User Manual

Version 1.1 – UMFC4301

Evercode™ Cell Fixation v4

Mid-Throughput Workflow

For use with

ECFC4301, ECFC4303
ECFC4305, ECFC4501
ECFC4503, ECFC4505

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 mid-throughput plate-based workflow enables more samples to be fixed in parallel, streamlining fixation when processing >12 samples at a time.

The mid-throughput plate-based workflow is compatible with both the 12 and 96 Reactions Cell Fixation kits.

If processing between 12 and 48 samples we suggest using ECFC4301 kit(s).

If processing 48 samples at a time, we suggest using half of the high throughput ECFC4501 kit(s). Please note that each high throughput kit should not be used more than twice.

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.

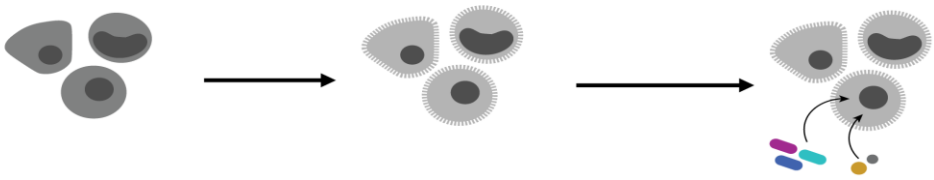


Figure 1: Evercode Cell Fixation v4. Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

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.

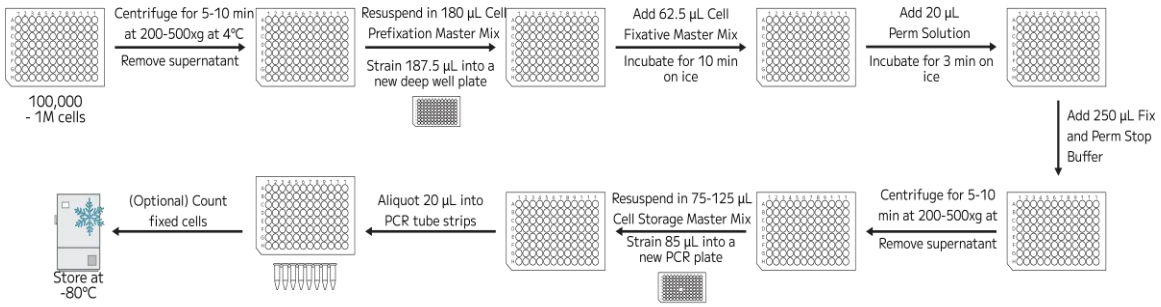


Figure 2: The Mid-Throughput Plate-Based Fixation Evercode Cell Fixation v4 Workflow

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 plates 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.
- Between 100,000 and 1 million cells can be fixed in a single reaction. However, we recommend using the highest number available up to 1 million total. Exceeding 1 million 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 A 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 Section 2. These factors should all be considered when determining how much sample input is needed for fixation. **Refer to Appendices A1 and A2 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

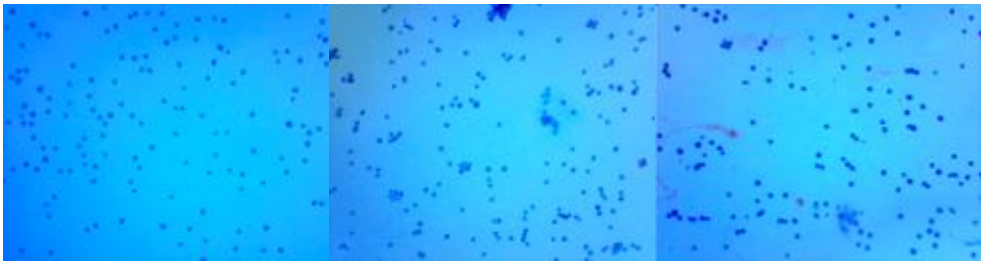


Figure 3: Example trypan blue stained fixed cells.

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.
- When using Evercode Fixation v4 kits for the first few times, we recommend retaining the supernatants removed in Section 2 steps 9 and 26. In the unlikely event of very high sample loss, these supernatants can be analyzed to identify points for optimization.

Removing Supernatant from Plates

- When using the plate-based Evercode Fixation
- workflow for the first time, we recommend practicing removing liquid from deep well plates without disturbing the bottom of the well.
- When removing supernatant, keep the pipette tips along the side of the wells to avoid disturbing the pellets. After removing most of the supernatant, we recommend tilting the plate at a 90 degree angle. This makes it easier to see the removal of residual supernatant from bottom and side of the plate.
- Less than 20 µL of supernatant should be left in each well after steps 10 and 26 in Section 2. The figure below shows different volumes of supernatant when viewed from the bottom of the deep well plate.

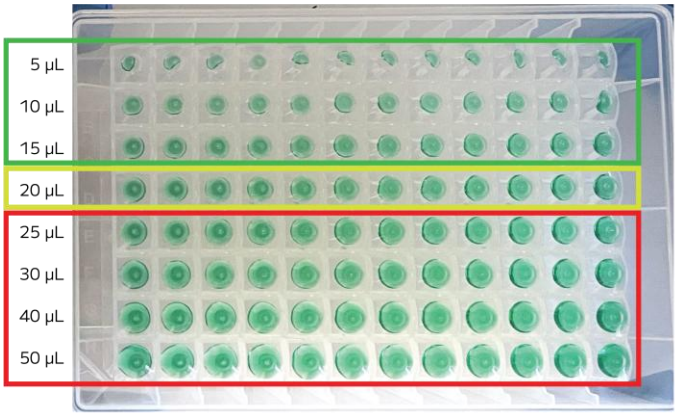


Figure 4: Bottom view of a deep well plate with different volumes of residual supernatant.

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 3 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 33. The aliquots should be at least 20 µL when stored in 0.2 mL PCR tubes or tube strip(s).
- 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. The Evercode Cell Fixation v4, mid-throughput plate workflow requires Cell Fixation Reagents and Cell Prefixation Enhancer boxes. If processing between 12 and 48 samples we suggest using the 12 reactions kit(s). If processing 48 samples at a time, we suggest using half of the high throughput kit(s).

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

Cell Fixation Reagents 96 Reactions Store at -20°C, PN CF700

LABEL	ITEM	PN	FORMAT	QTY
Perm	Permeabilization Solution	CF318	2 mL tube	2
Prefix	Prefixation Buffer	CF301	15 mL bottle	2
Storage	Storage Buffer	CF302	8 mL bottle	2
Fix A	Fixative Solution A	CF303	8 mL bottle	2
Fix B	Fixative Solution B	CF304	8 mL bottle	2
Stop	Fix and Perm Stop Buffer	CF306	15 mL bottle	2
RNase Inhib	RNase Inhibitor	CF307	1.5 mL tube	1
DMSO	DMSO	CF308	1.5 mL tube	1

Cell Prefixation Enhancer 96 Reactions Store at 4°C, PN CF800

LABEL	ITEM	PN	FORMAT	QTY
Prefix Enhan	Prefixation Enhancer	CF401	2 mL tube	1

30 µm Plate Strainer*. Store at Room Temperature

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

70 µm Plate Strainer*. Store at Room Temperature

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

100 µm Plate Strainer*. 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 Evercode Cell Fixation v4 workflow. 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. 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 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 device.
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 kit. 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.
Protein LoBind® Plate	Eppendorf®	951033308 (1 mL) 0030504305 (2 mL)	Or equivalent polypropylene, nuclease-free, v-bottom, 1 mL or 2 mL deep well plates. Do not substitute polystyrene plates as it will lead to substantial cell loss. If possible, we recommend using protein low-binding plates.
Reagent basins	Various Suppliers	Varies	(Optional) If blocking deep well plates with BSA. Sterile, nuclease-free reagent basins.
Eppendorf twin.tec® PCR Plate 96 LoBind®	Eppendorf	0030129504	Or equivalent DNA low-binding, nuclease-free PCR plate capable of holding 270 µL.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	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.
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.

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 Plates with BSA (Optional)

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

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

To block plates:

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

1% BSA			
Number of Samples	1	12	48
Nuclease-free water (not supplied)	1.82 mL	21.8 mL	87.2 mL
Gibco Bovine Albumin Fraction V (7.5% solution) (not supplied)	280 µL	3.4 mL	13.6 mL
Total Volume	2.1 mL	25.2 mL	100.8 mL

2. Fill a new basin with 1% BSA, refilling as needed to complete steps 3 and 4.
3. For each sample, add **1 mL** of 1% BSA to a well of a polypropylene, nuclease-free, v-bottom, 1 mL deep well plate or **2 mL** per well to a 2 mL deep well plate.
4. Repeat step 3 with a second deep well plate.
5. Add new plate seals and invert once to fully coat the wells.
6. Incubate for **30 minutes** at room temperature.
7. Remove the plate seals. Decant and discard the 1% BSA.
8. Add new plate seals.
9. Centrifuge the plates for **1 minute** at 100 x g at room temperature.

10. Remove the plates from the centrifuge and remove the plate seals.

11. Remove any remaining solution from the bottom of the wells with a multichannel P200.

12. Without sealing the plates, air dry for **30 minutes** in a biosafety cabinet at room temperature.

13. Proceed to Section 1.2 or store sealed BSA-coated plates at 4°C for up to 4 weeks.

1.2. Prepare Master Mixes

The reagents listed in this section and the master mix volumes are for the 12-reaction kit. For processing more than 48 samples using the 96-reaction kit, refer to the corresponding reagents and master mix in the High-Throughput Evercode Cell Fixation v4 user manual.

Master mixes are prepared and dispensed into 96 well PCR plates. This makes it easier to transfer reagents into 96 well plates during fixation. Plate Strainers are also pre-cut, if needed. 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.

Note: The reagents listed below refer to the Evercode Cell Fixation 12 Reactions Kit (boxes CF500 and CF600). For runs with more than 48 samples, use the Evercode Cell Fixation 96 Reactions Kit (boxes CF700 and CF800).

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	

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
Plate Strainer (30 µm, 70 µm, or 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 carefully avoiding to create bubbles, and store on ice.

CELL PREFIXATION MASTER MIX		
Number of Samples	1	12**
○ Prefixation 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

Note: If preparing the master mix for any number of samples, just multiply the volume of one sample by the total number of samples.

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. For each fixation reaction, dispense **210 µL** of the Cell Prefixation Master Mix into a new 96 well PCR plate. Seal, clearly label and store on ice.

CRITICAL! Do not dispense this master mix into a reagent reservoir because there is not sufficient overage to ensure recovery of the volume required for the fixation. This is true for all the master mixes and reagents dispensed into 96 well PCR plates in this section.

4. 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	40 µL	480 µL
Fixative Solution B	40 µL	480 µL
Total Volume	80 µL	960 µL

Note: If preparing the master mix for any number of samples, just multiply the volume of one sample by the total number of samples.

5. For each fixation reaction, dispense **77 µL** of the Cell Fixative Master Mix into a new 96 well PCR plate. Seal, clearly label and store on ice.

6. 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	131.25 µL	1.57 mL
RNase Inhibitor **	1.75 µL	21 µL
DMSO	7 µL	84 µL
Total Volume	140 µL	1.68 mL

Note: If preparing the master mix for any number of samples, just multiply the volume of one sample by the total number of samples.

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

7. For each fixation reaction, dispense **115 µL** of the Cell Storage Master Mix into a new 96 well PCR plate. Seal, clearly label and store on ice.

8. For each fixation reaction, dispense **40 µL** of the Permeabilization Solution into a new 96 well PCR plate. Seal, clearly label and store on ice.

9. Mix the OFix and Perm Stop Buffer by inverting the tube 5x. Do not vortex.

10. For each fixation reaction, dispense **270 µL** of the O Fix and Perm Stop Buffer into new 0.2 mL tube strip(s) or 96 well PCR plate. Clearly label and store on ice.

11. Each sample is strained twice into 2 different plates in Section 2, so Plate Strainer(s) may need to be cut so they can be applied separately in the two steps with 1 strainer well per sample at both steps. 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.

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.

12. Proceed immediately to Section 2.

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 stop these reactions. Cells are resuspended in Cell Storage Master Mix and stored at -80°C. This fixation protocol relies on multichannel pipettes and the reagents pre-dispensed 96 well plates in Section 1 to fix up to 48 samples in parallel.

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 100,000 to 1 million cells from each sample into the wells of polypropylene, nuclease-free, v-bottom, 1 mL or 2 mL deep well plate (or BSA-coated deep well plate if prepared in Section 1.1).
6. Add a new plate seal.
7. Centrifuge the plate 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 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 on ice.

9. With a multichannel P200, slowly aspirate and discard all but ~100 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.

10. Tilt the plate 90 degrees. With a multichannel P200, remove and discard the remaining supernatant. Keep the pipette tips along the side of the wells to avoid disturbing the pellets.

CRITICAL! Less than 20 µL of supernatant should be left in each well. See the Important Guidelines section for an example image with varying amounts of residual supernatant.

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

11. With the plate on ice, fully resuspend each pellet in **180 µL** of Cell Prefixation Master Mix.

12. Apply a Plate Strainer to a new 96 deep well plate (or BSA-coated deep well plate if prepared in Section 1.1) by peeling off the backing, carefully aligning over the wells, and placing on the surface of the plate.

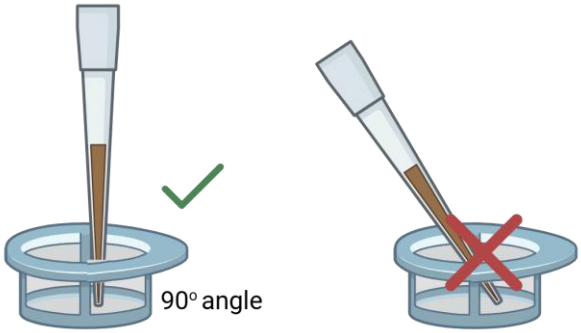
CRITICAL! Do not directly touch the mesh of Plate Strainer(s) to anything prior to straining.

Note: Plate Strainer(s) will need to be cut if processing fewer than 96 samples. See Step 11 in Section 1.2 for details.

13. 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.

14. With both plates on ice, pipette **187.5 µL** of each sample through the strainer into the new 96 deep well plate. Discard the original plate.

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.**



15. Tap the plate 3x on the benchtop to move liquid to the bottom of the wells.

16. Carefully peel off the Plate Strainer and discard.

17. With the plate on ice, add **62.5 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.

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. With the plate on ice, add **20 µL** of ● Permeabilization Solution to each well with a multichannel P20 and mix immediately by pipetting 3x with a multichannel P200 set to 180 µL.

20. 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 up to a total of **5 minutes** without negatively impacting performance.

21. With the plate on ice and changing tips between additions, add **250 µL** of ○ Fix and Perm Stop Buffer to each well with a multichannel P200 set to 125 µL and mix immediately by gently pipetting 3x.

22. Add a new plate seal.

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

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

25. With a multichannel P200 set to 200 µL, slowly aspirate and discard **400 µL** of supernatant from each well. Keep the pipette tips along the side of the wells to avoid disturbing the pellets.

26. Tilt the plate 90 degrees. With a multichannel P200, remove and discard the remaining supernatant from each well. Keep the pipette tips along the side of the wells to avoid disturbing the pellets.

CRITICAL! Less than 20 µL of supernatant should be left in each well after steps 25-26. See the Important Guidelines section for example images of varying volumes of residual supernatant.

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

27. With the plate on ice, fully resuspend each pellet in an additional **75-125 µL** of Cell Storage Master Mix.

Note: Choose a resuspension volume appropriate for the expected cell output, experimental design, and downstream Evercode kits. See Appendix A1 for additional details. If using the MAX WT V4 kits, refer to Appendix A2 to choose the most appropriate cell concentration for the MAX kits size selected.

28. Apply a Plate Strainer to a new 0.2 mL 96 well PCR plate by peeling off the backing, carefully aligning over the wells, and placing on the surface of the plate.

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

29. Without touching the mesh, fully adhere the Plate Strainer by pressing a pipet tip or plate sealer cleaned with RNaseZap along the green plastic on the edges and between the wells of the plate.

30. With both plates on ice, pipette **the whole volume** of each sample through the strainer into the new PCR plate. Discard the original plate.

31. Carefully peel off the Plate Strainer and discard.

32. With a multichannel P20 set to 20 µL, mix the samples by pipetting 5x then immediately transfer **20 µL** to a new 0.2 mL tube strip(s). Store on ice.

Note: These aliquots can be thawed and counted separately from the remaining sample for downstream processing with Evercode kits.

33. Seal the original plate with a seal that can withstand storage at -80°C or transfer the remainder of the sample to 0.2 mL tube strip(s).

CRITICAL! 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.

34. 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 6 months at -80°C.

35. The day before running the downstream Evercode kit, thaw the 20 µL aliquots in a water bath set to 37°C in sets of 2-4. 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.

Appendix A: Cell Number and Concentration Reference Tables

Appendix A1: Cell Concentrations for Evercode WT v4 Kits

The final concentration of cells post-fixation is influenced by the resuspension volume used in Step 27 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 A2: Cell Concentration 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 2.1.27, 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 B: Revision History

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

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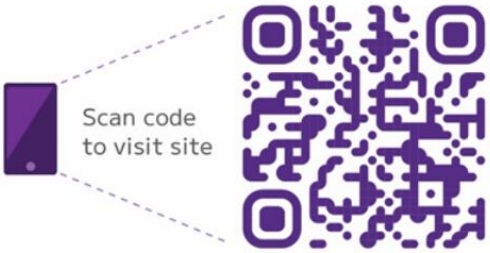

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