User Manual

Version 1.0 – UMMF4100

Evercode™ MAX WT FFPE Barcoding S

For use with

ECMF4100

Support Suite

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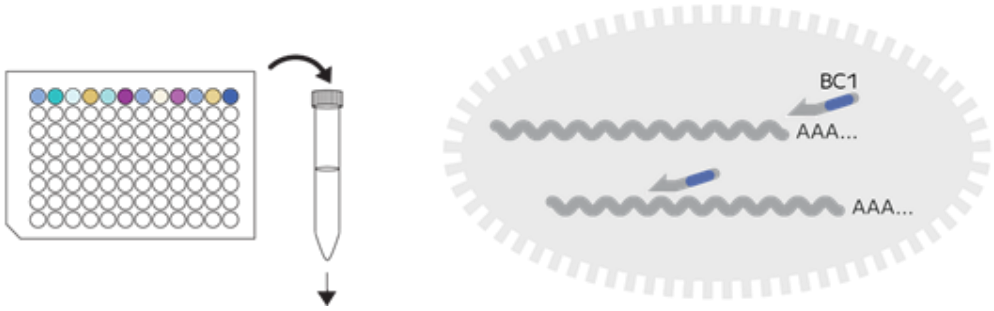
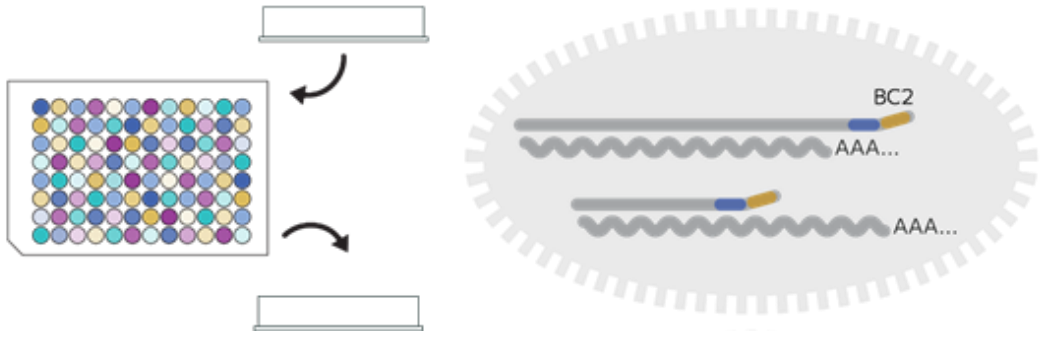
Overview

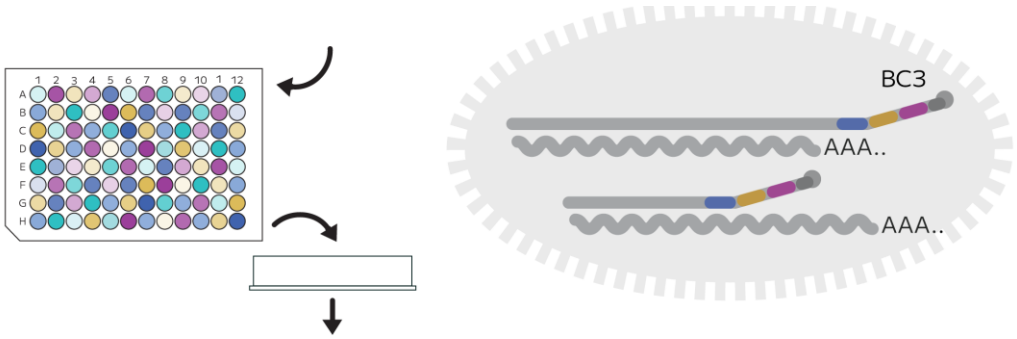
Workflow

Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode MAX WT FFPE S kit can profile up to 64,000 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 64,000 nuclei while avoiding doubtlets. 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 MAX WT FFPE barcoding workflow. These steps are completed after the pre-barcoding sections which are not depicted here.

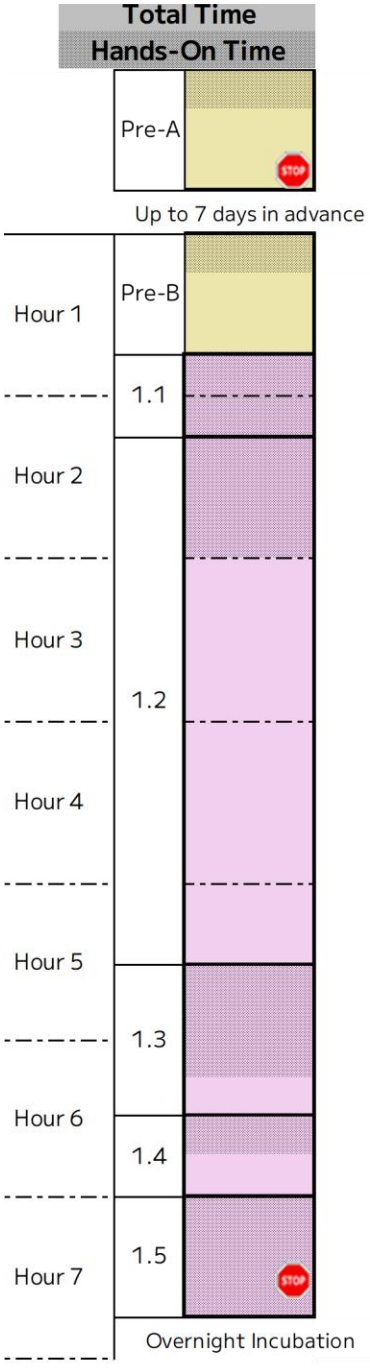
Section 1: *In situ* Nuclei Barcoding	
1	 **Round 1 Barcoding:** Fixed and permeabilized nuclei are loaded into 12 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with a well-specific barcode that is associated with specific samples.
2	 **Round 2 Barcoding:** The nuclei are pooled and loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode.

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

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


Total Time
Hands-On Time
Pre-A
Up to 7 days in advance
Hour 1
Pre-B
1.1
Hour 2
Hour 3
1.2
Hour 4
Hour 5
1.3
Hour 6
1.4
Hour 7
1.5
Overnight Incubation

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 MAX 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.

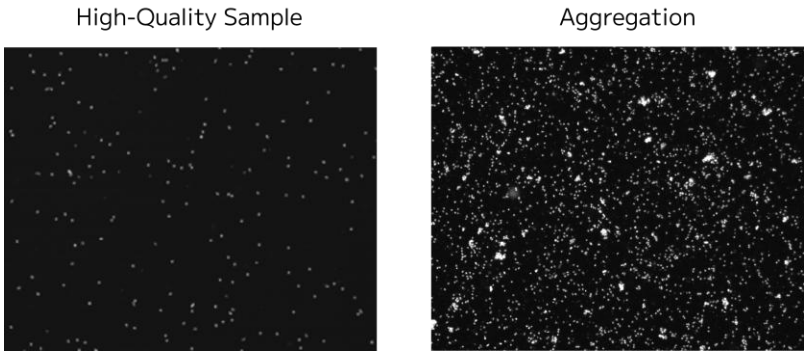


Figure 1: Example of AO fluorescent stained fixed nuclei

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 MAX 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 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 on 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.

Sublibrary Loading

- The Evercode MAX WT FFPE Barcoding S kit generates 12 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 B.
- 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 8,000 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.

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 MAX WT FFPE Barcoding S 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 XFMS400

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	PT109	1.5 mL tube	1
	FFPE Lysis Buffer	PT152	1.5 mL tube	1

4°C Reagents. Store 4°C, PN XFMS500

LABEL	ITEM	PN	FORMAT	QTY
	Spin Additive	MN201	1.5 mL tube	1
	Retention Enhancer	MN147	8 mL tube	1

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	CHECK LIST
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.	☐
Fluorescent Cell Counter	Various Suppliers	Varies		☐
96-well plate adapter	Various Suppliers	Varies	Capable of holding semi-skirted 96 well PCR plates and a tight fitting lid.	☐
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, 0.2 mL 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.	☐
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		

Consumables

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free, 10 mL or 25 mL 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 they 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.	☐
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	CHECK LIST
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.	☐
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.	☐
AO/PI	Various Suppliers	Varies	Or alternative dyes to assess viability.	☐

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 Part B 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 Mini 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 a 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 8 mL bottle of ○ FFPE Resuspension Buffer and 2 mL tube of ● FFPE Enhancement Buffer.
- The 96-Reactions kit (ECNP4500) includes 15 mL bottle of ○ FFPE Resuspension Buffer and 15 mL bottle of ○ FFPE Enhancement Buffer.

7. Thaw the PCR strip tube(s) containing the dissociated nuclei in a 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 dissociation were not recorded, count using a fluorescent counter, and input 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-minute** 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 fluorescence-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 the 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.

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.

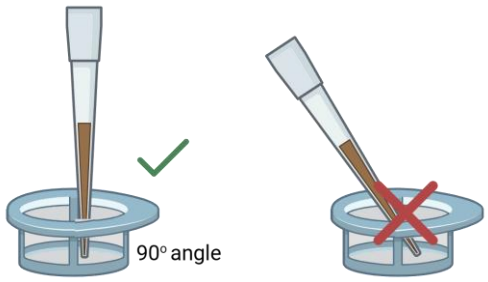
    ii. Transfer **50 µL** from row A to the basin.

    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 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 FFPE Pre-Lysis Dilution Buffer. The nuclei are counted and divided into sublibraries based on the Sublibrary Generation Table in Appendix B. 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 ● FFPE 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 fluorescence-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 B to determine the volume of sample and ● FFPE 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 10,000 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				
Number of Sublibraries	2	4	6	8
● Lysis Solution	55 µL	110 µL	165 µL	220 µL
● Lysis Enzyme	11 µL	22 µL	33 µL	44 µL
Total	66 µL	132 µL	198 µL	264 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 of the Evercode MAX WT FFPE Library Prep.

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

This ends the Barcoding portion of the Evercode assay. To continue with the Sublibrary Preparation portion of the workflow, refer to the Evercode MAX WT FFPE Library Prep user manual.

Appendices

Appendix A: Determine Sublibraries Number

Before proceeding with barcoding, determine the appropriate number of sublibraries (SL) to prepare. The table shows the maximum number of nuclei that can be used for a given number of sublibraries (SL). Maximum nuclei number for one SL is 10,000.

NUCLEI MAX NUMBER PER TOTAL SUBLIBRARIES				
KIT	**2 SL**	**4 SL**	**6 SL**	**8 SL**
MAX FFPE S	20,000	40,000	60,000	80,000

Appendix B: 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 FFPE 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.

Cell Stock Conc. (cells/uL)	Target Sublibrary Cell Count (cells/sublibrary)						
	200	500	1,000	2,000	5,000	8,000	10,000
50	4.0	10.0	20.0	N/A	N/A	N/A	N/A
	21.0	15.0	5.0	N/A	N/A	N/A	N/A
100	Dilute	5.0	10.0	20.0	N/A	N/A	N/A
	N/A	20.0	15.0	5.0	N/A	N/A	N/A
200	Dilute	2.5	5.0	10.0	25.0	N/A	N/A
	N/A	22.5	20.0	15.0	0.0	N/A	N/A
400	Dilute	Dilute	2.5	5.0	12.5	20.0	25.0
	N/A	N/A	22.5	20.0	12.5	5.0	0.0
600	Dilute	Dilute	Dilute	3.3	8.3	13.3	16.7
	N/A	N/A	N/A	21.7	16.7	11.7	8.3
800	Dilute	Dilute	Dilute	2.5	6.3	10.0	12.5
	N/A	N/A	N/A	22.5	18.8	15.0	12.5
1,000	Dilute	Dilute	Dilute	Dilute	5.0	8.0	10.0
	N/A	N/A	N/A	N/A	20.0	17.0	15.0
1,200	Dilute	Dilute	Dilute	Dilute	4.2	6.7	8.3
	N/A	N/A	N/A	N/A	20.8	18.3	16.7
1,400	Dilute	Dilute	Dilute	Dilute	3.6	5.7	7.1
	N/A	N/A	N/A	N/A	21.4	19.3	17.9
1,600	Dilute	Dilute	Dilute	Dilute	3.1	5.0	6.3
	N/A	N/A	N/A	N/A	21.9	20.0	18.8
1,800	Dilute	Dilute	Dilute	Dilute	2.8	4.4	5.6
	N/A	N/A	N/A	N/A	22.2	20.6	19.4
2,000	Dilute	Dilute	Dilute	Dilute	2.5	4.0	5.0
	N/A	N/A	N/A	N/A	22.5	21.0	20.0
2,500	Dilute	Dilute	Dilute	Dilute	Dilute	3.2	4.0
	N/A	N/A	N/A	N/A	N/A	21.8	21.0
3,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.7	3.3
	N/A	N/A	N/A	N/A	N/A	22.3	21.7
3,500	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.9
	N/A	N/A	N/A	N/A	N/A	N/A	22.1
4,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.5
	N/A	N/A	N/A	N/A	N/A	N/A	22.5
4,500	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5,500	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A
9,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute
	N/A	N/A	N/A	N/A	N/A	N/A	N/A

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 minutes	
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

Appendix D: Revision History

Version	Description	Date
1.0	Initial release	November 2026

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