User Manual

Version 1.4 – UMWF4500

PARSE BIOSCIENCES
A QIAGEN company

Evercode™ WT FFPE Mega

For use with

ECWF4500

Support Suite

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Overview

Workflow

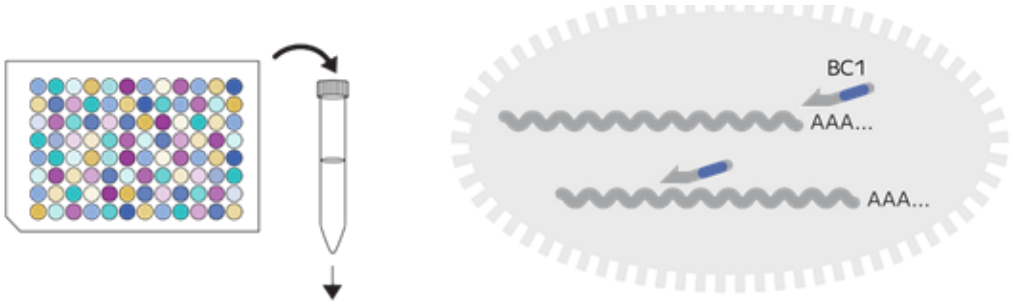
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT FFPE Mega kit can profile up to 1,000,000 nuclei across up to 96 different biological samples or experimental conditions.

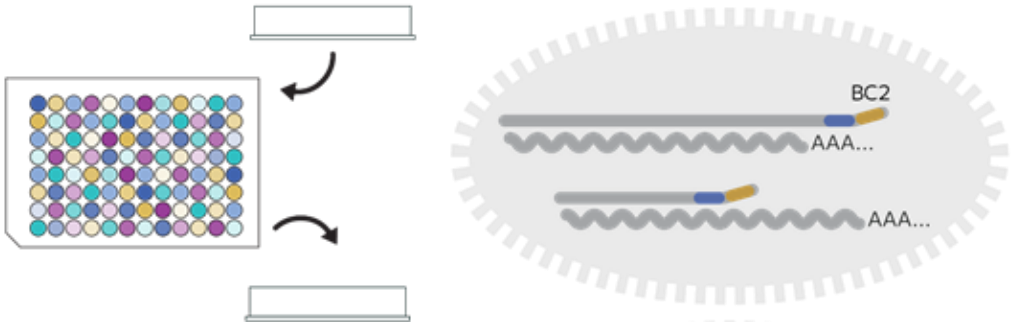
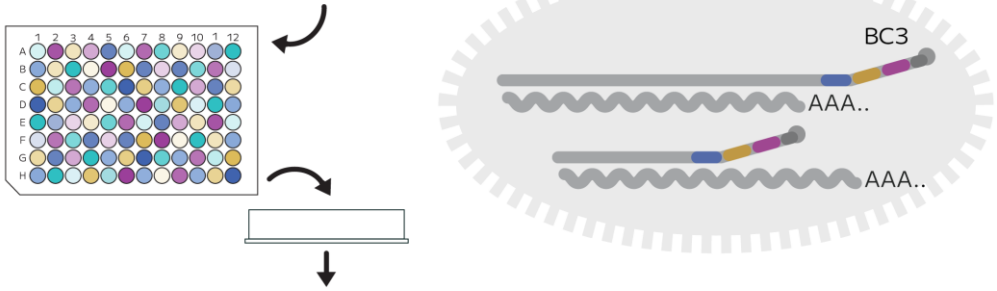
Dissociated nuclei act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed nuclei is uniquely labeled.

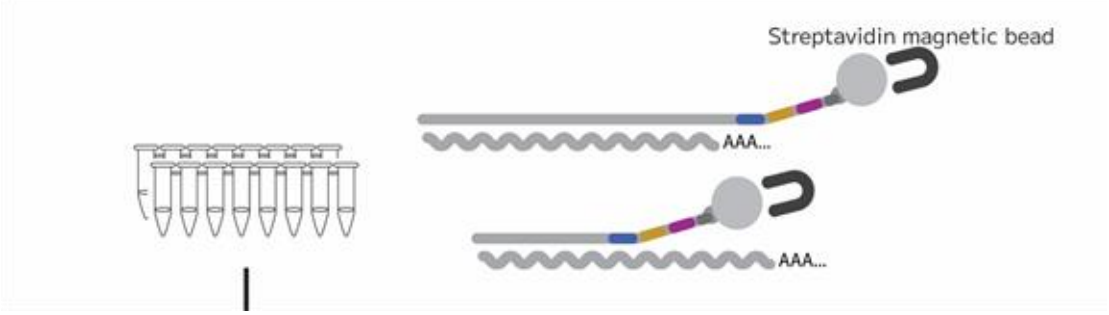
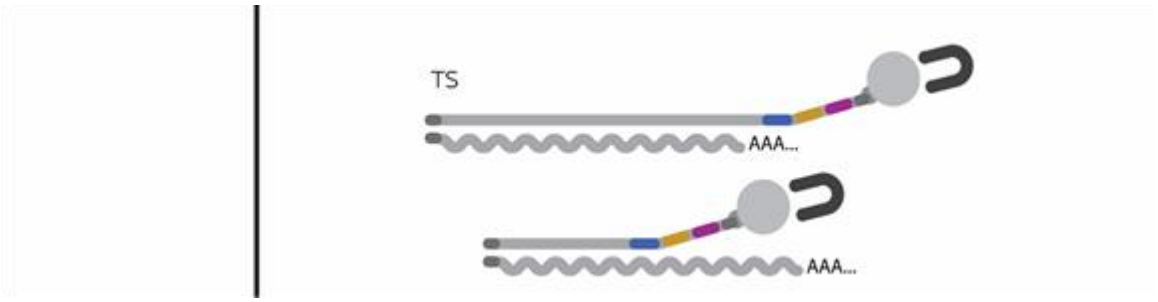
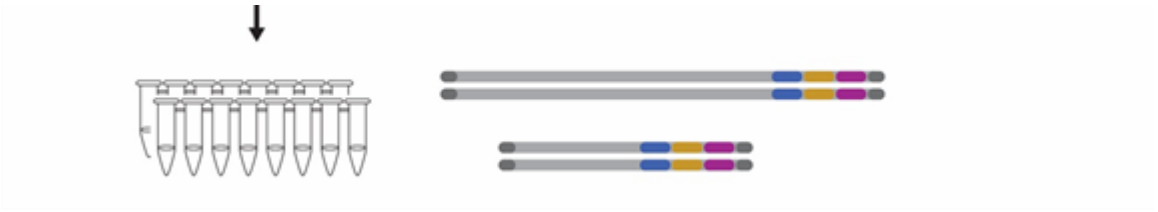
The four rounds of barcoding can yield a very large number of possible barcode combinations which is more than sufficient to uniquely label up to 1,000,000 nuclei while avoiding doublets.

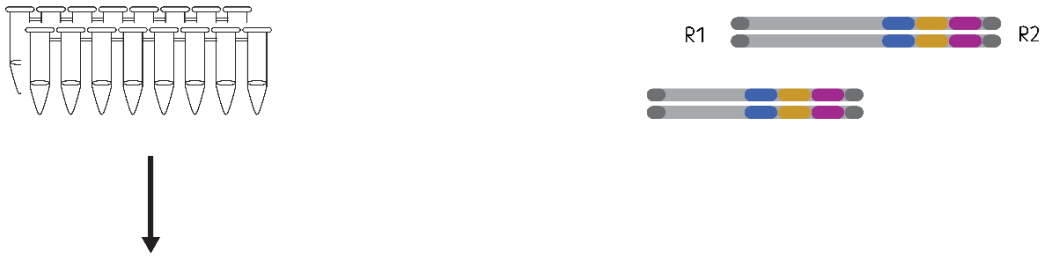
After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same 4 barcode combination to a single nuclei.

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	 **Round 1 Barcoding:** Fixed and permeabilized nuclei are loaded into 96 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.

Section 1: *In situ* Nuclei Barcoding	
2	 **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	 **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 Read 2 sequence, and biotin.
4	 **Lysis and Sublibrary Generation:** Nuclei are split across 16 sublibraries and lysed.

Section 2: cDNA Capture and Amplification	
1	 **cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads.
2	 **cDNA Template Switch:** A template switch reaction adds an adapter to the 3' end of the cDNA.
3	 **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	 **Fragmentation and End Repair:** DNA is fragmented, ends are repaired, and A-tailed.
2	 **Adapter Ligation:** An Illumina TruSeq Read 1 Adapter is ligated to the 5′ end of the DNA.
3	 **Round 4 Barcoding:** The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.

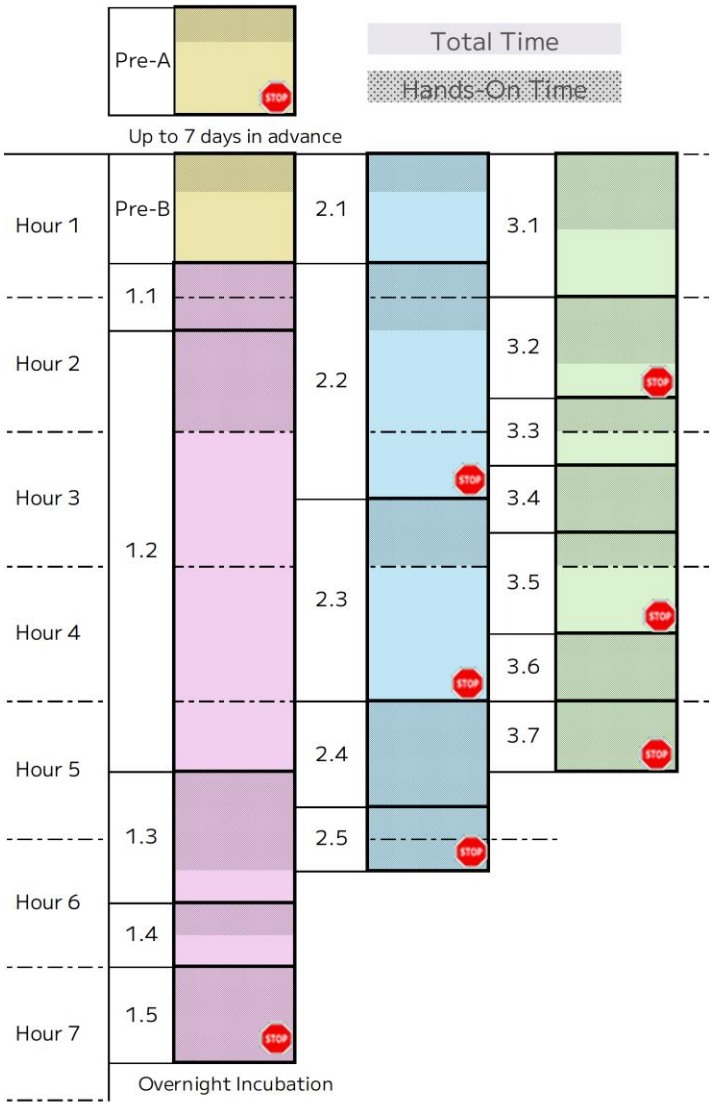
Protocol Timing

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

DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
3.5 Barcoding Round 4	45 min	15 min	4°C ≤ 18 hrs in the thermocycler
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.

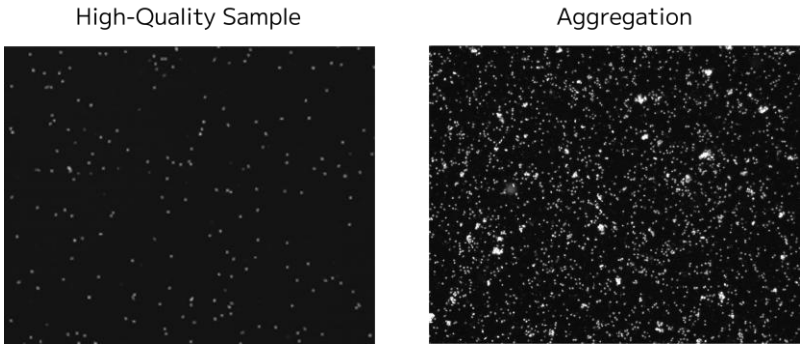


Figure 1: Example of AO fluorescent stained fixed nuclei

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 Mega 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.

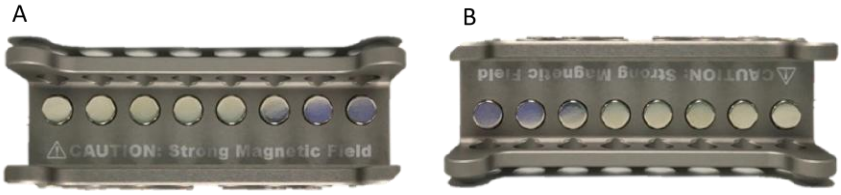


Figure 2: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.

- 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 Mega kit generates 16 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 78,125 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 Mega 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 FMG400

LABEL	ITEM	PN	FORMAT	QTY
	FFPE Round 1 Plate	MG301	Green semi-skirted 96 well plate	1
	Round 2 Plate v4	MG142	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Resuspension Buffer	MG104	5 mL tube	1
	Sample Dilution Buffer	MG105	2 mL tube	1
	Round 2 Ligation Buffer	MG106	5 mL tube	1
	Ligation Enzyme	MG140	1.5 mL tube	1
	Round 2 Stop Buffer	MG108	2 mL tube	1
	Round 3 Stop Buffer	MG109	5 mL tube	1
	Pre-Lysis Wash Buffer	MG110	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	FFPE Pre-Lysis Dilution Buffer	MG151	2 mL tube	1
	Lysis Enzyme	MG113	1.5 mL tube	1
	FFPE Lysis Buffer	MG152	1.5 mL tube	1
	Bead Wash Buffer	MG114	5 mL tube	1
	Wash Buffer 1	MG115	5 mL tube	1
	Wash Buffer 2	MG116	5 mL tube	1
	Capture Enhancer	MG117	1.5 mL tube	1
	Binding Buffer	MG118	1.5 mL tube	1
	Wash Buffer 3	MG119	5 mL tube	1
	Template Switch Buffer	MG120	2 mL tube	1
	Template Switch Enzyme	MG121	1.5 mL tube	1
	Template Switch Primer	MG122	1.5 mL tube	1
	cDNA Amp Mix	MG123	1.5 mL tube	1
	cDNA Amp Primers	MG124	1.5 mL tube	1
	Fragm/End Prep Buffer	MG125	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Fragm/End Prep Enzymes	MG126	1.5 mL tube	1
	Ligation Adapter	MG127	1.5 mL tube	1
	Adapter Ligation Buffer	MG128	1.5 mL tube	1
	Adapter Ligation Enzyme	MG129	1.5 mL tube	1
	Library Amp Mix	MG130	1.5 mL tube	1
	PCR Additive	MG150	1.5 mL tube	1

4°C Reagents. Store 4°C, PN FMG500

LABEL	ITEM	PN	FORMAT	QTY
	Spin Additive	MG201	1.5 mL tube	1
	Streptavidin Beads	MG203	1.5 mL tube	1
	Retention Enhancer	MG147	8 mL tube	1

The Evercode WT FFPE Mega 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 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). We do not recommend FlowMi Cell Strainers (SP Bel-Art).
pluriStrainer® Mini	pluriSelect®	43-10020-40 43-10040-40	
Falcon®Cell Strainer	Corning®	431750	
EASYstrainer™, small	Greiner Bio-One™	542120 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.

ITEM	SUPPLIER	PN	NOTES
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.

Reagents

ITEM	SUPPLIER	PN	NOTES
AMPure® XP Reagent	Beckman Coulter®	A63880 (5 mL) A63881 (60mL)	Choose one. We do not recommend substituting other magnetic beads.
SPRIselect Reagent	Beckman Coulter	B23317 (5mL) B23318 (60mL)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	
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) 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) 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.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ FFPE Resuspension Buffer	-20°C Reagents*	1	Thaw at 37°C, then place on ice.
● 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 Mega 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

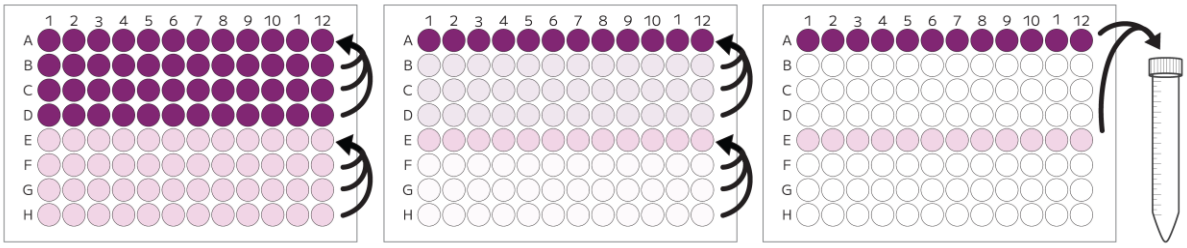
6. Remove the Round 1 Plate from the thermocycler, place in a PCR tube rack, and store on ice.

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 as follows:



i. With a multichannel P200 set to 30 µL, mix the sample in row B by pipetting 3x.

ii. Transfer **30 µL** from row B to row A.

iii. Repeat i-ii for rows C-D to mix the sample then transfer to row A.

iv. Transfer any residual liquid in rows B-D to row A with a multichannel P20 set to 10 µL.

v. Ensure the nuclei in row A are in suspension as described in i. Then, transfer the total volume of each well in row A into the same 15 mL tube with a single channel P200 set to 200 µL.

vi. Repeat i-ii for rows F-H to mix the sample then transfer to row E.

vii. Transfer any residual liquid in rows F-H to row E with a multichannel P20 set to 10 µL.

viii. Ensure the nuclei in row E are in suspension as described in i. Then, transfer the total volume of each well in row E into the same 15 mL tube with a single channel P200 set to 200 µL.

10. Add **19.2 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.

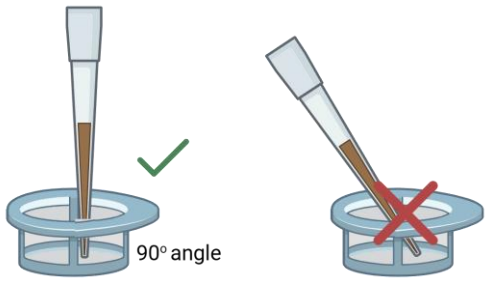
    ii. Transfer **50 µL** from row A to the basin.

    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 Section 1.3 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 in **200 µL** of ● FFPE Pre-Lysis Dilution Buffer for a final total volume of **240 µL**. Store on ice.



Note: Do not discard ● 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 78,125 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 200 µL prior to each transfer. Add the appropriate volume of sample to 16 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 P1000 set to 425 µL. Store at room temperature.

LYSIS MASTER MIX	
● FFPE Lysis Buffer	440 µL
● Lysis Enzyme	88 µL
Total Volume	528 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 **1 minute**. 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	16
● Streptavidin Beads	44 µL	704 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 OBead Wash Buffer as follows:

Lysates being processed	1	16
O Bead Wash Buffer	50 µL	800 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	16
● Binding Buffer	55 µL	880 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. Prepare the Template Switch Master Mix in a new 2 mL tube as follows, depending on the number of sublibraries being processed. Mix by pipetting 10x and store on ice.

TEMPLATE SWITCH MASTER MIX		
Number of Samples	1	16
● Template Switch Buffer	101.75 µL	1628 µL
● Template Switch Primer	2.75 µL	44 µL
● Template Switch Enzyme	5.5 µL	88 µL
Total	110 µL	1760 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 O Wash Buffer 3 to each tube.

CRITICAL! Do not discard the OWash 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 O 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** O 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 2 mL tube as follows. Mix by pipetting 10x and store on ice.

cDNA AMPLIFICATION MASTER MIX		
Number of Sublibraries	1	16
cDNA Amp Mix	60 µL	960 µL
cDNA Amp Primers	57.5 µL	920 µL
PCR Additive	2.50 µL	40 µL
Total	120 µL	1920 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
6,250-15,625	13
15,625-31,250	12
31,250-56,250	11
56,250-78,125	10

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

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 **25 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 **25 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.

STOP 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.

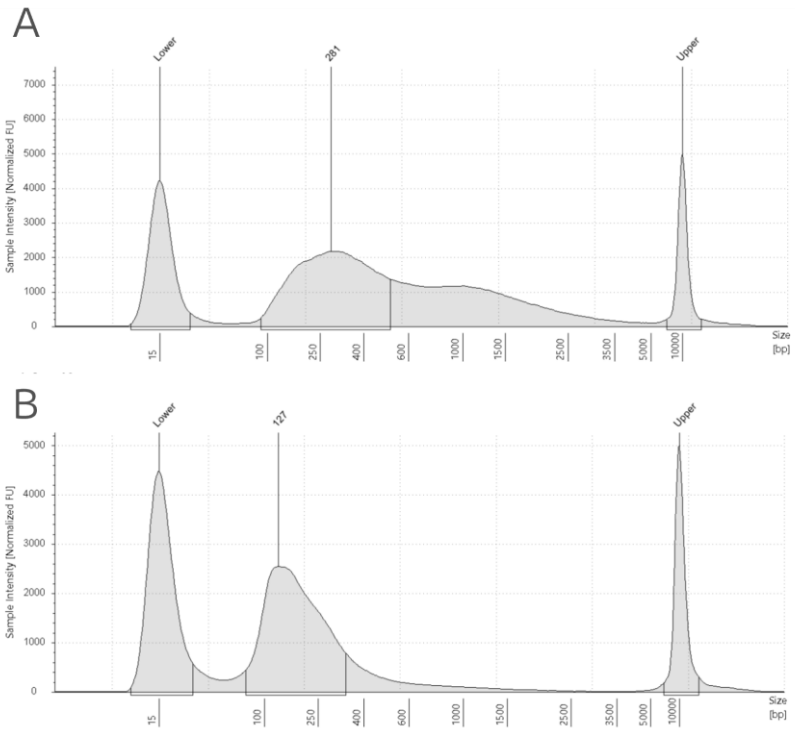


Figure 4: Expected post-amplification sublibrary cDNA size distribution. A: example of the trace of Human cDNA with expected size distribution run on a TapeStation. B: another example where

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 to a final volume of 35 µL and store on ice. Store any remaining purified cDNA at -20°C.

DILUTED cDNA	
Purified cDNA	10 µL
Nuclease-free water	25 µL
Total Volume	35 µL

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	16
● Fragm/End Prep Buffer	5.5 µL	88 µL
● Fragm/End Prep Enzymes	11 µL	176 µL
Total	16.5 µL	264 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	16
Nuclease-free water	19.25 µL	308 µL
Adapter Ligation Buffer	22 µL	352 µL
Adapter Ligation Enzyme	11 µL	176 µL
Ligation Adapter	2.75 µL	44 µL
Total	55 µL	880 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.

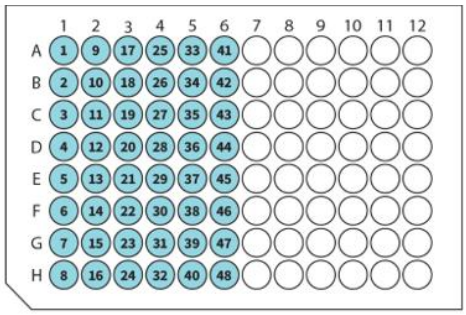
17. Proceed immediately to Section 3.5.

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
100-299	9
300-999	7
1,000 or more	6

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.

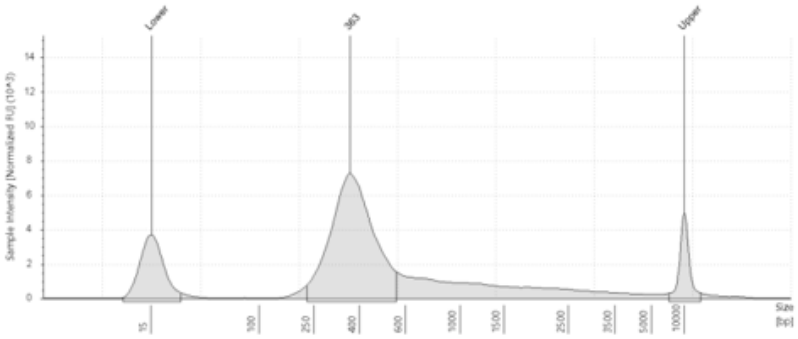


Figure 5: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

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	6,250	12,500	15,625	18,750	25,000	31,250	37,500	78,125
50	5	12.5	25									
	20	12.5	0									
100	2.5	6.3	12.5	25								
	22.5	18.8	12.5	0								
200	1.3	3.1	6.3	12.5								
	23.8	21.9	18.8	12.5								
300		2.1	4.2	8.3	20.8							
		22.9	20.8	16.7	4.2							
400		1.6	3.1	6.3	15.6							
		23.4	21.9	18.8	9.4							
800			1.6	3.1	7.8	15.6	19.5	23.4				
			23.4	21.9	17.2	9.4	5.5	1.6				
1,000			1.3	2.5	6.3	12.5	15.6	18.8	25			
			23.8	22.5	18.8	12.5	9.4	6.3	0			
1,200			1	2.1	5.2	10.4	13	15.6	20.8			
			24	22.9	19.8	14.6	12	9.4	4.2			
1,400				1.8	4.5	8.9	11.2	13.4	17.9	22.3		
				23.2	20.5	16.1	13.8	11.6	7.1	2.7		
1,600				1.6	3.9	7.8	9.8	11.7	15.6	19.5	23.4	
				23.4	21.1	17.2	15.2	13.3	9.4	5.5	1.6	
1,800				1.4	3.5	6.9	8.7	10.4	13.9	17.4	20.8	
				23.6	21.5	18.1	16.3	14.6	11.1	7.6	4.2	
2,000				1.3	3.1	6.3	7.8	9.4	12.5	15.6	18.8	
				23.8	21.9	18.8	17.2	15.6	12.5	9.4	6.3	
2,500				1	2.5	5	6.3	7.5	10	12.5	15	
				24	22.5	20	18.8	17.5	15	12.5	10	
3,000					2.1	4.2	5.2	6.3	8.3	10.4	12.5	
					22.9	20.8	19.8	18.8	16.7	14.6	12.5	
3,500					1.8	3.6	4.5	5.4	7.1	8.9	10.7	22.3
					23.2	21.4	20.5	19.6	17.9	16.1	14.3	2.7
4,000					1.6	3.1	3.9	4.7	6.3	7.8	9.4	19.5
					23.4	21.9	21.1	20.3	18.8	17.2	15.6	5.5
4,500					1.4	2.8	3.5	4.2	5.6	6.9	8.3	17.4
					23.6	22.2	21.5	20.8	19.4	18.1	16.7	7.6
5,000					1.3	2.5	3.1	3.8	5	6.3	7.5	15.6
					23.8	22.5	21.9	21.3	20	18.8	17.5	9.4
5,500					1.1	2.3	2.8	3.4	4.5	5.7	6.8	14.2
					23.9	22.7	22.2	21.6	20.5	19.3	18.2	10.8
6,000					1	2.1	2.6	3.1	4.2	5.2	6.3	13
					24	22.9	22.4	21.9	20.8	19.8	18.8	12
7,000						1.8	2.2	2.7	3.6	4.5	5.4	11.2
						23.2	22.8	22.3	21.4	20.5	19.6	13.8
8,000						1.6	2	2.3	3.1	3.9	4.7	9.8
						23.4	23	22.7	21.9	21.1	20.3	15.2
9,000						1.4	1.7	2.1	2.8	3.5	4.2	8.7
						23.6	23.3	22.9	22.2	21.5	20.8	16.3
#####						1.3	1.6	1.9	2.5	3.1	3.8	7.8
						23.8	23.4	23.1	22.5	21.9	21.3	17.2

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.

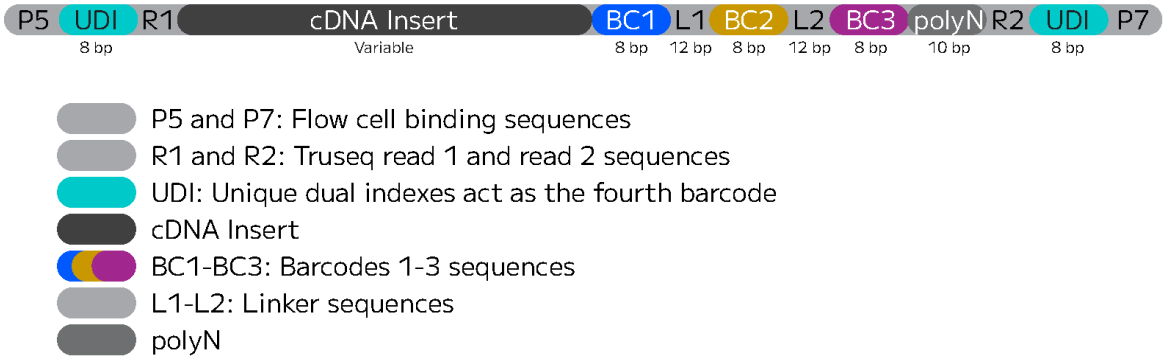


Figure 6: Whole transcriptome sequencing library structure.

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 min	
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 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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