

User Manual

Version 1.1 – UM0028



**Evercode™**

**Cell Fixation v3**

**Mid-Throughput Plate Workflow**

For use with

ECFC3301

ECFC3303

ECFC3305

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## Overview

### Workflow

From a single cell suspension, the Evercode Cell Fixation v3 kit generates fixed and permeabilized cells ready for use in Evercode Whole Transcriptome or Evercode TCR kits.

This mid-throughput plate-based workflow enables more samples to be fixed in parallel, streamlining fixation when processing  $>12$  samples at a time. If processing  $\leq 12$  samples, we recommend using the 1.5 mL tube-based workflow. If processing  $\geq 48$  samples, we recommend using the Evercode Cell Fixation v3, HT 96 reactions kit and its high-throughput plate-fixation protocol. Fixation maintains cell structure, prevents RNA degradation, and locks the RNA inside the cells, which are 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  $-80^{\circ}\text{C}$ , Evercode Cell Fixation v3 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.

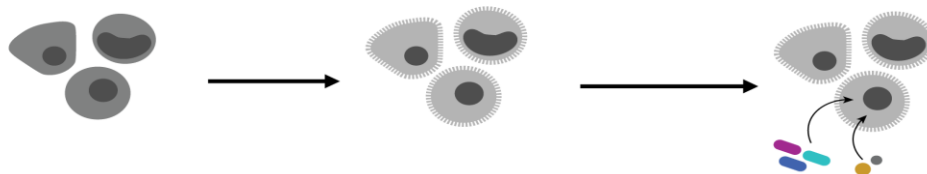


Figure 1: Evercode Cell Fixation v3. Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

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.

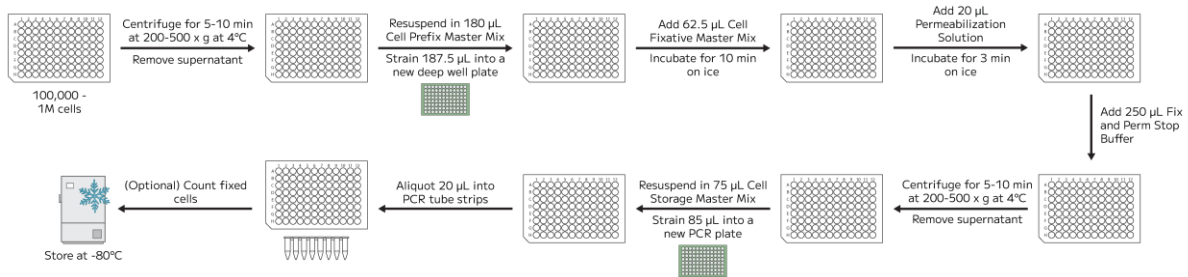


Figure 2: The Mid-Throughput Plate-Based Fixation Evercode Cell Fixation v3 Workflow

## 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 | 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](mailto: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 length of time samples are stored on ice prior to fixation, as it can negatively impact results.
- 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 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 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 18 of Section 2. These factors should all be taken into account when determining how much sample input is needed for fixation.

| CELL CONCENTRATIONS |                                    |   |
|---------------------|------------------------------------|---|
| Kit                 | Target Post-Fixation Concentration | Minimum Post-Thaw Concentration to Fully Load Kit |
| Evercode WT Mini    | ≥ 500 cells/ μL                    | 298 cells/ μL                                     |
| Evercode WT         | ≥ 1,000 cells/ μL                  | 520 cells/ μL                                     |
| Evercode WT Mega    | ≥ 3,000 cells/ μL                  | 2,126 cells/ μL                                   |



**Note:** These recommendations also apply to the Evercode TCR Kits.

### 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.

### Plate Strainers

- An example video of using a Plate Strainer can be found in our support site. We recommend watching this video and practicing using Plate Strainers before processing your samples.
- 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. We do not recommend using Plate Strainers more than one day after the plastic sleeve has been opened.
- Plate Strainers may need to be cut prior to processing samples. Plate Strainers should be cut with sterile scissors, razor blade, or scalpel that have been cleaned with RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific) immediately prior to use.
- Plate Strainers should be thoroughly adhered to 96 well plates prior to use. After being placed by hand, a plate seal applicator should be used to completely seal the strainer over each well by pressing along the outside of the wells. The plate seal applicator should be cleaned with RNaseZap RNase Decontamination Solution (Thermo Fisher



Scientific) immediately prior to use. Applicators should never be pressed directly onto the mesh.

- To maximize cell retention with Plate Strainers, press pipette tips 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.

### 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 v3 kits.
- When first using Evercode Fixation v3 kits, we suggest saving images at each counting step.
- 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

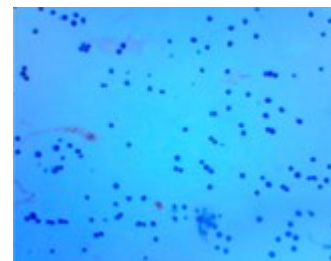


Figure 3: Example trypan blue stained fixed cells

## Centrifugation

- A range of centrifugation speeds and durations are given in this protocol rather than a single speed. When using Evercode Fixation v3 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 all high-speed 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.
- To minimize cell loss from cell adherence to the plate, carefully pipette along the bottom and sides of wells in the plate.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.
- When using Evercode Fixation v3 kits for the first few times, we recommend retaining the supernatants removed in Section 2 steps 9 and 29. 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 v3 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  $\mu$ L of supernatant should be left in each well after steps 10 and 30 in Section 2. Figure 4 shows different volumes of supernatant when viewed from the bottom of the deep well plate.

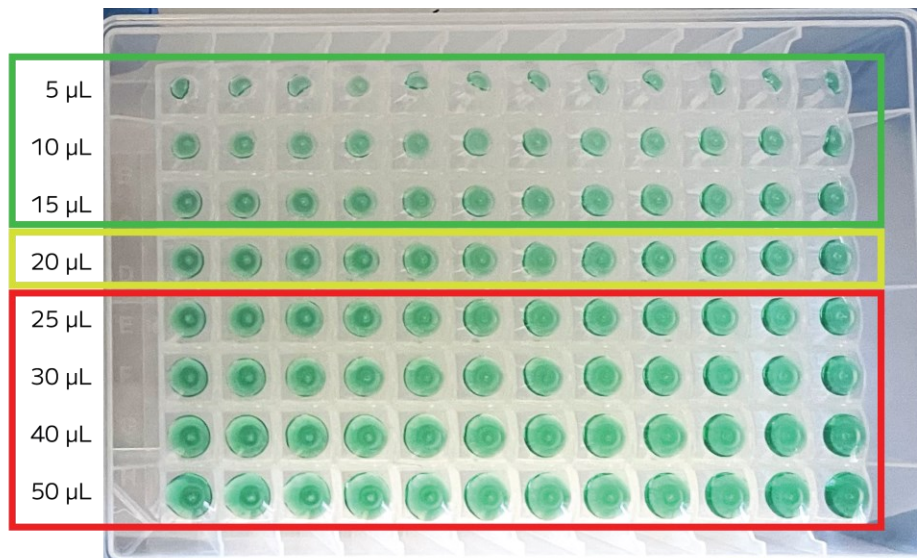


Figure 4: Bottom view of a deep well plate with different volumes of residual supernatant.

### 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 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 6 months. Fixed samples should not be refrozen after thawing.
- When possible, we recommend splitting samples into aliquots after fixation in Section 2 Step 37. Aliquots should be at least 20 µL when stored in 0.2 mL PCR tubes or tube strip(s).

## Parts List

The Evercode Cell Fixation v3, 12 reactions tube workflow requires Cell Fixation Reagents and Cell Fixation Enhancer boxes.

**Cell Fixation Reagents, 12 reactions.** Store at -20°C, PN CF100

| 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 | CF105 | 1.5 mL tube | 1   |
|  | Fix and Perm Stop Buffer  | CF106 | 8 mL bottle | 1   |
|  | RNase Inhibitor           | CF107 | 1.5 mL tube | 1   |
|  | DMSO                      | CF108 | 1.5 mL tube | 1   |

**Cell Prefixation Enhancer, 12 reactions.** Store at 4°C, PN CF200

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

**30 µM Plate Strainer\*.** Store at Room Temperature

| LABEL | ITEM                           | PN    | FORMAT         | QTY |
|-------|--------------------------------|-------|----------------|-----|
| N/A   | Adhesive Plate Strainer, 30 µM | PS030 | Plastic sleeve | 2   |

**70 µM Plate Strainer\*.** Store at Room Temperature

| LABEL | ITEM                           | PN    | FORMAT         | QTY |
|-------|--------------------------------|-------|----------------|-----|
| N/A   | Adhesive Plate Strainer, 70 µM | PS070 | Plastic sleeve | 2   |

**100 µM Plate Strainer\*.** Store at Room Temperature

| LABEL | ITEM                            | PN    | FORMAT         | QTY |
|-------|---------------------------------|-------|----------------|-----|
| N/A   | Adhesive Plate Strainer, 100 µM | PS100 | Plastic sleeve | 2   |



**Note:** \* Only one mesh size of Plate Strainer is required for the mid-throughput plate-based Evercode Cell Fixation v3 workflow. Select an appropriate mesh size for each sample type.

## User Supplied Equipment and Materials

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

### Equipment

| ITEM   | SUPPLIER          | PN      | NOTES   |
|--|-------------------|---------|---|
| 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<br>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 Whole Transcriptome kit.   |
| Water bath   | Various Suppliers | Varies  | (Optional) If preparing aliquots to count the day before running a downstream Evercode Whole Transcriptome or Evercode TCR 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)<br>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.   |
| RNaseZap™ RNase Decontamination Solution        | Thermo Fisher Scientific | AM9780                                | Or equivalent RNase decontamination solution.   |

| ITEM   | SUPPLIER                 | PN                                 | NOTES   |
|--|--------------------------|------------------------------------|---|
| Pipette Tips TR LTS<br>20 $\mu$ L, 200 $\mu$ L,<br>1,000 $\mu$ L | Rainin®                  | 17014961,<br>17014963,<br>17014967 | Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.                     |
| Trypan Blue  | Various Suppliers        | Varies                             | Or alternative viability dyes, such as AO/PI.   |
| TrypLE™ Express<br>OR TrypLE Select                              | Thermo Fisher Scientific | 12605010<br>OR 12563011            | (Optional) If using adherent cells. Trypsin is not recommended due to variable RNase levels.                                |
| 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. Contact applications support for alternatives. |



## Section 1: Set Up

### 1.1. Block Plates with BSA

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.

#### 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)                             | 1.82 mL | 21.8 mL |
| Gibco Bovine Albumin Fraction V (7.5% solution) (not supplied) | 280 µL  | 3.4 mL  |
| Total Volume   | 2.1 mL  | 25.2 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 or 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

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.

### 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 |   |

| ITEM   | SOURCE                            | FORMAT         | HANDLING AND STORAGE      |
|--|-----------------------------------|----------------|---------------------------|
| Plate Strainer (30 $\mu\text{m}$ , 70 $\mu\text{m}$ , or 100 $\mu\text{m}$ ) | Plate Strainer (Room Temperature) | Plastic sleeve | Keep at room temperature. |

- 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        | 203.5 $\mu\text{L}$ | 2.44 mL           |
| ● RNase Inhibitor           | 2.75 $\mu\text{L}$  | 33 $\mu\text{L}$  |
| ● Prefixation Enhancer      | 13.75 $\mu\text{L}$ | 165 $\mu\text{L}$ |
| Total Volume                | 220 $\mu\text{L}$   | 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.

- For each fixation reaction, dispense **210  $\mu\text{L}$**  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.

- 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 $\mu\text{L}$ | 480 $\mu\text{L}$ |
| ● Fixative Solution B    | 40 $\mu\text{L}$ | 480 $\mu\text{L}$ |
| Total Volume             | 80 $\mu\text{L}$ | 960 $\mu\text{L}$ |

- For each fixation reaction, dispense **77  $\mu\text{L}$**  of the Cell Fixative Master Mix into a new 96 well PCR plate. Seal, clearly label and store on ice.
- 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 $\mu\text{L}$ | 1.35 mL          |
| ● RNase Inhibitor       | 1.5 $\mu\text{L}$   | 18 $\mu\text{L}$ |
| ● DMSO                  | 6 $\mu\text{L}$     | 72 $\mu\text{L}$ |
| Total Volume            | 120 $\mu\text{L}$   | 1.44 mL          |

- For each fixation reaction, dispense **115  $\mu\text{L}$**  of the Cell Storage Master Mix into a new 96 well PCR plate. Seal, clearly label and store on ice.
- For each fixation reaction, dispense **40  $\mu\text{L}$**  of the ● Permeabilization Solution into a new 96 well PCR plate. Seal, clearly label and store on ice.
- Mix the ● Fix and Perm Stop Buffer by inverting the tube 5x. Do not vortex.
- For each fixation reaction, dispense **270  $\mu\text{L}$**  of the ● Fix and Perm Stop Buffer into new 0.2 mL tube strip(s) or 96 well PCR plate. Clearly label and store on ice.
- 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.

- 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  $-80^{\circ}\text{C}$ . 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  $4^{\circ}\text{C}$ .
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  $4^{\circ}\text{C}$ .

**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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$**  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  $\mu$ L** 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  $\mu$ L** 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  $\mu$ L** of ●Permeabilization Solution to each well with a multichannel P20 and mix immediately by pipetting 3x with a multichannel P200 set to 180  $\mu$ L.

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  $\mu$ L** of OFix and Perm Stop Buffer to each well with a multichannel P200 set to 125  $\mu$ L 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  $\mu\text{L}$ , slowly aspirate and discard **400  $\mu\text{L}$**  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  $\mu\text{L}$  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 **75  $\mu\text{L}$**  of Cell Storage Master Mix.
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 **85  $\mu\text{L}$**  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  $\mu\text{L}$ , mix the samples by pipetting 5x then immediately transfer 20  $\mu\text{L}$  to 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  $-80^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$ , 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  $-80^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$ .

35. The day before running the downstream Evercode Whole Transcriptome kit, thaw the 20  $\mu\text{L}$  aliquots in a water bath set to  $37^{\circ}\text{C}$  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: Revision History

| Version | Description                                  | Date       |
|---------|--|------------|
| 1.0     | Initial release                              | Feb 2024   |
| 1.1     | Improved readability and clarified language. | March 2024 |





[parsebiosciences.com](https://parsebiosciences.com)

[support@parsebiosciences.com](mailto:support@parsebiosciences.com)

