User Manual

Version 1.3 - UMLCN3301

Evercode™ Low Input Cell and Nuclei Fixation v3

For use with

ECLC3301, ECLC3303, ECLC3305, ECLC3501, ECLC3503, ECLC3505, ECLN3301, ECLN3501

Legal Notices

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Overview

Workflow

From a single cell suspension, the Evercode Low Input Fixation v3 kits generate fixed and permeabilized cells/nuclei ready for use in the appropriate Evercode assays.

This workflow is designed to efficiently process between 10,000 and 100,000 cells/nuclei, accommodating up to 12 samples or as many as 96 samples simultaneously. The fixation protocol preserves cell/nuclei structure, prevents RNA degradation, and locks RNA inside the cells/nuclei, essential for downstream processing with Evercode's split-pool combinatorial barcoding technology (Figures 1, 2).

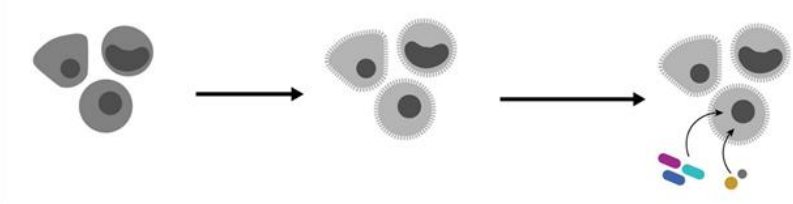

Figure 1: Evercode Cell Fixation. Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

Figure 2: Evercode Nuclei Fixation. Nuclei in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

Fixed samples are stable for up to 4 months at -80°C, providing flexibility by decoupling sample collection from library preparation. This allows samples to be stored and batched post-fixation, enabling simultaneous library preparation and minimizing batch effects.

The workflow facilitates parallel fixation of multiple samples, streamlining the process when handling up to 96 samples at a time.

The figure below provides an overview of the fixation workflow. Between 10,000 and 100,000 cells/nuclei can be fixed in a single reaction. After fixation, cells/nuclei can either be stored at -80°C or immediately proceed with capture and barcoding (Figure 3).

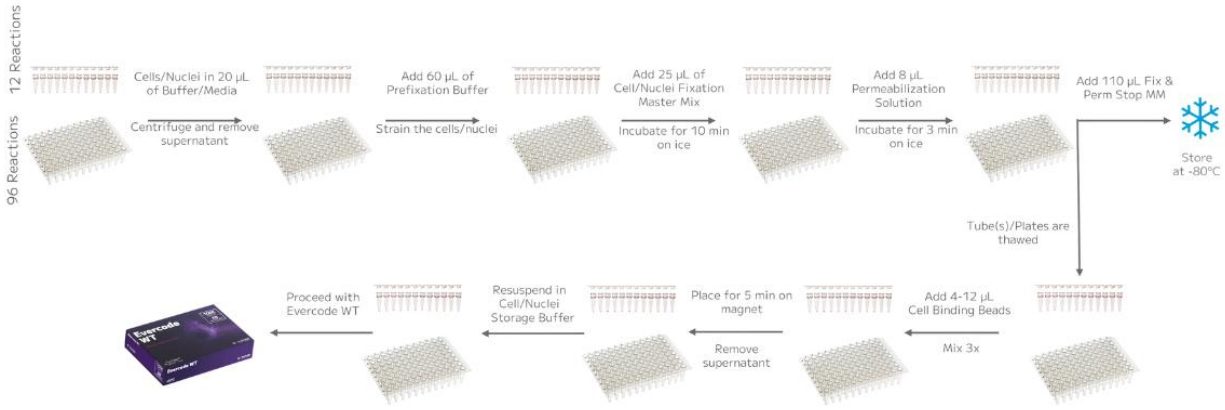

Figure 3: Low Input Cell/Nuclei Fixation workflow, designed for 12 or 96 reactions.

Protocol Timing

The table below provides details of the total and hands-on time required for the cell/nuclei fixation workflow.

SECTION	TOTAL TIME	HANDS-ON TIME	STOPPING POINTS
Sections 1 and 2			
1.1 / 2.1: Prepare Master Mixes	15 min	15 min	
1.2 / 2.2: Cell/Nuclei Fixation	60 min	60 min	-80°C ≤ 4 months
1.3 / 2.3: Cell/Nuclei Capture	30 min	30 min	

Important Guidelines

These guidelines provide additional information to obtain optimal performance beyond the detailed instructions in the protocol. 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 a previously prepared single cell/nuclei suspension. We recommend suspensions with <5% aggregation/debris.
- If cells were previously frozen, ensure the suspension is completely thawed and in suspension before beginning fixation.
- We recommend minimizing the length of time samples are stored on ice prior to fixation, as it can negatively impact results.
- Between 10,000 and 100,000 cells/nuclei can be fixed in a single reaction. Exceeding 100,000 cells/nuclei in a single fixation will result in substantially elevated doublet rates.
- The minimum input into fixation should also be determined based on how the samples will be processed downstream. The table below provides guidance on the post-fixation concentrations needed for downstream kits. However, more or less sample input may be required depending on the exact experimental design. To accurately determine required post-fixation concentrations and volumes, reference the relevant Sample Loading Table.
- Note that some cells/nuclei will be lost when freezing and thawing fixed samples, typically between 5-15%. Also consider the average bead binding retention being 65% for small cells/nuclei and 80% for large cells/nuclei. These factors should be taken into account when determining how much sample input is needed for fixation. There is one centrifugation step in this workflow. In this step cell loss will vary depending on cell type and spin speeds. We recommend optimizing centrifugation speed to minimize cell loss. See "Centrifugation" for more details.

CELL/NUCLEI CONCENTRATIONS	
Evercode Kit size	Minimum Post-Bind Concentration to Fully Load Kit per µL
Evercode Mini	298 cells/nuclei
Evercode WT	520 cells/nuclei
Evercode Mega	2,126 cells/nuclei

CELL/NUCLEI CONCENTRATIONS	
Evercode Penta	4,114 cells/nuclei

Avoiding RNase Contamination

- Standard precautions should be taken 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.

Cell/Nuclei Strainers

- To maximize cell/nuclei retention with 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.
- A strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 µm is appropriate for most cells/nuclei, the mesh size should be chosen based on your sample type.

Cell/Nuclei Counting and Quality Assessment

- We recommend a hemocytometer for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices to a hemocytometer when first using Evercode Fixation kits.
- When first using Evercode Fixation kits, we suggest saving images at each counting step.
- After fixation, the cells/nuclei are permeabilized and should appear dead with viability stains. We recommend Trypan Blue for all counting up until the bead binding step. After the beads are bound, we recommend using fluorescent staining such as Acridine Orange/Propidium Iodide (AO/PI) or Acridine Orange/DAPI (AO/DAPI) (Figure 4).

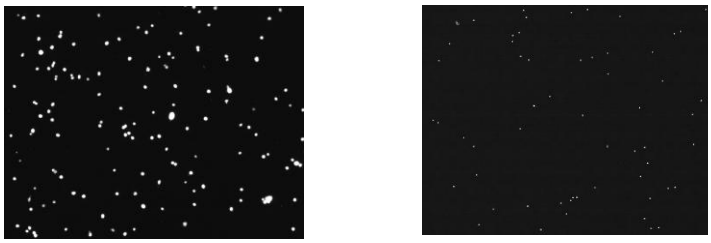

Figure 4: Example of post-bind AO/DAPI stained HEK cells (left) and PBMCs (right).

- Examples of stained fixed cells are shown below. High quality fixed samples have single distinct cells/nuclei with <5% aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing and may indicate a poor quality cell/nuclei isolation. When quantifying fixed cells/nuclei, it is critical to avoid counting debris to avoid overestimating the number of cells/nuclei (Figure 5).

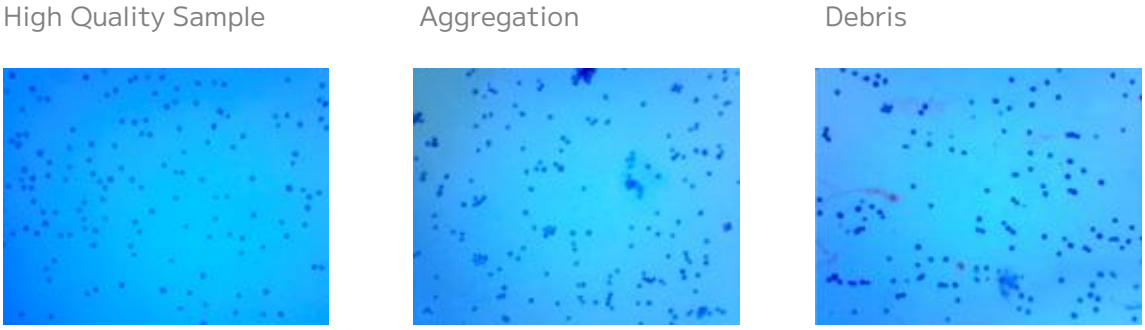

Figure 5: Standard Fixation: example of trypan blue stained fixed cells.

Centrifugation

- There is only one centrifugation step in this protocol. A range of centrifugation speeds and durations are given rather than a single speed. When using Evercode Low Input Fixation kits for the first time or when testing a new sample type, we recommend optimizing centrifugation conditions in 1.5 mL tubes before using the plate-based workflows. See the tube-based protocol in our support site, which includes detailed optimization recommendations.

- A swinging bucket rotor should be used for the high-speed centrifugation step in this protocol. The use of a fixed-angle rotor will lead to substantial cell/nuclei loss.

Maximizing Cells/Nuclei Recovery

- It is critical to thoroughly resuspend the cells/nuclei after centrifugation. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Ideally this should be verified with microscopy.

- To minimize cell/nuclei loss from cell/nuclei adherence to tubes, carefully pipette up and down along the bottom and sides of tubes.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell/nuclei pellets adequately.
- Ensure that the 0.2 mL centrifuge tubes/plates are polypropylene, as polystyrene tubes/plates will lead to substantial sample loss.
- When using Evercode Fixation kits for the first few times, we recommend retaining the supernatants removed in Sections 1.2.7 and 2.2.9. In the unlikely event of very high sample loss, these supernatants can be analyzed to identify points for optimization.

Reagent Stability

- The Prefixation Enhancer and the Cell/Nuclei Binding Beads must be stored at 4°C and should not be frozen. It is critical to never freeze the beads or vortex them for an extended period of time.
- Reagents in the Fixation Reagents box can be frozen and thawed up to 3 times.
- If the kit is going to be used more than 4 times, the reagents should be aliquoted into nuclease-free 1.5 mL tubes and stored at -20°C until use. We do not recommend making single use aliquots to minimize the impact of evaporation during storage.
- Reagent master mixes should be made fresh and used the same day.

Storage of Fixed Samples

- Fixed samples can be stored at -80°C for up to 4 months. Fixed samples should not be refrozen after thawing.

Section 1: 12 Reactions - Cell/Nuclei Fixation

Part List

Evercode Low Input Cell Fixation Kit Part List, 12 Reactions

The Evercode Low Input Cell Fixation, 12 reactions workflow requires Low Input Cell Fixation Reagents, Low Input Cell Prefixation and Binding Reagents, and Plate Strainer boxes. The Plate Strainer box should have an appropriate mesh size for the cell type being fixed.

Low Input Cell Fixation Reagents. Store at -20°C, LCF100

LABEL	ITEM	PN	FORMAT	QTY
Prefix	Prefixation Buffer	CF101	8 mL bottle	1
Storage	Storage Buffer	CF102	2 mL tube	1
Fix A	Fixative Solution A	CF103	1.5 mL tube	1
Fix B	Fixative Solution B	CF104	1.5 mL tube	1
Perm	Permeabilization Solution	CF110	1.5 mL tube	1
Stop	Fix and Perm Stop Buffer	CF106	8 mL bottle	1
RNase Inhib	RNase Inhibitor	CF111	1.5 mL tube	1
DMSO	DMSO	CF112	1.5 mL tube	1
CBB Wash Buffer	Cell Binding Bead Wash Buffer	CF113	1.5 mL tube	1

Low Input Cell Prefixation and Binding Reagents. Store at 4°C, LCF200

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Enhancer	CF201	1.5 mL tube	1
	Cell Binding Beads	CF109	1.5 mL tube	1

Plate Strainer 30 µM*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 30 µM	PCS1030	Plastic sleeve	1

Plate Strainer 70 µM*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 70 µM	PCS1070	Plastic sleeve	1

Plate Strainer 100 µM*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 100 µM	PCS1100	Plastic sleeve	1

Note: * Only one mesh size of Plate Strainer is required for the Evercode Low Input Cell Fixation kit. Select an appropriate mesh size for each sample type.

Evercode Low Input Nuclei Fixation Kit Part List, 12 Reactions

The Evercode Low Input Nuclei Fixation, 12 reactions workflow requires Low Input Nuclei Fixation Reagents, Low Input Nuclei Prefixation and Binding Reagents, and Plate Strainer boxes.

Low Input Nuclei Fixation Reagents. Store at -20°C, LNF100

LABEL	ITEM	PN	FORMAT	QTY
Prefix	Prefixation Buffer	NF101	8 mL bottle	1
Storage	Storage Buffer	NF102	2 mL tube	1
Fix	Fixative Solution	NF103	1.5 mL tube	1
Perm	Permeabilization Solution	NF109	1.5 mL tube	1
Stop	Fix and Perm Stop Buffer	NF105	8 mL bottle	1
RNase Inhib	RNase Inhibitor	NF110	1.5 mL tube	1
DMSO	DMSO	NF111	1.5 mL tube	1
NBB Wash Buffer	Nuclei Binding Bead Wash Buffer	NF112	1.5 mL tube	1

Low Input Nuclei Prefixation and Binding Reagents. Store at 4°C, LNF200

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Enhancer	NF201	1.5 mL tube	1
	Nuclei Binding Beads	NF108	1.5 mL tube	1

Plate Strainer 30 µM. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 30 µM	PCS1030	Plastic sleeve	1

User Supplied Equipment and Reagents

The following materials and equipment are required to perform the protocol but are not provided within the kit. This list does not include standard laboratory equipment, such as freezers.

Equipment

ITEM	SUPPLIER	PN	NOTES
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.
Plate Magnet	Permagen® or Alpaqua®	s500 or A000405	96-well Ring Plate Magnet or Magnum FLX®
Centrifuge with Swinging Bucket Rotor	Various Suppliers	Varies	Compatible with 0.2 mL tubes and 96 well plates, capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
1-channel: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting devices.
Styrofoam Cooler	Various Suppliers	Varies	(Optional) If storing fixed samples before processing with an Evercode kit.
Water bath	Various Suppliers	Varies	(Optional) If preparing aliquots to count the day before running a downstream Evercode kits. Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.

Consumables

ITEM	SUPPLIER	PN	NOTES
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free polypropylene 0.2 mL PCR tubes.
PCR Plates	Various Suppliers	Varies	Or equivalent nuclease-free polypropylene 0.2 mL PCR plates.
Reagents basins	Various Suppliers	Varies	12-Channel basins, capable of holding 5 mL, 25 mL
Falcon® High Clarity PP Centrifuge Tubes, 15 mL and 50 mL	Corning®	352097 (15 mL) 352098 (50 mL)	Or equivalent polypropylene centrifuge tubes.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Pipette Tips TR LTS 20 µL, 200 µL, 1,000 µL	Rainin®	17014961, 17014963, 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
AO/PI	Various Suppliers	Varies	Or alternative viability dyes
TrypLE™ Express OR TrypLE Select	Thermo Fisher Scientific	12605010 OR 12563011	(Optional) If using adherent cells. Trypsin is not recommended due to variable RNase levels.

1.1: 12 Reactions - Prepare Master Mixes

Prior to initiating the cell/nuclei fixation process, master mixes are prepared. Master mixes are tailored for each sample type.

The Evercode Low Input Cell Fixation kit necessitates the preparation of 3 distinct master mixes:

- Cell Prefixation Master Mix
- Cell Fixative Master Mix
- Cell Fix and Perm Stop Master Mix

The Cell Fixation master mixes should be prepared immediately prior to the cell fixation process.

The Evercode Input Nuclei Fixation kit necessitates the preparation of 2 distinct master mixes:

- Nuclei Prefixation Master Mix
- Nuclei Fix and Perm Stop Master Mix

The Nuclei Fixation master mixes should be prepared immediately prior to the nuclei fixation process.

1.1.1: Prepare Cell Master Mix

To prepare master mixes for Cell Fixation:

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	Low Input Cell Fixation Reagents (-20°C)	8 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex.
● Fixative Solution A	Low Input Cell Fixation Reagents (-20°C)	1.5 mL tube	
● Fixative Solution B	Low Input Cell Fixation Reagents (-20°C)	1.5 mL tube	
● Permeabilization Solution	Low Input Cell Fixation Reagents (-20°C)	1.5 mL tube	
○ Fix and Perm Stop Buffer	Low Input Cell Fixation Reagents (-20°C)	8 mL bottle	
● DMSO	Low Input Cell Fixation Reagents (-20°C)	1.5 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
○ RNase Inhibitor	Low Input Cell Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Low Input Cell Prefixation and Binding Reagents (4°C)	1.5 mL tube	Store on ice. Do not vortex.
Plate Strainer (30 µm, 70 µm, 100 µm)	Plate Strainer (Room Temperature)	Plastic sleeve	Keep at room temperature.

2. Prepare the Cell Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

CELL PREFIXATION MASTER MIX		
Number of samples	1	12
○ Prefixation Buffer	74 µL	888 µL
○ RNase Inhibitor	1.1 µL	13.2 µL
● Prefixation Enhancer	5 µL	60 µL
Total volume	80.1 µL	961.2 µL

Note: To avoid pipetting <2 µL of RNase Inhibitor, we do not recommend preparing less than 2 reactions of Cell Prefixation Master Mix. See Reagent Stability in Important Guidelines for additional details.

CRITICAL! Reagents in the Low Input Cell Fixation Reagents box can be frozen and thawed 3 times. If the kit will be used more than 4 times, aliquots should be made. See Reagent Stability in Important Guidelines for details.

3. Prepare the Cell Fixative Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

CELL FIXATIVE MASTER MIX		
Number of samples	1	12
● Fixative Solution A	15 µL	180 µL
● Fixative Solution B	15 µL	180 µL
Total volume	30 µL	360 µL

4. Prepare the Cell Fix and Perm Stop Master Mix in a new tube as follows. Mix thoroughly by pipetting and store in ice.

CELL FIX AND PERM STOP MASTER MIX		
Number of samples	1	12
O Fix and Perm Stop Buffer	117 µL	1.4 mL
● DMSO	13 µL	156 µL
Total volume	130 µL	1.56 mL

5. With the Plate Strainers still in the plastic sleeve, cut along the green plastic with sterile scissors, a razor blade, or a scalpel that has been cleaned with RNaseZap. Cut the strip according to the number of wells intended to use.

CRITICAL! Do not directly touch the mesh of Plate Strainer(s) to anything prior to straining.

CRITICAL! After opening their plastic sleeves, Plate Strainers should be stored in their original plastic sleeve until use and used on the same day they were opened.

6. Proceed immediately to Section 1.2.

1.1.2: Prepare Nuclei Master Mix

To prepare master mixes for Nuclei Fixation:

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	Low Input Nuclei Fixation Reagents (-20°C)	8 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex.
● Fixative Solution	Low Input Nuclei Fixation Reagents (-20°C)	1.5 mL tube	
● Permeabilization Solution	Low Input Nuclei Fixation Reagents (-20°C)	1.5 mL tube	
○ Fix and Perm Stop Buffer	Low Input Nuclei Fixation Reagents (-20°C)	8 mL bottle	
● DMSO	Low Input Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
● RNase Inhibitor	Low Input Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Low Input Nuclei Prefixation and Binding Reagents (4°C)	1.5 mL tube	
Plate Strainer (30 µm, 70 µm, or 100 µm)	Plate Strainer (Room Temperature)	Plastic sleeve	Keep at room temperature.

2. Prepare the Nuclei Prefixation Master Mix Stop in a new tube as follows. Mix thoroughly by pipetting and store on ice.

NUCLEI PREFIXATION MASTER MIX		
Number of samples	1	12
○ Prefixation Buffer	74 µL	888 µL
● RNase Inhibitor	1.1 µL	13.2 µL
● Prefixation Enhancer	5 µL	60 µL
Total Volume	80.1 µL	961.2 µL

Note: To avoid pipetting <2 µL of RNase Inhibitor, we do not recommend preparing less than 2 reactions of Nuclei Prefixation Master Mix. See Reagent Stability in Important Guidelines for additional details.

CRITICAL! Reagents in the Nuclei Fixation Reagents box can be frozen and thawed 3 times. If the kit will be used more than 4 times, aliquots should be made. See Reagent Stability in Important Guidelines for details.

3. Prepare the Nuclei Fix and Perm Stop Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

NUCLEI FIX AND PERM STOP MASTER MIX		
Number of samples	1	12
○ Fix and Perm Stop Buffer	117 µL	1.40 mL
● DMSO	13 µL	156 µL
Total	130 µL	1.56 mL

4. With the Plate Strainers still in the plastic sleeve, cut along the green plastic with sterile scissors, a razor blade, or a scalpel that has been cleaned with RNaseZap. Cut the strip according to the number of wells intended to use.

CRITICAL! Do not directly touch the mesh of Plate Strainer(s) to anything prior to straining.

CRITICAL! After opening their plastic sleeves, Plate Strainers should be stored in their original plastic sleeve until use and used on the same day they were opened.

5. Proceed immediately to Section 1.2.

1.2: 12 Reactions - Cell/Nuclei Fixation

After the initial centrifugation to remove the buffer/medium from the single cell suspension, Prefixation Master Mix are added to the cells/nuclei. Reagents are added to fix and permeabilize cells/nuclei, and then stop these reactions. Fixed cells/nuclei are stored at -80°C.

To fix cells/nuclei:

1. Cool the centrifuge with a swinging bucket rotor to 4°C.
2. Fill a bucket with ice.
3. Prepare a hemocytometer, flow cytometer, or other cell/nuclei counting device.
4. Count the cells/nuclei in the single cell suspension with a hemocytometer or alternative counting device and record the count. Keep cells/nuclei on ice during counting and work quickly to minimize time on ice prior to fixation.
5. Transfer 10,000 to 100,000 cells/nuclei from each sample into 0.2 mL tube(s)/plate. Seal the tube(s)/plate with caps or an adhesive seal.
6. Centrifuge the 0.2 mL tube(s)/plate in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

Note: A small cell/nuclei loss should be expected after centrifugation.

CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial cells/nuclei loss. Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See the Important Guidelines section for details.

CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

7. Slowly aspirate then discard the supernatant, leaving no more than **20 µL** of supernatant.

Note: Do not reuse any tips across wells throughout this protocol. Never place a tip that has entered one of the wells into a different well or back into a master mix.

8. Fully resuspend each pellet in **60 µL** of Cell/Nuclei Prefixation Master Mix.

9. Apply a Plate Strainer to a new 0.2 mL tube(s)/plate by peeling off the backing, carefully aligning over the wells, and placing on the surface of the tube(s)/plate.

Note: The Plate Strainer fits a 96 well plate. It will need to be cut when processing 12 samples.

10. Without touching the mesh, fully adhere the Plate Strainer by pressing a pipette tip or plate sealer cleaned with RNaseZap along the green plastic on the edges and between the wells of the tube(s)/plate.

11. Pipette **75 µL** of each sample through the strainer into the new 0.2 mL tube(s)/plate and store on ice.

CRITICAL! Do not directly touch the mesh of the strainer(s) with anything except the pipette tip.

Note: To ensure that all of the liquid passes through the strainer, firmly press the tip of the pipette against the filter and steadily depress down the pipette plunger. All of the liquid should pass through the strainer in ~1 second.

12. Carefully peel off the Plate Strainer and discard.

13. Add **25 µL** of Cell Fixative Master Mix/● Fixative Solution to each well and mix immediately by pipetting exactly 3x.

CRITICAL! Do not perform additional mixing at this step.

14. Incubate on ice for **10 minutes**.

15. Add **8 µL** of ● Cell/● Nuclei Permeabilization Solution to each well. Immediately mix thoroughly by pipetting 3x with a P200 set to 80 µL.

16. Incubate on ice for **3 minutes**.

17. Mix the Cell/Nuclei Fix and Perm Stop Master Mix by inverting the tube 5x. Do not vortex.

18. Add **110.4 µL** of Cell/Nuclei Fix and Perm Stop Master Mix to each well. Gently pipette 3x.

19. Proceed to Section 1.3: 12 Reactions - Cell/Nuclei Capture, if immediately processing samples with an Evercode Whole Transcriptome kit. Otherwise, proceed to the next step to freeze and save the fixed cells/nuclei.

20. Place the samples in a room temperature styrofoam cooler, close the lid, and store at -80°C to slowly cool the samples.

CRITICAL! Storing samples directly in the freezer without controlled cooling may lead to cell/nuclei damage and compromise data quality.

Safe stopping point: Samples are stable for up to 4 months at -80°C.

1.3: 12 Reactions - Cell/Nuclei Capture

After fixation, cells/nuclei need to be captured using magnetic beads and resuspended in Storage Buffer prior to barcoding. If fixed samples were stored at -80°C, they will need to be thawed before the capture step. It is recommended to count the captured cells/nuclei using fluorescent-based dyes on an automated cell counter or trypan blue on a hemocytometer prior to input into barcoding. Alternatively, you may extrapolate the remaining cells/nuclei based on the previous count (Section 1.2, Step 4) and assume 65% retention for small cells/nuclei and 80% retention for large cells/nuclei.

To capture cells/nuclei:

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
● Cell/● Nuclei Storage Buffer	Low Input Cell/Nuclei Fixation Reagents (-20°C)	2 mL tube	Thaw at room temperature then immediately store on ice. Mix by inverting. Do not vortex.
● Cell/● Nuclei Binding Beads	Low Input Cell/Nuclei Prefixation and Binding Reagents (4°C)	1.5 mL tube	**Do not freeze.** Gently pulse-vortex until resuspended and store at room temperature. Do not let settle for >3 minutes before pipetting.
● Cell/● Nuclei Binding Bead Wash Buffer	Low Input Cell/Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Thaw at room temperature then store on ice. Mix by inverting 3x.

CRITICAL! Do not use ● Cell/● Nuclei Binding Beads that are frozen or dried and settled at the bottom of the tube within ~10 seconds after pulse-vortexing.

2. Gently pulse-vortex ● Cell/● Nuclei Binding Beads until resuspended. Add the appropriate volume of ● Cell/● Nuclei Binding Beads to a new 0.2 mL PCR tube as follows, depending on the number of samples being processed:

BINDING BEADS VOLUMES		
Number of samples	1	12
● Cell/● Nuclei Binding Beads	15 µL	180 µL

3. Place the tube on the magnetic rack for 0.2 mL PCR tubes until the solution clears (~2 minutes).

4. Remove and discard the supernatant.

5. Remove the tube from the magnetic rack and fully resuspend the bead pellet in the appropriate volume of ● Cell/● Nuclei Binding Bead Wash Buffer.

BEAD WASH BUFFER VOLUMES		
Number of samples	1	12
● Cell/● Nuclei Binding Bead Wash Buffer	15 µL	180 µL

Note: Ensure no beads are stuck to the sides of the 0.2 mL tube.

6. Place the tube on the magnetic rack for 0.2 mL PCR tubes until the solution clears (~2 minutes).

7. Remove and discard the supernatant.

8. Repeat steps 5-7 twice for a total of 3 washes.

9. Remove the tube from the magnetic rack. Fully resuspend the pellet in the appropriate volume of ● Cell/● Nuclei Storage Buffer.

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

CELLS/NUCLEI STORAGE BUFFER		
Number of Samples	1	12
● Cell/● Nuclei Storage Buffer	15 µL	180 µL

10. Remove the tube(s)/plate of fixed cells/nuclei from -80°C storage. Set thermocycler to the following protocol:

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

11. To thaw, place the tube(s)/plate of frozen cells/nuclei in the thermocycler and start the program.

12. Once thaw protocol has finished, check that all tube(s)/wells are fully thawed. If ice remains, place the tube(s)/plate back into the thermocycler for another minute at 37°C. Once fully thawed, place the tube(s)/plate into a PCR plate holder, remove the cap(s)/plate seal, and store on ice.

13. To capture cells/nuclei, add ● Cell/● Nuclei Binding Beads in the ● Cell/● Nuclei Storage Buffer to each fixed sample according to the table below. It is critical to mix the beads thoroughly with a pipette to fully resuspend them before using them for cell capture. Discard unused beads.

Note: Do not let the beads settle for more than 2 minutes before pipette mixing them.

CELL/NUCLEI BINDING BEAD ADDITION	
Cell/Nuclei Input Number	Volume of 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

14. 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**.

15. Place the tube(s) on a 0.2 mL tube magnet or the plate on a plate magnet and bind at room temperature for **5 minutes**.

16. With a P200 pipette set to 200 µL, remove supernatant from each sample, being careful not to disturb the pellet.

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

17. Vigorously resuspend cells/nuclei in the ● Cell/● Nuclei Storage Buffer and mix well. The table below shows the minimum volume required for resuspension. Refer to the Sample Loading Table for exact dilutions for your Whole Transcriptome kit.

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

Note: If you have <30,000 cells/nuclei and prefer not to count, proceed assuming 65% retention for small cells/nuclei and 80% retention for large cells/nuclei. Note that these retention assumptions are an estimate and may result in under/overloading the barcoding plate.

MINIMUM CELL/NUCLEI STORAGE BUFFER RESUSPENSION	
Cell/Nuclei Number Input	Minimum required Cell/Nuclei Storage Buffer per Resuspension (µL)
10,000 - 49,999	20
50,000 - 74,999	22
75,000 - 89,999	32
90,000 - 100,000	46

CRITICAL! Storage buffer resuspension volumes in the table above are the minimum volumes needed to resuspend samples. Depending on sample concentration, further dilution prior to proceeding with barcoding is likely required. See Sample Loading Table for required dilution amounts.

18. Proceed to cell/nuclei counting and/or to Evercode Whole Transcriptome User Guide Section 1.1 Barcoding.

Section 2: 96 Reactions - Cell/Nuclei Fixation

Part List

Evercode Low Input Cell Fixation Kit Part List, 96 Reactions

The High Throughput Evercode Low Input Cell Fixation kit requires Low Input Cell Fixation Reagents, Low Input Cell Prefixation and Binding Reagents, and Plate Strainer boxes. The Plate Strainer box should have an appropriate mesh size for the cell type being fixed.

Low Input Cell Fixation Reagents. Store at -20°C, LCF300

LABEL	ITEM	PN	FORMAT	QTY
Prefix	Prefixation Buffer	CF301	15 mL bottle	1
Storage	Storage Buffer	CF302	8 mL bottle	2
Fix A	Fixative Solution A	CF303	8 mL bottle	1
Fix B	Fixative Solution B	CF304	8 mL bottle	1
Perm	Permeabilization Solution	CF310	8 mL bottle	1
Stop	Fix and Perm Stop Buffer	CF306	15 mL bottle	1
RNase Inhib	RNase Inhibitor	CF311	1.5 mL tube	1
DMSO	DMSO	CF312	2 mL tube	1
CBB Wash Buffer	Cell Binding Bead Wash Buffer	CF313	8 mL bottle	1

Low Input Cell Prefixation and Binding Reagents. Store at 4°C, LCF400

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Enhancer	CF401	2 mL tube	1
	Cell Binding Beads	CF309	2 mL tube	1

30 µM Plate Strainer 2x30 µM*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 30 µM	PCS1030	Plastic sleeve	2

Plate Strainer 2x70 µM*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 70 µM	PCS1070	Plastic sleeve	2

Plate Strainer 2x100 µM*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 100 µM	PCS1100	Plastic sleeve	2

Note: * Only one mesh size of Plate Strainer is required for the High Throughput Evercode Low Input Fixation kit. Select an appropriate mesh size for each sample type.

Evercode Low Input Nuclei Fixation Kit Part List, 96 Reactions

The High Throughput Evercode Low Input Nuclei Fixation kit requires Low Input Nuclei Fixation Reagents, Low Input Nuclei Prefixation and Binding Reagents, and Plate Strainer boxes.

Low Input Nuclei Fixation Reagents. Store at -20°C, LNF300

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Buffer	NF301	15 mL bottle	1
	Storage Buffer	NF302	8 mL bottle	2
	Fixative Solution	NF303	8 mL bottle	1
	Permeabilization Solution	NF309	8 mL bottle	1
	Fix and Perm Stop Buffer	NF305	15 mL bottle	1
	RNase Inhibitor	NF310	1.5 mL tube	1
	DMSO	NF311	2 mL tube	1
	Nuclei Binding Bead Wash Buffer	NF312	8 mL tube	1

Low Input Nuclei Prefixation and Binding Reagents. Store at 4°C, LNF400

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Enhancer	NF401	2 mL tube	1
	Nuclei Binding Beads	NF308	2 mL tube	1

30 µM Plate Strainer 2x30 µM*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 30 µM	PCS1030	Plastic sleeve	2

User Supplied Equipment and Reagents

The following materials and equipment are required to perform the protocol but are not provided within the kit. This list does not include standard laboratory equipment, such as freezers.

Equipment

ITEM	SUPPLIER	PN	NOTES
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.
Plate Magnet	Permagen® or Alpaqua®	s500 or A000405	96-well Ring Plate Magnet or Magnum FLX®
Centrifuge with Swinging Bucket Rotor	Various Suppliers	Varies	Compatible with 96 deep well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
1-channel: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting devices.
Styrofoam Cooler	Various Suppliers	Varies	(Optional) If storing fixed samples before processing with an Evercode kit.
Water bath	Various Suppliers	Varies	(Optional) If preparing aliquots to count the day before running a downstream Evercode Whole Transcriptome assay. Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.

Consumables

ITEM	SUPPLIER	PN	NOTES
SealPlate®	Excel Scientific®	100-SEAL-PLT	Or equivalent PCR plate seals. Note that many clear plastic seals are not designed for storage at -80°C, so we recommend using foil plate seals if storing fixed samples in PCR plates.
TempPlate® EXT Sealing Foil	USA Scientific®	2998-0100	(Optional) If storing fixed samples in a PCR plate. Note that many clear plastic seals are not designed for storage at -80°C.
Reagent basins	Various Suppliers	Varies	
Eppendorf twin.tec® PCR Plate 96 LoBind®	Eppendorf	0030129504	Or equivalent DNA low-binding, nuclease-free PCR plate or 0.2 mL tube strips.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL and 50 mL	Corning®	352097 (15mL) 352098	Or equivalent polypropylene centrifuge tubes.
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Pipette Tips TR LTS 20 µL, 200 µL, 1,000 µL	Rainin®	17014961, 17014963, 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
AO/PI	Various Suppliers	Varies	(Optional) Recommended for post-capture cell/nuclei counting.
Trypan Blue	Various Suppliers	Varies	Or alternative viability dyes.
TrypLE™ Express OR TrypLE Select	Thermo Fisher Scientific	12605010 OR 12563011	(Optional) If using adherent cells. Trypsin is not recommended due to variable RNase levels.

2.1: 96 Reactions - Prepare Master Mixes

Prior to initiating the cell/nuclei fixation process, master mixes are prepared. Master mixes are tailored for each sample type.

The Evercode Low Input Cell Fixation workflow necessitates the preparation of 3 distinct master mixes:

- Cell Prefixation Master Mix
- Cell Fixative Master Mix
- Cell Fix and Perm Stop Master Mix

The Cell Fixation master mixes should be prepared immediately prior to the cell fixation process.

The Evercode Low Input Nuclei Fixation workflow necessitates the preparation of 2 distinct master mixes:

- Nuclei Prefixation Master Mix
- Nuclei Fix and Perm Stop Master Mix

The Nuclei Fixation master mixes should be prepared immediately prior to the nuclei fixation process.

2.1.1 Prepare Cell Master Mix

To prepare master mixes for cell fixation:

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	Low Input Cell Fixation Reagents (-20°C)	15 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex.
○ Fixative Solution A	Low Input Cell Fixation Reagents (-20°C)	8 mL bottle	
○ Fixative Solution B	Low Input Cell Fixation Reagents (-20°C)	8 mL bottle	
○ Permeabilization Solution	Low Input Cell Fixation Reagents (-20°C)	2 mL tube	
○ Fix and Perm Stop Buffer	Low Input Cell Fixation Reagents (-20°C)	8 mL bottle	
● DMSO	Low Input Cell Fixation Reagents (-20°C)	2 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
○ RNase Inhibitor	Low Input Cell Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Low Input Cell Prefixation and Binding Reagents (4°C)	2 mL tube	
Plate Strainer (30 µm, 70 µm, 100 µm)	Plate Strainer (Room Temperature)	Plastic sleeve	Keep at room temperature.

2. Prepare the Cell Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

CELL PREFIXATION MASTER MIX		
Number of samples	1	96
○ Prefixation Buffer	74 µL	8.7 mL
○ RNase Inhibitor	1.1 µL	109 µL
● Prefixation Enhancer	5 µL	590 µL
Total Volume	80.1 µL	9.4 mL

Note: To avoid pipetting <2 µL of RNase Inhibitor, we do not recommend preparing less than 2 reactions of Cell Prefixation Master Mix. See Reagent Stability in Important Guidelines for additional details.

3. Prepare the Cell Fixative Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

CELL FIXATIVE MASTER MIX		
Number of samples	1	96
○ Fixative Solution A	26 µL	2.5 mL
○ Fixative Solution B	26 µL	2.5 mL
Total volume	52 µL	5.0 mL

4. Prepare the Cell Fix and Perm Stop Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

CELL FIX AND PERM STOP MASTER MIX		
Number of samples	1	96
○ Fix and Perm Stop Buffer	117 µL	11.23 mL
● DMSO	13 µL	1.25 mL
Total volume	130 µL	12.48 mL

5. With the Plate Strainers still in the plastic sleeve, cut along the green plastic with sterile scissors, a razor blade, or a scalpel that has been cleaned with RNaseZap. Cut the strip according to the number of rows intended to use.

CRITICAL! Do not directly touch the mesh of Plate Strainer(s) to anything prior to straining.

CRITICAL! After opening their plastic sleeves, Plate Strainers should be stored in their original plastic sleeve until use and used on the same day they were opened.

6. Proceed immediately to Section 2.2.

2.1.2 Prepare Nuclei Master Mix

To prepare master mixes for nuclei fixation:

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	Low Input Nuclei Fixation Reagents (-20°C)	15 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex.
○ Fixative Solution	Low Input Nuclei Fixation Reagents (-20°C)	8 mL bottle	
○ Permeabilization Solution	Low Input Nuclei Fixation Reagents (-20°C)	2 mL tube	
○ Fix and Perm Stop Buffer	Low Input Nuclei Fixation Reagents (-20°C)	15 mL bottle	
● RNase Inhibitor	Low Input Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● DMSO	Low Input Nuclei Fixation Reagents (-20°C)	2 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
● Prefixation Enhancer	Low Input Nuclei Prefixation and Binding Reagents (4°C)	2 mL tube	Store on ice immediately before use. Do not vortex.
Plate Strainer	Plate Strainer (Room Temperature)	Plastic sleeve	Keep at room temperature

2. Prepare the Nuclei Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

NUCLEI PREFIXATION MASTER MIX		
Number of samples	1	96
○ Prefixation Buffer	74 µL	8.7 mL
● RNase Inhibitor	1 µL	109 µL
● Prefixation Enhancer	5 µL	590 µL
Total Volume	80 µL	9.4 mL

Note: To avoid pipetting <2 µL of RNase Inhibitor, we do not recommend preparing less than 2 reactions of Nuclei Prefixation Master Mix. See Reagent Stability in Important Guidelines for additional details.

CRITICAL! Reagents in the Nuclei Fixation Reagents box can be frozen and thawed 3 times. If the kit will be used more than 4 times, aliquots should be made. See Reagent Stability in Important Guidelines for details.

3. Prepare the Nuclei Fix and Perm Stop Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

NUCLEI FIX AND PERM STOP MASTER MIX		
Number of samples	1	96
○ Fix and Perm Stop Buffer	117 µL	11.23 mL
● DMSO	13 µL	1.25 mL
Total	130 µL	12.48 mL

4. With the Plate Strainers still in the plastic sleeve, cut along the green plastic with sterile scissors, a razor blade, or a scalpel that has been cleaned with RNaseZap. Cut the strip according to the number of rows intended to use.

CRITICAL! Do not directly touch the mesh of Plate Strainer(s) to anything prior to straining.

CRITICAL! After opening their plastic sleeves, Plate Strainers should be stored in their original plastic sleeve until use and used on the same day they were opened.

5. Proceed immediately to Section 2.2.

2.2: 96 Reactions - Cell/Nuclei Fixation

After the initial centrifugation to remove the buffer/medium from the single cell/nuclei suspension, the Prefixation Master Mix is added to cells/nuclei. Reagents are added to fix and permeabilize cells/nuclei, and then stop these reactions. Fixed cells/nuclei are stored at -80°C for up to 4 months.

To fix cells/nuclei:

1. Cool the centrifuge with a swinging bucket rotor to 4°C.
2. Fill a bucket with ice.
3. Prepare a hemocytometer, flow cytometer, or other cell/nuclei counting device.
4. Count the cells/nuclei in the single cell/nuclei suspension with a hemocytometer or alternative counting device and record the count. Keep cells/nuclei on ice during counting and work quickly to minimize time on ice prior to fixation.
5. With the plate on ice, transfer 10,000 to 100,000 cells/nuclei from each sample into the wells of polypropylene, nuclease-free PCR plate.
6. Seal the plate with an adhesive seal.
7. Centrifuge the plate in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

Note: A small cell/nuclei loss should be expected after centrifugation.

CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial cell/nuclei loss. Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See the Important Guidelines section for details.

CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

8. Remove the plate from the centrifuge, remove the plate seal, and store it on ice.

9. With a multichannel P200, slowly aspirate and discard all but ~20 µL of supernatant from each well. Keep the pipette tips along the side of the wells to avoid disturbing the pellets.

CRITICAL! Do not reuse any tips across rows throughout this protocol. Never place a tip that has entered one of the wells into a different well or back into a master mix.

Note: Tilting the plate 90 degrees makes it easier to visualize removal of residual supernatant.

10. Transfer the Cell/Nuclei Prefixation Master Mix to a new basin with a pipette.

11. In order to prepare to strain the prefix samples, apply the Plate Strainer to a new PCR plate, carefully aligning over the wells. Without touching the mesh, fully adhere the Plate Strainer by pressing a pipette tip or plate sealer cleaned with RNaseZap along the green plastic on the edges and between the wells of the plate. Keep the plate on ice.

12. Using a multichannel P200 pipette, fully resuspend each pellet in **60 µL** of Cell/Nuclei Prefixation Master Mix. Using the same set of tips, strain **75 µL** of the resuspended cells/nuclei into the corresponding wells of the new PCR plate from step 11.

13. Repeat Step 12 for the next row until all samples are resuspended and strained. Keep strained cells/nuclei on ice.

CRITICAL! Do not directly touch the mesh of Plate Strainer(s) to anything prior to straining.

Note: Plate Strainer(s) will need to be cut if processing fewer than 96 samples.

Note: To ensure that all of the liquid passes through the strainer, firmly press the tip of the pipette against the filter and steadily depress down the pipette plunger. All of the liquid should pass through the strainer in ~1 second.

14. Tap the plate 3x on the benchtop to move liquid to the bottom of the wells. Check to ensure there are no bubbles in the bottom of the wells.

15. Carefully peel off the Plate Strainer and discard.

16. Transfer the Cell Fixative Master Mix/O Fixative Solution to a basin with a pipette.

FIXATIVE VOLUMES		
Number of samples	1	96
Cell Fixative Master Mix/O Fixative Solution	40 µL	5 mL

17. With the plate on ice, add **25 µL** of Cell Fixative Master Mix/O Fixative Solution to each well and mix immediately by pipetting exactly 3x.

18. Incubate on ice for **10 minutes**.

Note: Start the timer after adding Cell Fixative Master Mix/O Fixative Solution to the first row of the plate.

19. Transfer the O Cell/O Nuclei Permeabilization Solution to a new basin with a pipette as follows.

PERMEABILIZATION SOLUTION VOLUMES		
Number of samples	1	96
O Cell/Nuclei Permeabilization Solution	15 µL	1.44 mL

20. With a P20 multichannel pipette, add **8 µL** of O Cell/Nuclei Permeabilization Solution to each well with a multichannel P20 and mix immediately by pipetting 3x with a multichannel P200 set to 80 µL.

21. Repeat Step 20 for each row until all samples are permeabilized.

22. Incubate on ice for **3 minutes**.

Note: Start the timer after adding Permeabilization Solution to the first row of the plate. This incubation can be extended by 2 additional minutes and up to a total of **5 minutes** without negatively impacting performance.

23. Transfer the Cell/Nuclei Fix and Perm Stop Master Mix to a new basin with a pipette as follows:

FIX AND PERM STOP MASTER MIX VOLUMES		
Number of samples	1	96
Cell/Nuclei Fix and Perm Stop Master Mix	113 µL	12 mL

24. With the plate on ice, add **110.4 µL** of Cell/Nuclei Fix and Perm Stop Master Mix to each well and mix immediately by gently pipetting 3x with a multichannel P200 set to 100.4 µL, the same volume of the master mix.

25. Proceed to Section 2.3: 96 Reactions - Cell/Nuclei Capture if immediately processing samples with an Evercode Whole Transcriptome kit. Otherwise, proceed to the next step.

26. Seal the PCR plate with a seal that can withstand storage at -80°C.

CRITICAL! Many clear plastic seals are not designed for storage at -80°C, so we recommend using foil plate seals.

27. Place the samples in a room temperature styrofoam cooler, close the lid, and store at -80°C to slowly cool the samples.

CRITICAL! Storing samples directly in the freezer without controlled cooling may lead to cell/nuclei damage and compromise data quality.

Safe stopping point: Samples are stable for up to 4 months at -80°C.

2.3: 96 Reactions - Cell/Nuclei Capture

After fixation, cells/nuclei need to be captured using magnetic beads and resuspended in Storage Buffer prior to barcoding. If fixed samples were stored at -80°C, they will need to be thawed before the capture step. Capture of fixed cells/nuclei is performed using magnetic beads. It is recommended to count the captured cells/nuclei using fluorescent-based dyes on an automated cell/nuclei counter or trypan blue on a hemocytometer prior to input into barcoding. Alternatively, you may extrapolate the remaining cells/nuclei based on the previous count (Section 2.2, step 4) and assume 65% retention for small cells/nuclei and 80% retention for large cells/nuclei.

Note: If performing the downstream barcoding with 384 samples, **skip this section** and proceed with the "Set Up for Low Input Fixation Samples" section of the WT 384 or the Penta 384 user manuals.

To capture cells/nuclei:

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Storage Buffer	Low Input Cell/Nuclei Fixation Reagents (-20°C)	8 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting. Do not vortex.
● Cell/● Nuclei Binding Beads	Low Input Cell/Nuclei Prefixation and Binding Reagents (4°C)	2 mL tube	**Do not freeze.** Gently pulse-vortex until resuspended and store at room temperature. Do not let it settle for >3 minutes before pipetting.
○ Cell/Nuclei Binding Bead Wash Buffer	Low Input Cell/Nuclei Fixation Reagents (-20°C)	8 mL bottle	Thaw at room temperature then store on ice. Mix by inverting 3x.

CRITICAL! Do not use ● Cell/● Nuclei Binding Beads that are frozen or dried and settled at the bottom of the tube within ~10 seconds after pulse-vortexing.

2. Gently pulse-vortex ● Cell/● Nuclei Binding Beads until resuspended. Add the appropriate volume of ● Cell/● Nuclei Binding Beads to a new 1.5 mL tube as follows, depending on the number of samples being processed:

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

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

4. Remove and discard the supernatant.

5. Remove the tube from the magnetic rack and fully resuspend the bead pellet in the appropriate volume of ○ Cells/Nuclei Binding Bead Wash Buffer.

CELL/NUCLEI BINDING BEAD WASH BUFFER		
Number of Samples	1	96
○ Cell/Nuclei Binding Bead Wash Buffer	15 µL	1.44 mL

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

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

7. Remove and discard the supernatant.

8. Repeat steps 5-7 twice for a total of 3 washes.

9. Remove the tube from the magnetic rack. Fully resuspend the pellet in the appropriate volume of ○Storage Buffer.

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

STORAGE BUFFER		
Number of Samples	1	96
○ Storage Buffer	15 µL	1.44 mL

10. Remove the plate of fixed cells/nuclei from -80°C storage. Set thermocycler to the following protocol:

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

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

12. 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, remove the plate seal, and store on ice.

13. To capture cells/nuclei add ● Cell/● Nuclei Binding Beads in the ○ Storage Buffer to each fixed sample according to the table below. It is critical to mix the beads thoroughly with a pipette to fully resuspend them before using for cell capture. Discard unused beads.

Note: Do not let the beads settle for more than 2 minutes before pipette mixing them.

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

14. With a P200 pipette set to 120 µL, vigorously pipette 5x to ensure beads are fully suspended in cell/nuclei samples, and incubate the beads/cells/nuclei mix on ice for **5 minutes**.

15. Place the plate on a plate magnet and bind at room temperature for **5 minutes**.

16. With a P200 set to 200 µL, remove the supernatant from the wells, careful not to disturb the pellet.

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

17. Vigorously resuspend cells/nuclei in the **O** Storage Buffer and mix well. The table below shows the minimum volume required for resuspension. Refer to the Sample Loading Table for exact dilutions for your Whole Transcriptome kit.

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

Note: If you have <30,000 cells/nuclei and prefer not to count, you can proceed assuming 65% retention for small cells/nuclei and 80% retention for large cells/nuclei. Note that these retention assumptions are estimates and may result in under/overloading the barcoding plate.

MINIMUM STORAGE BUFFER RESUSPENSION	
Cell/Nuclei Number Input	Minimum Required Storage Buffer per Resuspension (µL)
10,000 - 49,999	20
50,000 - 74,999	22
75,000 - 89,999	32
90,000 - 100,000	46

CRITICAL! Storage buffer resuspension volumes in the table above are the minimum volumes needed to resuspend samples. Depending on sample concentration, further dilution prior to proceeding with barcoding is likely required. See sample loading table for required dilution amounts.

18. Proceed to cell/nuclei counting and/or to Evercode Whole Transcriptome User Guide Section 1.1 Barcoding.

Appendices

Appendix A: Centrifugation Optimization

When using Evercode Fixation kits for the first time or when testing a new sample type, we recommend optimizing centrifugation conditions. This appendix provides guidelines for optimization, suggestions for common sample types, and an example experiment to optimize centrifugation speed. Note that physical properties of cells/nuclei may change after the fixation process, which requires centrifugation conditions to be optimized during fixation.

Important Guidelines

A range of centrifugation speeds should be tested to identify a speed that maximizes sample retention and permits thorough resuspension into a high quality single cell/nuclei solution. Cells/nuclei should be examined under a microscope before and after centrifugation to calculate cell/nuclei retention and assess any aggregation or morphological changes. After determining the appropriate centrifugation conditions, we recommend using the same speed and duration throughout this and downstream Evercode User Guides.

Typical Sample Retention

Across a range of samples, cell/nuclei retention post-fixation typically varies between 40-60% of the initial input. Retention is impacted by sample type, sample preparation method, centrifugation conditions, and sample handling.

Speed

Increasing centrifugation speeds can improve cell/nuclei retention, but high speeds can complicate the pellet resuspension and damage or even lyse cells/nuclei. The optimal centrifugation speed will generally achieve a greater than 50% retention through the centrifugation step while maintaining membrane integrity.

Centrifugation speed depends on cell/nuclei size. Smaller nuclei need faster speeds, and larger nuclei need slower speeds.

Duration

If cells/nuclei are damaged by increased centrifugation speed, centrifugation duration can be adjusted to increase retention without cell/nuclei damage.

Temperature

For most sample types, the centrifugation should be done at 4°C. However, some sample types may require different temperatures to maximize cell/nuclei quality prior to fixation. All

centrifugation steps in the Evercode User Guide should be done at 4°C to maintain cell/nuclei and RNA integrity.

Aggregates After Centrifugation

If the pellet cannot be resuspended back into a single cell/nuclei suspension and there are aggregates where there were previously not, this is an indication that the sample may have been over centrifuged.

Aggregates may also be an indication of insufficient pipette mixing. Gently resuspend the pellet by slowly and repeatedly pipetting until no clumps are visible. This can be visually inspected via microscopy.

Aggregates at this stage may also be a result of the sample preparation method used. If none of the above have been successful in removing the aggregates, a filtering step may help remove aggregates or the sample preparation may require additional optimization.

Debris After Centrifugation

Samples with low quality cells/nuclei may lead to excessive debris in your fixed sample. Ideally, measures should be taken to optimize sample quality prior to proceeding into fixation. The Parse Biosciences applications support team can provide sample preparation optimization techniques.

If a sample with minimal debris has significant debris after centrifugation, this may be an indication that the sample has lysed due to over centrifugation and/or overly aggressive resuspension. The centrifugation speed should be reduced and/or pellets should be less aggressively pipetted.

Recommendations for Common Sample Types

These centrifugation conditions can be used as a starting point for common sample types. However, as samples vary, we still recommend following using the optimization guidance below.

SAMPLE TYPE	SPEED	TIME	TEMPERATURE
Cells: PBMCs	200-400 x g	10 min	4°C
Cells: HEK293, 3T3, and other cell lines	200 x g	10 min	4°C
Nuclei: Mammalian nuclei	200-400 x g	10 min	4°C

Centrifugation Optimization Method

When using Evercode Fixation kits for the first time or when testing a new sample type, we recommend using 1-2 samples to optimize centrifugation conditions prior to processing samples of interest. When this is not possible, centrifugation conditions can be determined while fixing samples of interest.

Appendix B: Revision History

Version	Description	Date
1.0	Initial Release	November 2024
1.1	Sections 1.3 and 2.3: Recommended gentle pulse-vortex of the Binding Beads	November 2024
1.2	Updated to accommodate WT 384 samples workflows	February 2025
1.3	Sections 1.3 and 2.3: Updated Binding Beads mixing steps	October 2025

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