User Manual

Version 1.0 – UMMF4080

Evercode™ MAX WT FFPE Library Prep

8 Reactions

For use with

ECMF4080

Support Suite

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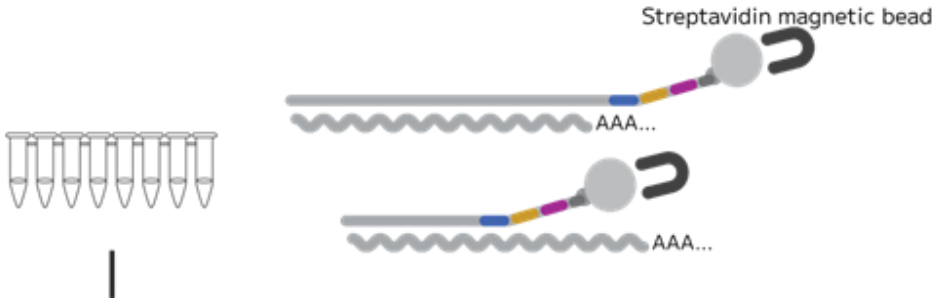
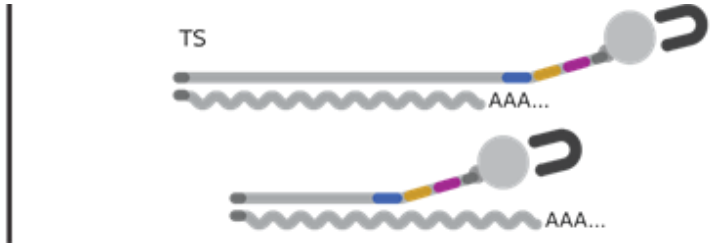
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Overview

Workflow

This manual accompanies the Evercode MAX WT FFPE Library Prep, and it picks up where the Evercode MAX WT FFPE Cell Barcoding user guide left off. In the Cell Barcoding guide's Section 1, samples underwent three rounds of barcoding before being divided into sublibraries and lysed. Sections 2 and 3 of this manual complete the workflow by amplifying the cDNA and preparing the sequencing libraries. This guide therefore should be completed after the Evercode MAX FFPE Cell Barcoding workflow.

This user guide can be used to prepare between 2-8 sublibraries concurrently for sequencing.

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.

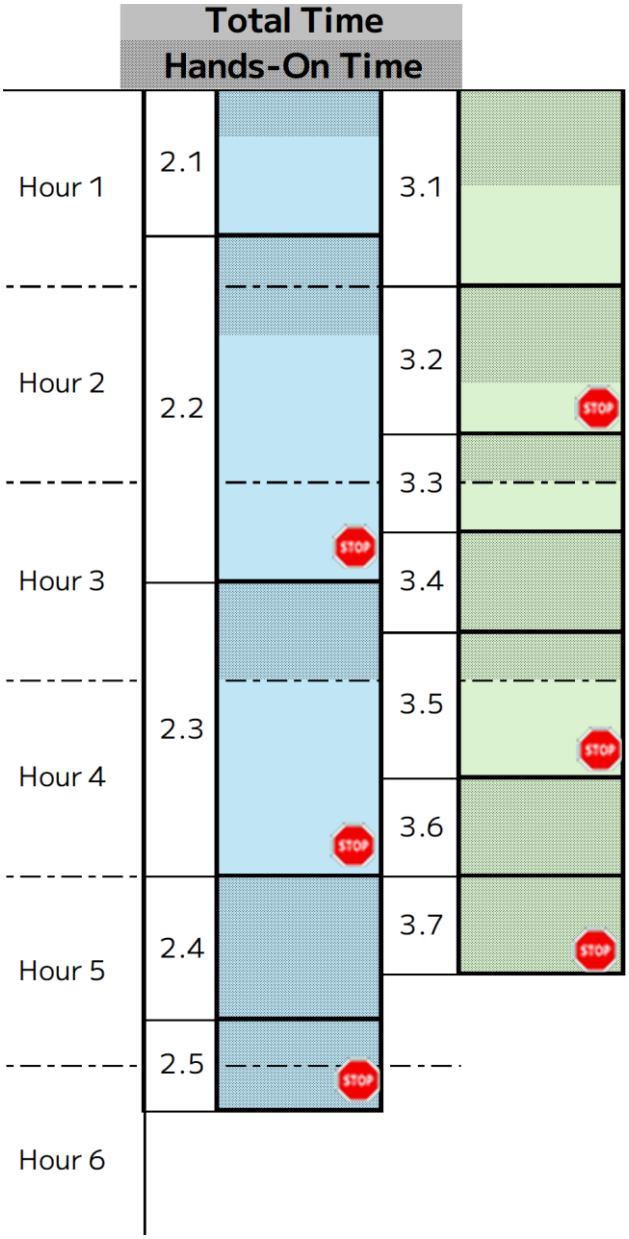
Section 3: Sequencing Library Preparation	
1	 **Fragmentation and End Repair:** DNA is fragmented, ends are repaired, and A-tailed.
2	 **Adapter Ligation:** An Illumina TruSeq Read 1 Adapter is ligated to the 5′ end of the DNA.
3	 **Round 4 Barcoding:** The sequencing library is amplified, adding P5/P7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

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
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	60 min	30 min	
2.2 cDNA Template Switch	120 min	30 min	4°C ≤ 18 hrs
2.3 cDNA Amplification	90 min	15 min	4°C ≤ 18 hrs in the thermocycler
2.4 Post-Amplification Purification	30 min	30 min	
2.5 cDNA Quantification	30 min	30 min	4°C ≤ 48 hrs or -20°C ≤ 3 months
Section 3: Sequencing Library Preparation			
3.1 Fragmentation and End Prep	65 min	30 min	
3.2 Post-Fragmentation and End Prep Size Selection	30 min	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
3.3 Adapter Ligation	30 min	15 min	
3.4 Post-Ligation Purification	30 min	30 min	
3.5 Barcoding Round 4	45 min	15 min	4°C ≤ 18 hrs in the thermocycler
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


Total Time
Hands-On Time
Hour 1
Hour 2
Hour 3
Hour 4
Hour 5
Hour 6
2.1
2.2
2.3
2.4
2.5
3.1
3.2
3.3
3.4
3.5
3.6
3.7
STOP

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/.

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. All user-supplied consumables and reagents should be RNase-free.
- 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.

Vortexing

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

Magnetic Racks and Bead Cleanups

- The Evercode MAX WT FFPE Library Prep workflow uses magnetic beads to clean up libraries. A magnetic rack capable of holding 1.5 mL tubes and the Parse Biosciences Magnetic Rack for 0.2 mL tubes are required for this protocol.
- When aspirating supernatants in the magnetic rack, angle pipette tips away from beads. Ensure tips are at the bottom of each tube to ensure all supernatant is removed.
- If beads are disturbed, dispense solution again and wait for 1 minute before trying to aspirate again.
- 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.

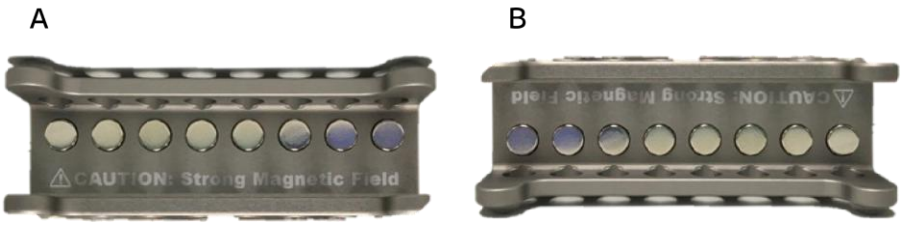


Figure 1: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- 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 cell.



Figure 2: Clear supernatant with compact bead pellets.

Sublibrary Loading

- 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.

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 Whole Transcriptome kits.

- Depending on your sequencing instrument and number of sublibraries, consider base diversity when selecting UDI combinations for sequence information. See Appendix A for index sequencing.

- 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 A.
- Record which UDI is added to each sublibrary. This information will be critical for the sequencing provider.

Part List

The Evercode MAX WT FFPE Library Prep 8 Reactions kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents. Store -20°C, PN XM080

LABEL	ITEM	PN	FORMAT	QTY
Bead Wash	Bead Wash Buffer	WT114	5 mL tube	1
Wash 1	Wash Buffer 1	WT115	5 mL tube	1
Wash 2	Wash Buffer 2	WT116	5 mL tube	1
Enhancer	Capture Enhancer	WT117	1.5 mL tube	1
Binding Buf	Binding Buffer	WT118	1.5 mL tube	1
Wash 3	Wash Buffer 3	WT119	5 mL tube	1
TS Buffer	Template Switch Buffer	WT120	1.5 mL tube	2
TS Enzyme	Template Switch Enzyme	WT121	1.5 mL tube	1
TS Primer	Template Switch Primer	MG122	1.5 mL tube	1
cDNA Amp Mix	cDNA Amp Mix	WT123	1.5 mL tube	1
cDNA Amp Primers	cDNA Amp Primers	MG124	1.5 mL tube	1
Fragm/End Prep Buf	Fragm/End Prep Buffer	WT125	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Fragm/End Prep Enzymes	WT126	1.5 mL tube	1
	Ligation Adapter	WT127	1.5 mL tube	1
	Adapter Ligation Buffer	WT128	1.5 mL tube	1
	Adapter Ligation Enzyme	WT129	1.5 mL tube	1
	Library Amp Mix	WT130	1.5 mL tube	1
	PCR Additive	MG150	1.5 mL tube	1

4°C Reagents. Store 4°C, PN XMF090

LABEL	ITEM	PN	FORMAT	QTY
	Streptavidin Beads	PT201	2 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
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.	☐
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.	☐
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.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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.	☐
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	CHECK LIST
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free, 10 mL or 25 mL reagent basins.	
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial nuclei loss.	☐
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.	☐
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.	☐
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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
AMPure® XP Reagent	Beckman Coulter®	A63880 (5 mL) A63881 (60 mL)	Choose one. We do not recommend substituting other magnetic beads.	☐
SPRIselect Reagent	Beckman Coulter	B23317 (5 mL) B23318 (60 mL)		☐
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30 mL)		☐
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.	☐
TE Buffer pH 8.0	Various Suppliers	Varies	Optional. Choose one that is RNase-free.	☐
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)		☐

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 a 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	2	4	6	8
● Streptavidin Beads	132 µL	264 µL	396 µL	528 µL

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

12. Remove and discard the supernatant.

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

Lysates being processed	2	4	6	8
○ Bead Wash Buffer	100 µL	200 µL	300 µL	400 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	2	4	6	8
● Binding Buffer	110 µL	220 µL	330 µL	440 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. The Wash Buffer 1 should remain at **room temperature** prior to addition to the sample.

27. After incubation, 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 ○ Wash Buffer 1.

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

Note: Save ○ 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 3′ 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 1.5 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	2	4	6	8
● Template Switch Buffer	203.5 µL	407 µL	610.5 µL	814 µL
● Template Switch Primer	5.5 µL	11 µL	16.5 µL	22 µL
● Template Switch Enzyme	11 µL	22 µL	33 µL	44 µL
Total	220 µL	440 µL	660 µL	880 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 O Wash Buffer 3 as it will be used in another step.

6. Incubate for **1 minute** at room temperature.
7. While still on the magnetic rack, remove and discard the 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 1.5 mL tube as follows. Mix by pipetting 10x and store on ice.

cDNA AMPLIFICATION MIX				
Number of Libraries	2	4	6	8
cDNA Amp Mix	120 µL	240 µL	360 µL	480 µL
cDNA Amp Primers	115 µL	230 µL	345 µL	460 µL
PCR Additive	5 µL	10 µL	15 µL	20 µL
Total	240 µL	480 µL	720 µL	960 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
2,000-4,000	15
4,000-8,000	14
8,000-16,000	13
16,000-32,000	12
32,000-64,000	11
64,000-80,000	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.

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

2.4. Post-Amplification Purification

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

To purify the cDNA:

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

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

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

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

4. While still on the magnetic rack, transfer **90 µL** of the supernatant containing the cDNA into 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.

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.

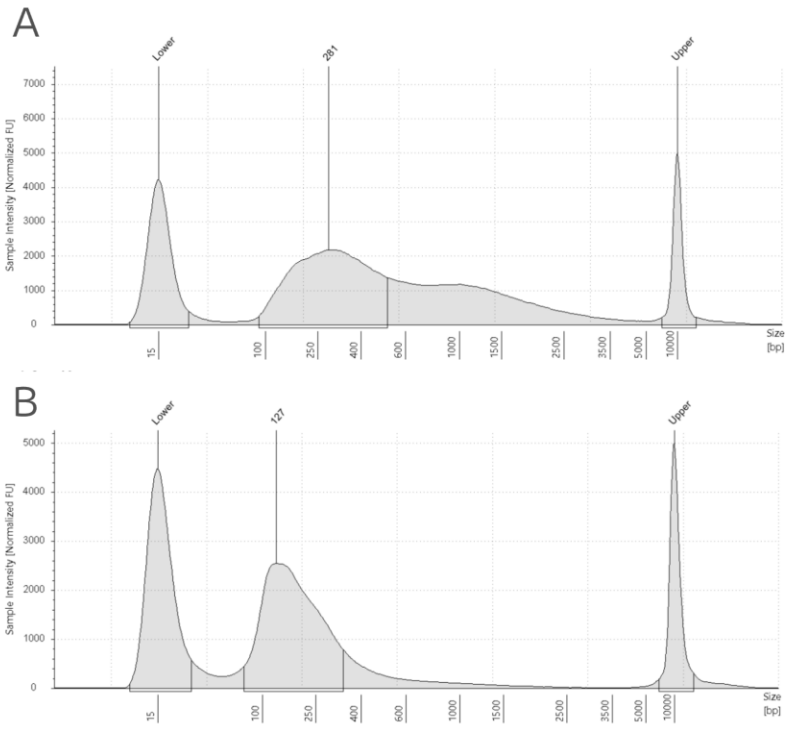


Figure 3: Expected post-amplification sublibrary cDNA size distribution. A: example of the trace of human cDNA with expected size distribution run on a TapeStation. B: Another example where the cDNA size distribution may shift toward lower sizes for FFPE samples and still produce high-quality data.

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

Note: The traces above are representative of typical TapeStation cDNA traces. The shape and prominence of the trace are 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	2	4	6	8
● Fragm/End Prep Buffer	11 µL	22 µL	33 µL	44 µL
● Fragm/End Prep Enzymes	22 µL	44 µL	66 µL	88 µL
Total	33 µL	66 µL	99 µL	132 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 is 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 Libraries	2	4	6	8
Nuclease-free water	38.5 µL	77 µL	115.5 µL	154 µL
Adapter Ligation Buffer	44 µL	88 µL	132 µL	176 µL
Adapter Ligation Enzyme	22 µL	44 µL	66 µL	88 µL
Ligation Adapter	5.5 µL	11 µL	16.5 µL	22 µL
Total	110 µL	220 µL	330 µL	440 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.

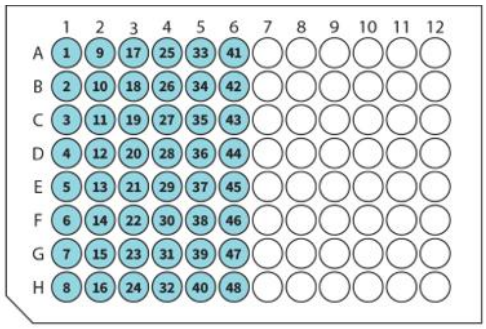
17. Proceed immediately to Section 3.5.

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 or TE Buffer (pH 8.0).
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.

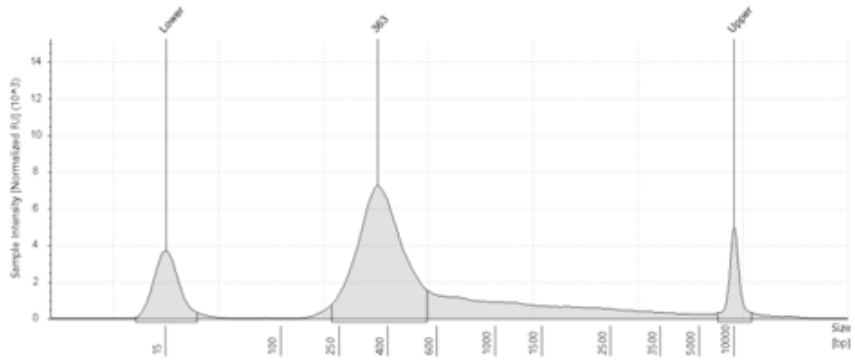


Figure 4: Expected Size Distribution before Illumina Sequencing. Example trace of human DNA from indexed sublibraries run on a TapeStation.

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

Note: The traces above are representative of typical TapeStation traces of DNA from indexed sublibraries. There should be a peak between 400-500 bp. The prominence of the trace is dependent on the 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: 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 heterogeneous population, deeper sequencing is required. Alternatively, when analyzing a homogeneous 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.

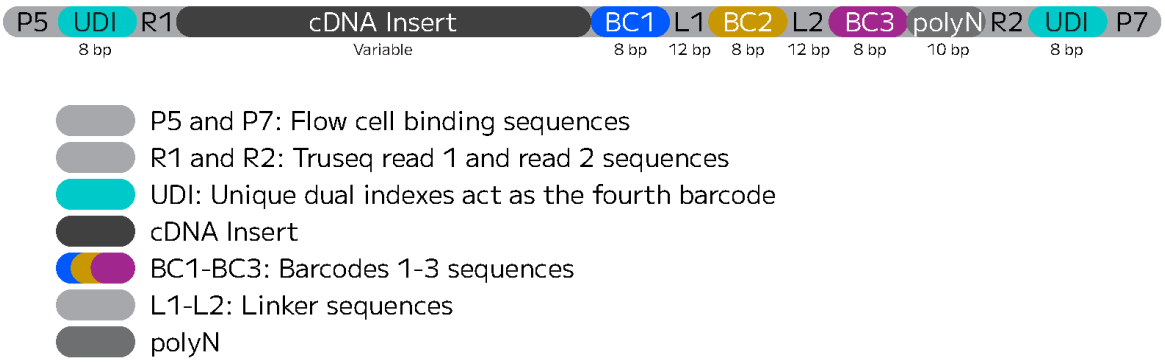


Figure 5: Whole transcriptome sequencing library structure.

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 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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
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 B: Thermocycling Programs

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 C: Revision History

Version	Description	Date
1.0	Initial release	November 2026

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