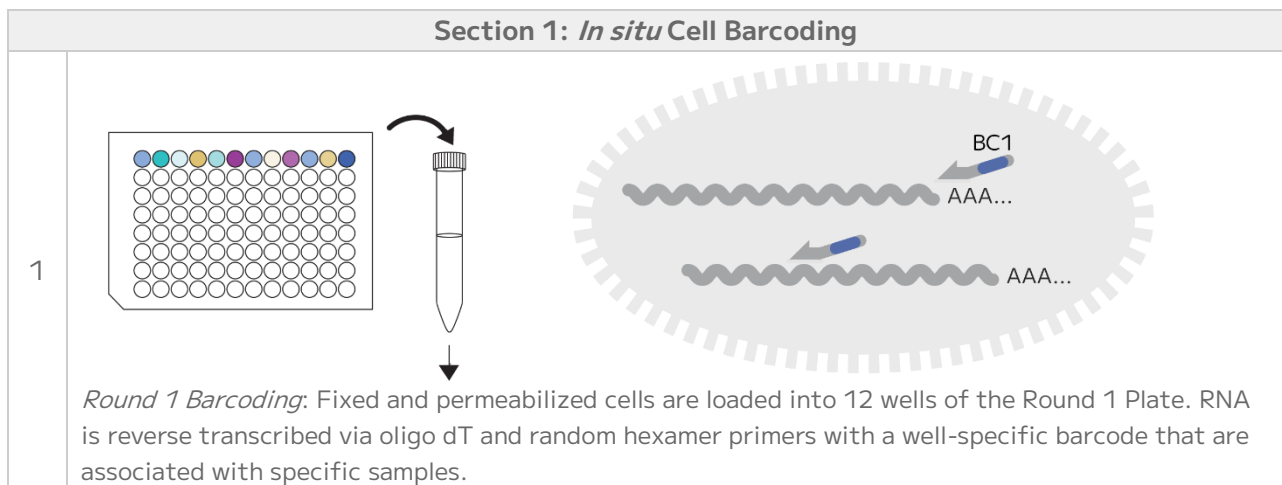
Overview

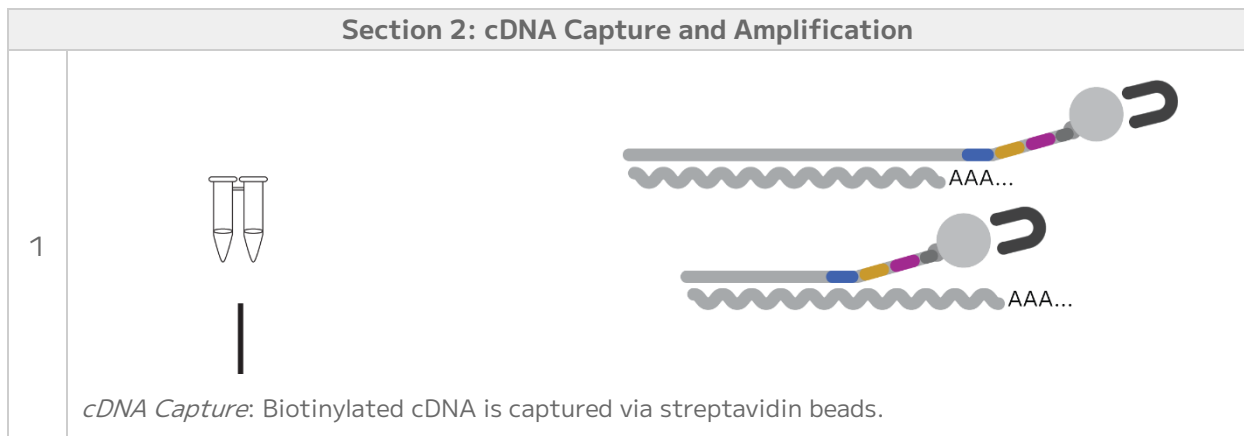
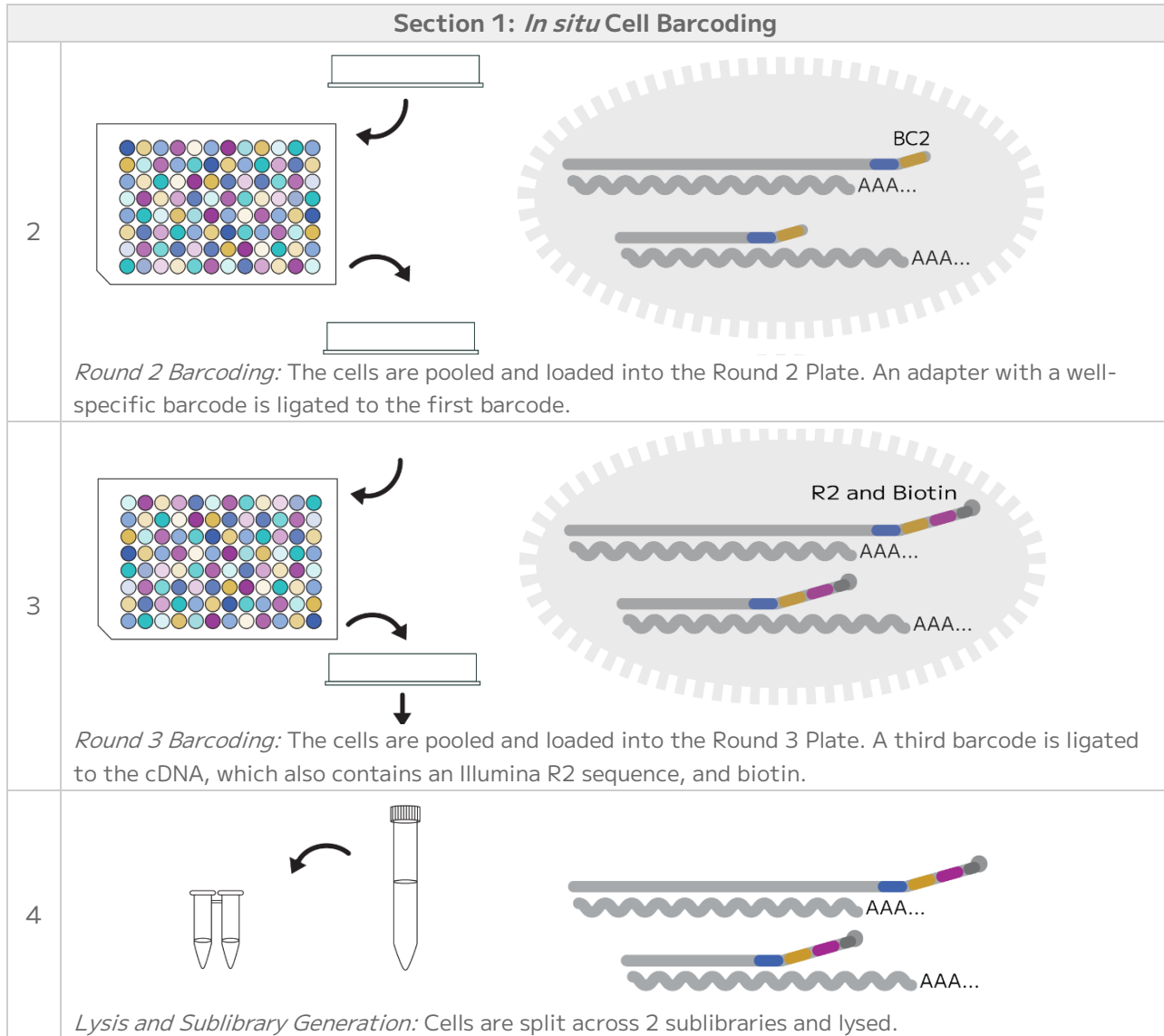
Workflow

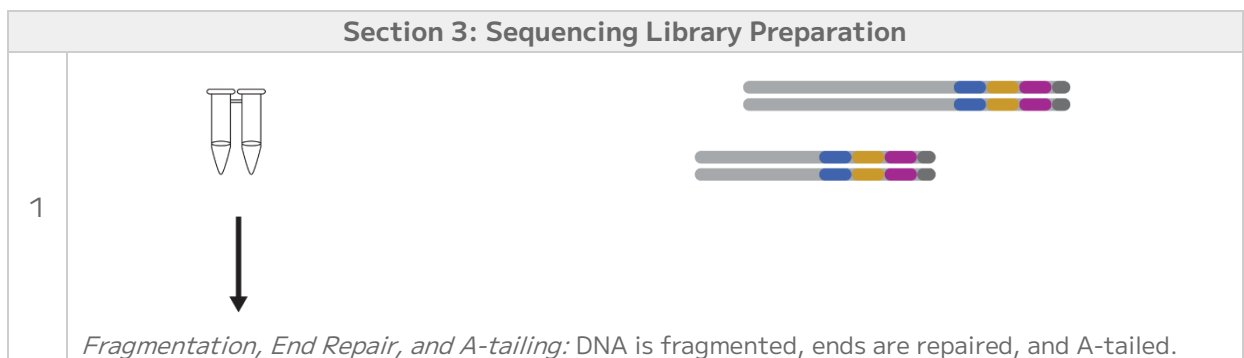
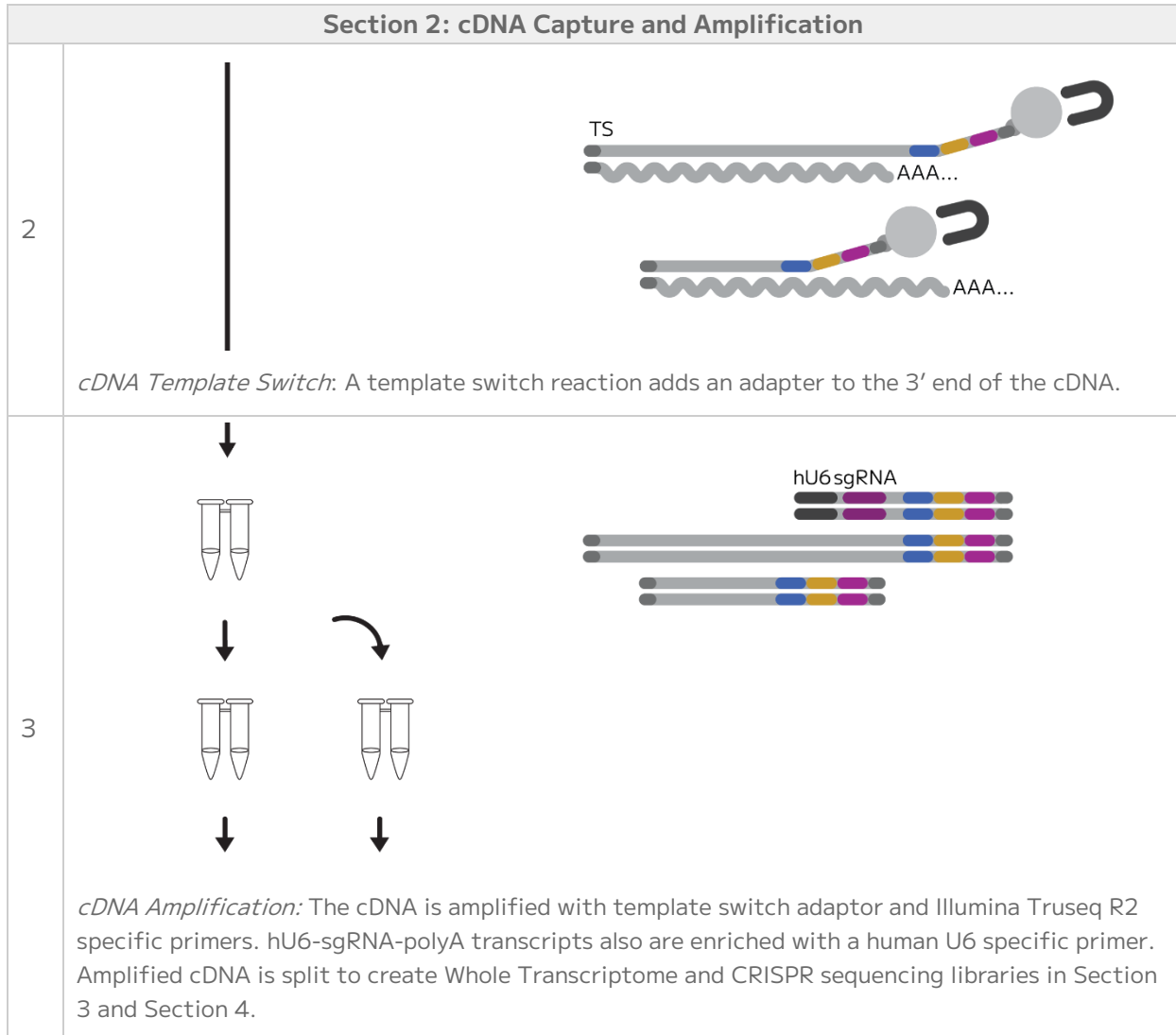
CRISPR Detect enables analysis of single guide RNAs (sgRNAs) in studies using CROP-seq or similar methods. Compatible methods generate a polyadenylated transcript containing the sgRNA sequence downstream of a human U6 promoter. When CRISPR Detect is combined with Evercode WT Mini v2, paired sgRNA detection and whole transcriptome expression can be analyzed in up to 10,000 cells across up to 12 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 barcode combination to a single cell and associates sgRNAs to the appropriate cell.





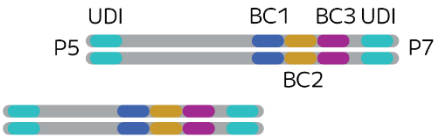
The tables below provide a high-level overview of the workflow.












Section 3: Sequencing Library Preparation

| | | |
|---|---|--|
| 2 |  |  |
| |  | |
| | | <p><i>Adapter Ligation:</i> An Illumina Truseq R1 Adapter is ligated to the 5' end of the DNA.</p> |
| 3 |  |  |
| | | <p><i>Round 4 Barcoding:</i> The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.</p> |

Section 4: CRISPR Sequencing Library Preparation

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

Protocol Timing

The table below provides details of the total and hands-on time required for the whole transcriptome workflow.

| DAY | SECTION | TOTAL TIME | HANDS-ON TIME | SAFE STOPPING POINTS |
|-----|--|----------------------|----------------------|----------------------------------|
| 1 | 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 | 30 min | |
| | 1.3: Barcoding Round 2 | 90 min | 30 min | |
| | 1.4: Barcoding Round 3 | 45 min | 15 min | |
| | 1.5 Lysis and Sublibrary Generation | 105 min | 45 min | -80°C ≤ 6 months |
| 2 | Section 2: cDNA Capture and Amplification | | | |
| | 2.1 Binder Bead Preparation | 30 min | 30 min | |
| | 2.2: cDNA Capture | 60 min | 15 min | |
| | 2.3 cDNA Template Switch | 150 min | 30 min | 4°C ≤ 18 hrs |
| | 2.4: cDNA Amplification | 120 min | 30 min | 4°C ≤ 18 hrs |
| | 2.5 Post-Amplification Purification | 30 min | 30 min | |
| | 2.6 cDNA Quantification | 30 min | 30 min | 4°C ≤ 48 hrs or -20°C ≤ 3 months |
| 3 | Section 3: WT Sequencing Library Preparation | | | |
| | 3.1 Fragmentation, End Repair, and A-Tailing | 60 min | 30 min | |
| | 3.2 Post-A-tailing Size Selection | 30 min | 30 min | 4°C ≤ 18 hrs or -20°C ≤ 2 weeks |

| DAY | SECTION | TOTAL TIME | HANDS-ON TIME | SAFE STOPPING POINTS |
|-----|--|------------|---------------|----------------------------------|
| | 3.3 Adaptor 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 |
| | 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 |
| 4 | 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 |

Important Guidelines

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

Sample Input

- This protocol begins with cells fixed with an Evercode Cell Fixation kit.
- 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. 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.

Cell Counting and Quality Assessment

- We recommend a hemocytometer for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices to a hemocytometer when using Evercode Whole Transcriptome kits for the first time.
- When first using Evercode Whole Transcriptome kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- After fixation, the cells are permeabilized and will appear dead with viability stains. Examples of trypan blue stained fixed cells are shown below.
- High quality fixed samples have single distinct cells with <5% cell aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed cells, it is critical to avoid counting cell debris to avoid overestimating the number of cells (Figure 1).

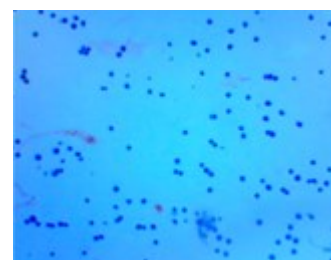
High Quality Sample



Aggregation



Debris



Avoiding RNase Contamination

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

Centrifugation

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

Optimizing Cell Recovery

- It is critical to thoroughly resuspend the cells after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to cell adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell loss.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.
- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- The first time using an Evercode 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 Parse Biosciences Evercode WT Mini Sample Loading Table V1.2.0 (Excel spreadsheet) should be completed before starting the experiment.

- If the table isn't working as expected, ensure that Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in the table to avoid disturbing any calculations.
- In the unlikely event that your sample is 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 40 μm cell strainer will be used in multiple steps. To maximize cell retention, 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. Recover any residual liquid on the bottom of the strainer.
- For cells larger than 40 μm , the provided strainers should be replaced throughout the protocol with the appropriate size mesh (70 μm or 100 μm).

Vortexing

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

Plate Sealing

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

Magnetic Racks and Bead Cleanups

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

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

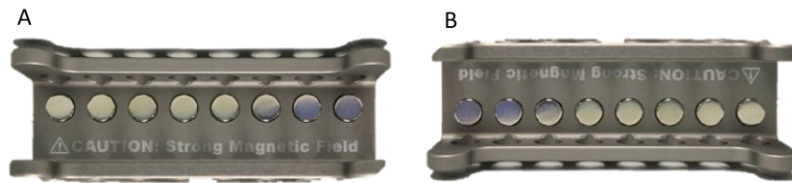


Figure 2: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.

- To ensure material is not lost during bead purifications, ensure supernatants are completely clear before moving to the next step. The incubation times at each step are recommendations, but visual confirmation of clearing should be used to make the final determination. See the figure below for an example of completely cleared supernatants. Discarding any beads in supernatants will result in a reduction of transcripts and genes detected per cell.



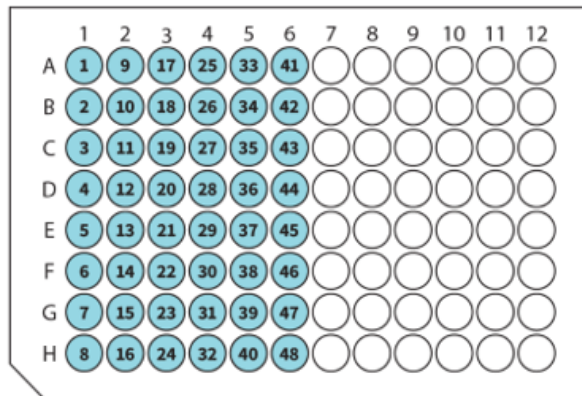
Figure 3: Clear supernatant with compact bead pellets.

Sublibrary Loading

- The Evercode WT kit generates 8 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.
- The number of cells per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix A.
- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.
- Sublibraries can be loaded with different numbers of cells, and the maximum number of cells that can be analyzed is the sum of cells across all sublibraries.
- Asymmetric sublibrary loading can enable cost-effective sequencing quality control. One sublibrary can be loaded with a few hundred cells and sequenced very deeply. This data can be used to choose an appropriate sequence depth for the remaining sublibraries.

Indexing Primers

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



Thermocycling Programs

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

Part List

Safety Data Sheets for these reagents can be provided upon request.

CRISPR Detect

Store at -20°C, PN CRS1010

| LABEL | ITEM | PN | FORMAT | QTY |
|--|--------------------------|-------|-------------|-----|
|  | Human U6 Primer 1 | CS101 | 1.5 mL tube | 1 |
|  | Human U6 Primer 2 | CS102 | 1.5 mL tube | 1 |
|  | Universal Primer | CS103 | 1.5 mL tube | 1 |
|  | 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. |
| Centrifuge with Swinging Bucket Rotors | Various Suppliers | Varies | Compatible with 15 mL centrifuge tubes and 96 well plates and capable of reaching 4°C. |
| Microcentrifuge | Various Suppliers | Varies | Compatible with 1.5 mL and 0.2 mL tubes. |
| Heat Block | Various Suppliers | Varies | Or equivalent water bath, bead bath, or thermomixer capable of holding temperature at 37°C. |
| Hemocytometer | Sigma-Aldrich® | Z359629 | Or other cell counting device. We recommend validating alternatives relative to a hemocytometer. |
| Single Channel Pipettes: P20, P200, P1000 12-channel: P20, P200 | Various Suppliers | Varies | Or 8-channel pipettes can be substituted for 12-channel pipettes. |
| T100 Thermal Cycler | Bio-Rad Laboratories® | 1861096 | Or an equivalent thermocycler compatible with unskirted 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. |

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

Consumables

| ITEM | SUPPLIER | PN | NOTES |
|----------------------|-------------------|----------------------------|--|
| Evercode™ WT Mini v2 | Parse Biosciences | ECW02110 or ECW02115 | |
| UDI Plate - WT | Parse Biosciences | UDI1001 | Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Whole Transcriptome sequencing libraries (48 sublibraries). |
| 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 |
|---|--------------------------|----------------------------------|---|
| Falcon® High Clarity PP Centrifuge Tubes, 15 mL | Corning® | 352097 | Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial cell loss. |
| DNA LoBind® Tubes, 1.5 mL, Snap Cap | Eppendorf® | 022431021 | Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes. |
| TempAssure® PCR 8-Tube Strips, 0.2 mL | USA Scientific® | 1402-4700 | Or equivalent nuclease-free 0.2 mL PCR tubes. |
| AMPure® XP Reagent | Beckman Coulter® | A63880 (5 mL) A63881 (60mL) | Choose one. We do not recommend substituting other magnetic beads. |
| SPRIselect Reagent | Beckman Coulter | B23317 (5mL) B23318 (60mL) | |
| KAPA® Pure Beads | Roche® | KK8000 (5 mL) KK8001 (30mL) | |
| Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL | Rainin® | 17014961 17014963 17014967 | Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips. |
| RNaseZap™ RNase Decontamination Solution | Thermo Fisher Scientific | AM9780 | Or equivalent RNase decontamination solution. |
| Ethyl alcohol, Pure | Sigma-Aldrich | 459844 | Or equivalent 100% non-denatured ethanol. |

| ITEM | SUPPLIER | PN | NOTES |
|--|-------------------|---|--|
| Nuclease-Free Water | Sigma-Aldrich | W4502 | Or equivalent nuclease-free water. |
| Trypan Blue | Various Suppliers | Varies | Or alternative dyes to assess cell viability, such as AO/PI. |
| High Sensitivity DNA Kit | Agilent | 5067-4626 | Choose one that corresponds to the chosen Bioanalyzer or Tapestation. |
| High Sensitivity D5000 ScreenTape and Reagents | Agilent | 5067-5592 (screen tape) 5067-5593 (sample buffer and ladder) | |
| Corning Cell Strainer | Corning | 431751 (70µm) 431752 (100µm) | (Optional) For cells larger than 40 µm, the included strainers should be replaced throughout the protocol with an appropriate size mesh. |

Section 1: in Situ Cell Barcoding

1.1. Set up and Sample Counting

Before barcoding, cells are thawed and counted. The Sample Loading Table uses these counts to calculate appropriate dilutions, loading concentrations, and loading positions of the samples.

To set up for barcoding:

1. Open the “WT Mini Sample Loading Table V1.2.0” (Excel spreadsheet), which will guide sample dilutions and plate loading in later steps.
2. Cool a centrifuge with swinging bucket rotors to 4°C.
3. Set a heat block to 37°C.
4. Fill a bucket with ice.
5. Prepare a hemocytometer, flow cytometer, or other cell counting device.
6. Gather the following items and handle as indicated below.

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---------------------------|----------------------------|-----|---|
| PCR plate seals | Accessories (Room Temp) | 1 | Keep at room temperature. |
| ● Spin Additive | 4°C Reagents (4°C) | 1 | |
| ● Dilution Buffer | Barcoding Reagents (-20°C) | 1 | Thaw at room temperature then store on ice. Mix by inverting 3 times. |
| ● Resuspension Buffer | Barcoding Reagents (-20°C) | 1 | |
| ● Ligation Mix | Barcoding Reagents (-20°C) | 1 | |
| ● Round 2 Ligation Enzyme | Barcoding Reagents (-20°C) | 1 | Place directly on ice. Briefly centrifuge before use. |
| Round 1 Plate | Barcoding Plates (-20°C) | 1 | Place directly on ice. |
| Round 2 Plate | Barcoding Plates (-20°C) | 1 | |

- Remove the Round 1 Plate from the green rack. Place it into a thermocycler and run the following program. Upon completion, proceed immediately to the next step.

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

- Thaw the previously fixed cell samples in a heat block set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.
- While minimizing time on ice, count the number of cells in your sample with a hemocytometer or alternative cell counting device. Record the cell count.
- Record the sample names and cell count in the Sample Loading Table (Parse Biosciences Evercode WT Mini Sample Loading Table V1.2.0.xlsm).
- Based on the values defined in the Sample Loading Table, dilute each sample with
 - Dilution Buffer and store it on ice. Do not discard the ● Dilution Buffer.



Note: If there are errors in the Sample Loading Table, see the Important Guidelines section for suggestions. Proceed immediately to Section 1.2.

- Proceed immediately to Section 1.2.

1.2. Barcoding Round 1

Samples are loaded into Round 1 Plate. An *in situ* reverse transcription reaction adds well-specific barcodes that also serve as sample barcodes. Cells are then pooled, centrifuged, and resuspended.

To add round 1 barcodes:

1. Gently remove the Round 1 Plate from the thermocycler, return to the green rack, and centrifuge for **1 minute** at 100 x g at 4°C.
2. Remove the Round 1 Plate from the centrifuge. While secured in the green 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 of wells).

3. Based on the positions defined in the Sample Loading Table and without reusing tips, add **14 µL** of each diluted sample to the appropriate wells of Round 1 Plate. Mix immediately after dispensing each sample by pipetting 3x and keeping the plate on ice.



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 a different well.



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

4. Remove the Round 1 Plate from ice. While secured in the green rack on a flat surface, add a new PCR plate seal.

- Place the Round 1 Plate into a thermocycler and run the program below. Upon completion, proceed immediately to the next step.

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

- Remove the Round 1 Plate from the thermocycler, return to the green rack, and store on ice.
- Remove the Round 2 Plate from the blue rack, place it into a thermocycler, and run the following program. Proceed immediately to the next step while the program is still running.

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

8. While secured in the green rack on a flat surface, remove the plate seal from the Round 1 Plate and store the uncovered plate on ice.
9. With both on ice, transfer all the liquid in the Round 1 Plate to a 15 mL tube. To optimize cell recovery, use a single channel pipette as follows:
 - i. With a single channel pipette set to 30 μL , pool Row A into a 15 mL centrifuge tube. To maximize cell retention while pooling, pipette up and down 3x in the middle of the well, 3x on the front side of the well, and 3x on the back side of the well before transferring the volume of Row A into the 15 mL tube.
 - ii. Recover residual liquid from Row A using the single channel pipette set to **10 μL** . Do not be concerned if there are a few μL of residual volume in the wells after pooling.



CRITICAL! Keep the Round 1 Plate and the 15 mL tube with pooled cells on ice during pooling.

10. Add **2.4 μL** of ●Spin Additive to the 15 mL tube with pooled cells. Do not discard the ●Spin Additive, as it will be needed in another step.
11. Mix by gently inverting the tube just once.
12. Centrifuge the 15 mL tube in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Immediately move to the next step after centrifugation.



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

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

13. Remove the supernatant until about ~40 μL of liquid remains above the pellet.



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

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

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

16. Proceed immediately to Section 1.3.

1.3. Barcoding Round 2

The pooled cells are added to the Ligation Master Mix, which is loaded into the Round 2 Plate. An *in situ* ligation reaction adds a well-specific barcode to the 3' end of the cDNA. The ligation reaction is quenched with Round 2 Stop 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 |
|--------------------------------------|----------------------------|-----|--|
| PCR plate seal | Accessories (Room Temp) | 3 | Keep at room temperature. |
| 40 µm Strainer (or alternative size) | Accessories (Room Temp) | 2 | |
| Basins | Accessories (Room Temp) | 6 | |
| ● Round 2 Stop Mix | Barcoding Reagents (-20°C) | 1 | Thaw at room temperature then store on ice. Mix by inverting 3x. |
| ● Round 3 Stop Mix | Barcoding Reagents (-20°C) | 1 | |
| ● Pre-Lyse Wash Buffer | Barcoding Reagents (-20°C) | 1 | |
| ● Round 3 Ligation Enzyme | Barcoding Reagents (-20°C) | 1 | Place directly on ice. Briefly centrifuge before use. |
| Round 3 Plate | Barcoding Reagents (-20°C) | 1 | Place directly on ice. |

2. On ice, add **20 µL** of ● Round 2 Ligation Enzyme directly into the ● Ligation Mix tube to create the Ligation Master Mix.
3. Using a P1000 pipette, add **2 mL** of sample in ● Resuspension Buffer into the Ligation Master Mix. Mix thoroughly by pipetting 10x with a P1000 set to 1000 µL. Store on ice.
4. Remove the Round 2 Plate from the thermocycler, return to the blue rack, and centrifuge for **1 minute** at 100 x g at 4°C. Keep at room temperature.

5. While secured in the blue rack on a flat surface, remove the plate seal from the Round 2 Plate.
6. Transfer the entire volume in the Ligation Master Mix tube to a basin with a P1000.
7. At room temperature and without reusing tips, transfer **40 µL** from the basin to each well in the Round 2 Plate. To optimize cell recovery, use a multichannel pipette as follows:
 - i. Mix the sample in the basin by pipetting 2x with a multichannel P200 set to 40 µL.
 - ii. Transfer **40 µL** of the mix to Row A of the Round 2 Plate and mix by pipetting 2x.
 - iii. Repeat i-ii for 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 insufficient to fill every well, a few can be left empty without impacting experimental results.

8. While secured in the blue rack on a flat surface, add a new plate seal to the Round 2 Plate.
9. Place the Round 2 Plate into a thermocycler and run the following program. Upon completion, proceed immediately to the next step.

| BARCODING ROUND 2 | | |
|-------------------|-----------------|---------------|
| Run time | Lid Temperature | Sample Volume |
| 30 min | 50°C | 50 µL |
| Step | Time | Temperature |
| 1 | 30 min | 37°C |
| 2 | Hold | 4°C |

10. Briefly vortex the ●Round 2 Stop Mix. Transfer the entire volume of this tube to a new basin with a P1000.

11. Remove the Round 2 Plate from the thermocycler and return to the blue rack.
12. While secured in the blue rack on a flat surface, remove the plate seal from the Round 2 Plate.
13. At room temperature and without reusing tips, transfer **10 μ L** of the ●Round 2 Stop Mix from the basin to each well in the Round 2 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.



CRITICAL! Carefully hold the strainer using the outside casing without touching the mesh. Different tips must be used when pipetting ●Round 2 Stop Mix 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.

14. While secured in the blue rack on a flat surface, add a new plate seal to the Round 2 Plate.
15. Place the Round 2 Plate into a thermocycler and run the following program. Upon completion, proceed immediately to the next step.

| ROUND 2 STOP | | |
|--------------|-----------------|---------------|
| Run time | Lid Temperature | Sample Volume |
| 30 min | 50°C | 60 μ L |
| Step | Time | Temperature |
| 1 | 30 min | 37°C |
| 2 | Hold | 4°C |

16. Transfer the Round 2 Plate from the thermocycler to the blue rack and store it at room temperature.

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

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

18. While secured in the blue rack on a flat surface, remove the plate seal from Round 2 Plate.

19. At room temperature, transfer all the liquid in the Round 2 Plate into a new basin. To optimize cell recovery, use a multichannel pipette as follows:

- i. With a multichannel P200 set to 50 µL, mix the sample in Row A by pipetting 3x in the middle of the well, then repeating on the front and the back of the wells.
- ii. Transfer **50 µL** from Row A to the basin.
- iii. Repeat for Rows B-H.
- iv. Transfer any residual liquid in the Round 2 Plate to the basin with a multichannel pipette set to 10 µL.



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

20. Pipette the sample through a 40 μm strainer into a new basin with a P1000. Before each transfer, gently mix the cells in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.



CRITICAL! Carefully hold the strainer using the outside casing without touching the mesh. To ensure that all of the liquid passes through the strainer, press the tip of the pipette against the mesh to create a tight seal and press the pipette plunger down steadily. All of the liquid should pass through the strainer in ~ 1 second.



Note: For cells larger than 40 μm , all strainers in the protocol should be replaced with an appropriate size mesh. Bubbles may form while straining. They will not affect the quality of the experiment.

21. Proceed immediately to Section 1.4.

1.4. Barcoding Round 3

Round 3 Ligation Enzyme is added to the pooled cells, which are then loaded into the Round 3 Plate. A second in situ ligation reaction adds a third well-specific barcode, the Illumina Truseq R2 sequence, and a biotin. The sample is then pooled and strained.

To add round 3 barcodes:

1. Add **20 µL** of ●Round 3 Ligation Enzyme to the basin containing the strained sample. Mix by gently pipetting 20x with a P1000 set to 1000 µL.
2. Remove the Round 3 Plate from the thermocycler, return to the orange rack, and centrifuge for **1 minute** at 100 x g at 4°C.
3. While secured in the orange rack on a flat surface, remove the plate seal from the Round 3 Plate.
4. At room temperature and without reusing tips, transfer **50 µL** from the basin to each well in the Round 3 Plate. To optimize cell recovery, use a multichannel pipette as follows:
 - i. Mix the sample in the basin by pipetting 2x pipetting with a multichannel P200 set to 50 µL.
 - ii. Transfer **50 µL** of the mix to Row A of the Round 3 Plate and mix by pipetting 2x.
 - iii. Repeat i-ii for 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 for transferring the last row with a multichannel, tilt the basin and transfer the remaining volume with a single-channel pipette. If the volume is insufficient to fill every well, a few can be left empty without impacting experimental results.

5. While secured in the orange rack on a flat surface, add a new plate seal to the Round 3 Plate.

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

| BARCODING ROUND 3 | | |
|-------------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 30 min | 50°C | 60 µL |
| Step | Time | Temperature |
| 1 | 30 min | 37°C |
| 2 | Hold | 4°C |

7. Briefly vortex the ●Round 3 Stop Mix. Transfer the entire volume to a new basin with a P1000.
8. Remove the Round 3 Plate from the thermocycler and return it to the orange rack.
9. While secured in the orange rack on a flat surface, remove the plate seal from the Round 3 Plate.
10. At room temperature and without reusing tips, transfer **20 µL** of the ●Round 3 Stop Mix from the basin to each well in the Round 3 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.



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

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

12. At room temperature, transfer all the liquid in the Round 3 Plate into a new basin. To optimize cell recovery, use a multichannel pipette as follows:
- i. With a multichannel P200 set to 70 μL , mix the sample in Row A by pipetting 3x in the middle of the well, then repeating on the front and the back of the wells.
 - ii. Transfer **70 μL** from Row A to the basin.
 - iii. Repeat for Rows B-H.
 - iv. Transfer any residual liquid in the Round 3 Plate to the basin with a multichannel pipette set to 10 μL .



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

13. Pipette the sample through a 40 μm strainer into a new 15 mL tube with a P1000. Before each transfer, gently mix the cells in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.



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

14. Proceed immediately to Section 1.5.

1.5. Lysis and Sublibrary Generation

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

To generate and lyse sublibraries:

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|-------------------|----------------------------|-----|---|
| ● 2x Lysis Buffer | 4°C Reagents (4°C) | 1 | Place in a 37°C heat block until use. |
| ● Lysis Enzyme | Barcoding Reagents (-20°C) | 1 | Place directly on ice. Briefly centrifuge before use. |
| ● Dilution Buffer | Barcoding Reagents (-20°C) | 1 | Thaw at room temperature, then place on ice. Mix by inverting 3x. |



Note: Ensure there is no precipitate when using the ● 2x Lysis Buffer.

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



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

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



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

5. Fully but gently resuspend the pellet in **1 mL** of ● Pre-Lyse Wash Buffer.
6. Add an additional **3 mL** of ● Pre-Lyse Wash Buffer for a total addition of 4 mL.

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



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

8. Remove the supernatant until about ~40 μL of liquid remains above the pellet. Use a P1000 for the first 3 mL and then a P200 for the remaining volume.
9. Fully but gently resuspend the pellet with the remaining supernatant in the 15mL tube. Measure the volume of the resuspended cells with a P200 pipette. If less than 60 μL are measured, add ●Dilution Buffer to a total of **60 μL** . If more than 60 μL are measured, proceed without adding ●Dilution Buffer. Store on ice.



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

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



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

11. Decide how to divide cells across the sublibraries. See the “Sublibrary Loading” in the important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix A to determine the volume of sample and ●Dilution Buffer that should be added to each sublibrary.



CRITICAL! Do not overload a sublibrary. The recommended loading is up to 5,000 cells/sublibrary, with a maximum of 10,000 cells/sublibrary. Adding additional cells will result in an increased multiplet rates.

12. Ensure the cells are in suspension by pipetting 5x with a P200 set to **45 μL** before each transfer. Add the appropriate volume of sample to 2 separate 0.2 mL tubes.
13. Add the appropriate volume of ●Dilution Buffer to the 0.2 mL tubes for a total volume of 25 μL .

14. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P200 set to **50 μ L**. Store at room temperature.

| LYSIS MASTER MIX | |
|-------------------|------------|
| ● 2x Lysis Buffer | 55 μ L |
| ● Lysis Enzyme | 11 μ L |
| Total Volume | 66 μ L |



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

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

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

17. Place the tubes into a thermocycler and run the following program.

| CELL LYSIS | | |
|------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 60 min | 80°C | 55 μ L |
| Step | Time | Temperature |
| 1 | 60 min | 65°C |
| 2 | Hold | 4°C |

18. Freeze the lysate(s) at -80°C.

Safe stopping point: Sublibrary lysates are stable for up to 6 months at -80°C.

Section 2: cDNA Capture and Amplification

2.1. Binder Bead Preparation

During the Binder Bead Preparation step, Streptavidin-coated Binder Beads are washed.

To prepare for cDNA capture and amplification:

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|-----------------------|-------------------------------------|-----|--|
| ● Binder Beads | 4°C Reagents (4°C) | 1 | Keep at room temperature. |
| ● Bead Wash Buffer | cDNA Amplification Reagents (-20°C) | 1 | Thaw and store at room temperature. Mix by inverting 3x. |
| ● Bind Buffer A | cDNA Amplification Reagents (-20°C) | 1 | |
| ● Bind Buffer B | cDNA Amplification Reagents (-20°C) | 1 | |
| ● Bind Buffer C | cDNA Amplification Reagents (-20°C) | 1 | |
| ● Bead Storage Buffer | cDNA Amplification Reagents (-20°C) | 1 | |

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---------------------|-------------------------------------|-----|---|
| ● TS Buffer | cDNA Amplification Reagents (-20°C) | 1 | Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use. |
| ● Lysis Neutralizer | cDNA Amplification Reagents (-20°C) | 1 | Place directly on ice. Briefly centrifuge before use. |

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

| | | |
|-------------------|------------|------------|
| Lysates processed | 1 | 2 |
| ● Binder Beads | 44 μ L | 88 μ L |

7. Place the tube on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).
8. Remove and discard the supernatant.
9. Remove the tube from the magnetic rack and fully resuspend the bead pellet in the appropriate volume of ●Bead Wash Buffer as follows:

| | | |
|--------------------|------------|-------------|
| Lysates processed | 1 | 2 |
| ● Bead Wash Buffer | 50 μ L | 100 μ L |



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

10. Place the tube on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).
11. Remove and discard the supernatant.
12. Repeat steps 9-11 twice for a total of 3 washes.

13. Remove the tube from the magnetic rack. Fully resuspend the pellet in the appropriate volume of ● Bind Buffer A as follows:

| | | |
|-------------------|------------|-------------|
| Lysates processed | 1 | 2 |
| ● Bind Buffer A | 55 μ L | 110 μ L |

14. Store the 1.5 mL tube at room temperature and proceed immediately to Section 2.2.

2.2. cDNA Capture

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

To capture the cDNA:

1. Remove the desired tube(s) of lysate from -80°C .
2. Incubate the tube(s) in a heat block or thermocycler at 37°C for **5 minutes**.



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

3. Briefly centrifuge and store at room temperature.
4. Briefly centrifuge ●Lysis Neutralizer and gently mix by pipetting 2x with a P20 set to $15\ \mu\text{L}$.
5. Add **$2.5\ \mu\text{L}$** of ●Lysis Neutralizer to each tube of lysate. Mix 5x with a P200 pipette set to $40\ \mu\text{L}$. Briefly centrifuge.
6. Incubate for **10 minutes** at room temperature.
7. Fully mix the ●Binder Beads prepared in Section 2.1 by pipetting 3x.
8. Add **$50\ \mu\text{L}$** of ●Binder Beads to each tube of lysate. Fully mix by pipetting 5x with a P200 set to $90\ \mu\text{L}$.
9. 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.
10. Place the rack onto a vortex mixer with a plate adaptor. Push to secure. Vortex on 20% power ($\sim 800\text{-}1000\ \text{RPM}$) for **60 minutes** at room temperature.



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

12. Remove the tube(s) from the vortex mixer with a plate adaptor.
13. Briefly vortex the tube(s) on a standard vortex adaptor. Briefly centrifuge without letting beads collect at the bottom of the tube(s).

14. 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.

15. While still on the magnetic rack, remove and discard the supernatant.
16. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ● Bind Buffer B.
17. Incubate for **1 minute** at room temperature.
18. Return the tube(s) to the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
19. While still on the magnetic rack, remove and discard the supernatant.
20. Repeat steps 16-19 once for a total of 2 washes with ● Bind Buffer B.
21. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ● Bead Storage Buffer.
22. Incubate for **1 minute** at room temperature.
23. Proceed immediately to Section 2.3.

2.3. cDNA Template Switch

After an additional wash, a template switching reaction is added to the captured cDNA. This template switching reaction adds a 5' adaptor to the cDNA.

To template switch:

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|-----------------|-------------------------------------|-----|---|
| ● TS Primer Mix | cDNA Amplification Reagents (-20°C) | 1 | Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use. |
| ● TS Enzyme | cDNA Amplification Reagents (-20°C) | 1 | Keep on ice. Briefly centrifuge before use. |



Note: Ensure there is no precipitate in the ●TS Buffer before proceeding.

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

| TEMPLATE SWITCH MASTER MIX | | |
|----------------------------|-----------|----------|
| Number of Samples | 1 | 2 |
| ● TS Buffer | 101.75 µL | 203.5 µL |
| ● TS Primer Mix | 2.75 µL | 5.5 µL |
| ● TS Enzyme | 5.5 µL | 11 µL |
| Total | 110 µL | 220 µL |

3. Place each tube of captured cDNA from Section 2.2 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.

- While still on the magnetic rack, add **125 μ L** of ●Bind Buffer C to each tube.



CRITICAL! Do not discard the ●Bind Buffer C as it will be used in another step.

- Incubate for **1 minute** at room temperature.
- While still on the magnetic rack, remove and discard the ●Bind Buffer C.
- Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 μ L** of the Template Switch Master Mix.



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

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

| cDNA TEMPLATE SWITCH | | |
|----------------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 90 min | 70°C | 100 μ L |
| Step | Time | Temperature |
| 1 | 90 min | 42°C |
| 2 | Hold | 4°C |

- If not immediately continuing to cDNA amplification, proceed to step 14. Otherwise, proceed immediately to Section 2.4.
- Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).



Note: You may need to pipette mix to resuspend settled beads.

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

16. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 μ L** ●Bead Storage Buffer.

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

2.4. cDNA Amplification

The captured cDNA is washed and amplified with TS- and Illumina Truseq R2- specific primers. The sgRNA transcripts are enriched with Human U6 primers.

To amplify the cDNA:

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|-------------------------------|-------------------------------------|-----|---|
| ● Amplification Master Buffer | cDNA Amplification Reagents (-20°C) | 1 | Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use. |
| ● Amplification Primer Mix | cDNA Amplification Reagents (-20°C) | 1 | |
| ● Human U6 Primer 1 | CRISPR Detect (-20°C) | 1 | |

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

| AMPLIFICATION REACTION MASTER MIX | | |
|-----------------------------------|----------|----------|
| Number of sublibraries | 1 | 2 |
| ● Amplification Master Buffer | 60 µL | 120 µL |
| ● Amplification Primer Mix | 60 µL | 120 µL |
| ● Human U6 Primer 1 | 2.4 µL | 4.8 µL |
| Total | 122.4 µL | 244.8 µL |

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



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

4. While still on the magnetic rack, remove and discard the supernatant.
5. While still on the magnetic rack, add **125 μ L** of ●Bind Buffer C to each tube.
6. Incubate for **1 minute** at room temperature.
7. While still on the magnetic rack, remove and discard the ●Bind Buffer C.
8. Remove tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 μ L** of the Amplification Reaction Master Mix. Store on ice.
9. Determine the number of PCR cycles required for cDNA amplification based on the table below. Although these recommendations are appropriate for many cell types, the number of cycles may need to be optimized for your 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 | 12 | 14 |
| 1,000-2,000 | 10 | 12 |
| 2,000-6,000 | 8 | 10 |
| 6,000-10,000 | 6 | 8 |

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

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



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



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

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

2.5. Post-Amplification Purification

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

To purify the cDNA:

1. Gather 400 μL of freshly prepared 85% ethanol per tube of amplified cDNA with nuclease-free water.
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 2.4 on the high position of the Parse Biosciences Magnetic Rack. Incubate until the solution clears (~2 minutes).
4. While still on the magnetic rack, transfer **90 μL** of the supernatant containing the cDNA into a new 0.2 mL tube(s). Store at room temperature.
5. Vortex the SPRI beads until fully mixed. Add **72 μL** of SPRI beads to each tube with amplified cDNA.
6. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
7. Incubate for **5 minutes** at room temperature.
8. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
9. While still on the magnetic rack, remove and discard the supernatant.
10. While still on the magnetic rack, add **180 μL** of 85% ethanol to each tube.
11. Incubate for **1 minute** at room temperature.
12. While still on the magnetic rack, remove and discard the supernatant.
13. Repeat steps 10-12 once for a total of 2 washes. Remove any residual ethanol with a P20.

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



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

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

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

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



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

18. While still on the magnetic rack, transfer **20 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.

2.6. 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 2.5 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. Samples may need to be diluted to be within the manufacturer's recommended concentration range.



Note: Typically, between a 1:3 to 1:10 dilution is appropriate.

3. Purified 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.

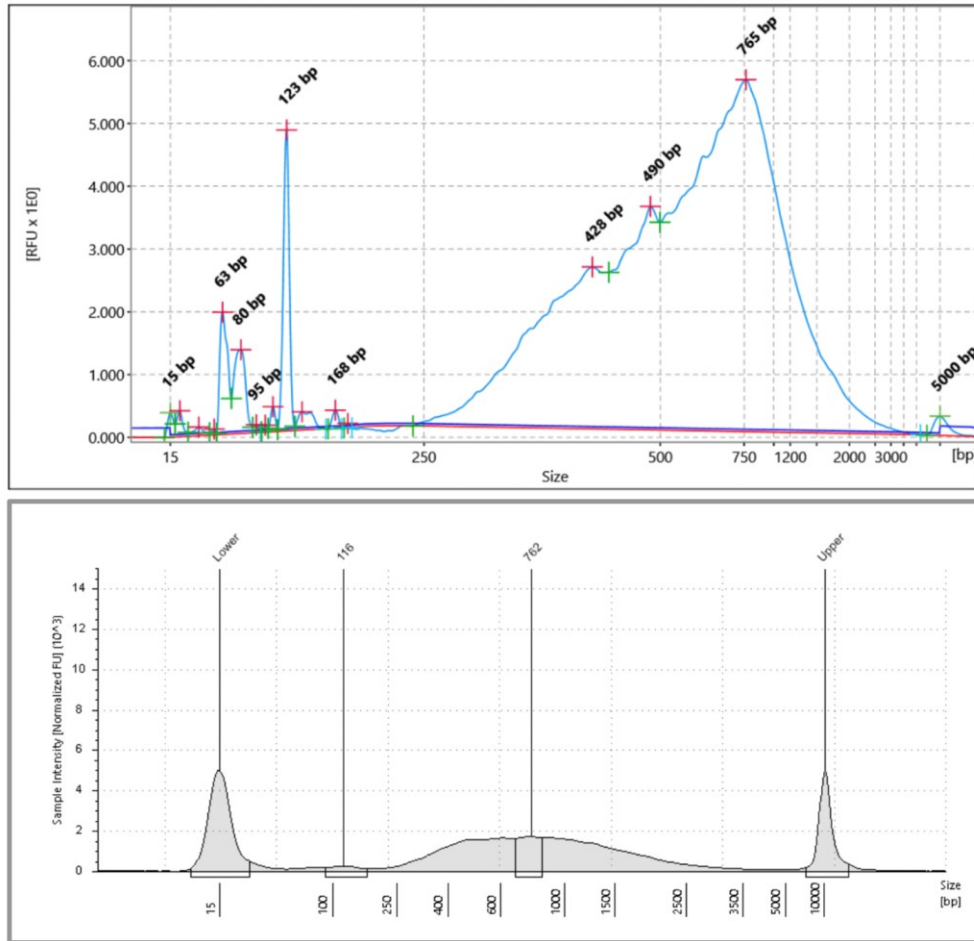


Figure 4: Expected post-amplification sublibrary cDNA size distribution. The expected cDNA Size Distribution after cDNA Amplification. Example trace of cDNA run on a Bioanalyzer (top). Example trace of cDNA run on a TapeStation (bottom). It is normal for libraries to be shifted to the left on a TapeStation relative to a Bioanalyzer



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

Section 3: WT Sequencing Library Preparation

3.1. Fragmentation, End Repair, and A-Tailing

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

To prepare for sequencing library preparation:

1. Prepare 1.2 mL 85% ethanol per sublibrary with nuclease-free water.
2. Equilibrate approximately 180 μ L 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 2.6.
6. Gather the following items and handle as indicated below.

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

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 and store on ice. Bring the total volume up to **35 µL**. Store any remaining sublibrary cDNA at -20°C.

| DILUTED cDNA | |
|---------------------|----------|
| Sublibrary cDNA | 100 ng |
| Nuclease-free water | Variable |
| Total Volume | 35 µL |

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

| SUBLIBRARY FRAGMENTATION, END REPAIR AND A-TAILING | | |
|--|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 40 min | 70°C | 50 µL |
| Step | Time | Temperature |
| 1 | Hold* | 4°C |
| 2 | 10 min | 32°C |
| 3 | 30 min | 65°C |
| 4 | Hold | 4°C |

* This ensures that the thermal cycler is cooled and ready for step 13.

10. Vortex the ●Fragmentation Buffer for **5 seconds**. Briefly centrifuge.



Note: Confirm the ●Fragmentation Buffer is fully thawed without precipitation.

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

| FRAGMENTATION MASTER MIX | | |
|--------------------------|--------------|------------|
| Number of Sublibraries | 1 | 2 |
| ● Fragmentation Buffer | 5.5 μ L | 11 μ L |
| ● Fragmentation Enzyme | 11 μ L | 22 μ L |
| Total | 16.5 μ L | 33 μ L |

12. Add **15 μ L** of Fragmentation Master Mix to each tube of diluted cDNA. Mix by pipetting 10x with a P200 multichannel pipette set to 40 μ L. Briefly centrifuge.

13. Place the tube(s) into the cooled thermocycler. When the tube(s) are cooled, skip Step 1 of the thermal cycler program (4°C hold step) so the thermocycler proceeds to Step 2 of the Sublibrary Fragmentation, End Repair, and A-Tailing program.



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

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

3.2. Post-A-tailing Size Selection

Fragmented and A-tailed DNA is size selected with a double sided SPRI cleanup.

To size select the DNA:

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



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

3. Vortex the SPRI beads until fully mixed. Add **30 μL** of SPRI beads to each tube of A-tailed 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 A-tailed DNA into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **10 μL** of SPRI beads to each tube.
9. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~3 minutes).



CRITICAL! This may take longer due to the low volume of beads. Ensure 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 **50 μ L** 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 μ L** of the supernatant into new 0.2 mL tube(s).

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

3.3. Adaptor Ligation

Adaptors with an Illumina Truseq R2 sequence are ligated to the 5' end of the fragmented DNA.

To ligate adaptors:

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

| ADAPTOR LIGATION MASTER MIX | | |
|------------------------------------|---------------|--------------|
| Number of Sublibraries | 1 | 2 |
| Nuclease-free water (not supplied) | 19.25 μ L | 38.5 μ L |
| ● Adaptor Ligation Buffer | 22 μ L | 44 μ L |
| ● Adaptor Ligase | 11 μ L | 22 μ L |
| ● Adaptor DNA | 2.75 μ L | 5.5 μ L |
| Total | 55 μ L | 110 μ L |

2. Add **50 μ L** of Adaptor Ligation Master Mix to each tube of purified A-tailed DNA from Section 3.2. Mix by pipetting 10x with a P200 multichannel pipette set to 80 μ L. Briefly centrifuge.

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

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

- As soon as the program reaches 4°C, store the adaptor ligated DNA on ice and proceed immediately to section 3.4.

3.4. Post-Ligation Purification

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

To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~90 μ L 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 μ L** of SPRI beads to each tube of adaptor 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 μ L** 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 μ L** 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 μ L** of the supernatant containing the purified adaptor 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 adaptor ligated DNA is PCR amplified with Illumina Truseq R1 and R2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.

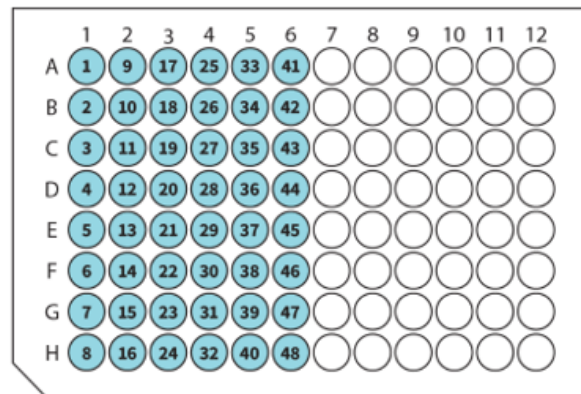
To add round 4 barcodes:

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



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

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



4. With a multichannel P20, pierce the seal of the chosen wells in the UDI Plate - WT.
5. With a multichannel P20 and new tips, add **4 µL** from a different well of the UDI Plate - WT to each tube of purified adaptor ligated DNA.



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

6. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C.
7. Add **25 µL** of ● Index PCR 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 indexing PCR based on the concentration of cDNA added to the fragmentation reaction as previously recorded in Section 3.1.

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

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

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



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

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

3.6. Post-Round 4 Barcoding Size Selection

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

To size select the sequencing libraries:

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



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

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



CRITICAL! This may take longer due to the low volume of beads. Ensure 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).
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 all elutant into new 0.2 mL tube(s). Store on ice.

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

3.7. Sequencing Library Quantification

The concentration and size distribution of the sequencing libraries are measured with fluorescent dyes and capillary electrophoresis. Libraries are then stored at 4°C for up to 48 hours or 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. Samples may need to be diluted to be within the manufacturer's recommended concentration range.



Note: Typically, between a 1:3 to 1:10 dilution is appropriate.

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

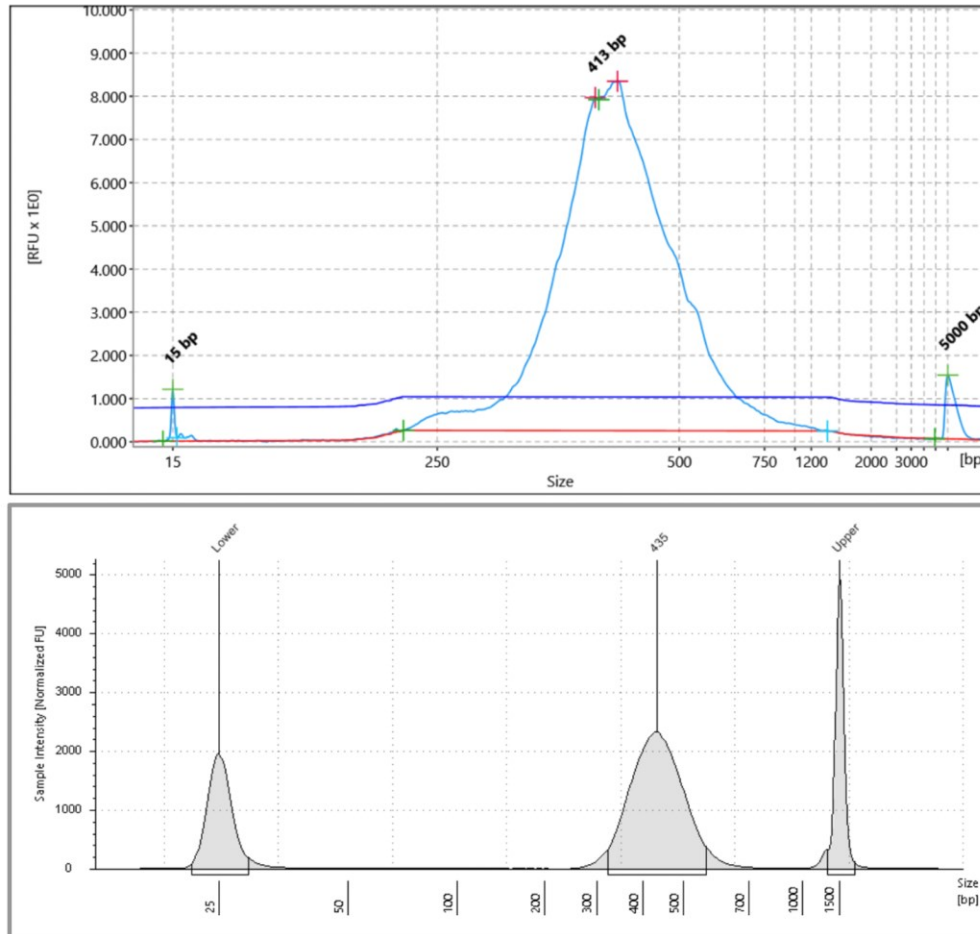


Figure 5: Expected Size Distribution before Illumina Sequencing. Example trace of DNA from indexed sublibraries run on a Bioanalyzer (top). Example trace of DNA from indexed sublibraries run on a TapeStation (bottom).

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



Note: There may be an additional peak present on the Bioanalyzer. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 400-500 bp). Do not use this additional peak when estimating amplicon size.

Section 4: CRISPR Sequencing Library Preparation

4.1 CRISPR PCR

A PCR reaction amplifies a subset of the cDNA enriched for sgRNA transcripts.

To amplify the sgRNA transcripts:

1. Prepare 1.2 mL 85% ethanol per sublibrary with nuclease-free water.
2. Equilibrate approximately 90 μ L of SPRI beads per sublibrary to room temperature.
3. Fill an ice bucket.
4. Take out the Parse Biosciences magnetic rack for 0.2 mL PCR tubes.
5. Obtain amplified cDNA from Section 2.6.
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. 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 | 2 |
| ● CRISPR Amplification Mix | 27.5 μL | 55 μL |
| ● Universal Primers | 2.2 μL | 4.4 μL |
| ● Human U6 Primer 2 | 2.2 μL | 4.4 μL |
| Total Volume | 31.9 μL | 63.8 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 | Lid Temperature | Sample Volume | |
| ~50 min | 105°C | 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.

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.

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).

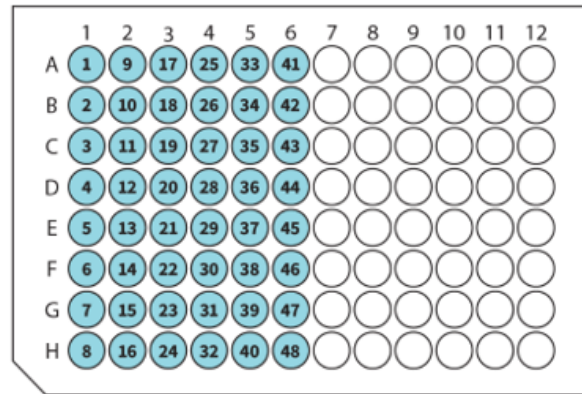
4. Centrifuge the UDI Plate - EC at 100 x g for **1 minute**.



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.

- 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.



- With a multichannel P20, pierce the seal of the chosen wells of the UDI Plate - EC.
- With a multichannel P20 and new tips, add **4 μ L** from a different well of the UDI Plate - EC to each tube of diluted CRISPR cDNA.



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

- If any unused wells remain in the UDI Plate - EC, store the plate at -20°C .
- Add **25 μ L** of CRISPR Amplification Master Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 40 μ L. Briefly centrifuge.

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

| SUBLIBRARY INDEX AMPLIFICATION | | | |
|--------------------------------|-----------------|---------------|-------|
| Run Time | Lid Temperature | Sample Volume | |
| ~30 min | 105°C | 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 μ L** 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 μ L** 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.

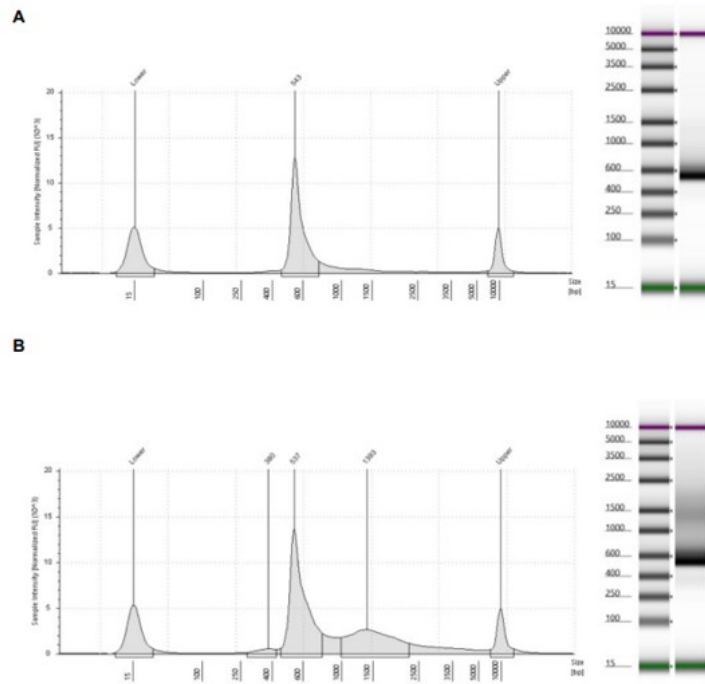
To quantify the CRISPR sequencing libraries:

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.



Note: Typically, between a 1:3 to 1:10 dilution is appropriate.

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



*Figure 6: **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: 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.

Please note the high molecular weight products when estimating amplicon size to ensure that CRIPR 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 Dilution Buffer in μL to add to each sublibrary.

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

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

| Stock con (cells/ μL) | 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 |
| 50 | 4 | 10 | 20 | | | | | | | | | |
| | 25 | 25 | 23 | | | | | | | | | |
| 100 | 2 | 5 | 10 | 20 | | | | | | | | |
| | 23 | 20 | 15 | 5 | | | | | | | | |
| 200 | | 2.5 | 5 | 10 | 15 | 20 | 25 | | | | | |
| | | 22.5 | 20 | 15 | 10 | 5 | 0 | | | | | |
| 300 | | | 3.33 | 6.67 | 10 | 13.33 | 16.67 | 20 | 23.33 | | | |
| | | | 21.67 | 18.33 | 15 | 11.67 | 8.33 | 5 | 1.67 | | | |
| 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 |
| | | | 23 | 21 | 19 | 17 | 15 | 13 | 11 | 9 | 7 | 5 |
| 600 | | | | 3.33 | 5 | 6.67 | 8.33 | 10 | 11.67 | 13.33 | 15 | 16.67 |
| | | | | 21.67 | 20 | 18.33 | 16.67 | 15 | 13.33 | 11.67 | 10 | 8.33 |
| 700 | | | | 2.86 | 4.29 | 5.71 | 7.14 | 8.57 | 10 | 11.43 | 12.86 | 14.29 |
| | | | | 22.14 | 20.71 | 19.29 | 17.86 | 16.43 | 15 | 13.57 | 12.14 | 10.71 |
| 800 | | | | 2.5 | 3.75 | 5 | 6.25 | 7.5 | 8.75 | 10 | 11.25 | 12.5 |
| | | | | 22.5 | 21.25 | 20 | 18.75 | 17.5 | 16.25 | 15 | 13.75 | 12.5 |
| 900 | | | | 2.22 | 3.33 | 4.44 | 5.56 | 6.67 | 7.78 | 8.89 | 10 | 11.11 |
| | | | | 22.78 | 21.67 | 20.56 | 19.44 | 18.33 | 17.22 | 16.11 | 15 | 13.89 |
| 1,000 | | | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | | | | 23 | 22 | 21 | 20 | 19 | 18 | 17 | 16 | 15 |
| 1,100 | | | | | 2.73 | 3.64 | 4.55 | 5.45 | 6.36 | 7.27 | 8.18 | 9.09 |
| | | | | | 22.27 | 21.36 | 20.45 | 19.55 | 18.64 | 17.73 | 16.82 | 15.91 |
| 1,200 | | | | | 2.50 | 3.33 | 4.17 | 5 | 5.83 | 6.67 | 7.5 | 8.33 |
| | | | | | 22.50 | 21.67 | 20.83 | 20 | 19.17 | 18.33 | 17.5 | 16.67 |
| 1,300 | | | | | 2.31 | 3.08 | 3.85 | 4.62 | 5.38 | 6.15 | 6.92 | 7.69 |
| | | | | | 22.69 | 21.92 | 21.15 | 20.38 | 19.62 | 18.85 | 18.08 | 17.31 |
| 1,400 | | | | | 2.14 | 2.86 | 3.57 | 4.29 | 5 | 5.71 | 6.43 | 7.14 |
| | | | | | 22.86 | 22.14 | 21.43 | 20.71 | 20 | 19.29 | 18.57 | 17.86 |
| 1,500 | | | | | 2.00 | 2.67 | 3.33 | 4.00 | 4.67 | 5.33 | 6.00 | 6.67 |
| | | | | | 23.00 | 22.33 | 21.67 | 21.00 | 20.33 | 19.67 | 19.00 | 18.33 |

Appendix B: Illumina Run Configuration

Evercode sequencing libraries should be diluted and denatured according to the instruction for the relevant sequencing instrument.

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. If sequenced alone or with CRISPR libraries, we recommend using a 5% PhiX spike for Whole Transcriptome libraries. For CRISPR libraries sequenced alone, we suggest using a minimum of 10% PhiX due to their limited complexity.

| READ | CYCLE |
|--------------------|-------|
| Read 1 | 66 |
| i7 Index (Index 1) | 8 |
| i5 Index (Index 2) | 8 |
| Read 2 | 86 |

Appendix C1: Unique Dual Index (UDI) Plate - WT Sequences

The fourth barcode that tags each sublibrary acts as a standard Illumina Unique Dual Index. It has associated i7 and i5 indexes that barcodes sublibraries and enables demultiplexing with increased accuracy. Please refer to the following table to demultiplex Whole Transcriptome sublibraries that have been sequenced together in the same run.



Note: Refer to “Appendix C2: Unique Dual Index (UDI) Plate- EC Sequences” to demultiplex CRISPR sequencing 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 |

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---------------------|---------------|---------------------|--------------------------------|---------------------|
| UDI_Plate_WT_19 | C3 | CTCAGAGT | TGGAGAGT | ACTCTCCA |
| UDI_Plate_WT_20 | D3 | TAATCGAC | TGACTTCG | CGAAGTCA |
| UDI_Plate_WT_21 | E3 | CATTTTCT | GGAAGGAT | ATCCTTCC |
| UDI_Plate_WT_22 | F3 | CTATACTC | TGTTTCGAG | 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 |

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---------------------|---------------|---------------------|--------------------------------|---------------------|
| UDI_Plate_WT_44 | D6 | GACACTTA | AGCAGGAA | TTCCTGCT |
| UDI_Plate_WT_45 | E6 | GAACGACA | ACGCTCGA | TCGAGCGT |
| UDI_Plate_WT_46 | F6 | AAGGCGAT | ACAGCAGA | TCTGCTGT |
| UDI_Plate_WT_47 | G6 | ATGCTTGA | ACAAGCTA | TAGCTTGT |
| UDI_Plate_WT_48 | H6 | AGTATCTG | CATCAAGT | ACTTGATG |

Appendix C2: Unique Dual Index (UDI) Plate- EC Sequences

The fourth barcode that tags each sublibrary acts as a standard Illumina Unique Dual Index. It has associated i7 and i5 indexes that barcodes sublibraries and enables demultiplexing with increased accuracy.

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



Note: Refer to “Appendix C1: Unique Dual Index (UDI) Plate - WT Sequences” to demultiplex Whole Transcriptome sublibraries.

| Sublibrary Index ID | Well Position | i7 Forward Sequence (for sample sheet) | i5 Reverse Complementary Sequence | i5 Forward Sequence |
|---------------------|---------------|--|-----------------------------------|---------------------|
| UDI_EC_1 | A1 | AACAGATC | TGCCATGA | TCATGGCA |
| UDI_EC_2 | B1 | CCTGTCTT | CTAACGAT | ATCGTTAG |
| UDI_EC_3 | C1 | ATATCGAG | TTCCGGAA | TTCCGGAA |
| UDI_EC_4 | D1 | TAGTCCGC | TCTCCATA | TATGGAGA |
| UDI_EC_5 | E1 | TGCTGTTA | AGTGACGT | ACGTCACT |
| UDI_EC_6 | F1 | TAGCGAAT | CTCTGGCA | TGCCAGAG |
| UDI_EC_7 | G1 | AGGACCGT | AGAAGATG | CATCTTCT |
| UDI_EC_8 | H1 | TTATCAGG | TCTAACAG | CTGTTAGA |
| UDI_EC_9 | A2 | CCTCGGAA | TACGTAGA | TCTACGTA |
| UDI_EC_10 | B2 | TTGATCGA | TAAGCTCT | AGAGCTTA |
| UDI_EC_11 | C2 | TCAACCTC | TTGGTCGA | TCGACCAA |
| UDI_EC_12 | D2 | CGGAATAA | CGGTAATA | TATTACCG |
| UDI_EC_13 | E2 | TATGAGAC | CACAGTGG | CCACTGTG |
| UDI_EC_14 | F2 | CCTACCAT | AGACGCGA | TCGCGTCT |
| UDI_EC_15 | G2 | CATAGGCC | AGCAATGG | CCATTGCT |
| UDI_EC_16 | H2 | CACGATAA | CCTCTTGA | TCAAGAGG |

| Sublibrary Index ID | Well Position | i7 Forward Sequence (for sample sheet) | i5 Reverse Complementary Sequence | i5 Forward Sequence |
|---------------------|---------------|--|-----------------------------------|---------------------|
| UDI_EC_17 | A3 | TTCTGGCG | TTGTCTAG | CTAGACAA |
| UDI_EC_18 | B3 | TCGTAACT | TGTTGCTA | TAGCAACA |
| UDI_EC_19 | C3 | TCCGTGGT | TACTGTCA | TGACAGTA |
| UDI_EC_20 | D3 | AAGACAAC | AAGCCATA | TATGGCTT |
| UDI_EC_21 | E3 | ATTGCTTC | CGGCACTT | AAGTGCCG |
| UDI_EC_22 | F3 | AGAGAAGT | TCATGGAT | ATCCATGA |
| UDI_EC_23 | G3 | TACGTCTT | TAATCAGG | CCTGATTA |
| UDI_EC_24 | H3 | TCCACGTT | CATCTGAG | CTCAGATG |
| UDI_EC_25 | A4 | CAATGAGT | CAGAATCG | CGATTCTG |
| UDI_EC_26 | B4 | CGTCAGTT | TTGATGCA | TGCATCAA |
| UDI_EC_27 | C4 | TTCACTCC | ACGTATGG | CCATACGT |
| UDI_EC_28 | D4 | CTACCTGA | TGCGGATA | TATCCGCA |
| UDI_EC_29 | E4 | AGGTGATT | TAACAGCA | TGCTGTTA |
| UDI_EC_30 | F4 | TCTCACAT | AATGTTCG | CGAACATT |
| UDI_EC_31 | G4 | TTAGTGAG | TTACGTGT | ACACGTAA |
| UDI_EC_32 | H4 | TGTTCACT | ATCTACTG | CAGTAGAT |
| UDI_EC_33 | A5 | CGAAGCCT | ATACCTCT | AGAGGTAT |
| UDI_EC_34 | B5 | TGGCCGTA | TACATCTG | CAGATGTA |
| UDI_EC_35 | C5 | CGCTTCAC | AAGGAGCA | TGCTCCTT |
| UDI_EC_36 | D5 | CGGAGAAC | CTCCTAGA | TCTAGGAG |
| UDI_EC_37 | E5 | AAGTTCAG | TCAGCCTA | TAGGCTGA |
| UDI_EC_38 | F5 | AAGCCTTC | TTCGCTCA | TGAGCGAA |
| UDI_EC_39 | G5 | ACACCTCA | CGGATTAA | TTAATCCG |

| Sublibrary Index ID | Well Position | i7 Forward Sequence (for sample sheet) | i5 Reverse Complementary Sequence | i5 Forward Sequence |
|---------------------|---------------|--|-----------------------------------|---------------------|
| UDI_EC_40 | H5 | TTCTCCTA | ATTAGAGG | CCTCTAAT |
| UDI_EC_41 | A6 | TGACACGC | CGTGTGAA | TTCACACG |
| UDI_EC_42 | B6 | AAGCGCCT | TGTCACGG | CCGTGACA |
| UDI_EC_43 | C6 | TGATAACC | CGTAATCT | AGATTACG |
| UDI_EC_44 | D6 | AAGAGTGT | CCTTCTGG | CCAGAAGG |
| UDI_EC_45 | E6 | TGCTACCG | AATCGCTA | TAGCGATT |
| UDI_EC_46 | F6 | CCATCGTC | TGGACCAA | TTGGTCCA |
| UDI_EC_47 | G6 | CCACGAGA | TGTGCACT | AGTGCACA |
| UDI_EC_48 | H6 | ACGGTCAT | CTTATGGA | TCCATAAG |

Appendix D: Thermocycling Programs

Section 1: *In Situ* Cell Barcoding

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

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

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

| BARCODING ROUND 2 | | |
|-------------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 30 min | 50°C | 50 µL |
| Step | Time | Temperature |
| 1 | 30 min | 37°C |
| 3 | Hold | 4°C |

| ROUND 2 STOP | | |
|--------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 30 min | 50°C | 60 µL |
| Step | Time | Temperature |
| 1 | 30 min | 37°C |
| 3 | Hold | 4°C |

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

| BARCODING ROUND 3 | | |
|-------------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 30 min | 50°C | 60 µL |
| Step | Time | Temperature |
| 1 | 30 min | 37°C |
| 2 | Hold | 4°C |

| CELL LYSIS | | |
|------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 60 min | 80°C | 55 µL |
| Step | Time | Temperature |
| 1 | 60 min | 65°C |
| 2 | Hold | 4°C |

Section 2: cDNA Capture and Amplification

| cDNA TEMPLATE SWITCH | | |
|----------------------|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 90 min | 70°C | 100 µL |
| Step | Time | Temperature |
| 1 | 90 min | 42°C |
| 2 | Hold | 4°C |

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

A-TAILING Section 3: WT Sequencing Library Preparation

| SUBLIBRARY FRAGMENTATION, END REPAIR, AND A-TAILING | | |
|---|-----------------|---------------|
| Run Time | Lid Temperature | Sample Volume |
| 40 min | 70°C | 50 µL |
| Step | Time | Temperature |
| 1 | Hold | 4°C |
| 2 | 10 min | 32°C |
| 3 | 30 min | 65°C |
| 4 | Hold | 4°C |

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

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

Section 4: CRISPR Sequencing Library Preparation

| CRISPR PCR | | | |
|------------|-----------------|---------------|-------|
| Run Time | Lid Temperature | Sample Volume | |
| ~50 min | 105°C | 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 | Lid Temperature | Sample Volume | |
| ~30 min | 105°C | 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 E: 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.



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