User Manual

Version 1.3 - UMLC3301M

Evercode™ Low Input Mouse TCR/BCR Cell Fixation

For use with

ECLC3301, ECLC3303
ECLC3305, ECLC3501
ECLC3503, ECLC3505

parsebiosciences.com support@parsebiosciences.com

Legal Notices

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Overview

Workflow

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

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

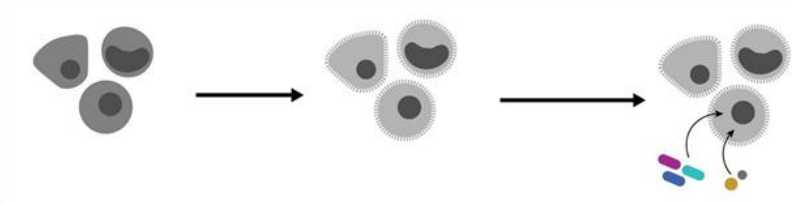

Figure 1: Evercode Cell Fixation. Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

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 can be fixed in a single reaction. After fixation, cells can either be stored at -80°C or immediately proceed with capture and barcoding (Figure 2).

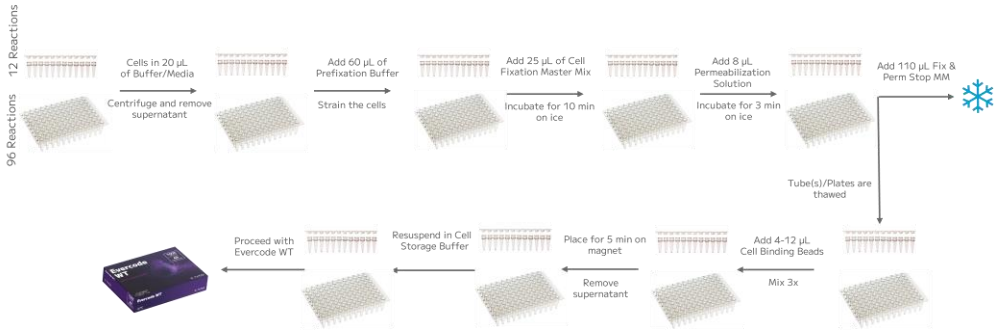

Figure 2: Low Input Mouse TCR/BCR Fixation workflow, designed for 12 or 96 reactions.

Protocol Timing

The table below provides details of the total and hands-on time required for the cell 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 Fixation	60 min	60 min	-80°C ≤ 4 months
1.3 / 2.3: Cell 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 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 can be fixed in a single reaction. Exceeding 100,000 cells 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 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 and 80% for large cells. 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 CONCENTRATIONS	
Evercode Kit size	Minimum Post-Bind Concentration to Fully Load Kit per µL
Evercode TCR or BCR Mini	298 cells
Evercode TCR or BCR	520 cells

CELL CONCENTRATIONS	
Evercode TCR or BCR Mega	2,126 cells

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 Strainers

- To maximize cell 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, the mesh size should be chosen based on your sample type.

Cell 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 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 3).

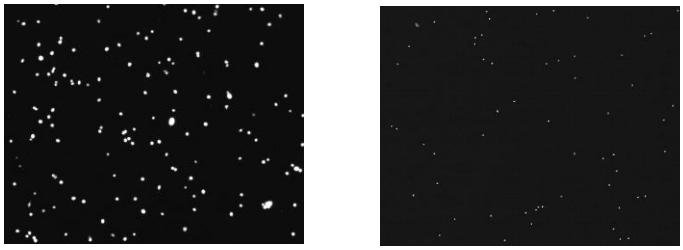

Figure 3: Example of post-bind AO/DAPI stained HEK cells (left) and PBMCs (right).

- Examples of stained fixed cells are shown below. High quality fixed samples have single distinct cells with <5% aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing and may indicate a poor quality cell isolation. When quantifying fixed cells, it is critical to avoid counting debris to avoid overestimating the number of cells (Figure 4).

High Quality Sample Aggregation Debris

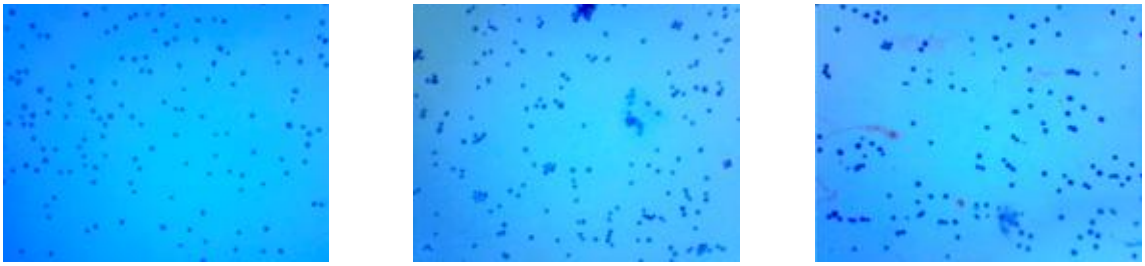

Figure 4: Example trypan blue stained fixed cells.

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 loss.

Maximizing Cells Recovery

- It is critical to use the recommended NEB RNase Inhibitor, Murine (New England Biolabs®), in addition to the RNase Inhibitor present in the Evercode Mouse TCR/BCR Low Input Cell Fixation kit to maximize RNA integrity and quality.

- It is critical to thoroughly resuspend the cells 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 loss from cell 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 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 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 Fixation

Part List

Low Input Evercode 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, PN LCF100

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

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

User Supplied Equipment and Consumables

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.

Reagents

ITEM	SUPPLIER	PN	NOTES
NEB RNase Inhibitor, Murine	New England Biolabs®, Inc.	M0314S M0314L	Choose based on experimental size. Do NOT substitute RNase Inhibitor as it will lead to decrease in sample quality.

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,

ITEM	SUPPLIER	PN	NOTES
			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.
Isopropyl alcohol	Various Suppliers	Varies	

1.1. 12 Reactions - 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.
● NEB RNase Inhibitor, Murine	Purchased Separately	1.5 mL	
● Prefixation Enhancer	Low Input Cell Prefixation and Binding Reagents (4°C)	1.5 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	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
Cell Fix and Perm Stop Buffer	117 µL	1.4 mL
DMSO	13 µL	156 µL
NEB RNase Inhibitor, Murine	13 µL	156 µL
Total volume	143 µL	1.71 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.

1.2. 12 Reactions - Cell Fixation

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

To fix cells:

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 counting device.
4. Count the cells in the single cell suspension with a hemocytometer or alternative counting device and record the count. Keep cells on ice during counting and work quickly to minimize time on ice prior to fixation.
5. Transfer 10,000 to 100,000 cells 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 loss should be expected after centrifugation.

CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial cells 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 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 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 Permeabilization Solution to each tube. Immediately mix thoroughly by pipetting 3x with a P200 set to 80 µL.

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

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

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

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

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 damage and compromise data quality.

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

1.3. 12 Reactions - Cell Capture

After fixation, cells 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 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 based on the previous count (Section 1.2, Step 4) and assume 65% retention for small cells and 80% retention for large cells.

To capture cells:

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

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

2. Gently pulse-vortex ● Cell Binding Beads until resuspended. Add the appropriate volume of ● Cell 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 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 Binding Bead Wash Buffer.

BEAD WASH BUFFER VOLUMES		
Number of samples	1	12
● Cell 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 Storage Buffer.

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

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

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

THAW CELLS		
Run Time	Lid Temperature	Sample Volume
1 min	40°C	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 in the thermocycler and start the program.

12. Once thaw protocol has finished, check that all 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, add ● Cell Binding Beads in the ● Cell Storage Buffer to each fixed sample according to the table below. Discard unused beads.

CELL BINDING BEAD ADDITION	
Cell Input Number	Volume of Cell Binding Beads (µL)
10,000 - 49,999	4
50,000 - 74,999	6
75,000 - 89,999	9
90,000 - 100,000	12

Note: With a pipette mix the beads thoroughly to fully resuspend them before using for cell capture.

14. With a P200 pipette set to 120 µL, pipette 3x to ensure beads are fully suspended in cell samples. The beads will begin binding to the cells almost immediately.

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 in the ● Cell 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 and prefer not to count, proceed assuming 65% retention for small cells and 80% retention for large cells. Note that these retention assumptions are an estimate and may result in under/overloading the barcoding plate.

MINIMUM CELL STORAGE BUFFER RESUSPENSION	
Cell Number Input	Minimum required Cell 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 counting and/or to Evercode TCR or BCR User Guides Section 1.1 Barcoding.

Section 2: 96 Reactions - Cell Fixation

Part List

Low Input Evercode 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, PN 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, PN LCF400

LABEL	ITEM	PN	FORMAT	QTY
Prefix Enhan	Prefixation Enhancer	CF401	2 mL tube	1
Cell Binding Beads	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.

User Supplied Equipment and Consumables

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.

Reagents

ITEM	SUPPLIER	PN	NOTES
NEB RNase Inhibitor, Murine	New England Biolabs®, Inc.	M0314S M0314L	Choose based on experimental size. Do NOT substitute RNase Inhibitor as it will lead to decrease in sample quality.

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 Whole Transcriptome kit.
Water bath	Various Suppliers	Varies	(Optional) If preparing aliquots to count the day before running a downstream

ITEM	SUPPLIER	PN	NOTES
			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 and 50 mL	Corning®	352097 (15mL) 352098 (50 mL)	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	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.

ITEM	SUPPLIER	PN	NOTES
AO/PI	Various Suppliers	Varies	(Optional) Recommended for post-capture cell 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 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.
◐ NEB RNase Inhibitor, Murine	Purchased Separately	1.5 mL tube	
● 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
◐ NEB RNase Inhibitor, Murine	13 µL	1.25 mL
Total volume	143 µL	13.73 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.

2.2. 96 Reactions - Cell Fixation

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

To fix cells:

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 counting device.
4. Count the cells in the single cell suspension with a hemocytometer or alternative counting device and record the count. Keep cells 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 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 loss should be expected after centrifugation.

CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial cell 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 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 Prefixation Master Mix. Using the same set of tips, strain **75 µL** of the resuspended cells into the corresponding wells of the new PCR plate from step 12.

13. Repeat Step 13 for the next row until all samples are resuspended and strained. Keep strained cells 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 to a basin with a pipette.

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

17. With the plate on ice, add **25 µL** of Cell Fixative Master Mix 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 to the first row of the plate.

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

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

20. With a P20 multichannel pipette, add **8 µL** of O Cell 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 21 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 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 Fix and Perm Stop Master Mix	113 µL	12 mL

24. With the plate on ice, add **110.4 µL** of Cell Fix and Perm Stop Master Mix to each well and mix immediately by gently pipetting 3x with a multichannel P200 set to 100 µL.

25. Proceed to Section 2.3: 96 Reactions - Cell Capture if immediately processing samples with an Evercode TCR/BCR 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 damage and compromise data quality.

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

2.3. 96 Reactions - Cell Capture

After fixation, cells 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 is performed using magnetic beads. It is recommended to count the captured cells 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 based on the previous count (Section 2.2, step 4) and assume 65% retention for small cells and 80% retention for large cells.

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 user manual(s).

To capture cells:

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

2. Gently pulse-vortex ● Cell Binding Beads until resuspended. Add the appropriate volume of ● Cell 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 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 O Cells Binding Bead Wash Buffer.

CELL BINDING BEAD WASH BUFFER		
Number of Samples	1	96
O Cell 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 O 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
O Storage Buffer	15 µL	1.44 mL

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

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

THAW CELLS		
2	Hold	4°C

11. To thaw, place the plate of frozen cells 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, add ● Cell Binding Beads in the O Storage Buffer to each fixed sample according to the table below.

CELL BINDING BEADS ADDITION	
Cell Input Number	Cell 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 for cell capture.

14. With a P200 pipette set to 120 µL, pipette 3x to ensure beads are fully suspended in cell samples. The beads will begin binding to the cells almost immediately.

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

MINIMUM STORAGE BUFFER RESUSPENSION	
Cell 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 counting and/or to Evercode TCR or BCR User Guides 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 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 solution. Cells should be examined under a microscope before and after centrifugation to calculate cell 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 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 retention, but high speeds can complicate the pellet resuspension and damage or even lyse cells. 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 size.

Duration

If cells are damaged by increased centrifugation speed, centrifugation duration can be adjusted to increase retention without cell 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 quality prior to fixation. All centrifugation steps in the Evercode User Guide should be done at 4°C to maintain cell and RNA integrity.

Aggregates After Centrifugation

If the pellet cannot be resuspended back into a single cell 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 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
Purified Primary Mouse T cells	200 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 Mouse BCR cell fixation	December 2024
1.3	Updated to accommodate WT 384 samples workflows	February 2025

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