

User Manual

Version 1.0 – UMCRS1010-WT4500



# CRISPR Detect for Evercode™ WT Mega v4

For use with

CRS1010

ECWT4500



Support Suite

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## Overview

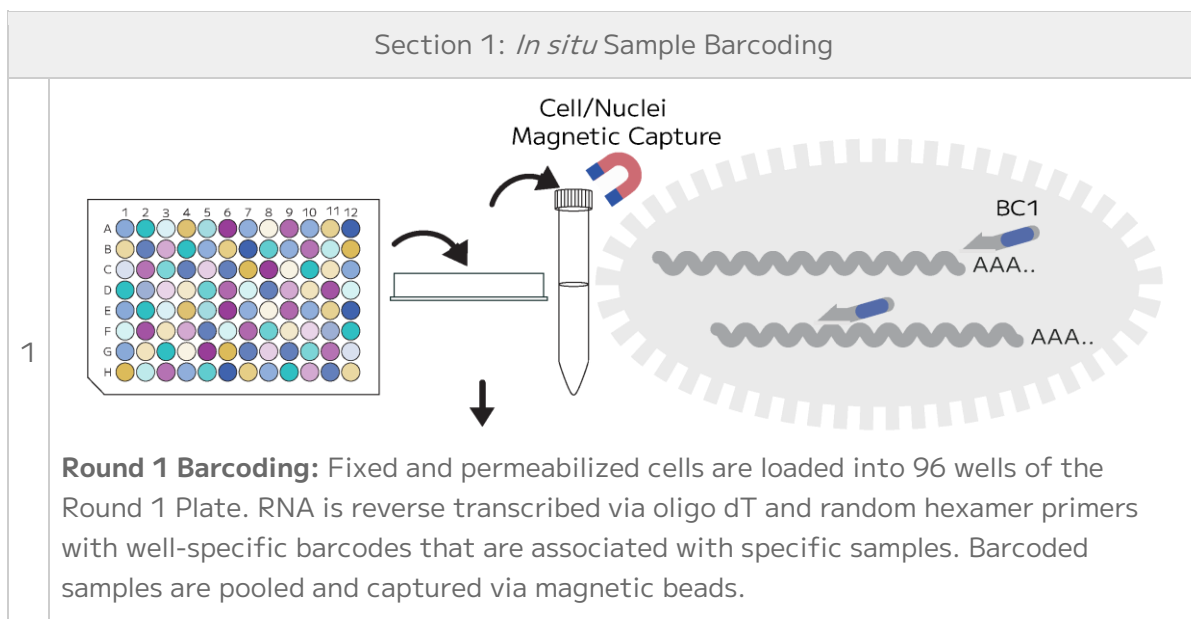
### Workflow

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 v4, paired sgRNA detection and whole transcriptome expression can be analyzed in up to 1,000,000 cells across up to 96 different biological samples or experimental conditions.

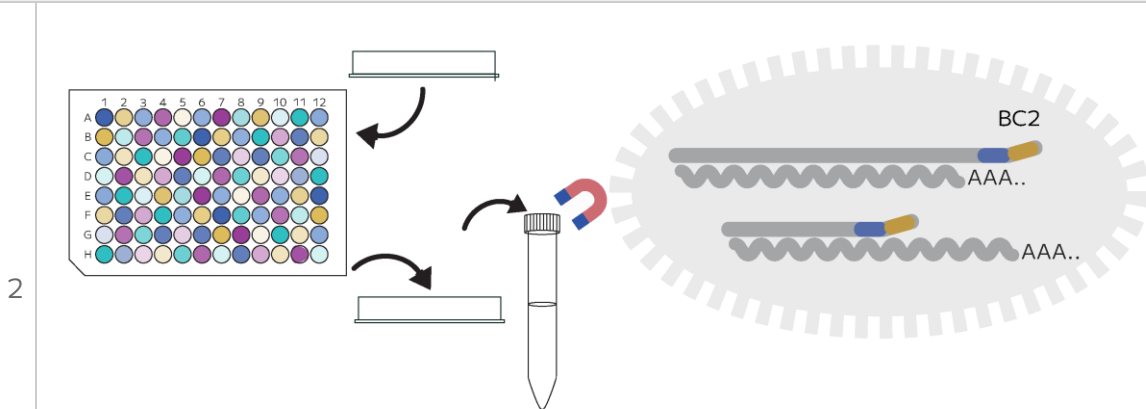
Evercode Cell Fixation kits convert the cells into individualized reaction compartments, thus avoiding the requirement for dedicated microfluidics hardware. Through three rounds of barcoding, the transcriptome of each fixed cell is uniquely labeled. In each round, pooled cells are randomly distributed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

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

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

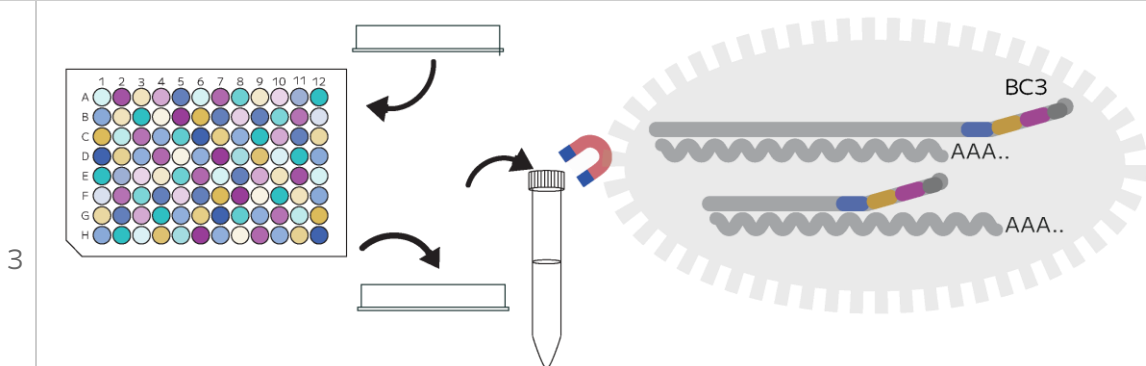


Section 1: *In situ* Sample Barcoding



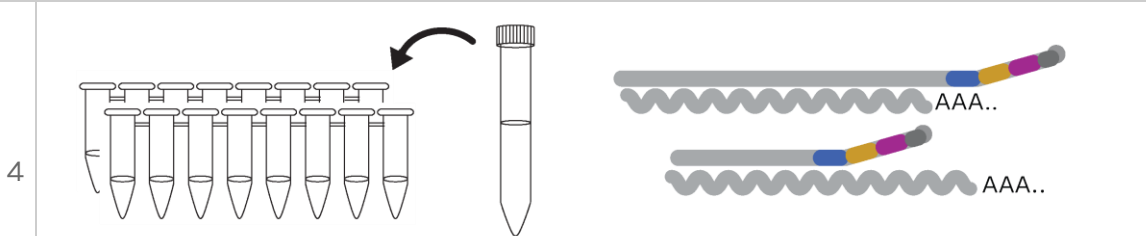
2

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



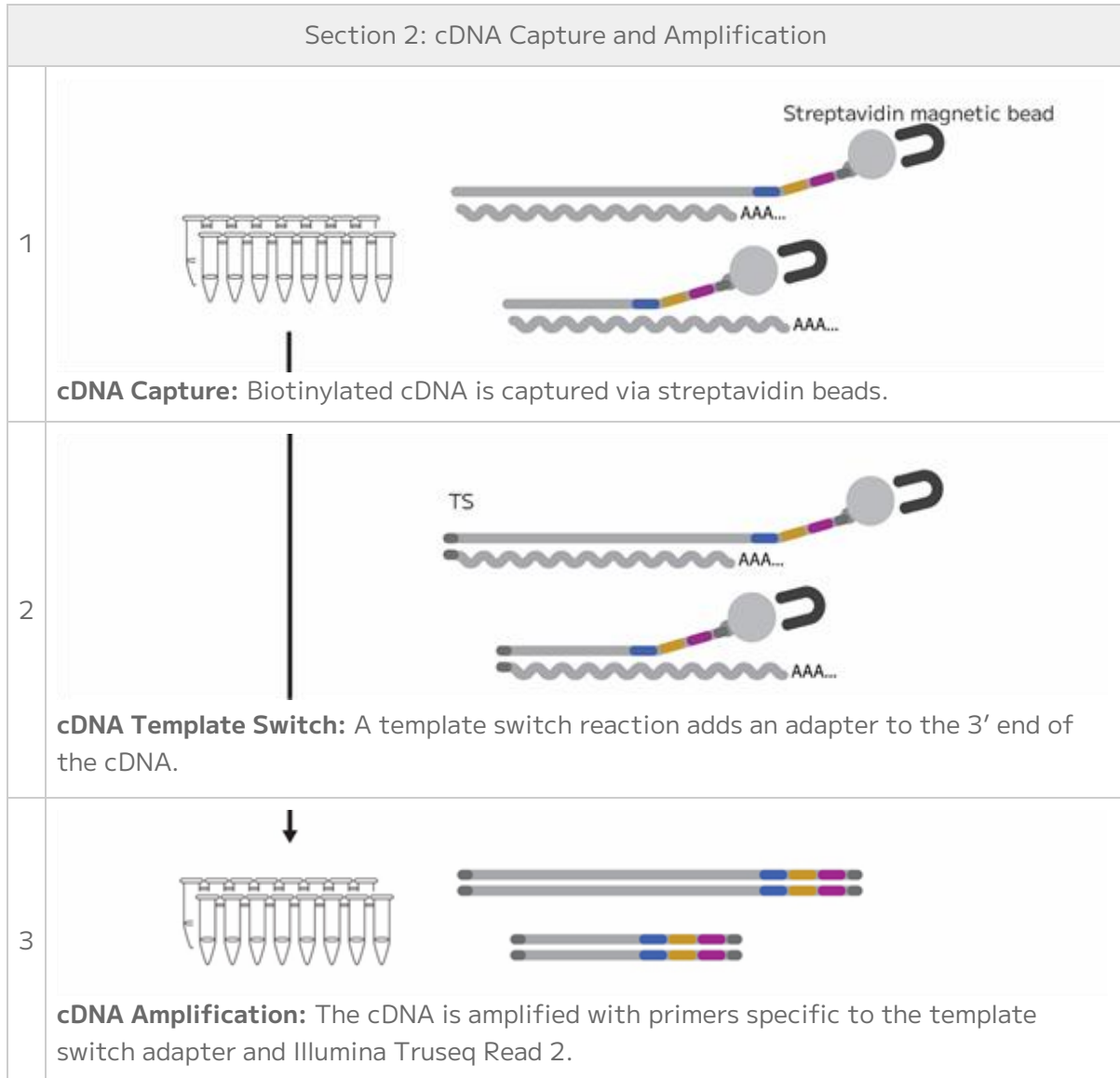
3

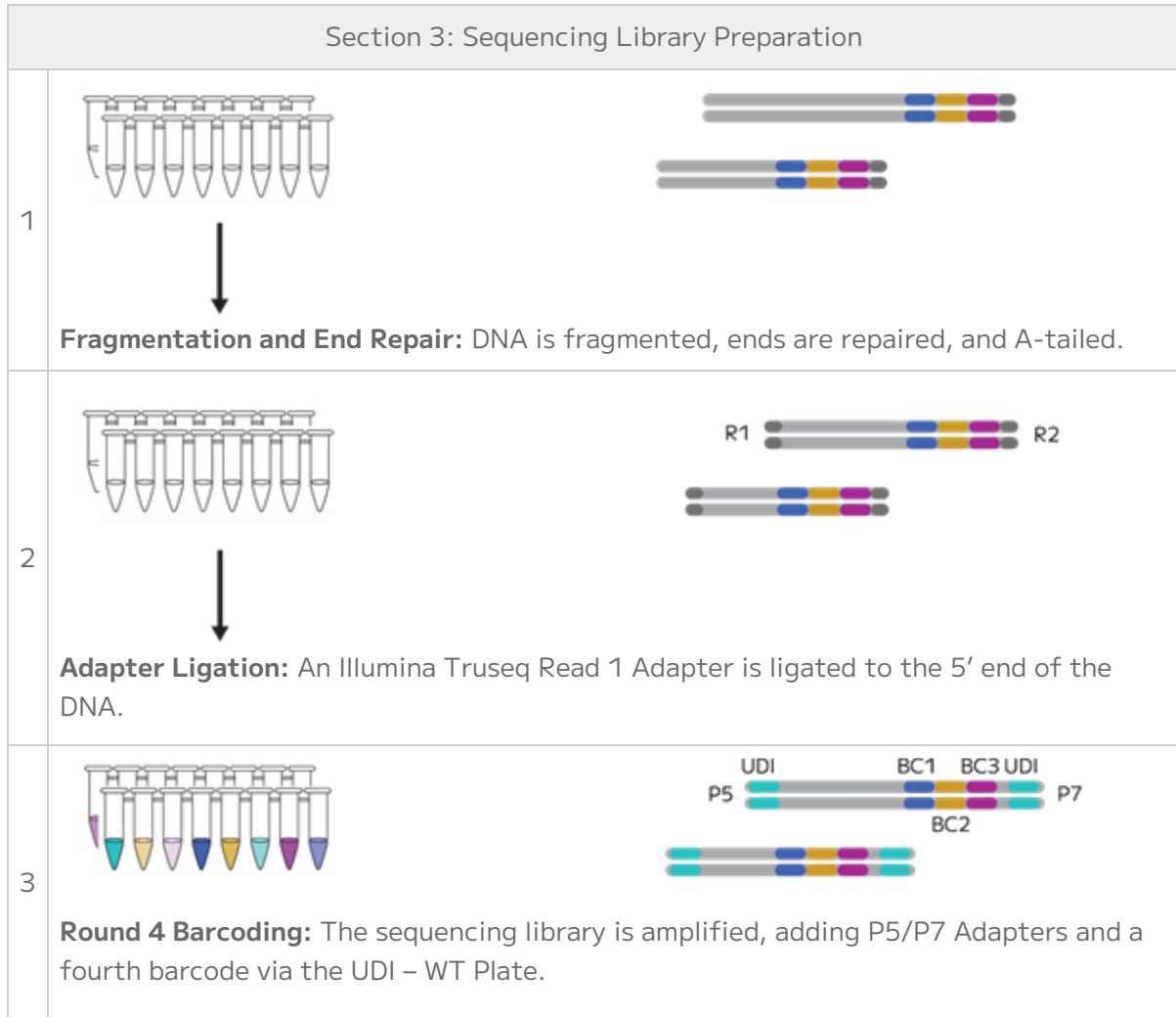
**Round 3 Barcoding:** The cells are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina Read 2 sequence, and biotin. Cells are pooled and then captured via magnet.

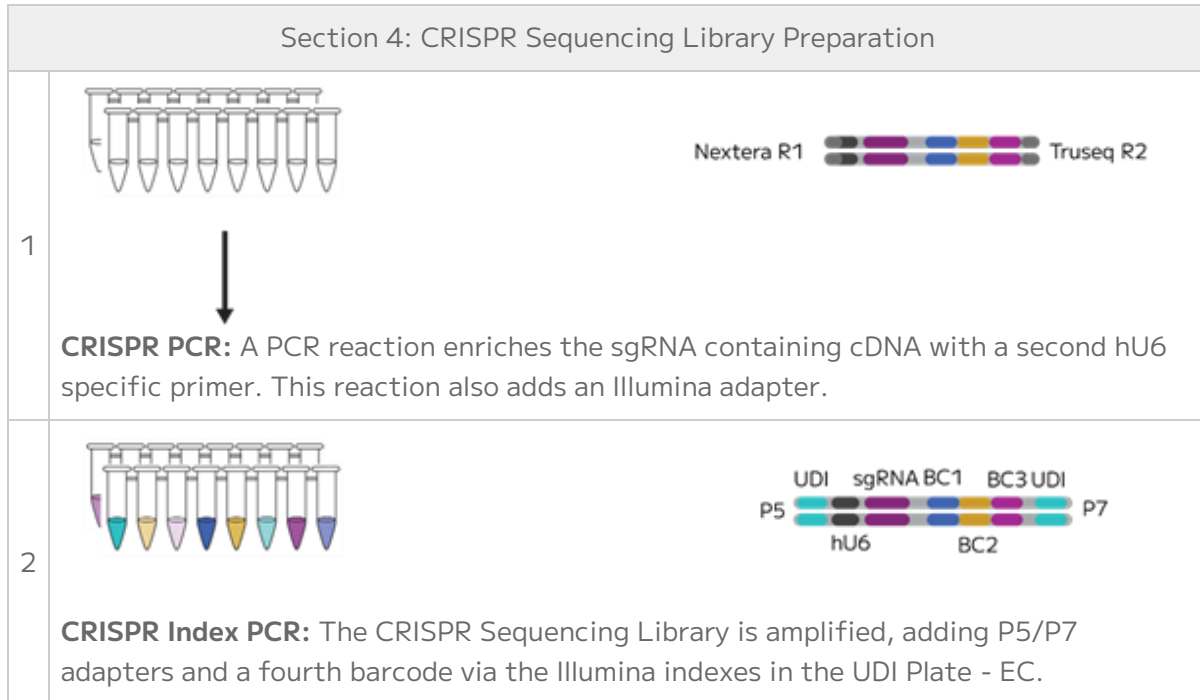


4

**Lysis and Sublibrary Generation:** Cells are split across 16 sublibraries and lysed.







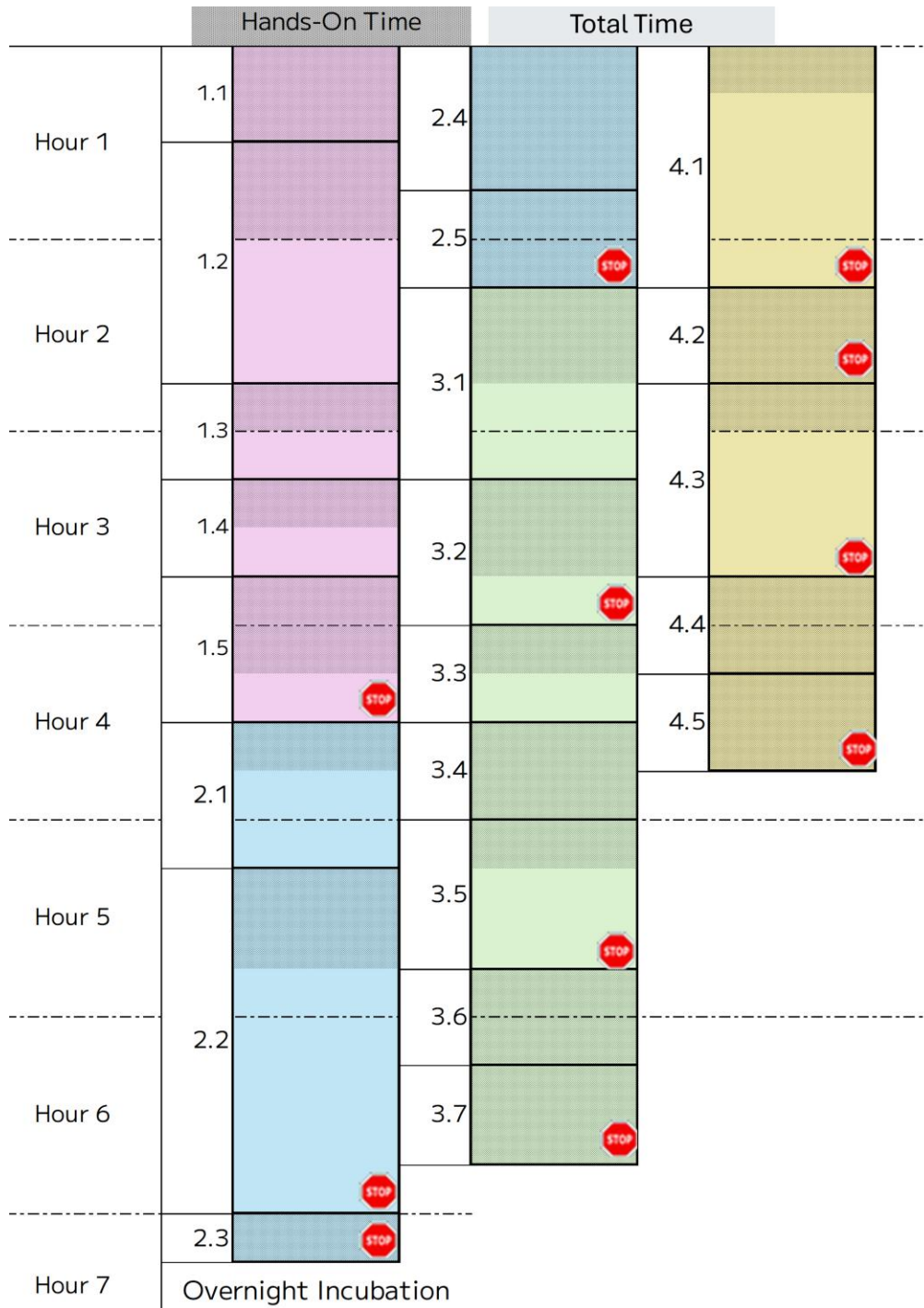
## Protocol Timing

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

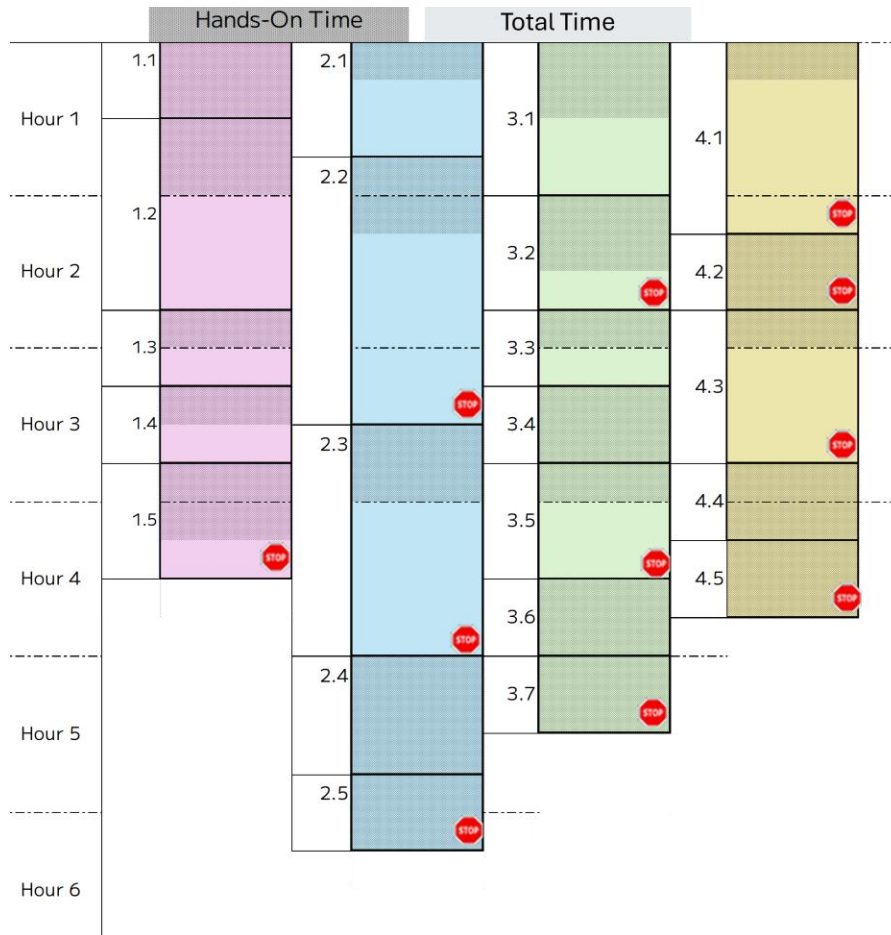
DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
Section 1: <i>In Situ</i> Cell Barcoding			
1.1 Set up and Sample Counting	Variable (30-90 min)	Variable (30-90 min)	
1.2 Barcoding Round 1	75 min	45 min	
1.3 Barcoding Round 2	30 min	15 min	
1.4 Barcoding Round 3	30 min	15 min	
1.5 Lysis and Sublibrary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

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

3-days workflow



4-days workflow



## Important Guidelines

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

### Sample Input

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

### Cell Counting and Quality Assessment

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

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

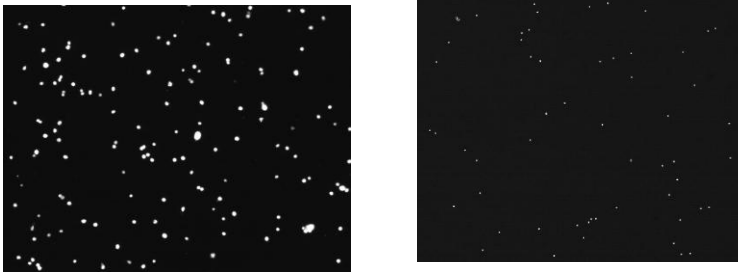


Figure 1: example of AO/DAPI stained HEK cells (left) and PBMCs (right).

High Quality Sample

Aggregation

Debris



Figure 2: Example of trypan blue stained fixed cells.

### Avoiding RNase Contamination

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

### Optimizing Cell Recovery

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

- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.
- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- The first time using an Evercode Whole Transcriptome kit, we recommend retaining supernatants after each barcoding step. In the unlikely event of unexpectedly high sample loss, these supernatants can be analyzed to identify points for optimization.

### Sample Loading Table

- The "Evercode WT Mega v4 Sample Loading Table RevA" should be completed before starting the experiment
- If the table isn't working as expected, ensure that Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in the table to avoid disturbing any calculations.
- In the unlikely event that samples are not concentrated enough according to the Sample Loading Table (Excel spreadsheet), choose to either:
  - Decrease the "Max number barcoded cells" until the Sample Loading Table no longer gives an error, which will maintain the desired proportion between samples but barcode fewer cells.
  - Add 14  $\mu$ L of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded cells but change the desired proportion between samples.

### Cell Strainers

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40  $\mu$ m is appropriate for many cell types, the mesh size should be chosen based on the sample type.
- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. An example video can be found in our support site.

### Vortexing

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

## Plate Sealing

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

## Magnetic Racks and Bead Cleanups

- The Evercode WT v4 workflow uses magnetic beads to clean up cells after each round of barcoding. A magnetic rack capable of holding 15 mL conical tubes and one capable of holding 1.5 mL tubes are needed throughout Section 1 of this protocol.
- A list of three validated 15 mL conical tube magnets are described in "User Supplied Equipment and Reagents" section of this manual to choose from.
- When aspirating supernatants in the magnetic rack, angle pipette tips away from beads. Ensure tips are at the bottom of each tube to ensure all supernatant is removed.
- If beads are disturbed, dispense solution again and wait for 1 minute before trying to aspirate again.
- The Parse Biosciences Magnetic Rack uses powerful rare earth magnets for rapid and efficient magnetic bead purifications for 0.2 mL tubes. The rack has high and low magnet positions important for optimal yield at key steps. We do not recommend substituting alternative racks.
- To alternate between the positions, the rack can be flipped upside down so the magnet is closer to the top (high) or bottom (low) of the 0.2 mL tubes. See the figure below for clarification

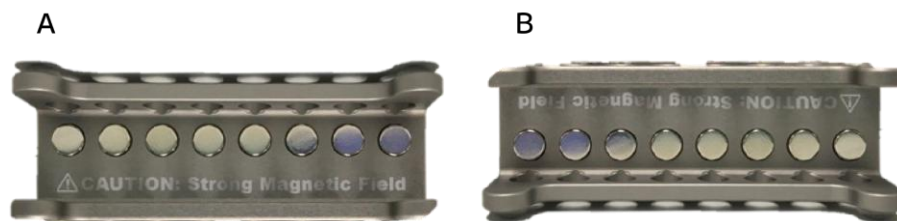


Figure 3: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- To ensure material is not lost during bead purifications, ensure supernatants are completely clear before moving to the next step. The incubation times at each step are recommendations, but visual confirmation of clearing should be used to make the final determination. See the figure below for an example of completely cleared supernatants.

Discarding any beads in supernatants will result in a reduction of transcripts and genes detected per cell.



Figure 4: Clear supernatant with compact bead pellets.

### Sublibrary Loading

- This Evercode WT Mega kit generates 16 sublibraries with distinct Illumina indexing barcodes can be processed and sequenced independently or concurrently after Section 1 of the protocol.
- The number of cells per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix A.
- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.
- Sublibraries can be loaded with different numbers of cells, and the maximum number of cells that can be analyzed is the sum of cells across all sublibraries.
- Asymmetric sublibrary loading can enable cost-effective sequencing quality control. One sublibrary can be loaded with a few hundred cells and sequenced very deeply. This data can be used to choose an appropriate sequence depth for the remaining sublibraries.

### Indexing Primers

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

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

### Thermocycling Programs

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

## Part List

Safety Data Sheets for these reagents can be provided upon request. This manual requires the use of Evercode WT Mega v4 barcoding kit. Part list for the appropriate WT kit are in manual Evercode WT Mega v4, UMWT4500. Note that the Evercode WT Mega reagent "PCR Additive" will not be used in the CRISPR workflow.

**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.
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.
15 mL Conical Tube Magnet (choose one)	Permagen	MSR6X15	6 X 15 mL Centrifuge Magnetic Separation Rack.
	Millipore	LSKMAGS15	PureProteome Magnetic Stand.
	STEMCELL Technologies	18103	EasyEights™ EasySep™ Magnet.
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 96 well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL and 0.2 mL tubes.
Water bath	Various Suppliers	Varies	Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. We recommend validating alternatives relative to a hemocytometer.
Fluorescence Cell Counter	Various Suppliers	Varies	
PCR tube rack	Various Suppliers	Varies	Capable of holding semi-skirted 96 well PCR plates and a tight fitting lid.
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.

ITEM	SUPPLIER	PN	NOTES
Single Channel Pipettes: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.
T100 Thermal Cycler	Bio-Rad Laboratories®	1861096	Or an equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM.
6-inch Platform	Scientific Industries	146-6005-00	
Microplate Foam Insert	Scientific Industries	504-0235-00	
Qubit™ Flex Fluorometer	Thermo Fisher Scientific®	Q33327	
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.
4200 TapeStation System	Agilent	G2991BA	
Serological Pipette Controller	Various Suppliers	Varies	Optional.

### Consumables

ITEM	SUPPLIER	PN	NOTES
Evercode WT Mega v4	Parse Biosciences	ECWT4500	
UDI Plate - WT	Parse Biosciences	UDI1001	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode Whole Transcriptome kits (48 sublibraries). UDI Plate - WT can be purchased separately or bundled with Evercode Whole Transcriptome kits.
UDI Plate - EC	Parse Biosciences	UDI1002	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple CRISPR sequencing libraries (48 sublibraries).

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

## Reagents

ITEM	SUPPLIER	PN	NOTES
SPRIselect Reagent	Beckman Coulter®	B23317 (5mL) B23318 (60mL)	Choose one. We do not recommend substituting other magnetic beads.
AMPure® XP Reagent	Beckman Coulter	A63880 (5 mL) A63881 (60mL)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	
MAGFLO NGS Size Selection	INTEGRA	7000 (1 mL) 7002 (5 mL)	
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Ethyl alcohol, Pure	Sigma-Aldrich	459844	Or equivalent ethanol.
TE Buffer pH 8.0	Various Suppliers	Varies	Optional. Choose one that is RNase-free.
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
AO/PI	Various Suppliers	Varies	Or alternative viability dyes.
Qubit dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific	Q33230 (100 assays) Q33231 (500 assays)	Or equivalent DNA quantifier.
High Sensitivity DNA Kit	Agilent	5067-4626	Choose one that corresponds to the chosen Bioanalyzer or TapeStation.
High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	
High Sensitivity D1000 ScreenTape and Reagents	Agilent	5067-5584 (screen tape) 5067-5585 (sample buffer and ladder)	

## Section 1: In Situ Cell Barcoding

### 1.1. Set up and Sample Counting

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






#### To set up for barcoding:

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



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

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

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

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

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

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

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

13. Proceed immediately to Section 1.2.

## 1.2. Barcoding Round 1

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

### To add round 1 barcodes:

1. Gently remove the Round 1 Plate from the thermocycler, place in a 0.2 mL tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.
2. Remove the Round 1 Plate from the centrifuge, place in a PCR tube rack on a flat surface, remove the plate seal, and store on ice.



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

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



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



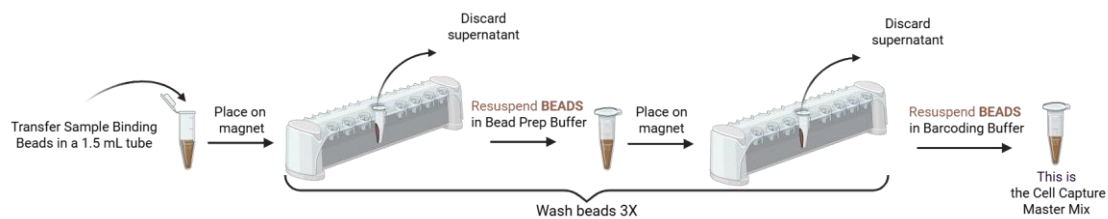
**Note:** When pipetting the same sample into many wells, the sample must be mixed by gentle pipetting prior to each transfer to avoid cells settling. Do not vortex the samples.

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

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

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

- If using **low input samples**, the cells are already attached to the binding beads and don't need further washing. **Proceed directly to step 16.**
- If using **standard fixation samples**, proceed to the next step to prepare the ● Sample Binding Beads to capture the cells. See workflow below.



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

8. While the thermocycling program is still running, gently pulse-vortex ● Sample Binding Beads to resuspend and store at room temperature. Do not let them settle for >3 minutes before pipetting. Transfer **400 µL** of ● Sample Binding Beads to a 1.5 mL Eppendorf tube.
9. Place the tube on the magnetic rack compatible with 1.5 mL tubes and wait for the solution to clear (~2 minutes).
10. While still on the magnet, remove and discard the supernatant.
11. Remove the tube from the magnetic rack and fully resuspend the bead pellet in **400 µL** of ● **Bead Prep Buffer**.
12. Place the tube on the magnetic rack until the solution clears (~2 minutes).
13. While still on the magnet, remove and discard the supernatant.
14. Repeat steps 11-13 twice for a total of 3 washes.
15. Remove the tube from the magnetic rack. Fully resuspend the pellet in **400 µL** of ○ **Barcoding Buffer** and store on ice. This is your Cell Capture Master Mix.



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

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

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

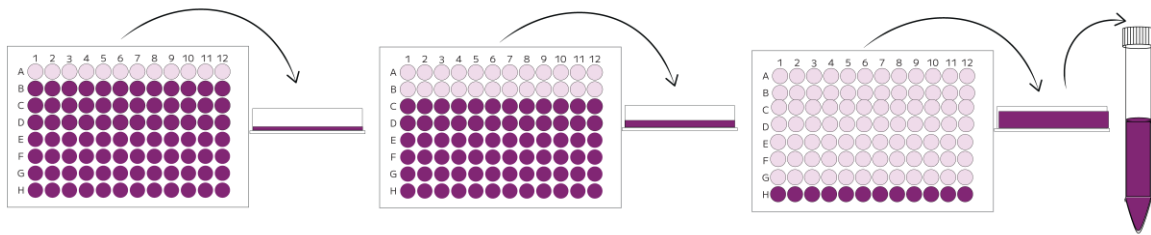
17. Mix the Ligation Master Mix by inverting the tube 10x. Store on ice.



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

18. Remove the Round 1 Plate from the thermocycler, place it in a PCR tube rack on the benchtop. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 1 Plate on ice.
19. Transfer all the ● Round 1 Stop Mix into a new basin with a P1000.

20. With a 12-channel multi-pipette transfer **10  $\mu\text{L}$**  of ● Round 1 Stop Mix into Row A of the Round 1 Plate, still keeping the plate on ice.
- Repeat step 20, **7 more times** using the same pipette tips for Rows B-H to dispense the Round 1 Stop Mix into all wells of the Round 1 Plate.
  - With a multichannel P200 set to 50  $\mu\text{L}$ , mix the sample in row A by pipetting 3x.
  - Transfer **50  $\mu\text{L}$**  from **row A** into a clean basin (bubbles will be generated, this will not impact experimental results).
  - Repeat b-c for **rows B-H**.
  - Once cells from all wells are in the basin, using a P1000 transfer the mix into a 15 mL conical tube. Keep on ice.



21. **If using low input fixation samples**, cells have been already captured. Place the 15 mL conical tube **magnet on ice** and wait **1 minute** to let it pre-cool. **Then proceed directly to step 27.**
22. **If using standard fixation samples**, proceed to the next step to capture the cells.
23. Resuspend any settled beads in the Cell Capture Master Mix from step 15 by pipette mixing.
24. Using a P1000, transfer **384  $\mu\text{L}$**  of the Cell Capture Master Mix to the pooled Round 1 cells. Pipette mix 10x with the P1000 set to 900  $\mu\text{L}$ .
25. Incubate the cells on ice for **5 minutes** to allow beads to bind.



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

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

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

28. With a P1000 set to 1000  $\mu$ L or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids. Holding the tube in the magnet will ensure the tube is kept from moving.
29. With the tube still on the magnet, add **5 mL** of **O Barcoding Buffer**. Do not resuspend. Incubate on ice for **1 minute** before removing and discarding the supernatant with a P1000 or serological pipette.
30. Remove tube from magnet and resuspend the beads-bound cells in **4.2 mL** of Ligation Master Mix. Using a P1000 set to 1000  $\mu$ L, pipette mix 10x to fully resuspend the beads into the Ligation Master Mix. Keep the sample on ice before proceeding to the next step.



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

### 1.3. Barcoding Round 2

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

#### To add Round 2 barcodes:

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

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

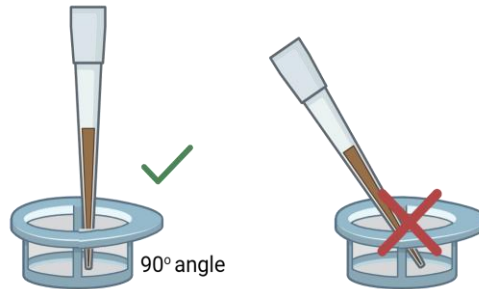
2. Place the Round 2 Plate into a thermocycler and run the following program. Proceed immediately to the next step while the program is still running.

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

3. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.
4. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate and store on ice.
5. Pipette all of the sample in Ligation Master Mix (**4.2 mL**) through a cell strainer into a new basin with a P1000.



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



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



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



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

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

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

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

9. Briefly vortex the ● Round 2 Stop Mix and ensure there is no precipitate. Transfer **1 mL** of this tube to a new basin with a P1000.
10. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.
11. With the Round 2 Plate on ice and the basin on the bench, transfer the contents of the Round 2 Barcoding plate into the ● Round 2 Stop Mix as follows:
- With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.
  - Transfer the entire content (**50 µL**) from row A to the basin containing ● Round 2 Stop Mix and mix 2x.
  - Repeat steps a-b for **rows B-H** to mix the sample then transfer to the basin. The same pipette tips may be used while pooling.
12. Transfer the Round 2 pool into a 15 mL conical tube and apply to the magnet. Loosen or remove the cap to avoid a rotational motion may cause the pellet to shift. Keep the magnet on ice. Allow beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear.
13. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.
14. Keep the 15 mL conical tube on the magnet on ice and with a P1000 set to 1000 µL or a 5 mL serological pipette, add **5 mL** of the ○ Barcoding Buffer to rinse the pellet. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound.






15. Remove and discard all the supernatant with a P1000 set to 1000  $\mu$ L or a 5 mL serological pipette.
16. Remove tube from magnet and resuspend the beads with **4.2 mL** of the Ligation Master Mix. Store the sample on ice and proceed to section 1.4.

## 1.4. Barcoding Round 3

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

### To add round 3 barcodes:

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

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

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

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

- Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.
- While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 3 Plate.
- Pipette all of the sample in Ligation Master Mix (**4.2 mL**) through a cell strainer into a new basin with a P1000.



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

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



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



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

7. While secured in a PCR tube rack on a flat surface, add a new plate seal to Round 3 Plate.
8. Place the Round 3 Plate into a thermocycler and run the following program.

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

9. Briefly vortex the **OFinal Stop Mix** and ensure there is no precipitate. Transfer **2 mL** to a new basin with a P1000.
10. Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.
11. With the Round 3 Plate on ice and the basin on the bench, transfer all the liquid in the Round 3 Plate into the basin with the Final Stop Mix as follows:
  - a. With a multichannel P200 set to 50 µL, mix the sample in row **A** by pipetting 3x.
  - b. Transfer **50 µL** from row **A** to the basin and mix 2x.
  - c. Repeat steps a-b for rows **B-H** to mix the sample then transfer to the basin.
12. Pipette the pooled sample through a cell strainer into a new 15 mL tube with a P1000. Before each transfer, gently mix the cells in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.
13. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind to the magnet for **5 minutes**, or until supernatant is clear.

14. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000  $\mu$ L.
15. Remove the 15 mL tube from the magnet and fully resuspend the cells in **4 mL** of **O Pre-Lysis Wash Buffer**. Do not invert.
16. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind for **5 minutes**, until supernatant is clear.
17. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000  $\mu$ L or serological pipette.
18. Remove the tube from the magnet and resuspend the cells in **200  $\mu$ L** of **● Pre-Lysis Dilution Buffer**.



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

19. Proceed to Lysis and Sublibrary Generation Step 1.5.

## 1.5. Lysis and Sublibrary Generation

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

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



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

2. Decide how to divide cells across the 16 sublibraries. See the “Sublibrary Loading” in the Important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix A to determine the volume of sample and ●Pre-Lysis Dilution Buffer that should be added to each sublibrary.



**CRITICAL!** Do not add more than 62,500 cells to a sublibrary. Adding additional cells will result in an increased multiplet rate.

3. Ensure the cells are in suspension by pipetting 5x with a P200 set to 200  $\mu\text{L}$  prior to each transfer. Add the appropriate volume of sample to 16 different 0.2 mL PCR tubes.
4. Keeping the samples on ice, add the appropriate volume of ●Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of **25  $\mu\text{L}$** .
5. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P1000 set to 425  $\mu\text{L}$ . Store at room temperature.

LYSIS MASTER MIX	
● Lysis Solution	440 $\mu\text{L}$
● Lysis Enzyme	88 $\mu\text{L}$
Total	528 $\mu\text{L}$



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



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

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



- Vortex the 0.2 mL tube(s) for **1 minute**. Briefly centrifuge.
- Place the tube(s) into a thermocycler and run the following program. If continuing to Section 2 without freezing the sample, proceed to Section 2 while the program is still running.

CELL LYSIS		
Run Time	15 minutes	
Lid Temperature	80°C	
Sample Volume	55 $\mu$ L	
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C

- Remove samples from the thermocycler and place the tubes on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
- While still on the magnetic rack, transfer **55  $\mu$ L** of the **supernatant** containing lysed cells into new 0.2 mL tubes.



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

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



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

## Section 2: cDNA Capture and Amplification

### 2.1. cDNA Capture

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



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

#### To capture the cDNA:

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

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

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

Lysates being processed	1	16
● Streptavidin Beads	66 µL	1056 µL

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

Lysates being processed	1	16 $\mu$ L
○ Bead Wash Buffer	50 $\mu$ L	800 $\mu$ L



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

8. Place the tube on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).
9. Remove and discard the supernatant.
10. Repeat steps 7-9 twice for a total of 3 washes.
11. Remove the tube from the magnetic rack. Fully resuspend the pellet in the appropriate volume of ● Binding Buffer as follows and store at room temperature.

Lysates being processed	1	16
● Binding Buffer	55 $\mu$ L	880 $\mu$ L

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

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

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



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

14. Briefly centrifuge the lysates and store at room temperature.
15. Add **50 µL** of ● Streptavidin Beads in ● Binding Buffer to each tube of lysate.
16. Place the tube(s) into a 96 well PCR tube rack, press to secure, and ensure the caps are secured tightly. Place the lid on the rack.
17. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex plastic holder on 100% power for **1 minute**.
18. Vortex on 20% power (~800-1000 RPM) for **30 minutes** at room temperature.



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

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

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



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

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

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



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

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

25. Proceed immediately to Section 2.2.

## 2.2. cDNA Template Switch

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



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

### To perform template switch:

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

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



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

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

TEMPLATE SWITCH MASTER MIX		
Number of Samples	1	16
● Template Switch Buffer	87 µL	1392 µL
● Template Switch Primer	3 µL	48 µL
● Template Switch Enzyme	6 µL	96 µL
Total	96 µL	1536 µL

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

4. While still on the magnetic rack, remove and discard the supernatant.
5. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125  $\mu$ L O Wash Buffer B** by vortexing for **10 seconds** (100% power).



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

6. Place the tube(s) on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the **O Wash Buffer B**.
8. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **20  $\mu$ L Nuclease-Free water** by vortexing for **10 seconds**, followed by a brief centrifugation.
9. Add **80  $\mu$ L** of the Template Switch Master Mix to each resuspended pellet and mix 5x with a P200 set to 80  $\mu$ L.



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

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

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

14. Proceed immediately to Section 2.3. Alternatively, proceed to step 15 to store samples prior to cDNA amplification.
15. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).



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

16. While still on the magnetic rack, remove and discard the supernatant.
17. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125  $\mu$ L O Wash Buffer A** by vortexing for **10 seconds** (100% power).



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

## 2.3. cDNA Amplification

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



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

### To amplify the cDNA:

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

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

2. Equilibrate **80 µL** of SPRI beads per lysate to room temperature. Immediately proceed to step 3 after placing SPRI beads at room temperature for later use.
3. Prepare the cDNA Amplification Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 10x and store on ice.

cDNA AMPLIFICATION MASTER MIX		
Number of Sublibraries	1	16
● cDNA Amp Mix	60.5 µL	968 µL
● cDNA Amp Primers	60.5 µL	968 µL
● Human U6 Primer 1	2.4 µL	38.4 µL
Total	123.4 µL	1,974.4 µL

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



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

5. While still on the magnetic rack, remove and discard the supernatant.
6. While still on the magnetic rack, add **125 µL** of **○** Wash Buffer B to each tube.
7. Incubate for **1 minute** at room temperature.
8. While still on the magnetic rack, remove and discard the **○**Wash Buffer B.
9. Remove tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 µL** of the cDNA Amplification Master Mix. Store on ice.
10. Determine the number of PCR cycles required for cDNA amplification based on the table below. Although these recommendations are appropriate for many cell types, the number of cycles may need to be optimized for each sample type.

NUMBER OF PCR CYCLES		
Cells in Sublibrary	High RNA content such as cell lines	Low RNA content such as PBMCs
200-1,000	11	13
1,000-2,000	9	11
2,000-5,000	7	9
5,000-12,500	6	8
12,500-25,000	4	6
25,000-62,500	3	5

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

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



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



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



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

## 2.4. Post-Amplification Purification

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



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

### To purify the cDNA:

1. Gather **400  $\mu\text{L}$**  of freshly prepared 85% ethanol for each tube of amplified cDNA.
2. Gather room temperature SPRI beads (**80  $\mu\text{L}$**  per tube of amplified cDNA).



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

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



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

4. While still on the magnetic rack, transfer **90  $\mu\text{L}$**  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  $\mu\text{L}$**  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  $\mu\text{L}$**  of 85% ethanol to each tube.
11. Incubate for **1 minute** at room temperature.
12. While still on the magnetic rack, remove and discard the supernatant.

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

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



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

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

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

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



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

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



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

## 2.5. cDNA Quantification

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

### To quantify the cDNA:

1. Measure the concentration of each tube of purified cDNA from Section 2.4 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions. Record the concentration(s), which will be used in Section 3.
2. Assess the size distribution of each tube of purified cDNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D5000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

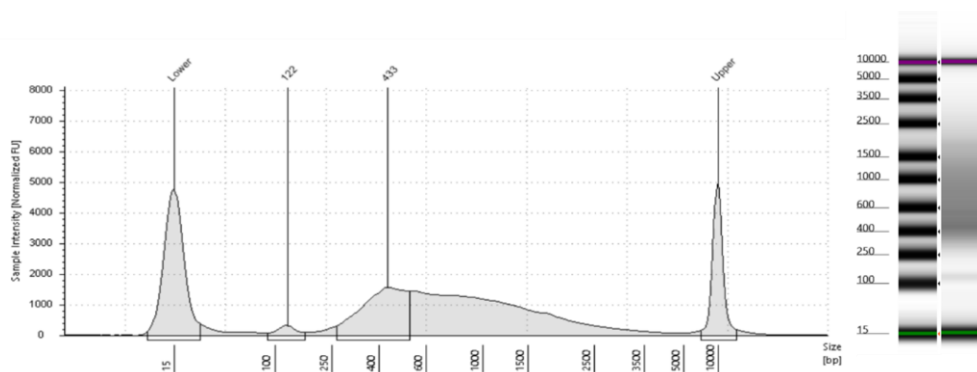


Figure 5: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a TapeStation.

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



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

## Section 3: WT Sequencing Library Preparation

### 3.1. Fragmentation and End Prep

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



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

To prepare for fragmentation and end prep:

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

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

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

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



**Note:** A recommended input of 5 ng of cDNA is needed for CRISPR library preparation in Section 4. If you have less than 105 ng of cDNA for a given sublibrary, less cDNA can

be aliquoted towards the Whole Transcriptome library to ensure sufficient cDNA material for Section 4. Successful Whole Transcriptome libraries can be prepared from as little as 10 ng of cDNA. Record the amount added to each tube as subsequent PCR cycles be adjusted based on cDNA concentration.

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

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



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

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



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

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

FRAGMENTATION AND END PREP MASTER MIX		
Number of Sublibraries	1	16
● Fragm/End Prep Buffer	6 µL	96 µL
● Fragm/End Prep Enzymes	12 µL	192 µL
Total	18 µL	288 µL

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



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

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

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

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



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

### To size select the fragmented and end prepped DNA:

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



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

3. Vortex the SPRI beads until fully mixed. Add **30  $\mu\text{L}$**  of SPRI beads to each tube of fragmented and end prepped DNA.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

7. While still on the magnetic rack, transfer **75  $\mu\text{L}$**  of the supernatant containing the fragmented and end prepped DNA into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **20  $\mu\text{L}$**  of SPRI beads to each tube.
9. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~3 minutes).



**CRITICAL!** Ensure the solution is completely clear before proceeding.

12. While still on the magnetic rack, remove and discard the supernatant.
13. While still on the magnetic rack, add **180  $\mu\text{L}$**  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  $\mu\text{L}$**  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  $\mu\text{L}$**  of the supernatant into new 0.2 mL tube(s).



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

### 3.3. Adapter Ligation

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



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

#### To ligate adapters:

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

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

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

ADAPTER LIGATION MASTER MIX		
Number of Sublibraries	1	16
Nuclease-free water	19.25 µL	308 µL
● Adapter Ligation Buffer	22 µL	352 µL
● Adapter Ligation Enzyme	11 µL	176 µL
● Ligation Adapter	2.75 µL	44 µL
Total	55 µL	880 µL

3. Add **50  $\mu$ L** of Adapter Ligation Master Mix to each tube of purified fragmented and end prepped DNA from Section 3.2. Mix by pipetting 10x with a P200 multichannel pipette set to 80  $\mu$ L. Briefly centrifuge.
4. Place the tube(s) into a thermocycler and run the program below.

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



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

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

### 3.4. Post-Ligation Purification

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



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

#### To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~**100  $\mu$ 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  $\mu$ L** of SPRI beads to each tube of adapter ligated DNA from Section 3.3.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180  $\mu$ 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  $\mu$ 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  $\mu$ L** of the supernatant containing the purified adapter ligated DNA into new 0.2 mL tube(s). Store on ice.
17. Proceed immediately to Section 3.5.

### 3.5. Barcoding Round 4

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



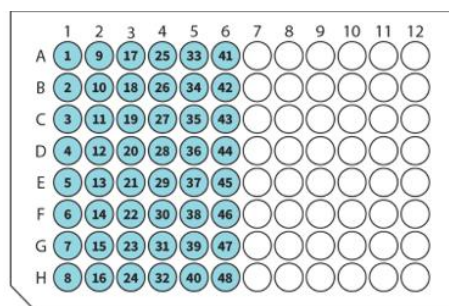
**CRITICAL!** Always use new tips for each UDI Plate's well and corresponding sublibrary.

#### To add round 4 barcodes:

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



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



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

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



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

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

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

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

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



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



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

### 3.6. Post-Barcoding Round 4 Size Selection

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



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

#### To size select the sequencing libraries:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~**50  $\mu$ 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 **30  $\mu$ L** 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  $\mu$ L** of the supernatant containing the DNA into a new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **10  $\mu$ L** of SPRI beads to each tube.
9. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~3 minutes).



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

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

13. While still on the magnetic rack, add **180  $\mu$ L** 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  $\mu$ L** of nuclease-free water or TE Buffer.
19. Incubate for **5 minutes** at room temperature.
20. Place the tube(s) on the low position of the magnetic rack. Incubate until the solution clears (~2 minutes).
21. While still on the magnetic rack, transfer the supernatant into a new 0.2 mL tube(s). Store on ice.



Safe stopping point: Sequencing libraries can be stored at  $-20^{\circ}\text{C}$  for up to 3 months.

### 3.7. Sequencing Library Quantification

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

#### To quantify the sequencing libraries:

1. Measure the concentration of each purified sequencing library from Section 3.6 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer’s instructions.
2. Assess the size distribution of each purified sequencing library with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D1000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer’s instructions.

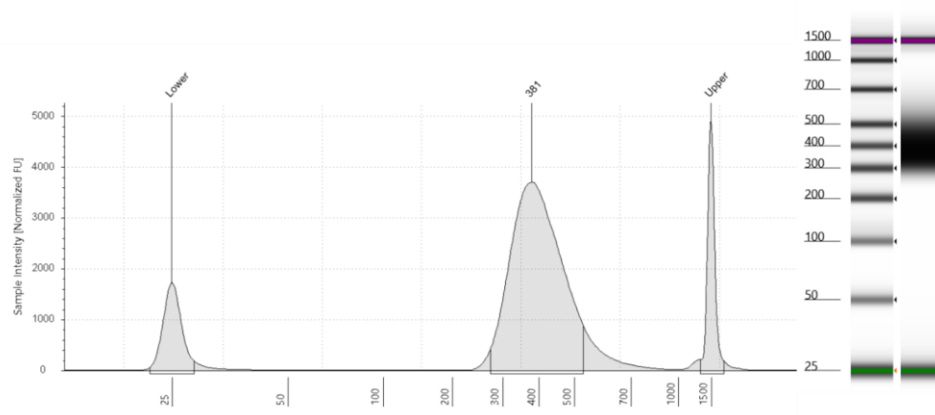


Figure 6: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

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

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



**Note:** If using a Bioanalyzer, there may be an additional peak present. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 300-450 bp). Do not use this additional peak when estimating amplicon size.

## Section 4: CRISPR Sequencing Library Preparation

### 4.1 CRISPR PCR

A PCR reaction further enriches sgRNA containing transcripts.

#### To amplify the sgRNA transcripts:

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

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

7. Vortex the tube(s) of amplified cDNA for **2-3 seconds**. Briefly centrifuge.

8. Prepare diluted cDNA in a new 0.2 mL tube(s) as follows and store on ice. Bring the total volume up to 21  $\mu\text{L}$  using nuclease-free water. Store any remaining cDNA at  $-20^{\circ}\text{C}$ .

DILUTED cDNA	
Sublibrary cDNA	50 ng
Nuclease-free water	Variable
Total Volume	21 $\mu\text{L}$

**Note:** If the concentration of cDNA is higher than 25 ng/ $\mu\text{L}$ , a dilution is recommended to ensure sufficient volume is used while pipetting (at least 2  $\mu\text{L}$  used).



**Note:** If you have less than 50 ng of cDNA after Whole Transcriptome Library Preparation for a given sublibrary, use all remaining cDNA at this step. We recommend a minimum amount of 5 ng/ $\mu\text{L}$ . If the input is greater than 5 ng/ $\mu\text{L}$ , 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	16
● CRISPR Amplification Mix	27.5 $\mu\text{L}$	440 $\mu\text{L}$
● Universal Primer	2.2 $\mu\text{L}$	35.2 $\mu\text{L}$
● Human U6 Primer 2	2.2 $\mu\text{L}$	35.2 $\mu\text{L}$
Total Volume	31.9 $\mu\text{L}$	510.4 $\mu\text{L}$

10. Add **29  $\mu\text{L}$**  of the CRISPR PCR Reaction Solution to each sublibrary. Mix sublibraries 10x with a P200 pipette set to 40  $\mu\text{L}$ . Briefly centrifuge.

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



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

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



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



**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  $\mu$ 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 **30  $\mu$ L** 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  $\mu$ L** 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  $\mu$ L** 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  $\mu$ L** 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  $\mu$ 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  $\mu$ L** 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



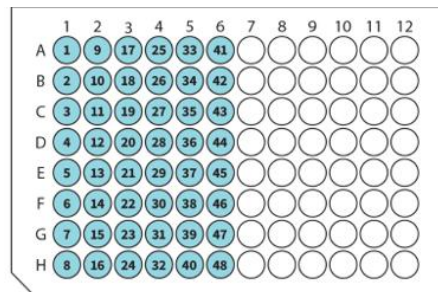
**Note:** If you have less than 10 ng of input for a given sublibrary, add the entire amount of cDNA at this step. This is unlikely to affect the library quality. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on cDNA concentration (See Appendix D for more 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.
6. Orient the UDI Plate - EC with the notch on the bottom left as pictured below. For each sublibrary being processed, choose one unused well of the UDI Plate - EC and record the well position and number for each sublibrary.



7. With a multichannel P20, pierce the seal of the chosen wells of the UDI Plate - EC.
8. With a multichannel P20 and new tips, mix by pipetting 5x then immediately transfer **4  $\mu$ L** from a chosen unused well of the UDI Plate - EC to its corresponding tube of diluted CRISPR cDNA.



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

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

SUBLIBRARY INDEX AMPLIFICATION			
Run Time		~30 min	
Lid Temperature		105°C	
Sample Volume		50 $\mu$ L	
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  $4^{\circ}\text{C}$  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  $\mu\text{L}$  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 **30  $\mu\text{L}$**  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  $\mu\text{L}$**  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  $\mu\text{L}$**  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  $\mu\text{L}$**  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  $\mu$ 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  $\mu$ 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.



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

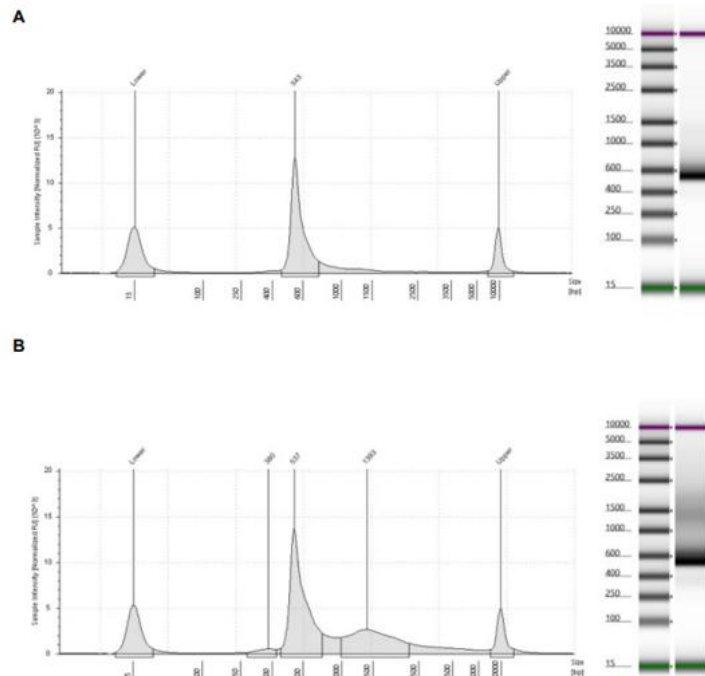


Figure 7: Expected Size Distribution of CRISPR Libraries before Illumina Sequencing. (A) Example trace of a CRISPR sequencing library with a minimal higher molecular weight product.

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

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



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

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

# Appendices

## Appendix A: Sublibrary Generation Table

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

Green text (top): Volume of cell suspension in  $\mu\text{L}$  (from step 1.5.10) to add to each sublibrary.

Purple text (bottom): Volume of Pre-Lysis Dilution Buffer in  $\mu\text{L}$  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/ $\mu\text{L}$ )	Target Sublibrary Cell Count (cells/sublibrary)														
	200	500	1,000	2,000	5,000	10,000	12,000	12,500	15,000	20,000	25,000	30,000	31,250	62,500	
50	4	10	20												
	21	15	5												
100	2	5	10	20											
	23	20	15	5											
200		2.5	5	10	25										
		22.5	20	15	0										
400			2.5	5	12.5	25									
			22.5	20	12.5	0									
600				3.33	8.33	16.67	20	20.83	25						
				21.67	16.67	8.33	5	4.17	0						
800				2.5	6.25	12.5	15	15.63	18.75	25					
				22.5	18.75	12.5	10	9.37	6.25	0					
1,000				2	5	10	12	12.5	15	20	25				
				23	20	15	13	12.5	10	5	0				
1,200					4.17	8.33	10	10.42	12.5	16.67	20.83	25			
					20.83	16.67	15	14.58	12.5	8.33	4.17	0			
1,400					3.57	7.14	8.57	8.93	10.71	14.29	17.86	21.43	22.32		
					21.43	17.86	16.43	16.07	14.29	10.71	7.14	3.57	2.68		
1,600					3.13	6.25	7.5	7.81	9.38	12.5	15.63	18.75	19.53		
					21.87	18.75	17.5	17.19	15.63	12.5	9.38	6.25	5.47		
1,800					2.78	5.56	6.67	6.94	8.33	11.11	13.89	16.67	17.36		
					22.22	19.44	18.33	18.06	16.67	13.89	11.11	8.33	7.64		
2,000					2.5	5	6	6.25	7.5	10	12.5	15	15.63		
					22.5	20	19	18.75	17.5	15	12.5	10	9.38		
2,500					2	4	4.8	5	6	8	10	12	12.5	25	
					23	21	20.2	20	19	17	15	13	12.5	0	
3,000						3.33	4	4.17	5	6.67	8.33	10	10.42	20.83	
						21.67	21	20.83	20	18.33	16.67	15	14.58	4.17	
3,500						2.86	3.43	3.57	4.29	5.71	7.14	8.57	8.93	17.86	
						22.14	21.57	21.43	20.71	19.29	17.86	16.43	16.07	7.14	
4,000						2.5	3	3.13	3.75	5	6.25	7.5	7.81	15.63	
						22.5	22	21.88	21.25	20	18.75	17.5	17.19	9.38	
4,500						2.22	2.67	2.78	3.33	4.44	5.56	6.67	6.94	13.89	
						22.78	22.33	22.22	21.67	20.56	19.44	18.33	18.06	11.11	
5,000						2	2.4	2.5	3	4	5	6	6.25	12.5	
						23	22.6	22.5	22	21	20	19	18.75	12.5	
5,500						2.18	2.27	2.73	3.64	4.55	5.45	5.68	5.68	11.36	
						22.82	22.73	22.27	21.36	20.45	19.55	19.32	13.64		
6,000						2	2.08	2.5	3.33	4.17	5	5.21	10.42		
						23	22.92	22.5	21.67	20.83	20	19.79	14.58		
7,000										2.14	2.86	3.57	4.29	4.46	8.93
										22.86	22.14	21.43	20.71	20.54	16.07
8,000											2.5	3.13	3.75	3.91	7.81
											22.5	21.88	21.25	21.09	17.19
9,000											2.22	2.78	3.33	3.47	6.94
											22.78	22.22	21.67	21.53	18.06
10,000											2	2.5	3	3.13	6.25
											23	22.5	22	21.88	18.75

## Appendix B: Sequencing Information

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

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

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

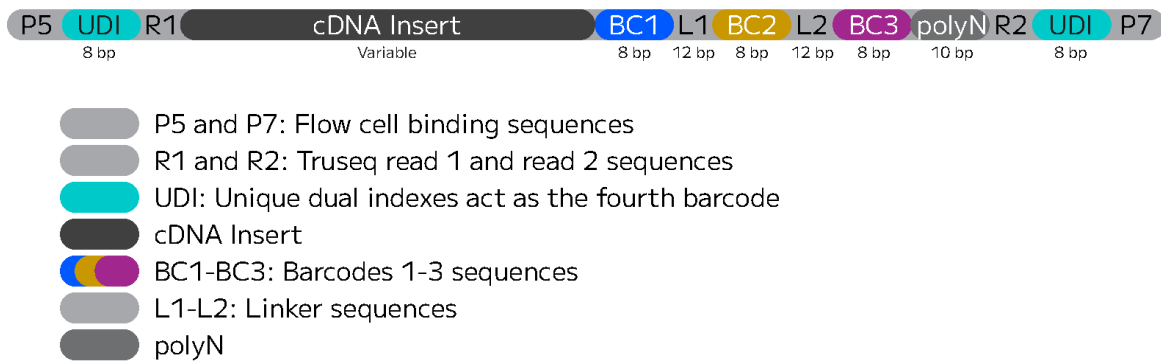


Figure 8: Whole transcriptome sequencing library structure.

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

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

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

CRISPR sequencing libraries can be sequenced together or separately from Whole Transcriptome libraries. We recommend sequencing CRISPR libraries at 2,000-5,000 reads per

cell, so the ratio between the libraries may need to be adjusted if they are sequenced together. For CRISPR libraries sequenced alone, we suggest using a minimum of 10% PhiX due to their limited complexity.

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

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

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

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

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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_22	F3	CTATACTC	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
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT

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

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

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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_EC_1	A1	AACAGATC	TGCCATGA	TCATGGCA
UDI_Plate_EC_2	B1	CCTGTCTT	CTAACGAT	ATCGTTAG
UDI_Plate_EC_3	C1	ATATCGAG	TTCCGGAA	TTCCGGAA
UDI_Plate_EC_4	D1	TAGTCCGC	TCTCCATA	TATGGAGA
UDI_Plate_EC_5	E1	TGCTGTTA	AGTGACGT	ACGTCACT
UDI_Plate_EC_6	F1	TAGCGAAT	CTCTGGCA	TGCCAGAG
UDI_Plate_EC_7	G1	AGGACCGT	AGAAGATG	CATCTTCT
UDI_Plate_EC_8	H1	TTATCAGG	TCTAACAG	CTGTTAGA
UDI_Plate_EC_9	A2	CCTCGGAA	TACGTAGA	TCTACGTA
UDI_Plate_EC_10	B2	TTGATCGA	TAAGCTCT	AGAGCTTA
UDI_Plate_EC_11	C2	TCAACCTC	TTGGTCGA	TCGACCAA
UDI_Plate_EC_12	D2	CGGAATAA	CGGTAATA	TATTACCG
UDI_Plate_EC_13	E2	TATGAGAC	CACAGTGG	CCACTGTG
UDI_Plate_EC_14	F2	CCTACCAT	AGACGCGA	TCGCGTCT
UDI_Plate_EC_15	G2	CATAGGCC	AGCAATGG	CCATTGCT
UDI_Plate_EC_16	H2	CACGATAA	CCTCTTGA	TCAAGAGG
UDI_Plate_EC_17	A3	TTCTGGCG	TTGTCTAG	CTAGACAA
UDI_Plate_EC_18	B3	TCGTAACT	TGTTGCTA	TAGCAACA
UDI_Plate_EC_19	C3	TCCGTGGT	TACTGTCA	TGACAGTA
UDI_Plate_EC_20	D3	AAGACAAC	AAGCCATA	TATGGCTT

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_EC_21	E3	ATTGCTTC	CGGCACTT	AAGTGCCG
UDI_Plate_EC_22	F3	AGAGAAGT	TCATGGAT	ATCCATGA
UDI_Plate_EC_23	G3	TACGTCTT	TAATCAGG	CCTGATTA
UDI_Plate_EC_24	H3	TCCACGTT	CATCTGAG	CTCAGATG
UDI_Plate_EC_25	A4	CAATGAGT	CAGAATCG	CGATTCTG
UDI_Plate_EC_26	B4	CGTCAGTT	TTGATGCA	TGCATCAA
UDI_Plate_EC_27	C4	TTCACTCC	ACGTATGG	CCATACGT
UDI_Plate_EC_28	D4	CTACCTGA	TGCGGATA	TATCCGCA
UDI_Plate_EC_29	E4	AGGTGATT	TAACAGCA	TGCTGTTA
UDI_Plate_EC_30	F4	TCTCACAT	AATGTTCG	CGAACATT
UDI_Plate_EC_31	G4	TTAGTGAG	TTACGTGT	ACACGTAA
UDI_Plate_EC_32	H4	TGTTCACT	ATCTACTG	CAGTAGAT
UDI_Plate_EC_33	A5	CGAAGCCT	ATACCTCT	AGAGGTAT
UDI_Plate_EC_34	B5	TGGCCGTA	TACATCTG	CAGATGTA
UDI_Plate_EC_35	C5	CGCTTCAC	AAGGAGCA	TGCTCCTT
UDI_Plate_EC_36	D5	CGGAGAAC	CTCCTAGA	TCTAGGAG
UDI_Plate_EC_37	E5	AAGTTCAG	TCAGCCTA	TAGGCTGA
UDI_Plate_EC_38	F5	AAGCCTTC	TTCGCTCA	TGAGCGAA
UDI_Plate_EC_39	G5	ACACCTCA	CGGATTAA	TTAATCCG
UDI_Plate_EC_40	H5	TTCTCCTA	ATTAGAGG	CCTCTAAT
UDI_Plate_EC_41	A6	TGACACGC	CGTGTGAA	TTCACACG
UDI_Plate_EC_42	B6	AAGCGCCT	TGTCACGG	CCGTGACA
UDI_Plate_EC_43	C6	TGATAACC	CGTAATCT	AGATTACG
UDI_Plate_EC_44	D6	AAGAGTGT	CCTTCTGG	CCAGAAGG

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

## Appendix C: Thermocycling Programs

### Section 1: In situ Cell Barcoding

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

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

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

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

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

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

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

## Section 2: cDNA Capture and Amplification

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

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

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

### Section 3: Sequencing Library Preparation

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

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

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

#### Section 4: CRISPR Sequencing and Library Preparation

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

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

## Appendix D: CRISPR-Specific Troubleshooting

### Adjusting PCR Cycles:

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

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



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

CRISPR SUBLIBRARY INDEX PCR (Step 4.3.12)	
Input (ng)	Total number of cycles
10	9
5-9	10



**Note:** Having less than 5 nanograms of input material for CRISPR PCR or CRISPR Sublibrary Index PCR may result in suboptimal sequencing results.

## Appendix E: Revision History

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



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