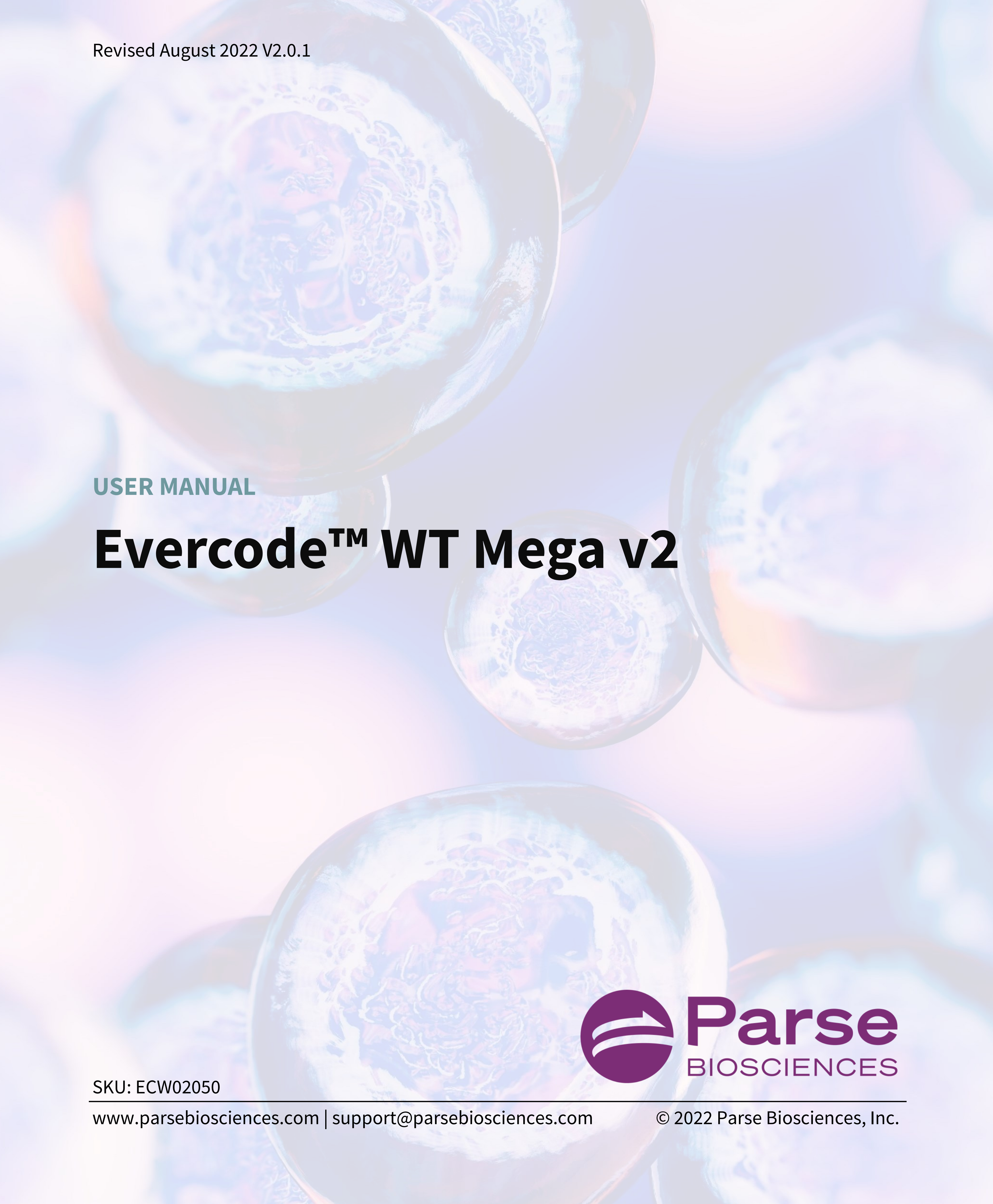

Revised August 2022 V2.0.1

USER MANUAL

Evercode™ WT Mega v2

SKU: ECW02050

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U.S. Pat. No. 10,900,065
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U.S. Pat. No. 11,427,856

Patents pending in the U.S. and other countries

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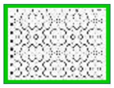
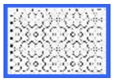
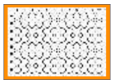
Parts List

Barcoding Plates (-20°C) RP100

Label	Component	Format	Quantity	Part Number
	Round 1 Plate	96-Well Plate	1	RP101
	Round 2 Plate	96-Well Plate	1	RP102
	Round 3 Plate	96 Well Plate	1	RP103

Barcoding Reagents (-20°C) RB100

Label	Component	Format	Quantity	Part Number
Dilution Buffer	Dilution Buffer	2 mL tube	2	RB101
Resusp. Buffer	Resuspension Buffer	5 mL tube	1	RB102
Ligation Mix	Ligation Mix	5 mL tube	1	RB103
R2 Lig. Enzyme	Round 2 Ligation Enzyme	1.5 mL tube	1	RB104
R2 Stop Mix	Round 2 Stop Mix	2 mL tube	1	RB105
R3 Lig. Enzyme	Round 3 Ligation Enzyme	1.5 mL tube	1	RB106
R3 Stop Mix	Round 3 Stop Mix	5 mL tube	1	RB107
Pre-Lyse Wash	Pre-Lyse Wash Buffer	5 mL tube	1	RB108
Lysis Enzyme	Lysis Enzyme	1.5 mL tube	1	RB109

● cDNA Amplification Reagents (-20°C) RC100

Label	Component	Format	Quantity	Part Number
Lysis Neut	Lysis Neutralizer	1.5 mL tube	1	RC101
Bead Wash	Bead Wash Buffer	5 mL tube	1	RC102
Bind Buf. A	Bind Buffer A	1.5 mL tube	1	RC103
Bind Buf. B	Bind Buffer B	5 mL tube	1	RC104
Bind Buf. C	Bind Buffer C	5 mL tube	1	RC105
Bead Storage	Bead Storage Buffer	5 mL tube	1	RC106
TS Buffer	TS Buffer	2 mL tube	1	RC107
TS Enzyme	TS Enzyme	1.5 mL tube	1	RC108
TS Primer	TS Primer Mix	1.5 mL tube	1	RC109
Amp Master	Amplification Master Buffer	1.5 mL tube	1	RC110
Amp Primer	Amplification Primer Mix	1.5 mL tube	1	RC111

● Fragmentation Reagents (-20°C) RX100

Label	Component	Format	Quantity	Part Number
Frag Buf.	Fragmentation Buffer	1.5 mL tube	1	RX101
Frag Enzyme	Fragmentation Enzyme	1.5 mL tube	1	RX102
Adapt DNA	Adaptor DNA	1.5 mL tube	1	RX103
Adapt Buffer	Adaptor Ligation Buffer	1.5 mL tube	1	RX104
Adapt Ligase	Adaptor Ligase	1.5 mL tube	1	RX105
Index PCR Mix	Index PCR Mix	1.5 mL tube	1	RX106
Univ Ind Primer	Universal Index Primer	1.5 mL tube	1	RX107
Index 1	Sublibrary Index Primer 1	1.5 mL tube	1	RX108
Index 2	Sublibrary Index Primer 2	1.5 mL tube	1	RX109
Index 3	Sublibrary Index Primer 3	1.5 mL tube	1	RX110
Index 4	Sublibrary Index Primer 4	1.5 mL tube	1	RX111
Index 5	Sublibrary Index Primer 5	1.5 mL tube	1	RX112
Index 6	Sublibrary Index Primer 6	1.5 mL tube	1	RX113
Index 7	Sublibrary Index Primer 7	1.5 mL tube	1	RX114
Index 8	Sublibrary Index Primer 8	1.5 mL tube	1	RX115
Index 9	Sublibrary Index Primer 9	1.5 mL tube	1	RX116

Fragmentation Reagents (-20°C) RX100

Label	Component	Format	Quantity	Part Number
Index 10	Sublibrary Index Primer 10	1.5 mL tube	1	RX117
Index 11	Sublibrary Index Primer 11	1.5 mL tube	1	RX118
Index 12	Sublibrary Index Primer 12	1.5 mL tube	1	RX119
Index 13	Sublibrary Index Primer 13	1.5 mL tube	1	RX120
Index 14	Sublibrary Index Primer 14	1.5 mL tube	1	RX121
Index 15	Sublibrary Index Primer 15	1.5 mL tube	1	RX122
Index 16	Sublibrary Index Primer 16	1.5 mL tube	1	RX123

Accessory Box 1 (Room Temp) RA100

Label	Component	Format	Quantity	Part Number
	40 µm strainers	Plastic Bag	2	RA101
	Basins	Plastic Bag	2	RA102
	96 Well Plate Seal Cover	Plastic Bag	5	RA103
	Plate Sealer	Plastic Sealer	1	RA104

Accessory Box 2 (4°C) RA200

Label	Component	Format	Quantity	Part Number
Spin Additive	Spin Additive	1.5 mL tube	1	RA201
2x Lysis	2x Lysis Buffer	1.5 mL tube	1	RA202
Binder Beads	Binder Beads	1.5 mL tube	1	RA203

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.

Equipment

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	

Consumables

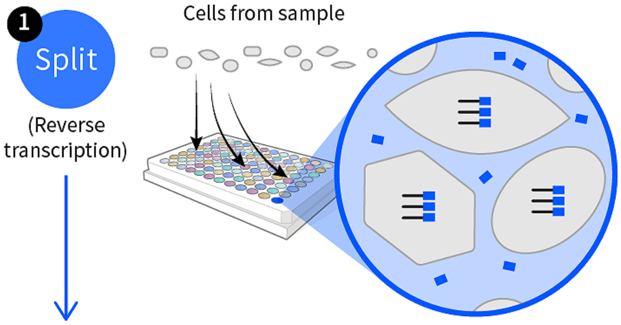
Item	Supplier	Part Number	Notes
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes as it will lead to substantial cell loss.
Corning Cell Strainer (70 µm or 100 µm)	Corning	431751 (70 µm) 431752 (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)	

Background

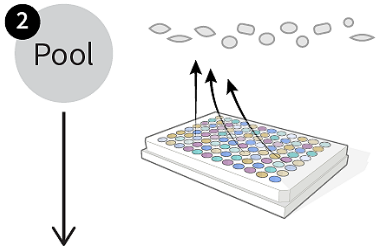
Evercode Combinatorial Barcoding Technology

Parse offers a new strategy for single cell RNA sequencing that can profile up to 1,000,000 cells in parallel across up to 96 samples. Our pioneering technology uses combinatorial cDNA barcoding within cells (or nuclei) themselves, and thus does not require complex cell partitioning instruments. Individual transcriptomes are uniquely labeled by passing fixed cells or nuclei through four rounds of barcoding. In each round, pooled cells are randomly distributed into different wells, and transcripts are labeled with well-specific barcodes. Using next-generation sequencing, each transcriptome is assembled by combining reads containing the same four-barcode combination. Four rounds of barcoding can yield 14,155,776 possible barcode combinations (three rounds of barcoding in 96x96x96 wells followed by a fourth round with 16 PCR reactions), enough to uniquely label up to 1,000,000 cells while avoiding doublets.

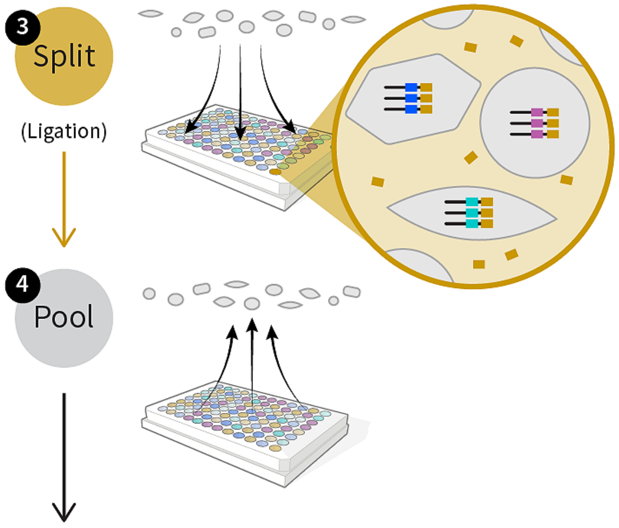
In the first round of barcoding, fixed cell samples are distributed into 96 wells, and cDNA is generated with an in-cell reverse transcription (RT) reaction using well-specific barcoded primers.



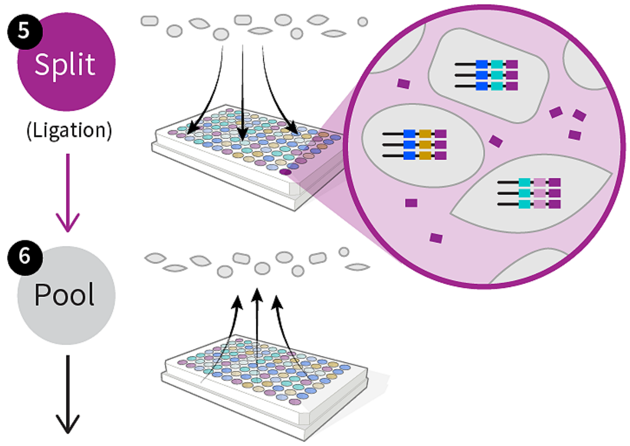
Cells from each well are pooled back together.



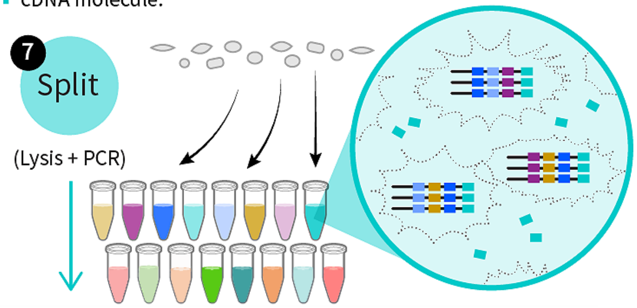
Cells are then distributed into 96 wells, and an in-cell ligation reaction appends a second well-specific barcode to the cDNA.



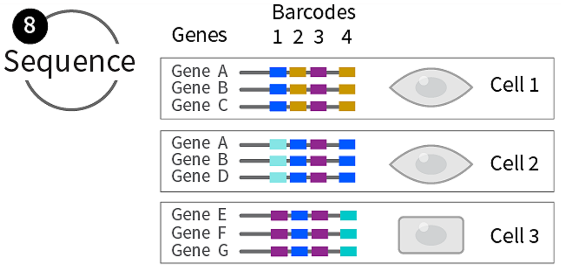
The third-round barcode, which also contains a unique molecular identifier (not depicted), is appended with another round of in-cell ligation.



After three rounds of barcoding, the cells are pooled and split into 16 distinct populations we term sublibraries. The user can choose the number of cells in each sublibrary to control the depth of sequencing. Cells will not be pooled again after this step. After this final split cells are lysed and the barcoded cDNA is isolated. A fourth sublibrary-specific barcode is introduced by PCR to each cDNA molecule.



After sequencing, each single cell transcriptome is assembled by combining reads containing the same four-barcode combination.



Background Continued

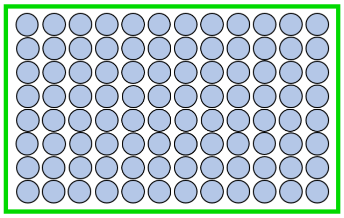
Unlike other scRNA-seq methods that physically separate individual cells into different compartments to label transcripts with cell-specific barcodes, Evercode WT Mega uses the cells (or nuclei) themselves as “containers” in which intracellular mRNA-transcripts are labeled using combinatorial indexing. 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.

Overview of Four Rounds of Barcoding in the Evercode WT Mega

Each cell will be barcoded four times throughout the kit process, which will generate 14,155,776 (96 x 96 x 96 x 16) possible barcode combinations. Each barcoding round is outlined below:

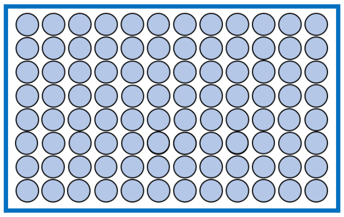
Round 1 Barcoding (96 barcodes):

Cells are distributed into 96 different wells within the Round 1 Barcoding Plate. Barcodes are added through an *in situ* reverse transcription reaction using barcoded primers.
Note: Check the “WT Mega - Sample Loading Table V1.2.0” (Excel spreadsheet) to determine which sample to add to each well.



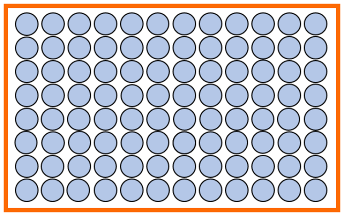
Round 2 Barcoding (96 barcodes):

Cells are distributed into 96 different wells within the Round 2 Barcoding Plate. The second barcode is added to each transcript via an *in situ* ligation reaction.



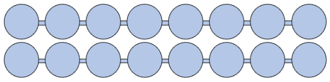
Round 3 Barcoding (96 barcodes):

Cells are distributed into 96 different wells within the Round 3 Barcoding Plate. The third barcode is added to each transcript via an *in situ* ligation reaction.

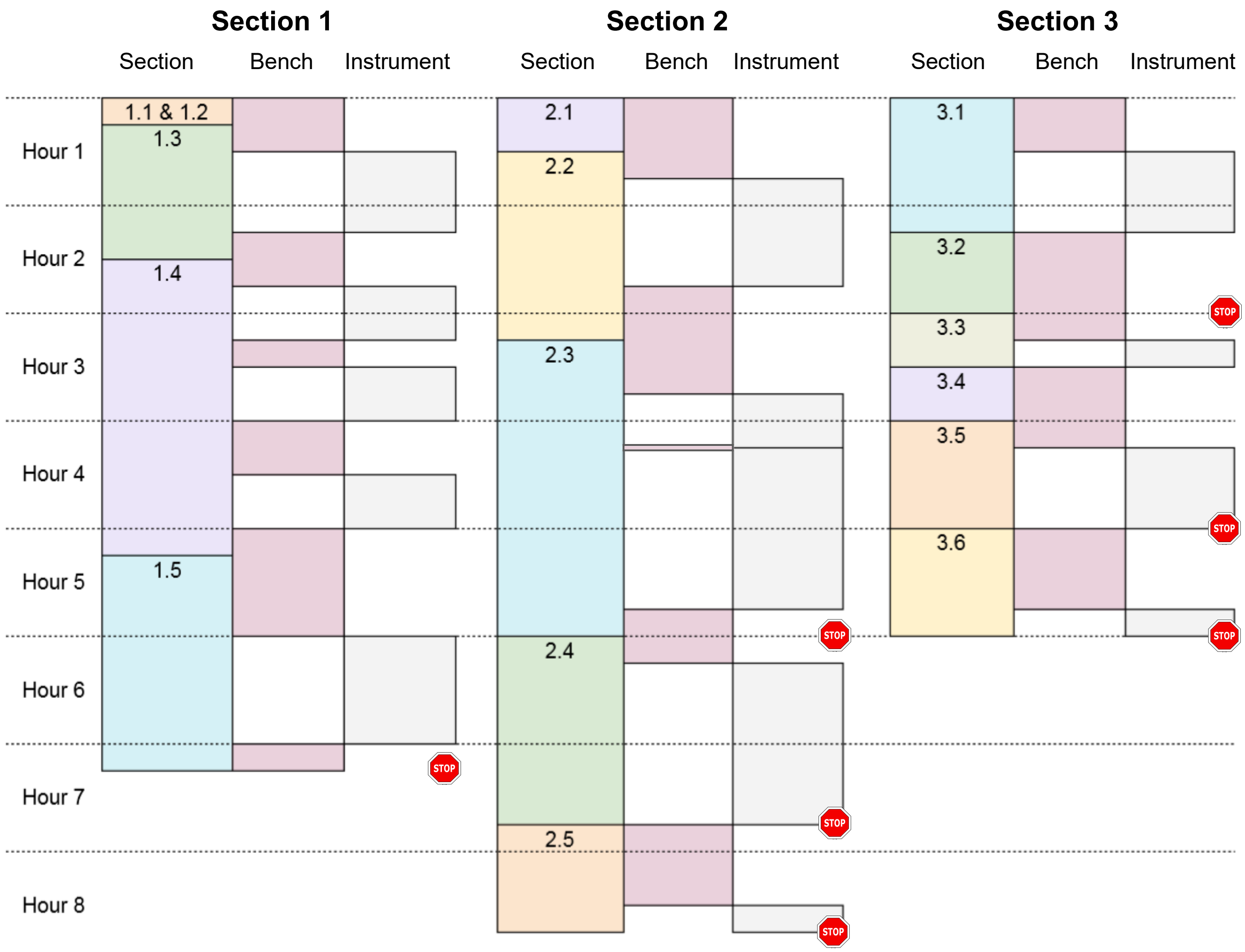


Sublibrary Barcoding (16 Illumina Indices):

Cells are distributed into 16 different tubes (sublibraries). While all the sublibraries can be processed together, each sublibrary can be sequenced separately. Different numbers of cells can be added to each sublibrary (see Section 1.5: Lysis and Sublibrary Generation) as desired by the user. Sublibraries with small cell numbers will be easier to sequence to saturation and can serve as a good QC measure before sequencing additional sublibraries with much larger cell numbers.



Protocol Timing



🛑 = **Optional** stopping point

Notes Before Starting

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 pipettes. 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 that 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 centrifuge tubes to minimize cell loss.
Sample Loading Table	The “WT Mega - Sample Loading Table V1.2.0” (Excel spreadsheet) should be completed before starting the experimental workflow. If not working properly, ensure that Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in 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 pipette 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 in wells.
- 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

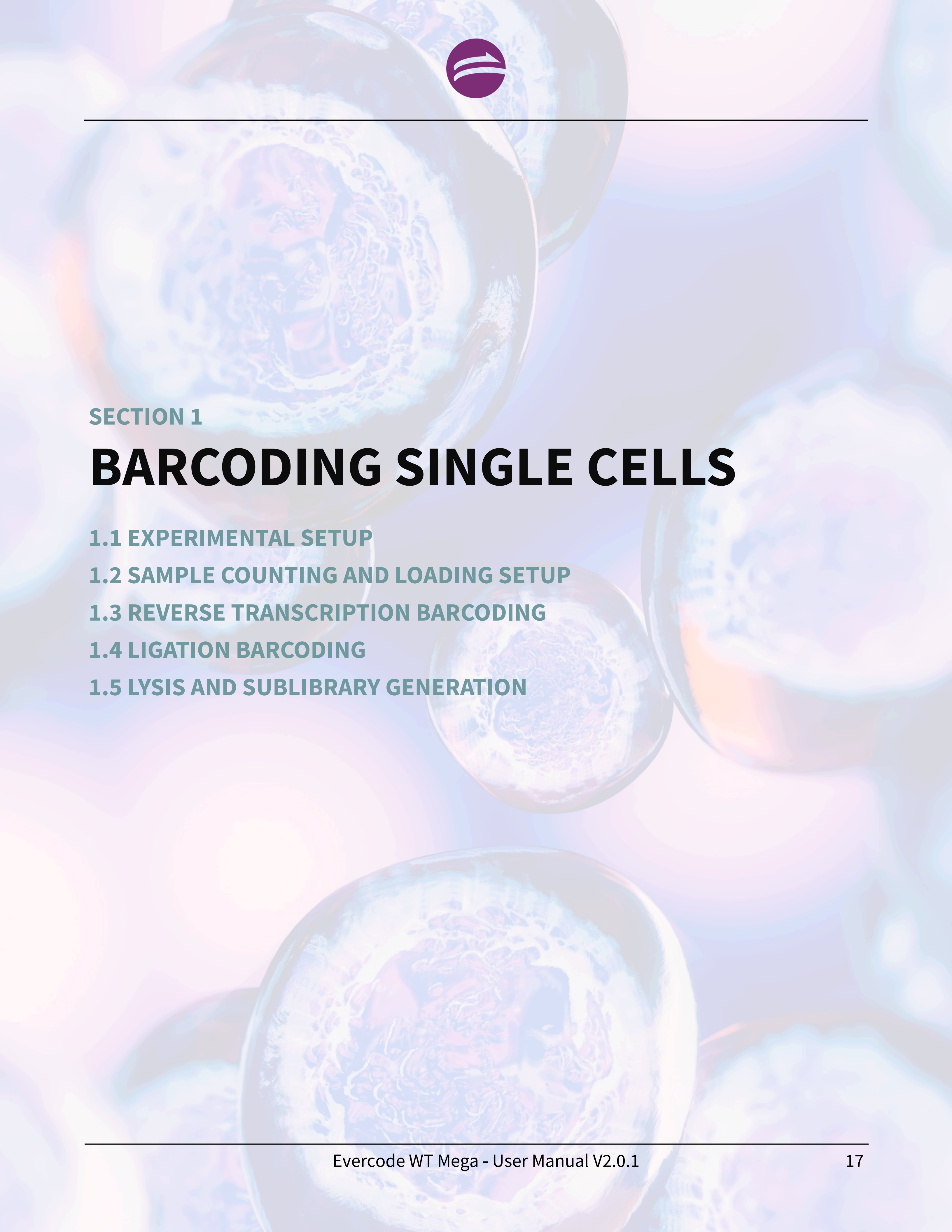
A 40 µm cell strainer will be used in multiple steps. To maximize cell retention, press the pipette tip directly against the strainer. Ensure that ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. For cells larger than 40 µm, the 40 µm strainer 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 37°C for 5 minutes should resolubilize solution. If precipitate remains, warm 2x Lysis Buffer at 37°C 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.



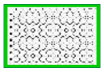
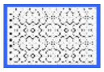
SECTION 1

BARCODING SINGLE CELLS

1.1 Experimental Setup

1. Prepare for the first round of barcoding with the following checklist:

    - ☐ Add each of your sample names to “WT Mega - Sample Loading Table V1.2.0” (Excel spreadsheet). In Section 1.2, you will add the concentrations of each sample to this spreadsheet and use it to make appropriate dilutions to each sample. The plate configuration in this spreadsheet will also tell you which wells to add each sample to during the first round of barcoding in Section 1.3.
    - ☐ Set your swinging-bucket centrifuge to **4°C.**
    - ☐ Prepare a **37°C** water bath.
    - ☐ Fill an ice bucket, large enough to hold two 96-well plates and several tubes.
    - ☐ Prepare a flow cytometer, hemocytometer, or other device for cell counting.

Item	Location	Quantity	Format	After taking out
 Adhesive 96-well plate cover	Accessory Box 1 (Room Temp)	1	With white protector	Keep at room temperature.
Spin Additive Spin Additive	Accessory Box 2 (4°C)	1	1.5 mL tube	Keep at room temperature.
Dilution Buffer Dilution Buffer	Barcoding Reagents (-20°C)	2	2 mL tube	Thaw, then place on ice.
Resusp. Buffer Resuspension Buffer	Barcoding Reagents (-20°C)	1	5 mL tube	Thaw, then place on ice.
Ligation Mix Ligation Mix	Barcoding Reagents (-20°C)	1	5 mL tube	Thaw, then place on ice.
R2 Lig. Enzyme Round 2 Ligation Enzyme	Barcoding Reagents (-20°C)	1	1.5 mL tube	Place directly on ice.
 Round 1 Plate	Barcoding Plates (-20°C)	1	96-well plate	Place directly on ice.
 Round 2 Plate	Barcoding Plates (-20°C)	1	96-well plate	Place directly on ice.

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

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.

Round 1 Plate Thaw Protocol		
Run Time	**Lid Temperature**	**Sample Volume**
10 min	70°C	26 µL
Step	**Time**	**Temperature**
1	10 min	25°C
2	Hold	4°C

1.2 Sample Counting and Loading Setup

1. Thaw the fixed cell samples in a **37°C** water bath until all ice crystals dissolve, then place on ice. It is important to fully thaw samples before placing on ice.
2. Using an automated cell counter, hemocytometer, or flow cytometer, count the number of cells in each sample.
3. Fill out the cell concentrations of each sample in the “WT Mega - Sample Loading Table V1.2.0” (Excel spreadsheet).

Dilution Buffer

4. Dilute samples in **Dilution Buffer** according to the Sample Loading Table and place on ice.

1.3 Reverse Transcription Barcoding

During this section, cDNA will be reverse transcribed from RNA with barcoded RT primers specific to each well. It is critical to add the samples to the wells specified in the plate configuration within the “WT Mega - Sample Loading Table V1.2.0” document.

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**.
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. **Add diluted samples to wells in the Round Plate 1.**

   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 P20 pipette, add **14 µL** cells 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 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.

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 adhesive 96-well plate seal cover.

   Note: Plate sealer is included in Accessory Box 1.

~40 min

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

Round 1 Plate Barcoding Protocol		
Run Time	**Lid Temperature**	**Sample Volume**
~40 min	70°C	40 µL
Step	**Time**	**Temperature**
1	10 min	50°C
	Begin Cycling	
2	12 sec	8°C
3	45 sec	15°C
4	45 sec	20°C
5	30 sec	30°C
6	2 min	42°C
7	3 min	50°C
	Go to step 2, repeat 2 times (3 cycles total)	
8	5 min	50°C
9	Hold	4°C

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

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.

Round 2 Plate Thaw Protocol		
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. Place the **Round 1 Plate** (and holder) on a flat surface and remove adhesive seal. Place back on ice.

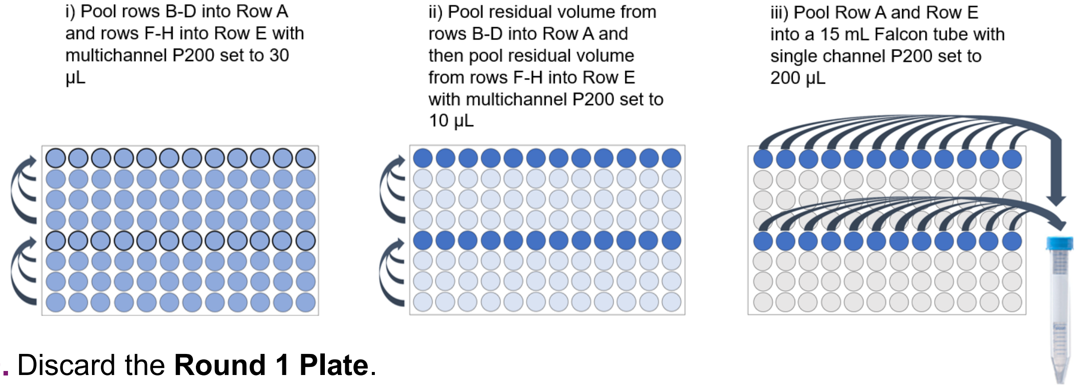
9. Pool all wells from the Round 1 Plate into a single 15 mL centrifuge tube on ice.

<u>Note</u>: Proper mixing is required to <u>prevent substantial cell loss</u> during pooling. See "Maximizing Cell Retention During Pooling Steps" in <u>Notes Before Starting</u>.

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, then pool rows F-H into the wells in Row E. 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 or Row E. Recover residual liquid across rows B-D and F-H 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 the same with Row E. Do not be concerned if there are a few µL of residual volume in the wells after pooling.

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

<u>CRITICAL!</u> Do NOT pool all eight rows into a single row or the liquid may overflow. Keep the Round 1 Plate and the 15 mL falcon tube with pooled cells on ice during the pooling step.


i) Pool rows B-D into Row A and rows F-H into Row E with multichannel P200 set to 30 µL

ii) Pool residual volume from rows B-D into Row A and then pool residual volume from rows F-H into Row E with multichannel P200 set to 10 µL

iii) Pool Row A and Row E into a 15 mL Falcon tube with single channel P200 set to 200 µL

10. Discard the **Round 1 Plate**.

Spin Additive

11. Add **19.2 µL** of **Spin Additive** to the 15 mL tube with pooled cells. Gently invert the tube once to mix.

<u>CRITICAL!</u> Do NOT discard the **Spin Additive** as it will be needed in another step.

12. Centrifuge the pooled cells in a <u>swinging bucket</u> centrifuge cooled to **4°C** for **10 minutes** at **200 x *g***.

<u>CRITICAL!</u> 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.

13. Using a P1000 pipette for the first 3 mL, then a P200 pipette for remaining volume, aspirate supernatant such that about ~40 µL of liquid remains above the pellet (see image on 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.

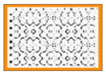
14. *<u>Note</u>: To <u>prevent substantial cell loss</u> during resuspension, see "Sample Handling" in <u>Notes Before Starting</u>.*

Resusp. Buffer

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.

1.4 Ligation Barcoding

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

Item	Location	Quantity	Format	After taking out
 Adhesive 96-well plate cover	Accessory Box 1 (Room Temp)	3	With white protector	Keep at room temperature.
 40 µm strainer	Accessory Box 1 (Room Temp)	2	In plastic bag	Keep at room temperature.
 Basins	Accessory Box 1 (Room Temp)	6	In plastic bag	Keep at room temperature.
R2 Stop Mix Round 2 Stop Mix	Barcoding Reagents (-20°C)	1	2 mL tube	Thaw, then place on ice.
R3 Stop Mix Round 3 Stop Mix	Barcoding Reagents (-20°C)	1	5 mL tube	Thaw, then place on ice.
Pre-Lyse Wash Pre-Lyse Wash Buffer	Barcoding Reagents (-20°C)	1	5 mL tube	Thaw, then place on ice.
R3 Lig. Enzyme Round 3 Ligation Enzyme	Barcoding Reagents (-20°C)	1	1.5 mL tube	Place directly on ice.
 Round 3 Plate	Barcoding Plates (-20°C)	1	96-well plate	Place directly on ice.

R2 Lig. Enzyme

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**.

Ligation Mix

3. Using a P1000 pipette, add **2 mL** of cells in **Resuspension Buffer** into the **Ligation Mix + Enzyme** tube. Mix 10x with a P1000 set to 1000 µL and place back on ice.

CRITICAL! Do NOT vortex the **Ligation Mix**.

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).

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

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.

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

30 min

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

Round 2 Ligation Barcoding Protocol		
Run Time	**Lid Temperature**	**Sample Volume**
30 min	50°C	50 µL
Step	**Time**	**Temperature**
1	30 min	37°C
2	Hold	4°C

R2 Stop Mix

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

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.

R2 Stop Mix

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.

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

30 min

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

Round 2 Stop Protocol		
Run Time	**Lid Temperature**	**Sample Volume**
30 min	50°C	60 µL
Step	**Time**	**Temperature**
1	30 min	37°C
2	Hold	4°C

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

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.

Round 3 Plate Thaw Protocol		
Run Time	**Lid Temperature**	**Sample Volume**
10 min	70°C	10 µL
Step	**Time**	**Temperature**
1	10 min	25°C
2	Hold	4°C

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

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 side 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.

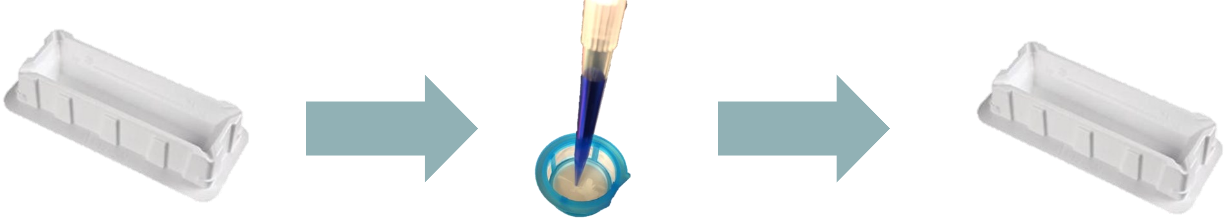
    Note: Bubbles may form while pooling. They will not affect the quality of the experiment.

18. Discard the **Round 2 Plate**.

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.



R3 Lig. Enzyme

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

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.** 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).

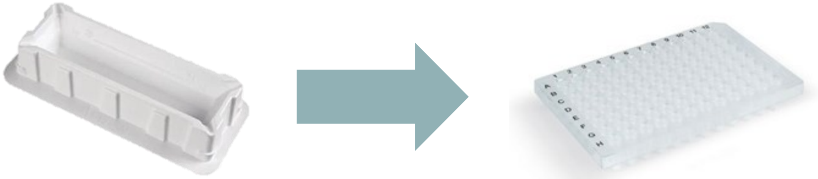
22. **Add pooled cells to the Round 3 Plate.**

    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 the 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, 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.



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

30 min

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

Round 3 Ligation Barcoding Protocol		
Run Time	**Lid Temperature**	**Sample Volume**
30 min	50°C	60 µL
Step	**Time**	**Temperature**
1	30 min	37°C
2	Hold	4°C

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.

R3 Stop Mix

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.

R3 Stop Mix

27. Add Round 3 Stop Mix to each well.

Note: This step requires a new box 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 **Round 3 Stop Mix** to each well. No incubation required after this step, proceed directly to the next step.

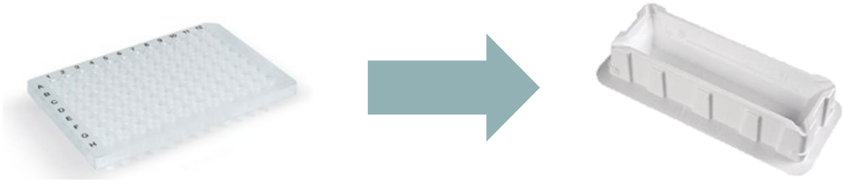
CRITICAL! Different tips must be used when pipetting stop mix into the 96-well plate. Never place a tip that has entered one of the 96 wells back into the basin.

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 side 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.

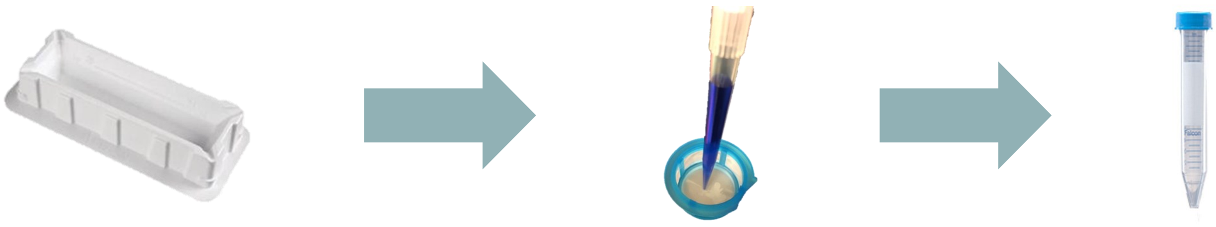
Note: Bubbles may form while pooling. They will not affect the quality of the experiment.



29. Discard the **Round 3 Plate**.

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 cells from this basin through a 40 µm strainer into a new 15 mL tube on ice. Mix cells in the basin 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.



1.5 Lysis and Sublibrary Generation

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

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

Spin Additive

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

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

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

   Pre-Lyse Wash

   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.

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

7. Using a P1000 pipette 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 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.

8. *<u>Note</u>: To <u>prevent substantial cell loss</u> during resuspension, see "Sample Handling" in <u>Notes Before Starting</u>.*

Dilution Buffer

Using a P200 pipette, gently resuspend the pellet with an additional **200 µL** of **Dilution Buffer**, bringing the final volume to ~240 µL. When resuspending the pellet, pipette slowly to prevent mechanical damage to cells. Keep the tube on ice.

<u>CRITICAL!</u> Do NOT discard **Dilution Buffer** as it will be needed in another step.

9. Using a P200 pipette set to 200 µL, gently pipette up and down 5x and immediately use **5 µL** of the mixed cells to count using a hemocytometer. Keep the 15 mL tube on ice.

*<u>Note</u>: When using a hemocytometer, dilute **5 µL** of the mixed cell solution into **5 µL** of Trypan Blue. Mix well and load onto 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.*

<u>Choosing Sublibrary Sizes</u>: In the following step, cells will be aliquoted into different sublibraries that will be prepared for sequencing. At the end of 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.

10. **Determine sublibrary size(s) and dilutions**. Up to 16 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. <u>Make sure to record which sublibrary sizes correspond to what labels</u>. Label both the top and side of the PCR tube with those labels.

<u>CRITICAL!</u> Do NOT overload a sublibrary. 62,500 cells/sublibrary is the maximum. Overloading a sublibrary lysate with too many cells will result in increased doublets.

Dilution Buffer

11. Using a P200 pipette set to 200 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.

2x Lysis

Lysis Enzyme

12. Make a **Lysis Master Mix.** Ensure there is no precipitate present in the **2x Lysis Buffer**. Add **440 µL** of **2x Lysis Buffer** to **88 µL** of **Lysis Enzyme** in a 1.5 mL tube.

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

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.

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

1 hr

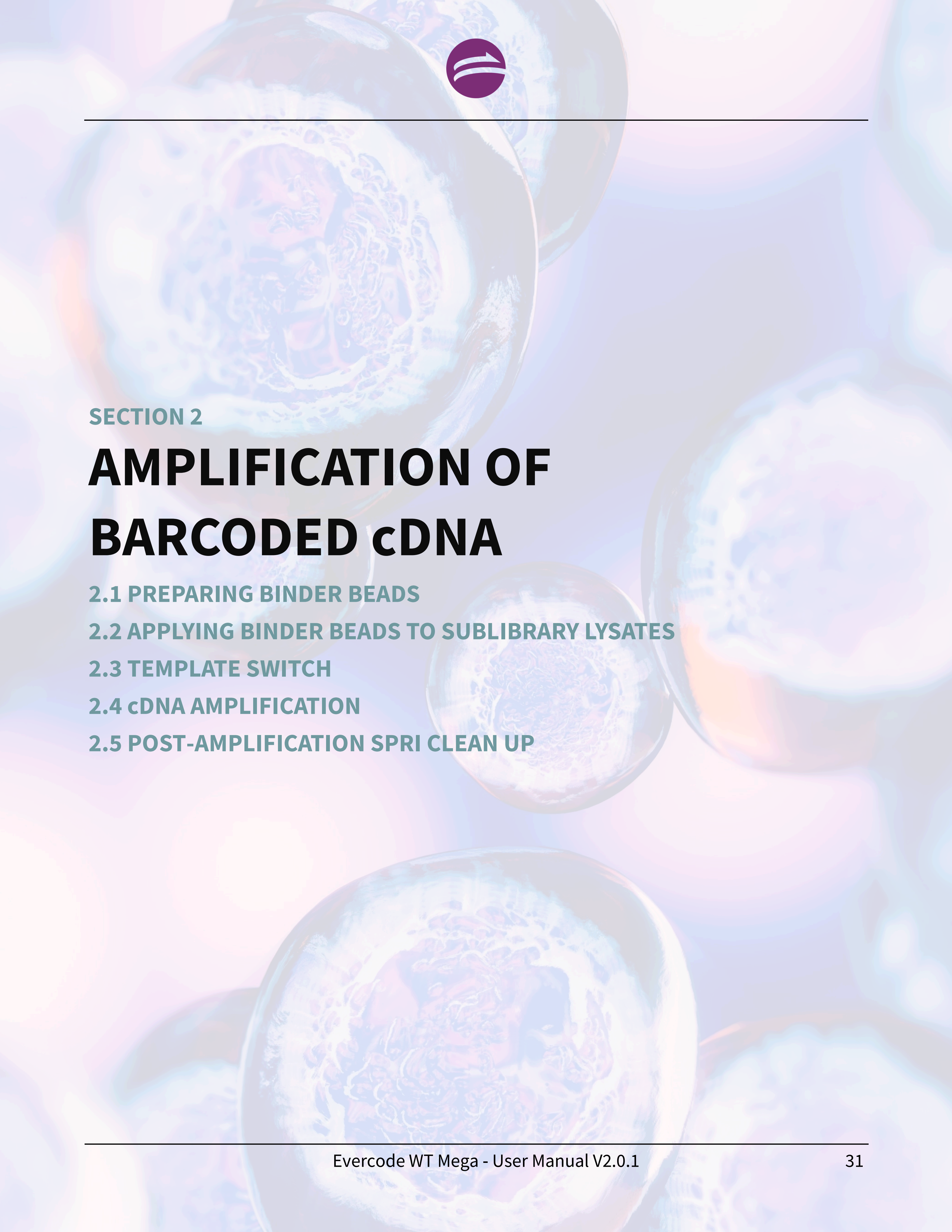
15. Incubate the sublibrary lysates in a thermocycler with the following protocol:

Sublibrary Lysis Protocol		
Run Time	**Lid Temperature**	**Sample Volume**
60 min	80°C	55 µL
Step	**Time**	**Temperature**
1	60 min	65°C
2	Hold	4°C

STOP

16. Freeze sublibrary lysate(s) at -80°C. Sublibrary lysates can be stored for up to 6 months.

[STOPPING POINT]



SECTION 2

AMPLIFICATION OF BARCODED cDNA

Any number of sublibraries (1-16) 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 Binder Beads	Accessory Box 2 (4°C)	1	1.5 mL tube	Keep at room temperature.
Bead Wash Bead Wash Buffer	cDNA Amplification Reagents (-20°C)	1	5 mL tube	Keep at room temperature.
Bind Buf. A Bind Buffer A	cDNA Amplification Reagents (-20°C)	1	1.5 mL tube	Keep at room temperature.
Bind Buf. B Bind Buffer B	cDNA Amplification Reagents (-20°C)	1	5 mL tube	Keep at room temperature.
Bind Buf. C Bind Buffer C	cDNA Amplification Reagents (-20°C)	1	5 mL tube	Keep at room temperature.
Bead Storage Bead Storage Buffer	cDNA Amplification Reagents (-20°C)	1	5 mL tube	Keep at room temperature.
TS Buffer TS Buffer	cDNA Amplification Reagents (-20°C)	1	2 mL tube	Thaw, then place on ice.
Lysis Neut Lysis Neutralization	cDNA Amplification Reagents (-20°C)	1	1.5 mL tube	Place directly on ice.

2.1 Preparing Binder Beads

Binder Beads

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:

	Volume to Add by Number of Sublibrary Lysates (µL)								
# Sublibrary Lysates	1	2	3	4	5	6	7	8	16
Binder Beads (µL)	44	88	132	176	220	264	308	352	704

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).
3. Remove the clear supernatant with a pipette and discard.

Bead Wash

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.

	Volume to Add by Number of Sublibrary Lysates (µL)								
# Sublibrary Lysates	1	2	3	4	5	6	7	8	16
Bead Wash Buffer (µL)	50	100	150	200	250	300	350	400	800

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. Remove the clear supernatant with a pipette and discard.
7. Repeat steps 4-6 twice more for a total of three washes.

Bind Buf. A

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.

	Volume to Add by Number of Sublibrary Lysates (µL)								
# Sublibrary Lysates	1	2	3	4	5	6	7	8	16
Bind Buffer A (µL)	55	110	165	220	275	330	385	440	880

2.2 Applying Binder Beads to Sublibrary Lysates

5 min

1. Remove the desired sublibrary lysates from the -80°C freezer and incubate at **37°C** for **5 minutes**, ensuring that no precipitate is present before proceeding. If precipitate is still present, incubate at 37°C for 5 more minutes.
2. Briefly centrifuge sublibrary lysates (~2 sec).

Lysis Neut

10 min

3. Lightly centrifuge the **Lysis Neutralizer**, mix gently with a pipette, and add **2.5 µL** to each sublibrary lysate. Place tubes into a 96-well plastic plate holder (press tubes securely into holder) and put plate holder lid back on. Vortex the plastic holder on 10 (or equivalent max setting) for **1 minute**. Remove tubes from plate holder. Briefly centrifuge (~2 sec), and incubate at room temperature for **10 minutes**.
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 without pipette mixing the lysates. Place tubes in a plastic plate holder (press tubes securely into holder) and put plate holder lid back on. Vortex plastic holder on 10 (or equivalent max setting) for **1 minute**. Discard the tube with any excess **Binder Beads**.

CRITICAL! After adding beads to sublibrary lysates, mix by vortexing and not by pipetting.

60 min

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).
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. 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.

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

Bind Buf. B

9. Remove tubes from the magnetic rack and resuspend beads with **125 µL** of **Bind Buffer B**.
10. Keep tubes at room temperature for **1 minute**.

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

Bind Buf. B

13. Repeat steps 9-12 for a second wash using **Bind Buffer B**.

Bead Storage

14. Remove the tubes from the magnetic rack and resuspend beads with **125 µL** of **Bead Storage Buffer**.
15. Keep tubes at room temperature for **1 minute**.
16. Proceed directly to Section 2.3: Template Switch.

2.3 Template Switch

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

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

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

CRITICAL! If processing more than 10 sublibrary lysates at one time, use a new 5 mL or 15 mL tube to make the **Template Switch Mix**.

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
TS Buffer TS Buffer	101.75	203.5	305.25	407	508.75	610.5	712.25	814	1,628
TS Primer TS Primer Mix	2.75	5.5	8.25	11	13.75	16.5	19.25	22	44
TS Enzyme TS Enzyme	5.5	11	16.5	22	27.5	33	38.5	44	88
Total	**110**	**220**	**330**	**440**	**550**	**660**	**770**	**880**	**1,760**

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.

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

Bind Buf. C

6. Without resuspending beads, add **125 µL** of **Bind Buffer C** and wait **1 minute.**

CRITICAL! Do NOT discard the supplied stock tube of **Bind Buffer C** as it will be used again in a later step.

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

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.*

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

30 min

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

90 min

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:

Sublibrary Template Switching		
Run Time	**Lid Temperature**	**Sublibrary Volume**
90 min	70°C	100 µL
Step	**Time**	**Temperature**
1	90 min	42°C
2	Hold	4°C

STOP

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 beads in **125 µL** of **Bead Storage Buffer**.
    d. Store tubes at 4°C overnight. Do not freeze sublibraries.

Bead Storage

[STOPPING POINT]

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 (next page) to determine how many thermocyclers are needed.

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

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

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.

CRITICAL! If processing more than 10 sublibrary lysates at one time, use a new 5 mL tube or 15 mL tube to make the **Amplification Reaction Solution.**

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
Amp Master Amplification Master Buffer	60.5	121	181.5	242	302.5	363	423.5	484	968
Amp Primer Amplification Primer Mix	60.5	121	181.5	242	302.5	363	423.5	484	968
Total	**121**	**242**	**363**	**484**	**605**	**726**	**847**	**968**	**1,936**

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.

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

Bind Buf. C

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.

6. Remove the clear supernatant with a pipette and discard.
7. Remove the tubes from the magnetic rack and resuspend beads in each tube with **100 µL** of **Amplification Reaction Solution**. Place tubes with **Amplification Reaction Solution** on ice.
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.

	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
	12,500-25,000	5	5
	25,000-62,500	5	4
Nuclei	200-1,000	5	13
	1,000-2,000	5	11
	2,000-6,000	5	9
	6,000-12,500	5	7
	12,500-25,000	5	6
	25,000-62,500	5	5
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
	12,500-25,000	5	7
	25,000-62,500	5	6

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.

50-100 min

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 (previous page).

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.

Amplification Protocol		
Run Time	**Lid Temperature**	**Sublibrary Volume**
50-70 min	105°C	100 µL
Step	**Time**	**Temperature**
1	3 min	95°C
	Begin 1st Cycling	
2	20 sec	98°C
3	***45 sec**	***65°C**
4	3 min	72°C
	Go to step 2, repeat 4 times (5 cycles total)	
	Begin 2nd Cycling	
5	20 sec	98°C
6	***20 sec**	***67°C**
7	3 min	72°C
	*Go to step 5, repeat **X - 1** times (**X** cycles total)*	
8	5 min	72°C
9	Hold	4°C

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)”.*

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

[STOPPING POINT]

2.5 Post-Amplification SPRI Clean Up

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.

2. Transfer 90 µL of the clear supernatant into new 200 µL PCR tubes. Discard the original tubes with the magnetic beads.
3. Remove SPRI beads (Ampure XP or KAPA Pure Beads) from the 4°C fridge and aliquot the following amount into a 1.5 mL tube (this accounts for 10% extra volume):

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
SPRI Beads Needed	79	158	238	317	396	475	554	634	1266

4. Prepare a fresh 85% ethanol solution (400 µL) for each sublibrary.
5. Add **72 µL** of SPRI beads to each sublibrary (90 µL) for a total volume of 162 µL.
6. Close the tops of all the tubes securely, vortex (~5 sec), then centrifuge briefly (~2 sec).
7. Incubate at room temperature for **5 minutes.**
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).
9. With SPRI beads still against the magnetic rack, aspirate and discard the clear supernatant with a pipette.
10. Without resuspending beads, add **180 µL** of 85% ethanol and wait for **1 minute**.
11. Using a pipette, aspirate and discard the ethanol from each tube.
12. Without resuspending beads, add another **180 µL** of 85% ethanol and wait for **1 minute**.
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). 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.

14. Remove the tubes from the magnetic rack and resuspend beads from each tube in **25 µL** of molecular biology grade water.
15. Incubate the tubes at **37°C** for **10 minutes** to maximize elution of amplified cDNA.

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).

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.

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).

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 (next page) for the expected cDNA size distribution.

STOP

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

[STOPPING POINT]

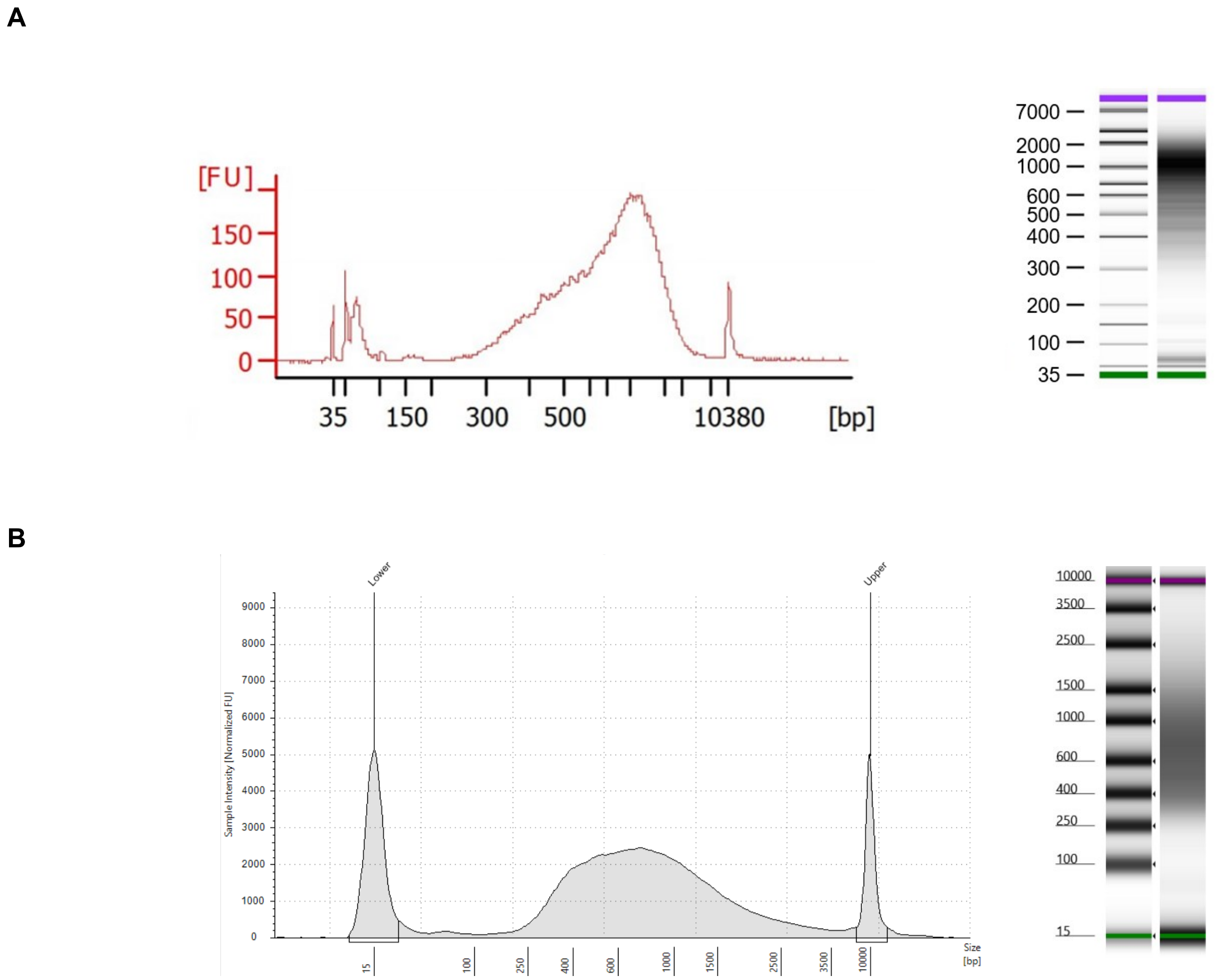
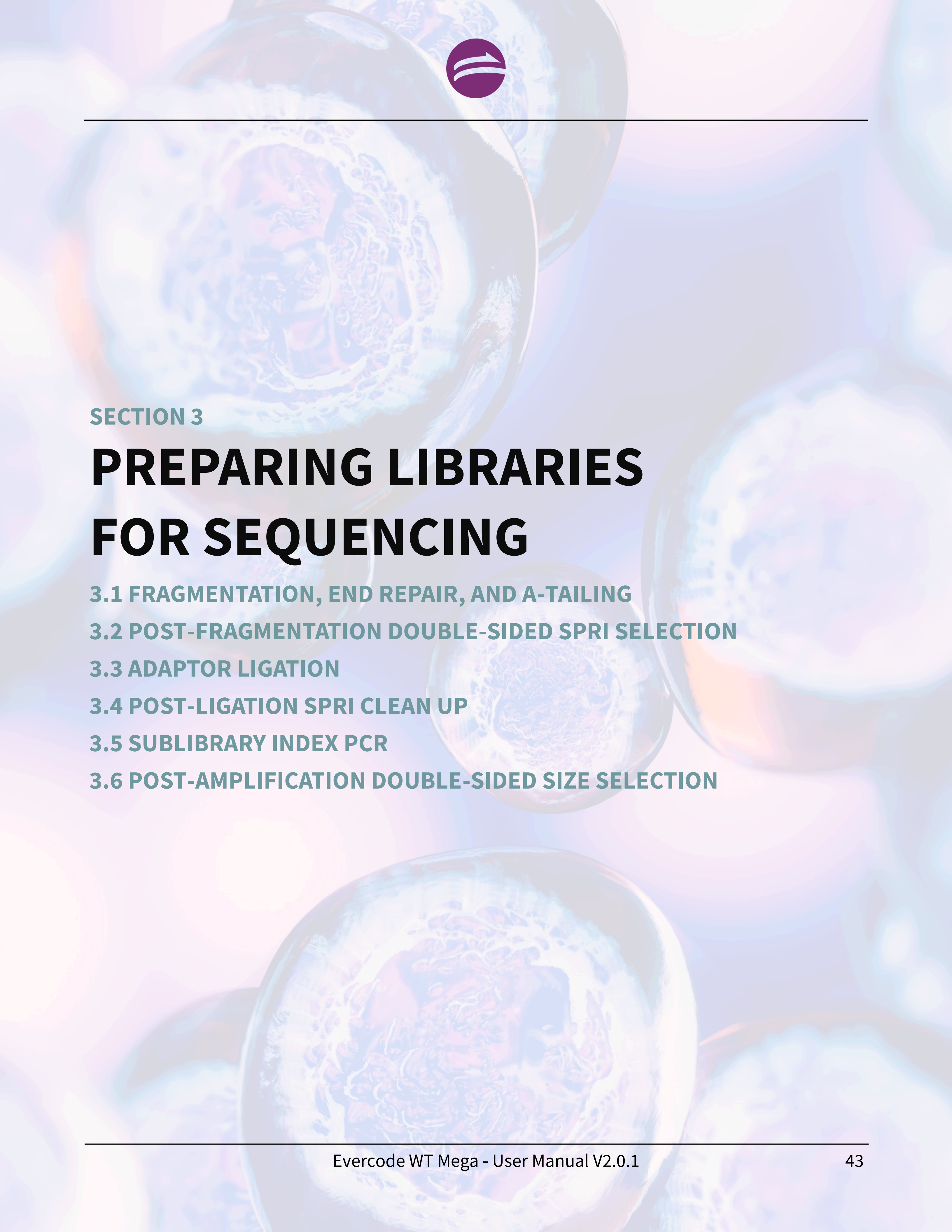


Fig. 1: Expected cDNA Size Distribution after cDNA Amplification. (A) Example trace of cDNA run on a Bioanalyzer. (B) Example trace of cDNA run on a TapeStation (it is normal for libraries to be shifted to the left on a TapeStation relative to a Bioanalyzer).

<u>Note</u>: 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

PREPARING LIBRARIES FOR SEQUENCING

3.1 FRAGMENTATION, END REPAIR, AND A-TAILING
3.2 POST-FRAGMENTATION DOUBLE-SIDED SPRI SELECTION
3.3 ADAPTOR LIGATION
3.4 POST-LIGATION SPRI CLEAN UP
3.5 SUBLIBRARY INDEX PCR
3.6 POST-AMPLIFICATION DOUBLE-SIDED SIZE SELECTION

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
Frag Buf. Fragmentation Buffer	Fragmentation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
Adapt DNA Adaptor DNA	Fragmentation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
Index PCR Mix Index PCR Mix	Fragmentation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
Adapt Buffer Adaptor Ligation Buffer	Fragmentation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
Univ Ind Primer Universal Index Primer	Fragmentation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
Index # Sublibrary Index Primers	Fragmentation Reagents (-20°C)	1-16	1.5 mL tubes	Thaw, then place on ice.
Frag Enzyme Fragmentation Enzyme	Fragmentation Reagents (-20°C)	1	1.5 mL tube	Place directly on ice.
Adapt Ligase Adaptor Ligase	Fragmentation Reagents (-20°C)	1	1.5 mL tube	Place directly on ice.

3.1 Fragmentation, End Repair, and A-Tailing

1. Vortex amplified cDNA briefly (2-3 sec). Be sure to keep caps closed on tubes. Briefly centrifuge tubes (~2 sec).
2. For each sublibrary to be sequenced, aliquot **10 µL** of cDNA into a PCR strip tube, then add **25 µL** of 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 -20°C to be used for future experiments.

   Note: Keep these tubes on ice.
3. Set the thermocycler to the following program:

Sublibrary Fragmentation, End Repair, and A-Tailing		
Run Time	**Lid Temperature**	**Sublibrary 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

4. Initiate the thermocycling program such that the machine is pre-cooled to 4°C.
5. Vortex the **Fragmentation Buffer** followed by a brief centrifugation (~2 sec) and confirm it is fully thawed (no precipitate).
6. Make the **Fragmentation Mix**, ensuring the **Fragmentation Buffer** and **Fragmentation Enzyme** blend are well mixed before using (mix ~10x with a pipette after adding **Fragmentation Enzyme**):

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	1	2	3	4	5	6	7	8	16
Frag Buf. Fragmentation Buffer	5.5	11	16.5	22	27.5	33	38.5	44	88
Frag Enzyme Fragmentation Enzyme	11	22	33	44	55	66	77	88	176
Total	**16.5**	**33**	**49.5**	**66**	**82.5**	**99**	**115.5**	**132**	**264**

7. Add **15 µL** of **Fragmentation Mix** to each sublibrary (should still be in cold block), bringing the total volume to 50 µL.
8. Mix sublibraries 10x with a P200 multichannel pipette set to 40 µL. Briefly centrifuge sublibraries (~2 sec) and place back on ice.

40 min

9. Place tubes in the chilled thermocycler and press “skip” or similar option to allow the machine to proceed to next step. Confirm that the thermocycler has elevated to 32°C and has proceeded to the rest of the protocol before leaving the machine.
10. Proceed directly to Section 3.2 after the thermocycling protocol finishes.

3.2 Post-Fragmentation Double-Sided SPRI Selection

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

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
SPRI Beads Needed	44	88	132	176	220	264	308	352	704

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).
3. Incubate at room temperature for **5 minutes.**
4. Place the sublibrary 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: liquid should be clear).

CRITICAL! Do NOT discard the supernatant at this step.

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.
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).
7. Incubate at room temperature for **5 minutes.**
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.

9. With SPRI Beads still against the magnetic rack, aspirate and discard the clear supernatant with a pipette.
10. Without resuspending beads, add **180 µL** of 85% ethanol and wait for **1 minute**.
11. Using a pipette, aspirate and discard the ethanol from each tube.
12. Without resuspending beads, add another **180 µL** of 85% ethanol and wait for **1 minute**.

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.

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

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

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).

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

18. Sublibraries can be stored at this point at 4°C overnight or at -20°C for up to 2 weeks. If you wish to continue, proceed directly to section 3.3: Adaptor Ligation.

[STOPPING POINT]

3.3 Adaptor Ligation

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

		Volume to Add by Number of Sublibraries (µL)								
# Sublibraries		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
Water	Nuclease-free water (not supplied)	19.25	38.5	57.75	77	96.25	115.5	134.75	154	308
Adapt Buffer	Adaptor Ligation Buffer	22	44	66	88	110	132	154	176	352
Adapt Ligase	Adaptor Ligase	11	22	33	44	55	66	77	88	176
Adapt DNA	Adaptor DNA	2.75	5.5	8.25	11	13.75	16.5	19.25	22	44
Total		**55**	**110**	**165**	**220**	**275**	**330**	**385**	**440**	**880**

2. Add **50 µL** of the **Adaptor Ligation Mix** to the 50 µL of the eluted DNA (from the end of Section 3.2).
3. Mix sublibraries 10x with a P200 pipette set to 80 µL. Briefly centrifuge sublibraries (~2 sec).

15 min

4. Put the tubes into a thermocycler with the following protocol:

Sublibrary Adaptor Ligation		
Run Time	**Lid Temperature**	**Sublibrary Volume**
15 min	30°C	100 µL
Step	**Time**	**Temperature**
1	15 min	20°C
2	Hold	4°C
Proceed Directly to Next Step		

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

3.4 Post-Ligation SPRI Clean Up

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

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
SPRI Beads Needed	88	176	264	352	440	528	616	704	1408

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).
3. Incubate at room temperature for **5 minutes.**
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).
5. With SPRI beads still against the magnetic rack, aspirate and discard the clear supernatant with a pipette.
6. Without resuspending beads, add **180 µL** of 85% ethanol and wait for **1 minute**.
7. Using a pipette, aspirate and discard the ethanol from each tube.
8. Without resuspending beads, add another **180 µL** of 85% ethanol and wait for **1 minute**.
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.

10. Remove the tubes from the magnetic rack and resuspend beads from each tube in **23 µL** of molecular biology grade water.
11. Incubate the tubes at room temperature for **5 minutes** to elute DNA.
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).
13. Transfer exactly **21 µL** of the eluted DNA into new PCR tubes. Discard the tubes with the SPRI beads.

3.5 Sublibrary Index PCR

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 3.5.4 (next page) to determine how many thermocyclers are needed.

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

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
Index PCR Mix Index PCR Mix	27.5	55	82.5	110	137.5	165	192.5	220	440
Univ Ind Primer Universal Index Primer	2.2	4.4	6.6	8.8	11	13.2	15.4	17.6	35.2
Total	**29.7**	**59.4**	**89.1**	**118.8**	**148.5**	**178.2**	**207.9**	**237.6**	**475.2**

Index #

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.
3. Add **27 µL** of the **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).

~30 min

4. 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.

Sublibrary Index Amplification		
Run Time	**Lid Temperature**	**Sublibrary Volume**
~30 min	105°C	50 µL
Step	**Time**	**Temperature**
1	3 min	95°C
	Begin Cycling	
2	20 sec	98°C
3	20 sec	67°C
4	1 min	72°C
	*Go to step 2, repeat **X-1** times (**X** cycles total)*	
5	5 min	72°C
6	Hold	4°C

	PCR Cycles based on cDNA in Fragmentation					
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

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).

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 Selection.

[STOPPING POINT]

3.6 Post-Amplification Double-Sided Size Selection

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

	Volume to Add by Number of Sublibraries (μL)								
# Sublibraries	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**16**
SPRI Beads Needed	44	88	132	176	220	264	308	352	704

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).
3. Incubate at room temperature for **5 minutes.**
4. Place the 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.

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.
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).
7. Incubate at room temperature for **5 minutes.**
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.

9. With SPRI beads still against the magnetic rack, aspirate and discard the clear supernatant with a pipette.
10. Without resuspending beads, add **180 μL** of 85% ethanol and wait for **1 minute**.
11. Using a pipette, aspirate and discard the ethanol from each tube.
12. Without resuspending beads, add another **180 μL** of 85% ethanol and wait for **1 minute**.

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 (as little as 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.

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

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

16. Place the tubes against a magnetic 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).

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.

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

19. Run1 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.

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

[STOPPING POINT]

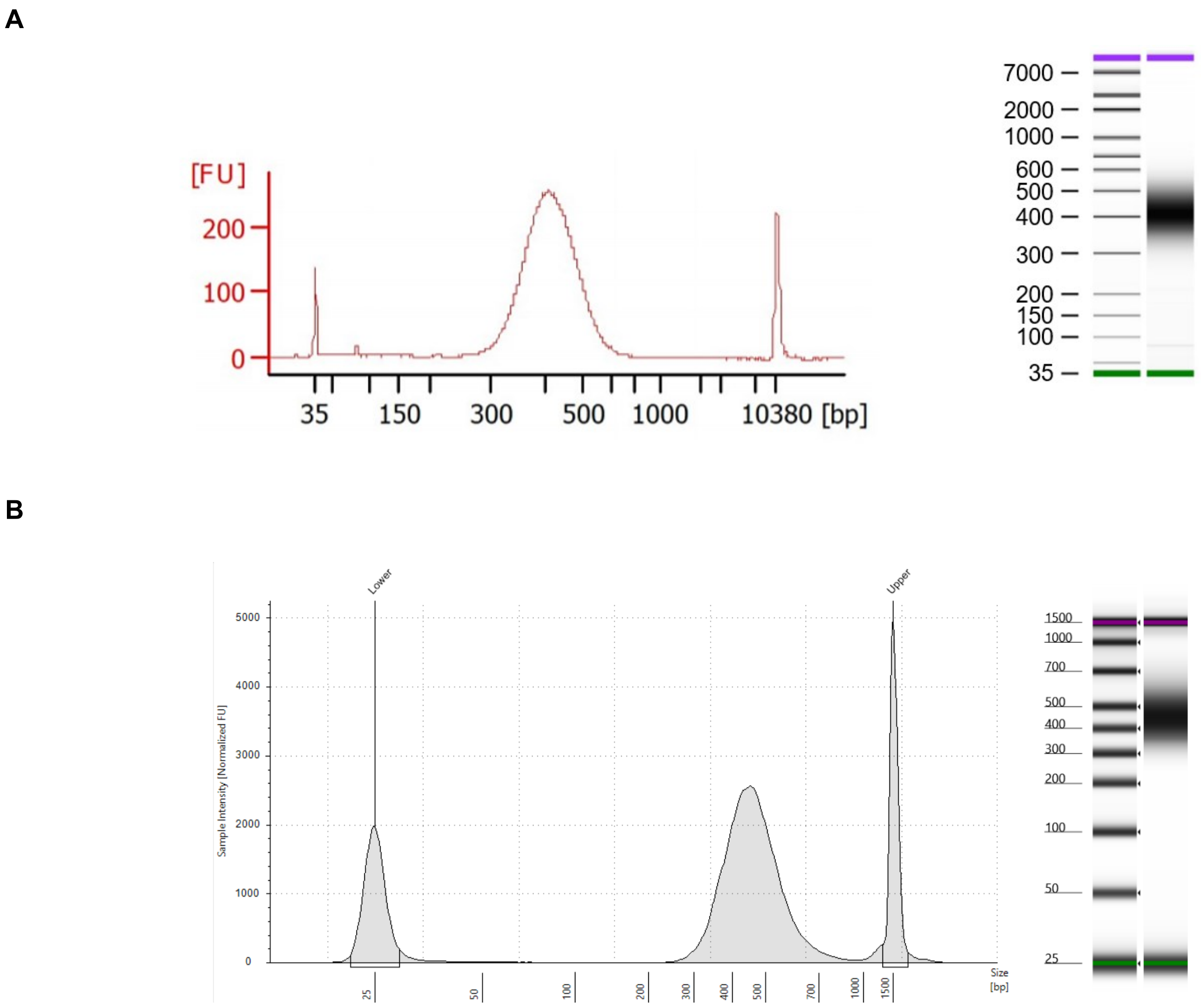


Fig. 2: Expected Size Distribution before Illumina Sequencing (A) Example trace of DNA from indexed sublibraries run on a Bioanalyzer. (B) Example trace of DNA from indexed sublibraries run on a TapeStation.

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.

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 400-500 bp). Do not use this additional peak when estimating amplicon size.

SECTION 4

SEQUENCING LIBRARIES

4.1 ILLUMINA RUN CONFIGURATION

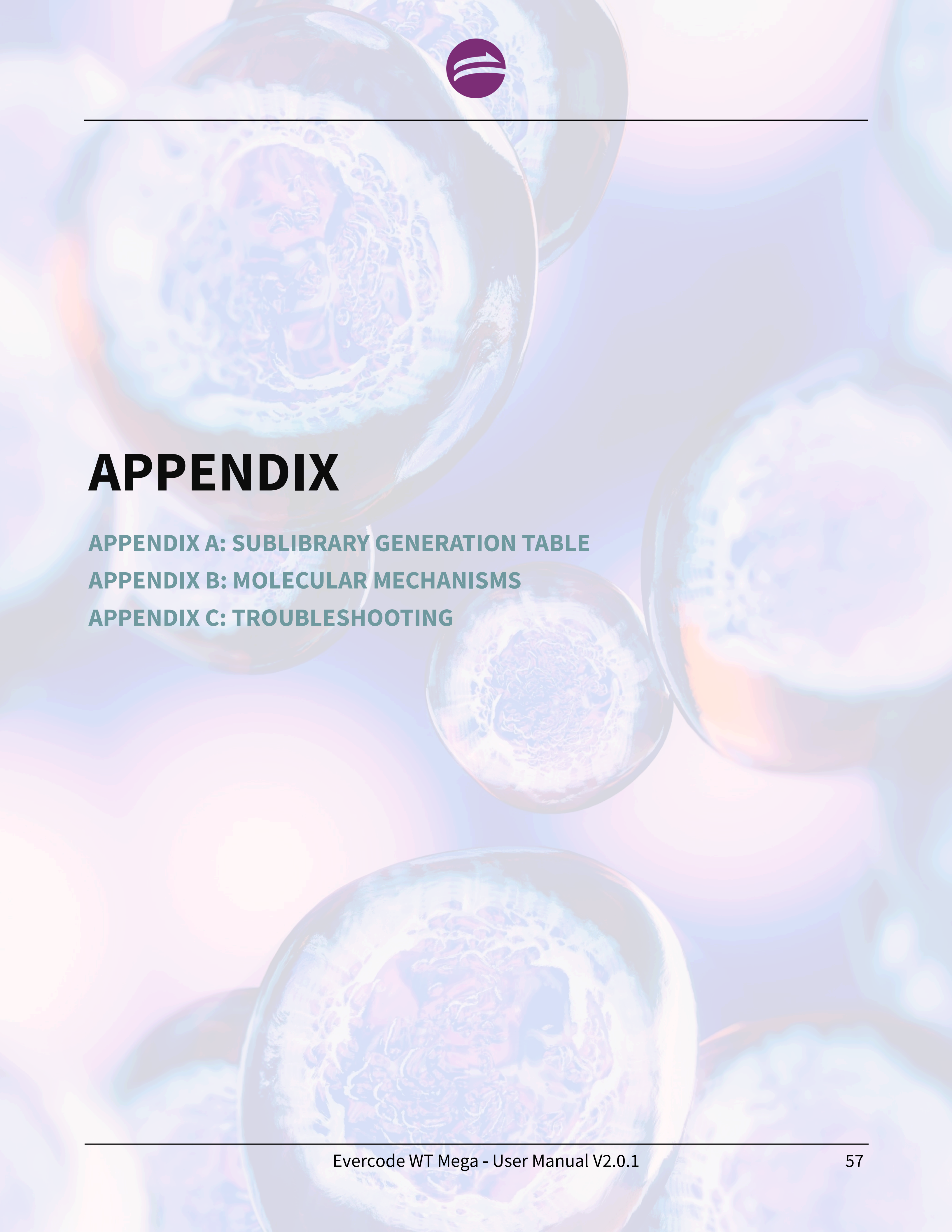
4.1 Illumina Run Configuration

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	74
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 (For Sample Sheet)	Reverse Complementary Sequence
1	CAGATC	GATCTG
2	ACTTGA	TCAAGT
3	GATCAG	CTGATC
4	TAGCTT	AAGCTA
5	ATGTCA	TGACAT
6	CTTGTA	TACAAG
7	AGTCAA	TTGACT
8	AGTTCC	GGAACT
9	GAGTGG	CCACTC
10	CCGTCC	GGACGG
11	GTAGAG	CTCTAC
12	GTCCGC	GCGGAC
13	GTGAAA	TTTCAC
14	GTGGCC	GGCCAC
15	GTTTCG	CGAAAC
16	CGTACG	CGTACG



APPENDIX

APPENDIX A: SUBLIBRARY GENERATION TABLE

APPENDIX B: MOLECULAR MECHANISMS

APPENDIX C: TROUBLESHOOTING

Appendix A: Sublibrary Generation Table

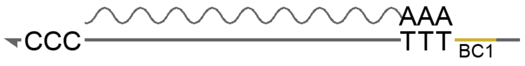
Green text (top): Volume of cell suspension (from step 1.5.8) to add to each sublibrary
Purple text (bottom): Volume of Dilution Buffer to add to each sublibrary
Blue Shading: **Serial dilution of cell stock is required to improve sublibrary cell count accuracy**
Red Shading: **Insufficient cell stock concentration for target sublibrary cell count**

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

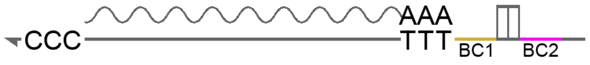
Appendix B: Molecular Mechanisms

Barcoding mRNA in Single Cells

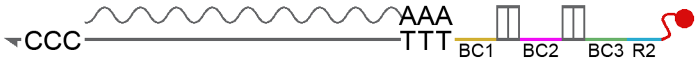
Polyadenylated mRNA



Reverse Transcription
Poly T and random hexamer primers anneal to mRNA within single cells. Each primer contains a barcode and a DNA linker. Reverse transcriptase extends cDNA to form a cDNA/mRNA hybrid.



R2 Ligation
A cDNA adaptor ligates to the DNA linker. The adaptor contains a second barcode and an additional DNA linker.



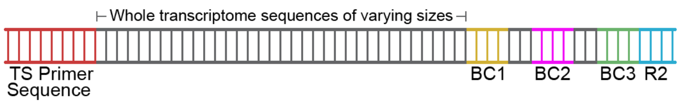
R3 Ligation
A second adaptor ligates to the growing cDNA. The adaptor contains a third barcode, an Illumina adaptor, and a biotin molecule.

Cell Lysis

cDNA Amplification

Apply Binder Beads
After cell lysis, the biotinylated cDNA/mRNA hybrid binds to a streptavidin binder bead. Molecules lacking biotin are lost.

Template Switch
A template switch reaction appends a primer binding site to the 3' end of the cDNA molecule.



cDNA Amplification PCR
A PCR reaction amplifies the cDNA using the TS primer and R2 Illumina primer. cDNA is now double stranded.

Quantify DNA

Preparing Sequencing Libraries

Fragmentation
Sublibrary cDNA is fragmented to a size compatible with Illumina sequencing.

Adaptor Ligation
A second Illumina adaptor ligates to the fragmented end of the cDNA.

Index PCR
A final PCR amplifies the fragmented cDNA and appends the fourth DNA barcode as well as the P5 and P7 adaptors.

Quantify DNA and Sequence

Appendix C: Troubleshooting

Assessing Quality of Fixed Samples

Several steps throughout your experiment require counting the cells or nuclei in your sample before progressing. If you are counting manually using a hemocytometer, it can be difficult to judge the quality of your sample. Below we provide examples of varying sample quality:

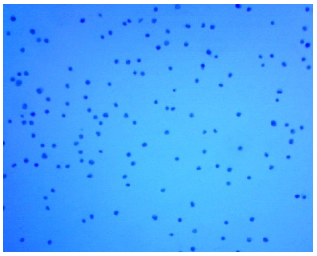
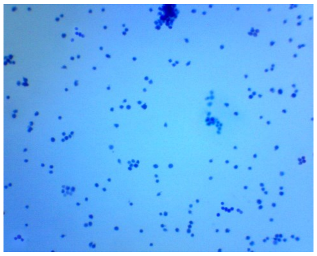
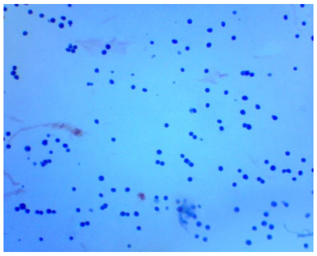
High Quality Sample		Single cells are easily distinguishable and no debris can be seen. Count each cell and proceed with your experiment as instructed.
Cell Clumps Have Formed		High quality cell samples should consist of > 95% single cells. Lower fractions of single cells may lead to elevated doublets.
Presence of Cell Debris		This is not a cause for concern assuming single cells are visible and debris is not causing cells to aggregate. Do not include any debris in your counting and continue your experiment as instructed.

Fig. 3: Photos Representative of Varying Qualities of Cell Samples Observed While Counting in the Presence of the Trypan Blue Stain.

Sample Loading Table

A situation may arise in which a cell sample is not concentrated enough according to the “WT Mega - Sample Loading Table V1.2.0” (Excel spreadsheet). The corrective action depends on your experimental goals. Some users will require constant ratios of all cell samples, while other users may allow for a decreased fraction in particular samples. Here we outline our recommended actions for each case:

A. If you require constant ratios for cell samples, you should decrease the “Max number barcoded cells” until the Sample Loading Table no longer gives an error.

B. If your experiment allows for a decreased fraction for the less concentrated samples, do not dilute these samples any further. Simply add 14 µL of undiluted sample into each designated well of the Round 1 Plate. This will result in fewer cells for the given sample, but the total number of barcoded cells will be greater than for option A.

Evercode WT Mega - User Manual V2.0.1

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