User Manual

Version 1.0 – UMMB4160

Evercode™ MAX BCR v4 Library Prep

16 Reactions

For use with

ECMB4160

ECMB4161

ECMB4162

Support Suite

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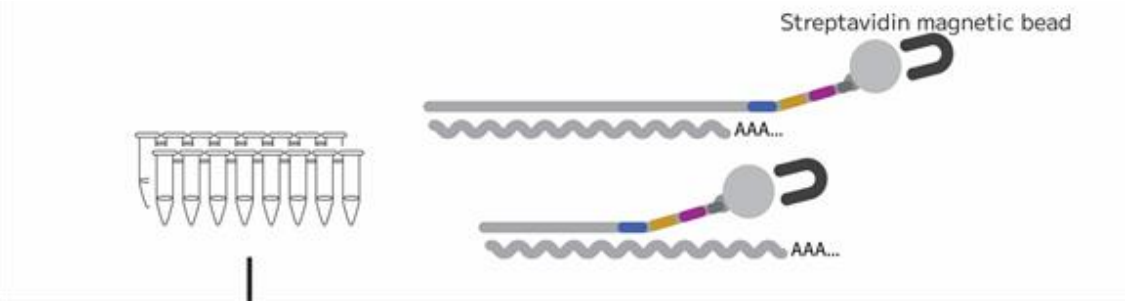
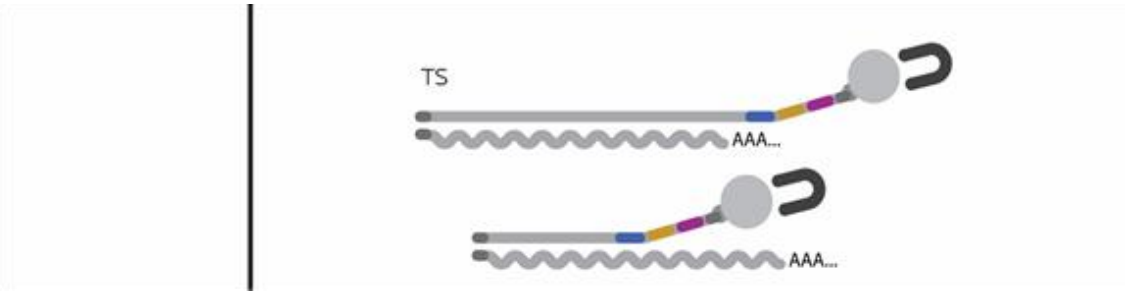
Overview

Workflow

This manual picks up where Evercode MAX BCR v4 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, 3, 4, and 5 of this manual complete the workflow by amplifying the cDNA, preparing the sequencing libraries, followed by BCR library preparation and amplification. This guide therefore should be completed after the Evercode MAX BCR v4 Cell Barcoding workflow.

This user guide can be used to prepare up to 16 sublibraries concurrently for sequencing.

This workflow supports the preparation of up to 32 sublibraries in a single run. For 32 reactions, two Evercode MAX BCR v4 Library Prep 16 Reactions kits are needed.

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.

Section 2: cDNA Capture and Amplification	
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.

Section 4: BCR Library Preparation	
1	  **WT Libraries Pool and Blocker Hybridization:** Up to 8 sublibraries are pooled per BCR capture reaction, and up to four separate BCR library pools are made to process up to 32 sublibraries. Evercode library-specific and repetitive-sequence blockers are hybridized.
2	  **BCR Hybridization:** Biotinylated probes in the BCR Panel are hybridized to the target DNA.

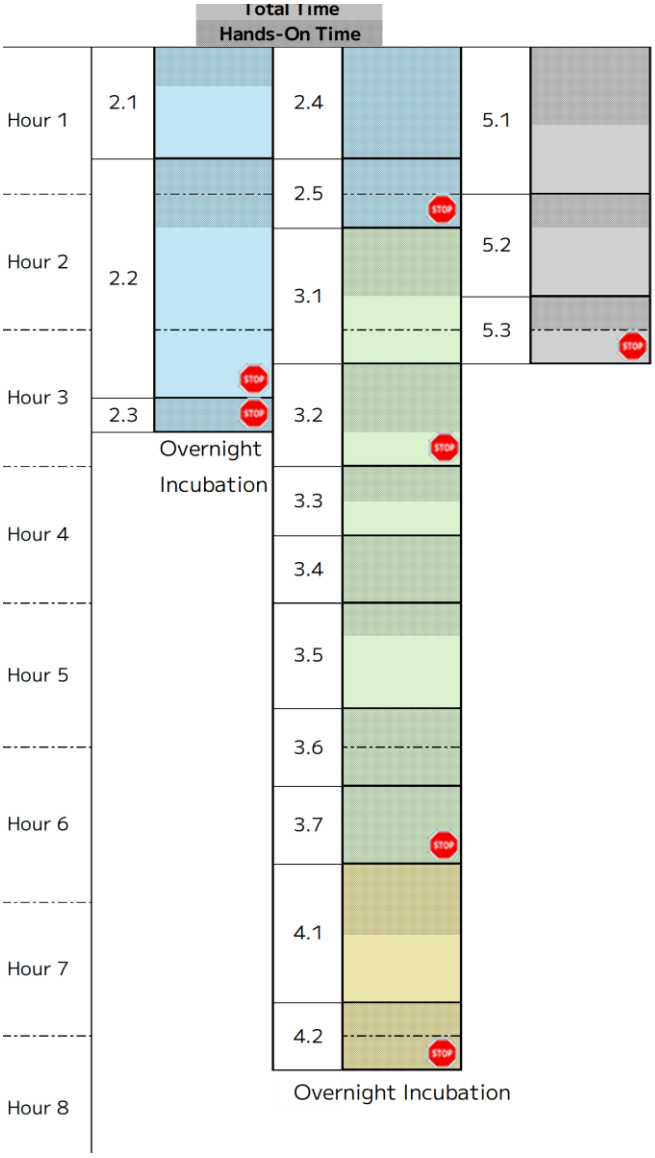
Section 5: BCR Amplification	
1	  **Bind and Wash:** Target molecules are captured with Streptavidin Binder Beads and washed.
2	  PCR-Amplified BCR Libraries **BCR Enrichment:** Captured molecules are enriched via PCR. The amplified double-stranded libraries are ready to sequence.

Protocol Timing

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

Preparing more than 16 sublibraries using plate magnets will add about 30 minutes to the workflow.

DESCRIPTION	TIME	HANDS-ON TIME	STOPPING POINTS
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	45 min	10 min	
2.2 cDNA Template Switch	105 min	15 min	4°C ≤ 18 hrs
2.3 cDNA Amplification	90 min	10 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	60 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
Section 4: BCR Library Preparation			
4.1 Pool Libraries	60 min	30 min	
4.2 Blocking & Hybridization	15-17 hrs	30 min	
Section 5: BCR Amplification			
5.1 Bind and Wash	60 min	30 min	
5.2 BCR Amplification	40 min	10 min	
5.3 BCR Library Quantification	20 min	10 min	-20°C ≤ 3 months


Total Time
Hands-On Time
Hour 1
Hour 2
Hour 3
Hour 4
Hour 5
Hour 6
Hour 7
Hour 8
2.1
2.2
2.3
2.4
2.5
3.1
3.2
3.3
3.4
3.5
3.6
3.7
4.1
4.2
5.1
5.2
5.3
STOP
Overnight Incubation
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/.

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.

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 on the benchtop and off ice will minimize this occurrence. PCR plate seals may be difficult to remove. Carefully peel the PCR plate seal while applying downward pressure on the plate to keep it in the PCR tube rack.

Magnetic Racks and Bead Cleanups

- The Evercode MAX BCR v4 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 figures below for clarification.

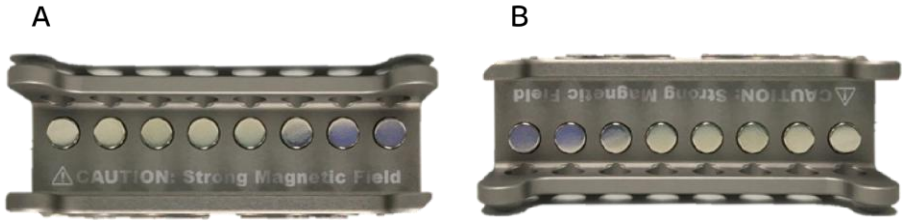


Figure 1: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- 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.

- The Evercode MAX BCR v4 workflow supports preparation of up to 32 sublibraries using two Evercode MAX BCR v4 Library Prep 16 Reactions kits. When planning to process samples for more than 16 sublibraries, we recommend using a PCR plate magnet for capturing lysed cells and library preparation steps. Dispense lysates into low-bind 96-well plates if using a 96-well plate magnetic rack.

Sublibrary Processing

- 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 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 sequences.

- 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.

BCR Library Generation

- The BCR library generation protocol begins with indexed sublibraries obtained with the Evercode BCR kit. For each library pool, up to 8 indexed sublibraries can be used for BCR library preparation. If processing more than 8 sublibraries, more than one BCR library pool should be prepared. Pooling sublibraries requires at least a concentration of 5 ng/µL per sublibrary. We recommend adding a minimum of 100 ng of each sublibrary to the BCR library pool for a maximum of 800 ng of total input per reaction.

- Each set of 16 sublibraries generates two BCR library pools (8 sublibraries per pool). To process up to 32 sublibraries in a single run, use two Evercode MAX BCR v4 Library Prep 16 Reactions kits, which will generate up to four BCR library pools.

- To ensure even sequencing coverage, sublibraries with similar cell numbers should be mixed in equal proportion. However, if any sublibraries have less than 100 ng of input, 50 ng of each sublibrary can be pooled in equal proportion to a total of 400 ng. Adjust the number of PCR cycles based on the indexed sublibraries' input amount to ensure optimal amplification.

Sublibrary Concentration

- SPRI beads are used to concentrate libraries during Section 4.1 of the Evercode BCR workflow and will remain in the solution through subsequent steps. These beads are

not removed immediately but will be removed during the post-PCR library cleanup in Section 5.2. In Section 5.1, streptavidin beads are introduced to capture target molecules. As a result, during Section 5 both SPRI and streptavidin beads will be present in the sample.



Thermocycling Programs

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

Part List BCR

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

-20°C Reagents. Store -20°C, XHB160 or XMB160 or XTMB160

LABEL	ITEM	PN	FORMAT	QTY
Wash A	Wash Buffer A	MG143	5 mL tube	1
Bead Wash	Bead Wash Buffer	MG114	5 mL tube	1
Binding Buf	Binding Buffer	MG118	1.5 mL tube	1
Wash B	Wash Buffer B	MG144	5 mL tube	1
TS Buffer	Template Switch Buffer	WT148	1.5 mL tube	4
TS Enzyme	Template Switch Enzyme	MG121	1.5 mL tube	1
TS Primer	Template Switch Primer	MG122	1.5 mL tube	1
PCR Additive	PCR Additive	MG150	1.5 mL tube	1
cDNA Amp Mix	cDNA Amp Mix	MG123	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	MG125	1.5 mL tube	1
Frag/End Prep Enzy	Fragm/End Prep Enzymes	MG126	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Ligation Adapter	MG127	1.5 mL tube	1
	Adapter Ligation Buffer	MG128	1.5 mL tube	1
	Adapter Ligation Enzyme	MG129	1.5 mL tube	1
	Library Amp Mix	MG130	1.5 mL tube	1
	Hybridization Mix	GC101	1.5 mL tube	1
	Hybridization Enhancer	GC102	1.5 mL tube	1
	Blocker Solution	GC103	1.5 mL tube	1
	Evercode Blocker Solution	GC108	0.5 mL tube	1
 or  or 	Human BCR Panel v4 or Mouse BCR Panel v4 or Transgenic Mouse BCR Panel v4	GC112 or GC113 or GC114	0.5 mL tube	1
	Enrichment Primer Mix	GC105	1.5 mL tube	1
	Enrichment Amplification Mix	GC106	0.5 mL tube	1

4°C Reagents. Store 4°C, PN XHB170 or XMB170 or XTMB170

LABEL	ITEM	PN	FORMAT	QTY
Strep Beads	Streptavidin Beads	PT201	2 mL tube	1
Bead Wash A	Bead Wash Buffer A	GC301	1.5 mL tube	1
Bead Wash B	Bead Wash Buffer B	GC302	2 mL tube	1
Strep Binding Buff	Streptavidin Binding Buffer	GC305	8 mL tube	1
Strep Bind Beads	Streptavidin Binder Beads	GC304	1.5 mL tube	1

User Supplied Equipment and Reagents

The following materials and equipment 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
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.	☐
Permagen Bar Magnet	Permagen®	MSPU650	Optional: if preparing >16 sublibraries. We do not recommend alternative magnets, as they may result in lower transcript and gene detection.	☐
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.	☐
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.	☐
PCR tube rack	Various Suppliers	Varies	Capable of holding semi-skirted 96-well PCR plates.	☐
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.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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		☐
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.	☐
4200 TapeStation System	Agilent	G2991BA		

Consumables

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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.	☐
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.	☐
twin.tec™ PCR Plates LoBind™	Eppendorf	0030129504	Optional if preparing more than 16 sublibraries: Or equivalent DNA low-binding, nuclease-free 96-well plate.	☐
SealPlate®	Excel Scientific	100-SEAL-PLT	Optional: Or equivalent PCR plate seals.	☐

Reagents

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
UDI Plate-WT	Parse Biosciences	UDI1001	Each 96-well plate contains 48 unique single-use reactions	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
			sufficient for multiple Evercode Library Prep kits (48 sublibraries). UDI Plate-WT can be purchased separately or bundled with Evercode kits.	
SPRIselect Reagent	Beckman Coulter®	B23317 (5 mL) B23318 (60 mL)	Choose one. We do not recommend substituting other magnetic beads.	☐
AMPure® XP Reagent	Beckman Coulter	A63880 (5 mL) A63881 (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 ethanol.	☐
TE Buffer pH 8.0	Various Suppliers	Varies	Optional. Choose one that is RNase-free.	☐
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.	☐
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)		
High Sensitivity D1000 ScreenTape and Reagents	Agilent	5067-5584 (screen tape) 5067-5585 (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 cellular debris. To prepare up to 32 sublibraries at once, use two Evercode MAX BCR v4 Library Prep 16 Reactions kits.



Note: Optional: if preparing more than 16 sublibraries, we recommend using a plate magnet for bead cleanup throughout Section 2. See "User Supplied Equipment and Reagents" for recommended plate magnetic racks.



CRITICAL! Always use new tips for each sublibrary.

To capture the cDNA:

1. Fill an ice bucket.
2. Gather the following equipment.
    a. Magnetic rack for 1.5 mL tubes
    b. Parse Biosciences magnetic rack for 0.2 mL PCR tubes
    c. 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 A	-20°C Reagents	1	
Wash Buffer B	-20°C Reagents	1	
Binding Buffer	-20°C Reagents	1	

4. 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. You will need 2 tubes if processing 32 samples.

Lysates Being Processed	4	8	12	16	32
● Streptavidin Beads	264 µL	528 µL	792 µL	1056 µL	2112 µL

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

6. Remove and discard the supernatant.

7. 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	4	8	12	16	32
○ Bead Wash Buffer	200 µL	400 µL	600 µL	800 µL	1600 µL

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

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

9. Remove and discard the supernatant.

10. Repeat steps 7-9 twice for a total of 3 washes.

11. 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	4	8	12	16	32
● Binding Buffer	220 µL	440 µL	660 µL	880 µL	1760 µL

12. Remove the desired tube(s) of lysate from the thermocycler (if continuing directly from Evercode MAX BCR v4 Cell Barcoding) or from storage at -80°C.

13. If previously frozen, incubate the tube(s) in a water bath or thermocycler at 37°C for **5 minutes**.

THAW LYSATES		
Run Time	5 minutes	
Lid Temperature	40°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C



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

14. Briefly centrifuge the lysates and store at room temperature.

15. Add **50 µL** of ● Streptavidin Beads in ●Binding Buffer to each tube of lysate.

16. 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.

17. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex plastic holder on 100% power for **1 minute.**

18. Vortex on 20% power (~800-1000 RPM) for **30 minutes** at room temperature.



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.

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

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

21. 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 cell. See "Magnetic Racks and Bead Cleanups" in the Important Guidelines section for representative images.

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

23. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ○ **Wash Buffer A** by vortexing for **10 seconds** (100% power).



Note: Save ○Wash Buffer A to use for optional storage before cDNA amplification.

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

25. 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.

CRITICAL! Always use new tips for each sublibrary.

To perform template switch:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Template Switch Buffer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by pipetting **5x**. Briefly centrifuge before use.
Template Switch Primer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by pipetting 3x. Briefly centrifuge before use.
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 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 Sublibraries	4	8	12	16	32
Template Switch Buffer	348 µL	696 µL	1044 µL	1392 µL	2784 µL
Template Switch Primer	12 µL	24 µL	36 µL	48 µL	96 µL
Template Switch Enzyme	24 µL	48 µL	72 µL	96 µL	192 µL
Total	384 µL	768 µL	1152 µL	1536 µL	3072 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. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** O **Wash Buffer B** by vortexing for **10 seconds** (100% power).



CRITICAL! Do not discard the remaining O Wash Buffer B as it will be used in another step.

6. 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).

7. While still on the magnetic rack, remove and discard the O **Wash Buffer B**.

8. Remove the tube(s) from the magnetic rack. Add **20 µL** nuclease-free water to each bead pellet and then fully resuspend by vortexing for **10 seconds**, followed by a brief centrifugation.

9. Add **80 µL** of the Template Switch Master Mix to each resuspended pellet and mix 5x with a P200 set to 80 µL.



Note: Because the Template Switch Master Mix is viscous, it may take time to fully mix the beads in solution.

10. Briefly centrifuge without letting beads collect at the bottom of the tube(s).

11. Incubate for **30 minutes** at room temperature.

12. Fully resuspend each bead pellet by mixing 5x with a P200 set to 75 µL.

13. 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

14. Proceed immediately to Section 2.3. Alternatively, proceed to step 15 to store samples prior to cDNA amplification.

15. 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 prior to placing on the magnet.

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

17. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** OWash Buffer A by vortexing for **10 seconds** (100% power).



Safe stopping point: Template-switched cDNA can be stored in Wash Buffer A 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.



CRITICAL! Always use new tips for each sublibrary.

To amplify the cDNA:

1. 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	

2. Equilibrate **80 µL** of SPRI beads per lysate to room temperature. Immediately proceed to step 3 after placing SPRI beads at room temperature. These will be used later.

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

cDNA AMPLIFICATION MIX					
Number of Sublibraries	4	8	12	16	32
cDNA Amp Mix	240 µL	480 µL	720 µL	960 µL	1920 µL
cDNA Amp Primers	230 µL	460 µL	690 µL	920 µL	1840 µL
PCR Additive	10 µL	20 µL	30 µL	40 µL	80 µL
Total	480 µL	960 µL	1440 µL	1920 µL	3840 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 prior to placing them on the magnet.

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

6. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** ○ **Wash Buffer B** by vortexing for **10 seconds** (100% power). Briefly centrifuge.

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

8. While still on the magnetic rack, remove and discard the ○ Wash Buffer B.

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

10. Determine the number of PCR cycles required for cDNA amplification based on the table below. Although these recommendations are appropriate for many cell types, the number of cycles may need to be optimized for each sample type.

NUMBER OF PCR CYCLES				
Cells in Sublibrary	Cell lines	Human Primary B Cells	Human PBMCs	Mouse Primary B Cells
200-1,000	11	12	13	14
1,000-2,000	9	10	11	12
2,000-4,000	8	9	10	11
4,000-8,000	7	9	10	11
8,000-16,000	6	7	8	9
16,000-32,000	4	5	6	7
32,000-64,000	3	4	5	6

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	Cycle
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 cells, they should be amplified in separate thermocyclers according to the recommendations above.



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



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

2.4. Post-Amplification Purification

Amplified cDNA is purified with a single-sided SPRI bead cleanup.



CRITICAL! Always use new tips for each sublibrary.

To purify the cDNA:

1. Gather **400 µL** of freshly prepared 85% ethanol for each tube of amplified cDNA.
2. Gather room temperature SPRI beads (**80 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, remove from the magnet and 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 **72 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. With a pipette, fully resuspend each bead pellet with **25 µL** of nuclease-free water.

16. Incubate for **5 minutes** at room temperature.

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 immediately to cDNA quantification.

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.

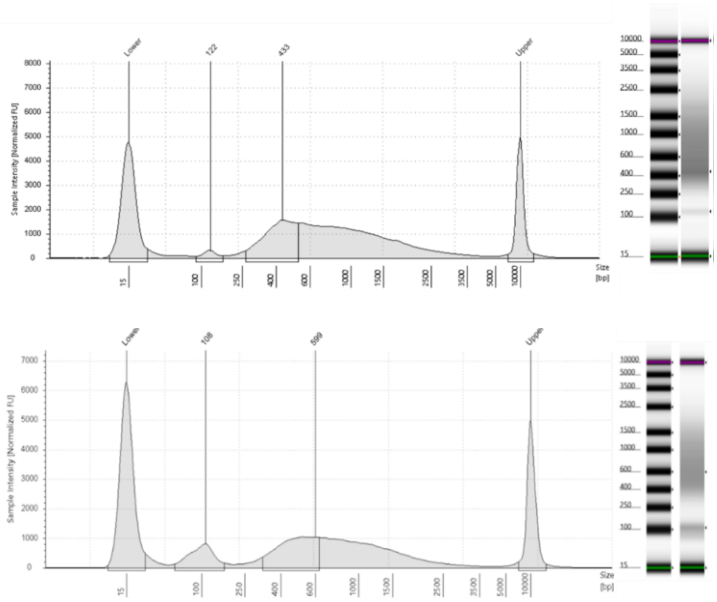


Figure 3: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a TapeStation.

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 cDNA traces. The shape and prominence of the trace is dependent on cell type, sublibrary size, and amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high-quality data.

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.



Note: Optional: if preparing more than 16 sublibraries, we recommend using a plate magnet for bead cleanup throughout Section 3. See "User Supplied Equipment and Reagents" for recommended plate magnetic racks.



CRITICAL! Always use new tips for each sublibrary.

To prepare for fragmentation and end prep:

1. For each sublibrary, prepare **1.2 mL** of fresh 85% ethanol with nuclease-free water. This will be used in Section 3.2.
2. Equilibrate **180 µL** of SPRI beads per sublibrary to room temperature. This will be used in Section 3.2.
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. Vortex to mix.
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		
Amount of cDNA	≥10 ng/µL	<10 ng/µL
Purified cDNA	10 µL	100 ng
Nuclease-free water	25 µL	Variable
Total Volume	35 µL	35 µL

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

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. Mix ● Fragm/End Prep Buffer by vortexing for **5 seconds**. Briefly centrifuge and store on ice.

Note: Confirm the ● Fragm/End Prep Buffer is fully thawed and mixed 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 Libraries	4	8	12	16	32
Fragm/End Prep Buffer	24 µL	48 µL	72 µL	96 µL	192 µL
Fragm/End Prep Enzymes	48 µL	96 µL	144 µL	192 µL	384 µL
Total	72 µL	144 µL	216 µL	288 µL	576 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.

CRITICAL! Always use new tips for each sublibrary.

To size-select the fragmented and end prepped DNA:

1. Gather freshly prepared 85% ethanol prepared in Section 3.1.
2. Gather room temperature SPRI beads prepared in Section 3.1.

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 **40 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).

CRITICAL! Ensure the solution is completely clear before proceeding.

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 tube as follows. Mix by pipetting 10x and store on ice.

ADAPTER LIGATION MASTER MIX					
Number of Libraries	4	8	12	16	32
Nuclease-free water	77 µL	154 µL	231 µL	308 µL	616 µL
Adapter Ligation Buffer	88 µL	176 µL	264 µL	352 µL	704 µL
Adapter Ligation Enzyme	44 µL	88 µL	132 µL	176 µL	352 µL
Ligation Adapter	11 µL	22 µL	33 µL	44 µL	88 µL
Total	220 µL	440 µL	660 µL	880 µL	1760 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 size-selected with a single-sided SPRI bead cleanup.

CRITICAL! Always use new tips for each sublibrary.

To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (**~90 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 **80 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

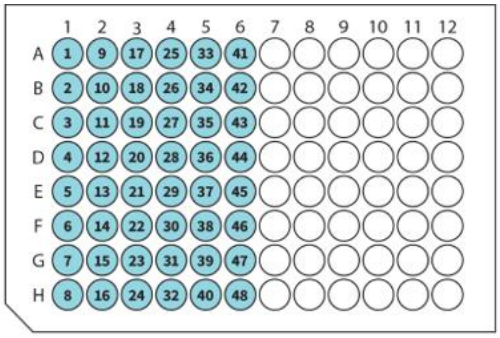
Purified adapter-ligated DNA is PCR amplified with Illumina TruSeq Read 1 and Read 2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.

Note: Depending on your sequencing instrument and number of sublibraries, consider base diversity when selecting UDI combinations (see Appendix A) for sequence information. If sequencing two libraries, select index adapters with diverse sequences or contact Parse Support at support@parsebiosciences.com.

CRITICAL! Always use new tips for each UDI plate's well and corresponding sublibrary.

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 it 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.



Note: cDNA Input (ng) = cDNA concentration (ng/µL) x volume added (µL). Example: 10 µL at 10 ng/µL = 100 ng total input.

NUMBER OF PCR CYCLES	
cDNA Input (ng)	PCR Cycles
10-24	13
25-49	12
50-99	11
100-199	10
200-399	9
400-999	8
1,000 or more	7

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 sec	98°C	Varies, see table above
3	20 sec	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.

CRITICAL! Always use new tips for each sublibrary.

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 **40 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).

CRITICAL! This may take longer due to the low volume of beads. Ensure the solution is completely clear before proceeding.

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 or D1000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

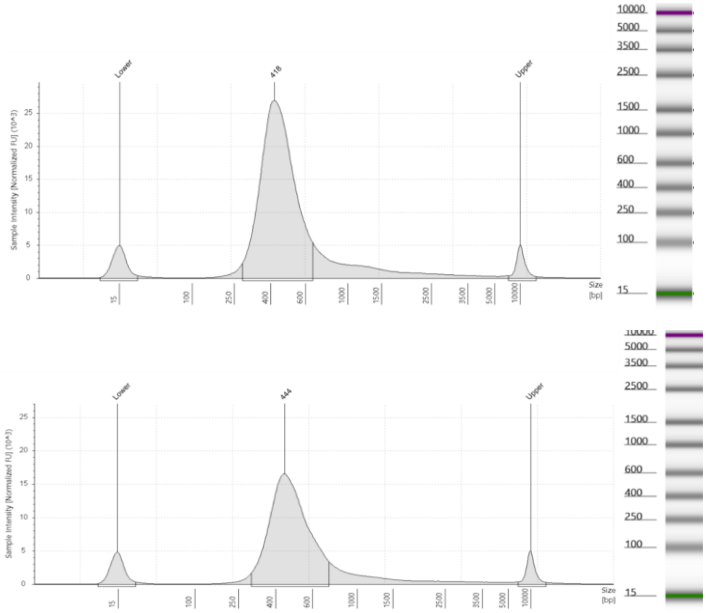


Figure 4: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA (Top) and Mouse DNA (Bottom) from indexed sublibraries run on a TapeStation.

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.

Section 4: BCR Sequencing Library Preparation

4.1. Pool Libraries

Libraries are pooled and prepared for the hybridization reaction. Up to 8 indexed sublibraries can be pooled per BCR library preparation. If processing more than 8 sublibraries, more than one BCR library pool should be prepared. No more than **12 µL** per sublibrary should be used to prepare the BCR library to ensure that there is sufficient input for WT sequencing. When pooling sublibraries in step 10, vacuum concentration can be used rather than SPRI bead concentration. See Appendix C for a protocol.



Note: When working with more than 16 sublibraries and two Library Prep 16 Reactions kits, prepare 3–4 BCR library pools. Up to 8 sublibraries can be pooled per BCR library pool.

To prepare for BCR library preparation:

1. Fill a bucket with ice.
2. Set a heat block to 65°C.
3. Program a thermocycler to 95°C and set the heated lid to 105°C.
4. Equilibrate SPRI beads (SPRIselect, AMPure XP, or KAPA Pure Beads) to room temperature for at least 30 minutes.
5. Prepare at least 700 µL of 85% ethanol per reaction and the magnetic rack for 1.5 mL tubes.
6. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Evercode BCR Indexed Sublibraries	User Stored Location (-20°C)	Up to 8 per reaction	Thaw on ice.
Hybridization Mix	-20°C Reagents	1	Heat at 65°C for 10 minutes, then keep at room temperature.
Hybridization Enhancer	-20°C Reagents	1	Thaw on ice. Briefly centrifuge before use.
Blocker Solution	-20°C Reagents	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Evercode Blocker Solution	-20°C Reagents	1	
○ Human or Mouse or Transgenic Mouse BCR Panel v4	-20°C Reagents	1	

7. Incubate the ● Hybridization Mix in the heat block at 65°C for **10 minutes** or until no precipitate is present before proceeding.

8. Incubate the ● Hybridization Mix at room temperature for **5 minutes** to equilibrate the solution.

9. Vortex sublibraries (2-3 seconds). Briefly centrifuge (~2 seconds).

10. Pool indexed sublibraries into a 1.5 mL tube to make the Library Pool(s). We recommend adding **100 ng** of each sublibrary going into BCR library preparation for a maximum of 800 ng of input per tube. If using 16 sublibraries, pool 8 sublibraries into one pool and the other 8 sublibraries into the other pool. For 17 to 32 sublibraries, 3 or 4 pools can be made.

Number of Sublibraries per Pool	
Sublibraries	BCR libraries
1-8	1 pool
9-16	2 pools
17-24	3 pools
25-32	4 pools

Note: See "BCR Library Generation" in Important Guidelines if your sublibraries contain less than 100 ng of total mass.

11. If the total volume of the Library Pool(s) is less than **100 µL**, bring the volume up to 100 µL with nuclease-free water.

12. Vortex the SPRI beads until fully mixed. Add **1.8x** of SPRI beads to the Library Pool(s).

Note: For example, if a Library Pool is 100 µL, add (1.8 x 100 µL) = 180 µL of SPRI beads.

13. Vortex the tube(s) for **5 seconds**. Briefly centrifuge (~2 seconds).

14. Incubate tube(s) at room temperature for **5 minutes**.

15. Place the tube(s) in a 1.5 mL magnetic rack and wait for all the beads to bind to the magnet (~3 minutes: ensure the liquid is clear).

16. With the tube(s) still on the magnetic rack, slowly remove and discard the clear supernatant.

17. Without resuspending beads, add **300 µL** of 85% ethanol to the tube(s) using a P1000 and wait **1 minute**.

18. Using a pipette, aspirate and discard the ethanol from the tube(s).

19. Without resuspending beads, add another **300 µL** of 85% ethanol to the tube(s) using a P1000 and wait **1 minute**.

20. Using a pipette, aspirate and discard all of the ethanol from the tube(s) (it may be necessary to remove the final few drops with a P20 pipette). With the tube(s) still on the rack, air dry the beads (~2 minutes).



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

21. Remove the tube(s) from the magnetic rack. Proceed directly to Section 4.2.

4.2. Blocking and Hybridization

1. Add the following reagents individually to each Library Pool from step 4.1.21.

ADD TO EACH LIBRARY POOL	
ITEM	VOLUME (µL)
Evercode Blocker Solution	7
Blocker Solution	5
TOTAL	12

2. Resuspend the beads by gentle pipetting with a P20 set to 10 µL.

3. Briefly centrifuge and carefully transfer **12 µL** into each new 0.2 mL tube using a P20 set to 15 µL.

4. Heat the Library Pool(s) for **5 minutes** at 95°C in a thermocycler with the lid heated to 105°C, then immediately place at room temperature.

5. Prepare a Probe Solution in a **new 0.2 mL tube**. Mix by pipetting.

PROBE SOLUTION				
ITEM	Volume for 1 Library Pool	Volume for 2 Library Pools	Volume for 3 Library Pools	Volume for 4 Library Pools
Hybridization Mix	21 µL	42 µL	63 µL	84 µL
Human, Mouse, or Transgenic Mouse BCR Panel v4	4.2 µL	8.4 µL	12.6 µL	16.8 µL
Nuclease-free Water	4.2 µL	8.4 µL	12.6 µL	16.8 µL
TOTAL	29.4 µL	58.8 µL	88.2 µL	117.6 µL

6. Heat the Probe Solution for **2 minutes** at 95°C in a thermocycler with the lid heated to 105°C, then immediately cool for **5 minutes** on ice.

7. Separately, incubate both the Probe Solution and Library Pool(s) at room temperature for **5 minutes**. For timing, see the figure below.



Figure 5: Heating and denaturing steps for Probe Solution and Library Pool are efficiently performed together.

8. Set a thermocycler to 70°C with the lid at 85°C for infinite time.
9. Vortex the Probe Solution for **5 seconds**. Briefly centrifuge (~2 seconds).
10. Transfer **28 µL** of the Probe Solution into each Library Pool to create the Hybridization Reaction.
11. Vortex the tube(s) for **5 seconds**. Briefly centrifuge (~2 seconds).
12. Without mixing, add **30 µL** of ● Hybridization Enhancer to the top of the meniscus of the Hybridization Reaction from step 11.

CRITICAL! Do not mix after adding the Hybridization Enhancer. It should form a distinct layer above the hybridization reaction.

13. Briefly centrifuge tube(s) to ensure there are no bubbles present.
14. Ensure the tube(s) are tightly capped to prevent excess evaporation.
15. Incubate the Hybridization Reaction(s) (72 µL) at 70°C for **15-17 hours** in a thermocycler with the lid at 85°C.

Note: Hybridizing for less than 15 hours or more than 17 hours will impact hybridization efficiency.

Section 5: BCR Amplification

5.1. Bind and Wash

Streptavidin Binder Beads capture the hybridized BCR repertoire prior to amplification.

To prepare the BCR capture pool for amplification:

1. Gather two heat blocks: set one to 68°C (for 1.5 mL tubes) and the other to 48°C (for 2 mL and 5 mL tubes).

2. Equilibrate ● Streptavidin Binder Beads and the SPRI beads to room temperature for at least **30 minutes**.

Note: See Important Guidelines, "Sublibrary Concentration" for clarifications about the Streptavidin Beads and the SPRI beads in the sample(s).

3. Prepare the magnetic rack for 1.5 mL tubes.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Bead Wash Buffer A	4°C Reagents	1	Heat tube at 68°C and keep at 68°C.
● Bead Wash Buffer B	4°C Reagents	1	Heat tube at 48°C and keep at 48°C.
○ Streptavidin Binding Buffer	4°C Reagents	1	Heat tube at 48°C for 5 minutes. Then keep at room temperature.
● Streptavidin Binder Beads	4°C Reagents	1	Equilibrate at room temperature for at least 30 minutes.
● Enrichment Primer Mix	-20°C Reagents	1	Thaw at room temperature, then place on ice.
● Enrichment Amplification Mix	-20°C Reagents	1	Thaw on ice. Briefly centrifuge before use.

5. Check if precipitate is dissolved, then equilibrate the ○Streptavidin Binding Buffer to room temperature. Keep the ● Bead Wash Buffer A and ● Bead Wash Buffer B in their respective heat blocks.

6. Ensure the ● Streptavidin Binder Beads have been equilibrated to room temperature for at least **30 minutes**. Vortex the ● Streptavidin Binder Beads until mixed.

7. For each pool, add **100 µL** of ● Streptavidin Binder Beads to new 1.5 mL tube(s) for the hybridization reaction.

8. Add **200 µL** of ○Streptavidin Binding Buffer to the tube(s) with ● Streptavidin Binder Beads. Mix thoroughly by pipetting 10x with a P200 set to 200 µL.

9. Place the tube(s) in a 1.5 mL magnetic rack and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).

10. Using a pipette, aspirate and discard the supernatant from the tube(s). Remove tube(s) from the magnetic rack.

11. Repeat steps 9-11 twice for a total of three washes.

12. Add **200 µL** of ○Streptavidin Binding Buffer and resuspend the beads by vortexing until fully homogenized.

13. Heat the resuspended beads at 68°C for **10 minutes** before proceeding to the next step.

14. Set a P200 pipette to 80 µL. After the hybridization reaction from Section 4.2 is complete, open the thermocycler lid and immediately transfer the entire volume of the Hybridization Reaction into a corresponding tube of preheated ● Streptavidin Binder Beads from step 5.1.14. Mix by pipetting.



CRITICAL! The rapid transfer from the 70°C thermocycler in step 4.2.15 is a critical step for minimizing off-target binding. Do not remove the Hybridization Reaction tube from the thermocycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptavidin Binder Beads. Allowing the Hybridization Reaction to cool to room temperature for more than 5 minutes will result in as much as a 10–20% increase in off-target binding.

15. Incubate the tube(s) containing the Hybridization Reaction and ● Streptavidin Binder Beads mixture at 68°C for **5 minutes**.

16. Remove the tube(s) from the heat block. Do not vortex. Briefly centrifuge (~2 seconds).

17. Place the tube(s) in a magnetic rack and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).

18. Using a pipette, aspirate and discard the clear supernatant from the tube(s).



Note: Some Hybridization Enhancer reagent residue may be visible after supernatant removal and throughout each wash step. This will not affect the final capture product.

19. Remove the tube(s) from the magnetic rack and add **200 µL** of 68°C ● Bead Wash Buffer A. Mix by pipetting.

20. Incubate the tube(s) at 68°C for **5 minutes**. Briefly centrifuge.

21. For the hybridization reaction, transfer the entire volume from step 21 (~200 µL) into new 1.5 mL tube(s).



CRITICAL! This step reduces background from non-specific binding to the surface of the tube.

22. Place the tube(s) in a 1.5 mL magnetic rack and wait for all the beads to bind to the magnet (~1 minute: liquid should be clear).

23. Using a pipette, aspirate and discard the clear supernatant from the tube(s).

24. Remove the tube(s) from the magnetic rack and add **200 µL** of 48°C ● Bead Wash Buffer B. Mix by pipetting and briefly centrifuge.

25. Incubate the tube(s) at 48°C for **5 minutes** and place ● Bead Wash Buffer B back into the 48°C heat block.

26. Place the tube(s) in a magnetic rack and wait for all the beads to bind to the magnet (~1 minute: liquid should be clear).

27. Using a pipette, aspirate and discard the clear supernatant from the tube(s).

28. Repeat steps 25-28 twice for a total of three washes.

29. Remove any residual supernatant with a P20 pipette. Proceed immediately to the next step.

30. Remove the tube(s) from the magnetic rack and resuspend in **45 µL** of nuclease-free water. Mix by pipetting until homogenized, then incubate on ice. This solution will be referred to as Streptavidin Binding Bead Slurry.

31. Proceed immediately to Section 5.2.

5.2. BCR Amplification

The BCR repertoire is amplified for sequencing.

1. Prepare 400 µL of 85% ethanol per reaction.
2. Prepare the magnetic rack for 1.5 mL tubes.
3. Mix the Streptavidin Binding Bead Slurry by pipetting.
4. For the hybridization reaction, prepare a PCR mixture in new 0.2 mL PCR tube(s) as follows. Then, mix by pipetting, centrifuge briefly, and store on ice.

PCR MIXTURE	
REAGENTS	VOLUME (µL)
Streptavidin Binding Bead Slurry	22.5*
● Enrichment Primer Mix	2.5
● Enrichment Amplification Mix	25
TOTAL	50

Note: *When preparing the PCR mixture, only half the slurry is necessary for immediate use. Store the unused slurry at -20°C.

5. Determine the number of PCR cycles required for the total amount of input into the library pool, as shown in the table below.

NUMBER OF PCR CYCLES	
Library Pool (ng)	PCR Cycles
800	13
400	14
200	15
100	16

Note: For inputs between listed values, round up to the higher cycle number. For example, an input of 600 ng should use the cycle number listed for 400 ng, not 800 ng.

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

BCR AMPLIFICATION			
Run Time		~30 min	
Lid Temperature		105°C	
Sample Volume		50 µL	
Step	Time	Temperature	Cycles
1	45 sec	98°C	1
2	15 sec	98°C	Variable, see above
3	30 sec	60°C	
4	30 sec	72°C	
5	1 min	72°C	1
6	Hold	4°C	1

7. As soon as the program reaches 4°C, place the tube(s) at room temperature and proceed immediately to the next step.

8. Ensure the SPRI beads have been equilibrated to room temperature for at least **30 minutes**. Vortex the SPRI beads until fully mixed. Add **90 µL** of SPRI beads (1.8x) to the tube(s). Vortex for **5 seconds**.

Note: It is not necessary to recover supernatant or remove Streptavidin Binder Beads from the amplified PCR product.

9. Incubate tube(s) at room temperature for **5 minutes**.

10. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).

11. With tube(s) still on the magnetic rack, remove and discard the clear supernatant.

12. With tube(s) still on the magnetic rack, add **180 µL** of 85% ethanol to the tube(s).

13. Incubate tube(s) at room temperature for **1 minute**.



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

14. With tube(s) still on the magnetic rack, remove and discard the supernatant.
15. Repeat steps 12-14 once more for a total of 2 washes. Remove any residual ethanol with a P20 pipette.
16. With tube(s) still on the magnetic rack, air dry the beads (~**30 seconds**).
17. Remove the tube(s) from the magnetic rack. Fully resuspend the bead pellet with **32 µL** of nuclease-free water or TE Buffer (pH 8.0).
18. Incubate the tube(s) at room temperature for **5 minutes**.
19. Place the tube(s) on the low magnet position of the magnetic rack for 0.2 mL tubes and wait for all the beads to bind to the magnet (~2 minutes: liquid should be clear).
20. With tube(s) still on the magnetic rack, transfer **30 µL** of the supernatant containing the purified BCR sublibraries into new 0.2 mL tube(s). Store on ice.
21. Proceed to Section 5.3.

5.3. BCR Library Quantification

The concentration and size distribution of the sequencing libraries are measured with fluorescent dyes and capillary electrophoresis.

1. Measure the concentration of the BCR library from Section 5.2 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

2. Assess the size distribution of the purified DNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System according to the manufacturer's instructions. See Figure 6 for the expected size distribution.

3. For sequencing, refer to run configuration details in Appendix A.

STOP Sequencing libraries can be stored at -20°C for up to 3 months.

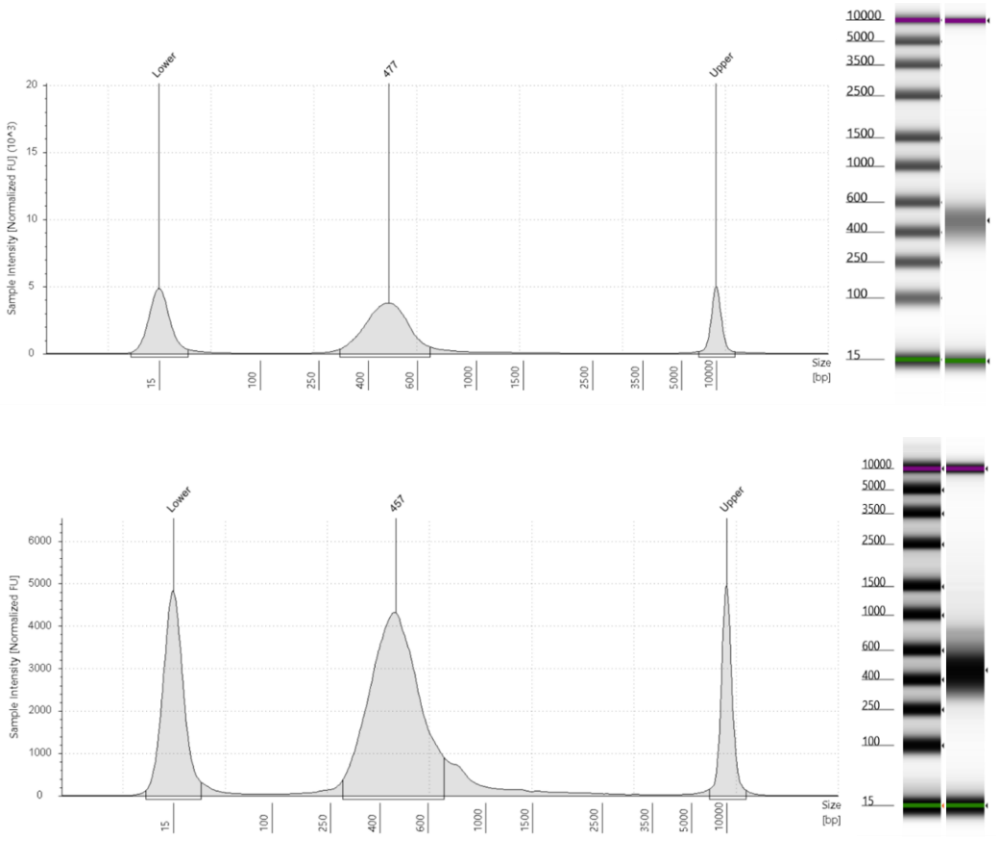


Figure 6: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA (top) and Mouse DNA (bottom) from indexed sublibraries run on a TapeStation.

Note: The traces above are representative of typical TapeStation traces of DNA from BCR 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 Bioanalyzer or TapeStation. Samples with minor deviations can still produce high-quality data.

Note: If quantifying DNA with a Bioanalyzer, there may be an additional peak present. If there is a 400-500 bp peak, it should not impact sequencing or data quality, but care should be taken when calculating the library concentration before sequencing.

Appendices

Appendix A: Sequencing Information

We recommend a minimum sequencing depth of 10,000 reads per cell for whole transcriptome libraries and 5,000 reads per cell for the BCR 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.

The diagram below illustrates the composition of the sequencing libraries generated. This library structure applies to both the whole transcriptome library and the BCR-enriched library.

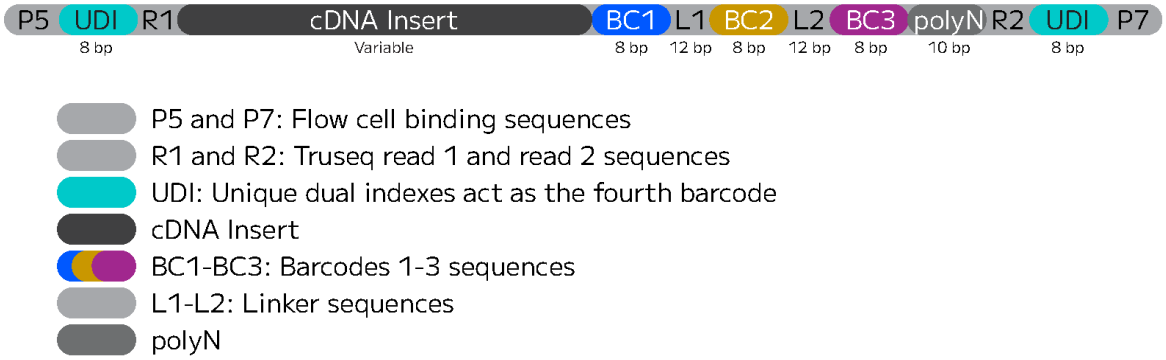


Figure 7: Whole transcriptome and BCR sequencing library structure.

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. Note that WT and BCR libraries have different sequencing requirements. The WT and BCR libraries should not be run together; however, they can be run in different lanes.

WHOLE TRANSCRIPTOME ILLUMINA RUN CONFIGURATION			
Run	**Function**	**WT Library Cycles**	**Notes**
Read 1	cDNA Insert	64	The DNA product used for this sequencing run should have come from Section 3.
i7 Index (Index 1)	Barcode 4 / UDI	8	
i5 Index (Index 2)	Barcode 4 / UDI	8	
Read 2	Barcodes 1-3	58	

Sequencing BCR libraries requires an Illumina 300-cycle kit. See the table below for details:

BCR ILLUMINA RUN CONFIGURATION			
Run	**Function**	**BCR Library Cycles**	**Note**
Read 1	cDNA Insert	242	The DNA product used for this sequencing run should have come from Section 5: Preparing BCR Libraries for Sequencing.
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 UDI Whole Transcriptome and BCR sublibraries.

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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_10	B2	TCATTCCT	AATGCCTG	CAGGCATT
UDI_Plate_WT_11	C2	GGTAGCAT	TGCTTGCC	GGCAAGCA
UDI_Plate_WT_12	D2	ACTTGATC	TTTGGGTG	CACCCAAA
UDI_Plate_WT_13	E2	ATGAGCAT	GAATCTGA	TCAGATTC
UDI_Plate_WT_14	F2	GCGCTATC	CGACTGGA	TCCAGTCG
UDI_Plate_WT_15	G2	TGACCAGT	ACATTGGC	GCCAATGT
UDI_Plate_WT_16	H2	TATAATCA	ACCACTGT	ACAGTGGT
UDI_Plate_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
UDI_Plate_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
UDI_Plate_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
UDI_Plate_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
UDI_Plate_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
UDI_Plate_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
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

THAW LYSATES		
Run Time	5 minutes	
Lid Temperature	40°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

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	Cycle
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

Section 3: Sequencing Library Preparation

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

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

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

Section 5: BCR Amplification

BCR AMPLIFICATION			
Run Time		~30 min	
Lid Temperature		105°C	
Sample Volume		50 µL	
Step	Time	Temperature	Cycles
1	45 sec	98°C	1
2	15 sec	98°C	Variable, see Section 5.2.5
3	30 sec	60°C	
4	30 sec	72°C	
5	1 min	72°C	1
6	Hold	4°C	1

Appendix C: Vacuum Concentration

Pooled sublibraries require at least a concentration of 5 ng/µL per sublibrary. We recommend adding a minimum of 100 ng of each sublibrary to the BCR library pool for a maximum of 800 ng of total input. We recommend adding a minimum of 50 ng of each sublibrary to the BCR library pool for a total of 100 ng minimum input. If you have enough material, up to 400 ng of each sublibrary can be added for a total of 800 ng maximum input.

When pooling sublibraries in Section 4.1, vacuum concentration can be used rather than SPRI bead concentration. See protocol below.

To vacuum concentrate the Pooled Sublibraries:

1. Pool 100 ng of each indexed sublibrary into a 0.2 mL PCR tube. Briefly centrifuge.
2. Dry the pool of sublibraries using a vacuum concentrator without heat or at the lowest heat setting.
3. The dried Library Pool can be stored at -20°C for 1 week. Otherwise, proceed directly to Section 4.2 Blocking and Hybridization and skip step 4.2.2.

Appendix D: Indexed SL Input and Pooling Guidelines for BCR Libraries

Indexed sublibraries generated in Section 3 are pooled before BCR library preparation. Up to 4 BCR libraries can be prepared per experiment. The acceptable range for each BCR library is 100–800 ng of total indexed sublibrary mass. 100 ng per sublibrary is recommended, but users can go as low as 50 ng per BCR pool.

To ensure even representation across sublibraries, we recommend adding a comparable mass of each indexed sublibrary to the pool. When the combined mass exceeds 800 ng, divide the sublibraries between two BCR libraries. Use the table below to determine the number of BCR libraries and the pooling strategy based on the number of indexed sublibraries.

When working with more than 16 sublibraries and two Library Prep 16 Reactions kits, prepare 3–4 BCR library pools.

Column 1 (Indexed SL): Indicates the total number of indexed sublibraries you have prepared for the BCR kit.

Column 2 (BCR Library Pools): Total number of pools that should be prepared based on the total number of indexed sublibraries. **Up to 8 indexed sublibraries can be combined per pool.**

Columns 3-6 (Pool 1-Pool 4): List the recommended number of indexed sublibraries to put in each pool and the total input mass for the pool. For example, for 9 indexed sublibraries you will need to create two pools. Pool 1 should contain 4 sublibraries (100 ng each) for a total of 400 ng input. Pool 2 should contain 5 sublibraries (100 ng each) for a total of 500 ng.

Indexed SL	BCR Library Pools	Pool 1	Pool 2	Pool 3	Pool 4
1	1	1 (100 ng)	—	—	—
2	1	2 (200 ng)	—	—	—
4	1	4 (400 ng)	—	—	—
6	1	6 (600 ng)	—	—	—
8	1	8 (800 ng)	—	—	—
9	2	4 (400 ng)	5 (500 ng)	—	—
12	2	6 (600 ng)	6 (600 ng)	—	—
13	2	6 (600 ng)	7 (700 ng)	—	—

Indexed SL	BCR Library Pools	Pool 1	Pool 2	Pool 3	Pool 4
14	2	7 (700 ng)	7 (700 ng)	—	—
15	2	7 (700 ng)	8 (800 ng)	—	—
16	2	8 (800 ng)	8 (800 ng)	—	—
17	3	5 (500 ng)	6 (600 ng)	6 (600 ng)	—
18	3	6 (600 ng)	6 (600 ng)	6 (600 ng)	—
20	3	6 (600 ng)	7 (700 ng)	7 (700 ng)	—
23	3	7 (700 ng)	8 (800 ng)	8 (800 ng)	—
24	3	8 (800 ng)	8 (800 ng)	8 (800 ng)	—
25	4	6 (600 ng)	6 (600 ng)	6 (600 ng)	7 (700 ng)
28	4	7 (700 ng)	7 (700 ng)	7 (700 ng)	7 (700 ng)
30	4	7 (700 ng)	7 (700 ng)	8 (800 ng)	8 (800 ng)
31	4	7 (700 ng)	8 (800 ng)	8 (800 ng)	8 (800 ng)
32	4	8 (800 ng)	8 (800 ng)	8 (800 ng)	8 (800 ng)

Appendix E: Revision History

Version	Description	Date
1.0	Initial release	October 2026

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