User Manual

Version 1.0 - UMMB4500

Evercode™ MAX BCR v4 Cell Barcoding L

For use with

ECMB4500

ECMB4510

ECMB4520

Support Suite

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Overview

Workflow

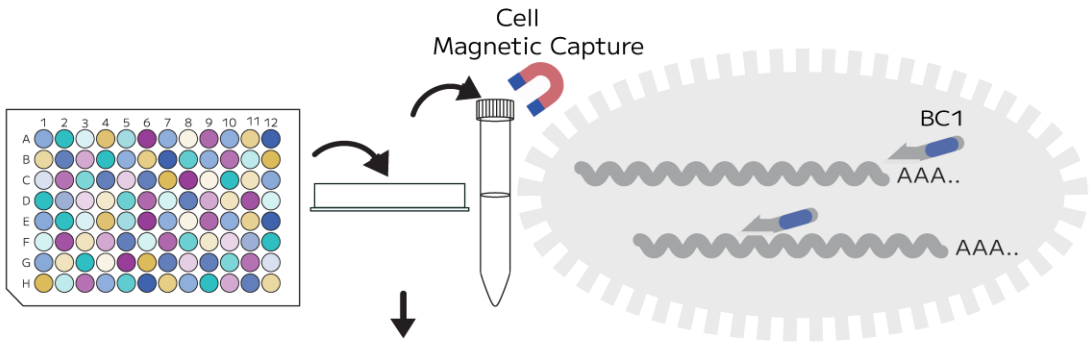
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. Evercode MAX BCR v4 Cell Barcoding kits add further flexibility by separating cell barcoding from library construction, so each dimension can be scaled independently based on experimental needs.

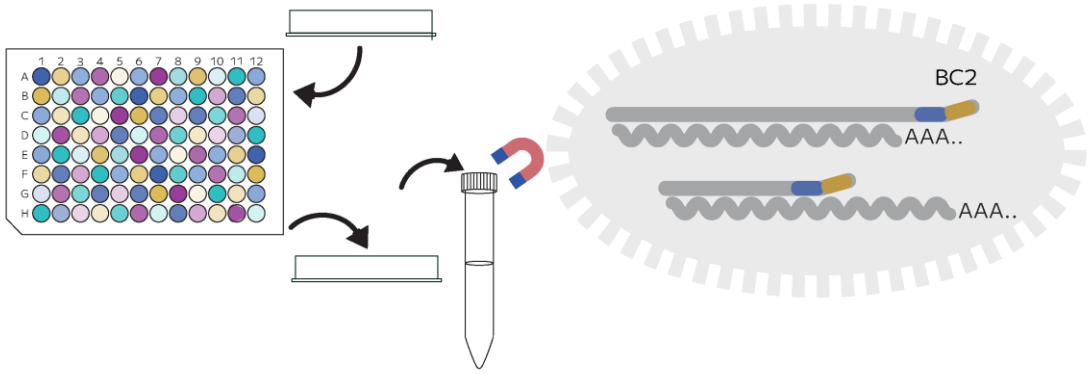
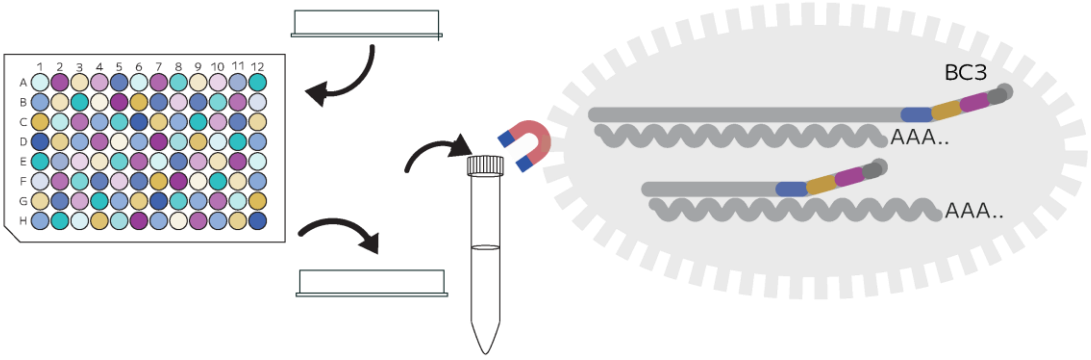
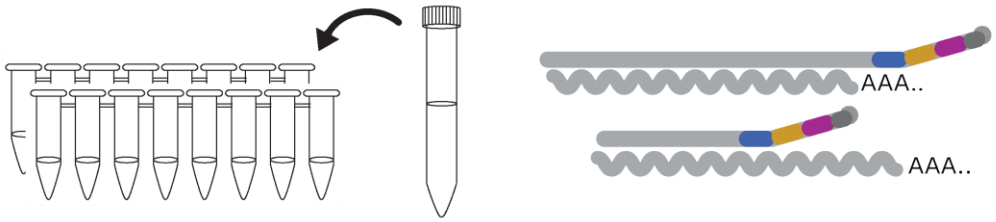
- Two sizes of Evercode MAX BCR v4 Cell Barcoding kits determine the number of samples that can be indexed in a given run.
- Two sizes of Evercode MAX BCR v4 Library Prep kits determine the number of sublibraries that can be prepared from the indexed samples.

Barcoding kits can be paired with any sublibrary kit. This lets users choose cell number and sublibrary count based on experimental design.

The Evercode MAX BCR v4 Cell Barcoding L kit can index up to 2,048,000 cells across up to 96 different samples. Anywhere between 2 and 32 sublibraries can be prepared following barcoding.

This manual describes the first 3 rounds of barcoding and the generation of sublibraries as lysates. Users can either store the lysates or proceed directly to library preparation.

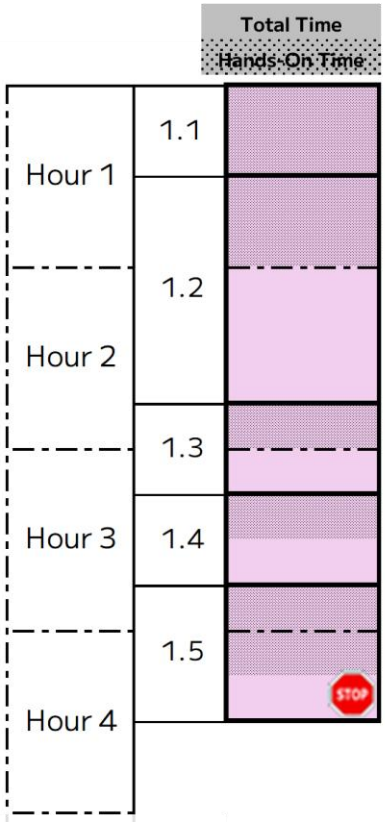
Section 1: *In situ* Sample Barcoding	
1	 **Round 1 Barcoding:** Fixed and permeabilized cells are loaded into 96 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with well-specific barcodes that are associated with specific samples. Barcoded samples are pooled and captured via magnetic beads.

Section 1: *In situ* Sample Barcoding	
2	 **Round 2 Barcoding:** The pooled cells are loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode. Cells are pooled and then captured via magnet.
3	 **Round 3 Barcoding:** The cells are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina TruSeq Read 2 sequence, and biotin. Cells are pooled and then captured via magnet.
4	 **Lysis and Sublibrary Generation:** Cells are split across the desired number of sublibraries and lysed.

Protocol Timing

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

DESCRIPTION	TIME	HANDS-ON TIME	STOPPING POINTS
Section 1: In situ Cell Barcoding			
1.1 Set up and Sample Counting	Variable (30-90 min)	Variable (30-90 min)	
1.2 Barcoding Round 1	75 min	35 min	
1.3 Barcoding Round 2	30 min	15 min	
1.4 Barcoding Round 3	30 min	15 min	
1.5 Lysis and Sublibrary Generation	40 min	25 min	-80°C ≤ 6 months



Important Guidelines

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

Sample Input

- When working with mouse B cells, use the Evercode Cell Fixation (Mouse TCR/BCR) workflow, which includes a murine RNase Inhibitor.
- This protocol begins with cells fixed with the Evercode Cell Fixation v3 and v4 kits, the Evercode Low Input Cell Fixation v3 and v4 kits, or the Evercode Whole Blood Fixation kit.
- Even if samples were counted before freezing, we strongly recommend counting cells again after thawing to account for any changes during storage and freeze-thaw. Typically, a 5-15% decrease after thawing should be expected. These counts are used to determine how cells are loaded in the Round 1 Plate, and their accuracy is critical to recover the desired number of cells.
- For standard fixed samples only: when processing many fixed samples (≥12), we strongly recommend generating counting aliquots of each sample after fixation and counting them the day before using an Evercode MAX Barcoding kit. The Evercode Fixation User Manuals outline recommendations for generating aliquots. Because the counting aliquots have undergone a similar storage time and a freeze-thaw, cell counts from these aliquots will be more representative than using counts obtained immediately after fixation. Aliquots should be thawed in a water bath set to 37°C in sets of 2-4 and counted with a hemocytometer or alternative counting device. Counts should be recorded in the Sample Loading Table, and any remaining counting aliquot should be discarded. Note that Low Input (LI) fixation reactions do not allow for a counting aliquot. Please follow guidance in the LI fixation manuals.
- Once fixed samples have been thawed, they should not be refrozen.

Cell Counting and Quality Assessment

- There is a count required at the pre-lysis sublibrary generation step. Due to the use of magnetic capture beads, a fluorescent counting device is required for this count. If possible, validate cell counts from an automatic counting device against a hemocytometer count ahead of use.

- Fluorescent stains such as acridine orange and propidium iodide (AO/PI) or DAPI should be used for counting.

- It is good practice to save images at each counting step for record keeping and troubleshooting purposes.

- Examples of AO/DAPI and trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells with <5% cell aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed samples, it is critical to avoid counting cell debris to avoid overestimating the number of cells.

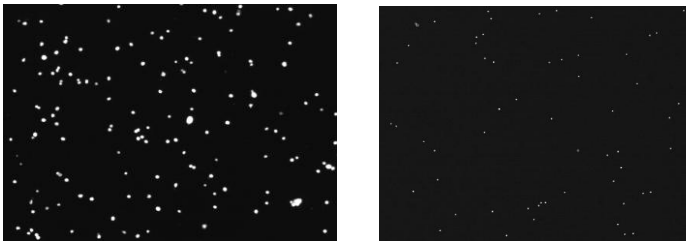


Figure 1: Example of AO/DAPI stained HEK cells (left) and PBMCs (right).

High Quality Sample Aggregation Debris

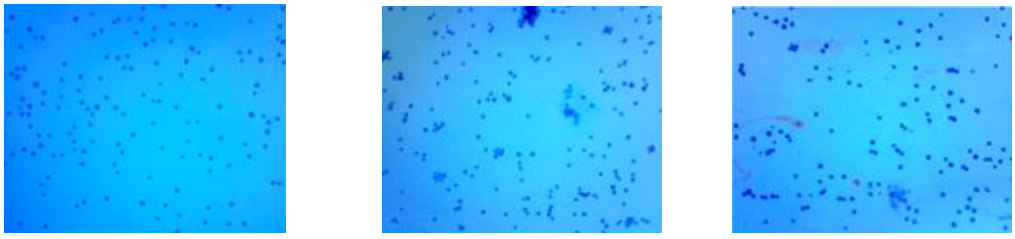


Figure 2: Example of trypan blue stained fixed cells.

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.

Optimizing Cell Recovery

- It is critical to thoroughly resuspend the cells after binding throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible.

Due to cell adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell loss.

- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.
- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- The first time using an Evercode MAX Barcoding kit, we recommend retaining supernatants after each barcoding step. In the unlikely event of unexpectedly high sample loss, these supernatants can be analyzed to identify points for optimization.

Sample Loading Table

- Ensure that the "Evercode™ MAX WT v4 Cell Barcoding L-Sample Loading Table" being used is the most up-to-date version. The Sample Loading Table can be downloaded from the Parse Biosciences Customer Support Suite. Customer log-in is required to access the Sample Loading Table.
- The "Evercode™ MAX WT v4 Cell Barcoding L-Sample Loading Table" should be completed before starting the experiment.
- If the table isn't working as expected, ensure that macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in the table to avoid disturbing any calculations.
- In the unlikely event that samples are not concentrated enough according to the Sample Loading Table, choose to either:
    - Decrease the "Max number barcoded cells" until the Sample Loading Table no longer gives an error, which will maintain the desired proportion between samples but barcode fewer cells.
    - Add 14 µL of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded cells but change the desired proportion between samples.

Cell Strainers

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 µm is appropriate for many cell types, the mesh size should be chosen based on the sample type.

- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second. An example video can be found on our support site.

Vortexing

- Unless specified in an individual step, do not vortex 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 Cell Barcoding L Barcoding workflow uses magnetic beads to clean up cells after each round of barcoding. A magnetic rack capable of holding 15 mL conical tubes, one capable of holding 1.5 mL tubes, and the Parse Biosciences Magnetic Rack for 0.2 mL tubes are required for this protocol.

- A list of validated 15 mL conical tube magnets is described in the "User Supplied Equipment and Reagents" section of this manual to choose from.

- When aspirating supernatant on the magnetic rack, angle pipette tips away from the beads. After removing most of the volume, position tips at the bottom of each tube to remove any remaining liquid.

- 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.

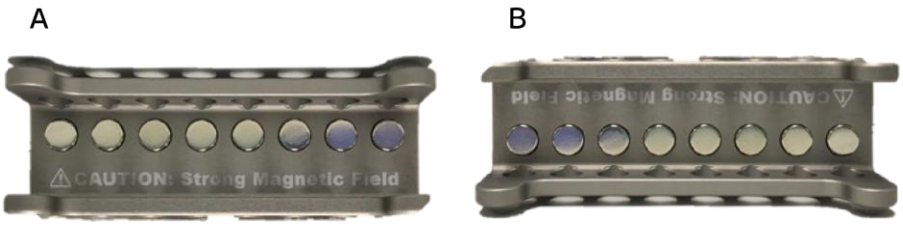


Figure 3: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- To ensure material is not lost during 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.



Figure 4: 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 M Library Prep 16-Reaction kits. When planning to barcode 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 Loading

- The Evercode MAX BCR v4 Cell Barcoding M kit generates sublibraries that can be processed and sequenced independently or concurrently after barcoding.
- Before proceeding with barcoding, refer to Appendix A to determine the number of sublibraries to prepare for your experimental needs.
- The number of cells per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix B.
- The maximum number of cells that can be barcoded is the sum of cells across all sublibraries.

Thermocycling Programs

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

Part List

The Evercode MAX BCR v4 Cell Barcoding L kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents. Store at -20°C, PN XHBL400 or XMBL400 or XTMBL400

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	HBG101 or MBG101	Green semi-skirted 96 well plate	1
	Round 2 Plate v4	MG142	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Bead Prep Buffer	MG136	1.5 mL tube	1
	Barcoding Buffer	MG137	15 mL bottle	1
	Round 1 Stop Mix	MG138	2 mL tube	1
	Round 2 Stop Mix	MG139	2 mL tube	1
	Ligation Enzyme	MG140	1.5 mL tube	1
	Sample Dilution Buffer	MG105	2 mL tube	1
	Ligation Buffer	MG134	15 mL bottle	1
	Final Stop Mix	MG141	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Pre-Lysis Dilution Buffer	MG112	2 mL tube	1
	Pre-Lysis Wash Buffer	MG110	5 mL tube	1
	Lysis Solution	PT146	1.5 mL tube	1
	Lysis Enzyme	PT109	1.5 mL tube	1

4°C Reagents. Store at 4°C, PN XHBL500 or XMBL500 or XTMBL500

LABEL	ITEM	PN	FORMAT	QTY
	Sample Binding Beads	PT149	0.5 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.	☐
Permagen Ring Magnet	Permagen®	S500	Needed only for processing low input samples.	☐
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.	☐
15 mL Conical Tube Magnet (choose one)	Permagen	MSR6X15	6 x 15 mL Centrifuge Magnetic Separation Rack.	☐
	Millipore	LSKMAGS15	PureProteome Magnetic Stand.	☐
	STEMCELL Technologies	18103	EasyEights™ EasySep™ Magnet.	☐
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 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.	☐
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. We recommend validating alternatives relative to a hemocytometer.	☐
Fluorescence Cell Counter	Various Suppliers	Varies		☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
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.	☐
N=2 T100 Thermal Cycler	Bio-Rad Laboratories®	1861096	Or an equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2 mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.	☐
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM.	☐
6-inch Platform	Scientific Industries	146-6005-00		
Microplate Foam Insert	Scientific Industries	504-0235-00		
Serological Pipette Controller	Various Suppliers	Varies	Optional.	☐

Consumables

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free, 10 mL or 25 mL polypropylene reagent basins. Do not substitute with polystyrene.	☐
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as they will lead to substantial cell loss.	☐
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-20 TC70-SWM-40 TC70-SWM-70 TC70-SWM-100	Choose one or an equivalent sterile cell strainer with an appropriate mesh size for	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
pluriStrainer® Mini	pluriSelect®	43-10020-40 43-10040-40 43-10070-40 43-10100-40	the cell type(s) being fixed (20 µm, 40 µm, 70 µm, 100 µm). We do not recommend FlowMi Cell Strainers (SP Bel-Art).	
Falcon® Cell Strainer	Corning®	431750 431751 431752		
EASYstrainer™, small	Greiner Bio-One™	542120 542140 542170 542100		
PCR Plate 96 LoBind® E	Eppendorf	0030129504	Optional: if preparing >16 sublibraries using plate magnet.	☐
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.	☐
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.	☐
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.	☐
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.	☐
5 mL serological pipettes	Various Suppliers	Varies	Optional. Use DNase/RNase-free pipettes.	☐

Reagents

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.	☐
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.	☐
AO/DAPI	Various Suppliers	Varies	Or alternative viability dyes.	☐

Section 1: In situ Cell Barcoding

1.1. Set up and Sample Counting

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

To set up for barcoding:

1. If working with standard fixation samples, remove the ● Sample Binding Beads from the 4°C Reagents box. Gently pulse-vortex (1-2 seconds per pulse) or pipette mix the beads until completely resuspended. Observe the beads to confirm they stay in solution for **at least 2-3 minutes** before beginning to settle. Store the ● Sample Binding Beads at room temperature until use.



CRITICAL! If you observe that the Sample Binding Beads are frozen or dried, and settled at the bottom of the tube within ~10 seconds after pulse-vortexing, do not proceed. Contact your FAS or support@parsebiosciences.com for assistance.

2. Open the "Evercode™ MAX WT v4 Cell Barcoding L-Sample Loading Table", which will guide sample dilutions and plate loading in later steps. The Sample Loading Table can be found on the Parse Biosciences Customer Support Suite. Customer log-in is required to access the Sample Loading Table.

3. Fill a bucket with ice.

4. Prepare a hemocytometer or other cell counting device.

5. Thaw the previously fixed cell samples in a water bath set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.

6. While minimizing time on ice, count the cells in the sample with a hemocytometer or alternative cell counting device.

7. Record the sample names and cell counts in the Sample Loading Table.



Note: If there are errors in the Sample Loading Table, see the Important Guidelines section for suggestions.

8. If cells show acceptable integrity and minimal debris, start setting up for barcoding and cool a centrifuge with a swinging bucket rotor to 4°C.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 1 Plate	-20°C Reagents	1	Place directly on ice.
Sample Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x. *Note: Bead Prep Buffer is only used for standard fixed samples.
Bead Prep Buffer*	-20°C Reagents	1	
Round 1 Stop Mix	-20°C Reagents	1	
Ligation Buffer	-20°C Reagents	1	
Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Barcoding Buffer	-20°C Reagents	1	Thaw in a 37°C water bath, then store on ice.
Sample Binding Beads*	4°C Reagents	1	*Used for standard fixed samples only*. Keep at room temperature. Ensure beads are not settled before use.

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

THAW ROUND 1 PLATE		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	26 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

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

12. Proceed immediately to Section 1.2.

1.2. Barcoding Round 1

Samples are loaded into the Round 1 Plate. An in situ reverse transcription reaction adds well-specific barcodes that also act as sample barcodes. The reverse transcription reaction is stopped, cells are pooled and then resuspended.

To add Round 1 barcodes:

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

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



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

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



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



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

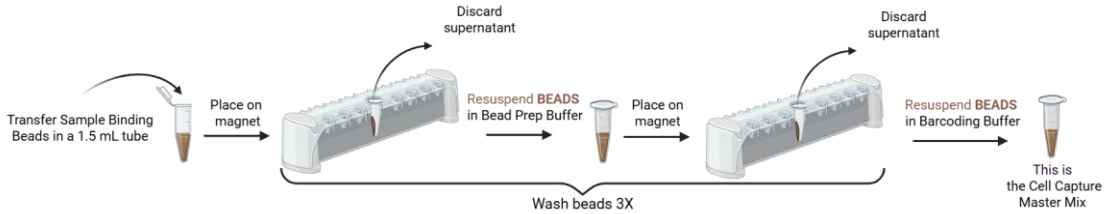
4. While secured in a PCR tube rack on a flat surface, add a new plate seal.

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

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

6. **If using low input samples**, the cells are already attached to the binding beads and don't need further washing. **Proceed directly to step 16**.

7. **If using standard fixation samples**, proceed to the next step to prepare the ● Sample Binding Beads to capture the cells. See workflow below.



Cell Capture Master Mix: Sample Binding Beads are washed three times, then Barcoding Buffer is added.

8. While the thermocycling program is still running, gently pulse-vortex ● Sample Binding Beads to resuspend and store at room temperature. Do not let them settle for >3 minutes before pipetting. Transfer **400 µL** of ● Sample Binding Beads to a 1.5 mL Eppendorf tube.

9. Place the tube on the magnetic rack compatible with 1.5 mL tubes and wait for the solution to clear (~2 minutes).

10. While still on the magnet, remove and discard the supernatant with a P1000.

11. Remove the tube from the magnetic rack and fully resuspend the bead pellet in **400 µL of ● Bead Prep Buffer**.

12. Place the tube on the magnetic rack until the solution clears (~2 minutes).

13. While still on the magnet, remove and discard the supernatant with a P1000.

14. Repeat steps 11-13 twice for a total of 3 washes.

15. Remove the tube from the magnetic rack. Fully resuspend the pellet in **400 µL** of ○ **Barcoding Buffer** and store on ice. This is your Cell Capture Master Mix.

Note: Save the Barcoding Buffer as it will be used later in this section.

16. Proceed to make the Ligation Master Mix for both Round 2 and Round 3 Ligation in a new tube:

LIGATION MASTER MIX	
○ Ligation Buffer	8.8 mL
● Ligation Enzyme	44 µL

17. Mix the Ligation Master Mix by inverting the tube 10x. Store on ice.

CRITICAL! 4.2 mL will be used for the Round 2 ligation barcoding step, and later **4.2 mL** will be used for the Round 3 ligation barcoding step.

18. Remove the Round 1 Plate from the thermocycler, place it in a PCR tube rack on the benchtop. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 1 Plate. Place the Round 1 Plate on ice.

19. Transfer all the Round 1 Stop Mix into a new basin with a P1000.

20. With a 12-channel multi-pipette, transfer **10 µL** of ● Round 1 Stop Mix into Row A of the Round 1 Plate, still keeping the plate on ice.

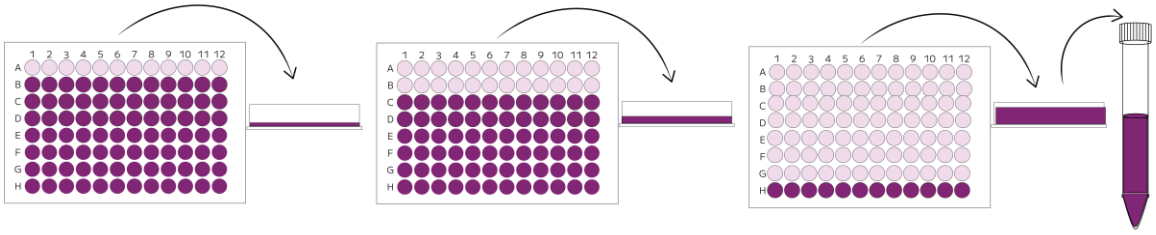
    a. Repeat step 20, **7 more times** using the same pipette tips for Rows B-H to dispense the Round 1 Stop Mix into all wells of the Round 1 Plate.

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

    c. Transfer **50 µL** from **row A** into a clean basin (bubbles will be generated, this will not impact experimental results).

    d. Using the same tips, repeat steps b-c for rows B-H.

    e. Once cells from all wells are in the basin, using a P1000 transfer the mix into a 15 mL conical tube. Keep on ice.



21. **If using low input fixation samples**, cells have already been captured. Place the 15 mL conical tube **magnet on ice**, and wait **1 minute** to let it pre-cool. **Then proceed directly to step 27**.

22. **If using standard fixation samples**, proceed to the next step to capture the cells.

23. Resuspend any settled beads in the Cell Capture Master Mix from step 15 by pipette mixing.

24. Transfer **384 µL** of the Cell Capture Master Mix to the pooled Round 1 cells. Pipette mix 10x with the P1000 set to 900 µL.

25. Incubate the cells on ice for **5 minutes** to allow beads to bind.

CRITICAL! Let beads incubate for the **entire 5 minutes**.

26. While cells are incubating, place the magnet on ice to pre-cool.

27. Remove or loosen the cap, then place the tube with the pooled cells in the 15 mL conical tube magnet to allow magnetic beads to bind to the magnet. If the cap is adjusted

afterward, the rotational motion may cause the pellet to shift. Keep the magnet on ice the entire time, covering the tube in ice up to the liquid level to keep the tube cold. Allow beads to bind to the magnet for **10 minutes**.



Note: At this point your cells are bound to beads. Preserve the beads in the subsequent rounds of barcoding, as loss of any beads from this point forward will result in loss of cells.

28. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids. Holding the tube on the magnet will ensure the tube is kept from moving.

29. With the tube still on the magnet, add **5 mL** of **O Barcoding Buffer**. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound to the magnet.

30. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.

31. Remove the tube from the magnet and resuspend the bead-bound cells in **4.2 mL** of Ligation Master Mix. Using a P1000 set to 1000 µL, pipette mix 10x to fully resuspend the beads into the Ligation Master Mix. Keep the sample on ice before proceeding to the next step.



CRITICAL! Do not throw away Ligation Master Mix after use, keep it on ice: the remainder will be used in Section 1.4.

1.3. Barcoding Round 2

The pooled bead-bound cells are loaded into the Round 2 Plate. An in situ ligation reaction adds a well-specific barcode to the 5′ end of the cDNA. The ligation reaction is quenched with Round 2 Stop Mix, and the cells are pooled and strained.

To add Round 2 barcodes:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
▭ Round 2 Plate v4	-20°C Reagents	1	Place directly on ice.
● Round 2 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.

2. Place the Round 2 Plate into a thermocycler and run the following program.

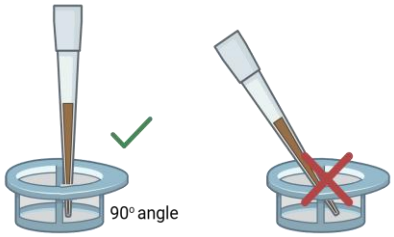
THAW ROUND 2 PLATE		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	10 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

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

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

5. Pipette all of the sample in Ligation Master Mix (**4.2 mL)** through a cell strainer into a new basin with a P1000.

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



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

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

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

    c. Changing tips between rows, repeat steps a-b to mix the sample in the basin then transfer to rows **B-H**.

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

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

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

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

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

9. Briefly vortex the ● Round 2 Stop Mix and ensure there is no precipitate. Transfer **1 mL** from this tube to a new basin with a P1000.

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

11. With the Round 2 Plate on ice and the basin on the bench, transfer the contents of the Round 2 Barcoding plate into the ● Round 2 Stop Mix as follows:

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

    b. Transfer the entire content (**50 µL)** from row A to the basin containing ● Round 2 Stop Mix and mix 2x.

    c. Repeat steps a-b for **rows B-H** to mix the sample then transfer to the basin. The same pipette tips may be used while pooling.

12. Transfer the Round 2 pool into a 15 mL conical tube and apply to the magnet. Loosen or remove the cap to avoid a rotational motion that may cause the pellet to shift. Keep the magnet on ice the entire time, covering the tube in ice up to the liquid level to keep the tube cold. Allow the beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear before proceeding to the next step. If the supernatant is not clear, continue binding to the magnet for an additional 5 minutes.

13. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.

14. While the tube is still on the magnet, add **5 mL** of ○ Barcoding Buffer. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound to the magnet.

15. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the beads, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.

16. Remove the tube from the magnet and resuspend bead-bound cells with **4.2 mL** of the Ligation Master Mix. Using a P1000 set to 1000 µL, pipette mix 10x to fully resuspend the beads into the Ligation Master Mix. Store the sample on ice and proceed to Section 1.4.

1.4. Barcoding Round 3

The cells are strained and loaded into the Round 3 Plate. A second in situ ligation reaction adds a third well-specific barcode, the Illumina TruSeq Read 2 sequence, and a biotin. The ligation reaction is stopped, and the sample is then pooled and strained.

To add Round 3 barcodes:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 3 Plate	-20°C Reagents	1	Place directly on ice.
Final Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
Pre-Lysis Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Pre-Lysis Dilution Buffer	-20°C Reagents	1	
Lysis Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Lysis Solution	-20°C Reagents	1	Thaw and store at room temperature.

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

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

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

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

5. Pipette all of the sample in Ligation Master Mix (**4.2 mL**) through a cell strainer into a new basin with a P1000.

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

6. With the Round 3 Plate on ice and the basin on the bench, transfer the cells in Ligation Master Mix to each well in the Round 3 Plate as follows:

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

    b. Transfer **40 µL** of the mix to row **A** of the Round 3 Plate and mix by pipetting 2x.

    c. Changing tips for each row, repeat steps a-b to mix the sample in the basin then transfer to rows **B-H**.

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

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

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

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

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

9. Briefly vortex the Final Stop Mix and ensure there is no precipitate. Transfer **2 mL** to a new basin with a P1000.

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

11. With the Round 3 Plate on ice and the basin on the bench, transfer all the liquid in the Round 3 Plate into the basin with the Final Stop Mix as follows:

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

    b. Transfer **50 µL** from row **A** to the basin and mix 2x.

    c. Repeat steps a-b for rows **B-H** to mix the sample then transfer to the basin. The same pipette tips may be used while pooling.

12. Pipette the pooled sample through a cell strainer into a new 15 mL tube with a P1000. Before each transfer, gently mix the cells in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.

13. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind to the magnet for **5 minutes**. Ensure the supernatant is clear before proceeding to the next step. If the supernatant is not clear, continue binding to the magnet for an additional 5 minutes.

14. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 µL.

15. Remove the 15 mL tube from the magnet and fully resuspend the cells in 4 **mL** of **Pre-Lysis Wash Buffer**. Do not invert.

16. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind for **5 minutes**, until supernatant is clear.

17. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 µL or serological pipette.

18. Remove the tube from the magnet and resuspend the cells in **200 µL of** ● Pre-Lysis Dilution Buffer.



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

19. Proceed to Section 1.5: Lysis and Sublibrary Generation.

1.5. Lysis and Sublibrary Generation

The cell pool resuspended in Pre-Lysis Dilution Buffer is counted and divided into sublibraries based on the Sublibrary Generation Table in Appendix B. These sublibraries are lysed, transferred into new tubes, and stored at -80°C.

Note: Optional: if preparing between 17 and 32 sublibraries, we recommend transferring the lysates into a 96 well plate and using a plate magnet for bead cleanup. See "User Supplied Equipment and Reagents" for recommended plate magnetic racks.

1. While minimizing time on ice, count the number of cells in the sample with a fluorescent (e.g., DAPI) cell counting device. Record the cell count.

CRITICAL! We strongly recommend carefully mixing the sample before collecting an aliquot for counting to ensure accurate sublibrary loading.

2. Decide how to divide cells across the desired number of sublibraries. See “Sublibrary Loading” in the Important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix B to determine the volume of sample and ●Pre-Lysis Dilution Buffer that should be added to each sublibrary.

CRITICAL! Do not add more than 64,000 cells to a sublibrary. Adding additional cells will result in an increased multiplet rate.

3. Ensure the cells are in suspension by pipetting 5x with a P200 set to 200 µL prior to each transfer. Add the appropriate volume of sample to the decided number of 0.2 mL PCR tubes (one tube per sublibrary).

4. Keeping the samples on ice, add the appropriate volume of ●Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of **25 µL**.

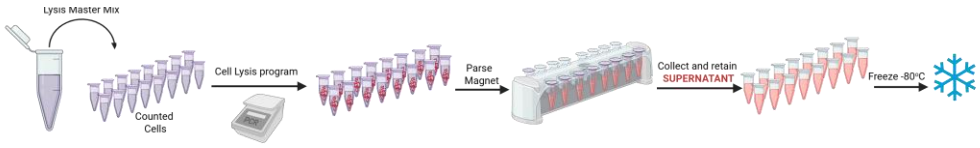
5. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x. Store at room temperature.

LYSIS MASTER MIX					
Number of Sublibraries	4	6	8	16	32
● Lysis Solution	110 µL	165 µL	220 µL	440 µL	880 µL
● Lysis Enzyme	22 µL	33 µL	44 µL	88 µL	176 µL
Total	132 µL	198 µL	264 µL	528 µL	1056 µL

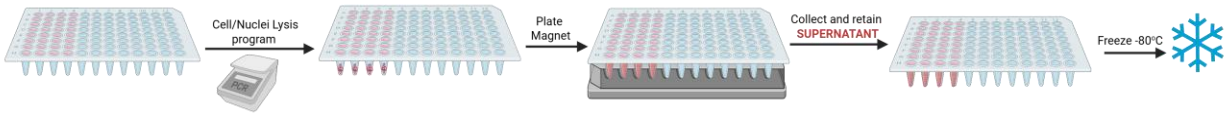
Note: Ensure that there is no precipitate before using the ● Lysis Solution.

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

6. For a visual representation of the next few steps see the workflow below. Add **30 µL** of Lysis Master Mix to each 0.2 mL tube with diluted cells. Store at room temperature.



OR



Use a plate magnet when preparing more than 16 sublibraries.

7. Vortex the 0.2 mL tube(s) for **1 minute**. Briefly centrifuge.

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

CELL LYSIS		
Run Time	15 minutes	
Lid Temperature	80°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C



CRITICAL! At this point, cells are lysed, so the supernatant contains the barcoded transcripts. Do **NOT** discard the supernatant. The beads are no longer needed and will be removed.

9. Remove samples from the thermocycler and place the tubes 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).

10. While still on the magnetic rack, transfer **55 µL** of the **supernatant** containing lysed cells into new 0.2 mL tubes.

11. Freeze the lysate(s) at -80°C or proceed to the appropriate Evercode MAX BCR v4 Library Prep User Manual.



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

This ends the Barcoding portion of the Evercode assay. To continue with the Sublibrary Preparation portion of the workflow, refer to the Evercode MAX BCR v4 Library Prep User Manual.

Appendices

Appendix A: Determine the Number of Sublibraries

Before proceeding with barcoding, determine the appropriate number of sublibraries (SL) to prepare. The table shows the maximum number of cells that can be used for a given number of sublibraries (SL). The maximum number of cells per SL is 64,000.

CELL MAX NUMBER PER TOTAL SUBLIBRARIES						
KIT	**2 SL**	**4 SL**	**8 SL**	**12 SL**	**16 SL**	**32 SL**
MAX BCR L	128,000	256,000	512,000	768,000	1,024,000	2,048,000

Appendix B: Sublibrary Generation Table

The Sublibrary Generation Table provides guidance for sample dilution in Section 1.5 to achieve a target sublibrary concentration.

Green text (top): Volume of cell suspension in µL (from step 1.5.2) to add to each sublibrary.

Purple text (bottom): Volume of Pre-Lysis Dilution Buffer in µL to add to each sublibrary.

Blue Shading: Serial dilution of cell stock is required to improve sublibrary cell count.

Red Shading: Insufficient cell stock concentration for target sublibrary cell count.

Cell Stock Conc. (cells/uL)	Target Sublibrary Cell Count (cells/sublibrary)																
	200	500	1,000	2,000	5,000	8,000	10,000	12,000	12,500	15,000	20,000	25,000	30,000	31,250	32,000	62,500	64,000
50	4	10	20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	21	15	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
100	Dilute	5	10	20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	20	15	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
200	Dilute	2.5	5	10	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	22.5	20	15	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
400	Dilute	Dilute	2.5	5	12.5	20	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	22.5	20	12.5	5	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
600	Dilute	Dilute	Dilute	3.33	8.33	13.33	16.67	20	20.83	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	21.67	16.67	11.67	8.33	5	4.17	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
800	Dilute	Dilute	Dilute	2.5	6.25	10	12.5	15	15.63	18.75	25	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	22.5	18.75	15	12.5	10	9.38	6.25	0	N/A	N/A	N/A	N/A	N/A	N/A
1,000	Dilute	Dilute	Dilute	Dilute	5	8	10	12	12.5	15	20	25	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	20	17	15	13	12.5	10	5	0	N/A	N/A	N/A	N/A	N/A
1,200	Dilute	Dilute	Dilute	Dilute	4.17	6.67	8.33	10	10.42	12.5	16.67	20.83	25	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	20.83	18.33	16.67	15	14.58	12.5	8.33	4.17	0	N/A	N/A	N/A	N/A
1,400	Dilute	Dilute	Dilute	Dilute	3.57	5.71	7.14	8.57	8.93	10.71	14.29	17.86	21.43	22.32	22.86	N/A	N/A
	N/A	N/A	N/A	N/A	21.43	19.29	17.86	16.43	16.07	14.29	10.71	7.14	3.57	2.68	2.14	N/A	N/A
1,600	Dilute	Dilute	Dilute	Dilute	3.13	5	6.25	7.5	7.81	9.38	12.5	15.63	18.75	19.53	20	N/A	N/A
	N/A	N/A	N/A	N/A	21.88	20	18.75	17.5	17.19	15.63	12.5	9.38	6.25	5.47	5	N/A	N/A
1,800	Dilute	Dilute	Dilute	Dilute	2.78	4.44	5.56	6.67	6.94	8.33	11.11	13.89	16.67	17.36	17.78	N/A	N/A
	N/A	N/A	N/A	N/A	22.22	20.56	19.44	18.33	18.06	16.67	13.89	11.11	8.33	7.64	7.22	N/A	N/A
2,000	Dilute	Dilute	Dilute	Dilute	2.5	4	5	6	6.25	7.5	10	12.5	15	15.63	16	N/A	N/A
	N/A	N/A	N/A	N/A	22.5	21	20	19	18.75	17.5	15	12.5	10	9.38	9	N/A	N/A
2,500	Dilute	Dilute	Dilute	Dilute	Dilute	3.2	4	4.8	5	6	8	10	12	12.5	12.8	25	N/A
	N/A	N/A	N/A	N/A	N/A	21.8	21	20.2	20	19	17	15	13	12.5	12.2	0	N/A
3,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.67	3.33	4	4.17	5	6.67	8.33	10	10.42	10.67	20.83	21.33
	N/A	N/A	N/A	N/A	N/A	22.33	21.67	21	20.83	20	18.33	16.67	15	14.58	14.33	4.17	3.67
3,500	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.86	3.43	3.57	4.29	5.71	7.14	8.57	8.93	9.14	17.86	18.29
	N/A	N/A	N/A	N/A	N/A	N/A	22.14	21.57	21.43	20.71	19.29	17.86	16.43	16.07	15.86	7.14	6.71
4,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3	3.13	3.75	5	6.25	7.5	7.81	8	15.63	16
	N/A	N/A	N/A	N/A	N/A	N/A	22.5	22	21.88	21.25	20	18.75	17.5	17.19	17	9.38	9
4,500	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.67	2.78	3.33	4.44	5.56	6.67	6.94	7.11	13.89	14.22
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.33	22.22	21.67	20.56	19.44	18.33	18.06	17.89	11.11	10.78
5,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3	4	5	6	6.25	6.4	12.5	12.8
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	22	21	20	19	18.75	18.6	12.5	12.2
5,500	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.73	3.64	4.55	5.45	5.68	5.82	11.36	11.64
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.27	21.36	20.45	19.55	19.32	19.18	13.64	13.36
6,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3.33	4.17	5	5.21	5.33	10.42	10.67
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	21.67	20.83	20	19.79	19.67	14.58	14.33
7,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.86	3.57	4.29	4.46	4.57	8.93	9.14
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.14	21.43	20.71	20.54	20.43	16.07	15.86
8,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3.13	3.75	3.91	4	7.81	8
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	21.88	21.25	21.09	21	17.19	17
9,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.78	3.33	3.47	3.56	6.94	7.11
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.22	21.67	21.53	21.44	18.06	17.89
10,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3	3.13	3.2	6.25	6.4
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	22	21.88	21.8	18.75	18.6

Appendix C: Thermocycling Programs

Section 1: In situ Cell Barcoding

THAW ROUND 1 PLATE		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	26 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

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

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

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

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

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

CELL LYSIS		
Run Time	15 minutes	
Lid Temperature	80°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C

Appendix D: Revision history

Version	Description	Date
1.0	Initial release	October 2026

PARSE BIOSCIENCES

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parsebiosciences.com

support@parsebiosciences.com

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