User Manual

Version 1.1 – UMFC4300

Evercode™ Cell Fixation

v4

12 Reactions

For use with

ECFC4300

ECFC4301

ECFC4303

ECFC4305

Support Suite

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Overview

Workflow

From a single cell suspension, the Evercode Cell Fixation v4 kit generates fixed and permeabilized cells ready for use in Evercode kits.

This 1.5 mL tube based workflow is recommended when processing ≤12 samples at a time. If processing >12 samples at a time, we recommend the mid-throughput plate-based workflow which streamlines fixation when processing more samples. If processing ≥48 samples, we recommend using the Evercode Cell Fixation, HT 96 reactions kit and associated high-throughput plate-fixation protocol. Fixation maintains cell structure, prevents RNA degradation, and locks the RNA inside the cells, which is crucial for downstream processing with Evercode split-pool combinatorial barcoding technology (Figure 1).

Because fixed samples are also stable for up to 6 months at -80°C, Evercode Cell Fixation provides flexibility by separating sample collection from library preparation. It also enables samples to be stored and batched after fixation so they can be processed through library preparation together, reducing the potential of batch effects.

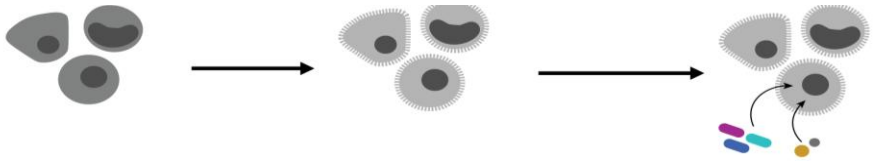


Figure 1: Evercode Cell Fixation v4. Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

The figure below provides an overview of the fixation workflow. Between 100,000 and 1 million cells can be fixed in a single reaction. Note that more than 100,000 cells may need to be fixed to fully utilize the capacity of the downstream Evercode kits. See Important Guidelines for additional details. If desired, 1-4 million cells can be fixed in a single reaction, see Appendix B for details.

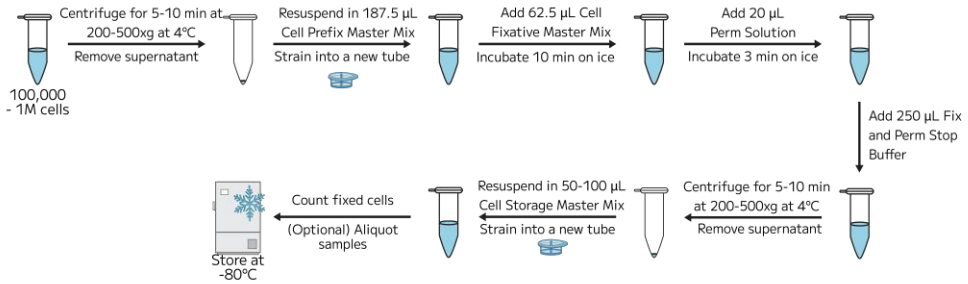


Figure 2: Evercode Cell Fixation Workflow.

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
Section 1: Set Up			
1.1 Block tubes with BSA (Optional)	65 min	5 min	4°C ≤ 1 month
1.2 Prepare Master Mixes	15 min	15 min	
Section 2: Fixation			
2.1 Cell Fixation	60 min	60 min	-80°C ≤ 6 months

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 >70% viability (ideally above 90%) and <5% aggregation/debris.
- If cells were previously frozen, ensure the suspension is completely thawed and in suspension before beginning fixation.
- We recommend minimizing the time between cell isolation and fixation, as it can negatively impact the results. Store the samples on ice the entire time.
- If fixing between 1-4 million cells, use the High Input workflow in Appendix B. Exceeding the maximum number of cells in a single fixation will result in substantially elevated doublet rates.
- The minimum input into fixation should be determined based on how the samples will be processed downstream. The table in Appendix C 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 cell concentrations and volumes, reference the relevant Sample Loading Table.
- Note that retention during fixation varies typically between 40-60%, and some cells will be lost when freezing and thawing fixed samples, typically between 5-15%.
- The final concentration of cells post-fixation is also influenced by the resuspension volume used in Step 19 of Section 2. These factors should all be considered when determining how much sample input is needed for fixation. **Refer to Appendices C1 and C2 for detailed cell number and concentration reference tables.**

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.

- Nuclease-free, filtered pipette tips should be used to reduce RNase contamination from pipettes.

Cell Detachment

- If using adherent cell line samples, we recommend TrypLE Express Enzyme (1X), phenol red (Thermo Fisher Scientific). Due to high RNase activity, we do not recommend dissociation with standard trypsin, which may reduce gene and transcript detection.

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 v4 kits.

- We suggest saving images at each counting step, especially when first using Evercode Fixation v4 kits.

- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).

- After fixation, the cells are permeabilized and should appear dead with viability stains. If using Acridine Orange/Propidium Iodide (AO/PI) stains, we suggest using the red (PI) channel to count to avoid the impact of any autofluorescence in the green (AO) channel.

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

High Quality Sample | Aggregation | Debris

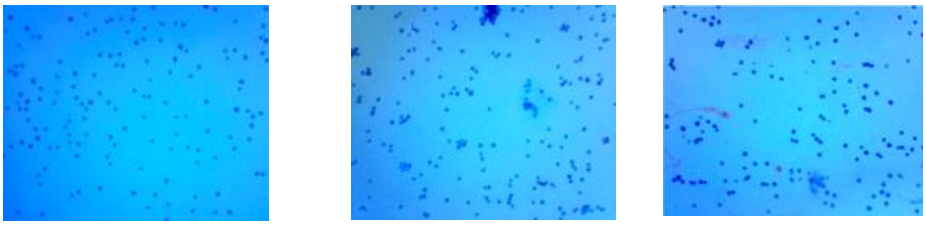


Figure 3: Example trypan blue stained fixed cells.

Centrifugation

- A swinging bucket rotor should be used for all centrifugation steps in this protocol. The use of a fixed-angle rotor will lead to substantial cell loss.

Maximizing Cell Recovery

- It is critical to thoroughly resuspend the cells after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting until no clumps are visible. Ideally this should be verified with microscopy.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.
- Ensure that the 1.5 mL and 15 mL centrifuge tubes are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- When using Evercode Cell Fixation v4 kits for the first few times, we recommend retaining the supernatants removed in Section 2 steps 8 and 18 and if relevant, steps 8 and 18 in Appendix B3. In the unlikely event of very high sample loss, these supernatants can be analyzed to identify points for optimization.

Reagent Stability

- Reagents in the Cell Fixation Reagents box should not be frozen and thawed more than 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.
- To avoid pipetting <2 µL of RNase Inhibitor, we do not recommend preparing less than 2 reactions of Cell Storage Master Mix. In addition, DMSO should be added fresh prior to use in the protocol to a final concentration of 5%. If aliquoting is required, the master mix can be prepared without DMSO, split into aliquots, and stored at -20°C for up to a month.

Storage of Fixed Samples

- Fixed samples can be stored at -80°C for up to 6 months. Fixed samples should not be refrozen after thawing.
- When possible, we recommend splitting samples into aliquots after fixation in Section 2 Step 21. The aliquots should be ≥50 µL when stored in 1.5 mL tubes. If aliquots are <50 µL, we recommend storing them in a PCR tube(s)/strip.

- We **strongly suggest** making a 10-20 µL counting aliquot for each sample. This aliquot should be used to update sample concentrations before starting the Evercode workflow. The loss after freeze/thaw should match the sample. This will minimize the time between sample thawing and the start of the Evercode workflow.

Part List

The Evercode Cell Fixation v4, 12 reactions tube workflow requires Cell Fixation Reagents and Cell Prefixation Enhancer boxes.

Cell Fixation Reagents 12 Reactions Store at -20°C, PN CF500

LABEL	ITEM	PN	FORMAT	QTY
Perm	Permeabilization Solution	CF114	1.5 mL tube	1
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
Stop	Fix and Perm Stop Buffer	CF106	8 mL bottle	1
RNase Inhib	RNase Inhibitor	CF107	1.5 mL tube	1
DMSO	DMSO	CF108	1.5 mL tube	1

Cell Prefixation Enhancer 12 Reactions Store at 4°C, PN CF600

LABEL	ITEM	PN	FORMAT	QTY
Prefix Enhan	Prefixation Enhancer	CF201	1.5 mL tube	1

User Supplied Equipment and Consumables

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

Equipment

ITEM	SUPPLIER	PN	NOTES
Centrifuge with Swinging Bucket Rotor	Various Suppliers	Varies	Compatible with 1.5 mL and 15 mL tubes and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
1-channel: P20, P200, P1000	Various Suppliers	Varies	
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device.
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific®	5100-0001	Needed if storing fixed samples before processing with an Evercode kit. Or an equivalent device that cools samples at about -1°C/minute to minimize cell damage.
Water bath	Various Suppliers	Varies	(Optional) If preparing aliquots to count the day before running a downstream Evercode kit. Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.

Consumables

ITEM	SUPPLIER	PN	NOTES
Protein LoBind® Tubes	Eppendorf®	022431081	Or equivalent protein low-binding, nuclease-free 1.5 mL tubes.
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-40 TC70-SWM-70 TC70-SWM-100	Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (30 µm, 40 µm, 70 µm, 100 µm). We do not recommend FlowMi Cell Strainers (SP Bel-Art).
pluriStrainer® Mini (Cell Strainer)	pluriSelect®	43-10040-40 43-10070-40 43-10100-40	
Falcon® Cell Strainer	Corning®	431750 431751 431752	

ITEM	SUPPLIER	PN	NOTES
EASYstrainer™, small	Greiner Bio-One™	542140 542170 542100	
Pipette Tips TR LTS 20 µL, 200 µL, 1,000 µL	Rainin®	17014961, 17014963, 17014967	Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning	352097	Or equivalent 15 mL centrifuge tubes. If using the high input protocol in Appendix B, do not use polystyrene tubes as it will lead to substantial cell loss.
Falcon® High Clarity PP Centrifuge Tubes, 50 mL	Corning	352070	(Optional, for BSA coating) or equivalent 50 mL centrifuge tubes.

Reagents

ITEM	SUPPLIER	PN	NOTES
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Trypan Blue	Various Suppliers	Varies	Or alternative viability dyes, such as AO/PI.
Isopropyl alcohol	Various Suppliers	Varies	(Optional) If using a Mr. Frosty Freezing Container.
Gibco™ Bovine Albumin Fraction V (7.5% solution)	Thermo Fisher Scientific	15260037	(Optional) If blocking tubes with BSA. Chosen due to its low RNase activity.

Section 1: Set Up

1.1. Block Tubes with BSA (Optional)

Although not required, blocking tubes with BSA can increase cell retention. When Protein LoBind tubes are not available, we recommend blocking tubes, especially for samples with low cell inputs or cells prone to aggregation.

Note: Do not use BSA coated tubes for storing fixed samples. Use regular tube if you don't have LoBind tubes.

To block tubes:

1. Prepare a fresh 1% BSA as follows, depending on the number of samples being processed.

1% BSA		
Number of Samples	1	12
Nuclease-free water (not supplied)	2.6 mL	31.2 mL
Gibco Bovine Albumin Fraction V (7.5% solution) (not supplied)	400 µL	4.8 mL
Total Volume	3 mL	36 mL

2. For each sample, fill two 1.5 mL tubes with 1.5 mL of 1% BSA and cap the tubes.
3. Invert once to fully coat the tubes.
4. Incubate the tubes for **30 minutes** at room temperature.
5. Remove the 1% BSA with a P1000 and discard.
6. Remove any remaining solution from the bottom of the tube with a P200.
7. With the caps removed, air dry the tubes for **30 minutes** in a biosafety cabinet at room temperature.
8. Proceed to Section 1.2 or store capped BSA-coated tubes at 4°C for up to 4 weeks.

1.2. Prepare Master Mixes

Master mixes should be prepared just prior to fixation.

To prepare master mixes:

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

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	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.
● Storage Buffer	Cell Fixation Reagents (-20°C)	2 mL tube	
● Fixative Solution A	Cell Fixation Reagents (-20°C)	1.5 mL tube	
● Fixative Solution B	Cell Fixation Reagents (-20°C)	1.5 mL tube	
● Permeabilization Solution	Cell Fixation Reagents (-20°C)	1.5 mL tube	
○ Fix and Perm Stop Buffer	Cell Fixation Reagents (-20°C)	8 mL bottle	
● DMSO	Cell Fixation Reagents (-20°C)	1.5 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
● RNase Inhibitor	Cell Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Cell Prefixation Enhancer (4°C)	1.5 mL tube	

2. Prepare the Cell Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting, carefully avoiding to create bubbles, and store on ice.

CELL PREFIXATION MASTER MIX		
Number of Samples	1	12
○ Pre-Fix Buffer	203.5 µL	2.44 mL
● RNase Inhibitor	2.75 µL	33 µL
● Prefixation Enhancer	13.75 µL	165 µL
Total Volume	220 µL	2.64 mL

CRITICAL! Reagents in the 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	36 µL	432 µL
● Fixative Solution B	36 µL	432 µL
Total Volume	72 µL	864 µL

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

CELL STORAGE MASTER MIX		
Number of Samples	1*	12
● Storage Buffer	112.5 µL	1.35 mL
● RNase Inhibitor **	1.5 µL	18 µL
● DMSO	6 µL	72 µL
Total Volume	120 µL	1.44 mL

Note: *To avoid pipetting 1.5 µL of RNase Inhibitor, we do not recommend preparing less than 2 reactions of Cell Storage Master Mix. See Reagent Stability in Important Guidelines for additional details.

Note: ** If fixing samples to use for mouse BCR or TCR barcoding, refer to the Mouse TCR-BCR Cell Fixation user guide.

Section 2: Fixation

2.1. Cell Fixation

After the initial centrifugation to remove the buffer/medium from the single cell suspension, cells are transferred to Cell Prefixation Master Mix. Reagents are added to fix and permeabilize cells, and then to stop these reactions. Cells are resuspended in Cell Storage Master Mix and stored at -80°C or processed immediately with a downstream Evercode kit.

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. Place a Mr. Frosty Freezing Container at room temperature.
5. 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.
6. Transfer 100,000 to 1 million cells from each sample into a Protein LoBind 1.5 mL tube (or a BSA coated tube if prepared in Section 1.1).
7. Centrifuge the tubes in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial cell loss.

CRITICAL! Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See Appendix A for details.

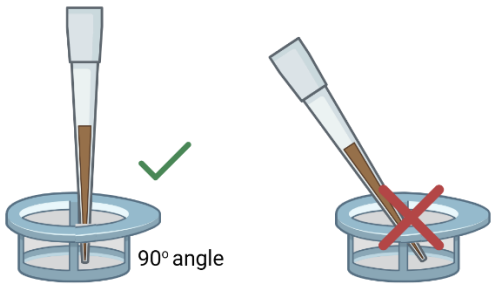
CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

8. Slowly aspirate then discard the supernatant. There should be less than 20 µL remaining in the tubes.
9. Fully resuspend each pellet in **187.5 µL** of Cell Prefixation Master Mix.

10. Pipette **187.5 µL** of each sample through a cell strainer into a new 1.5 mL tube and store on ice.

CRITICAL! Do not directly touch the mesh of cell strainer(s) with gloved hands.

Note: To ensure that all of the liquid passes through the strainer, 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.



11. Add **62.5 µL** of Cell Fixative Master Mix to each tube and mix immediately by pipetting exactly 3x.

CRITICAL! Do not perform additional mixing at this step.

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

13. Add **20 µL** of ● Permeabilization Solution to each tube. Immediately mix thoroughly by pipetting 3x with a P200 set to 180 µL.

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

15. Mix the ○ Fix and Perm Stop Buffer by inverting the tube 5x. Do not vortex.

16. Add **250 µL** of ○ Fix and Perm Stop Buffer to each tube and gently pipette mix 3x.

17. Centrifuge in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

18. With a P1000 set to 500 µL, slowly aspirate then discard the supernatant. There should be less than 20 µL remaining in the tubes.

19. Fully resuspend each pellet in an additional **50-100 µL** Cell Storage Master Mix and store on ice.

Note: Choose a resuspension volume appropriate for the expected cell output, experimental design, and downstream Evercode kits. See Appendix C1 for additional details. If using the MAX WT V4 kits, refer to Appendix C2 to choose the most appropriate cell concentration for the MAX kits size selected.

20. Pipette the sample through a cell strainer into a new, non BSA coated 1.5 mL tube and store on ice.

21. While minimizing time on ice, count the number of cells in the sample with a hemocytometer or alternative counting device and record the cell count.

22. Optional: If collecting and storing multiple samples over time, we recommend transferring **20 µL** to a new 0.2 mL PCR tube. These aliquots can be thawed and counted separately from the remaining sample for downstream processing with Evercode kits.

23. Proceed to the appropriate user guide if immediately processing samples with an Evercode kit. Otherwise, proceed to the next step.

24. Store tubes in a Mr. Frosty Freezing Container (or equivalent device) at -80°C, according to the manufacturer's instructions.

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 6 months at -80°C.

25. The day before running the downstream Evercode kit, thaw the 20 µL counting aliquots in a water bath set to 37°C. Count the cells in the single cell suspension with a hemocytometer or alternative counting device and record the count. Discard any remaining sample from the counting aliquot.

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. Smaller cells need faster speeds, and larger cells need slower speeds.

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 viability prior to fixation. For example,

isolated dendritic cells or myeloid-derived suppressor cells are sensitive to cold temperatures and should be processed at 25°C until the addition of the Cell Fixative Master Mix. After fixation, the final centrifugation step in this User Guide and 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 viability of <70% may result in 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 protocol below.

SAMPLE TYPE	SPEED	TIME	TEMPERATURE
HEK293, 3T3, and other cell lines	200 x g	10 min	4°C
PBMCs	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.

Figure 4 outlines suggested modifications to the fixation protocol to test different centrifugation conditions. This approach starts centrifugation at a low speed, but retains the supernatants after each spin. These supernatants are then centrifuged again to recover additional cells. After resuspension, each pellet should be assessed with microscopy to count cells, quantify debris, and assess aggregation. Resuspended pellets of high quality (minimal debris, minimal aggregation, and minimal evidence of cell damage) are pooled and can be used with downstream Evercode kits. If the retention is below 40-60% after pooling, we recommend contacting our applications support team for additional recommendations.

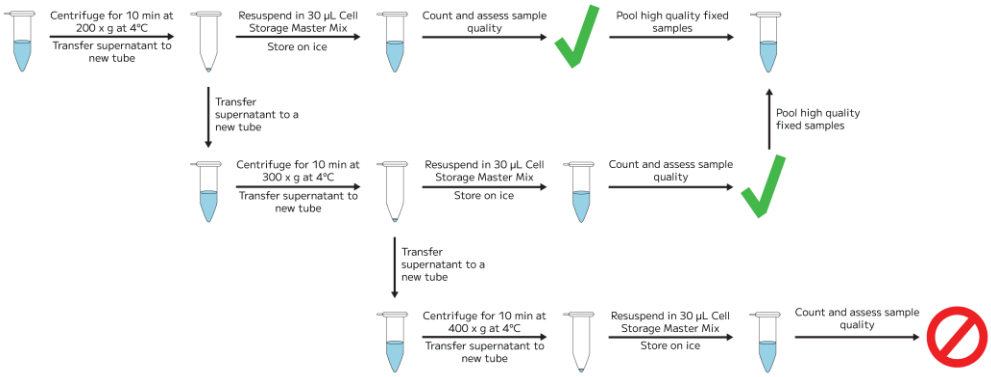


Figure 4: **Example Centrifugation Optimization Experiment.** In this example, the sample is first centrifuged at 200 x g for 10 minutes. The pellet is resuspended in Cell Storage Master Mix, and the first supernatant is centrifuged again at 300 x g for 10 minutes. The second pellet is resuspended in Cell Storage Master Mix, and the second supernatant is centrifuged again at 400 x g for 10 minutes. This final, third pellet is resuspended in Cell Storage Master Mix and the third supernatant is discarded. The three resuspended pellets are then counted with a hemocytometer. In this example, the cells centrifuged at 400 x g are aggregated with significant debris, so this resuspended pellet should be discarded. Conversely, the cells centrifuged at 200 x g and 300 x g were high quality, so they were pooled together. Once pooled, this sample has ~50% retention. These results suggest that this sample type should be centrifuged at 300 x g throughout all Evercode workflows.

To modify the fixation protocol and optimize the centrifugation as suggested, complete all the steps as outlined in Section 1, follow steps 1-16 in Section 2. Then follow steps 17-25 below.

17. Centrifuge in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.
18. Transfer each supernatant to a new 1.5 mL tube(s).
19. With a P200, fully resuspend each pellet in **30 µL** of Cell Storage Master Mix and store on ice.
20. Repeat steps 17-19 between 2-4x, increasing the centrifugation speed by 50-100 x g each centrifugation.
21. While minimizing time on ice, count the cells and assess their quality with a hemocytometer or alternative counting device and record the cell count.
22. Calculate the retention for each centrifugation condition by comparing the number of cells input into fixation and the number of cells recovered.
23. Assess the level of debris, aggregation, and cell damage in each resuspended cell pellet.
24. Pool the high quality resuspended pellets and discard any low quality ones.

Note: If the retention is below 40-60% after pooling, we recommend contacting our applications support team for additional recommendations.

25. Proceed to step 20 in Section 2.

Appendix B: High Input Workflow Set Up

If desired, 1-4 million cells can be fixed in a single reaction. However, this requires the reagent volume to be scaled up 4x, which reduces the total number of samples that can be fixed with a 12 reaction kit to 3 samples. The figure below outlines the protocol for the high input fixation workflow.

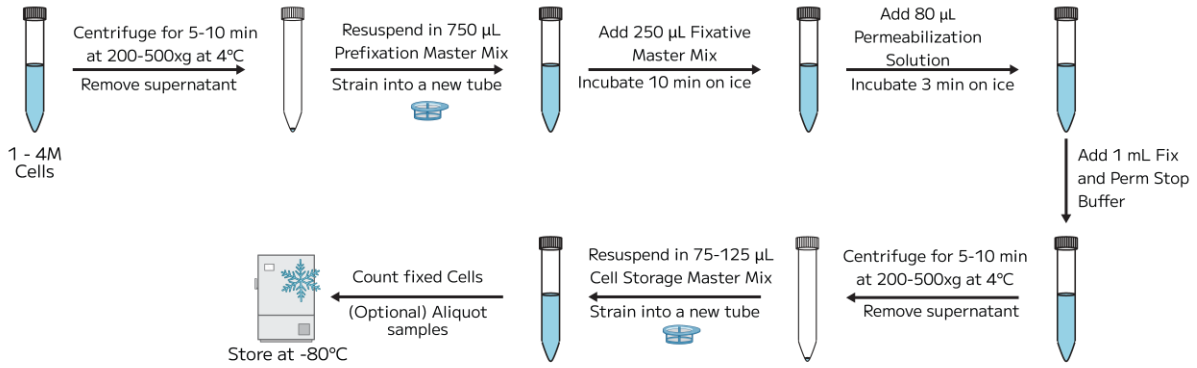


Figure 5: High Input Evercode Cell Fixation v4 Workflow.

Appendix B1: Block Tubes with BSA (Optional)

Although not required, blocking tubes with BSA can increase cell retention.

To block tubes:

1. Prepare a fresh 1% BSA as follows, depending on the number of samples being processed.

1% BSA			
Number of Samples	1	2	3
Nuclease-free water (not supplied)	26 mL	52 mL	78 mL
Gibco Bovine Albumin Fraction V (7.5% solution) (not supplied)	4 mL	8 mL	12 mL
Total Volume	30 mL	60 mL	90 mL

2. For each sample, fill two 15 mL polypropylene centrifuge tubes with 15 mL of 1% BSA and cap the tubes.
3. Invert once to fully coat the tubes.
4. Incubate the tubes for **30 minutes** at room temperature.
5. Decant and discard the 1% BSA. Remove any remaining solution from the bottom of the tube with a P1000.
6. With the caps removed, air dry the tubes for **30 minutes** in a biosafety cabinet at room temperature.
7. Proceed to Appendix B2 or store BSA-coated tubes at 4°C for up to 4 weeks.

Appendix B2: Prepare Master Mixes

Master mixes should be prepared just prior to fixation.

To prepare master mixes:

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

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	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.
● Storage Buffer	Cell Fixation Reagents (-20°C)	2 mL tube	
● Fixative Solution A	Cell Fixation Reagents (-20°C)	1.5 mL tube	
● Fixative Solution B	Cell Fixation Reagents (-20°C)	1.5 mL tube	
● Permeabilization Solution	Cell Fixation Reagents (-20°C)	1.5 mL tube	
○ Fix and Perm Stop Buffer	Cell Fixation JP Reagents (-20°C)	8 mL bottle	
● DMSO	Cell Fixation Reagents (-20°C)	1.5 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
● RNase Inhibitor	Cell Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Cell Prefixation Enhancer (4°C)	1.5 mL tube	

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	3
○ Prefixation Buffer	740 µL	2.22 mL
● RNase Inhibitor	10 µL	30 µL
● Prefixation Enhancer	50 µL	150 µL
Total Volume	800 µL	2.4 mL

Note: Reagents in the 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	3
● Fixative Solution A	144 µL	432 µL
● Fixative Solution B	144 µL	432µL
Total Volume	288 µL	864 µL

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

CELL STORAGE MASTER MIX		
Number of Samples	1	3
● Storage Buffer	300 µL	900 µL
● RNase Inhibitor	4 µL	12 µL
● DMSO	16 µL	48 µL
Total Volume	320 µL	960 µL

5. Proceed immediately to Appendix B3.

Appendix B3: Cell Fixation

After the initial centrifugation to remove the buffer/medium from the single cell suspension, cells are transferred to Cell Prefixation Master Mix. Reagents are added to fix and permeabilize cells, and then to stop these reactions. Cells are resuspended in Cell Storage Master Mix and 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. Place a Mr. Frosty Freezing Container at room temperature.
5. 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.
6. Transfer 1-4 million cells from each sample into a 15 mL polypropylene centrifuge tube (or BSA-coated polypropylene centrifuge tube if prepared in Appendix B1).
7. Centrifuge the tubes in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial cell loss.

CRITICAL! Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See Appendix A for details.

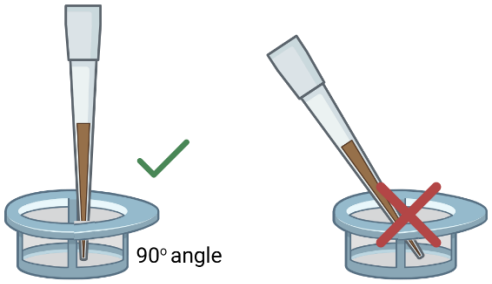
CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

8. Slowly aspirate then discard the supernatant. There should be less than 20 µL remaining in the tubes.
9. Fully resuspend each pellet in **750 µL** of Cell Prefixation Master Mix.

10. Pipette **750 µL** of each sample through a cell strainer into a new 15 mL polypropylene centrifuge tube (or BSA-coated polypropylene centrifuge tube) with a P1000 and store on ice.

CRITICAL! Do not directly touch the mesh of cell strainer(s) with gloved hands.

Note: To ensure that all of the liquid passes through the strainer, 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.



11. Add **250 µL** of Cell Fixative Master Mix to each tube and mix immediately by pipetting exactly 3x with a P1000 set to 250 µL.

CRITICAL! Do not perform additional mixing at this step.

12. Incubate on ice for **10 minutes**.
13. Add **80 µL** of ● Permeabilization Solution to each tube. Immediately mix thoroughly by pipetting 3x with a P1000 set to 250 µL.
14. Incubate on ice for **3 minutes.**
15. Mix the ○ Fix and Perm Stop Buffer by inverting the tube 5x. Do not vortex.
16. Add **1 mL** of ○ Fix and Perm Stop Buffer to each tube. Gently pipette 3x with a P1000 set to 1000 µL.
17. Centrifuge in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.
18. Remove and discard the supernatant. There should be less than 20 µL remaining in the tubes.

19. Fully resuspend each pellet in an additional **75-125 µL** Cell Storage Master Mix and store on ice.

Note: Choose a resuspension volume appropriate for the expected cell output, experimental design, and downstream Evercode kits. See Appendix C1 for additional details. If using the MAX WT V4 kits, refer to Appendix C2 to choose the most appropriate cell concentration for the MAX kits size selected.

20. Pipette each sample through a cell strainer into a new, non BSA coated 1.5 mL tube with a P1000 and store on ice.

21. While minimizing time on ice, count the number of cells in the sample with a hemocytometer or alternative counting device and record the cell count.

22. We recommend making 20 µL Counting Aliquots to streamline the downstream Evercode processing. Mix the sample at least 5x before aliquoting. See Important Guidelines for more information.

23. Proceed to the appropriate user guide if immediately processing samples with an Evercode kit. Otherwise, proceed to the next step.

24. Store tubes in a Mr. Frosty Freezing Container (or equivalent device) at -80°C, according to the manufacturer's instructions.

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 6 months at -80°C.

Appendix C: Cell Number and Concentration Reference Tables

Appendix C1: Cell Concentrations for Evercode WT v4 Kits

The final concentration of cells post-fixation is influenced by the resuspension volume used in Step 19 of Section 2. These factors should all be considered when determining how much sample input is needed for fixation.

When using the Evercode WT v4 kits, use the table below to target the post-fixation cell concentration.

CELL CONCENTRATIONS FOR EVERCODE WT V4 KITS		
Kit	Target Post-Fixation Concentration	Minimum Post-Thaw Concentration to Fully Load Kit
Evercode WT Mini	>500 cells/µL	238 cells/µL
Evercode WT	>1,000 cells/µL	373 cells/µL
Evercode WT Mega	>3,000 cells/µL	1,860 cells/µL
Evercode Mega 384	>1,000 cells/µL	456 cells/µL
Evercode WT Penta	4,500-5,000 cells/µL	3,720 cells/µL
Evercode WT Penta 384	4,200-5,000 cells/µL	2,325 cells/µL

Note: We do not recommend storing fixed samples at a concentration higher than 5,000 cells/µL after fixation. Higher concentrations could lead to clumping in certain sample types, such as HEK and 3T3 cell lines.

Appendix C2: Cell Concentrations for Evercode MAX WT v4 Kits

When using the Evercode MAX WT v4 Barcoding kits, the table below shows the range number of cells needed for each barcoding kit, and the range of post-fixation cell concentration.

Users should confirm both their target cell number and their sublibrary format before adding the Cell Storage Master Mix in Section 2.1.19, to ensure the fixed cell concentration meets both requirements.

We do not recommend preparing less than two sublibraries (2 SL).

LOADING CONCENTRATION GUIDANCE							
KIT	**METRIC**	**2 SL**	**4 SL**	**8 SL**	**12 SL**	**16 SL**	**32 SL**
MAX WT S	**MIN CONC.**	381	762	952	X	X	X
	TARGET CONC.	500	1,000	2,000	X	X	X
	MAX CELL	16,000	32,000	64,000	X	X	X
MAX WT M	**MIN CONC.**	238	476	952	1,429	1,905	X
	TARGET CONC.	500	1,000	2,000	2,000	3,000	X
	MAX CELL	64,000	128,000	256,000	384,000	512,000	X
MAX WT L	**MIN CONC.**	238	476	952	1,429	1,905	3910
	TARGET CONC.	500	1,000	2,000	2,000	3,000	5,000
	MAX CELL	128,000	256,000	512,000	768,000	1,024,000	2,048,000
MAX WT L-384	**MIN CONC.**	X	X	238	357	476	952
	TARGET CONC.	X	X	500	500	1,000	1,500
	MAX CELL	X	X	512,000	768,000	1,024,000	2,048,000

Appendix D: Revision History

Version	Description	Date
1.0	Initial release	March 2026
1.1	Updated for Evercode MAX	September 2026

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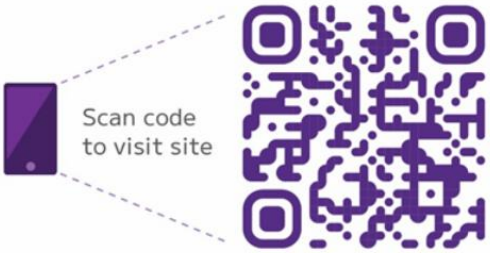

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