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Evercode WT v2.2.1

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Abstract

This protocol describes the original Parse Biosciences Evercode WT v2 protocol for single-nucleus or single-cell RNA-seq of 100,000 nominal nuclei or cells. Unlike other scRNA-seq methods that physically separate individual cells into different compartments to label transcripts with cell-specific barcodes, Evercode WT uses the cells (or nuclei) themselves as “containers” in which intracellular transcripts are labeled using combinatorial indexing. In practice, cells are split into different wells, a well-specific barcode is appended to intracellular transcripts, and cells are then pooled back together. Repeating this process several times ensures a high likelihood that each cell travels through a unique combination of wells. Consequently, the transcriptome of each individual cell is labeled with a unique combination of well-specific barcodes. Unlike previous methods that scale linearly with the number of available compartments and barcodes, this method scales exponentially with the number of barcoding rounds, enabling a massive increase in the number of cells that can be sequenced, while minimizing doublets.

The products of this protocol are up to 8 subpools of varying size, where each molecule has a cell/nucleus barcode and UMI, ready for Illumina short-read sequencing. The cell/nucleus barcode is unique within each subpool, so the subpol index acts as the fourth barcode to uniquely barcode all the cells/nuclei within an experiment. Please see the attachment for the original Parse Biosciences protocol. The main deviations from the original protocol are 1. loading **15 ul** rather than **14 ul** in the Round 1 barcoding plate and 2. **saving all "leftover" barcoded nuclei or cells at -80C** for potential repeats.

The first part of the protocol, Section 1, describes barcoding cells or nuclei. Briefly, fixed cells/nuclei are thawed and added to the Round 1 reverse transcription barcoding plate at 15,000 cells/nuclei per well across 48 wells. Individual samples from each tissue are distributed in the sample barcoding plate with at least 1 well per sample. Within the fixed cells/nuclei, RNA is reverse transcribed using oligodT and random hexamer primers and the first barcode is annealed. After RT, cells/nuclei are pooled and distributed in 96 wells of the Round 2 ligation barcoding plate for in situ barcode ligation. After Round 2, cells/nuclei are pooled and redistributed into 96 wells of the Round 3 ligation barcoding plate for barcode 3 and Illumina adapter ligation. Finally, cells/nuclei are counted using a hemocytometer, distributed into up to 8 subpools, and lysed. The second part, Section 2, describes template switching and amplification of the full-length cDNA. **Importantly, full-length cDNA from Section 2 is the input for cDNA Exome Capture. For non-exome captured Illumina libraries, proceed to Section 3: Preparing Libraries for Sequencing.** Section 3 describes Illumina library preparation with **100 ng** of full-length cDNA per subpool. Subpool cDNA is fragmented and Illumina P5/P7 adapters are ligated during the final amplification. **Importantly, if using single indices (WX100) please follow Appendix D: Single Indexing. If using dual indices (WX200) follow main protocol.** Libraries with 5% PhiX spike-in are sequenced on an Illumina sequencer as paired-end, single-index (140/86/6/0) or dual-index reads (130/86/8/8).

Attachments

PDF

Evercode+WT+User+Man...

5.8MB

Materials

User Supplied Equipment and Consumables

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. Any questions regarding these items can be directed to support@parsebiosciences.com.

	Item	Supplier	Part Number	Notes
	Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Capable of reaching 4°C. Compatible with 15 mL centrifuge tubes and 96-well plates.
	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.
	T100 Thermal Cycler	Bio-Rad Laboratories	1861096	Or an equivalent thermocycler compatible with unskirted 96-well plates and a heated lid capable of 50-105°C.
	Parse Biosciences Magnetic Rack	Parse Biosciences	SB1004	Magnetic strength is critical. If 3rd party magnetic racks are used, the number of transcripts and genes detected per cell will be compromised. This magnetic rack is compatible with most 0.2 mL PCR tubes.
	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	Compatible with a vortex adapter for 96-well plates. Or a shaker set to 800-1000 RPM. Part number varies with different lab voltage and frequency requirements.
	6-inch Platform	Scientific Industries	146-6005-00	Or an equivalent vortex adapter for 96-well plates.
	Microplate Foam Insert	Scientific Industries	504-0235-00	Or an equivalent vortex adapter for 96-well plates.
	Qubit Flex Fluorometer	Thermo Fisher Scientific	Q33327	Or an equivalent fluorometer.
	2100 Bioanalyzer	Agilent	G2939BA	Choose one.
	4200 TapeStation System	Agilent	G2991BA	

Equipment

	Item	Supplier	Part Number	Notes
	Falcon High Clarity PP Centrifuge	Corning	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes as it will lead to substantial cell loss.

	Item	Supplier	Part Number	Notes
	Tubes, 15 mL			
	Corning Cell Strainer (70 µm or 100 µm)	Corning	431751 (70 µm) 431752 (100 µm	For cells larger than 40 µm, the 40 µm strainer should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm).
	DNA LoBind Tubes, 1.5 mL, Snap Cap	Eppendorf	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.
	DNA LoBind Tubes, 5 mL, Snap Cap	Eppendorf	0030108310	Or equivalent DNA low-binding, 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.
	Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin	17014961 17014963 17014967	Or appropriate sterile, DNA low-binding, and filtered pipette tips. Do not use wide bore tips. Autoclaved pipette tips are not RNase and DNase free.
	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 that can be used to assess cell viability, such as AOPI.
	KAPA Pure Beads	Roche	KK8000 (5 mL) KK8001 (30 mL)	Choose one. We do not recommend substituting other magnetic beads, including SPRIselect (Beckman Coulter) and ProNex (Promega).
	AMPure XP Reagent	Beckman Coulter	A63880 (5 mL) A63881 (60 mL)	
	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 chosen Bioanalyzer or TapeStation.
	High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	

Consumables

Troubleshooting

Before start

User Supplied Equipment and Consumables: Before starting an experiment, check the "User Supplied Equipment and Consumables" section and confirm that your lab has all of the supplies that are not provided by the kit. Avoid substituting custom materials for those that are provided in the kit. Each item has been deliberately chosen to attain optimal results.

Avoiding RNase Contamination: Standard precautions should be taken to avoid introducing RNases into samples or reagents throughout the workflow. Always wear proper laboratory gloves and use aseptic technique. RNases are not inactivated by ethanol or isopropanol, but can be inactivated by specific products such as RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific). These can be sprayed on benchtops and used to clean pipette. It is recommended to use pre-sterilized, filter pipette tips to reduce RNase contamination from pipettes.

Centrifuges: Use a swinging bucket centrifuge for all high speed spin steps in this protocol. Use of a fixed-angle centrifuge will lead to substantial cell loss. Although the recommended centrifugation speeds are appropriate for most sample types, they can be adjusted to improve retention.

Centrifuge Tubes: Ensure that the tubes will be used are **polypropylene** and not polystyrene. Polystyrene tubes will lead to substantial cell loss.

Sample Handling: It is critical that cells are thoroughly resuspended after centrifugation. Resuspend cells by slowly (to prevent mechanical damage) and repeatedly pipetting up and down until no clumps are visible. Wide bore pipette tips are not recommended as they make it difficult to adequately resuspend cell pellets. Due to cell adherence to tubes, it is recommended to carefully pipette along the bottom and sides of the centrifuge tubes to minimize cell loss.

Sample Loading Table: The "Sample Loading Table V1.3.0" (Excel spreadsheet) should be completed before starting the experimental workflow. If not working properly, ensure that the Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells on the table to avoid disturbing the necessary formatting.

Maximizing Cell Retention During Pooling Steps: During the barcoding steps, some cells may stick to the side of the wells in the 96-well plates. To increase cell retention, it is important to pipette up and down several times in each well before removing and pooling cells. Note that additional pipetting may lead to increased bubbles while pooling. While bubbles will not affect results, we advise using caution when pipetting to prevent excess bubble formation and maintain experimental ease. We recommend the following procedure when pooling:

- Set the multichannel P200 to 10 µL less than the volume in each well. The volumes for Barcoding Rounds 1, 2, and 3 should be 30 µL, 50 µL, and 70 µL, respectively. This will avoid bubbles while pipetting up and down.
- Insert tips into the bottom of the wells. Pipette up and down 3x in the middle of the well, then pipette up and down 3x on the front side of the well, followed by 3x on the back side of the well, before proceeding with pooling cells.
- Pool any remaining liquid left in the wells (should be ~10 µL).

Sealing Plates in Original Container: There are multiple steps requiring the removal and application of seals to 96-well plates. In either motion, ensure that the plate is in its original container for best support. Failure to do so may result in plate slippage and loss, or swapping, of liquid between wells.

Cell Strainers: A 40 µm cell strainer will be used in multiple steps. To maximize cell retention, press the pipette tip directly against the stainer. Ensure that ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. For cells larger than 40 µm, the 40 µm strainer should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm).

Lysis Buffer Precipitate: Ensure that there is no precipitate when using the 2x Lysis Buffer. Warming the 2x Lysis Buffer at 37C for 5 minutes should resolubilize solution. If precipitate remains, warm 2x Lysis buffer at 37C for another 5 minutes.

Sequencing Libraries: Multiple sequencing libraries can be prepared from the same experiment. At the end of barcoding (Section 1), the recovered cells can be split across different sublibraries. The number of cells to be sequenced is determined when cells are divided into sublibraries at the lysis step. Thus, not all of the cells prepared in these steps must be sequenced together.

Section 1: Barcoding Single Cells

1 **1.1: Experimental Setup**

1.1 Prepare for the first round of barcoding with the following checklist:

- Each sample should have been counted after nuclei fixation and recorded on the spreadsheet in order to calculate volumes to normalize concentrations to 1,000 cells/nuclei per ul. Do math so that dilution buffer volume varies but nuclei/cell volume is the same. Adjust target volumes as necessary depending on the volume needed in the final plate(s).
- At least a day ahead of the experiment, distribute dilution buffer in a new sample normalization plate one well at a time (NOT the actual barcoding plate) and store at -20C.
- On the day of the experiment, take out sample normalization plate and thaw at room temperature. Centrifuge the plate at **100 x *g*** for **1 minute** and place on ice.
- Set your swinging bucket centrifuge to **4C.**
- Prepare a **37C** water bath.
- Fill some ice buckets, enough to hold 3 96-well plates and several tubes.

1.2

	Item	Location	Quantity	Format	After taking out
	Adhesive 96-well plate cover	Accessories (Room Temp)	1	With white protector	Keep at room temperature
	Spin Additive	4C Reagents (4C)	1	1.5 mL tube	Keep at room temperature.
	Dilution Buffer	Barcoding Reagents (-20C)	2	2 mL tube	Thaw, then place on ice
	Resuspension Buffer	Barcoding Reagents (-20C)	1	5 mL tube	Thaw, then place on ice
	Ligation Mix	Barcoding Reagents (-20C)	1	5 mL tube	Thaw, then place on ice
	Round 2 Ligation Enzyme	Barcoding Reagents (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Round 1 Plate	Barcoding Reagents (-20C)	1	96-well plate	Thaw, then place on ice
	Round 2 Plate	Barcoding Reagents (-20C)	1	96-well plate	Thaw, then place on ice

Critical! Only proceed if you have completed the checklist in step 1 and taken out all the items listed in step 2.

1.3 To thaw, place the **Round 1 Plate** into a thermocycler and set the following protocol below. The heated lid will force any liquid on the plastic plate seal back down into the well. Proceed to the next step while the thermocycler is running.

	Run Time	Lid Temperature	Sample Volume
	10 min	70C	26 µl

Round 1 Plate Thaw Protocol Overview

	Step	Time	Temperature
	1	10 min	25C
	2	Hold	4C

Round 1 Plate Thaw Protocol

2 **1.2: Sample Counting and Loading Setup**

2.1 Thaw the fixed cell samples in a **37C** water bath until all ice crystals dissolve, then place on ice. It is important to fully thaw samples before placing on ice.

2.2 Arrange samples in desired order in 1.5 mL plate racks on ice.
Critical! Double-check order of samples matches the order on the spreadsheet.

2.3 *Note*: *This step requires a new box of 20 µl tips.*
Using a Move-it multichannel pipette with adjustable spacer, pipette fixed nuclei/cells from 1.5 mL tubes to sample normalization plate. Should be same volume across entire plate. When pipetting cells/nuclei, mix gently by pipetting up and down when taking cells/nuclei and again when dispensing.

3 **1.3: Reverse Transcription Barcoding**
During this section, cDNA will be reverse transcribed from RNA with barcoded RT primers specific to each well.

3.1 Gently remove the **Round 1 Plate** from the thermocycler and place into the original green plastic plate holder. Centrifuge the plate at **100 x *g*** for **1 minute**.

3.2 Place the plate (and holder) on a flat surface and remove the plastic seal. Store on ice.
Note: Plate seals may be difficult to remove. Carefully peel the plate seal while applying downward pressure to keep the plates from moving (to minimize cross-contamination of wells).

3.3 **Add diluted samples to wells in the Round 1 Plate.**
Note: To prevent sample loss, mix cells as indicated below. Additionally, this step requires a new box of 20 µl tips.
Follow the Sample Loading Table during this step to determine which samples to add to each well. Using a multichannel pipette, add **15 µl** nuclei/cells from the sample normalization plate to each of the 96 wells in the **Round 1 Plate.** Immediately after dispensing cells, mix gently by pipetting up and down exactly 3x. When pipetting the same sample into many wells, the same sample should be periodically mixed by gentle pipetting to avoid cells settling. Do not vortex your cells.
Critical! Different tips must be used when pipetting cells into the 96-well plate. Never place a tip that has entered one of the 96 wells into a different well.

3.4 Remove the **Round 1 Plate** and holder from the ice bucket and place on a flat surface. Seal the **Round 1 Plate** with an additional 96-well plate seal cover.
Note: A plate sealer is included in the Accessories box.

3.5 **Start the reverse transcription reaction.** Put the **Round 1 Plate** with cells into a thermocycler with the following thermocycling protocol:

	Run Time	Lid Temperature	Sample Volume
	~40 min	70C	40 µl

Round 1 Plate Barcoding Protocol Overview

	Step	Time	Temperature
	1	10 min	50C
	2	12 sec	8C
	3	45 sec	15C
	4	45 sec	20C
	5	30 sec	30C
	6	2 min	42C
	7	3 min, then go to step 2 and repeat 2 times 3 cycles total)	50C
	8	5 min	50C
	9	Hold	4C

Round 1 Plate Barcoding Protocol

3.6 Transfer the **Round 1 Plate** from the thermocycler back to the original green plate holder and place on ice.

3.7 Thaw the **Round 2 Plate** by transferring the plate from the ice bucket into the thermocycler and running the following protocol. Proceed directly to the next step.

	Run Time	Lid Temperature	Sample Volume
	10 min	70C	10 µl

Round 2 Plate Thaw Protocol Overview

	Step	Time	Temperature
	1	10 min	25C
	2	Hold	4C

Round 2 Plate Thaw Protocol

3.8 Place the **Round 1 Plate** (and holder) on a flat surface and remove adhesive seal. Place back on ice.

3.9 **Pool all wells from the Round 1 Plate into a single 15-mL centrifuge tube on ice.**
Note: Proper mixing is required to prevent substantial cell loss during pooling. See "Maximizing Cell Retention During Pooling Steps" in Notes Before Starting.
The pooling process can be simplified (see figure below). With the multichannel pipette set to 30 µL, pool rows B-D into the wells in Row A. To maximize cell retention while pooling, pipette up and down 3x in the middle of the well, 3x on the front side of the well, and 3x on the back side of the well before transferring the volume of the rows to Row A. Recover residual liquid across rows B-D using the multichannel pipette set to 10 µL. Next, pipette the total volume in Row A up and down 3x, then transfer the total volume of each well in Row A into the same 15 mL centrifuge tube with a single channel P200 pipette set to 200 µL. Do not be concerned if there are a few µL of residual volume in the wells after pooling.
Note: Bubbles may form while pooling. They will not affect the quality of the experiment.
Critical! Both the Round 1 Plate and the 15 mL falcon tube with pooled cells should be kept on ice during the pooling step.

3.10 Discard the **Round 1 Plate.**

3.11 Add **9.6 µL** of **Spin Additive** to the 15 mL tube with pooled cells. Gently invert the tube once to mix.
Critical! Do NOT discard the **Spin Additive** as it will be needed in another step.

3.12 Centrifuge the pooled cells in a swinging bucket centrifuge cooled to **4C** for **10 minutes** at **200 x *g*.**
Critical! Move to the next step as soon as the centrifuge finishes and handle the tube gently to avoid dislodging the cell pellet. Waiting too long to aspirate supernatant increases the risk of dislodging the pellet.

3.13 Using a P1000 pipette for the first 1 mL, then a P200 pipette for remaining volume, aspirate supernatant such that about ~40 µL of liquid remains above the pellet. Do not disturb the pellet. Depending on the number of starting cells and cell types, the pellet may not be visible.

3.14 *Note: To prevent substantial cell loss during resuspension, see "Sample Handling" in Notes Before Starting.*
Gently resuspend cells with **1 mL** of **Resuspension Buffer.** Once cells are fully resuspended, add an **additional 1 mL** of **Resuspension Buffer** to make a total volume of 2 mL. Keep this solution on ice and proceed to Ligation Barcoding.

4 1.4: Ligation Barcoding

4.1 Gather the following items and handle as indicated below:

	Item	Location	Quantity	Format	After taking out
	Adhesive 96-well plate cover	Accessories (Room Temp)	3	With white protector	Keep at room temperature
	40 um strainer	Accessories (Room Temp)	2	In plastic bag	Keep at room temperature

	Item	Location	Quantity	Format	After taking out
	Basins	Accessories (Room Temp)	6	In plastic bag	Keep at room temperature
	Round 2 Stop Mix	Barcoding Reagents (-20C)	1	2 mL tube	Thaw, then place on ice
	Round 3 Stop Mix	Barcoding Reagents (-20C)	1	5 mL tube	Thaw, then place on ice
	Pre-Lyse Wash Buffer	Barcoding Reagents (-20C)	1	5 mL tube	Thaw, then place on ice
	Round 3 Ligation Enzyme	Barcoding Reagents (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Round 3 Plate	Barcoding Plates (-20C)	1	96-well plate	Place directly on ice

4.2 Lightly centrifuge the **Round 2 Ligation Enzyme** and add **20 µL** of **Round 2 Ligation Enzyme** directly into the cold **Ligation Mix** tube to make **Ligation Mix + Enzyme.**

4.3 Using a P1000 pipette, add **2 mL** of cells in **Resuspension Buffer** into the **Ligation Mix + Enzyme** tube. Mix 10x with a P1000 pipette set to 1000 µL and place back on ice.
Critical! Do NOT vortex the **Ligation Mix**.

4.4 Transfer the **Round 2 Plate** from the thermocycler back to its original blue plate holder and keep at room temperature. Centrifuge the plate at **100 x *g*** for **1 minute**. Place the plate (and holder) on a flat surface and remove the seal. Keep at room temperature.
Note: Plate seals may be difficult to remove. Carefully peel the plate seal while applying downward pressure to keep the plates from moving (to minimize cross-contamination of wells).

4.5 Using a P1000 pipette, add the entirety of cells in the **Ligation Mix + Enzyme** to a basin.

4.6 **Add pooled cells to the Round 2 Plate.**
Note: To prevent sample loss, mix cells as indicated below. Additionally, this step requires a new box of 200 µL tips.
Using a multichannel P200 pipette, add **40 µL** of mix in the basin to each of the 96 wells in the **Round 2 Plate.** As you add the 40 µL to each well, pipette up and down exactly 2x to ensure proper mixing. To avoid cells settling in the basin, also gently pipette up and down 2x with the multichannel pipette in the basin before transferring the cells from the basin to each row.
Note: Using a single channel pipette and tilting the basin may be required to fill the last row if volume in the basin is low. If volume is insufficient to fill every well, a few wells can be left empty without impacting experimental results.
Critical! Different tips must be used when pipetting cells into the 96-well plate. Never place a tip that has entered one of the 96 wells back into the basin.

4.7 Reseal the **Round 2 Plate** with an adhesive seal.

4.8 **Start the second round of barcoding.** Incubate the **Round 2 Plate** in a thermocycler with the following protocol:

	Run Time	Lid Temperature	Sample Volume
	30 min	50C	50 µL

Round 2 Ligation Barcoding Overview

	Step	Time	Temperature
	1	30 min	37C
	2	Hold	4C

Round 2 Ligation Barcoding Protocol

4.9 Vortex the **Round 2 Stop Mix** briefly (2-3 sec) and using a P1000 pipette, and the entirety (~1.4 mL) to a new basin.

4.10 Transfer the **Round 2 Plate** from the thermocycler back to its original blue plate holder and remove the seal. Keep the plate at room temperature.

4.11 **Add Round 2 Stop Mix to each well.**
Note: This step requires a new box of 20 µL tips.
Using a multichannel P20 pipette, add **10 µL** of the **Round 2 Stop Mix** in the basin to each of the 96 wells of the **Round 2 Plate.** Pipette up and down exactly 3x to ensure proper mixing after adding **Round 2 Stop Mix** to each well.
Critical! Different tips must be used when pipetting **Round 2 Stop Mix** into the 96-well plate. Never place a tip that has entered one of the 96 wells back into the basin.

4.12 Reseal the **Round 2 Plate** with an adhesive seal.

4.13 Incubate the **Round 2 Plate** in a thermocycler with the following protocol:

	Run Time	Lid Temperature	Sample Volume
	30 min	50°C	60 µL

Round 2 Stop Overview

	Step	Time	Temperature
	1	30 min	37°C
	2	Hold	

Round 2 Stop Protocol

4.14 Transfer the **Round 2 Plate** from the thermocycler to its original blue plate holder and keep at room temperature.

4.15 Thaw the **Round 3 Plate** by transferring it from the ice bucket into the thermocycler and running the following protocol. Proceed directly to the next step.

	Run Time	Lid Temperature	Sample Volume
	10 min	70C	10 µl

Round 3 Plate Thaw Overview

	Step	Time	Temperature
	1	10 min	Temperature
	2	Hold	4C

Round 3 Plate Thaw Protocol

4.16 Remove the seal on the **Round 2 Plate**.

4.17 **Pool all wells from the Round 2 Plate into a new basin.**
Note: Proper mixing is required to prevent substantial cell loss during pooling. See "Maximizing Cell Retention During Pooling Steps" in Notes Before Starting.
With the multichannel pipette set to 50 µL, pool volume from each well into a new basin. To maximize cell retention while pooling, pipette up and down 3x in the middle of the well, 3x on the front sides of the well, and 3x on the back side of the well before transferring the volume of rows A-H to the basin. Recover residual liquid across all rows using the multichannel pipette. Do not be concerned if there are a few µL of residual volume in the wells after pooling.
Note: Bubbles may form while pooling. They will not affect the quality of the experiment.

4.18 Discard the **Round 2 Plate.**

4.19 Remove the 40 µm strainer from the packaging and carefully hold the strainer using the outside casing without touching the mesh. Using a P1000 pipette set to 1000 µL, pass all cells from this basin through the 40 µm strainer into a new basin. Mix cells in the basin between passages. The original basin must be tilted in order to pipette the final volume.
Note: For cells larger than 40 µm, the 40 µm strainer should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm). Additionally, bubbles may form while straining. They will not affect the quality of the experiment.
Critical! To ensure that all of the liquid passes through the strainer, press the tip of the pipette against the filter and press the pipette plunger down steadily. All of the liquid should pass through the strainer in ~1 second.

4.20 Add **20 µL** of **Round 3 Ligation Enzyme** to the basin with the strained cells and mix by gently pipetting up and down ~20x with a P1000 pipette set to 1000 µL.

4.21 Transfer the **Round 3 Plate** from the thermocycler back to its original orange plate holder. Centrifuge the plate at **100 x *g*** for **1 minute.** Plate the plate (and holder) on a flat surface and remove the seal. Keep at room temperature.
Note: Plate seals may be difficult to remove. Carefully peel the plate seal while applying downward pressure the keep the plates from moving (to minimize cross-contamination of wells).

4.22 **Add pooled cells to the Round 3 Plate.**
Note: To prevent sample loss, mix cells as indicated below. Additionally, this step requires a new box of 200 µL tips.
Using a multichannel P200 pipette, add **50 µL** of mix in the basin to each of the 96 wells in the **Round 3 Plate.** As you add the 50 µL to each well, pipette up and down exactly 2x

to ensure proper mixing. To avoid cells settling in the basin, also gently pipette up and down 2x with the multichannel pipette in the basin before transferring the cells from the basin to each row.
Note: Using a single channel pipette and tilting the basin may be required to fill the last row if volume in the basin is low. If volume is insufficient to fill every well, a few wells can be left empty without impacting experimental results.
Critical! Different tips must be used when pipetting cells into the 96-well plate. Never place a tip that has entered one of the 96 wells back into the basin.

4.23 Reseal the **Round 3 Plate** with an adhesive seal.

4.24 **Start the third round of barcoding.** Incubate the **Round 3 Plate** in a thermocycler with the following protocol:

	Run Time	Lid Temperature	Sample Volume
	30 min	50C	60 µL

Round 3 Ligation Barcoding Overview

	Step	Time	Temperature
	1	30 min	37C
	2	Hold	4C

Round 3 Ligation Barcoding

4.25 Remove the **Round 3 Plate** from the thermocycler, place it in its original orange plate holder on a flat surface and remove the seal. Keep at room temperature.

4.26 Vortex the **Round 3 Stop Mix** briefly (2-3 sec) and using a P1000 pipette, add the entirety of the Round 3 Stop Mix to a new basin.

4.27 **Add Round 3 Stop Mix to each well.**
Note: This step requires of 20 µL tips.
Using a multichannel P20 pipette, add **20 µL** of the **Round 3 Stop Mix** in the basin to each of the 96 wells of the **Round 3 Plate.** Pipette up and down exactly 3x to ensure proper mixing after adding the **Round 3 Stop Mix** to each well. No incubation required after this step, proceed directly to the next step.
Critical! Different tips must be used when pipetting stop mix into the 96-well plate. Never place a tip that has entered one of the 96 wells back into the basin.

4.28 **Pool all cells from the Round 3 Plate into a new basin.**
Note: Proper mixing is required to prevent substantial cell loss during pooling. See "Maximizing Cell Retention During Pooling Steps" in Notes Before Starting.
With the multichannel pipette set to 70 µL, pool volume from each well into a new basin. To maximize cell retention while pooling, pipette up and down 3x in the middle of the well, 3x on the front sides of the well, and 3x on the back side of the well before transferring the volume of rows A-H to the basin. Recover residual liquid across all rows using the multichannel pipette. Do not be concerned if there are a few µL of residual volume in the wells after pooling.
Note: Bubbles may form while pooling. They will not affect the quality of the experiment.

4.29 Discard the **Round 3 Plate.**

4.30 Remove a 40 µm strainer from the packaging and carefully hold the strainer using the outside casing without touching the mesh. Using a P1000 pipette set to 1000 µL, pass all the cells from this basin through a 40 µm strainer into a new 15 mL tube on ice. Mix cells in the basin in between passages. The basin must be tilted in order to pipette the final volume. Keep the 15 mL tube on ice and proceed to lysis.
Note: Bubbles may form while straining. They will not affect the quality of the experiment.

5 **1.5: Lysis and Sublibrary Generation**

5.1 Gather the following items and handle as indicated below:

	Item	Location	Quantity	Format	After taking out
	2x Lysis Buffer	4C Reagents (4C)	1	1.5 mL tube	Keep warm at 37C until use.
	Lysis Enzyme	Barcoding Reagents (-20C)	1	1.5 mL tube	Place directly on ice.
	Dilution Buffer	Barcoding Reagents (-20C)	2	2 mL tube	Thaw, then place on ice.

5.2 Add **70 µL** of **Spin Additive** to your cells in a 15 mL centrifuge tube. Gently invert the tube once to mix.

5.3 Use a swinging bucket centrifuge to spin down the cells for **10 minutes** at **200 x *g*** at **4C**.

5.4 Using a P1000 pipette for the first 6 mL, then a P200 pipette for the remaining volume, aspirate supernatant such that ~40 µL of liquid remains above the pellet. Do not disturb the pellet. Depending on the number of starting cells and cell types, the pellet may not be visible.

5.5 *Note: To prevent substantial cell loss during resuspension, see "Sample Handling" in Notes Before Starting.*
Gently resuspend cells with **1 mL** of **Pre-Lyse Wash Buffer.** When resuspending the pellet, pipette slowly to prevent mechanical damage to cells. Once cells are fully resuspended, add an **additional 3 mL** of **Pre-Lyse Wash Buffer** for a total volume of 4 mL.

5.6 Use a swinging bucket centrifuge to spin down for **10 minutes** at **200 x *g*** at **4C**.

5.7 Using a P1000 for the first 3 mL, then a P200 pipette for the remaining volume, aspirate supernatant such that ~40 µL of liquid remains above the pellet (see image on the right for estimate of 40 µL). Do not disturb the pellet. Depending on the number of starting cells and cell types, the pellet may not be visible.

5.8 *Note: To prevent substantial cell loss during resuspension, see "Sample Handling" in Notes Before Starting.*

Using a P200 pipette, gently resuspend the pellet with an additional **60 µL** of **Dilution Buffer,** bringing the final volume to ~100 µL. When resuspending the pellet, pipette slowly to prevent mechanical damage to cells. Keep tubes on ice.
Critical! Do NOT discard **Dilution Buffer** as it will be needed in another step.

5.9 Using a P200 pipette set to 80 µL, gently pipette up and down the 5x and immediately use **5 µL** of the mixed cells to count using a hemocytometer. Keep the 15 mL tube on ice.
*Note: When using a hemocytometer, dilute **5 µL** of the mixed cell solution into **5 µL** of Trypan Blue or DAPI. Mix well and load onto the hemocytometer. Some level of debris is normal at this step. Alternatively, cells can be counted via flow cytometry, but using a hemocytometer is strongly recommended.*

Choosing Sublibrary SIzes: In the following step, cells will be aliquoted into different sublibraries that will be prepared for sequencing. At the end of the library prep, each sublibrary will have its own sublibrary index, making it possible to sequence each sublibrary with different numbers of reads. It is also possible to add different numbers of cells to each sublibrary. In practice it can be useful to have at least one sublibrary with very few cells (200-500) that can be sequenced deeply (>50,000 reads per cells) with a limited number of overall reads. This sublibrary then provides a good estimate of gene and transcript detection per cell that would be expected if the other sublibraries were also sequenced deeply. The maximum number of cells that can eventually be sequenced will be the sum of the number of cells across all sublibraries.

We slightly overload 8 subpools with 13,000 cells/nuclei. Any extra barcoded cells/nuclei are distributed in additional 13,000 aliquots and kept at -80C.

5.10 **Determine sublibrary size(s) and dilutions.** Up to 8 sublibraries, of varying sizes, can be made. Use the "Sublibrary Generation Table" (Appendix A) to determine the volume of cells and **Dilution Buffer** to add to each sublibrary (dependent on desired sublibrary size and the concentration of cells measured in the previous step). Give each sublibrary a unique label. Make sure to record which sublibrary sizes correspond to what labels. Label both the top and side of the PCR tube with those labels.
Critical! Do NOT overload a sublibrary. 12,500 cells/sublibrary is the maximum. Overloading a sublibrary lysate with too many cells will result in increased doublets.

5.11 Using a P200 pipette set to 75 µL, gently pipette up and down 5x. Aliquot the determined volume of cells (from the previous step) to each correctly labelled sublibrary PCR tube and add **Dilution Buffer** to bring to total volume to 25 µL. Between each aliquot, gently pipette mix the cells to avoid settling. Store sublibraries on ice.

5.12 Make a **Lysis Master Mix.** Ensure there is no precipitate present in the **2x Lysis Buffer.** Add **220 µL** of **2x Lysis Buffer** to **44 µL** of **Lysis Enzyme** in a 1.5 mL tube.
Critical! Do NOT place **Lysis Master Mix** on ice, as a precipitate will form.

5.13 **Add Lysis Master Mix to sublibraries.** Add **30 µL** of **Lysis Master Mix** to each tube, bringing the total volume to 55 µL. Keep sublibraries at room temperature.

5.14 Vortex samples for 10 sec to initiate lysis. Be sure to keep caps closed on tubes. Briefly centrifuge tubes (~2 sec).

5.15 Incubate the sublibrary lysates in a thermocycler with the following protocol.

	Run Time	Lid Temperature	Sample Volume
	60 min	80C	55 µL

Sublibrary Lysis Overview

	Step	Time	Temperature
	1	60 min	65C
	2	Hold	4C

Sublibrary Lysis Protocol

5.16 Freeze sublibrary lysate(s) at **-80C**. Sublibrary lysates can be stored for up to 6 months.
[STOPPING POINT]

Section 2: Amplification of Barcoded cDNA

6 **2.1 Preparing Binder Beads**
Any number of sublibraries (1-8) can be chosen for processing, where each sublibrary will ultimately be barcoded a fourth time with a sublibrary index. **Take care not to cross-contaminate any sublibraries for the remainder of the experiment.**
Setup
- Fill an ice bucket.
- Take out a magnetic rack for 1.5 mL tubes.
- Take out the Parse Biosciences magnetic rack for 0.2 mL PCR tubes.
- Ensure you have at least 79 µL of SPRI beads (Ampure XP or KAPA Pure Beads) per sublibrary.
Gather the following items and handle as indicated below:
Note: Do NOT remove sublibrary lysates from the freezer until the beginning of Section 2.2.

	Item	Location	Quantity	Format	After taking out
	Binder Beads	4C Reagents (4C)	1	1.5 mL tube	Keep at room temperature.
	Bead Wash Buffer	cDNA Amplification (-20C)	1	5 mL tube	Keep at room temperature.
	Bind Buffer A	cDNA Amplification (-20C)	1	1.5 mL tube	Keep at room temperature.
	Bind Buffer B	cDNA Amplification (-20C)	1	5 mL tube	Keep at room temperature.
	Bind Buffer C	cDNA Amplification (-20C)	1	5 mL tube	Keep at room temperature.
	Bead Storage Buffer	cDNA Amplification (-20C)	1	5 mL tube	Keep at room temperature.
	TS Buffer	cDNA Amplification (-20C)	1	2 mL tube	Thaw, then place on ice.
	Lysis Neutralizatio n	cDNA Amplification (-20C)	1	1.5 mL tube	Place directly on ice.

6.1 Vortex **Binder Beads** until fully mixed and add a volume to an empty 1.5 mL tube according to the number of sublibrary lysates that you plan to process:

	A	B	C	D	E	F	G	H	I
	# Sublibrary Lysates	1	2	3	4	5	6	7	8
	Binder Beads (µL)	44	88	132	176	220	264	308	352

Volume to Add by Number of Sublibrary Lysates (µL)

6.2 Capture the **Binder Beads** to a magnet using a magnetic rack (for 1.5 mL tubes) and wait until liquid becomes clear (~2 min).

6.3 Remove the clear supernatant with a pipette and discard.

6.4 Remove the tube from the magnetic rack and resuspend beads with the appropriate volume of **Bead Wash Buffer** (see table below). Ensure that all beads are fully resuspended and not stuck to the side of the tube.

	A	B	C	D	E	F	G	H	I
	# Sublibrary Lysates	1	2	3	4	5	6	7	8
	Bead Wash Buffer (µL)	100	200	300	400	500	600	700	800

Volume to Add by Number of Sublibrary Lysates (µL)

6.5 Capture the **Binder Beads** to a magnet using a magnetic rack (for 1.5 mL tubes) and wait until liquid becomes clear (~2 min).

6.6 Remove the clear supernatant with a pipette and discard.

6.7 Repeat steps 4-6 twice more for a total of three washes.

6.8 Remove the tube from the magnetic rack and resuspend beads in the appropriate volume of **Bind Buffer A** (see table below). Keep beads at room temperature and proceed to Section 2.2.

	A	B	C	D	E	F	G	H	I
	# Sublibrary Lysates	1	2	3	4	5	6	7	8
	Bind Buffer A (µL)	55	110	165	220	275	330	385	440

Volume to Add by Number of Sublibrary Lysates (µL)

7 **2.2 Applying Binder Beads to Sublibrary Lysates**

7.1 Remove the desired sublibrary lysates from the -80C freezer and incubate at **37C** for **5 minutes**, ensuring that no precipitate is present before proceeding. If precipitate is still present, incubate at **37C** for 5 more minutes.

7.2 Briefly centrifuge sublibrary lysates (~ 2 sec).

7.3 Lightly centrifuge the **Lysis Neutralizer,** mix gently with a pipette, and add **2.5 µL** to each sublibrary lysate. Mix 5x with a P200 pipette (set to 40 µL), taking care not to lose any volume. Briefly centrifuge (~2 sec), and incubate at room temperature for **10 minutes.**

7.4 **Add Binder Beads to sublibrary lysates.** First mix the **Binder Beads** suspended in **Bind Buffer A** by pipetting up and down. Then add **50 µL** to each sublibrary lysate. Mix 5x with. aP200 pipette (set to 90 µL), taking care not to lose any volume. Discard the tube with any excess **Binder Beads.**

7.5 Agitate the sublibrary lysates with **Binder Beads** at room temperature for **60 minutes.** Place the tubes in a 96-well plastic plate holder (press tubes securely into the holder) with the lid on and then put the plastic holder into a foam attachment for a vortexer. Vortex on 2 (out of 10) for the duration of the 60 minute incubation (~800-1000 RPM).

7.6 Take the tubes off of the vortexer (beads may have settled somewhat). Vortex briefly (~5 sec) and then briefly centrifuge (~1 sec) without letting beads collect at the bottom of the tubes.

7.7 Place the tubes against a magnetic rack (for 0.2 mL tubes) on the high position (with magnets closest to the top) and wait for all the beads to bind to the magnet (~2 min). *Critical!* The supernatant should be clear before proceeding. The cDNA is unamplified at this step, so discarding any beads in the supernatant will result in a reduction of transcripts and genes detected per cell.

7.8 Remove the clear supernatant with a pipette and discard, while still keeping the tubes in the magnetic rack.

7.9 Remove tubes from the magnetic rack and resuspend beads with **125 µL** of **Bind Buffer B.**

7.10 Keep tubes at room temperature for **1 minute.**

7.11 Place the tubes against a magnetic rack on the high position and wait for all the beads to bind to the magnet (~2 min; liquid should be clear).

7.12 Remove the clear supernatant with a pipette and discard., while still keeping the tubes in the magnetic rack.

7.13 Repeat steps 9-12 for a second wash during **Bind Buffer B.**

7.14 Remove the tubes from the magnetic rack and resuspend beads with **125 µL** of **Bead StorageBuffer.**

7.15 Keep tubes at room temperature for **1 minute.**

7.16 Proceed directly to Section 2.3: Template Switch

8 **2.3 Template Switch**

8.1 Gather the following items and handle as indicated below:

	Item	Location	Quantity	Format	After taking out
	TS Primer Mix	cDNA Amplification (-20C)	1	1.5 mL tube	Thaw, then place on ice
	TS Enzyme	cDNA Amplification (-20C)	1	1.5 mL tube	Place directly on ice

8.2 Ensure that the **TS Buffer** is fully thawed and has no white precipitate before proceeding.

8.3 In a new 1.5 mL tube, make the **Template Switch Mix** by adding the following volumes of **TS Buffer, TS Primer Mix,** and **TS Enzyme** together. Mix well and store on ice.

	A	B	C	D	E	F	G	H	I
	# Sublibrary Lysates	1	2	3	4	5	6	7	8
	TS Buffer	101.75	203.5	305.25	407	508.75	610.5	712.25	814
	TS Primer Mix	2.75	5.5	8.25	11	13.75	16.5	19.25	22
	TS Enzyme	5.5	11	16.5	22	27.5	33	38.5	44
	Total	110	220	330	440	550	660	770	880

Volume to Add by Number of Sublibrary Lysates (µL)

8.4 Place the sublibrary tubes against a magnetic rack (for 0.2 mL tubes) on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear). *Critical!* The supernatant should be clear before proceeding. The cDNA is unamplified at this step, so discarding any beads in the supernatant will result in a reduction of transcripts and genes detected per cell.

8.5 Remove the clear supernatant with a pipette and discard while still keeping the tubes in the magnetic rack.

8.6 Without resuspending beads, add **125 µL** of **Bind Buffer C** and wait **1 minute.** *Critical!* Do NOT discard the supplied stock of **Bind Buffer C** as it will be used again in a later step.

8.7 Without removing tubes (still in magnetic rack), remove and discard **Bind Buffer C** from each tube using a pipette.

8.8 Remove the tubes from the magnetic rack and resuspend beads with **100 µL** of **Template Switch Mix.**

*Note: **Template Switch Mix** is a viscous solution. Ensure that beads are fully resuspended and well mixed before progressing.*

8.9 Centrifuge tubes very briefly (~1 sec). Longer centrifugation times will cause beads to settle.

8.10 Incubate sublibraries at room temperature for **30 minutes.**

8.11 Mix sublibraries by pipetting 5x, ensuring that beads that may have settled are resuspended. Be careful to prevent any losses of bead volumes while pipetting. Incubate sublibraries in a thermocycler with the following protocol:

	Run Time	Lid Temperature	Sample Volume
	90 min	70C	100 µL

Sublibrary Template Switching Overview

	Step	Time	Temperature
	1	90 min	42C
	2	Hold	4C

Sublibrary Template Switching

8.12 If you would like to stop and store sublibraries, proceed with the following steps. If you are continuing the protocol, proceed directly to Section 2.4: cDNA Amplification.
Note: You may need to pipette mix to resuspend settled beads.
a. Place the tubes against a magnetic rack on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
b. Remove the clear supernatant with a pipette and discard, while still keeping the tubes in the magnetic rack.
c. Resuspend the beads in **125 µL** of **Bead Storage Buffer.**
d. Store tubes at 4C overnight. **Do not freeze sublibraries.**

9 **2.4 cDNA Amplification**
Multiple thermocyclers may be needed for this section depending on your sample types and sublibrary sizes. Refer to step 2.4.8 to determine how many thermocyclers are needed.

9.1 Gather the following items and handle as indicated below:

	Item	Location	Quantity	Format	After taking out
	Amplification Master Buffer	cDNA Amplification (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Amplification Primer Mix	cDNA Amplification (-20C)	1	1.5 mL tube	Thaw, then place on ice

9.2 In a new 1.5 mL tube, make the **Amplification Reaction Solution** by adding the following volumes of **Amplification Master Buffer** and **Amplification Primer Mix.** Mix well and store on ice.

	# Sublibraries	1	2	3	4	5	6	7	8
	Amplification Master Buffer	60.5	121	181.5	242	302.5	363	423.5	484
	Amplification Primer Mix	60.5	121	181.5	242	302.5	363	423.5	484
	Total	121	242	363	484	605	726	847	968

Volume to Add by Number of Sublibrary Lysates (µL).

9.3 Place the sublibrary tubes against a magnetic rack (for 0.2 mL tubes) on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
Note: You may need to pipette mix to resuspend settled beads.

9.4 Remove the clear supernatant with a pipette and discard while still keeping the tubes in the magnetic rack.

9.5 Without resuspending beads, add **125 µL** of **Bind Buffer C** and wait for **1 minute.** Do not remove the tubes from the magnetic rack during this time.

9.6 Remove the clear supernatant with a pipette and discard.

9.7 Remove the tubes from the magnetic rack and resuspend beads in each time with **100 µL** of **Amplification Reaction Solution.** Place tubes with **Amplification Reaction Solution** on ice.

9.8 For each sublibrary, determine the cDNA amplification cycling conditions. Only the number of 2nd cycles (**X**) changes with cell type and sublibrary size. Below are recommended cycling conditions for commonly used cell types.

	Cell Type	Number of Cells/Nuclei in Individual Sublibrary	Number of 1st Cycles (PCR Steps 2-4)	Number of 2nd cycles (X) (PCR Steps 5-7)
	Mammalian Cell Lines	200-1,000	5	12
		1,000-2,000	5	10
		2,000-6,000	5	8
		6,000-12,500	5	6
	Nuclei	200-1,000	5	13
		1,000-2,000	5	11
		2,000-6,000	5	9
		6,000-12,500	5	7
	Immune cells (PBMCs)	200-1,000	5	14
		1,000-2,000	5	12
		2,000-6,000	5	10
		6,000-12,500	5	8

Note: 1-2 cycles may need to be added (to 2nd cycling) to account for cells with low RNA content. The cycling protocol may need to be optimized for your sample type.

9.9 **Start cDNA amplification.** Group sublibraries with the same cycling conditions in their own thermocycler with the following protocol, adjusting the number of 2nd cycles (**X**) according to the table on step 2.4.8.

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

	Run Time	Lid Temperature	Sublibrary Volume
	50-70 min	105C	100 µL

Amplification Overview

	Step	Time	Temperature
	1	3 min	95C
	2	20 sec	98C
	3	***45 sec**	***65C**
	4	3 min, then go to step 2, repeat 4 times (5 cycles total)	72C
	5	20 sec	98C
	6	***20 sec**	***67C**
	7	3 min, then go to step 5, repeat X-1 times (X cycles total)	72C
	8	5 min	72C
	9	Hold	4C

Amplification Protocol

*Example: If you had 500 cells (with medium to high RNA content), your cycling conditions would be: 5 (first cycling) and 12 (second cycling). In this scenario, you would *Go to step 2, repeat 4 times (5 cycles total)" and "Go to step 5, repeat **11** times (**12** cycles total)".*

9.10 Remove tubes from the thermocycler. Sublibraries can be stored at this point at 4C overnight. If you wish to continue, proceed directly to Section 2.5: Post-Amplification SPRI Clean Up.
[STOPPING POINT]

10 **2.4 Post-Amplification SPRI Clean Up**

10.1 Place the sublibrary tubes against a magnetic rack (for 0.2 mL tubes) on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear). *Critical!* Do NOT discard the supernatant at this step.

10.2 Transfer 90 µL of the clear supernatant into new 200 µL PCR tubes. Discard the original tubes with the magnetic beads.

10.3 Remove SPRI beads (Ampure XP or KAPA Pure Beads) from the 4C fridge and aliquot the following amount into a 1.5 mL tube (this accounts for 10% extra volume):

	A	B	C	D	E	F	G	H	I
	# Sublibraries	1	2	3	4	5	6	7	8
	SPRI Beads Needed	79	158	238	317	396	475	554	634

Volume to Add by Number of Sublibrary Lysates (µL)

10.4 Prepare a fresh 85% ethanol solution (400 µL) for each sublibrary.

10.5 Add **72 µL** of SPRI beads to each sublibrary (90 µL) for a total volume of 162 µL.

10.6 Close the tops of the tubes securely, vortex (~5 sec), then centrifuge briefly (~2 sec).

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

10.8 Place the tubes against a magnetic rack on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).

10.9 With SPRI beads still against a magnetic rack, aspirate and discard the clear supernatant with a pipette.

10.10 Without resuspending beads, add **180 µL** of 85% ethanol and wait for **1 minute.**

10.11 Using a pipette, aspirate and discard the ethanol from each tube.

10.12 Without resuspending beads, add another **180 µL** of 85% ethanol and wait for **1 minute.**

10.13 Using a pipette, aspirate and discard the ethanol from each tube (it may be necessary to remove the final few drops with a P20 pipette). With the tube still on the rack, air dry the beads (~2 min).
Critical! Do NOT over-dry the beads. Over-drying of beads can lead to substantial losses in yield. "Cracking" of the beads is a sign of over-drying.

10.14 Remove the tubes from the magnetic rack and resuspend beads from each tube in **25 µL** of molecular biology grade water.

10.15 Incubate the tubes at **37C** for **10 minutes** to maximize elution of amplified cDNA.

10.16 Place the tubes against a magnetic rack on the **low** position (with magnets closest to the bottom) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).

10.17 Transfer **25 µL** of the eluted DNA into new PCR tubes with a P200 pipette. Discard the tubes with the SPRI beads. The amplified cDNA is now ready to be quantified.
Note: Label the new PCR tubes as cDNA to avoid confusion in subsequent steps.

10.18 Measure the concentration of the cDNA using the Qubit dsDNA HS protocol.
Note: Be sure to record sample concentrations as they will be needed for further downstream steps (Section 3.5: Sublibrary Index PCR).

10.19 Run 1 µL of the cDNA on a Bioanalyzer or TapeStation. Use the concentration obtained from the Qubit to determine the appropriate dilution necessary (check manufacturer specifications, 1:10 dilution is generally appropriate). See Figure 1 for the expected cDNA size distribution.

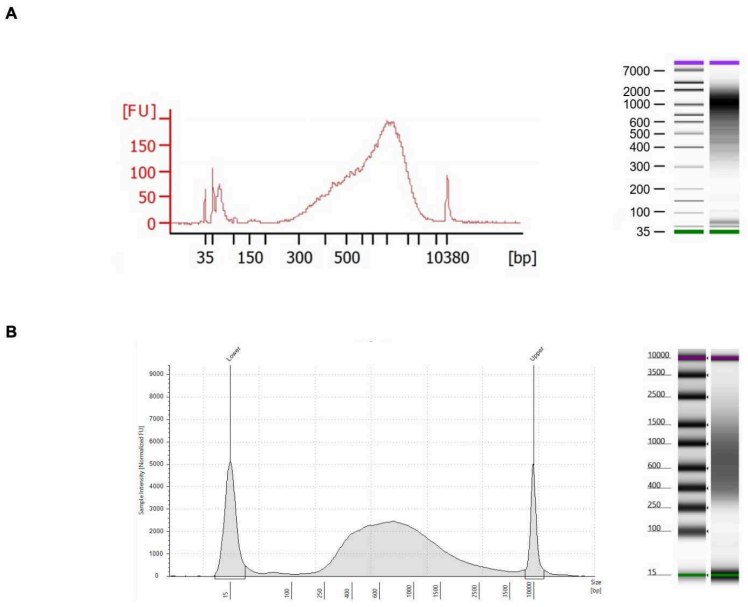


Fig. 1: **Expected cDNA Size Distribution after cDNA Amplification**. (A) Example trace of cDNA run on a Bioanalyzer. (B) Example trace of cDNA run on a TapeStation (it is normal for libraries to be shifted to the left on a TapeStation relative to a Bioanalyzer).

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 Bioanalzyer or TapeStation. Sublibraries with minor deviations can still produce high quality data.

10.20 Sublibraries can be stored at this point at 4C for up to 2 days or at -20C for up to 3 months. If you wish to continue, proceed directly to Section 3: Preparing Libraries for Sequencing.
[STOPPING POINT].

Section 3: Preparing Libraries for Sequencing

11 **Setup**
- Prepare ~1.2 mL 85% ethanol per sublibrary lysate (e.g. 2.4 mL for 2 sublibraries).
- Fill an ice bucket.
- Take out the Parse Biosciences magnetic rack for 0.2 mL PCR tubes.
- Ensure you have at least 176 µL of SPRI beads (Ampure XP or KAPA Pure Beads) per sublibrary. These will be used throughout Section 3.
- Obtain recorded cDNA concentrations from step 2.5.18.

Gather the following items and handle as indicated below:

	Item	Location	Quantity	Format	After taking out
	Fragmentation Buffer	Fragmentation (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Adaptor DNA	Fragmentation (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Index PCR Mix	Fragmentation (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Adaptor Ligation Buffer	Fragmentation (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Universal Index Primer	Fragmentation (-20C)	1	1.5 mL tube	Thaw, then place on ice
	Sublibrary Index Primers	Fragmentation (-20C)	1-16	1.5 mL tubes	Thaw, then place on ice
	Fragmentation Enzyme	Fragmentation (-20C)	1	1.5 mL tube	Place directly on ice
	Adaptor Ligase	Fragmentation (-20C)	1	1.5 mL tube	Place directly on ice

11.1 **3.1 Fragmentation, End Repair, and A-Tailing**
Vortex amplified cDNA briefly (2-3 sec). Be sure to keep caps closed on tubes. Briefly centrifuge tubes (~2 sec).

11.2 For each sublibrary to be sequenced, aliquot **100 ng** of cDNA into a PCR strip tube, then add molecular biology grade water to bring the total volume to 35 µL. Ensure that any concentrations obtained by the Qubit, not the Bioanalyzer, are recorded for further downstream steps (Section 3.5: Sublibrary Index PCR) and store any remaining cDNA at -20C to be used for future experiments.
Note: Keep these tubes on ice.

11.3 Set the thermocycler to the following program:

	Run Time	Lid Temperature	Sublibrary Volume
	40 min	70C	50 µL

Sublibrary Fragmentation, End Repair, and A-Tailing Overview

	Step	Time	Temperature
	1	Hold	4C
	2	10 min	32C
	3	30 min	65C
	4	Hold	4C

Sublibrary Fragmentation, End Repair, and A-Tailing Protocol

11.4 Initiate the thermocycling program such that the machine is pre-cooled to 4C.

11.5 Vortex the **Fragmentation Buffer** followed by a brief centrifugation (~2 sec) and confirm it is fully thawed (no precipitate).

11.6 Using a new 1.5 mL tube, combine the **Fragmentation Buffer** and **Fragmentation Enzyme** to make the **Fragmentation Mix**. Mix well by pipetting 10x and store on ice.

	A	B	C	D	E	F	G	H	I
	# Sublibraries	1	2	3	4	5	6	7	8
	Fragmentation Buffer	5.5	11	16.5	22	27.5	33	38.5	44
	Fragmentation Enzyme	11	22	33	44	55	66	77	88
	Total	16.5	33	49.5	66	82.5	99	115.5	132

Volume to Add by Number of Sublibrary Lysates (µL)

11.7 Add **15 µL** of **Fragmentation Mix** to each sublibrary (should still be in cold block), bringing the total volume to 50 µL.

11.8 Mix sublibraries with a P200 multichannel pipette set to 40 µL Briefly centrifuge sublibraries (~2 sec) and place back on ice.

11.9 Place tubes in the chilled thermocycler and press "skip" or similar option to allow the machine to proceed to the next step. Confirm that the thermocycler has elevated to 32C and has proceeded to the rest of the protocol before leaving the machine.

11.10 Proceed directly to Section 3.2 after the thermocycling protocol finishes.

12 **3.2 Post-Fragmentation Double-Sided SPRI Selection**

12.1 Remove your SPRI beads (Ampure XP or KAPA Pure Beads) from the 4C fridge and aliquot the following amount into a 1.5 mL tube (this accounts for 10% extra volume):

	A	B	C	D	E	F	G	H	I
	# Sublibraries	1	2	3	4	5	6	7	8
	SPRI Beads Needed	44	88	132	176	220	264	308	352

Volume to Add by Number of Sublibrary Lysates (µL)

12.2 Add **30 µL** of SPRI beads to the 50 µL of fragmented sublibraries and vortex briefly (2-3 sec) followed by a brief centrifugation (~2 sec).

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

12.4 Place the sublibrary tubes against a magnetic rack (for 0.2 mL tubes) on the high position (with magnet closest to the top) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
Critical! Do NOT discard the supernatant at this step.

12.5 With SPRI beads still against the magnetic rack, transfer **75 µL** of the clear supernatant into new 200 µL PCR tubes. Discard the tubes with the SPRI beads.

12.6 Add **10 µL** of SPRI beads to the 75 µL of supernatant and vortex briefly (2-3 sec) followed by a brief centrifugation (~2 sec).

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

12.8 Place the tubes against a magnetic rack on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
Critical! This may take longer than other SPRI bead binding due to the low volume of beads. Ensure that all of the beads have bound before proceeding.

12.9 With SPRI beads still against the magnetic rack, aspirate and discard the clear supernatant with a pipette.

12.10 Without resuspending beads, add **180 µL** of 85% ethanol and wait for **1 minute.**

12.11 Using a pipette, aspirate and discard the ethanol from each tube.

12.12 Without resuspending beads, add another **180 µL** of 85% ethanol and wait for **1 minute.**

12.13 Using a pipette, aspirate and discard all of the ethanol from each tube (it may be necessary to remove the final few drops with a P20 pipette). Air dry the beads (only ~30 seconds due to the small amount of beads).
Critical! Do NOT over-dry the beads. Over-drying of beads can lead to substantial losses in yield. "Cracking" of the beads is a sign of over-drying.

12.14 Remove the tubes form the magnetic rack and resuspend beads from each tube in **50 µL** of molecular biology grade water.

12.15 Incubate the tubes at room temperature for **5 minutes** to elute fragmented DNA.

12.16 Place the tubes against a magnetic rack on the high position and wait for all the beads to bind to. the magnet (~2 min: liquid should be clear).

12.17 Transfer exactly **50 µL** of the eluted DNA into new PCR tubes. Discard. the tubes with SPRI beads.

12.18 Sublibraries can be stored at this point at 4C overnight or at -20C or up to 2 weeks. If you wish to continue, proceed directly to section 3.3: Adaptor Ligation.
[STOPPING POINT].

13 **3.3 Adaptor Ligation**

13.1 Make the **Adaptor Ligation Mix** in the order shown below. Ensure that all the reagents are fully thawed and mixed well before using. Mis the **Adaptor Ligation Mix** by pipetting and store on ice.

	A	B	C	D	E	F	G	H	I
	# Sublibraries	1	2	3	4	5	6	7	8
	Nuclease-free water (not supplied)	19.25	38.5	57.75	77	96.25	115.5	134.75	154
	Adaptor Ligation Buffer	22	44	66	88	110	132	154	176
	Adaptor Ligase	11	22	33	44	55	66	77	88
	Adaptor DNA	2.75	22	8.25	11	13.75	16.5	19.25	22
	Total	55	110	165	220	275	330	385	440

Volume to Add by Number of Sublibrary Lysates (µL)

13.2 Add **50 µL** of the **Adaptor Ligation Mix** to the 50 µL of the eluted DNA (from the end of Section 3.2).

13.3 Mix sublibraries 10x with a P200 pipette set to 80 µL. Briefly centrifuge sublibraries (~2 sec).

13.4 Put the tubes into a thermocycler with the following protocol:

	Run Time	Lid Temperature	Sublibrary Volume
	15 min	30C	100 µL

Sublibrary Adaptor Ligation Overview

	Step	Time	Temperature
	1	15 min	20C
	2	Hold	4C

Sublibrary Adaptor Ligation

13.5 Proceed directly to the next step. Do NOT leave the tube in the thermocycler for longer than the indicated time.

14 **3.4 Post-Ligation SPRI Clean Up**

14.1 Remove your SPRI beads (Ampure XP or KAPA Pure Beads) from the 4C fridge and aliquot the following amount into a 1.5 mL tube (this accounts for 10% extra volume):

	A	B	C	D	E	F	G	H	I
	# Sublibraries	1	2	3	4	5	6	7	8

	A	B	C	D	E	F	G	H	I
	SPRI Beads Needed	88	176	264	352	440	528	616	704

Volume to Add by Number of Sublibrary Lysates (µL)

14.2 Add **80 µL** of SPRI beads to each sublibrary (100 µL) to a total volume of 180 µL Ensure the caps are secured and then vortex briefly (2-3 sec) followed by a brief centrifugation (~2 sec).

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

14.4 Place the sublibrary tubes against a magnetic rack (for 0.2 mL tubes) on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).

14.5 With SPRI beads still against the magnetic rack, aspirate and discard the clear supernatant with a pipette.

14.6 Without resuspending beads, add **180 µL** of 85% ethanol and wait for **1 minute.**

14.7 Using a pipette, aspirate and discard the ethanol from each tube.

14.8 Without resuspending beads, add another **180 µL** of 85% ethanol and wait for **1 minute.**

14.9 Using a pipette, aspirate and discard all of the ethanol from each tube (it may be necessary to remove the final few drops with a P20 pipette). Air dry the beads (~3 min). *Critical!* Do NOT over-dry the beads. Over-drying of beads can lead to substantial losses in yield. "Cracking" of the beads is a sign of over-drying.

14.10 Remove the tubes from the magnetic rack and resuspend beads from each tube in **23 µL** of molecular biology grade water.

14.11 Incubate the tubes at room temperature for **5 minutes** to elute DNA.

14.12 Place the tubes against a magnetic rack on the low position (with magnets closest to the bottom) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).

14.13 Transfer exactly **21 µL** of the eluted DNA into new PCR tubes. Discard the tubes with the SPRI beads.

15 **3.5 Sublibrary Index PCR**
If using the alternative version of Evercode WT v2 (ECW02030) that includes WX100 instead of WX200, follow the protocol modifications described in Appendix D.

Multiple thermocyclers may be needed for this section depending on the amount ofcDNA added to each sublibrary during the fragmentation reaction. Refer to the step 3.5.6.

15.1 Ensure that each well of the **UDI Plate - WT** is properly thawed. Centrifuge the plate at **100 x g** for **1 minute**.

Critical! Double-check the label on the plate as specific plates are used in different protocols.

15.2 Thoroughly wipe the UDI Plate - WT seal with 70% ethanol and allow it to dry completely.

Note: Before proceeding, ensure the UDI Plate is properly orientated. The notched corner should be in the bottom left (see image on the right). Only wells from columns 1-6, and only one well/sublibrary can be used.

15.3 Add well-specific index primers from the **UDI Plate - WT** to your sublibraries as follows:

Using a multichannel P20 pipette set to 4 µL, pierce new, unused wells of the **UDI Plate - WT**. Mix 5x then transfer **4 µL** of the index primer solution into your sublibraries.

Note: Ensure that no two sublibraries contain index primers from the same well. To minimize cross-contamination, use a new pipette tip for each sublibrary and avoid splashing or mixing the liquid between individual wells.

For each sublibrary, record the **UDI Plate - WT's** well position (e.g., 'A1', 'B1') and sublibrary index ID (see Section 4.1) for sequencing and demultiplexing purposes.

15.4 If the **UDI Plate - WT** has unused wells, store it at -20°C for future use.

15.5 Add **25 µL** of the **Index PCR Mix** to each sublibrary, bringing the total volume to 50 µL. Pipette up and down 10x with the pipette set to 25 µL to ensure proper mixing, followed by brief centrifugation (~2 sec).
Critical! Different tips must be used when pipetting **Index PCR Mix** into each sublibrary. Never place a tip that has entered a sublibrary back into the **Index PCR Mix**.

15.6 Place the sample(s) into a thermocycler and run the program below. The number of cycles (**X**) should be adjusted based on the amount of cDNA added to the fragmentation reaction.

	Run Time	Lid Temperature	Sublibrary Volume
	~30 min	105C	50 µL

Sublibrary Index Amplification Overview

	Step	Time	Temperature
	1	3 min	95C
	2	20 sec	98C
	3	20 sec	67C
	4	1 min, then go to step 2, repeat X-1 times (X cycles total)	72C
	5	5 min	72C
	6	Hold	4C

Sublibrary Index Amplification

	A	B	C	D	E	F	G
	cDNA in Fragmentation (ng)	10-24	25-49	50-99	100-299	300-899	1,000+
	Total PCR Cycles Required (X)	13	12	11	10	8	7

PCR Cycles based on cDNA in Fragmentation

Note: cDNA concentration was recorded in step 2.5.18, and 10 µL from each sublibrary should have been added into the fragmentation reaction (step 3.1.2).

15.7 Sublibraries can be stored at this point at 4C overnight. If you wish to continue, proceed directly to Section 3.6: Post-Amplification Double-Sided Selection.
[STOPPING POINT]

16 **3.6 Post-Amplification Double-Sided Size Selection**

16.1 Remove your SPRI beads (Ampure XP or KAPA Pure Beads) from the 4C fridge and aliquot the following amount into a 1.5 mL tube (this accounts for 10% extra volume):

	A	B	C	D	E	F	G	H	I
	# Sublibraries	1	2	3	4	5	6	7	8
	SPRI Beads Needed	44	88	132	176	220	264	308	352

Volume to Add by Number of Sublibrary Lysates (µL)

16.2 For each sublibrary, add 30 µL of SPRI beads to the 50 µL of fragmented sublibraries (80 µL total volume). Vortex briefly (2-3 sec) followed by a brief centrifugation (~2 sec).

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

16.4 Place the tubes against a magnetic rack on the high position and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
Critical! This may take longer than other SPRI bead binding due to the low volume of beads. Ensure that all of the beads have bound before proceeding.

16.5 With SPRI beads still against the magnetic rack, transfer **75 µL** of the clear supernatant into new PCR tubes. Discard the tubes with the SPRI beads.

16.6 Add **10 µL** of SPRI beads to the 75 µL of supernatant. Vortex briefly (2-3 sec) followed by a brief centrifugation (~2 sec).

16.7 Incubate the tubes at room temperature for **5 minutes.**

16.8 Place the tubes against a magnetic rack on the high position and wait for all the beads to bind to the magnet (~2 min; liquid should be clear).
Critical! This may take longer than other SPRI bead binding due to the low volume of beads. Ensure that all of the beads have bound before proceeding.

16.9 With SPRI beads still against the magnetic rack, aspirate and discard the clear supernatant with a pipette.

16.10 Without resuspending beads, add **180 µL** of 85% ethanol and wait for **1 minute.**

16.11 Using a pipette, aspirate and discard the ethanol from each tube.

16.12 Without resuspending beads, add another **180 µL** of 85% ethanol and wait for **1 minute.**

16.13 Using a pipette, aspirate and discard the ethanol from each tube (it may be necessary to remove the final few drops with a P20 pipette). With the tube still on the rack, air dry the beads (~2 min).
Critical! Do NOT over-dry the beads. Over-drying of beads can lead to substantial losses in yield. "Cracking" of the beads is a sign of over-drying.

16.14 Remove the tubes from the magnetic rack and resuspend beads from each tube in **20 µL** of molecular biology grade water.

16.15 Incubate the tubes at room temperature for **5 minutes** to elute DNA.

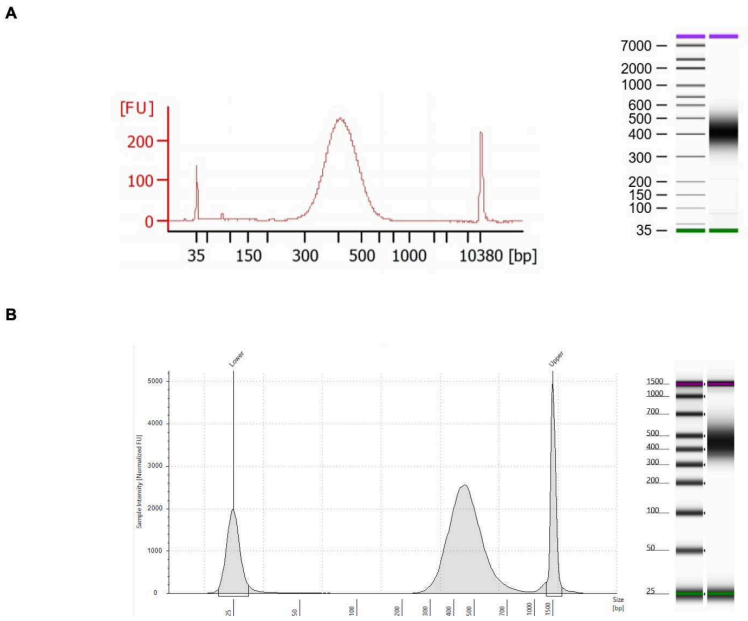
16.16 Place the tubes against a magnetic rack on the **low** position (with magnets closest to the bottom) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).

16.17 Transfer the eluted DNA into new PCR tubes. Discard the tubes with the SPRI beads. The products are now ready to be quantified for sequencing.

16.18 Measure the concentration of the fragmented DNA using the Qubit dsDNA HS protocol.

16.19 Run 1 µL of the DNA on a Bioanalyzer or TapeStation. Use the concentration obtained from the Qubit to determine the appropriate dilution necessary (check manufacturer specifications, 1:10 dilution is generally appropriate). There should be a peak between 400-500 bp. See Figure 2 (next page) for the expected DNA size distribution.

16.20 Sublibraries can be stored at this point at -20C for up to 3 months. If you wish to continue, proceed directly to Section 4: Sequencing Libraries.
[STOPPING POINT]



Note: The traces above are representative of typical Bioanalyzer and TapeStation 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 Bioanalzyer or TapeStation. Sublibraries with minor deviations can still produce high quality data.

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

Section 4: Sequencing Libraries

17 **4.1 Illumina Run Configuration**

If single indexing primers were used in Section 3, see Appendix D2. Otherwise, use the following UDI-specific Illumina run configuration and sequence information.

Evercode sequencing libraries should be diluted and denatured according to the instruction 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	130
	i7 Index (Index 1)	8
	i5 Index (Index 2)	8
	Read 2	86

The fourth barcode that tags each sublibrary acts as a standard Illumina UDI with i7 and

i5 indexes. Please refer to the following table to demultiplex Whole Transcriptome sublibraries with UDIs that were sequenced together in the same run.

	Sublibrary Index ID	Well Position	i7 Forward Sequence (for Sample Sheet)	i5 Reverse Complementary Sequence	i5 Forward Sequence
	UDI_WT_1	A1	CAGATCAC	ATGTGAAG	CTTCACAT
	UDI_WT_2	B1	ACTGATAG	GTCCAACC	GGTTGGAC
	UDI_WT_3	C1	GATCAGTC	AGAGTCAA	TTGACTCT
	UDI_WT_4	D1	CTTGTAAT	AGTTGGCT	AGCCAACT
	UDI_WT_5	E1	AGTCAAGA	ATAAGGCG	CGCCTTAT
	UDI_WT_6	F1	CCGTCCTA	CCGTACAG	CTGTACGG
	UDI_WT_7	G1	GTAGAGTA	CATTCATG	CATGAATG
	UDI_WT_8	H1	GTCCGCCT	AGATACGG	CCGTATCT
	UDI_WT_9	A2	GTGAAACT	TACAGACT	AGTCTGTA
	UDI_WT_10	B2	TCATTCCT	AATGCCTG	CAGGCATT
	UDI_WT_11	C2	GGTAGCAT	TGCTTGCC	GGCAAGCA
	UDI_WT_12	D2	ACTTGATC	TTTGGGTG	CACCCAAA
	UDI_WT_13	E2	ATGAGCAT	GAATCTGA	TCAGATTC
	UDI_WT_14	F2	GCGCTATC	CGACTGGA	TCCAGTCG
	UDI_WT_15	G2	TGACCAGT	ACATTGGC	GCCAATGT
	UDI_WT_16	H2	TATAATCA	ACCACTGT	ACAGTGGT
	UDI_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
	UDI_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
	UDI_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
	UDI_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
	UDI_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
	UDI_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
	UDI_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
	UDI_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
	UDI_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
	UDI_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
	UDI_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
	UDI_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
	UDI_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
	UDI_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT
	UDI_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
	UDI_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
	UDI_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA
	UDI_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA
	UDI_WT_35	C5	TTCCATTG	CTGGCATA	TATGCCAG

	Sublibrary Index ID	Well Position	i7 Forward Sequence (for Sample Sheet)	i5 Reverse Complementary Sequence	i5 Forward Sequence
	UDI_WT_36	D5	TAACGCTG	CTGAGCCA	TGGCTCAG
	UDI_WT_37	E5	TTGGTATG	CTCAATGA	TCATTGAG
	UDI_WT_38	F5	TGAACTGG	CGCATACA	TGTATGCG
	UDI_WT_39	G5	TCCAGTCG	CCGAAGTA	TACTTCGG
	UDI_WT_40	H5	TGTATGCG	CCAGTTCA	TGAACTGG
	UDI_WT_41	A6	TGGCTCAG	CAGCGTTA	TAACGCTG
	UDI_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
	UDI_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
	UDI_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
	UDI_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
	UDI_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
	UDI_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
	UDI_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix

18 **Appendix D: Single Indexing**

If using the alternative version of Evercode WT v2 (ECW02030) that includes WX100 instead of WX200, follow the protocol modifications described here.

	Original Section	Replacement Section
	3.5: Sublibrary Index PCR with UDIs	Appendix D1: Sublibrary Index PCR (see protocol on the next page)
	4.1: Illumina Run Configuration with UDIs	Appendix D2: Illumina Run Configuration with Single Indexing

Protocol Adjustments with Single Indexing

19 **Appendix D1: Part List**

	Component	Format	Quantity	Part Number
	Fragmentation Buffer	1.5 mL tube	1	WX101
	Fragmentation Enzyme	1.5 mL tube	1	WX102
	Adaptor DNA	1.5 mL tube	1	WX103
	Adaptor Ligation Buffer	1.5 mL tube	1	WX104

	Component	Format	Quantity	Part Number
	Adaptor Ligase	1.5 mL tube	1	WX105
	Index PCR Mix	1.5 mL tube	1	WX106
	Universal Index Primer	1.5 mL tube	1	WX107
	Sublibrary Index Primer 1	1.5 mL tube	1	WX108
	Sublibrary Index Primer 2	1.5 mL tube	1	WX109
	Sublibrary Index Primer 3	1.5 mL tube	1	WX110
	Sublibrary Index Primer 4	1.5 mL tube	1	WX111
	Sublibrary Index Primer 5	1.5 mL tube	1	WX112
	Sublibrary Index Primer 6	1.5 mL tube	1	WX113
	Sublibrary Index Primer 7	1.5 mL tube	1	WX114
	Sublibrary Index Primer 8	1.5 mL tube	1	WX115

Fragmentation (-20°C) WX100

20 **Appendix D2: Sublibrary Single Index PCR**
If using unique dual indexes (UDIs) instead of sublibrary single index primers for indexing, see Section 3.5. Otherwise, replace the entirety of Section 3.5 with the following steps.

Multiple thermocyclers may be needed for this section depending on the amount of cDNA added to each sublibrary during the fragmentation reaction. Refer to step next page to determine how many thermocyclers are needed.

20.1 Using a new 1.5 mL tube, combine the **Universal Index Primer** and **Index Primer Mix** to make the **Sublibrary Amplification Mix.** Mix well by pipetting and store on ice.

	A	B	C	D	E	F	G	H	I
	# Sublibraries	1	2	3	4	5	6	7	8
	Index PCR Mix	27.5	55	82.5	110	137.5	165	192.5	220
	Universal Index Primer	2.2	4.4	6.6	8.8	11	13.2	15.4	17.6

	A	B	C	D	E	F	G	H	I
	Total	29.7	59.4	89.1	118.8	148.5	178.2	207.9	237.6

20.2 **Add 2 µL of different index primers to each sublibrary** ensuring that no two sublibraries contain the same sublibrary index primer. Make sure to record which sublibrary contains which index primer.

20.3 Add **27 µL Sublibrary Amplification Mix** to the 23 µL sublibrary from the previous step. Pipette up and down 10x with the pipette set to 27 µL to ensure proper mixing, followed by brief centrifugation (~2 sec).

20.4 Place the samples(s) into a thermocycler and run the program below. The number of cycles (X) should be adjusted based on the amount of cDNA added to the fragmentation reaction.

	Run Time	Lid Temperature	Sublibrary Volume
	~30 min	105C	50 µL

Sublibrary Index Amplification Overview

	Step	Time	Temperature
	1	3 min	95°C
	2	20 sec	98°C
	3	20 sec	67°C
	4	1 min, then go to step 2, repeat X-1 times (X cycles total)	72°C
	5	5 min	72°C
	6	Hold	4°C

Sublibrary Index Amplification

	A	B	C	D	E	F	G
	cDNA in Fragmentation (ng)	10-24	25-49	50-99	100-299	300-999	1,000+
	Total PCR Cycles Required (X)	13	12	11	10	8	7

PCR Cycles based on cDNA in Fragmentation

Note: cDNA concentration was recorded in step 2.5.18, and 10 µL from each sublibrary should have been added into the fragmentation reaction (step 3.1.2).

20.5 Sublibraries can be stored at this point at 4°C overnight. If you wish to continue, proceed directly to Section 3.6: Post-Amplification Double-Sided Size Selection.

21 **Appendix D3: Illumina Run Configuration**
If unique dual indexes (UDIs) instead of sublibrary index primers were used for indexing, see Section 4.1. Otherwise, use the following single index Illumina run configuration and sequence information.

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

	Read	Cycles
	Read 1	140
	i7 Index (Index 1)	6
	Read 2	86
	i5 Index (Index 2)	0

The 4th barcode that tags each sublibrary acts as a standard Illumina index. Please refer to the following table to demultiplex sublibraries that have been sequenced together in the same run.

	Sublibrary Index	Forward Sequence
	1	CAGATC
	2	ACTTGA
	3	GATCAG
	4	TAGCTT
	5	ATGTCA
	6	CTTGTA
	7	AGTCAA
	8	AGTTCC

Protocol references

Please see the attachment for the original Parse Biosciences protocol with more information, including estimation of time required for each section.