User Manual

Version 2.3 – UM0021

PARSE BIOSCIENCES

Evercode™ WT Mini v2

For use with

ECW02110

ECW02115

ECW02010

Legal Notices

This document and its contents are proprietary to Parse Biosciences, Inc. (“Parse Biosciences”) and are intended solely for use by its customers in connection with the use of the product(s) described herein and for no other purpose. The products may be used solely FOR RESEARCH PURPOSES, AND MAY NOT BE USED IN ANY DIAGNOSTIC OR THERAPEUTIC USE IN HUMANS OR ANIMALS. This document and its contents shall not be used or distributed for any other purpose and/ or otherwise communicated, disclosed or reproduced in any way whatsoever without the prior written consent of Parse Biosciences.

No rights are granted under this document with respect to any of Parse Biosciences’ intellectual property rights. The license to use of any products described herein is subject to a separate written agreement between Parse Biosciences and the applicable user.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. Parse Biosciences shall have no liability for any direct, indirect, consequential or incidental damages arising out of any failure to use the product(s) in strict compliance with the terms herein.

This document may contain references to third-party sources of information, hardware or software, products, or services and/ or third-party web sites (collectively “Third Party Information”). Parse Biosciences does not control, and is not responsible for, any Third Party Information. The inclusion of Third Party Information in this document does not imply endorsement by Parse Biosciences of the Third Party Information or the third party in any way.

The product(s) described in this document are provided for one-time use by the purchaser and may not be re-used, refurbished or resold. In addition, such product(s) may not be altered, changed or modified by anyone other than Parse Biosciences and its authorized agents, and Parse Biosciences will not be liable for any such alterations, changes or modifications. The product(s) described herein may be covered by one or more of the following patents:

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

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

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

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

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

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

Patents pending in the U.S. and other countries

PARSE and EVERCODE are trademarks of Parse Biosciences, Inc. The information made available herein is the sole property of Parse Biosciences and its licensors.

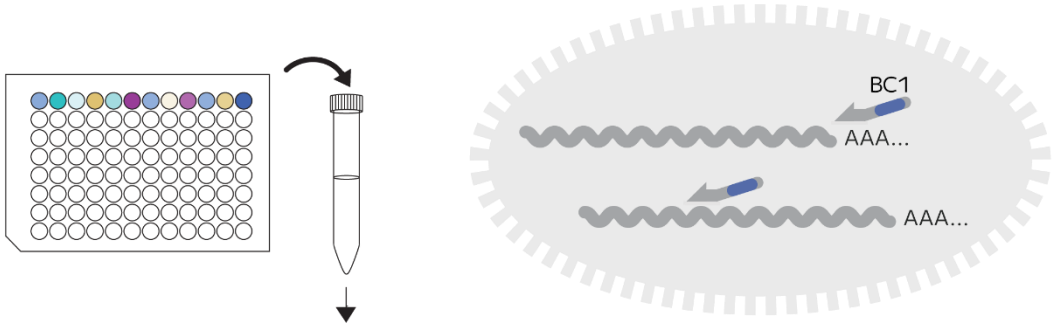
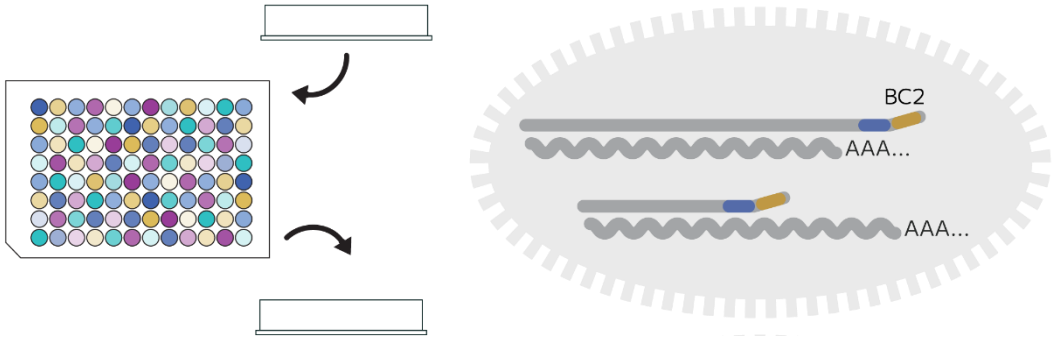
Table of Contents

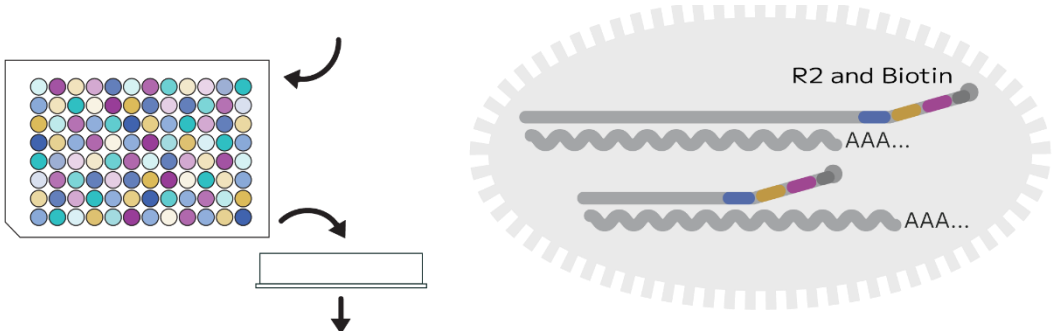
Overview

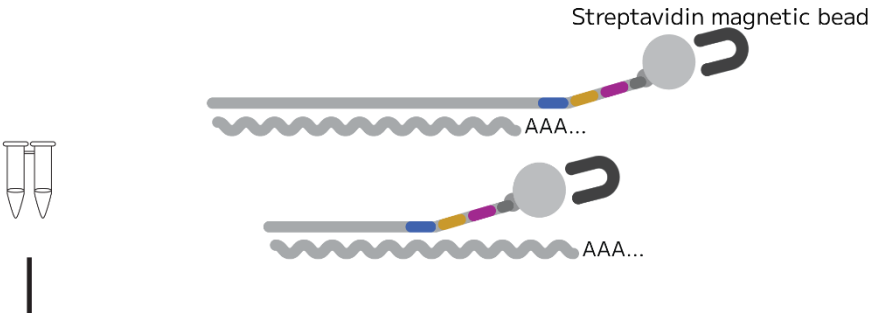
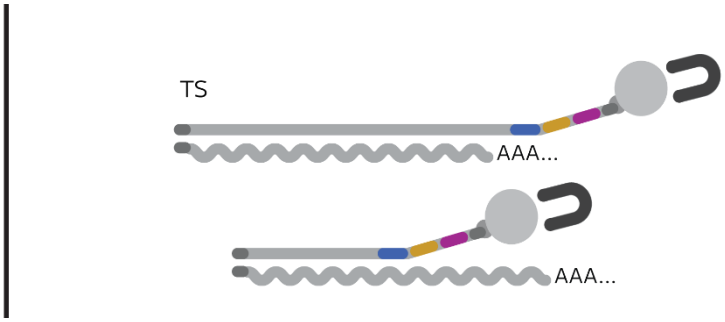
Workflow

Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT Mini v2 kit can profile up to 20,000 cells (10,000 cells is recommended) across up to 12 different biological samples or experimental conditions. Evercode fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments. This eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding can yield a vast number of possible barcode combinations, which is more than sufficient to uniquely label up to 20,000 cells/nuclei while avoiding doublets. After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same four barcode combination to a single cell/nuclei.

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

Section 1: *In situ* Cell/Nuclei Barcoding	
1	 *Round 1 Barcoding*: Fixed and permeabilized cells/nuclei are loaded into 12 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with a well-specific barcode that are associated with specific samples.
2	 *Round 2 Barcoding:* The cells/nuclei are pooled and loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode.

Section 1: *In situ* Cell/Nuclei Barcoding	
3	 *Round 3 Barcoding:* The cells/nuclei are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina R2 sequence, and biotin.
4	 *Lysis and Sublibrary Generation:* Cells/nuclei are split across 2 sublibraries and lysed.

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

Section 2: cDNA Capture and Amplification	
3	 *cDNA Amplification:* The cDNA is amplified with primers specific to the template switch adapter and Illumina Truseq R2.

Section 3: Sequencing Library Preparation	
1	 *Fragmentation, End Repair, and A-tailing:* DNA is fragmented, ends are repaired, and A-tailed.
2	 *Adapter Ligation:* An Illumina Truseq R1 Adapter is ligated to the 5' end of the DNA.
3	 *Round 4 Barcoding:* The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.

Protocol Timing

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

DAY	SECTION	TOTAL TIME	HANDS-ON TIME	SAFE STOPPING POINTS
1	Section 1: In Situ Cell/Nuclei Barcoding			
	1.1 Set up and Sample Counting	Variable (30-90 min)	Variable (30-90 min)	
	1.2: Barcoding Round 1	75 min	30 min	
	1.3: Barcoding Round 2	90 min	30 min	
	1.4: Barcoding Round 3	45 min	15 min	
	1.5 Lysis and Sublibrary Generation	105 min	45 min	-80°C ≤ 6 months
2	Section 2: cDNA Capture and Amplification			
	2.1 Binder Bead Preparation	30 min	30 min	
	2.2: cDNA Capture	60 min	15 min	
	2.3 cDNA Template Switch	150 min	30 min	4°C ≤ 18 hrs
	2.4: cDNA Amplification	120 min	30 min	4°C ≤ 18 hrs
	2.5 Post-Amplification Purification	30 min	30 min	
	2.6 cDNA Quantification	30 min	30 min	4°C ≤ 48 hrs or -20°C ≤ 3 months
3	Section 3: WT Sequencing Library Preparation			
	3.1 Fragmentation, End Repair, and A-Tailing	60 min	30 min	

DAY	SECTION	TOTAL TIME	HANDS-ON TIME	SAFE STOPPING POINTS
	3.2 Post-A-tailing Size Selection	30 min	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
	3.3 Adaptor Ligation	30 min	15 min	
	3.4 Post-Ligation Purification	30 min	30 min	
	3.5 Barcoding Round 4	45 min	15 min	4°C ≤ 18 hrs
	3.6 Post-Barcoding Round 4 Size Selection	30 min	30 min	
	3.7 Sequencing Library Quantification	30 min	30 min	20°C ≤ 3 months

Important Guidelines

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

Sample Input

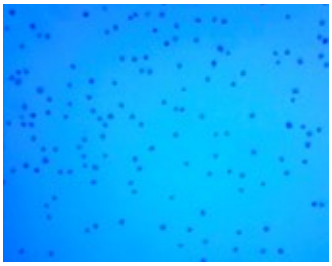
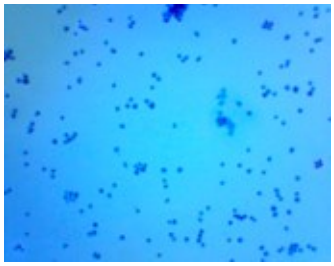
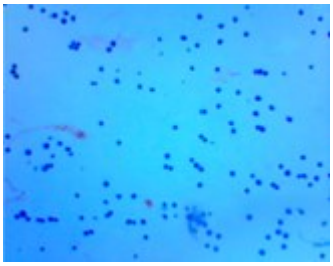
- This protocol begins with cells or nuclei fixed with an Evercode Cell Fixation or Evercode Nuclei Fixation kit.
- Even if samples were counted before freezing, we strongly recommend counting cells/nuclei again after thawing to account for any changes during storage and freeze thaw. These counts are used to determine how cells/nuclei are loaded in the Round 1 Plate, and their accuracy is critical to recover the desired number of cells/nuclei.

Cell/Nuclei Counting and Quality Assessment

- We recommend a hemocytometer for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices to a hemocytometer when using Evercode Whole Transcriptome kits for the first time.
- When first using Evercode Whole Transcriptome kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- After fixation, the cells/nuclei are permeabilized and will appear dead with viability stains. Examples of trypan blue stained fixed cells are shown below.

- High quality fixed samples have single distinct cells with <5% cell aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing (Figure 1). When quantifying fixed cells, it is critical to avoid counting cell debris to avoid overestimating the number of cells.

High Quality Sample | Aggregation | Debris

Avoiding RNase Contamination

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

Centrifugation

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

Optimizing Cell/Nuclei Recovery

- It is critical to thoroughly resuspend the cells/nuclei after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to cell/nuclei adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell/nuclei loss.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell/nuclei pellets adequately.

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

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

Sample Loading Table

- The Parse Biosciences Evercode WT Mini Sample Loading Table V1.2.0 (Excel spreadsheet) should be completed before starting the experiment.

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

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

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

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

Cell Strainers

- A 40 µm cell strainer will be used in multiple steps. To maximize cell retention, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. Recover any residual liquid on the bottom of the strainer.

- For cells larger than 40 µm, the provided strainers should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm).

Vortexing

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

Plate Sealing

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

Magnetic Racks and Bead Cleanups

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

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

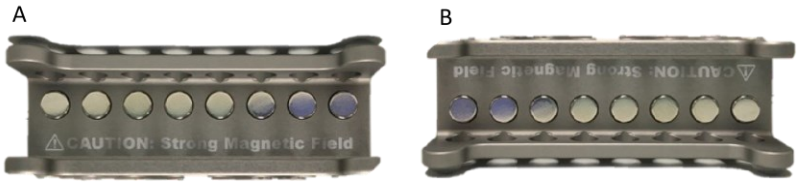


Figure 2: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.

- To ensure material is not lost during bead purifications, ensure supernatants are completely clear before moving to the next step. The incubation times at each step are recommendations, but visual confirmation of clearing should be used to make the final determination. See Figure 4 for an example of completely cleared supernatants. Discarding any beads in supernatants will result in a reduction of transcripts and genes detected per cell.

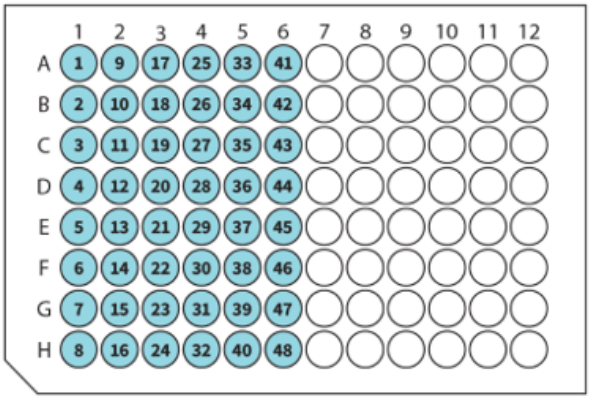
Figure 3: Clear supernatant with compact bead pellets.

Sublibrary Loading

- The Evercode WT Mini kit generates 2 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.
- The number of cells or nuclei 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/nuclei across all sublibraries.
- Asymmetric sublibrary loading can enable cost-effective sequencing quality control. One sublibrary can be loaded with a few hundred cells/nuclei and sequenced very deeply. This data can be used to choose an appropriate sequence depth for the remaining sublibraries.

Indexing Primers

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



Thermocycling Programs

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

Part List

The Evercode WT Mini v2 kit includes the following boxes.

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

Barcoding Plates. Store at -20°C, PN SP100.

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	SP101	96 well plate	1
	Round 2 Plate	SP102	96 well plate	1
	Round 3 Plate	SP103	96 well plate	1

Barcoding Reagents. Store at -20°C, PN SB100.

LABEL	ITEM	PN	FORMAT	QTY
	Dilution Buffer	SB101	1.5 mL tube	2
	Resuspension Buffer	SB102	5 mL tube	1
	Ligation Mix	SB103	5 mL tube	1
	Round 2 Ligation Enzyme	SB104	1.5 mL tube	1
	Round 2 Stop Mix	SB105	2 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Round 3 Ligation Enzyme	SB106	1.5 mL tube	1
	Round 3 Stop Mix	SB107	5 mL tube	1
	Pre-Lyse Wash Buffer	SB108	5 mL tube	1
	Lysis Enzyme	SB109	1.5 mL tube	1

cDNA Amplification. Store at -20°C, PN SC100.

LABEL	ITEM	PN	FORMAT	QTY
	Lysis Neutralizer	SC101	1.5 mL tube	1
	Bead Wash Buffer	SC102	1.5 mL tube	1
	Bind Buffer A	SC103	1.5 mL tube	1
	Bind Buffer B	SC104	1.5 mL tube	1
	Bind Buffer C	SC105	1.5 mL tube	1
	Bead Storage Buffer	SC106	1.5 mL tube	1
	TS Buffer	SC107	1.5 mL tube	1
	TS Enzyme	SC108	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	TS Primer Mix	SC109	1.5 mL tube	1
	Amplification Master Buffer	SC110	1.5 mL tube	1
	Amplification Primer Mix	SC111	1.5 mL tube	1

Fragmentation. Store at -20°C, PN SX200.

LABEL	ITEM	PN	FORMAT	QTY
	Fragmentation Buffer	SX101	1.5 mL tube	1
	Fragmentation Enzyme	SX102	1.5 mL tube	1
	Adaptor DNA	SX103	1.5 mL tube	1
	Adaptor Ligation Buffer	SX104	1.5 mL tube	1
	Adaptor Ligase	SX105	1.5 mL tube	1
	Index PCR Mix	SX106	1.5 mL tube	1

Accessories. Store at Room Temperature, PN WA100.

LABEL	ITEM	PN	FORMAT	QTY
N/A	40 µm strainers	WA101	N/A	2
N/A	Basins	WA102	N/A	2
N/A	PCR plate seal	WA103	N/A	5
N/A	Plate Sealer	WA104	N/A	1

4°C Reagents. Store at 4°C, PN SA200.

LABEL	ITEM	PN	FORMAT	QTY
	Spin Additive	SA201	1.5 mL tube	1
	2x Lysis Buffer	SA202	1.5 mL tube	1
	Binder Beads	SA203	1.5 mL tube	1

User Supplied Equipment and Reagents

The following materials and equipment are required to perform the protocol, but are not provided within the kit. Note that this list does not include standard laboratory equipment, such as freezers.

Equipment

ITEM	SUPPLIER	PN	NOTES
Parse Biosciences Magnetic Rack	Parse Biosciences	SB1004	We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection.
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 15 mL centrifuge tubes and 96 well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL and 0.2 mL tubes.
Heat Block	Various Suppliers	Varies	Or equivalent water bath, bead bath, or thermomixer capable of holding temperature at 37°C.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. We recommend validating alternatives relative to a hemocytometer.
Single Channel Pipettes: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.
T100 Thermal Cycler	Bio-Rad Laboratories®	1861096	Or an equivalent thermocycler compatible with unskirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.

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

Consumables

ITEM	SUPPLIER	PN	NOTES
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.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial cell loss.
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.

ITEM	SUPPLIER	PN	NOTES
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.
AMPure® XP Reagent	Beckman Coulter®	A63880 (5 mL) A63881 (60mL)	Choose one. We do not recommend substituting other magnetic beads.
SPRIselect Reagent	Beckman Coulter	B23317 (5mL) B23318 (60mL)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Ethyl alcohol, Pure	Sigma-Aldrich	459844	Or equivalent 100% non-denatured ethanol.
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
Trypan Blue	Various Suppliers	Varies	Or alternative dyes to assess cell viability, such as AO/PI.

ITEM	SUPPLIER	PN	NOTES
High Sensitivity DNA Kit	Agilent	5067-4626	Choose one that corresponds to the chosen Bioanalyzer or Tapestation.
High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	
Corning Cell Strainer	Corning	431751 (70µm) 431752 (100µm)	(Optional) For cells larger than 40 µm, the included strainers should be replaced throughout the protocol with an appropriate size mesh.

Section 1: In Situ Cell/Nuclei Barcoding

1.1: Set up and Sample Counting

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

To set up for barcoding:

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

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

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

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

8. Thaw the previously fixed cell/nuclei samples in a heat block set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.

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

10. Record the sample names and cell/nuclei count in the Sample Loading Table (Parse Biosciences Evercode WT Mini Sample Loading Table V1.2.0.xlsm).

11. Based on the values defined in the Sample Loading Table, dilute each sample with ● Dilution Buffer and store it on ice. Do not discard the ●Dilution Buffer.

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

12. Proceed immediately to Section 1.2.

1.2: Barcoding Round 1

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

To add round 1 barcodes:

1. Gently remove the Round 1 Plate from the thermocycler, return to the green rack, and centrifuge for **1 minute** at 100 x g at 4°C.
2. Remove the Round 1 Plate from the centrifuge. While secured in the green rack on a flat surface, remove the plate seal and store on ice.

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

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

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

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

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

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

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

6. Remove the Round 1 Plate from the thermocycler, return to the green rack, and store on ice.

7. Remove the Round 2 Plate from the blue rack, place it into a thermocycler, and run the following program. Proceed immediately to the next step while the program is still running.

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

8. While secured in the green rack on a flat surface, remove the plate seal from the Round 1 Plate and store the uncovered plate on ice.

9. Pool all wells from the Round 1 plate into a single 15 mL centrifuge tube on ice. To optimize cell recovery, use a single channel pipette as follows:

    i. With a single channel pipette set to 30 µL, pool Row A into a 15 mL centrifuge tube. To maximize cell retention while pooling, pipette 3x in the middle of the well, 3x on the front side of the well, and 3x on the back side of the well before transferring the volume of Row A into the 15 mL tube.

    ii. Recover residual liquid from Row A using the single channel pipette set to 10 µL. Do not be concerned if there are a few µL of residual volume in the wells after pooling.

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

10. Add **2.4 µL** of ●Spin Additive to the 15 mL tube with pooled cells. Do not discard the ●Spin Additive as it will be needed in another step.

11. Mix by gently inverting the tube just once.

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

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

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

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

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

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

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

16. Proceed immediately to Section 1.3

1.3. Barcoding Round 2

The pooled cells/nuclei are added to the Ligation Master Mix, which is loaded into the Round 2 Plate. An *in situ* ligation reaction adds a well-specific barcode to the 3′ end of the cDNA. The ligation reaction is quenched with Round 2 Stop Mix, and the cells/nuclei are pooled and strained.

To add round 2 barcodes:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
PCR plate seal	Accessories (Room Temp)	3	Keep at room temperature.
40 µm Strainer (or alternative size)	Accessories (Room Temp)	2	
Basins	Accessories (Room Temp)	6	
● Round 2 Stop Mix	Barcoding Reagents (-20°C)	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Round 3 Stop Mix	Barcoding Reagents (-20°C)	1	
● Pre-Lyse Wash Buffer	Barcoding Reagents (-20°C)	1	
● Round 3 Ligation Enzyme	Barcoding Reagents (-20°C)	1	Place directly on ice. Briefly centrifuge before use.
Round 3 Plate	Barcoding Reagents (-20°C)	1	Place directly on ice.

2. On ice, add **20 µL** of ●Round 2 Ligation Enzyme directly into the ●Ligation Mix tube to create the Ligation Master Mix.

3. Using a P1000 pipette, add **2 mL** of sample in ●Resuspension Buffer into the Ligation Master Mix. Mix thoroughly by pipetting 10x with a P1000 set to 1000 µL. Store on ice.

4. Remove the Round 2 Plate from the thermocycler, return to the blue rack, and centrifuge for **1 minute** at 100 x g at 4°C. Keep at room temperature.

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

6. Transfer the entire volume in the Ligation Master Mix tube to a basin with a P1000.

7. At room temperature and without reusing tips, transfer **40 µL** from the basin to each well in the Round 2 Plate. To optimize cell recovery, use a multichannel pipette as follows:

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

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

    iii. Repeat i-ii for Rows B-H.

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

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

8. While secured in the blue rack on a flat surface, add a new plate seal to the Round 2 Plate.

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

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

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

11. Remove the Round 2 Plate from the thermocycler and return to the blue rack.

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

13. At room temperature and without reusing tips, transfer **10 µL** of the ●Round 2 Stop Mix from the basin to each well in the Round 2 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.

CRITICAL! Carefully hold the strainer using the outside casing without touching the mesh. Different tips must be used when pipetting ●Round 2 Stop Mix into each well of the plate. Never place a tip that has entered one of the wells back into the basin or a different well.

14. While secured in the blue rack on a flat surface, add a new plate seal to the Round 2 Plate.

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

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

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

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

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

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

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

    i. With a multichannel P200 set to 50 µL, mix the sample in Row A by pipetting 3x in the middle of the well, then repeating on the front and the back of the wells.

    ii. Transfer **50 µL** from Row A to the basin.

    iii. Repeat for Rows B-H.

    iv. Transfer any residual liquid in the Round 2 Plate to the basin with a multichannel pipette set to 10 µL.

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

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

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

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

21. Proceed immediately to Section 1.4.

1.4. Barcoding Round 3

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

To add round 3 barcodes:

1. Add **20 µL** of Round 3 Ligation Enzyme to the basin containing the strained sample. Mix by gently pipetting 20x with a P1000 set to 1000 µL.
2. Remove the Round 3 Plate from the thermocycler, return to the orange rack, and centrifuge for **1 minute** at 100 x g at 4°C.
3. While secured in the orange rack on a flat surface, remove the plate seal from the Round 3 Plate.
4. At room temperature and without reusing tips, transfer **50 µL** from the basin to each well in the Round 3 Plate. To optimize cell recovery, use a multichannel pipette as follows:

    i. Mix the sample in the basin by pipetting 2x pipetting with a multichannel P200 set to 50 µL.

    ii. Transfer **50 µL** of the mix to Row A of the Round 3 Plate and mix by pipetting 2x.

    iii. Repeat i-ii for Rows B-H.

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

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

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

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

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

7. Briefly vortex the ●Round 3 Stop Mix. Transfer the entire volume to a new basin with a P1000.

8. Remove the Round 3 Plate from the thermocycler and return it to the orange rack.

9. While secured in the orange rack on a flat surface, remove the plate seal from the Round 3 Plate.

10. At room temperature and without reusing tips, transfer **20 µL** of the ●Round 3 Stop Mix from the basin to each well in the Round 3 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.

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

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

12. At room temperature, transfer all the liquid in the Round 3 Plate into a new basin. To optimize cell recovery, use a multichannel pipette as follows:

    i. With a multichannel P200 set to 70 µL, mix the sample in Row A by pipetting 3x in the middle of the well, then repeating on the front and the back of the wells.

    ii. Transfer **70 µL** from Row A to the basin.

    iii. Repeat for Rows B-H.

    iv. Transfer any residual liquid in the Round 3 Plate to the basin with a multichannel pipette set to 10 µL.

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

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

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

14. Proceed immediately to Section 1.5.

1.5. Lysis and Sublibrary Generation

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

To generate and lyse sublibraries:

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

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

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

2. Add **70 µL** of ●Spin Additive to the 15 mL tube with the sample. Gently invert once to mix.

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

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

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

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

5. Fully but gently resuspend the pellet in **1 mL** of ●Pre-Lyse Wash Buffer.

6. Add an additional **3 mL** of ●Pre-Lyse Wash Buffer for a total addition of 4 mL.

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

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

8. Remove the supernatant until about ~40 µL of liquid remains above the pellet. Use a P1000 for the first **3 mL** and then a P200 for the remaining volume.

9. Fully but gently resuspend the pellet with the remaining supernatant in the 15mL tube. Measure the volume of the resuspended cells with a P200 pipette. If less than 60 µL are measured, add ●Dilution Buffer to a total of **60 µL**. If more than 60 µL are measured, proceed without adding ●Dilution Buffer. Store on ice.

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

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

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

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

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

12. Ensure the cells/nuclei are in suspension by pipetting 5x with a P200 set to **50 µL** before each transfer. Add the appropriate volume of sample to 2 separate 0.2 mL tubes.

13. Add the appropriate volume of ●Dilution Buffer to the 0.2 mL tubes for a total volume of **25 µL**.

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

LYSIS MASTER MIX	
2x Lysis Buffer	55 µL
Lysis Enzyme	11 µL
Total Volume	66 µL

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

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

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

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

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

18. Freeze the lysate(s) at -80°C.

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

Section 2: cDNA Capture and Amplification

2.1. Binder Bead Preparation

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

To prepare for cDNA capture and amplification:

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

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

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

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

Lysates processed	1	2
Binder Beads	44 µL	88 µL

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

8. Remove and discard the supernatant.

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

Lysates processed	1	2
Bead Wash Buffer	50 µL	100 µL

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

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

11. Remove and discard the supernatant.

12. Repeat steps 9-11 twice for a total of 3 washes.

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

Lysates processed	1	2
● Bind Buffer A	55 µL	110 µL

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

2.2. cDNA Capture

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

To capture the cDNA:

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

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

3. Briefly centrifuge and store at room temperature.
4. Briefly centrifuge Lysis Neutralizer and gently mix by pipetting 2x with a P20 set to 15 µL.
5. Add **2.5 µL** of Lysis Neutralizer to each tube of lysate. Mix 5x with a P200 pipette set to 40 µL. Briefly centrifuge.
6. Incubate for **10 minutes** at room temperature.
7. Fully mix the Binder Beads prepared in Section 2.1 by pipetting 3x.
8. Add **50 µL** of Binder Beads to each tube of lysate. Fully mix by pipetting 5x with a P200 set to 90 µL.
9. Place the tube(s) into a 96 well PCR tube rack, press to secure, and ensure the caps are secured tightly. Place the lid on the rack.
10. Place the rack onto a vortex mixer with a plate adaptor. Push to secure. Vortex on 20% power (~800-1000 RPM) for **60 minutes** at room temperature.

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

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

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

CRITICAL! Ensure the supernatant is completely clear before proceeding. Discarding any beads in the supernatant will result in a reduction of transcripts and genes detected per cell.

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

16. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** Bind Buffer B.

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

18. Return the tube(s) to the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).

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

20. Repeat steps 16-19 once for a total of 2 washes with Bind Buffer B.

21. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** Bead Storage Buffer.

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

23. Proceed immediately to Section 2.3.

2.3. cDNA Template Switch

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

To template switch:

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

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

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

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

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

3. Place each tube of captured cDNA from Section 2.2 on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).

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

5. While still on the magnetic rack, add **125 µL** of ●Bind Buffer C to each tube.

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

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

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

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

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

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

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

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

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

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

2.4 cDNA Amplification

The captured cDNA is washed and amplified with TS- and Illumina Truseq R2- specific primers.

To amplify the cDNA:

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

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

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

AMPLIFICATION REACTION MASTER MIX		
Number of Sublibraries	1	2
● Amplification Master Buffer	60.5 µL	121 µL
● Amplification Primer Mix	60.5 µL	121 µL
Total	121 µL	242 µL

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

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

4. While still on the magnetic rack, remove and discard the supernatant.
5. While still on the magnetic rack, add **125 µL** of ●Bind Buffer C to each tube.
6. Incubate for **1 minute** at room temperature.

7. While still on the magnetic rack, remove and discard the ●Bind Buffer C.

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

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

NUMBER OF PCR CYCLES			
Cells/Nuclei in Sublibrary	High RNA content such as cell lines	Low RNA content such as PBMCs	Nuclei
200-1,000	12	14	13
1,000-2,000	10	12	11
2,000-6,000	8	10	9
6,000-10,000	6	8	7

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

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

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

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

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

2.5. Post-Amplification Purification

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

To purify the cDNA:

1. Gather 400 µL of freshly prepared 85% ethanol per tube of amplified cDNA with nuclease-free water.
2. Gather room temperature SPRI beads (80 µL per tube of amplified cDNA).

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

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

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

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

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

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

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

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

18. While still on the magnetic rack, transfer **20 µL** of the supernatant containing the purified cDNA into new 0.2 mL tube(s). Store on ice.

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

2.6. cDNA Quantification

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

To quantify the cDNA:

1. Measure the concentration of each tube of purified cDNA from Section 2.5 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions. Record the concentration(s), which will be used in Section 3.

2. Assess the size distribution of each tube of purified cDNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D5000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions. Samples may need to be diluted to be within the manufacturer's recommended concentration range.

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

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

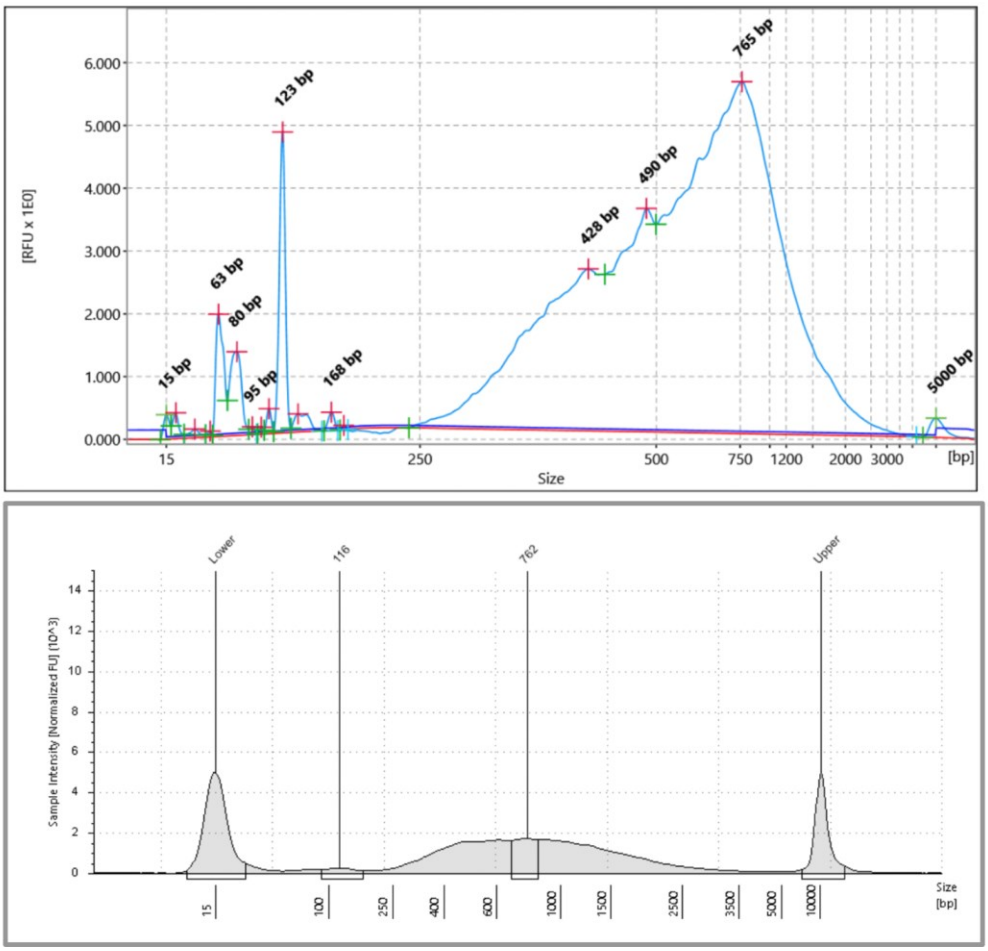


Figure 5: Expected post-amplification sublibrary cDNA size distribution. The expected cDNA Size Distribution after cDNA Amplification. Example trace of cDNA run on a Bioanalyzer (top). Example trace of cDNA run on a TapeStation (bottom). It is normal for libraries to be shifted to the left on a TapeStation relative to a Bioanalyzer

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

Section 3: Sequencing Library Preparation

3.1. Fragmentation, End Repair, and A-Tailing

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

To prepare for sequencing library preparation:

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

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

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

8. Prepare diluted cDNA in new 0.2 mL tube(s) as follows and store on ice. Bring the total volume up to **35 µL**. Store any remaining sublibrary cDNA at -20°C.

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

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

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

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

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

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

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

FRAGMENTATION MASTER MIX		
Number of Sublibraries	1	2
● Fragmentation Buffer	5.5 µL	11 µL
● Fragmentation Enzyme	11 µL	22 µL
Total	16.5 µL	33 µL

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

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

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

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

3.2. Post-A-tailing Size Selection

Fragmented and A-tailed DNA is size selected with 0.6x and 0.8x SPRI cleanups.

To size select the DNA:

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

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

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

CRITICAL! This may take longer due to the low volume of beads. Ensure all the solutions are completely clear before proceeding.

12. While still on the magnetic rack, remove and discard the supernatant.
13. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
14. Incubate for **1 minute** at room temperature.

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

16. Repeat steps 13-15 once for a total of 2 washes. Remove any residual ethanol with a P20.

17. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).

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

18. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **50 µL** of nuclease-free water.

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

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

21. While still on the magnetic rack, transfer **50 µL** of the supernatant into new 0.2 mL tube(s).

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

3.3. Adaptor Ligation

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

To ligate adaptors:

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

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

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

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

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

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

3.4. Post-Ligation Purification

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

To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~90 µL per sublibrary).

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

3. Vortex the SPRI beads until fully mixed. Add **80 µL** of SPRI beads to each tube of adaptor ligated DNA from Section 3.3.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~2 minutes).
13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **23 µL** of nuclease-free water.
14. Incubate for **5 minutes** at room temperature.
15. Place the tube(s) on the low magnet position of the magnetic rack so the magnet is closer to the bottom of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

16. While still on the magnetic rack, transfer exactly **21 µL** of the supernatant containing the purified adaptor ligated DNA into new 0.2 mL tube(s). Store on ice.

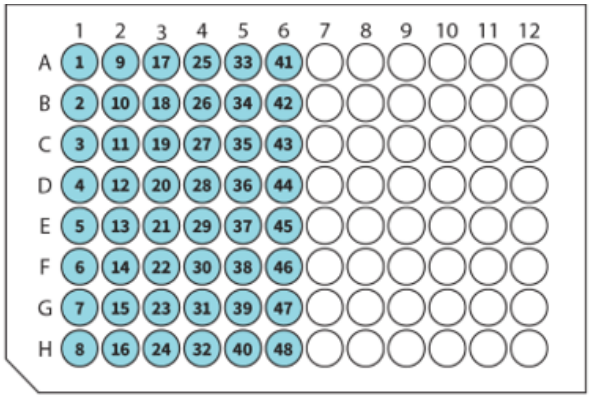
17. Proceed immediately to Section 3.5.

3.5. Barcoding Round 4

Purified adaptor ligated DNA is PCR amplified with Illumina Truseq R1 and R2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.

To add round 4 barcodes:

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



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

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

6. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C.
7. Add **25 µL** of Index PCR Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 25 µL. Briefly centrifuge.

8. Determine the number of PCR cycles required for indexing PCR based on the concentration of cDNA added to the fragmentation reaction as previously recorded in Section 3.1.

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

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

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

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

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

3.6. Post-Round 4 Barcoding Size Selection

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

To size select the sequencing libraries:

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

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

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

CRITICAL! This may take longer due to the low volume of beads. Ensure all the solutions are completely clear before proceeding.

12. While still on the magnetic rack, remove and discard the supernatant.
13. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
14. Incubate for **1 minute** at room temperature.

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

16. Repeat steps 13-15 once for a total of 2 washes. Remove any residual ethanol with a P20.

17. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).

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

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

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

21. While still on the magnetic rack, transfer all elutant into new 0.2 mL tube(s). Store on ice.

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

3.7. Sequencing Library Quantification

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

To quantify the sequencing libraries:

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

2. Assess the size distribution of each purified sequencing library with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D1000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions. Samples may need to be diluted to be within the manufacturer's recommended concentration range.

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

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

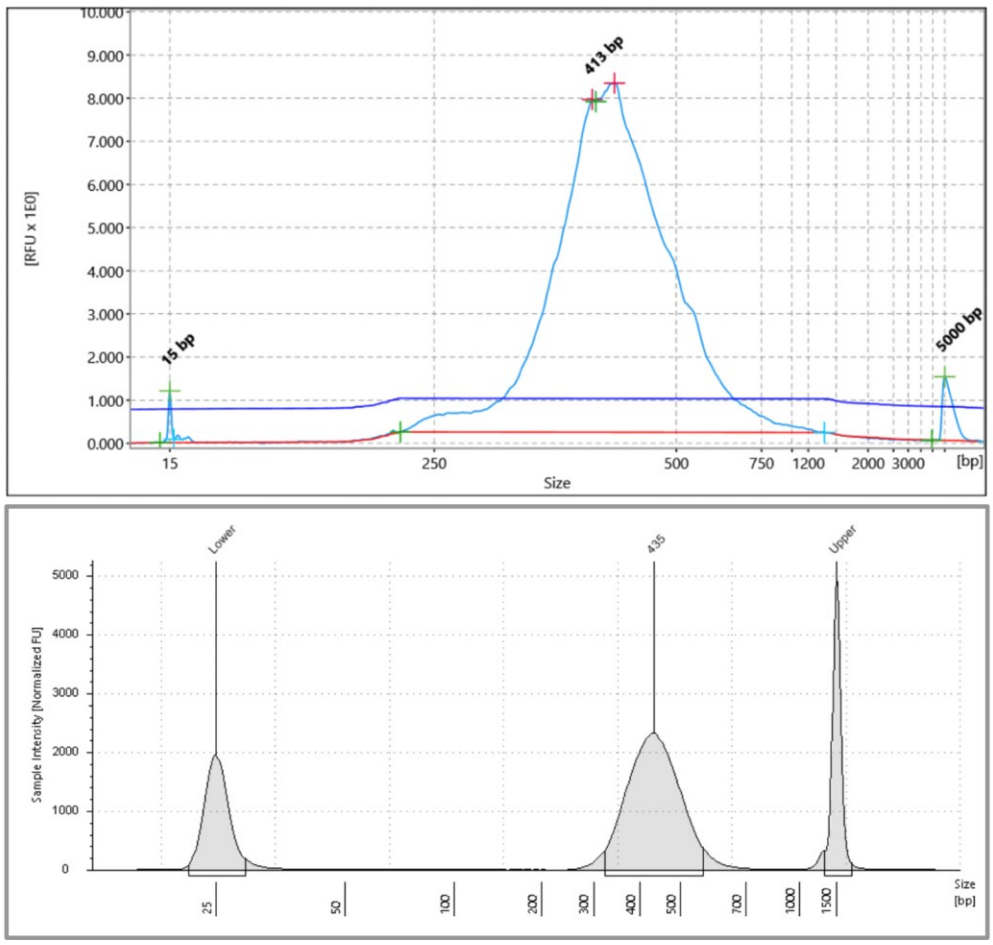


Figure 6: Expected Size Distribution before Illumina Sequencing. Example trace of DNA from indexed sublibraries run on a Bioanalyzer (top). Example trace of DNA from indexed sublibraries run on a TapeStation (bottom).

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

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

.

Appendices

Appendix A: Sublibrary Generation Table

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

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

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

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

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

Stock con (cells/uL)	Target Sublibrary Cell Count (cells/sublibrary)											
	200	500	1,000	2,000	3,000	4,000	5,000	6,000	7,000	8,000	9,000	10,000
50	4 25	10 25	20 23									
100	2 23	5 20	10 15	20 5								
200		2.5 22.5	5 20	10 15	15 10	20 5	25 0					
300			3.33 21.67	6.67 18.33	10 15	13.33 11.67	16.67 8.33	20 5	23.33 1.67			
400			2.5 22.5	5 20	7.5 17.5	10 15	12.5 12.5	15 10	17.5 7.5	20 5	22.5 2.5	25 0
500			2 23	4 21	6 19	8 17	10 15	12 13	14 11	16 9	18 7	20 5
600				3.33 21.67	5 20	6.67 18.33	8.33 16.67	10 15	11.67 13.33	13.33 11.67	15 10	16.67 8.33
700				2.86 22.14	4.29 20.71	5.71 19.29	7.14 17.86	8.57 16.43	10 15	11.43 13.57	12.86 12.14	14.29 10.71
800				2.5 22.5	3.75 21.25	5 20	6.25 18.75	7.5 17.5	8.75 16.25	10 15	11.25 13.75	12.5 12.5
900				2.22 22.78	3.33 21.67	4.44 20.56	5.56 19.44	6.67 18.33	7.78 17.22	8.89 16.11	10 15	11.11 13.89
1,000				2 23	3 22	4 21	5 20	6 19	7 18	8 17	9 16	10 15
1,100					2.73 22.27	3.64 21.36	4.55 20.45	5.45 19.55	6.36 18.64	7.27 17.73	8.18 16.82	9.09 15.91
1,200					2.50 22.50	3.33 21.67	4.17 20.83	5 20	5.83 19.17	6.67 18.33	7.5 17.5	8.33 16.67
1,300					2.31 22.69	3.08 21.92	3.85 21.15	4.62 20.38	5.38 19.62	6.15 18.85	6.92 18.08	7.69 17.31
1,400					2.14 22.86	2.86 22.14	3.57 21.43	4.29 20.71	5 20	5.71 19.29	6.43 18.57	7.14 17.86
1,500					2.00 23.00	2.67 22.33	3.33 21.67	4.00 21.00	4.67 20.33	5.33 19.67	6.00 19.00	6.67 18.33

Appendix B: Illumina Run Configuration

Evercode sequencing libraries should be diluted and denatured according to the manufacturer's instructions for the relevant sequencing instrument. We strongly recommend adding 5% PhiX for optimal sequencing quality. Libraries should be sequenced with paired reads using the following read structure.

READ	CYCLES
Read 1	66
i7 Index (Index 1)	8
i5 Index (Index 2)	8
Read 2	86

The fourth barcode that tags each sublibrary act as a standard Illumina i7 and i5 indexes. Please refer to the following table to demultiplex UDI Whole Transcriptome sublibraries.

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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_14	F2	GCGCTATC	CGACTGGA	TCCAGTCG
UDI_Plate_WT_15	G2	TGACCAGT	ACATTGGC	GCCAATGT
UDI_Plate_WT_16	H2	TATAATCA	ACCACTGT	ACAGTGGT
UDI_Plate_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
UDI_Plate_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
UDI_Plate_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
UDI_Plate_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
UDI_Plate_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
UDI_Plate_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_39	G5	TCCAGTCG	CCGAAGTA	TACTTCGG
UDI_Plate_WT_40	H5	TGTATGCG	CCAGTTCA	TGAACTGG
UDI_Plate_WT_41	A6	TGGCTCAG	CAGCGTTA	TAACGCTG
UDI_Plate_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
UDI_Plate_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix C: Thermocycling Programs

Section 1: In Situ Cell/Nuclei Barcoding

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

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

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

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

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

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

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

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

Section 2: cDNA Capture and Amplification

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

cDNA AMPLIFICATION			
Run Time	Lid Temperature	Sample Volume	
50-70 min	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 sec	98°C	5
3	45 sec	65°C	
4	3 min	72°C	
5	20 sec	98°C	Variable, see table in section 2
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

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

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

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

PARSE BIOSCIENCES

parsebiosciences.com

support@parsebiosciences.com

Scan code to visit site