

User Manual

Version 1.0 – UMMW4530



Evercode™ MAX WT v4

Cell Barcoding L-384

For use with
ECMW4530



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Table of Contents

Overview	4
Workflow	4
Protocol Timing.....	8
Important Guidelines	9
Part List	15
User Supplied Equipment and Reagents.....	18
Section 1: In situ Cell/Nuclei Barcoding.....	22
1.1 Sample Set Up and Barcoding Round 1.....	22
1.1a Set Up for Low Input Fixation Samples: Capture Step	23
1.1b Set Up for Standard Fixation Samples	31
1.2. Pooling Round 1 Plates.....	37
1.3. Barcoding Round 2	41
1.4. Barcoding Round 3	46
1.5. Lysis and Sublibrary Generation.....	50
Appendices	53
Appendix A: Determine Sublibraries Number.....	53
Appendix B: Sublibrary Generation Table	54
Appendix C: Thermocycling Programs	55
Appendix D: Revision history	57

Overview

Workflow

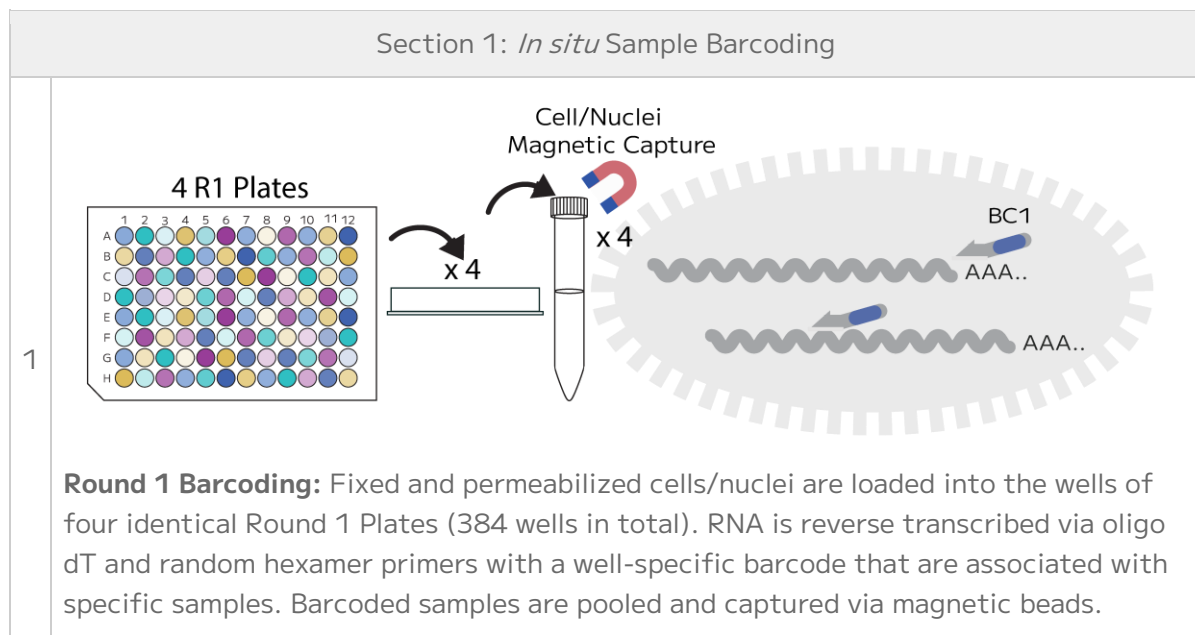
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. Evercode MAX WT v4 Cell Barcoding kits add further flexibility by separating cell barcoding from library construction, so each dimension can be scaled independently based on experimental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

- Four sizes of Evercode MAX WT v4 Cell Barcoding kits determine the number of samples that can be indexed in a given run.
- Two sizes of Evercode MAX WT 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 WT v4 Cell Barcoding L-384 Barcoding kit can index up to 2,048,000 cells/nuclei across up to 384 different samples. Anywhere between 2 to 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

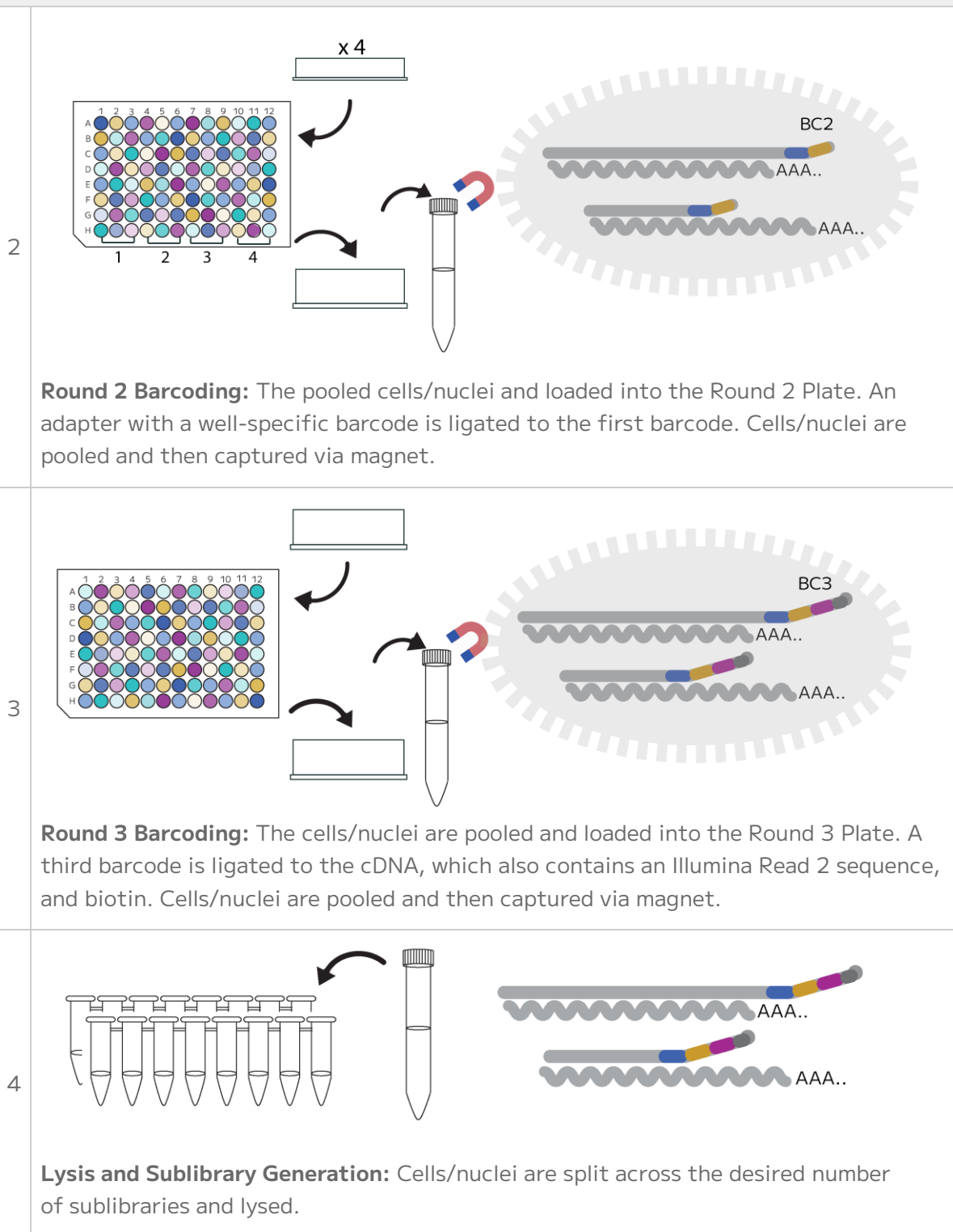


Figure 1 shows the L-384 workflow. This kit can barcode up to 2,048,000 cells/nuclei across 384 samples in a single reaction. The L-384 utilizes four identical Round 1 Plates, each holding a unique set of 96 samples, totaling 384 samples. These samples are pooled from the four Round 1 Plates into a single Round 2 Plate. The barcodes from Round 1 and Round 2 serve as sample identifiers.

Samples from Round 2 are pooled and distributed into the Round 3 Plate. Cells/nuclei are then pooled and subsequently split to generate sublibraries (Figure 1). Sample plates are easily identified by labeling color-coded stickers (Figure 2).

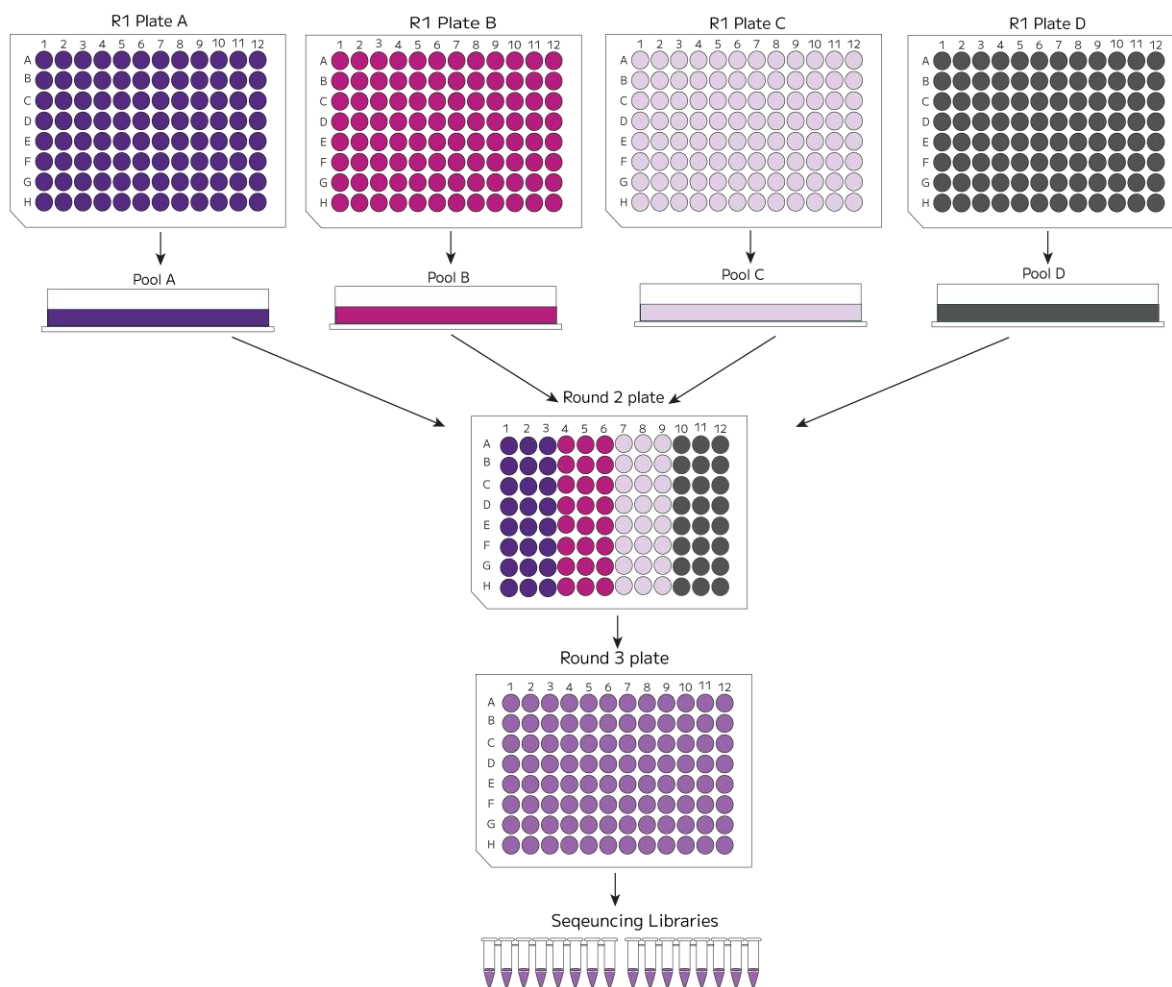


Figure 1: Evercode MAX WT L-384 workflow pools 384 samples from four Round 1 Barcoding plates into one Round 2 and Round 3 Barcoding Plates respectively, generating Sequencing Libraries

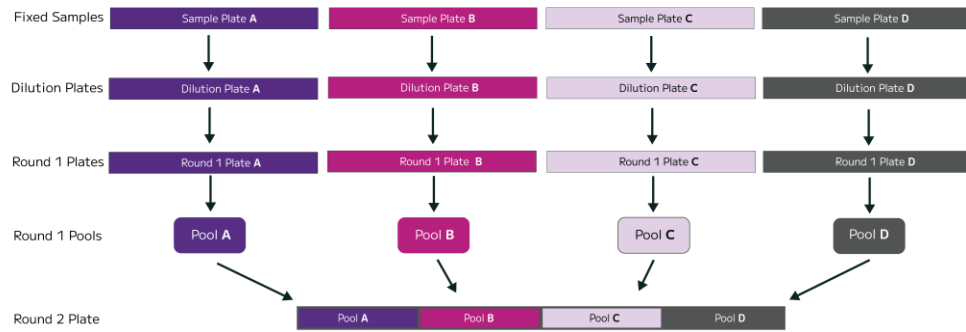
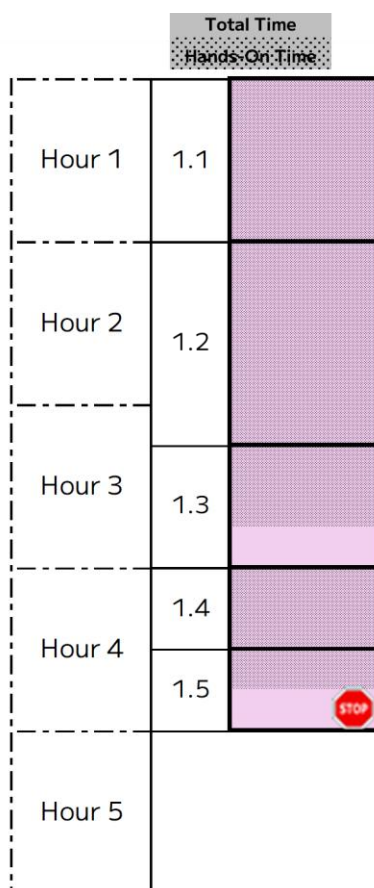


Figure 2: Plates are easy to handle with labeled color-coded stickers throughout the Round 1 Barcoding section.

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/Nuclei Barcoding			
1.1 Sample Set up and Barcoding Round 1	Variable (60-90 min)	Variable (60-90 min)	
1.2 Pooling Round 1 Plates	75 min	75 min	
1.3 Barcoding Round 2	40 min	25 min	
1.4 Barcoding Round 3	30 min	25 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

- This protocol begins with cells or nuclei fixed with an Evercode Cell Fixation v3 and v4 Kit, Evercode Nuclei Fixation v3 and v4 kit, Evercode Low-Input Cell Fixation Kit or Evercode Low-Input Nuclei Fixation v3 and v4 kits, or the Whole Blood Fixation kit.
- For the Standard Fixation kit only: aliquot the samples after fixation (counting aliquots) and count them the day before using an Evercode kit. Refer to the guidelines in the Evercode Fixation User Manuals for generating aliquots. Because aliquots have undergone a similar storage time and a freeze thaw, cell/nuclei counts from these aliquots will be more representative than using counts from immediately after fixation. Aliquots should be thawed in a thermocycler set to 37°C and counted with an automated counting device. Counts should be recorded in the Sample Loading Table, and any remaining counting aliquot should be discarded.

Note that Low Input fixation reactions do not allow for a counting aliquot. Please follow guidance in the LI fixation manuals.

- For the samples fixed with the Standard Fixation kit, after recording the concentrations of the counting aliquots in the Evercode™ MAX WT v4 Cell Barcoding L-384 Standard Fixation-Sample Loading Table RevA, create Sample Dilution Plates as shown on the "Dilution and R1 Plate Config." tab (see Section 1.1b for more information).
- For Low Input Fixation samples, use recommended volumes of sample input and dilution buffer from Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA based on prefixation cell input. We do not recommend counting the samples directly post cell capture due to time constraints.
- For Low Input fixed samples, bead and storage buffer volumes vary based on cell/nuclei input. With 384 samples, loading can become complex and time-consuming. To streamline bead capture and keep it within a manageable timeframe, we strongly recommend normalizing cell/nuclei counts across sets of 12 or 8 prior to fixation.
- Once fixed samples have been thawed, they should not be refrozen.

Cell/Nuclei Counting and Quality Assessment

- For the Standard Fixation kit we recommend using a plate based automated cell counter to count cells/nuclei from the counting plates of Standard Fixation samples due to the high-throughput nature of the workflow.
- For Low Input samples, we recommend counting pre-fixation and optionally spot-counting post fixation.
- Fluorescent sample counting is recommended for low input samples after beads have been added for ease of counting.
- When first using Evercode kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue (pre-bead capture only) or acridine orange and propidium iodide (AO/PI).
- Examples of AO/DAPI and trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells/nuclei 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/nuclei.

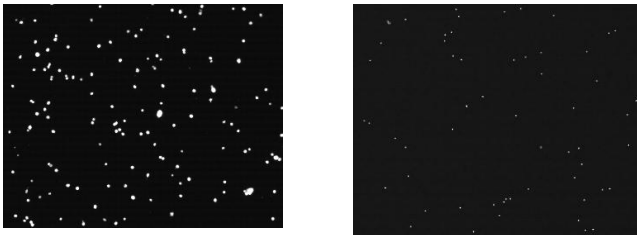


Figure 3: Low Input Fixation: example of post-bind AO/DAPI stained HEK cells (left) and PBMCs (right).

High Quality Sample

Aggregation

Debris

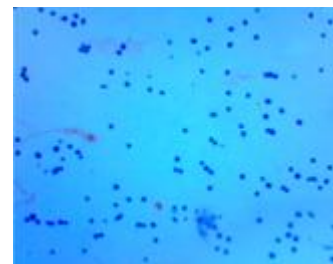


Figure 4: Example of trypan blue stained fixed cells.

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.
- 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/Nuclei Recovery

- It is critical to thoroughly resuspend the cells/nuclei after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to cell/nuclei adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell/nuclei loss.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell/nuclei pellets adequately.
- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- The first time using an Evercode 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.
- For Low Input Fixation samples only: if the bead/cell pellet is disturbed during the bead binding aspiration step, dispense all liquid back into the wells and wait an additional 2 minutes for the bead-bound cells to bind to the magnet again before re-aspirating.

Sample Loading Table

- The Sample Loading Table should be completed before starting the experiment. Be sure to use either the Evercode™ MAX WT v4 Cell Barcoding L-384 Standard Fixation-Sample Loading Table RevA or the Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA, since workflow requirements are different.
- Ensure that the 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.

- 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. Reach out to Support for any further question at support@parsebiosciences.com.
- 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 μ L 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.
- In the unlikely event that samples are **too highly concentrated** according to the Sample Loading Table, choose to either:
 - Standard Fixation Samples: dilute samples upstream of adding them into the Sample Dilution Plates. This can be done using the left over Cell/Nuclei Storage Buffer from Fixation.
 - For Low Input fixation, do not input more than 100,000 cells/nuclei per fixation sample because it will negatively impact the subsequent accuracy of the Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA.
- Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA only: sample concentrations used to input into the Sample Loading Table should be obtained from spot counts during fixation while samples are in Prefixation Buffer. Averaged spot count can be used to estimate the number of cells per well throughout the plate. Account for up to 10% cell/nuclei loss that can happen during freeze-thaw.

Cell Strainers

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 μ m 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 in our support site.

Vortexing

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

Plate Sealing

- While sealing or unsealing 96 well plates, do not splash liquid onto the PCR plate seal or between wells. Securing plates in PCR tube racks will minimize this occurrence. PCR plate seals may be difficult to remove. Carefully peel the PCR plate seal while applying downward pressure on the plate to keep it in the PCR tube rack.

Magnetic Racks and Bead Cleanups

- The Evercode MAX WT L-384 v4 workflow uses magnetic beads to clean up cells/nuclei after each round of barcoding. Magnetic racks capable of holding 15 mL conical tubes and one capable of holding 1.5 mL tubes are needed throughout this protocol.
- Three validated 15 mL conical tube magnets are described in the "User Supplied Equipment and Reagents" section of this manual to choose from.
- The Parse Biosciences Magnetic Rack uses powerful rare earth magnets for rapid and efficient magnetic bead purification 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.

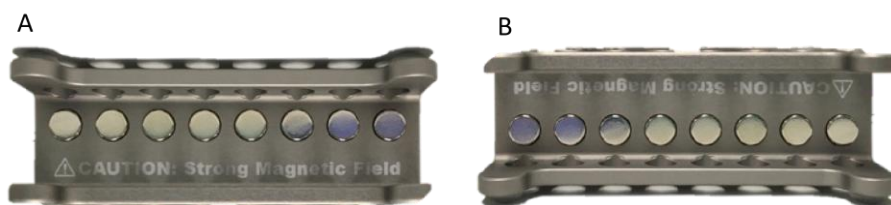


Figure 5: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.



Figure 6: Clear supernatant with compact bead pellets.

- The Low Input Fixation kit uses a plate magnetic rack. We validated the plate magnetic racks listed in the "User Supplied Equipment and Consumables". We do not recommend substituting alternative racks.
- The Evercode MAX WT v4 workflow supports preparation of up to 32 sublibraries using two Evercode MAX WT v4 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 when using a 96-well plate magnetic rack.

Sublibrary Loading

- The Evercode MAX WT v4 Cell Barcoding L-384 generates up to 32 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.
- Before proceeding with barcoding, refer to Appendix A to determine the number of sublibraries to prepare for your experimental needs.
- The number of cells or nuclei per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix B.
- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.
- Sublibraries can be loaded with different numbers of cells, and the maximum number of cells that can be analyzed is the sum of cells/nuclei across all sublibraries.

Thermocycling Programs

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

Part List

The Evercode MAX WT V4 Cell Barcoding L-384 includes the following boxes. Safety Data Sheets for these reagents can be provided upon request

-20°C Reagents Store -20°C, XMX400

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	MG101	Green semi-skirted 96 well plate	4
	Round 2 Plate	MG142	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Sample Dilution Buffer	MG132	5 mL tube	1
	Bead Prep Buffer	PT136	5 mL tube	1
	Barcoding Buffer	PT137	15 mL tube	3
	Round 1 Stop Mix	PTT138	5 mL tube	1
	Round 2 Stop Mix	MG139	2 mL tube	1
	Ligation Enzyme	MG140	1.5 mL tube	1
	Ligation Buffer	PT134	15 mL tube	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 Enzyme	PT109	1.5 mL tube	1
	Lysis Solution	PT146	2 mL tube	1

4°C Reagents. Store 4°C, XMX500

LABEL	ITEM	PN	FORMAT	QTY
	Sample Binding Beads	PT135	2 mL tube	1

4°C Reagents and Stickers. Store at room temperature, XMX400

LABEL	ITEM	PN	FORMAT	QTY
N/A	Sticker Labels	N/A	Sticker Sheet	1

To avoid confusion while handling the plates, specifically labeled color-coded stickers are provided with the kit. When working with four fixed sample plates, we recommend labeling each as Fixation Plate A, Fixation Plate B, Fixation Plate C, and Fixation Plate D respectively.

For subsequent plates, use the corresponding stickers (e.g., Sample Plate A, Dilution Plate A, Round 1 Plate A, Pool A). Below is a visual representation of the stickers.

Side Stickers for Plates (Section 1.1)

Fixed Samples

1.1a, step 23 or 1.1b, step 19	Sample Plate A	Sample Plate A
	Sample Plate B	Sample Plate B
	Sample Plate C	Sample Plate C
	Sample Plate D	Sample Plate D

Dilution Plates

1.1b, step 6	Dilution Plate A	Dilution Plate A
	Dilution Plate B	Dilution Plate B
	Dilution Plate C	Dilution Plate C
	Dilution Plate D	Dilution Plate D

Round 1 Plates (MG101)

1.1a, step 19 or 1.1b, step 17	Round 1 Plate A	Round 1 Plate A
	Round 1 Plate B	Round 1 Plate B
	Round 1 Plate C	Round 1 Plate C
	Round 1 Plate D	Round 1 Plate D

Stickers for Tubes

Stickers for Round 1 Pools (Section 1.2)

1.2, step 5	Pool A	A	Pool B	B	Pool C	C	Pool D	D
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Stickers for Strained Pools (Section 1.3)

1.3, step 3	Strained Pool A	A	Strained Pool B	B	Strained Pool C	C	Strained Pool D	D
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1.3, step 4	A A A A A A A A	B B B B B B B B	C C C C C C C C	D D D D D D D D
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Top Stickers for Round 2 Plate (MG102)

1.3, step 8	Pool A	Pool B	Pool C	Pool D
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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.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
Fluorescence Cell Counter	Various Suppliers	Varies		☐
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.	☐
2-4 T100 Thermal Cycler	Bio-Rad Laboratories®	1861096	Or an equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.	☐
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 it will lead to substantial cell loss.	☐
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-20	Choose one or an equivalent sterile cell strainer	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
		TC70-SWM-40 TC70-SWM-70 TC70-SWM-100	with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm, 70 µm, 100 µm). We do not recommend FlowMi Cell Strainers (SP Bel-Art).	
pluriStrainer® Mini	pluriSelect®	43-10020-40 43-10040-40 43-10070-40 43-10100-40		
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/Nuclei Barcoding

1.1 Sample Set Up and Barcoding Round 1

Prior to starting barcoding, fixed samples are thawed and prepared.

If the Low-Input Fixation workflow was performed, set up and prepare samples following Section 1.1a and using the "Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA".

If the Standard Fixation workflow was performed, set up and prepare samples following Section 1.1b and using the "Evercode™ MAX WT v4 Cell Barcoding L-384 Standard Fixation-Sample Loading Table RevA".

1.1a Set Up for Low Input Fixation Samples: Capture Step

Capture and barcoding of Low-Input samples requires planning to minimize sample processing time. Prior to barcoding, cells or nuclei are thawed and captured using magnetic beads. Due to the precise nature of the bead capture, sample input into Round 1 barcoding can be calculated based on known sample input prior to fixation. This is determined by the Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA. In the following protocol, the four plates with fixed samples will be labeled as A, B, C, and D. To minimize the processing time, we recommend capturing, loading and initiating Round 1 barcoding reactions for plates A and B first, then repeating the process for plates C and D.

To set up for barcoding:

1. Open the Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA, which will guide sample dilutions and plate loading in later steps. 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.
2. Cool a centrifuge with plate swinging bucket rotors to 4°C.
3. Fill a bucket with ice.
4. Set the thermocyclers at the temperatures needed.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 1 Plate	-20°C Reagents	4	Place directly on ice.
○ Ligation Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
 Cell/  Nuclei Binding Beads	Low Input Prefixation and Binding Reagents (4°C)	4*	Gently pulse-vortex or pipette mix until completely resuspended and store at room temperature. Do not let it settle for >3 minutes before pipetting.
○ Cell/Nuclei Binding Bead Wash Buffer	Low Input Fixation Reagents (-20°C)	4*	Thaw at room temperature then store on ice. Mix by inverting 3x.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Storage Buffer	Low Input Fixation Reagents (-20°C)	8**	Thaw at room temperature then immediately store on ice. Mix by inverting. Do not vortex.
Sticker Sheet	4°C Reagents and Stickers	1	Remove sheet and follow instructions for plate and tube labeling.

*The high throughput Low Input Fixation kit can prepare up to 96 samples. Reagents from four Low Input Fixation kits are needed to process 384 samples.

** The eight tubes come from four different Low Input Fixation kits.



Note: For every 96 samples being processed, a 1.5 mL tube is needed to resuspend and wash the beads. Therefore, up to 4 tubes would be needed to process all 384 samples.

- Gently pulse-vortex (1-2 seconds per pulse) or pipette mix ● Cell/● Nuclei Binding Beads until completely resuspended.
- Add **1.44 mL** of ● Cell/● Nuclei Binding Beads to **four** new 1.5 mL tubes.

BINDING BEADS VOLUMES		
Number of Samples	1	96 (x4)
● Cell/● Nuclei Binding Beads	15 µL	1.44 mL (x4)

- Place the four tubes on the 1.5 mL tube magnetic rack until the solution clears (~ 2 minutes).
- With the tubes still on the magnetic rack, remove and discard the supernatant.
- Remove the tubes from the magnetic rack and fully resuspend each bead pellet.

11. Add **1.44 mL** of **○** Cell/Nuclei Binding Bead Wash Buffer to each tube.

CELL/NUCLEI BINDING BEAD WASH BUFFER		
Number of Samples	1	96 (x4)
○ Cell/Nuclei Binding Bead Wash Buffer	15 μ L	1.44 mL (x4)



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

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

13. With the tubes still on the magnetic rack, remove and discard the supernatant.

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

15. Remove the tubes from the magnetic rack. Fully resuspend each pellet in the appropriate volume of **○** Storage Buffer following the table below.

STORAGE BUFFER (4)		
Number of Samples	1	96 (x4)
○ Storage Buffer	15 μ L	1.44 mL (x4)



CRITICAL! Do not discard the **○** Storage Buffer after this step as it is used again in the following steps.

16. Using a P200 pipette set to 110 μ L, aliquot **110 μ L** of beads resuspended in **○** Storage Buffer into each tube of **four** sets of 12 PCR tubes (48 tubes total).



CRITICAL! Ensure beads are thoroughly mixed several times before and throughout the aliquoting process.

17. Thaw the four Round 1 Plates in the thermocyclers according to the following protocol:

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

18. Gently remove the Round 1 Plates from the thermocyclers, place in PCR plate holder, and centrifuge for **1 minute** at 100 x g at 4°C.

19. Remove the Round 1 Plates from the centrifuge, store on ice, and label the four Round 1 Plates uniquely with "Round 1-Plate [A-D]" using the stickers provided.



Note: To minimize the processing time, we recommend capturing, loading and initiating Round 1 barcoding reactions for plates A and B first, then repeating the process for plates C and D.

20. Remove **two** of the four plates of fixed cells/nuclei from -80°C storage. Set the thermocycler to the following protocol and thaw cells/nuclei:

THAW CELLS/NUCLEI (2)		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	100 µL	
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

21. To thaw, place the plate of frozen cells/nuclei in the thermocycler and start the program.

22. Once the thaw protocol has finished, check that all wells are fully thawed. If ice remains, place the plate back into the thermocycler for another minute at 37°C. Once fully thawed, place the plate into a PCR plate holder, and store on ice.
23. Label the plates with the corresponding adhesive labels provided in the kit, following designations as filled out in the Sample Loading Table (i.e. "Sample Plate-[A-D]").
24. Use the table below as guidance for loading the binding beads into each sample starting with "Sample Plate A". See Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA for a visual diagram of loading the appropriate amount of beads to each sample.

CELL/NUCLEI BINDING BEADS ADDITION	
Cell/Nuclei Input Number	Cell/Nuclei Binding beads (μL)
10,000-49,999	4
50,000-74,999	6
75,000-89,999	9
90,000-100,000	12



Note: Be sure to mix beads by pipetting thoroughly to fully resuspend before using them for cell/nuclei capture.

25. With a P200 pipette set to 120 μL, vigorously pipette 5x to ensure beads are fully resuspended in cell/nuclei samples. Then, incubate the mixture on ice for **5 minutes**.
26. Place the plate on a plate magnet and bind at room temperature for **5 minutes**.
27. During the bind step, add **20 mL** of **O** Storage Buffer to a clean basin (refilling with more volume as needed). The Storage Buffer will be used in a later step.
28. With the plate still on the magnet, remove the supernatant from the wells, without disturbing the pellet with a multichannel P200 set to 200 μL and switching tips between columns.



CRITICAL! Move quickly to resuspend beads at this step to minimize time beads are out of liquid (<5 minutes).

29. Remove the plate from the magnetic rack and place it on ice.

30. Keep plate on ice while vigorously resuspending cells/nuclei in the ○ Storage Buffer with a P200 multichannel pipette to ensure no pellets remain. Switch tips between wells. Refer to the Sample Loading Table to determine ○ Storage Buffer volumes (ranges from 20-100 μ L).



CRITICAL! Resuspending the beads in less than the volume required for resuspension will harm the barcoding chemistry and result in a loss of transcript detection.

31. Repeat steps 24-30 for the second sample plate ("Sample Plate B").
32. Before loading the first and second sample plates (A and B) into the corresponding Round 1 Plate, remove the third and fourth sample plates (C and D) from the -80°C storage and thaw according to the following protocol. Once the program has completed, store plates on ice and label plates with corresponding adhesive labels.

THAW CELLS/NUCLEI (2)		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	200 μ L*	
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

* Or maximum volume allowed by the thermocycler.

33. Remove the plastic seal from a thawed Round 1 Plate (labeled "Round 1-Plate A").



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.

34. With the Round 1 Plate on ice, add **14 μ L** of "Sample Plate A" to the appropriate wells of "Round 1 Plate A" 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: Refer to the Evercode™ MAX WT v4 Cell Barcoding L-384 Low Input Fixation-Sample Loading Table RevA Plate Configuration tab for visual reference on how to load samples into Round 1 plates.

35. While secured in a PCR plate holder on a flat surface, add a new plate seal and place back on ice.
36. Repeat steps 33-35 for "Sample Plate-B" with "Round 1 Plate B".
37. Place both Round 1 Plates (A and B) into thermocyclers and run the program below for both plates using two separate thermocyclers. Once the program has been started, proceed immediately to the next step.

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

38. Repeat steps 20-37 to load third and fourth sample plates (C and D) with the corresponding Round 1 Plates (C and D).
39. Once the Round 1 Plates A and B have finished the "Barcoding Round 1" program, place them on ice.

40. When there are **10 minutes** remaining in "Barcoding Round 1" for Round 1 Plates C and D, proceed with Section 1.2, individually pooling Round 1 Plate A into Pool A and Round 1 Plate B into Pool B ([Section 1.2](#)).

1.1b Set Up for Standard Fixation Samples

Prior to barcoding, users are expected to count cells or nuclei using a counting plate in order to ascertain the concentration of all 384 samples. This will allow for using variable Sample Dilution Buffer volumes to dilute with a constant volume of fixed samples. This will streamline the loading process into the Round 1 Plate and minimize dedicated processing time. In the following protocol, we will designate the four plates of fixed samples as A, B, C and D. Appropriate dilutions, loading concentrations, and loading positions are determined by the Evercode™ MAX WT v4 Cell Barcoding L-384 Standard Fixation-Sample Loading Table RevA. Dilution plates may be made up to 48 hours in advance of barcoding.

Dilution plate set up:

1. Prepare an automated counter, hemocytometer, flow cytometer, or other plate-based counting device.
2. Using a counting plate aliquot of fixed samples, count the number of cells/nuclei in the sample with an automated cell counter or alternative cell counting device. Record the cell/nuclei count.
3. Fill out the Evercode™ MAX WT v4 Cell Barcoding L-384 Standard Fixation-Sample Loading Table RevA, which will guide sample dilutions and plate loading in later steps. 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.
4. Fill a bucket with ice and cool a centrifuge with plate swinging bucket rotors to 4°C.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Sample Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Sticker Sheet	4°C Reagents	1	Remove sheet and follow instructions for plate and tube labeling.

6. Label four (user-provided) PCR Plates uniquely with stickers "Dilution Plate [A-D]" provided in the 4°C Reagents and Stickers box.

7. Create the corresponding dilution plates of ○ Sample Dilution Buffer using the Evercode™ MAX WT v4 Cell Barcoding L-384 Standard Fixation-Sample Loading Table RevA.



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



Safe Stopping Point: Dilution plates can be made in advance, sealed, and frozen at -20°C for 48 hours prior to barcoding.

Barcoding plate set up:

8. 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 the tube within ~10 seconds after pulse-vortexing, do not proceed. Contact your FAS or support@parsebiosciences.com for assistance.

9. Open the Evercode™ MAX WT v4 Cell Barcoding L-384 Standard Fixation-Sample Loading Table RevA, which will guide sample dilutions and plate loading in later steps.
10. Fill a bucket with ice.
11. Have two or four thermocyclers on standby.



Note: If only 2 thermocyclers are available, process Plates A and B first, keeping Plates C and D on ice.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 1 Plate	-20°C Reagents	4	Place directly on ice.
○ Round 1 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Ligation Buffer	-20°C Reagents	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

13. If the Dilution Plates are stored at -20°C, thaw the four pre-prepared Dilution Plates in the thermocyclers according to the following protocol. Then gently remove the Dilution Plates from the thermocyclers, place in 0.2 mL tube racks, and centrifuge for **1 minute** at 100 x g at 4°C.

THAW DILUTION PLATES (4)		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	100 µL	
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

14. Place the Dilution Plates on ice, and label with corresponding adhesive labels, if not done previously.

15. Thaw the four Round 1 Plates in the thermocyclers according to the following protocol:

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

16. Gently remove the Round 1 Plates from the thermocyclers, place in 0.2 mL tube racks, and centrifuge for **1 minute** at 100 x g at 4°C.

17. Remove the Round 1 Plates from the centrifuge and store on ice.

18. Label Round 1 Plates with a sticker "Round 1 Plate [A-D]" sticker provided, taking care that each plate is associated with a unique letter from A-D.



Note: Each Round 1 Plate is identical until samples are loaded. Ensure traceability by using the provided adhesive labels.

19. From the -80°C storage remove the sample plate that is associated with Plate A loading in the Sample Loading Table, and thaw it according to the following protocol.

THAW CELLS/NUCLEI		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	100 µL	
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

20. Gently remove the sample plate from the thermocycler and place on ice. Label sample plate with "Sample Plate A" sticker provided.



Note: At this point, there will be 9 plates on ice: four Dilution Plates, four Round 1 Plates, and the Sample Plate A. Be sure to have each plate labeled for traceability using the provided sticker sheet. To avoid confusion, when not in use, Dilution Plates and Round 1 Plates can be stored in the fridge at 4°C.

21. Using a multichannel P200 set to 40 µL, mix cells/nuclei 3x in the Sample Plate A, Row A. Then, using a multichannel P20 set to 14 µL, transfer **14 µL** from Sample Plate A, Row A into Dilution Plate A, Row A.

22. Mix 3x with the same pipette tips to ensure even mixing, immediately transfer **14 µL** of the diluted cells/nuclei into the corresponding wells of the Round 1 Plate A, Row A and mix again 3x. Example of the workflow is shown in the diagram below. See Sample Loading Table plate configuration tab for sample location.

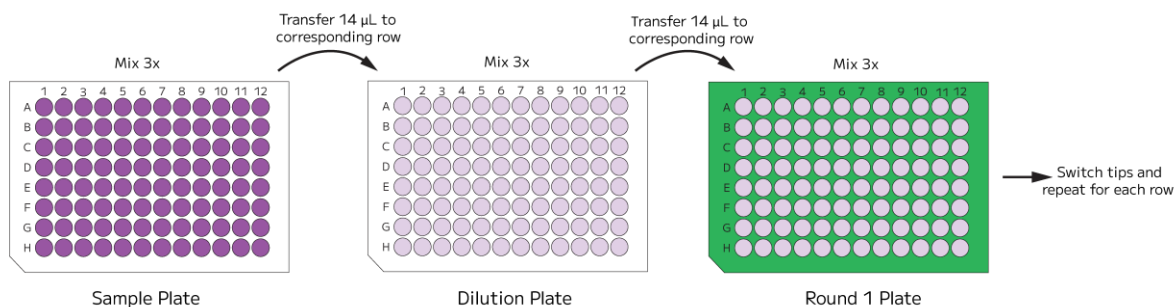


Figure 7: Samples in the sample plate are diluted and then transferred to the Round 1 Plate.

23. Repeat the process for the rest of rows B-H until all samples from Sample Plate A are diluted and transferred into Round 1 Plate A, switching tips after every transfer.



CRITICAL! Do not reuse any tips that have entered the Round 1 Plate wells. Do not reuse tips across non-correlating rows.

24. Place the Round 1 Plate A into the thermocycler and run the program below.

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

25. Repeat steps 19-24 for Plates B, C, and D labeling the plates accordingly.
26. When there are **10 minutes** remaining in "Barcoding Round 1" for Round 1 Plates C and D, proceed with Section 1.2, individually pooling Round 1 Plate A into Pool A and Round 1 Plate B into Pool B (Section 1.2).



Note: If only 2 thermocyclers are available, store the completed Plates A and B on ice, while Plates C and D undergo Barcoding Round 1.

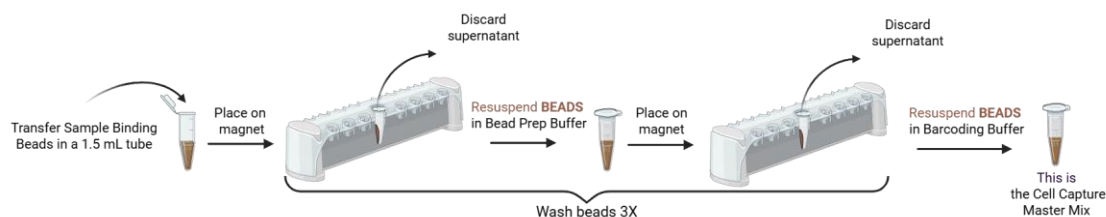
1.2. Pooling Round 1 Plates

After the Round 1 barcoding, the reverse transcription reaction is stopped, cells/nuclei are pooled into four Pools, and then cell/nuclei are resuspended.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Bead Prep Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Round 1 Stop Mix	-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 37°C water bath, then store on ice.
● Sample Binding Beads	4°C Reagents	1	Keep at room temperature. Ensure beads are not settled before use.

2. **If using low input samples**, the cells/nuclei are already attached to the binding beads and don't need further washing. **Proceed directly to step 12.**
3. **If using standard fixation samples**, proceed to the next step to prepare the ● Sample Binding Beads to capture the cells/nuclei. See workflow below.



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

4. 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 **800 µL** of ● Sample Binding Beads into two 1.5 mL Eppendorf tubes (for a total of **1,600 µL** of ● Sample Binding Beads used).
5. Place the tubes on the magnetic rack compatible with 1.5 mL tubes and wait for the solution to clear (~2 minutes).

6. While still on the magnet, remove and discard the supernatants.
7. Remove the tubes from the magnetic rack and fully resuspend each bead pellet in **800 µL** of **○ Bead Prep Buffer**.
8. Place the tube on the magnetic rack until the solution clears (~2 minutes).
9. While still on the magnet, remove and discard the supernatant.
10. Repeat steps 7-9 twice for a total of 3 washes.
11. Remove the tubes from the magnetic rack. Fully resuspend each pellet in **800 µL** of **○ Barcoding Buffer** and store on ice. This is your Cell Capture Master Mix (for a total of 1,600 µL of resuspended beads).



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

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

LIGATION MASTER MIX	
○ Ligation Buffer	9.8 mL
● Ligation Enzyme	44 µL

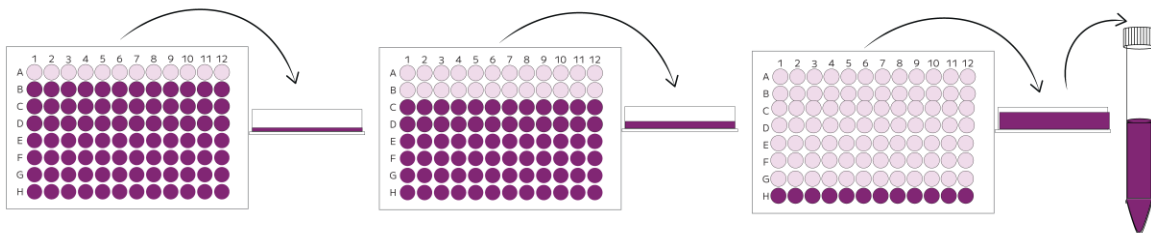
13. Mix the Ligation Master Mix by inverting the tube 10x. Store on ice.



CRITICAL! **4.8 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.

14. Remove the Round 1 Plate A 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. Keep the Round 1 Plate A on ice.
15. Transfer all the ○ Round 1 Stop Mix in a new basin with a P1000.
16. With a 12-channel multi-pipette transfer **10 µL** of ○ Round 1 Stop Mix into Row A of the Round 1 Plate A, still keeping the plate on ice.
17. Repeat step 16, **7 more times** for Rows B-H to dispense the ○ Round 1 Stop Mix into all wells of the Round 1 Plate A.

18. Switching tips between plates, repeat steps 16-17 with Plates B, C, and D to add the **○ Round 1 Stop Mix** to all samples. Keep plates on ice.
19. Label four 15 mL conical tubes with the provided stickers as Pool A, Pool B, Pool C, and Pool D.
20. Start pooling the samples from Round 1 Plate A **into Pool A tube** as follows:
 - a. With a multichannel P200 set to 50 μ L, mix the sample in row A by pipetting 3x.
 - b. Transfer **50 μ L** from **row A** into a clean basin (bubbles will be generated, this will not impact experimental results).
 - c. Repeat steps b-c for **rows B-H**.
 - d. Once cells from all wells are in the basin, using a P1000 transfer the mix into a new 15 mL conical tube. Keep on ice.
 - e. Repeat steps a-d with the remaining three Round 1 Plates B-D to generate four separate pools of cells/nuclei: Pool A, Pool B, Pool C, and Pool D.



Round 1 Plate [A-D] pooling. Pool Round 1 Plate A sample into Tube A, store on ice, then proceed with [B-D].

21. **If using low input fixation samples**, cells/nuclei have been already captured. Place the 15 mL conical tube **magnet on ice**, and wait **1 minute** to let it pre-cool. **Proceed directly to step 27.**
22. **If using standard fixation samples**, proceed to the next step to capture the cells/nuclei.
23. Resuspend any settled beads in the Cell Capture Master Mix from step 12 by pipette mixing.
24. Using a P1000, transfer **384 μ L** of the Cell Capture Master Mix into each Pool [A-D] tube containing the pooled Round 1 cells/nuclei. Mix each pool 10x with the P1000 set to 900 μ L. Switch pipette tips between each Pool.

25. Incubate the cells/nuclei 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.



Note: In the next step, if the magnet cannot accommodate all four Pools simultaneously, **process one or two Pools at a time, keeping the others on ice.**

27. Remove or loosen the cap, then place the Pool tubes on 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 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 the cells/nuclei 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/nuclei.

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

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 μ L 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 beads-bound cells/nuclei in **1.2 mL** of Ligation Master Mix. Using a P1000 set to 1000 μ L, pipette mix 10x to fully resuspend the beads into the Ligation Master Mix. Keep the sample on ice before proceeding to the next step.

32. If processing one or two Pools at the time, repeat steps 28-31 with the remaining Pools. Then proceed to Section 1.3.



CRITICAL! Do not throw away Ligation Master Mix after use, keep it on ice: the remainder will be used in Section 1.3.

1.3. Barcoding Round 2

The four Pools of beads-bound cells/nuclei are loaded **column-wise** 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/nuclei are pooled and strained.

To add Round 2 barcodes:

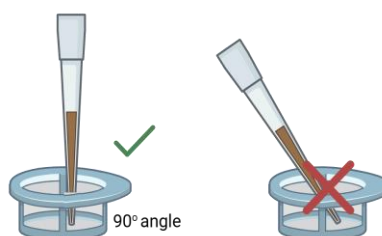
1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 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. Label four new 1.5 mL tubes with the provided stickers.
3. Pipette the entire content of each Pool sample in Ligation Master Mix (**1.2 mL** each Pool) through a cell strainer into the corresponding 1.5 mL tube with a P1000.



CRITICAL! To ensure that all of the liquid passes through the strainer, keep the tip or 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.



4. Label four 8-tubes PCR strips with the provided stickers. You can either label only the first tube of each strip or all 8 tubes in the strip.
5. Using a P200 single-channel, aliquot **139 µL** of the resuspended pool (from Pool A) into 8 labeled PCR tubes (strip format), mixing between each transfer step.
6. Repeat previous step for the remaining three resuspended pools corresponding to Pools B-D, changing tips between each pool.

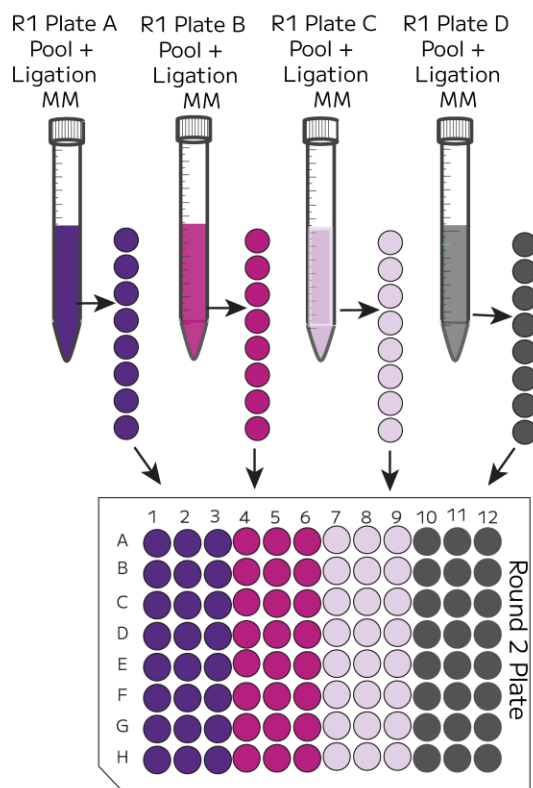
7. Place the Round 2 Plate into a thermocycler and run the following program.

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

8. 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.
9. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate and apply the provided appropriate adhesive labels to assist with loading. Store on ice.
10. With the labeled Round 2 Plate on ice and PCR strip tubes on ice, transfer the cells in Ligation Master Mix to each well in the Round 2 Plate as follows:
- Mix Pool A in the PCR strip tube by pipetting 2x with a multichannel P200 set to 40 µL.
 - Transfer **40 µL** of the mix to column 1 of the Round 2 Plate and mix by pipetting 2x.
 - Switching tips between each column, repeat steps a-b to mix Pool A in the basin then transfer to columns 2-3.
 - Repeat steps a-c to add the remaining 3 samples to columns 4-6, 7-9, and 10-12, respectively.



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.



After resuspending the four Round 1 pools with Ligation Master Mix, each pool is strained and aliquoted across **four** 8-tubes PCR strips respectively. Each pool is then loaded into three columns of the Round 2 Plate.



Note: If when loading column 3, 6, 9, and 12 (last column loaded for each pool), the volume remaining is less than 40 μ L, the wells can be partially loaded without impacting experimental results.

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

12. 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

13. 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.
14. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.
15. 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:
- With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.
 - Transfer the entire content (**50 µL**) from row A to the basin containing ● Round 2 Stop Mix.
 - 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.
16. 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 beads to bind to the magnet for **5 minutes**, or until supernatant is clear. 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.
17. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the pellet, remove and discard the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.

18. With the tube still on the magnet add **5 mL** of the **○** Barcoding Buffer. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound to the magnet.
19. With a P1000 set to 1000 μ L 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.
20. Remove tube from magnet and resuspend the beads with **4.2 mL** of the Ligation Master Mix. Using a P1000 set to 1000 μ L, 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/nuclei 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 μ L.
 - b. Transfer **40 μ L** 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 **O**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:
- With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.
 - Transfer **50 µL** from row **A** to the basin and mix 2x.
 - 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/nuclei in **4 mL** of **O 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 μ L or serological pipette.
18. Remove the tube from the magnet and resuspend the cells in **200 μ L** of ● Pre-Lysis Dilution Buffer.



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

19. Proceed to Lysis and Sublibrary Generation Step 1.5.

1.5. Lysis and Sublibrary Generation

The cell/nuclei 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 use a plate magnet for beads clean up. See "User Supplied Equipment and Reagents" for recommended plate magnetic racks.

1. While minimizing time on ice, count the number of cells/nuclei in the sample with a fluorescent (e.g. DAPI) cell counting device. Record the cell/nuclei 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/nuclei across the desired number of sublibraries. See the "Sublibrary Loading" in the Important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix A to determine the volume of sample and ●Pre-Lysis Dilution Buffer that should be added to each sublibrary.



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

3. Ensure the cells/nuclei are in suspension by pipetting 5x with a P200 set to 200 µL prior to each transfer. Add the appropriate volume of sample to 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	2	6	8	16	32
● Lysis Solution	55 µL	165 µL	220 µL	440 µL	880 µL
● Lysis Enzyme	11 µL	33 µL	44 µL	88 µL	176 µL
Total	66 µL	198 µL	264 µL	528 µL	1056 µL

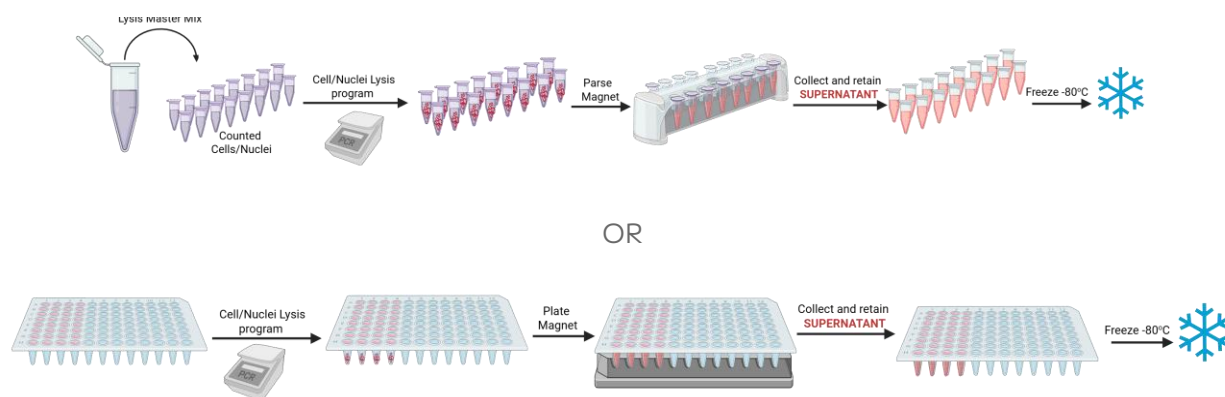


Note: Ensure that there is no precipitate before using the ● Lysis Solution.



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/nuclei. Store at room temperature.



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/NUCLEI 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/nuclei 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 WT v4 Library Prep User Guide.



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

This ends the Barcoding portion of the Evercode assay. To continue with the Sublibrary Preparation portion of the workflow, refer to the Evercode Max WT v4 Library Prep Reactions user manual.

Appendices

Appendix A: Determine Sublibraries Number

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

CELL/NUCLEI MAX NUMBER PER TOTAL SUBLIBRARIES				
KIT	8 SL	12 SL	16 SL	32 SL
MAX WT L-384	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/ μL)	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/Nuclei 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/NUCLEI 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	September 2026



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