User Manual

Version 1.4 – UMWF4100

PARSE BIOSCIENCES
A QIAGEN company

# Evercode™ WT FFPE Mini

For use with

ECWF4100

Support Suite



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# Overview

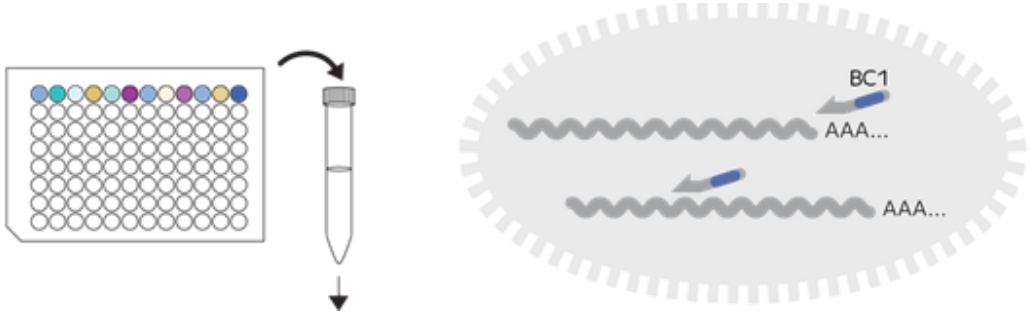
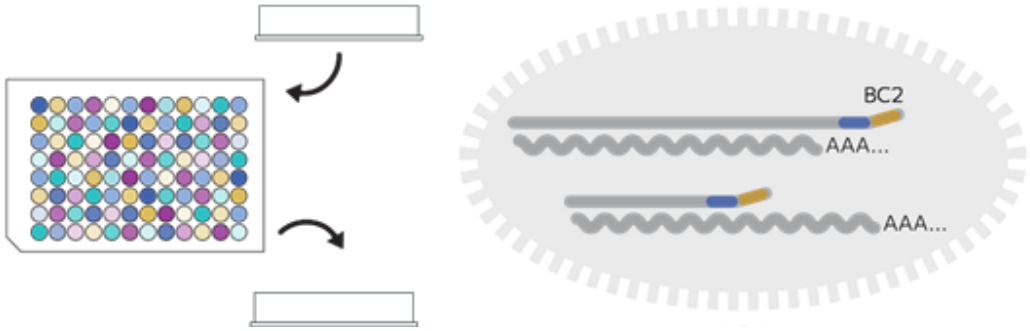
## Workflow

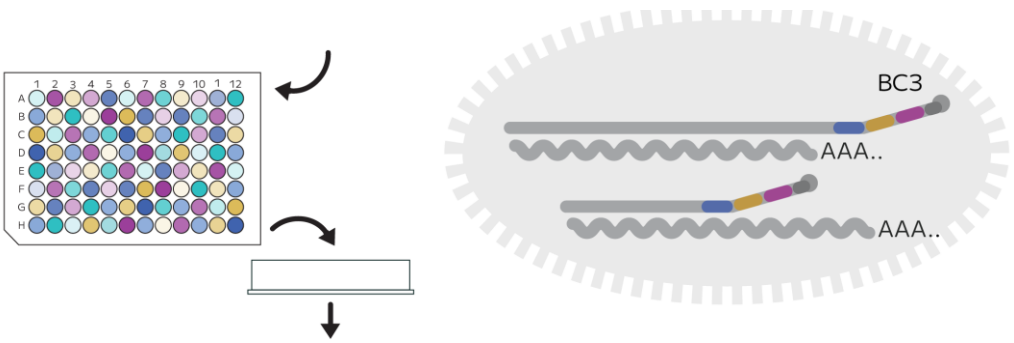
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT FFPE Mini kit can profile up to 10,000 nuclei across up to 12 different biological samples or experimental conditions.

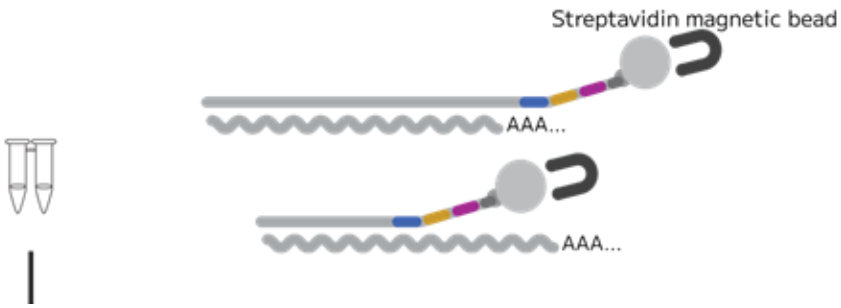
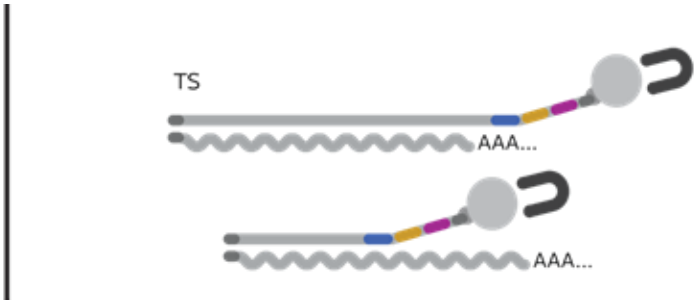
Dissociated nuclei act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed nuclei is uniquely labeled. The four rounds of barcoding can yield a very large number of possible barcode combinations which is more than sufficient to uniquely label up to 10,000 nuclei while avoiding doublets.

After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same 4 barcode combination to a single nuclei.

The tables below provide a high-level overview of the Evercode WT FFPE barcoding workflow. These steps are completed after the Pre-Barcoding sections which are not depicted here.

| Section 1: In situ Nuclei Barcoding | |
|---|---|
| 1 | <br>**Round 1 Barcoding:** Fixed and permeabilized nuclei are loaded into 12 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with a well-specific barcode that are associated with specific samples. |
| 2 |  |

| Section 1: In situ Nuclei Barcoding | |
|---|---|
| | **Round 2 Barcoding:** The nuclei are pooled and loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode. |
| 3 | <br>**Round 3 Barcoding:** The nuclei are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina TruSeq Read 2 sequence, and biotin. |
| 4 | <br>**Lysis and Sublibrary Generation:** Nuclei are split across 2 sublibraries and lysed. |

| Section 2: cDNA Capture and Amplification | |
|---|---|
| 1 | <br>**cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads. |
| 2 | <br>**cDNA Template Switch:** A template switch reaction adds an adapter to the 3′ end of the cDNA. |
| 3 | <br>**cDNA Amplification:** The cDNA is amplified with primers specific to the template switch adapter and Illumina TruSeq Read 2. |

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

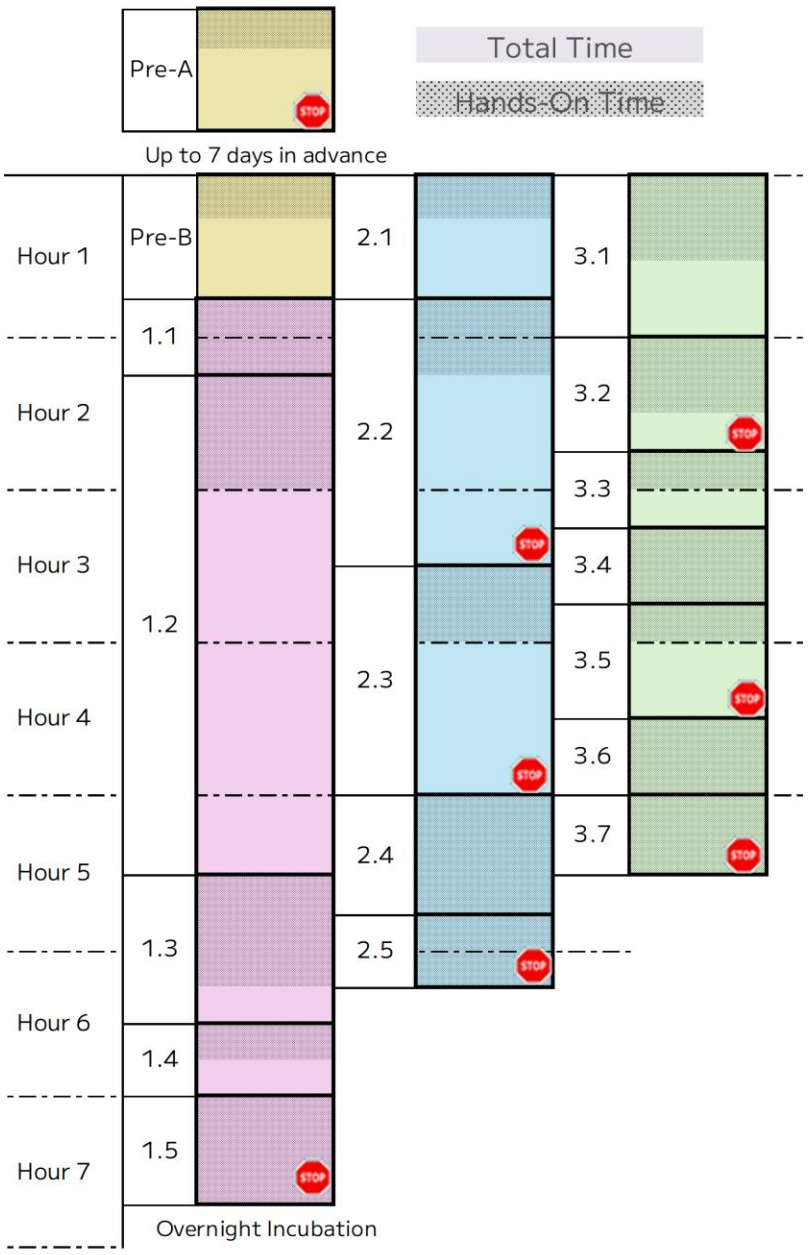
## Protocol Timing

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

| DESCRIPTION | TIME | HANDS-ON TIME | STOPPING POINTS |
|---|---|---|---|
| Pre-Barcoding Part A: Retention Enhancer Coating | 35 min | 5 min | 4°C ≤ 7 days |
| Pre-Barcoding Part B: FFPE Sample Preparation | 45 min | 15 min | |
| Section 1: In Situ Nuclei Barcoding | | | |
| 1.1 Set up and Sample Counting | Variable (30-90 min) | Variable (30-90 min) | |
| 1.2 Barcoding Round 1 | 190 min | 45 min | |
| 1.3 Barcoding Round 2 | 60 min | 45 min | |
| 1.4 Barcoding Round 3 | 30 min | 15 min | |
| 1.5 Lysis and Sublibrary Generation | 16 hours | 45 min | -80°C ≤ 6 months |
| Section 2: cDNA Capture and Amplification | | | |
| 2.1 cDNA Capture | 60 min | 30 min | |
| 2.2 cDNA Template Switch | 120 min | 30 min | 4°C ≤ 18 hrs |
| 2.3 cDNA Amplification | 90 min | 15 min | 4°C ≤ 18 hrs in the thermocycler |
| 2.4 Post-Amplification Purification | 30 min | 30 min | |
| 2.5 cDNA Quantification | 30 min | 30 min | 4°C ≤ 48 hrs or -20°C ≤ 3 months |
| Section 3: Sequencing Library Preparation | | | |
| 3.1 Fragmentation and End Prep | 65 min | 30 min | |
| 3.2 Post-Fragmentation and End Prep Size Selection | 30 min | 30 min | 4°C ≤ 18 hrs or -20°C ≤ 2 weeks |
| 3.3 Adapter Ligation | 30 min | 15 min | |
| 3.4 Post-Ligation Purification | 30 min | 30 min | |
| 3.5 Barcoding Round 4 | 45 min | 15 min | 4°C ≤ 18 hrs in the thermocycler |

| DESCRIPTION | TIME | HANDS-ON TIME | STOPPING POINTS |
|---|---|---|---|
| 3.6 Post-Barcoding Round 4 Size Selection | 30 min | 30 min | |
| 3.7 Sequencing Library Quantification | 30 min | 30 min | -20°C ≤ 3 months |

Workflow



# Important Guidelines

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

## Sample Input

- This protocol begins with nuclei that have been dissociated from FFPE scrolls and stored in Evercode Nuclei Storage Master Mix.
- Stored Reactions per sample should be thawed at 37°C before beginning Pre-Barcoding Part B: FFPE Sample Preparation. Input the counts of previously dissociated nuclei into the FFPE Sample Prep Planning loading table to ensure an adequate number of nuclei for barcoding.
- Once the FFPE Sample Preparation is complete, count nuclei with a fluorescence based cell counter. These counts are used to determine how nuclei are loaded in the Round 1 Plate, and their accuracy is critical to recover the desired number of nuclei.
- Once dissociated samples have been thawed, they should not be refrozen.

## RNA Integrity

- We recommend performing an RNA quality assessment to determine the DV200 value, defined as the percentage of RNA fragments longer than 200 nucleotides, prior to running the Evercode WT FFPE assay. In general, higher DV200 values are associated with improved data quality. For optimal performance, we recommend using tissue blocks with a DV200 of at least 30%.

## Nuclei Counting and Quality Assessment

- We recommend a fluorescence based counting device.
- When first using Evercode WT FFPE kits, we suggest saving images at each counting step.
- Examples of dissociated nuclei are shown below. High quality samples have single distinct nuclei with <5% aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying samples, it is critical to avoid counting debris to avoid overestimating the number of nuclei.

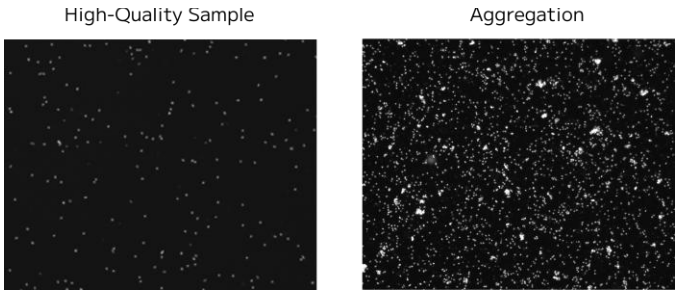



Figure 1: Example of AO fluorescent stained fixed nuclei

## Avoiding RNase Contamination

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

## Centrifugation

- For the centrifugation steps in this protocol we recommend 300 x g for 10 minutes. Note that some sample types may need further optimization of centrifugation speeds.
- Use a swinging bucket rotor for all high-speed centrifugation steps in this protocol. A fixed-angle rotor will lead to substantial nuclei loss.

## Optimizing Nuclei Recovery

- It is critical to thoroughly resuspend the nuclei after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to nuclei adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize nuclei loss.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend nuclei pellets adequately.
- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.

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

### Sample Loading Table

- The Parse Biosciences Evercode WT FFPE Mini Sample Loading Table RevA should be completed after completing FFPE pre-barcoding part B and before section 1.
- If the table isn't working as expected, ensure that Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in the table to avoid disturbing any calculations.
- In the unlikely event that samples are not concentrated enough according to the Sample Loading Table, choose to either:
    - Decrease the "Max number barcoded nuclei" until the Sample Loading Table no longer gives an error, which will maintain the desired proportion between samples but barcode fewer nuclei.
    - Add 14 µL of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded nuclei but change the desired proportion between samples.

### Cell Strainers

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

### Vortexing

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

### Plate Sealing

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

## Magnetic Racks and Bead Cleanups

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

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

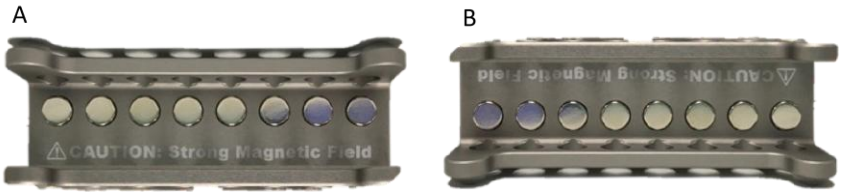



Figure 2: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.

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



Figure 3: Clear supernatant with compact bead pellets.

## Sublibrary Loading

- The Evercode WT FFPE Mini kit generates 2 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.

- The number of nuclei per sublibrary is determined when the nuclei are divided into sublibraries in Section 1.5 according to the guidelines in Appendix A.

- Due to the challenging nature of FFPE samples and variability in recovered nuclei quality, we recommend increasing sublibrary loading by up to 25% (see FFPE sublibrary loading

table), with a maximum of 6,250 nuclei per sublibrary, to help ensure sufficient data is retained after computational data filtering.

- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.
- Sublibraries can be loaded with different numbers of nuclei and the maximum number of nuclei that can be analyzed is the sum of nuclei across all sublibraries.

### Indexing Primers

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

### Thermocycling Programs

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

## Part List

The Evercode WT FFPE Mini kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

**Note:** Pre-Barcoding Part B uses FFPE Enhancement Buffer and FFPE Resuspension Buffer that are components of the FFPE Nuclei Preparation Kit. Please refer to ECNP4300 (12-reactions) or ECNP4500 (96-reactions) for the complete part list.

**-20°C Reagents** Store -20°C, PN FMN400

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
| | FFPE Round 1 Plate | MN301 | Green semi-skirted 96 well plate | 1 |
| | Round 2 Plate v4 | MN142 | Blue semi-skirted 96 well plate | 1 |
| | Round 3 Plate | MN103 | Yellow semi-skirted 96 well plate | 1 |
| Resuspen Buf | Resuspension Buffer | MN104 | 5 mL tube | 1 |
| Sample Dil | Sample Dilution Buffer | MN105 | 2 mL tube | 1 |
| R2 Lig Buf | Round 2 Ligation Buffer | MN106 | 5 mL tube | 1 |
| Lig Enzy | Ligation Enzyme | MN140 | 1.5 mL tube | 1 |
| R2 Stop | Round 2 Stop Buffer | MN108 | 2 mL tube | 1 |
| R3 Stop | Round 3 Stop Buffer | MN109 | 5 mL tube | 1 |
| Pre-Lysis Wash | Pre-Lysis Wash Buffer | MN110 | 5 mL tube | 1 |

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|   | FFPE Pre-Lysis Dilution Buffer | MN151 | 2 mL tube | 1 |
|   | Lysis Enzyme | MN113 | 1.5 mL tube | 1 |
|   | FFPE Lysis Buffer | MN152 | 1.5 mL tube | 1 |
|   | Bead Wash Buffer | MN114 | 1.5 mL tube | 1 |
|   | Wash Buffer 1 | MN115 | 1.5 mL tube | 1 |
|   | Wash Buffer 2 | MN116 | 1.5 mL tube | 1 |
|   | Capture Enhancer | MN117 | 1.5 mL tube | 1 |
|   | Binding Buffer | MN118 | 1.5 mL tube | 1 |
|   | Wash Buffer 3 | MN119 | 1.5 mL tube | 1 |
|   | Template Switch Buffer | MN120 | 1.5 mL tube | 1 |
|   | Template Switch Enzyme | MN121 | 1.5 mL tube | 1 |
|   | Template Switch Primer | MN122 | 1.5 mL tube | 1 |
|   | cDNA Amp Mix | MN123 | 1.5 mL tube | 1 |
|   | cDNA Amp Primers | MN124 | 1.5 mL tube | 1 |
|   | Fragm/End Prep Buffer | MN125 | 1.5 mL tube | 1 |

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|   | Fragm/End Prep Enzymes | MN126 | 1.5 mL tube | 1 |
|   | Ligation Adapter | MN127 | 1.5 mL tube | 1 |
|   | Adapter Ligation Buffer | MN128 | 1.5 mL tube | 1 |
|   | Adapter Ligation Enzyme | MN129 | 1.5 mL tube | 1 |
|   | Library Amp Mix | MN130 | 1.5 mL tube | 1 |
|   | PCR Additive | MN150 | 1.5 mL tube | 1 |

**4°C Reagents.** Store 4°C, PN FMN500

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|   | Spin Additive | MN201 | 1.5 mL tube | 1 |
|   | Streptavidin Beads | MN203 | 1.5 mL tube | 1 |
|   | Retention Enhancer | MN147 | 8 mL tube | 1 |

The Evercode WT FFPE Mini kit requires the following item to be purchased separately. Store at -20°C.

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| UDI Plate - WT | Parse Biosciences | UDI1001 | Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode kits (48 sublibraries). UDI Plate - WT can be purchased separately or bundled with Evercode kits. |

## User Supplied Equipment and Reagents

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

**Equipment**

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| Parse Biosciences Magnetic Rack | Parse Biosciences | SB1004 | We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection. |
| Centrifuge with Swinging Bucket Rotors | Various Suppliers | Varies | Compatible with 15 mL centrifuge tubes and 96 well plates and capable of reaching 4°C. |
| Microcentrifuge | Various Suppliers | Varies | Compatible with 1.5 mL and 0.2 mL tubes. |
| Water bath | Various Suppliers | Varies | Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C. |
| Two heat blocks | Various Suppliers | Varies | Or equivalent water bath, bead bath, or thermomixer capable of holding temperatures from 48°C to 68°C and compatible with 1.5 mL, 2 mL, and 5 mL tubes. |
| Fluorescence Cell Counter | Various Suppliers | Varies | |
| PCR plate adaptor | Various Suppliers | Varies | Capable of holding semi-skirted 96 well PCR plates and a tight fitting lid. |
| 96-well plate adapter | Various Suppliers | Varies | |
| Plate Seal Applicator | Various Suppliers | Varies | Capable of adhering plate sealing films to 96 well plates. |
| Single Channel Pipettes: P20, P200, P1000. 12-channel: P20, P200 | Various Suppliers | Varies | Or 8-channel pipettes can be substituted for 12-channel pipettes. |
| T100 Thermal Cycler | Bio-Rad Laboratories® | 1861096 | Or an equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C. |

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

**Consumables**

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| Reagent basins | Various Suppliers | Varies | Sterile, nuclease-free, 10 mL or 25 mL polypropylene reagent basins. |
| SWiSH™ Mini Cell Strainer | Stellar Scientific® | TC70-SWM-20<br>TC70-SWM-40 | Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm).<br>We do not recommend FlowMi Cell Strainers (SP Bel-Art). |
| pluriStrainer® Mini | pluriSelect® | 43-10020-40<br>43-10040-40 | |
| Falcon®Cell Strainer | Corning® | 431750 | |
| EASYstrainer™, small | Greiner Bio-One™ | 542120<br>542140 | |
| 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 nuclei loss. |
| DNA LoBind® Tubes, 1.5 mL, Snap Cap | Eppendorf® | 022431021 | Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes. |
| TempAssure® PCR 8-Tube Strips, 0.2 mL | USA Scientific® | 1402-4700 | Or equivalent nuclease-free 0.2 mL PCR tubes. |
| SealPlate® | Excel Scientific | 100-SEAL-PLT | Or equivalent PCR plate seals. |

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL | Rainin® | 17014961<br>17014963<br>17014967 | Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips. |

**Reagents**

| ITEM | SUPPLIER | PN | NOTES |
|---|---|---|---|
| AMPure® XP Reagent | Beckman Coulter® | A63880 (5 mL)<br>A63881 (60mL) | Choose one. We do not recommend substituting other magnetic beads. |
| SPRIselect Reagent | Beckman Coulter | B23317 (5mL)<br>B23318 (60mL) | |
| KAPA® Pure Beads | Roche® | KK8000 (5 mL)<br>KK8001 (30mL) | |
| 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. |
| AO/PI | Various Suppliers | Varies | Or alternative dyes to assess viability. |
| Qubit dsDNA HS (High Sensitivity) Assay Kit | Thermo Fisher Scientific | Q33230 (100 assays)<br>Q33231 (500 assays) | Or equivalent DNA quantifier. |
| High Sensitivity DNA Kit | Agilent | 5067-4626 | Choose one that corresponds to the chosen Bioanalyzer or TapeStation. |
| High Sensitivity D5000 ScreenTape and Reagents | Agilent | 5067-5592 (screen tape)<br>5067-5593 (sample buffer and ladder) | |

# Pre-Barcoding Part A: Retention Enhancer Coating

Coating tubes with Retention Enhancer increases nuclei retention and is required for advance preparation, so this step is included in the pre-barcoding section.
Tubes can be prepared up to one week in advance and stored at 4 °C.

**To coat tubes:**

1. Prepare a fresh Retention Enhancer Solution as follows:

| RETENTION ENHANCER SOLUTION | |
|---|---|
| O Retention Enhancer | 2 mL |
| Nuclease-free water | 18 mL |

2. Fill two 15 mL tubes with 10 mL of O Retention Enhancer Solution and cap the tubes.
3. Invert once to fully coat the tubes.
4. Incubate the tubes for **30 minutes** in a water bath at 37°C.
5. Discard the Retention Enhancer Solution by decanting the liquid. Remove any remaining solution from the bottom of the tube with a P200.
6. With the caps removed, air dry the tubes for **30 minutes** in a biosafety cabinet at room temperature.

**Note:** If additional liquid accumulates at the bottom of the tube after air drying, carefully discard the liquid.

7. Proceed to Section 1.1, or store capped Retention Enhancer-coated tubes at 4°C, protected from light, for up to **7 days.**

# Pre-Barcoding Part B: FFPE Sample Preparation

The nuclei previously dissociated from FFPE tissue blocks and stored in 0.2 mL PCR strip tube(s) are thawed and treated for Evercode barcoding.

1. Open the "Evercode WT FFPE Sample Loading Table RevA" and completely fill out the "FFPE Sample Prep Planning" tab. Use the "Stored Reactions required for Pre-barcoding part B: sample preparation" (Column D) to determine the minimum number of Stored Reactions per sample to thaw in Part B, step 7.

2. Cool a centrifuge with swinging bucket rotors to 4°C.

3. Fill a bucket with ice.

4. Set a water bath to 37°C.

5. Start the following program on thermocycler.

| FFPE Enhancement | | |
|---|---|---|
| Run Time | 5 minutes | |
| Lid Temperature | 105°C | |
| Sample Volume | 100 µL | |
| Step | Time | Temperature |
| 1 | Hold* | 80°C* |
| 2 | 5 min | 80°C |
| 3 | Hold | 4°C |

**Note:** * Hold the temperature until ready to start the thermocycler. When the samples are loaded and ready to incubate, press "skip" to start the program from step 2.

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ●/○ FFPE Enhancement Buffer | -20°C Reagents* | 1 | Thaw at 37°C and keep at 37°C for 5 minutes, vortex, then place on ice. |
| ○ FFPE Resuspension Buffer | -20°C Reagents* | 1 | Thaw at 37°C, then place on ice. |

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ● Sample Dilution Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by inverting 3x |

**Note: *** ●/○ FFPE Enhancement Buffer and ○ FFPE Resuspension Buffer are components of the FFPE Nuclei Preparation Kit.

The FFPE Nuclei Preparation Kit is available in two sizes:
The 12-Reactions kit (ECNP4300) includes an 8 mL tube of ○ FFPE Resuspension Buffer and a 2 mL tube of ● FFPE Enhancement Buffer.

The 96-Reactions kit (ECNP4500) includes a 15 mL tube of ○ FFPE Resuspension Buffer and a 15 mL tube of ○ FFPE Enhancement Buffer.

7. Thaw the PCR strip tube(s) containing the dissociated nuclei in the water bath or thermocycler set to 37°C for **3 minutes** or until ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.

**CRITICAL!** If counts from the dissociation were not recorded, count using a fluorescent counter, and input the count into FFPE Sample Prep Planning (column D).

8. Switching tips between each tube, add **100 µL** of ○ FFPE Resuspension Buffer to each tube and mix with the P200 pipette set to 180 µL.

9. Cap the PCR strip tubes and place them in a 96-well plate adapter.

10. Centrifuge the tube(s) in the PCR plate adapter in a swinging bucket rotor for **10 minutes** at 300 x g at 4°C. Immediately move to the next step after centrifugation.

11. When the spin is complete, remove and discard the supernatant until ~20 µL remain above the pellet, using a new tip for each tube.

12. Switching tips between tubes, resuspend the nuclei with **100 µL** of ●/○ FFPE Enhancement Buffer and mix the nuclei until fully resuspended.

13. Cap the PCR strip tubes and place them into the pre-heated thermocycler. Press skip to move to step 2 and start the **5 minutes** incubation.

14. Once the program is complete place the samples on ice. Switching tips between tubes, add **100 µL** of ○ FFPE Resuspension Buffer to each tube, mixing 2x with a P200 pipette set to 180 µL.

15. Centrifuge the tube(s) in the PCR plate adapter in a swinging bucket rotor for **10 minutes** at 300 x g at 4°C. Immediately move to the next step after centrifugation.

16. When the spin is complete, remove and discard the supernatant until ~20 µL remain above the pellet.

17. Resuspend the samples in ● Sample Dilution Buffer using the per Stored Reactions volumes specified in the FFPE Sample Prep Planning table. For samples split across multiple Stored Reactions, pool into a single tube per sample after resuspending in ● Sample Dilution Buffer.

18. Proceed immediately to Section 1.1.

# Section 1: In Situ Nuclei Barcoding

## 1.1. Set up and Sample Counting

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

**To set up for barcoding:**

1. Open the "Evercode WT FFPE Mini Sample Loading Table RevA", which will guide sample dilutions and plate loading in later steps.
2. Prepare a fluorescence counter, or other cell counting device.
3. Gather the following items and handle as indicated below.

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| FFPE Round 1 Plate | -20°C Reagents | 1 | Place directly on ice. |
| Round 2 Plate v4 | -20°C Reagents | 1 | |
| Resuspension Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by inverting 3x. |
| Sample Dilution Buffer | -20°C Reagents | 1 | |
| Round 2 Ligation Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice until use in section 1.3. Mix by inverting 3x before use. |
| Spin Additive | 4°C Reagents | 1 | Keep at room temperature. |

4. While minimizing time on ice, count the number of nuclei in each sample with a fluorescent based counting device. Record the nuclei count.

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

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

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

7. Proceed immediately to Section 1.2.

## 1.2. Barcoding Round 1

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

**To add round 1 barcodes:**

1. Gently remove the Round 1 Plate from the thermocycler, place in a 0.2 mL tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.

2. Remove the Round 1 Plate from the centrifuge, place in a PCR tube rack, remove the plate seal, and store on ice.

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

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

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

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

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

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

| FFPE BARCODING ROUND 1 | | | |
|---|---|---|---|
| Run Time | 105 minutes | | |
| Lid Temperature | 70°C | | |
| Sample Volume | 40 µL | | |
| Step | Time | Temperature | Cycles |
| 1 | 10 min | 50°C | 1 |
| 2 | 12 s | 8°C | 12 |
| 3 | 30 s | 15°C | |
| 4 | 45 s | 20°C | |
| 5 | 1 min | 25°C | |
| 6 | 1 min | 30°C | |
| 7 | 2 min | 42°C | |
| 8 | 30 min | 42°C | 1 |
| 9 | Hold | 4°C | 1 |

6. Remove the Round 1 Plate from the thermocycler, place in a PCR tube rack, and store on ice.

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

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

8. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 1 Plate.

9. With the plate and tube on ice, pool all wells from the Round 1 plate into a retention enhancer coated 15 mL centrifuge tube. With a P200 set to 30 µL, mix the sample in row A by pipetting 3x then transfer to a 15 mL conical tube.

**Note:** Using a P20 pipette set to 10 µL, transfer any residual liquid left in the 15 mL tube.

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

11. Mix by gently inverting the tube just once.

12. Centrifuge the 15 mL tube in a swinging bucket rotor for **10 minutes** at 300 x g at 4°C. Immediately move to the next step after centrifugation.

**CRITICAL!** Centrifugation speed and duration can be adjusted to meet the specific samples' optimal retention and resuspension efficiencies. Overly aggressive centrifugation can damage nuclei integrity and decrease data quality.

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

13. Remove the supernatant until ~40 µL of liquid remains above the pellet. Use a P1000 for the first 1 mL and then a P200 for the remaining volume.

**Note:** Depending on the number of nuclei types, a pellet may or may not be visible.

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

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

16. Proceed immediately to Section 1.3.

## 1.3. Barcoding Round 2

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

**To add round 2 barcodes:**

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ● Ligation Enzyme | -20°C Reagents | 1 | Place directly on ice. Briefly centrifuge before use. |
| ● Round 2 Stop Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by vortexing. |
| ▭ Round 3 Plate | -20°C Reagents | 1 | Place directly on ice. |

2. On ice, prepare the Round 2 Ligation Master Mix by adding the following to the samples in ○ Resuspension Buffer prepared in Section 1.2. Mix thoroughly by pipetting 10x with a P1000 set to 1000 µL. Store on ice.

**Note:** Prior to use, mix by inverting 3x the Round 2 Ligation Buffer that was gathered and stored on ice in Section 1.1.

| ROUND 2 LIGATION MASTER MIX | |
|---|---|
| Sample in Resuspension Buffer | 2 mL |
| ○ Round 2 Ligation Buffer | 1.95 mL |
| ● Ligation Enzyme | 20 µL |
| Total Volume | 3.97 mL |

**CRITICAL!** Return the ● Ligation Enzyme on ice. Do **NOT** discard it as it will be used in Round 3.

3. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.

4. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate and store on ice.

5. Transfer the Round 2 Ligation Master Mix to a basin with a P1000.

6. With the Round 2 Plate on ice and the basin on the bench, transfer Round 2 Ligation Master Mix to each well in the Round 2 Plate as follows:

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

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

    iii. Repeat i-ii to mix the sample in the basin then transfer to rows B-H.

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

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

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

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

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

9. Briefly vortex the ● Round 2 Stop Buffer and ensure there is no precipitate. Transfer the entire volume of this tube to a new basin with a P1000.

10. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.

11. With the Round 2 Plate on ice and the basin on the bench, transfer **10 µL** of the ● Round 2 Stop Buffer to each well in the Round 2 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.

**CRITICAL!** Do not reuse any tips during this step. Different tips must be used when pipetting ● Round 2 Stop Buffer into each well of the plate. Never place a tip that has entered one of the wells back into the basin or a different well.

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

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

| ROUND 2 STOP | | |
|---|---|---|
| Run Time | 5 minutes | |
| Lid Temperature | 50°C | |
| Sample Volume | 60 µL | |
| Step | Time | Temperature |
| 1 | 5 min | 16°C |
| 2 | Hold | 4°C |

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

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

**Note:** If a second thermocycler is not available, the same thermocycler used in step 13 can subsequently be used in step 14. However, the Round 2 Plate should be stored on ice until the Thaw Round 3 Plate program is complete.

15. Immediately upon completion of the Round 2 Stop program, transfer the Round 2 Plate from the thermocycler to a PCR tube rack, remove the plate seal, and store on ice.

16. With the Round 2 Plate on ice and the basin on the bench, transfer all the liquid in the Round 2 Plate into a new basin as follows:

    i. With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.

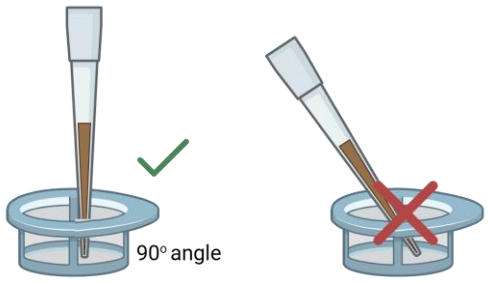
    ii. Transfer **50 µL** from row A to the basin.

    iii. Repeat i-ii for rows B-H to mix the sample then transfer to the basin.

    iv. Transfer any residual liquid in the Round 2 Plate to the basin with a multichannel P20 set to 10 µL.

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

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




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

18. Proceed immediately to Section 1.4.

## 1.4. Barcoding Round 3

The Ligation Enzyme is added to the pooled nuclei, which are then loaded into the Round 3 Plate. A second *in situ* ligation reaction adds a third well-specific barcode, the Illumina TruSeq Read 2 sequence, and a biotin. The sample is then pooled and strained.

**To add round 3 barcodes:**

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ○ Round 3 Stop Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by vortexing. |
| ○ Pre-Lysis Wash Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by inverting 3x. |
| ● FFPE Pre-Lysis Dilution Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by inverting 3x. |
| ● Lysis Enzyme | -20°C Reagents | 1 | Place directly on ice. Briefly centrifuge before use. |
| ● FFPE Lysis Buffer | -20°C Reagents | 1 | Place in a 37°C water bath until use. |

2. Add **20 µL** of ● Ligation Enzyme from the previous section to the basin containing the strained sample. Mix by gently pipetting 20x with a P1000 set to 1000 µL.

3. Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.

4. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 3 Plate.

5. With the Round 3 Plate on ice and the basin on the bench, transfer **50 µL** from the basin to each well in the Round 3 Plate as follows:

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

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

    iii. Repeat i-ii to mix the sample in the basin then transfer to rows B-H.

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

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

6. While secured in a PCR tube rack on a flat surface, add a new plate seal to Round 3 Plate.

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

| BARCODING ROUND 3 | | |
|---|---|---|
| Run Time | 15 minutes | |
| Lid Temperature | 50°C | |
| Sample Volume | 60 µL | |
| Step | Time | Temperature |
| 1 | 15 min | 16°C |
| 2 | Hold | 4°C |

8. Briefly vortex the O Round 3 Stop Buffer and ensure there is no precipitate. Transfer the entire volume to a new basin with a P1000.

9. Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.

10. With the Round 3 Plate on ice and the basin on the bench, transfer **20 µL** of the O Round 3 Stop Buffer from the basin to each well in the Round 3 Plate with a multichannel P20. After each transfer, mix by pipetting exactly 3x.

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

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

12. With the Round 3 Plate on ice and the basin on the bench, transfer all the liquid in the Round 3 Plate into a new basin as follows:

i. With a multichannel P200 set to 70 µL, mix the sample in row A by pipetting 3x.

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

iii. Repeat i-ii for rows B-H to mix the sample then transfer to the basin.

iv. Transfer any residual liquid in the Round 3 Plate to the basin with a multichannel P20 pipette set to 10 µL.

13. Pipette the sample through a cell strainer into a new retention enhancer-coated 15 mL tube with a P1000. Before each transfer, gently mix the nuclei in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.



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

14. Proceed immediately to Section 1.5.

## 1.5. Lysis and Sublibrary Generation

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

**To generate and lyse sublibraries:**

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

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



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

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



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

4. Fully but gently resuspend the pellet in **1 mL** of ○ Pre-Lysis Wash Buffer.

5. Add an additional **3 mL** of ○ Pre-Lysis Wash Buffer for a total addition of 4 mL.

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



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

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

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



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

9. While minimizing time on ice, count the number of nuclei in the sample with a fluorescence based counting device. Record the nuclei count.

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

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

**CRITICAL!** Due to the challenging nature of FFPE samples and the variable quality of recovered nuclei, we recommend increasing sublibrary loading by up to 25% to help ensure sufficient data is retained after computational data filtering. Do not add more than 6,250 nuclei to a sublibrary. Adding additional nuclei will result in an increased multiplet rate.

11. Ensure the nuclei are in suspension by pipetting 5x with a P200 set to 50 µL prior to each transfer. Add the appropriate volume of sample to 2 different 0.2 mL PCR tubes.

12. Add the appropriate volume of ● FFPE Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of 25 µL.

13. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P200 set to 55 µL. Store at room temperature.

| LYSIS MASTER MIX | |
|---|---|
| ● FFPE Lysis Buffer | 55 µL |
| ● Lysis Enzyme | 11 µL |
| Total Volume | 66 µL |

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

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

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

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

16. Place the tube(s) into a thermocycler and run the following overnight incubation program.

**Note:** The nuclei lysis incubation time is 15 hours. After incubation, the sublibrary lysates can be stored at 4°C in the thermocycler for up to 12 hours before freezing at –80°C.

| FFPE NUCLEI LYSIS | | |
|---|---|---|
| Run Time | 15 hours | |
| Lid Temperature | 80°C | |
| Sample Volume | 55 µL | |
| Step | Time | Temperature |
| 1 | 15 hours | 37°C |
| 2 | Hold | 4°C |

**CRITICAL!** Freeze the lysate(s) at -80°C for at least **15 minutes** before proceeding to Section 2.

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

# Section 2: cDNA Capture and Amplification

## 2.1. cDNA Capture

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

**To capture the cDNA:**

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| Streptavidin Beads | 4°C Reagents | 1 | Keep at room temperature. |
| Bead Wash Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by inverting 3x. |
| Wash Buffer 1 | -20°C Reagents | 1 | |
| Wash Buffer 2 | -20°C Reagents | 1 | |
| Capture Enhancer | -20°C Reagents | 1 | Place directly on ice. Briefly centrifuge before use. |
| Binding Buffer | -20°C Reagents | 1 | Thaw at room temperature then store on ice. Mix by inverting 3x. |

4. Remove the desired tube(s) of lysate from storage at -80°C.
5. Incubate the tube(s) in water bath or thermocycler at 37°C for **5 minutes**.

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

6. Briefly centrifuge and store at room temperature.

7. Briefly centrifuge ● Capture Enhancer and gently mix by pipetting 2x with a P20 set to 15 µL.

8. Add **2.5 µL** of ● Capture Enhancer to each tube of lysate and mix by pipetting 5x with a P200 set to 40 µL. Briefly centrifuge.

9. Incubate for **10 minutes** at room temperature. Proceed immediately to the next step during the incubation.



**Note:** This incubation can be extended by 5 additional minutes (up to a total of 15 minutes) without negatively impacting performance.

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

| Lysates being processed | 1 | 2 |
|---|---|---|
| ● Streptavidin Beads | 44 µL | 88 µL |

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

12. Remove and discard the supernatant.

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

| Lysates being processed | 1 | 2 |
|---|---|---|
| ○ Bead Wash Buffer | 50 µL | 100 µL |



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

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

15. Remove and discard the supernatant.

16. Repeat steps 13-15 twice for a total of 3 washes.

17. Remove the tube from the magnetic rack. Fully resuspend the pellet in the appropriate volume of ● Binding Buffer as follows and store at room temperature.

| Lysates being processed | 1 | 2 |
|---|---|---|
| ● Binding Buffer | 55 µL | 110 µL |

18. Add **50 µL** of ● Streptavidin Beads in ● Binding Buffer to each tube of lysate and fully mix by pipetting 2x with a P200 set to 90 µL.

19. Place the tube(s) into a 96 well PCR tube rack, press to secure, and ensure the caps are secured tightly. Place the lid on the rack.

20. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex on 20% power (~800-1000 RPM) for **30 minutes** at room temperature. During this step, preheat a thermocycler to 70°C with the heated lid set to 80°C.

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

21. Remove the tube(s) from the vortex mixer.

22. Briefly vortex the tube(s) on a standard vortex adapter. Briefly centrifuge without letting beads collect at the bottom of the tube(s).

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

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

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

25. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ○ Wash Buffer 1.

26. Place the tube(s) in the thermocycler preheated to 70°C in step 2.1.20, close the lid, and incubate for **5 minutes**.

**CRITICAL! Do not** preheat the Wash Buffer 1. Wash Buffer 1 should remain at **room temperature** prior to addition to the sample.

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

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

29. Repeat steps 25-28 once for a total of 2 washes with O Wash Buffer 1.

30. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** O Wash Buffer 2.



**Note:** Save O Wash Buffer 2 to use for optional storage before cDNA amplification.

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

32. Proceed immediately to Section 2.2.

## 2.2. cDNA Template Switch

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

**To perform template switch:**

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ○ Wash Buffer 3 | -20°C Reagents | 1 | Thaw and store at room temperature. Mix by inverting 3x. |
| ● Template Switch Buffer | -20°C Reagents | 1 | Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use. |
| ● Template Switch Primer | -20°C Reagents | 1 | |
| ● Template Switch Enzyme | -20°C Reagents | 1 | Keep on ice. Briefly centrifuge before use. |

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

2. Mix by pipetting 10x and store on ice.

| TEMPLATE SWITCH MASTER MIX | | |
|---|---|---|
| Number of Samples | 1 | 2 |
| ● Template Switch Buffer | 101.75 µL | 203.5 µL |
| ● Template Switch Primer | 2.75 µL | 5.5 µL |
| ● Template Switch Enzyme | 5.5 µL | 11 µL |
| Total | 110 µL | 220 µL |

3. Place each tube of captured cDNA from Section 2.1 on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
4. While still on the magnetic rack, remove and discard the supernatant.
5. While still on the magnetic rack, add **125 µL** of ○ Wash Buffer 3 to each tube.

**CRITICAL!** Do not discard the ○ Wash Buffer 3 as it will be used in another step.

6. Incubate for **1 minute** at room temperature.
7. While still on the magnetic rack, remove and discard the ○ Wash Buffer 3.
8. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 µL** of the Template Switch Master Mix.

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

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

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

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

13. Proceed immediately to Section 2.3. Alternatively, proceed to step 14 to store samples prior to cDNA amplification.

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

**Note:** Beads may need to be resuspended if they have settled.

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

16. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** Wash Buffer 2.

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

## 2.3. cDNA Amplification

The captured cDNA is washed and amplified with Template Switch Primer and Illumina TruSeq Read 2 specific primers.

**To amplify the cDNA:**

1. Equilibrate **85 µL** of SPRI beads per lysate to room temperature. Set aside for future use.

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| cDNA Amp Mix | -20°C Reagents | 1 | Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use. |
| cDNA Amp Primers | -20°C Reagents | 1 | |
| PCR Additive | -20°C Reagents | 1 | |

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

| cDNA AMPLIFICATION MASTER MIX | | |
|---|---|---|
| Number of Sublibraries | 1 | 2 |
| cDNA Amp Mix | 60 µL | 120 µL |
| cDNA Amp Primers | 58.75 µL | 117.5 µL |
| PCR Additive | 1.25 µL | 2.5 µL |
| Total | 120 µL | 247.5 µL |

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

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

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

6. While still on the magnetic rack, add **125 µL** of O Wash Buffer 3 to each tube.

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

8. While still on the magnetic rack, remove and discard the **O** Wash Buffer 3.

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

10. Determine the number of PCR cycles required for cDNA amplification based on the table below.

| NUMBER OF PCR CYCLES | |
|---|---|
| Nuclei in Sublibrary | Cycles |
| 200-1,000 | 16 |
| 1,000-2,000 | 15 |
| 2,000-6,250 | 14 |

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

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

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

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

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

## 2.4. Post-Amplification Purification

Amplified cDNA is purified with a 0.9x SPRI bead cleanup.

**To purify the cDNA:**

1. For each tube of amplified cDNA, gather **400 µL** of freshly prepared 85% ethanol.
2. Gather the SPRI beads equilibrated and set aside in 2.3.1 (**85 µL** per tube of amplified cDNA).



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

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



**Note:** If beads remain in solution after 2-3 minutes, pipette 3x in the bottom of the PCR tube with a P200 set to 40 µL. Then return to the magnet and incubate until the solution clears.

4. While still on the magnetic rack, transfer **90 µL** of the supernatant containing the cDNA into a new 0.2 mL tube(s). Store at room temperature.
5. Vortex the SPRI beads until fully mixed. Add **81 µL** of SPRI beads to each tube with amplified cDNA.
6. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
7. Incubate for **5 minutes** at room temperature.
8. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
9. While still on the magnetic rack, remove and discard the supernatant.
10. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
11. Incubate for **1 minute** at room temperature.
12. While still on the magnetic rack, remove and discard the supernatant.
13. Repeat steps 10-12 once for a total of 2 washes. Remove any residual ethanol with a P20.

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

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

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

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

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

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

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

Safe stopping point: Amplified cDNA can be stored at 4°C for up to 48 hours or at -20°C for up to 3 months. Otherwise, proceed to Section 2.5 to quantify the cDNA before proceeding to Section 3.

## 2.5. cDNA Quantification

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

**To quantify the cDNA:**

1. Measure the concentration of each tube of purified cDNA from Section 2.4 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions. Record the concentration(s), which will be used in Section 3.

2. Assess the size distribution of each tube of purified cDNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D5000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

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

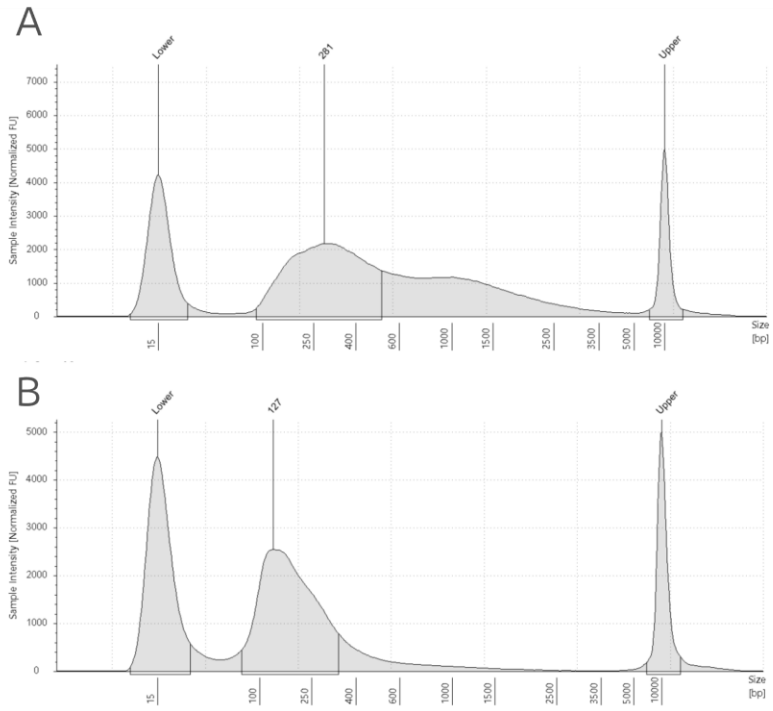



Figure 4: Expected post-amplification sublibrary cDNA size distribution. A: example of the trace of Human cDNA with expected size distribution run on a TapeStation. B: another example where the cDNA size distribution may shift toward lower sizes for FFPE samples and still produce high quality data.

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

**Note:** The traces above are representative of typical TapeStation cDNA traces. The shape and prominence of the trace is dependent on cell type, sublibrary size, and amount of DNA loaded into the TapeStation.

# Section 3: Sequencing Library Preparation

## 3.1. Fragmentation and End Prep

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

**To prepare for fragmentation and end prep:**

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

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

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

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

**Note:** If you have less than 100 ng of cDNA, add the entire amount of cDNA at this step. This will not affect the quality of your libraries. Successful libraries can be

prepared from as little as 10 ng of cDNA. Record the amount added to each tube as subsequent PCR cycles will have to be adjusted based on cDNA concentration.

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

| FFPE FRAGMENTATION AND END PREP | | |
|---|---|---|
| Run Time | 35 minutes | |
| Lid Temperature | 70°C | |
| Sample Volume | 50 µL | |
| Step | Time | Temperature |
| 1 | Hold* | 4°C |
| 2 | 5 min | 32°C |
| 3 | 30 min | 65°C |
| 4 | Hold | 4°C |

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

10. Vortex the ● Fragm/End Prep Buffer for **5 seconds**. Briefly centrifuge.

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

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

| FRAGMENTATION AND END PREP MASTER MIX | | |
|---|---|---|
| Number of Sublibraries | 1 | 2 |
| ● Fragm/End Prep Buffer | 5.5 µL | 11 µL |
| ● Fragm/End Prep Enzymes | 11 µL | 22 µL |
| Total | 16.5 µL | 33 µL |

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

13. Place the tube(s) into the cooled thermocycler. Skip the 4°C hold in Step 1 so the thermocycler proceeds to Step 2 of the Fragmentation and End Prep program.



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

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

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

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

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

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



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

3. Vortex the SPRI beads until fully mixed. Add **45 µL** of SPRI beads to each tube of fragmented and end prepped DNA.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).



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

13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **50 µL** of nuclease-free water.
14. Incubate for **5 minutes** at room temperature.
15. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).

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



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

## 3.3. Adapter Ligation

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

**To ligate adapters:**

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

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

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

| ADAPTER LIGATION MASTER MIX | | |
|---|---|---|
| Number of Sublibraries | 1 | 2 |
| Nuclease-free water | 19.25 µL | 38.5 µL |
| Adapter Ligation Buffer | 22 µL | 44 µL |
| Adapter Ligation Enzyme | 11 µL | 22 µL |
| Ligation Adapter | 2.75 µL | 5.5 µL |
| Total | 55 µL | 110 µL |

3. Add **50 µL** of Adapter Ligation Master Mix to each tube of purified fragmented and end prepped DNA from Section 3.2. Mix by pipetting 10x with a P200 multichannel pipette set to 80 µL. Briefly centrifuge.

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

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



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

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

## 3.4. Post-Ligation Purification

Adapter ligated DNA is purified with a 0.9x SPRI bead cleanup.

**To purify the ligated DNA:**

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~100 µL per sublibrary)

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

3. Vortex the SPRI beads until fully mixed. Add **90 µL** of SPRI beads to each tube of adapter ligated DNA from Section 3.3.
4. Vortex the tube(s) for **5 seconds.** Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~2 minutes).
13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **23 µL** of nuclease-free water.
14. Incubate for **5 minutes** at room temperature.
15. Place the tube(s) on the low magnet position of the magnetic rack, so the magnet is closer to the bottom of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

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

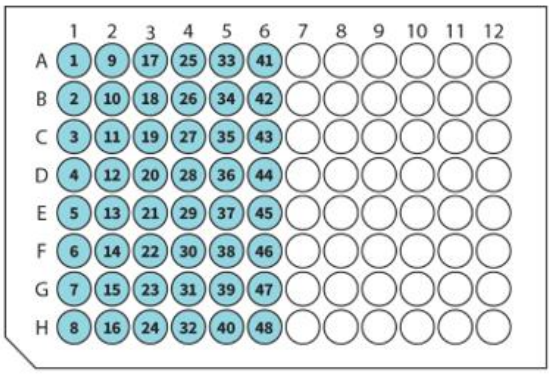
17. Proceed immediately to Section 3.5.

## 3.5. Barcoding Round 4

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

**To add round 4 barcodes:**

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




4. With a multichannel P20, pierce the seal of the chosen wells of the UDI Plate - WT.
5. With a multichannel P20 and new tips, mix by pipetting 5x then immediately transfer **4 µL** from a chosen unused well of the UDI Plate - WT to its corresponding tube of purified adapter ligated DNA from Section 3.4.

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

6. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C. Do not reuse wells.
7. Add **25 µL** of Library Amp Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 25 µL. Briefly centrifuge.

8. Determine the number of PCR cycles required for the Indexing PCR based on the amount of cDNA added to the fragmentation and end prep reaction as recorded in Section 2.5.

| NUMBER OF PCR CYCLES | |
|---|---|
| cDNA Input (ng) | PCR Cycles |
| 10-24 | 12 |
| 25-49 | 11 |
| 50-99 | 10 |

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

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

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

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

## 3.6. Post-Barcoding Round 4 Size Selection

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

**To size select the sequencing libraries:**

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

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

3. Vortex the SPRI beads until fully mixed. Add **45 µL** of SPRI beads to each sequencing library tube.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 µL** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).
13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **20 µL** of nuclease-free water.
14. Incubate for **5 minutes** at room temperature.
15. Place the tube(s) on the low position of the magnetic rack. Incubate until the solution clears (~2 minutes).
16. While still on the magnetic rack, transfer the supernatant into new 0.2 mL tube(s). Store on ice.

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

## 3.7. Sequencing Library Quantification

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

**To quantify the sequencing libraries:**

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

2. Assess the size distribution of each purified sequencing library with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D5000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

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

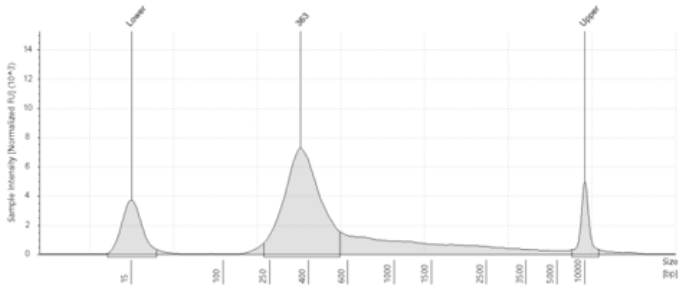


Figure 5: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

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

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

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

# Appendices

## Appendix A: Sublibrary Generation Table

The Sublibrary Generation Table provides guidance for sample dilution in Section 1.5 to achieve a target sublibrary concentration.
Green text (top): Volume of nuclei suspension in µL (from step 1.5.10) to add to each sublibrary.
Purple text (bottom): Volume of Pre-Lysis Dilution Buffer in µL to add to each sublibrary.
Blue Shading: Serial dilution of nuclei stock is required to improve sublibrary nuclei count.
Red Shading: Insufficient nuclei stock concentration for target sublibrary nuclei count.

**Note:** Due to the challenging nature of FFPE samples and the variable quality of recovered nuclei, we recommend increasing sublibrary loading by up to 25% to help ensure sufficient data is retained after computational data filtering.

| Stock Conc. (cells/uL) | Target Sublibrary Cell Count (cells/sublibrary) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 250 | 625 | 1,250 | 2,500 | 3,750 | 5,000 | 6,250 | 7,500 | 8,750 | 10,000 | 11,250 | 12,500 |
| 50 | 5 | 12.5 | | | | | | | | | | |
| | 20 | 12.5 | | | | | | | | | | |
| 100 | 2.5 | 6.3 | 12.5 | | | | | | | | | |
| | 22.5 | 18.8 | 12.5 | | | | | | | | | |
| 200 | 1.3 | 3.1 | 6.3 | 12.5 | 18.8 | | | | | | | |
| | 23.8 | 21.9 | 18.8 | 12.5 | 6.3 | | | | | | | |
| 300 | | 2.1 | 4.2 | 8.3 | 12.5 | 16.7 | | | | | | |
| | | 22.9 | 20.8 | 16.7 | 12.5 | 8.3 | | | | | | |
| 400 | | 1.6 | 3.1 | 6.3 | 9.4 | 12.5 | 15.6 | 18.8 | | | | |
| | | 23.4 | 21.9 | 18.8 | 15.6 | 12.5 | 9.4 | 6.3 | | | | |
| 500 | | 1.3 | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 17.5 | 20 | | |
| | | 23.8 | 22.5 | 20 | 17.5 | 15 | 12.5 | 10 | 7.5 | 5 | | |
| 600 | | 1 | 2.1 | 4.2 | 6.3 | 8.3 | 10.4 | 12.5 | 14.6 | 16.7 | 18.8 | |
| | | 24 | 22.9 | 20.8 | 18.8 | 16.7 | 14.6 | 12.5 | 10.4 | 8.3 | 6.3 | |
| 700 | | | 1.8 | 3.6 | 5.4 | 7.1 | 8.9 | 10.7 | 12.5 | 14.3 | 16.1 | 17.9 |
| | | | 23.2 | 21.4 | 19.6 | 17.9 | 16.1 | 14.3 | 12.5 | 10.7 | 8.9 | 7.1 |
| 800 | | | 1.6 | 3.1 | 4.7 | 6.3 | 7.8 | 9.4 | 10.9 | 12.5 | 14.1 | 15.6 |
| | | | 23.4 | 21.9 | 20.3 | 18.8 | 17.2 | 15.6 | 14.1 | 12.5 | 10.9 | 9.4 |
| 900 | | | 1.4 | 2.8 | 4.2 | 5.6 | 6.9 | 8.3 | 9.7 | 11.1 | 12.5 | 13.9 |
| | | | 23.6 | 22.2 | 20.8 | 19.4 | 18.1 | 16.7 | 15.3 | 13.9 | 12.5 | 11.1 |
| 1,000 | | | 1.3 | 2.5 | 3.8 | 5 | 6.3 | 7.5 | 8.8 | 10 | 11.3 | 12.5 |
| | | | 23.8 | 22.5 | 21.3 | 20 | 18.8 | 17.5 | 16.3 | 15 | 13.8 | 12.5 |
| 1,100 | | | 1.1 | 2.3 | 3.4 | 4.5 | 5.7 | 6.8 | 8 | 9.1 | 10.2 | 11.4 |
| | | | 23.9 | 22.7 | 21.6 | 20.5 | 19.3 | 18.2 | 17 | 15.9 | 14.8 | 13.6 |
| 1,200 | | | 1 | 2.1 | 3.1 | 4.2 | 5.2 | 6.3 | 7.3 | 8.3 | 9.4 | 10.4 |
| | | | 24 | 22.9 | 21.9 | 20.8 | 19.8 | 18.8 | 17.7 | 16.7 | 15.6 | 14.6 |
| 1,300 | | | | 1.9 | 2.9 | 3.8 | 4.8 | 5.8 | 6.7 | 7.7 | 8.7 | 9.6 |
| | | | | 23.1 | 22.1 | 21.2 | 20.2 | 19.2 | 18.3 | 17.3 | 16.3 | 15.4 |
| 1,400 | | | | 1.8 | 2.7 | 3.6 | 4.5 | 5.4 | 6.3 | 7.1 | 8 | 8.9 |
| | | | | 23.2 | 22.3 | 21.4 | 20.5 | 19.6 | 18.8 | 17.9 | 17 | 16.1 |
| 1,500 | | | | 1.7 | 2.5 | 3.3 | 4.2 | 5 | 5.8 | 6.7 | 7.5 | 8.3 |
| | | | | 23.3 | 22.5 | 21.7 | 20.8 | 20 | 19.2 | 18.3 | 17.5 | 16.7 |

# Appendix B: Sequencing Information

We recommend a minimum sequencing depth of 20,000 reads per nuclei for whole transcriptome libraries. However, ideal sequencing depth is dependent on the sample type and experimental goals. For example, if trying to capture rare cell types in a heterogenous population, deeper sequencing is required. Alternatively, analyzing a homogenous population, more shallow sequencing may be appropriate.

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

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

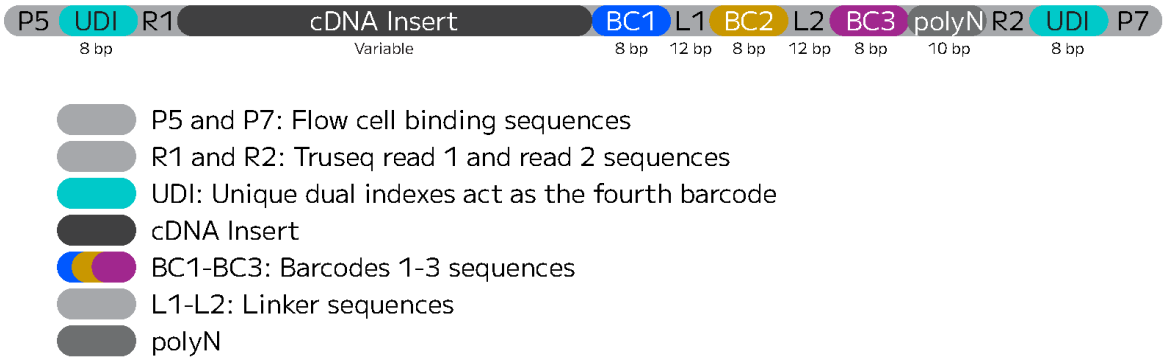



Figure 6: Whole transcriptome sequencing library structure.

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

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

The fourth barcode that tags each sublibrary acts as a standard Illumina i7 and i5 indexes. Please refer to the following table to demultiplex the whole transcriptome libraries.

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---|---|---|---|---|
| UDI_Plate_WT_1 | A1 | CAGATCAC | ATGTGAAG | CTTCACAT |
| UDI_Plate_WT_2 | B1 | ACTGATAG | GTCCAACC | GGTTGGAC |
| UDI_Plate_WT_3 | C1 | GATCAGTC | AGAGTCAA | TTGACTCT |
| UDI_Plate_WT_4 | D1 | CTTGTAAT | AGTTGGCT | AGCCAACT |
| UDI_Plate_WT_5 | E1 | AGTCAAGA | ATAAGGCG | CGCCTTAT |
| UDI_Plate_WT_6 | F1 | CCGTCCTA | CCGTACAG | CTGTACGG |
| UDI_Plate_WT_7 | G1 | GTAGAGTA | CATTCATG | CATGAATG |
| UDI_Plate_WT_8 | H1 | GTCCGCCT | AGATACGG | CCGTATCT |
| UDI_Plate_WT_9 | A2 | GTGAAACT | TACAGACT | AGTCTGTA |
| UDI_Plate_WT_10 | B2 | TCATTCCT | AATGCCTG | CAGGCATT |
| UDI_Plate_WT_11 | C2 | GGTAGCAT | TGCTTGCC | GGCAAGCA |
| UDI_Plate_WT_12 | D2 | ACTTGATC | TTTGGGTG | CACCCAAA |
| UDI_Plate_WT_13 | E2 | ATGAGCAT | GAATCTGA | TCAGATTC |
| UDI_Plate_WT_14 | F2 | GCGCTATC | CGACTGGA | TCCAGTCG |
| UDI_Plate_WT_15 | G2 | TGACCAGT | ACATTGGC | GCCAATGT |
| UDI_Plate_WT_16 | H2 | TATAATCA | ACCACTGT | ACAGTGGT |
| UDI_Plate_WT_17 | A3 | CAAAAGTC | CGGTTGTT | AACAACCG |
| UDI_Plate_WT_18 | B3 | CGATGTCA | CATGAGGA | TCCTCATG |
| UDI_Plate_WT_19 | C3 | CTCAGAGT | TGGAGAGT | ACTCTCCA |
| UDI_Plate_WT_20 | D3 | TAATCGAC | TGACTTCG | CGAAGTCA |
| UDI_Plate_WT_21 | E3 | CATTTTCT | GGAAGGAT | ATCCTTCC |
| UDI_Plate_WT_22 | F3 | CTATACTC | TGTTCGAG | CTCGAACA |
| UDI_Plate_WT_23 | G3 | CACTCACA | AAGGCTGA | TCAGCCTT |
| UDI_Plate_WT_24 | H3 | CTCGAACA | CTCGAGTG | CACTCGAG |
| UDI_Plate_WT_25 | A4 | CTCTATCG | ATCGGTGG | CCACCGAT |

| Sublibrary Index ID | Well Position | i7 Forward Sequence | i5 Reverse Complement Sequence | i5 Forward Sequence |
|---|---|---|---|---|
| UDI_Plate_WT_26 | B4 | TCCTCATG | AGGTCTTG | CAAGACCT |
| UDI_Plate_WT_27 | C4 | AACAACCG | AGGAAGCG | CGCTTCCT |
| UDI_Plate_WT_28 | D4 | GCCAATGT | ACATGTGT | ACACATGT |
| UDI_Plate_WT_29 | E4 | TGGTTGTT | ATACAGTT | AACTGTAT |
| UDI_Plate_WT_30 | F4 | TCTGCTGT | ATCGCCTT | AAGGCGAT |
| UDI_Plate_WT_31 | G4 | TTGGAGGT | TTCGACGC | GCGTCGAA |
| UDI_Plate_WT_32 | H4 | TCGAGCGT | TGTCGTTC | GAACGACA |
| UDI_Plate_WT_33 | A5 | TGCGATCT | TCCATAGC | GCTATGGA |
| UDI_Plate_WT_34 | B5 | TTCCTGCT | TAAGTGTC | GACACTTA |
| UDI_Plate_WT_35 | C5 | TTCCATTG | CTGGCATA | TATGCCAG |
| UDI_Plate_WT_36 | D5 | TAACGCTG | CTGAGCCA | TGGCTCAG |
| UDI_Plate_WT_37 | E5 | TTGGTATG | CTCAATGA | TCATTGAG |
| UDI_Plate_WT_38 | F5 | TGAACTGG | CGCATACA | TGTATGCG |
| UDI_Plate_WT_39 | G5 | TCCAGTCG | CCGAAGTA | TACTTCGG |
| UDI_Plate_WT_40 | H5 | TGTATGCG | CCAGTTCA | TGAACTGG |
| UDI_Plate_WT_41 | A6 | TGGCTCAG | CAGCGTTA | TAACGCTG |
| UDI_Plate_WT_42 | B6 | TATGCCAG | CAATGGAA | TTCCATTG |
| UDI_Plate_WT_43 | C6 | GGTTGGAC | ATCCTGTA | TACAGGAT |
| UDI_Plate_WT_44 | D6 | GACACTTA | AGCAGGAA | TTCCTGCT |
| UDI_Plate_WT_45 | E6 | GAACGACA | ACGCTCGA | TCGAGCGT |
| UDI_Plate_WT_46 | F6 | AAGGCGAT | ACAGCAGA | TCTGCTGT |
| UDI_Plate_WT_47 | G6 | ATGCTTGA | ACAAGCTA | TAGCTTGT |
| UDI_Plate_WT_48 | H6 | AGTATCTG | CATCAAGT | ACTTGATG |

# Appendix C: Thermocycling Programs

### Pre-Barcoding Part B: FFPE Sample Preparation

| FFPE Enhancement | | |
|---|---|---|
| Run Time | 5 minutes | |
| Lid Temperature | 105°C | |
| Sample Volume | 100 µL | |
| Step | Time | Temperature |
| 1 | Hold | 80°C |
| 2 | 5 min | 80°C |
| 3 | Hold | 4°C |

### Section 1: *In Situ* Nuclei Barcoding

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

| FFPE BARCODING ROUND 1 | | | |
|---|---|---|---|
| Run Time | | 105 minutes | |
| Lid Temperature | | 70°C | |
| Sample Volume | | 40 µL | |
| Step | Time | Temperature | Cycles |
| 1 | 10 min | 50°C | 1 |
| 2 | 12 s | 8°C | 12 |
| 3 | 30 s | 15°C | |
| 4 | 45 s | 20°C | |
| 5 | 1 min | 25°C | |
| 6 | 1 min | 30°C | |
| 7 | 2 min | 42°C | |
| 8 | 30 min | 42°C | 1 |
| 9 | Hold | 4°C | 1 |

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

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

| ROUND 2 STOP | | |
|---|---|---|
| Run Time | 5 min | |
| Lid Temperature | 50°C | |
| Sample Volume | 60 µL | |
| Step | Time | Temperature |
| 1 | 5 min | 16°C |
| 2 | Hold | 4°C |

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

| BARCODING ROUND 3 | | |
|---|---|---|
| Run Time | 15 minutes | |
| Lid Temperature | 50°C | |
| Sample Volume | 60 µL | |
| Step | Time | Temperature |
| 1 | 15 min | 16°C |
| 2 | Hold | 4°C |

| FFPE NUCLEI LYSIS | | |
|---|---|---|
| Run Time | 15 hours | |
| Lid Temperature | 80°C | |
| Sample Volume | 55 µL | |
| Step | Time | Temperature |
| 1 | 15 hours | 37°C |
| 2 | Hold | 4°C |

**Section 2: cDNA Capture and Amplification**

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

| cDNA AMPLIFICATION | | | |
|---|---|---|---|
| Run Time | | 50-70 minutes | |
| Lid Temperature | | 105°C | |
| Sample Volume | | 100 µL | |
| Step | Time | Temperature | Cycles |
| 1 | 3 min | 95°C | 1 |
| 2 | 20 s | 98°C | 5 |
| 3 | 45 s | 65°C* | |
| 4 | 3 min | 72°C | |
| 5 | 20 s | 98°C | Variable, see section 2.3 |
| 6 | 20 s | 67°C* | |
| 7 | 3 min | 72°C | |
| 8 | 5 min | 72°C | 1 |
| 9 | Hold | 4°C | 1 |



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

**Section 3: Sequencing Library Preparation**

| FFPE FRAGMENTATION AND END PREP | | |
|---|---|---|
| Run Time | 35 minutes | |
| Lid Temperature | 70°C | |
| Sample Volume | 50 µL | |
| Step | Time | Temperature |
| 1 | Hold | 4°C |
| 2 | 5 min | 32°C |
| 3 | 30 min | 65°C |
| 4 | Hold | 4°C |

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

| INDEXING PCR | | | |
|---|---|---|---|
| Run Time | | ~30 minutes | |
| Lid Temperature | | 105°C | |
| Sample Volume | | 50 µL | |
| Step | Time | Temperature | Cycles |
| 1 | 3 min | 95°C | 1 |
| 2 | 20 s | 98°C | Variable, see table in Section 3.5 |
| 3 | 20 s | 67°C | |
| 4 | 1 min | 72°C | |
| 5 | 5 min | 72°C | 1 |
| 6 | Hold | 4°C | 1 |

## Appendix D: Revision History

| Version | Description | Date |
|---|---|---|
| 1.0 | Initial release | April 2026 |
| 1.1 | Updated column reference to Sample Loading Table | April 2026 |
| 1.2 | Important Guidelines section: Added guidance on recommended RNA integrity | May 2026 |
| 1.3 | Step 2.1.20: added thermocycler's instruction. Step 2.1.26: updated incubation timing and temperature. | September 2026 |
| 1.4 | Section 1.3: Added important instruction on reagent handling | October 2026 |

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