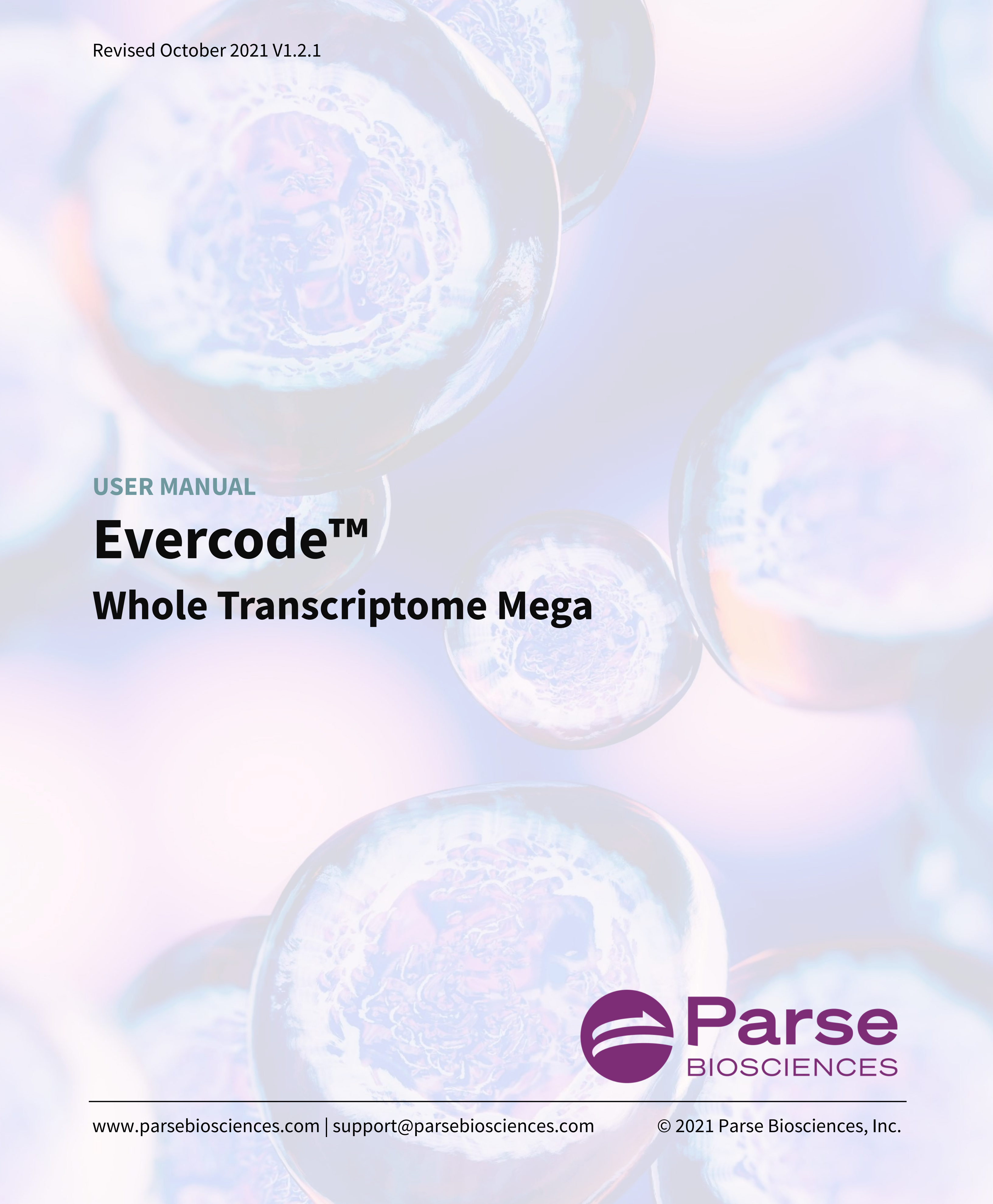

Revised October 2021 V1.2.1

USER MANUAL

Evercode™

Whole Transcriptome Mega

Parse
BIOSCIENCES

www.parsebiosciences.com | support@parsebiosciences.com

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U.S. Pat. No. 11,168,355
Patents pending in the U.S. and other countries

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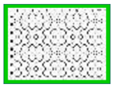
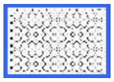
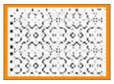
Parts List

● 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	1.5 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

Additional Materials and Equipment

The following materials and equipment are required to perform the protocol, but are not provided within the kit. Refer to Appendix D for vendor and catalog numbers for these items. Any questions regarding these items can be directed to support@parsebiosciences.com.

Benchtop

Pipettes: P1000, P200, P20, 12-channel P200, 12-channel P20

Pipette tips: P20, P200, P1000

100% ethanol

Water bath for thawing cells

Nuclease-free water

Tubes

- 0.2 mL PCR tubes
- Eppendorf DNA/RNA LoBind 1.7 mL tubes
- Eppendorf DNA/RNA LoBind 5 mL tubes
- Falcon 15 mL **polypropylene** centrifuge tubes
  Note: centrifuge tubes must be polypropylene and not polystyrene. Polystyrene centrifuge tubes will lead to substantial cell loss.

Beads and Racks

SPRI beads: AMPure XP Beads or KAPA Pure Beads will both work

Magnetic rack for PCR tubes with high and low setting (available for purchase from Parse Biosciences)
Note: Magnetic strength is critical. If 3rd party magnetic racks are used, the number of transcripts and genes detected per cell will be compromised.

Magnetic rack for 1.7 mL tubes

Centrifuging

Swinging bucket centrifuge that can hold 15 mL tubes, and is capable of reaching 4°C

Tabletop centrifuge (for spinning down residual liquid in 1.7 mL and 0.2 mL tubes)

Mechanism for spinning down 96 well plates

Equipment

Vortexer with foam insert for a 96 well plate (see image top right). Foam insert should be compatible with vortexer. A shaker set to 800-1000 RPM may be used instead.

Thermocycler, capable of holding a 96 well plate

Cell counting device (e.g. hemocytometer, flow cytometer, etc.)

Bioanalyzer or TapeStation

Qubit and reagents

Access to next generation sequencer (Illumina)

Storage

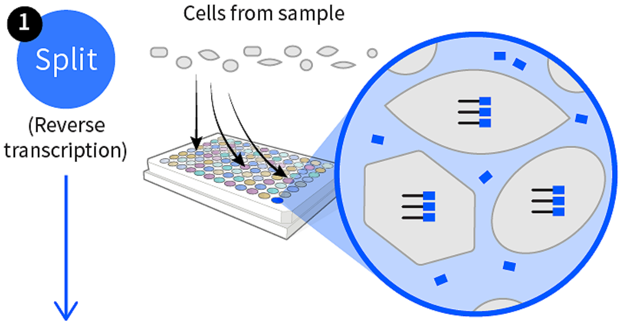
Access to a -80°C freezer, -20°C freezer, and 4°C fridge.

Background

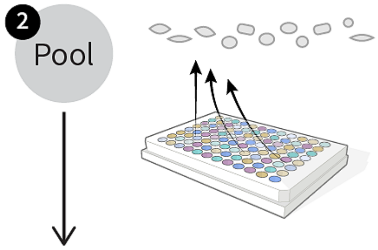
Evercode Combinatorial Barcoding Technology

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 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.

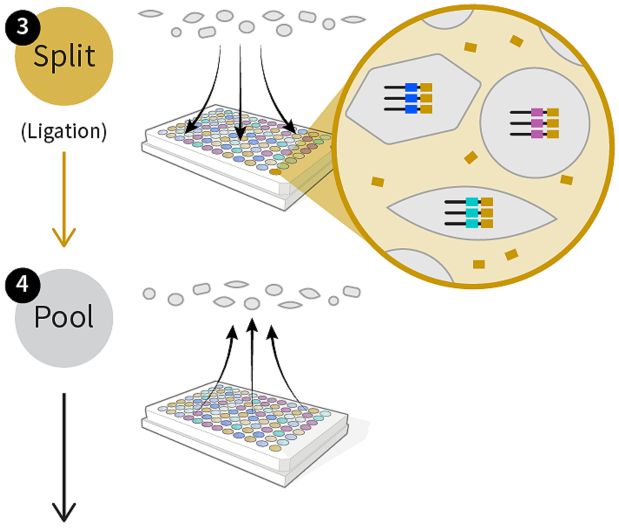
In the first round of barcoding, fixed cell samples are distributed into 96 wells, and cDNA is generated with an in-cell reverse transcription (RT) reaction using well-specific barcoded primers.



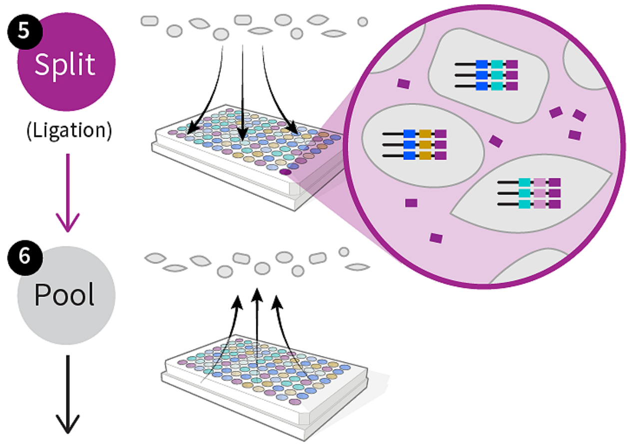
Cells from each well are pooled back together.



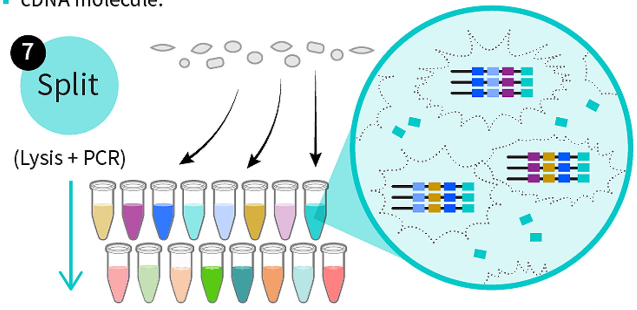
Cells are then distributed into 96 wells, and an in-cell ligation reaction appends a second well-specific barcode to the cDNA.



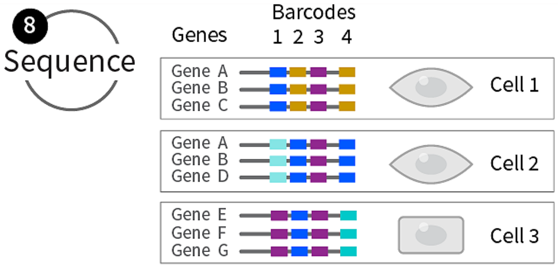
The third-round barcode, which also contains a unique molecular identifier (not depicted), is appended with another round of in-cell ligation.



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.



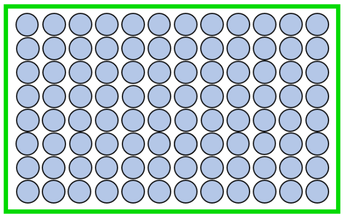
Background Continued

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 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.

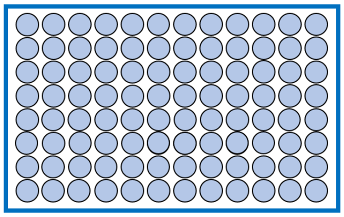
Overview of Four Rounds of Barcoding in the Evercode WT Mega:

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:

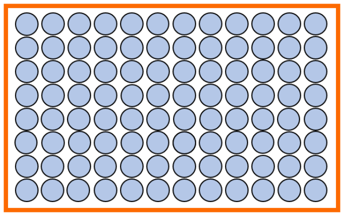
Round 1 Barcoding (96 barcodes):
Cells are distributed into 96 different wells (blue wells) within the Round 1 Barcoding Plate. Barcodes are added through an *in situ* reverse transcription reaction using barcoded primers.
<u>Note:</u> Check the "WT Mega - Sample Loading Table V1.2.0" (Excel spreadsheet)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



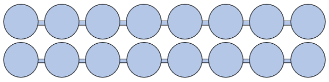
Round 2 Barcoding (96 barcodes):
Cells are distributed into 96 different wells within the Round 2 Barcoding Plate. Barcodes are added to transcripts with an *in situ* ligation reaction.



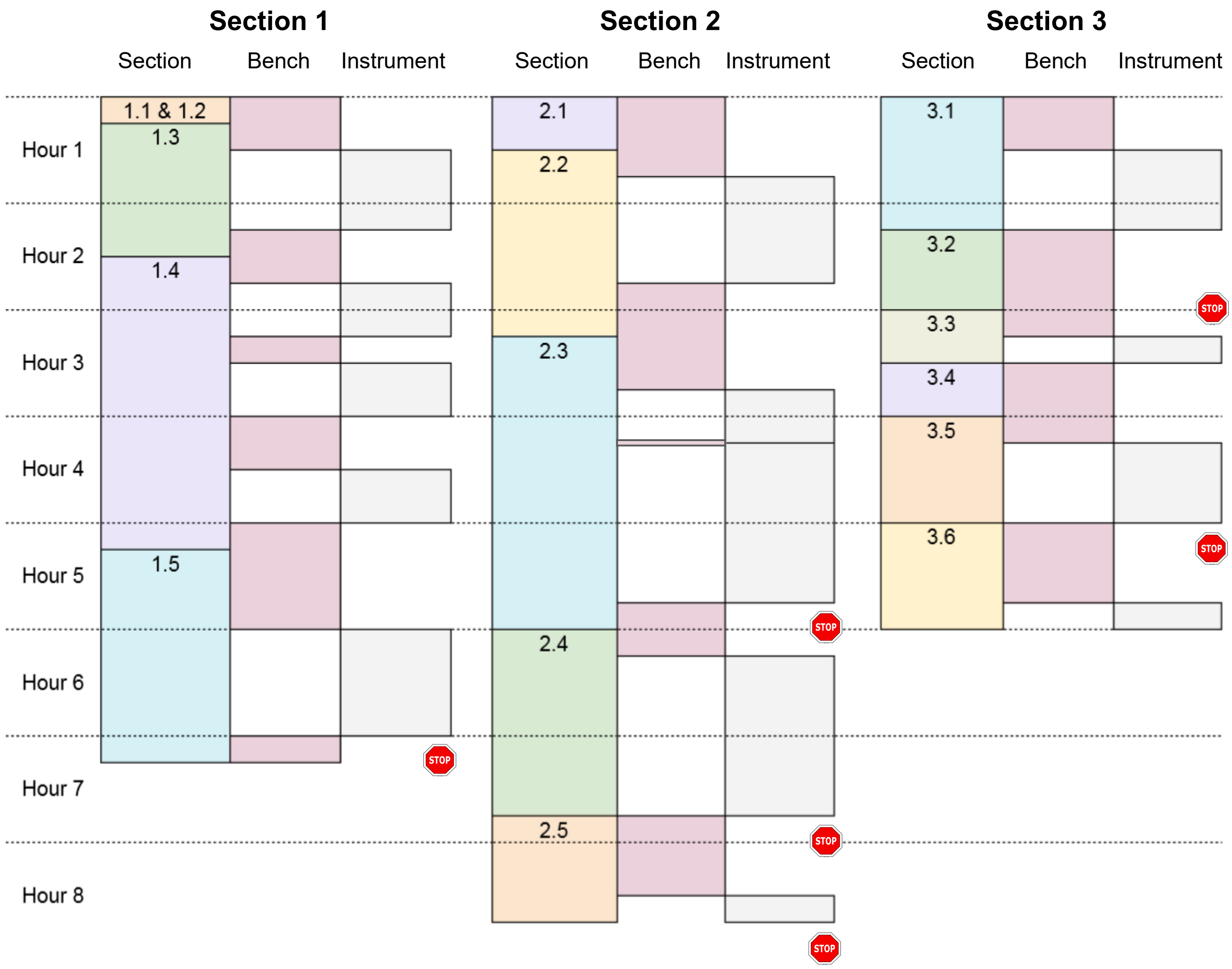
Round 3 Barcoding (96 barcodes):
Cells are distributed into 96 different wells within the Round 3 Barcoding Plate. Additional barcodes are added to transcripts with another *in situ* ligation reaction.



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



STOP = **Optional** stopping point

Notes Before Starting

User Materials	Before starting an experiment, check the "Additional Materials and Equipment" 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 that can be sprayed on benchtops and used to clean pipettes. Using filtered pipette tips is also recommended to reduce RNase contamination on 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.
Centrifuge Tubes	Ensure that the tubes that will be used are **polypropylene** and not polystyrene. Polystyrene tubes will lead to substantial 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 before using. Be sure to only edit the colored cells in the table to avoid disturbing the necessary formatting.
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.

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 of cells in each well. For Round 1/2/3 respectively this will be 30 µL / 50 µL / 70 µL. 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).

Cell Strainers

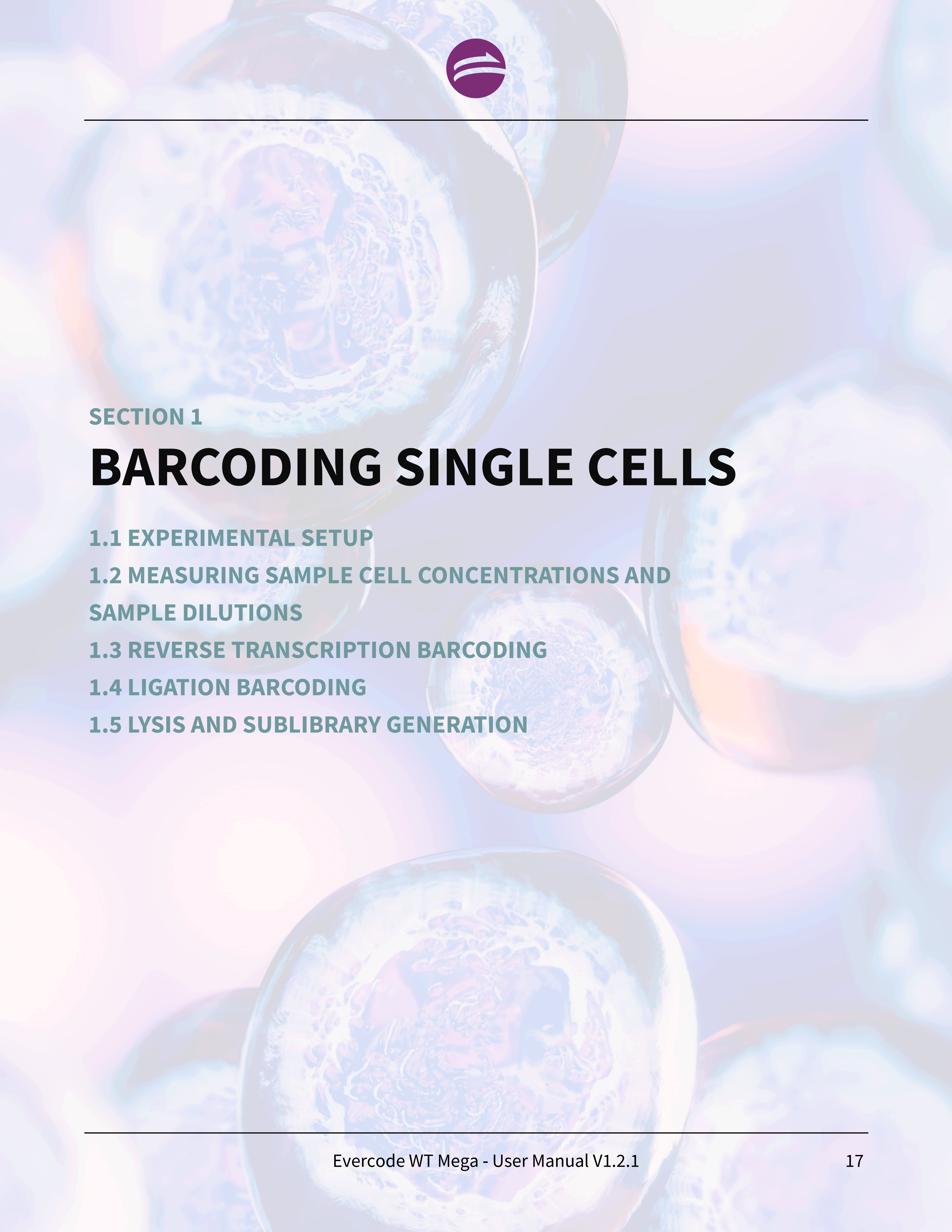
A 40 µm cell strainer will be used in multiple steps. To maximize cell retention, press the pipette tip directly against the strainer. Ensure that ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second.

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:

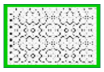
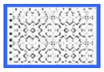
   ☐ Add each of your sample names to “WT Mega - Sample Loading Table V1.2.0” (Excel spreadsheet). In Section 1.2, you will add the concentrations of each sample to this document and use it to make appropriate dilutions to each sample. The plate configuration in this document will also tell you which wells to add each sample to during the first round of barcoding in Section 1.3.

   ☐ Set your swinging-bucket centrifuge to **4°C.**

   ☐ Warm 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 Measuring Sample Cell Concentrations and Sample Dilutions

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 part of the protocol, cDNA will be reverse transcribed from RNA with barcoded RT primers specific to each well. This adds the first round barcode to each molecule in the fixed cells or nuclei. 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 min**.
2. Place the plate (and holder) on a flat surface and remove the plastic seal, then place the plate on ice.
3. *Note: This step requires at least 96 unused 20 µL tips.*

   Add diluted samples to wells in Plate 1. 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 3 times. When pipetting the same sample into many wells, the sample should be periodically mixed by gentle pipetting to avoid cells from 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.

   <u>Note:</u> 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. <u>Proceed directly to the next step.</u>

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.

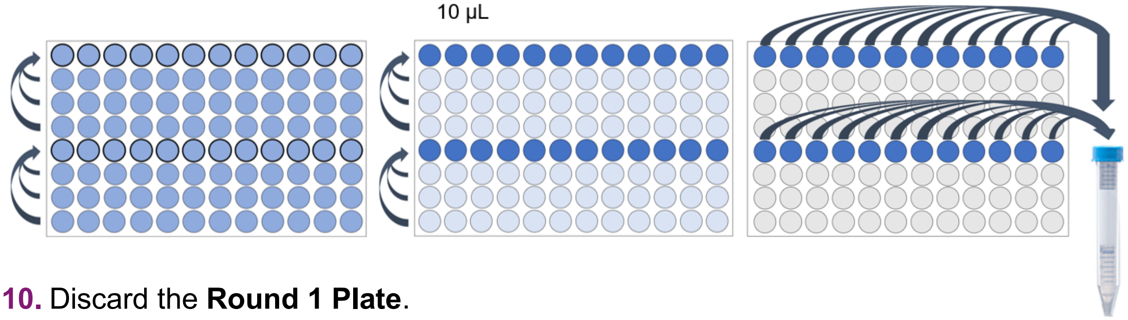
9. **Pool all the cells into a single 15 mL centrifuge tube on ice.**

   Note: 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, 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. 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.

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

CRITICAL! 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.

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

12. Centrifuge the pooled cells in a swinging bucket centrifuge cooled to **4°C** for **10 minutes** at **200 x *g***. Depending on the number of starting cells and cell types, the pellet may not be visible.

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.

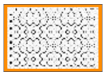
13. Using a P1000 pipette for the first 3 mL, then a P200 pipette for remaining volume, aspirate supernatant such that about ~40 µL of liquid remains above the pellet (see image on right for estimate of 40 µL). For some cell types, the pellet may be hard to see or not visible.

Resusp. Buffer

14. Gently resuspend cells with **1 mL** of **Resuspension Buffer**. When resuspending the pellet, pipette slowly to prevent mechanical damage to cells. 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. Take the following items out:

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	1.5 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** tube and add **20 µL** of **Round 2 Ligation Enzyme** to the **Ligation Mix**.

Ligation Mix

3. Using a P1000 pipette, add the **2 mL** of cells in **Resuspension Buffer** into the tube containing both the **Ligation Mix** and the **Round 2 Ligation Enzyme**. 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 min**. Place the plate on a flat surface and remove the seal.

5. Using a P1000 pipette, add the entirety of cells in the **Ligation Mix** (made in the previous step) to a basin.

6. *Note: This step requires a new box of 200 µL tips.*

   Using a 12-channel 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 from settling in the basin, also gently pipette up and down 2x with the 12-channel 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. Place the **Round 2 Plate** (and holder) on a flat surface and reseal the 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.

11. *Note: This step requires a new box of 20 µL tips.*

    Using a 12-channel 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 blocking solution into the 96 well plate. Never place a tip that has entered one of the 96 wells back into the basin.

12. Place the **Round 2 Plate** (and holder) on a flat surface and reseal the 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. Place the **Round 2 Plate** (and holder) on a flat surface and remove the seal.

17. Pool all cells from the Round 2 Plate into a new basin.

Note: 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.

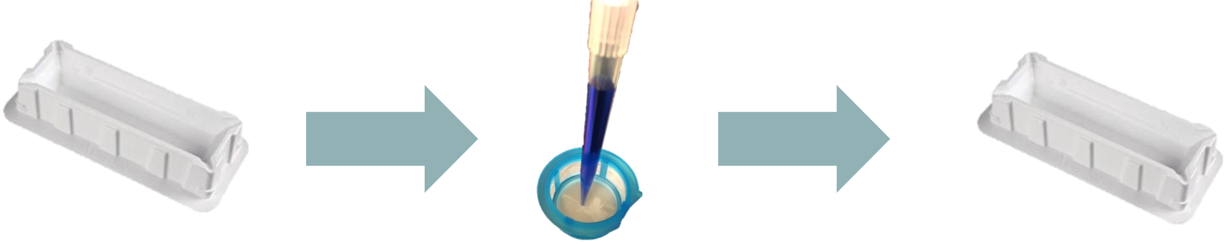
Note: Bubbles may form while pooling. They will not affect the quality of the experiment.

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

19. Remove the 40 µm strainer out of 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 the 40 µm strainer into a new basin. The original basin must be tilted in order to pipette the final volume.

    Note: 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 ~20 times with a P1000 pipette set to 1000 µL.

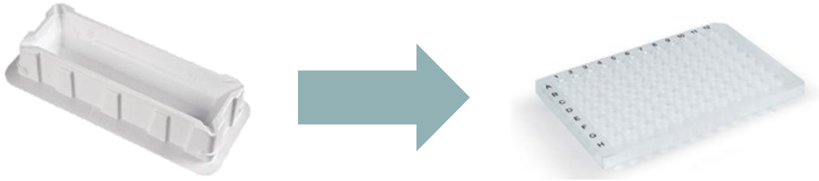
21. Transfer the **Round 3 Plate** from the thermocycler back to its original orange plate holder. Centrifuge the plate at **100 x *g*** for **1 min.** Place the plate on a flat surface at room temperature and remove the seal.

22. *Note: This step requires a new box of 200 µL tips.*

    Add cells to the Round 3 Plate. Using a 12-channel 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 from settling, also gently pipette up and down 2x with the 12-channel 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. Place the **Round 3 Plate** (and holder) on a flat surface and reseal the 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.

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.

27. *<u>Note:</u> This step requires a new box of 20 µL tips.*

Using a 12-channel 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.

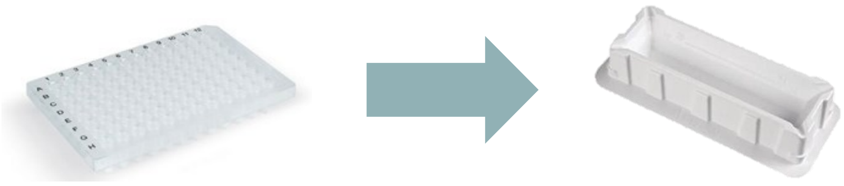
CRITICAL! Different tips must be used when pipetting stop mix into the 96 well plate. Never place a tip that has entered one of the 96 wells back into the basin.

28. Pool all cells from the Round 3 Plate into a new basin.

Note: 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.

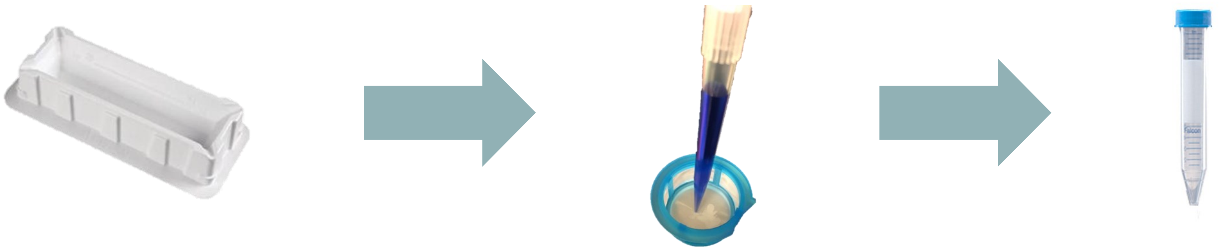
Note: Bubbles may form while pooling. They will not affect the quality of the experiment.



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

30. Remove a 40 µm strainer out of 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 15 mL tube. The basin must be tilted in order to pipette the final volume. Keep the tube on ice and proceed to lysis.

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



1.5 Lysis and Sublibrary Generation

1. Take the following items out:

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**. The pellet may be very small and hard to see, or not visible.
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).

Pre-Lyse Wash

5. 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** to make 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).

Dilution Buffer

8. 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.

CRITICAL! Do NOT discard **Dilution Buffer**.

9. Count the cells through one of two approaches:

Dilution Buffer

    a. **Flow Cytometry:** Dilute **5 µL** of the cell solution by mixing into 195 µL **Dilution Buffer**, and count via flow cytometry (only FSC vs. SSC, no fluorescence required).
    b. **Hemocytometer:** Dilute **5 µL** into 5 µL of **Dilution Buffer** and load onto hemocytometer. Some level of debris is normal at this step.

Note: 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 Illumina 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 (100-500) 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.

Dilution Buffer

10. **Add cells to sublibraries.** First label 16 PCR tubes and determine how many cells will be added to each of the 16 sublibraries. Use the "Sublibrary Generation Table" (Appendix A) to determine the volume of cells and **Dilution Buffer** that must be added to each sublibrary (this will depend on the concentration of cells you measured in the previous step). Add the cells to each tube and then add the **Dilution Buffer** to bring the total volume to 25 µL.

CRITICAL! 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.

2x Lysis

Lysis Enzyme

11. 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 1.7 mL tube.

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

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

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

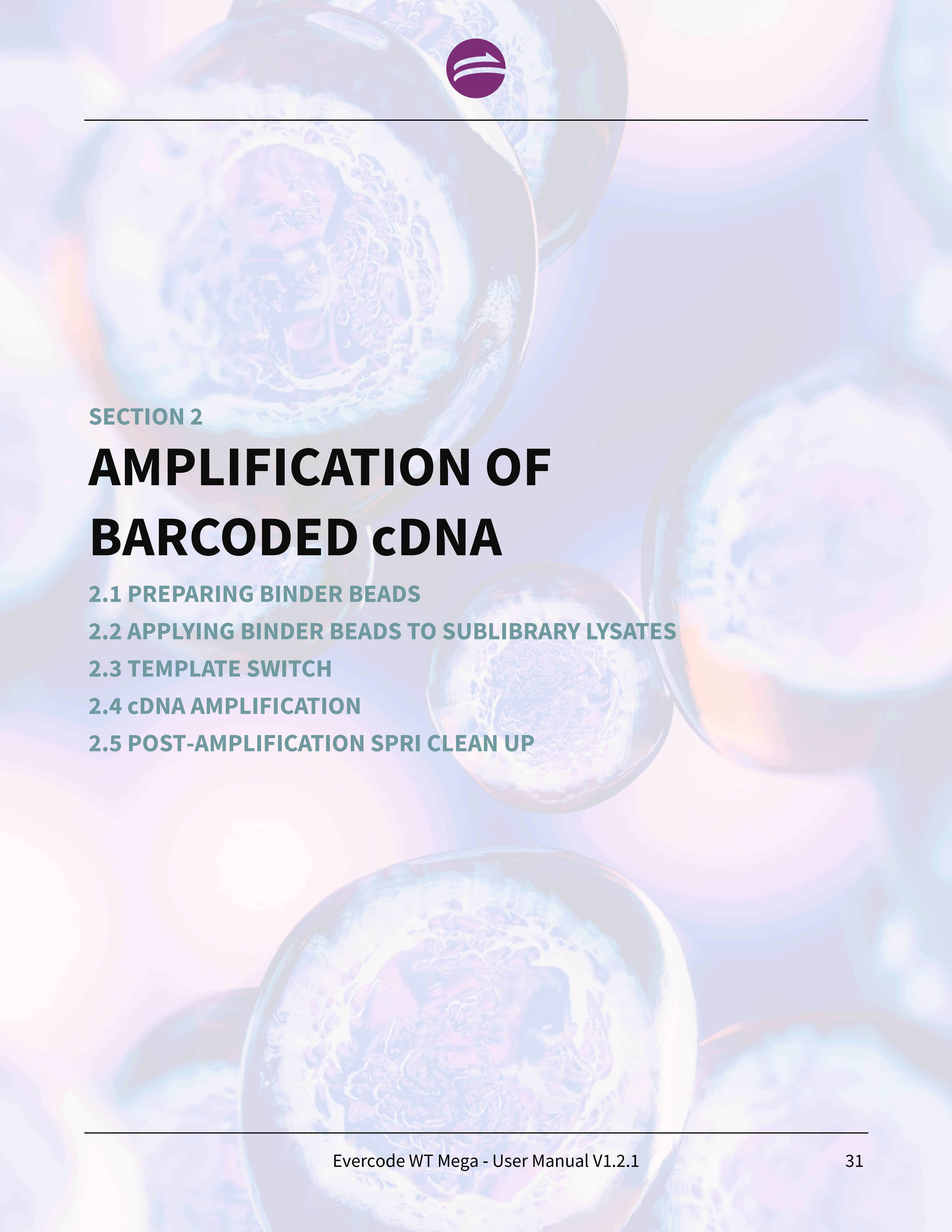
1 hr

14. Incubate the 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

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

STOP **[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 sequencing index. Take care not to cross-contaminate any sublibraries for the remainder of the experiment.

Setup

- ☐ Prepare a **37°C** water bath or incubator.
- ☐ Fill an ice bucket.
- ☐ Take out a magnetic rack (with high and low setting) for PCR tubes.
- ☐ Take out a magnetic rack for 1.7 mL tubes.

2.1 Preparing Binder Beads

1. Remove these mixes, following directions for what to do after taking each one out:

   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

Binder Beads

2. Vortex **Binder Beads** until fully mixed and add a volume to an empty 1.7 mL tube according to the number of lysates that you plan to process:

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

3. Capture the **Binder Beads** to a magnet using a magnetic rack and wait until liquid becomes clear (~2 min).
4. Remove the clear supernatant with a pipette and discard.

Bead Wash

5. Remove tube from magnetic rack and resuspend beads with the volume of **Bead Wash Buffer** according to the number of lysates that you plan to process (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 Sublibraries (µL)								
# Sublibraries	1	2	3	4	5	6	7	8	16
Bead Wash Buffer (µL)	50	100	150	200	250	300	350	400	800

6. Capture the **Binder Beads** to a magnet using a magnetic rack and wait until liquid becomes clear (~2 min).
7. Remove the clear supernatant with a pipette and discard.
8. Repeat steps 5-7 twice more for a total of three washes.

Bind Buf. A

9. Remove tube from magnetic rack and resuspend beads in the appropriate amount of **Bind Buffer A**, as designated by the table below. Keep beads at room temperature and proceed to Section 2.2.

	Volume to Add by Number of Sublibraries (µL)								
# Sublibraries	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 lysates (~2 sec).

Lysis Neut

10 min

3. Gently centrifuge the **Lysis Neutralizer**, mix gently with a pipette, and add **2.5 µL** to each sublibrary. 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 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 lysates, mix by vortexing and not by pipetting.

60 min

5. Agitate the tubes containing cell lysate 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 (high setting) 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 magnetic rack and resuspend beads with **125 µL Bind Buffer B**.
10. Keep tubes at room temperature for **1 minute**.

11. Place the tubes against a magnetic rack (high setting) 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.
13. Repeat steps 9-12 for a second wash using **Bind Buffer B**.
14. Remove tubes from magnetic rack and resuspend beads with **125 µL 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. Remove the following items:

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 1.7 mL tube, make the **Template Switch Mix** by adding the following volumes of **TS Buffer**, **TS Primer Mix**, and **TS Enzyme** together. Mix well by pipetting and keep on ice.

CRITICAL! If processing more than 10 sublibrary lysates at one time, use a single 5 mL tube or 15 mL tube to make **Template Switch Mix** according to the number of lysates that you are processing.

	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 tubes against a magnetic rack (high setting) 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 removing tubes (still in magnetic rack), add **125 µL 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 tubes from magnetic rack and resuspend beads with **100 µL** of the **Template Switch Mix**.

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

30 min

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

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 continuing the protocol, proceed directly to Section 2.4: cDNA Amplification.
    a. Place the tubes against a magnetic rack (high setting) 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 Bead Storage Buffer**.
    d. Store tubes at 4°C overnight. Do not freeze sublibraries.

Bead Storage

[STOPPING POINT]

2.4 cDNA Amplification

1. Remove the following items:

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. Using a new 1.7 mL tube, combine the **Amplification Master Buffer** and **Amplification Primer Mix** to make the **Amplification Reaction Solution**:

CRITICAL! If processing more than 10 sublibrary lysates at one time, use a single 5 mL tube or 15 mL tube to make **Amplification Reaction Solution** according to the number of lysates that you are processing.

	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 tubes against a magnetic rack (high setting) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
4. Remove the clear supernatant with a pipette and discard while still keeping the tubes in the magnetic rack.

Bind Buf. C

5. Without removing tubes (still in magnetic rack), add **125 µL Bind Buffer C** and wait **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 tubes from magnetic rack and resuspend beads in each tube with **100 µL** of the **Amplification Reaction Solution**. Place tubes with **Amplification Reaction Solution** on ice.

50-70 min

8. **Start cDNA amplification.** Put the tubes in a thermocycler with the following protocol, adjusting the number of cycles according to the table below.

   Note: 1-2 cycles may need to be added to account for cells with low RNA content (e.g. lymphocytes). For more information refer to Appendix C.

Cells in Sublibrary	Total PCR Cycles
200-1,000	5 (first cycling) + 13 (second cycling)
1,000-2,000	5 (first cycling) + 11 (second cycling)
2,000-6,000	5 (first cycling) + 9 (second cycling)
6,000-12,500	5 (first cycling) + 7 (second cycling)
12,500-25,000	5 (first cycling) + 6 (second cycling)
25,000-62,500	5 (first cycling) + 5 (second cycling)

   Note: For 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 for X total cycles	
8	5 min	72°C
9	Hold	4°C

9. 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.

STOP **[STOPPING POINT]**

2.5 Post-Amplification SPRI Clean Up

1. Place the tubes with amplified cDNA against a magnetic rack (high setting) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).

CRITICAL! Do NOT discard the supernatant.

2. Transfer 90 µL of the clear supernatant into new 200 µL PCR tubes. Discard the original tubes with the magnetic beads.
3. Remove your SPRI beads (Ampure XP or KAPA Pure Beads) from the 4°C fridge and aliquot the following amount into a 1.7 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 (1 mL 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 (high setting) 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 magnet 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 (**low setting**) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).

17. Transfer **25 µL** of the elutant 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 elutant 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 below for expected 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 three months. If you wish to continue, proceed directly to Section 3: Preparing Libraries for Sequencing.

[STOPPING POINT]

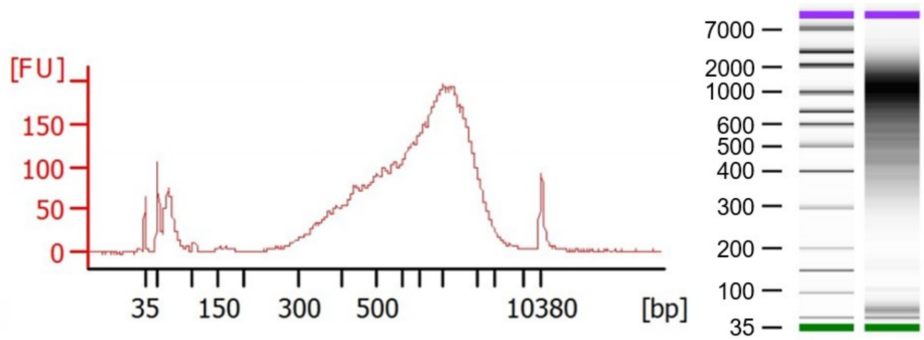
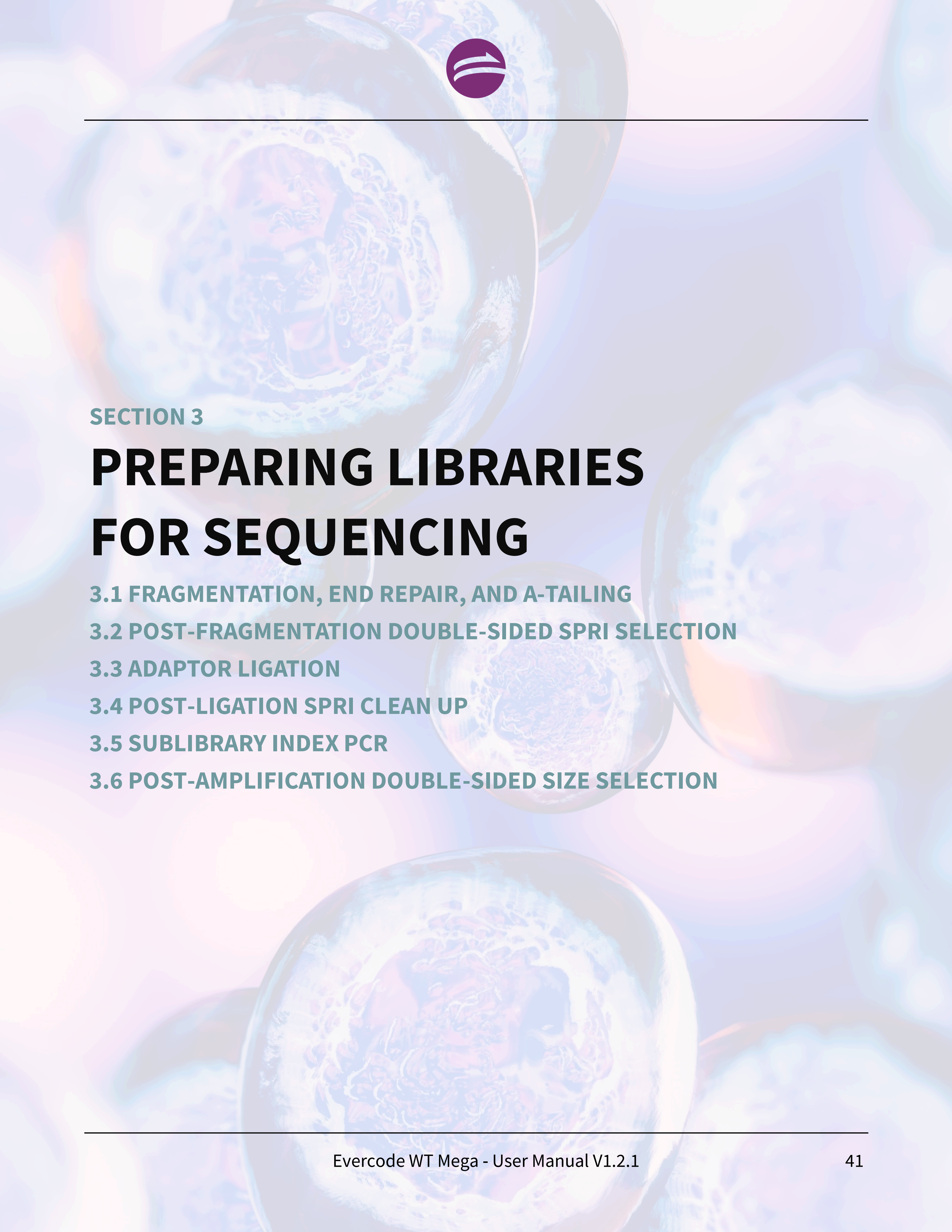


Fig. 1: Expected cDNA Size Distribution after cDNA Amplification.



SECTION 3

PREPARING LIBRARIES FOR SEQUENCING

Setup

- ☐ Prepare ~1.2 mL 85% ethanol per sublibrary lysate (e.g. 2.4 mL for 2 sublibraries).
- ☐ Fill an ice bucket.
- ☐ Take out a magnetic rack (with high and low setting) for PCR tubes.
- ☐ Ensure you have at least 176 µL SPRI beads (Ampure XP or KAPA Pure Beads) per sublibrary. These will be used throughout Section 3.
- ☐ Obtain recorded cDNA concentrations from step 18 of Section 2.5: Amplification of barcoded DNA.

1. Remove these mixes, following directions for what to do after taking each one out:

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 1.7 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 tubes against a magnetic rack (high setting) 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 (high setting) and wait for all the beads to bind to the magnet (~3 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 tubes from the magnet and resuspend beads from each tube in **50 µL** of molecular biology grade water.
15. Incubate the tube at room temperature for **5 minutes** to elute fragmented DNA.
16. Place the tubes against a magnetic rack (high setting) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
17. Transfer exactly **50 µL** of the elutant into new PCR tubes. Discard the tubes with SPRI beads.

STOP

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.

		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 SPRI elutant in each PCR tube 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 1.7 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 tubes against a magnetic rack (high setting) 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 tubes from the magnet and resuspend beads from each tube in **23 µL** of molecular biology grade water.
11. Incubate the tube at room temperature for **5 minutes** to elute DNA.
12. Place the tubes against a magnetic rack (**low setting**) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
13. Transfer exactly **21 µL** of the elutant into a new PCR tube. Discard the tubes with the SPRI beads.

3.5 Sublibrary Index PCR

1. Make the **Sublibrary Amplification Mix**. Ensure that all reagents are fully thawed and mixed well before using. Place the mix on ice after making.

	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. <u>Make sure to record which sublibrary contains which index primer.</u>

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 PCR tube(s) in the thermocycler and run the following program:

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, cycle according to the table below	
5	5 min	72°C
6	Hold	4°C

Adjust PCR cycles depending on the amount of cDNA added during the fragmentation reaction. The required PCR cycles are as follows:

	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	13	12	11	10	8	7

STOP

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 1.7 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 (high setting) 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 (high setting) 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 tubes from magnet and resuspend beads from each tube in **20 µL** of molecular biology grade water.
15. Incubate the tube at room temperature for **5 minutes** to elute DNA.
16. Place the tubes against a magnetic rack (**low setting**) and wait for all the beads to bind to the magnet (~2 min: liquid should be clear).
17. Transfer the elutant 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 cDNA using the Qubit dsDNA HS protocol.
19. Run 1 µL of the elutant on a Bioanalyzer or TapeStation. Use the concentrations 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 below for expected size distribution).

    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.

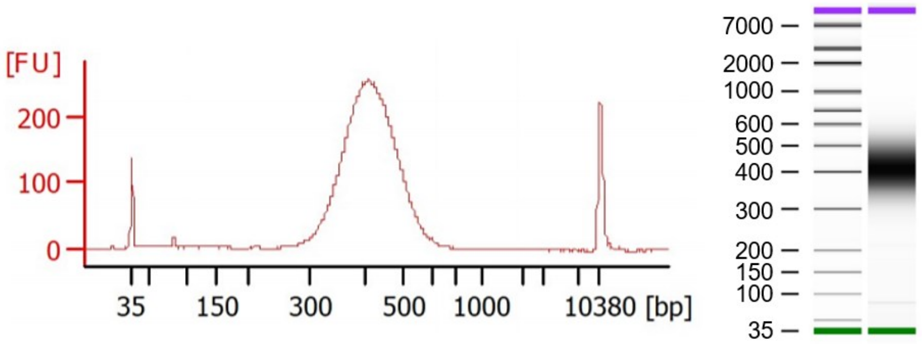


Fig. 2: Expected Size Distribution before Illumina Sequencing.

SECTION 4

SEQUENCING LIBRARIES

4.1 ILLUMINA RUN CONFIGURATION

4.1 Illumina Run Configuration

Parse Biosciences libraries do not require custom primers to sequence. Sequencing must be run with paired reads (and an i7 index read if you are sequencing multiple sublibraries together). The following is the recommended cycles per read:

Read	Cycles
Read 1	74
i7 Index	6
Read 2	86
i5 Index	0

Libraries should be diluted and denatured according to the standard instructions from Illumina. It is **strongly recommended** to include 5% phiX with your libraries to improve sequencing quality.

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

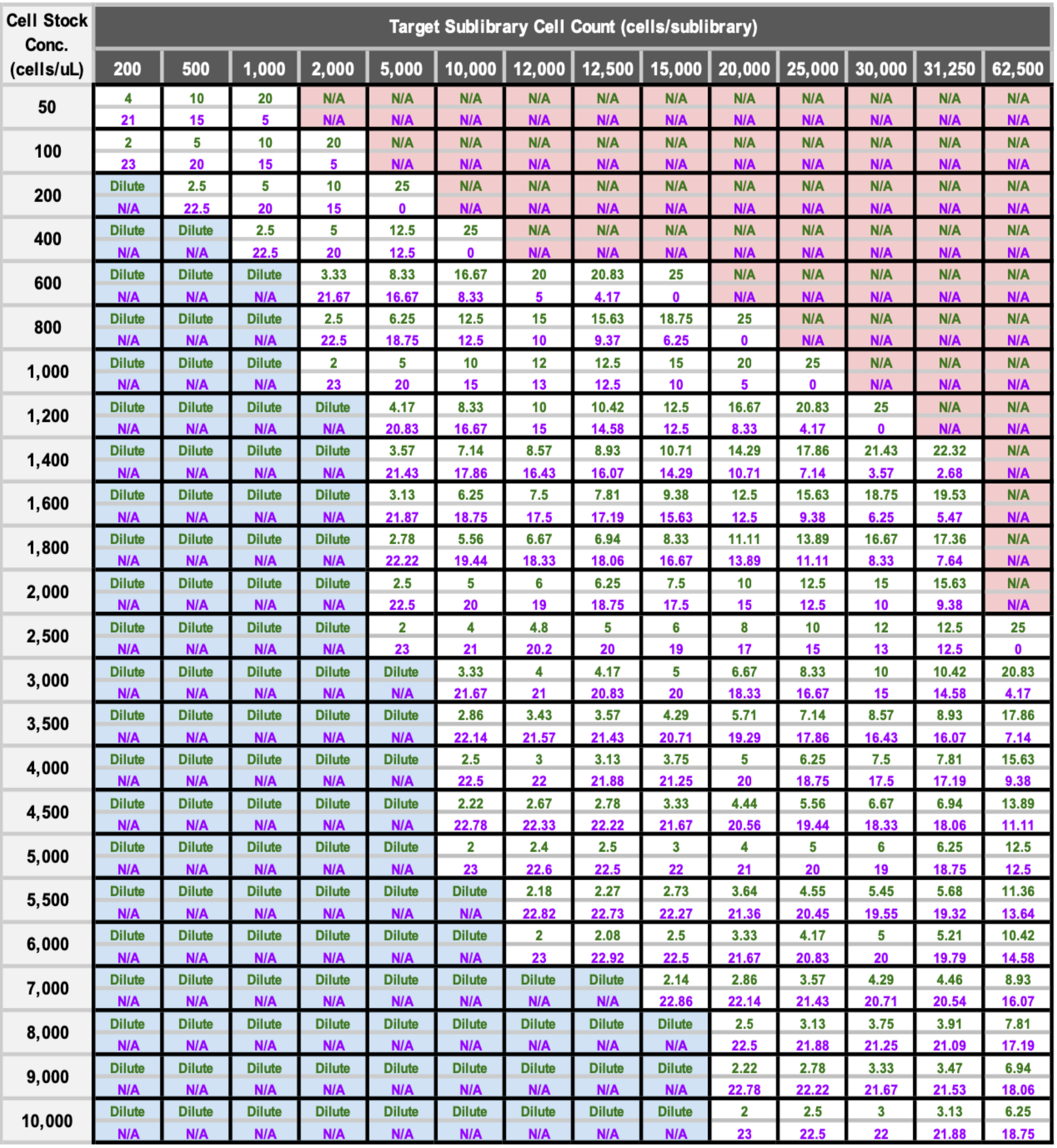
APPENDIX B: MOLECULAR MECHANISMS

APPENDIX C: TROUBLESHOOTING

APPENDIX D: ORDERING ADDITIONAL MATERIALS

Appendix A: Sublibrary Generation Table

Green text (top): Volume of cells to add to each sublibrary
Purple text (bottom): Volume of Dilution Buffer to add to each sublibrary



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

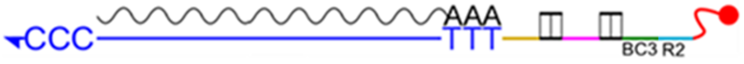
Appendix B: Molecular Mechanisms

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.

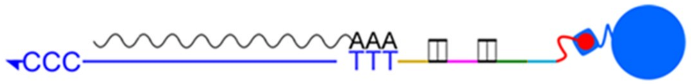
R2 Ligation
A cDNA adaptor ligates to the DNA linker. The adaptor contains a second barcode and an additional DNA linker.



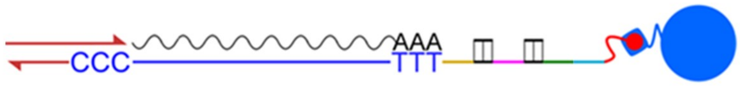
R3 Ligation
A second adaptor ligates to the growing cDNA. The adaptor contains a third barcode, an Illumina adaptor, and a biotin molecule.

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 cDNA

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.

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:

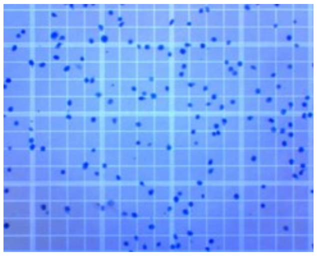
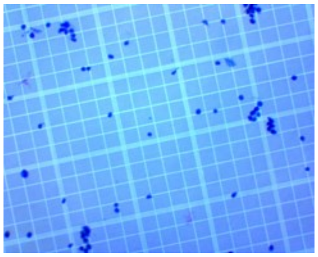
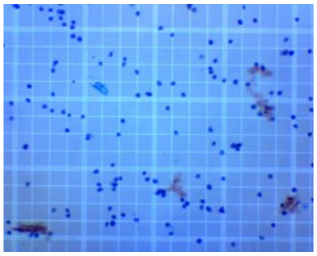
High Quality Sample		Single cells are easily distinguishable and no debris can be seen. Count each cell and proceed with your experiment as instructed.
Cell Clumps Have Formed		High quality cell samples should consist of >95% single cells. Lower fractions of single cells may lead to elevated doublets.
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.

cDNA Amplification PCR Cycling

The cDNA Amplification PCR (step 2.4.8) is critical to ensure that enough cDNA is amplified to represent each sublibrary, but overamplification can lead to unwanted products. Different cell types express varying amounts of mRNA, and the cycling protocol may need to be optimized to fit your experiment. Below are recommended cycling protocols for commonly used cell types:

	Number of Cells	Number of Total PCR Cycles
Mammalian Cell Lines	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
Nuclei	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
Immune Cells (PBMCs)	200-1,000	5+15
	1,000-2,000	5+13
	2,000-6,000	5+11
	6,000-12,500	5+9
	12,500-25,000	5+8
	25,000-62,500	5+7

Low DNA Concentration

DNA is quantified both after cDNA Amplification (step 2.5.19) and after Library Preparation (step 3.6.18). After cDNA Amplification, your concentrations of DNA should be above 0.5 ng/µL. After Library Preparation, your concentrations of DNA should be above 5 ng/µL. If your samples fall below these values, we advise the following:

- Ensure that enough cycles were included in the cDNA Amplification PCR (step 2.4.8). The number of cycles required may change depending on your cell type (see “cDNA Amplification PCR Cycling” section above).
- If fewer than 100 ng was added to the fragmentation, ensure that you included additional cycles during the Sublibrary Index PCR (step 3.5.4).

Appendix D: Ordering Additional Materials

Material	Vendor	Catalog Number
15 mL Polypropylene Falcon tubes	Corning	352097
1.7 mL Eppendorf tubes	Eppendorf	0030108418
5 mL Eppendorf tubes	Eppendorf	0030108310
KAPA Pure Beads	KAPA Biosystems	KK8001
1.7 mL magnetic rack	New England Biosystems	S1506S
0.2 mL magnetic rack	Parse Biosciences	PB1004
Filter pipette tips 20 µL	Rainin	17014961
Filter pipette tips 200 µL	Rainin	17014963
Filter pipette tips 1000 µL	Rainin	17014967
Vortexer	Scientific Industries	SI-0236
Foam insert for vortexer	Scientific Industries	504-0235-00
100% ethanol	Sigma-Aldrich	459844
Nuclease-free water	Sigma-Aldrich	W4502-1L
Hemocytometer	Sigma-Aldrich	Z359629-1EA
0.2 mL PCR tubes	USA Scientific	1402-4700
RNaseZap	Thermo Fisher Scientific	AM9780

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