User Manual

Version 2.3 – UM0039

Evercode™ Cell Fixation v2

For use with

ECF2101

ECF2001

Legal Notices

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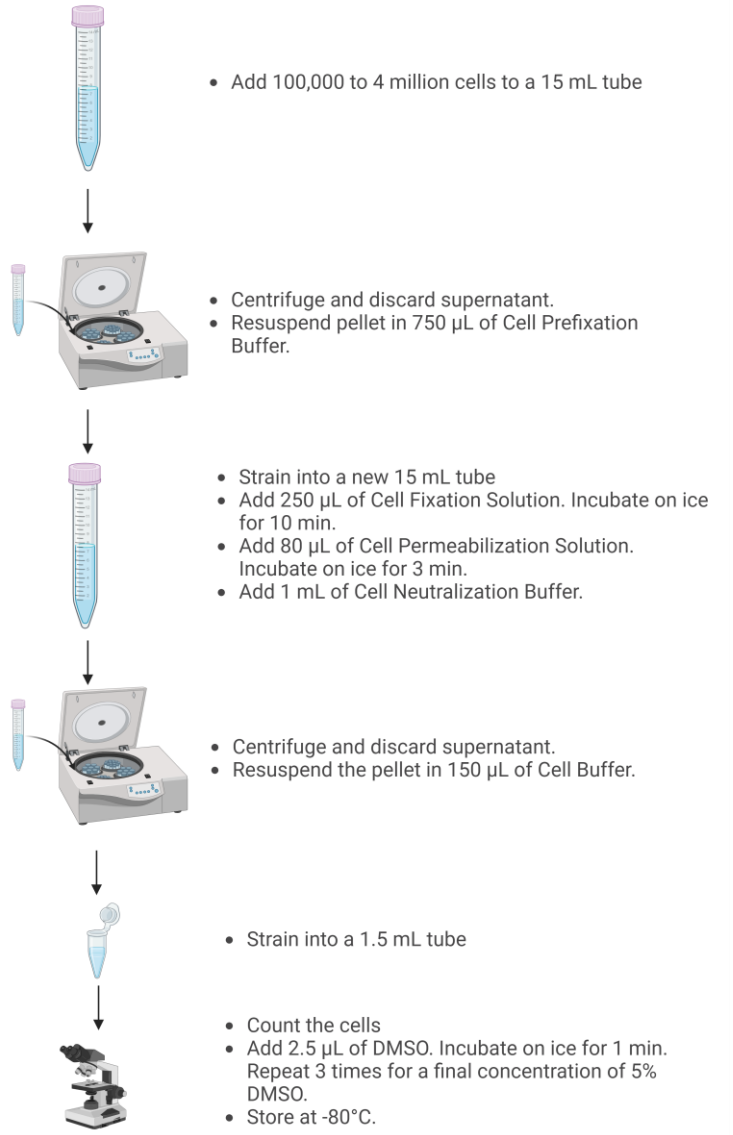
Overview

Workflow

From a single cell suspension, the Evercode Cell Fixation v2 kit generates fixed and permeabilized cells ready for use in Evercode Whole Transcriptome kits. Fixation maintains cell structure and prevents RNA degradation, which are crucial for downstream processing with Evercode split pool combinatorial barcoding technology. Because fixed samples are also stable for up to 6 months at -80°C, Evercode Cell Fixation v2 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 potential batch effects.

Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

The figure below provides a high-level overview of the cell fixation workflow.



Protocol timing

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

SECTION	TOTAL TIME	HANDS-ON TIME	SAFE STOPPING POINTS
1.1 Block Tubes with BSA	30 min	5 min	Blocked tubes: 4°C ≤ 1 month
1.2 Prepare Reagent Mixes	5 min	5 min	Reagent mixes: -20°C ≤ 1 month
2.1 Cell Fixation	45 min	45 min	-80°C ≤ 6 months

Important Guidelines

Although this user guide provides detailed instructions, the following guidelines provide additional information to obtain optimal performance. 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%), <5% aggregation/debris, and >100,000 cells. If more cells are available, we recommend using the highest number available up to 4 million total.
- Exceeding 4 million cells in a single fixation will result in substantially elevated doublet rates.
- If cells were previously frozen, ensure the suspension is completely thawed and in suspension before beginning fixation.
- We recommend minimizing the time samples are stored on ice before fixation, as it can negatively impact results.

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, as it may reduce gene and transcript detection.

Avoiding RNase Contamination

- Take standard precautions 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.
- Use filtered pipette tips to reduce RNase contamination from pipettes.

Cell Strainers

- A 40 µm cell strainer will be used in multiple steps. To maximize cell retention, press the pipette tip directly against the strainer. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second.
- For cells larger than 40 µm, the provided strainers should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm).

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 Cell Fixation v2 kits.
- When first using Evercode Cell Fixation v2 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 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 (Figure 1).

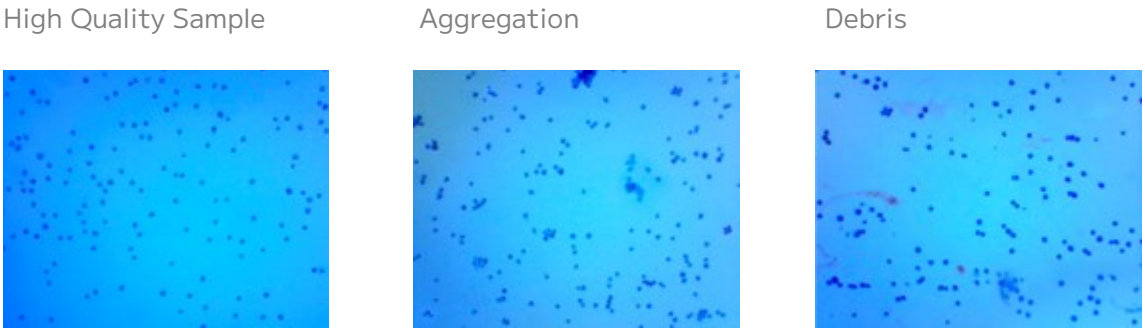


Figure 1: Example trypan blue stained fixed cells

Centrifugation

- This protocol specifies a spectrum of centrifugation speeds and durations instead of prescribing a single set speed. We strongly recommend optimizing centrifugation conditions for each sample type to balance retention and resuspension efficiencies. See the Appendix for additional details on optimizing centrifugation speeds.
- For most cell types, the centrifuge should be set to 4°C to help maintain cell integrity.
- Use a swinging bucket rotor 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 up and down until no clumps are visible. To minimize cell loss from adherence to tubes, carefully pipette up and down along the bottom and sides of tubes.
- We do not recommend wide bore pipette tips as they make it difficult to adequately resuspend pellets.
- Ensure that the 15 mL centrifuge tubes used are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- The 15 mL polypropylene tubes can also be blocked with Bovine Serum Albumin (BSA) to prevent cell adhesion to plastics and improve retention. The recommended protocol is described in Section 1.1.
- BSA can prevent aggregation of cell types prone to clumping, including PBMCs. If your cell type is prone to clumping or has low cell numbers, we strongly recommend adding BSA to the Cell Prefixation Buffer as described in the protocol.
- When using Evercode Cell Fixation v2 kits for the first few times, we recommend retaining the supernatants removed in Section 2 steps 10 and 19. In the unlikely event of very high sample loss, these supernatants can be analyzed to identify points for optimization.

Reagent Mix Stability

- After Cell Fixation Solution Additive or RNase Inhibitor is added to Cell Fixation Solution, Cell Prefixation Buffer, and Cell Buffer, as indicated in the protocol, the mixed reagents are stable for 1 month at -20°C and can be freeze-thawed once. Additional storage or freeze-thaws will compromise data quality.

Storage of Fixed Samples

- Fixed samples can be stored at -80°C for up to 6 months. Do not refreeze them after thawing.
- When possible, we recommend splitting samples into aliquots after fixation before adding DMSO in Section 2 Step 21. Aliquots should contain at least 75 µL. Note that the volume of DMSO added in Section 2 Steps 23-25 should be adjusted so the final concentration of DMSO after the three additions is 5%.
- To streamline downstream processing, samples can be diluted and aliquoted before adding DMSO. The following table shows the minimum input concentration required for each kit. Because some cells are lost after thawing, we strongly recommend targeting a higher concentration.

CELL CONCENTRATION		
Kit	**Target Cell Concentrations**	**Minimum Cell Concentration to Fully Load Kit**
Evercode WT Mini	≥ 500 cells/ µL	360 cells/ µL
Evercode WT	≥ 1,000 cells/ µL	625 cells/ µL
Evercode WT Mega	≥ 3,000 cells/ µL	2,250 cells/ µL

Part List

The Evercode Cell Fixation v2 kit includes the following two boxes. Safety Data Sheets for these reagents can be provided upon request.

Fixation Reagents Store at -20°C, PN WF400

LABEL	ITEM	PN	FORMAT	QTY
Cell Prefix	Cell Prefixation Buffer	WF301	5 mL tube	1
Buffer	Cell Buffer	WF302	2 mL tube	1
Cell Fix	Cell Fixation Solution	WF303	1.5 mL tube	1
Cell Fix Addit	Cell Fixation additive	WF304	1.5 mL tube	1
Cell Perm	Cell Permeabilization Solution	WF305	1.5 mL tube	1
Cell Neut	Cell Neutralization Buffer	WF306	5 mL tube	1
Rnase Inhib	RNase Inhibitor	WF307	1.5 mL tube	1
DMSO	DMSO	WF308	1.5 mL tube	1

Note: An alternative version of Evercode Cell Fixation v2 (ECF2001) includes WF300 instead of WF310. This configuration includes 4 Cell Neutralization Buffer tubes, but only 1 will be used in this protocol. The other 3 tubes can be discarded.

Fixation Accessory Box. Store at Room Temp, PN WF200

LABEL	ITEM	PN	FORMAT	QTY
N/A	40 µm strainers	WF201	Plastic bag	8

User Supplied Equipment and Reagents

The following materials and equipment are required to perform the protocol but are not provided with 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 15 mL centrifuge tubes and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
Single channel pipettes P20, P200, P1000	Various Suppliers	Varies	
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device.
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific®	5100-0001	(Optional) Use if storing fixed samples before processing with Evercode Whole Transcriptome. Or an equivalent device that cools samples at about -1°C/minute to minimize cell damage.

Consumables

ITEM	SUPPLIER	PN	NOTES
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL or 5 mL polypropylene centrifuge tubes. Do not substitute polystyrene tubes as it will lead to substantial cell loss.
DNA LoBind® Tubes	Eppendorf®	22431021	Or equivalent 15 mL or 5 mL polypropylene centrifuge tubes. Do not substitute polystyrene tubes as it will lead to substantial cell loss.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Pipette Tips TR LTS 20 µL, 200 µL, 1,000 µL	Rainin®	17014961, 17014963, 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
Trypan Blue	Various Suppliers	Varies	Or alternative viability dyes, such as AO/PI.
Gibco™ Bovine Albumin Fraction V (7.5% solution)	Thermo Fisher Scientific	15260037	Chosen due to its low RNase activity. Contact applications support for alternatives.
TrypLE™ Express OR TrypLE Select	Thermo Fisher Scientific	12605010 OR 12563011	(Optional) If using adherent cells, Trypsin is not recommended due to variable RNase levels.

ITEM	SUPPLIER	PN	NOTES
Isopropyl alcohol	Various Suppliers	Varies	(Optional) If using a Mr. Frosty Freezing Container.
Corning Cell Strainers	Corning	431751 (70 µm) or 431752 (100 µm)	(Optional) For cells larger than 40 µm, included strainers should be replaced throughout with those of an appropriate size mesh.

Section 1: Set Up

1.1 Block Tubes with BSA

Although not required, blocking centrifuge tubes with BSA can increase cell retention. This is especially helpful for cells prone to aggregation or when working with low cell counts.

To block tubes:

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

1% BSA				
Number of Samples	1	2	3	4
Nuclease-free water (not supplied)	26 mL	52 mL	78 mL	104 mL
Gibco™ Bovine Albumin Fraction V (7.5% solution) (not supplied)	4 mL	8 mL	12 mL	16 mL
Total Volume	30 mL	60 mL	90 mL	120 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 the fixation protocol in Section 1.2 or store BSA-coated tubes at 4°C for up to 4 weeks.

1.2 Prepare Reagent Mixes

If using this kit for the first time, follow the instructions in this section to prepare reagent mixes. Otherwise, proceed to Section 2. These reagent mixes are stable for 1 month at -20°C and can be freeze-thawed once.

To prepare reagent mixes:

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

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
● Cell Prefixation Buffer	Cell Fixation Reagents (-20°C)	5 mL	Store on ice. Briefly centrifuge before use. Mix by inverting 3x.
● Cell Buffer	Cell Fixation Reagents (-20°C)	2 mL	
● Cell Fixation Solution	Cell Fixation Reagents (-20°C)	1.5 mL	
● Cell Fixation Additive	Cell Fixation Reagents (-20°C)	1.5 mL	
● RNase Inhibitor	Cell Fixation Reagents (-20°C)	1.5 mL	Store on ice. Briefly centrifuge before use.

2. Add **550 µL** of ●Cell Fixation Additive directly into the ●Cell Fixation Solution. Mix thoroughly by pipetting 5x with a P1000 set to 750 µL. Store on ice.
3. Record the addition of ●Cell Fixation Additive by marking the cap of the ●Cell Fixation Solution tube.
4. Add **50 µL** of ●RNase Inhibitor directly into the ●Cell Prefixation Buffer tube. Mix thoroughly by pipetting 5x with a P1000 set to 750 µL. Store on ice.
5. Record the addition of ●RNase Inhibitor by marking the cap of the ●Cell Prefixation Buffer tube.
6. Add **17 µL** of ●RNase Inhibitor directly into the ●Cell Buffer tube. Mix thoroughly by pipetting 5x with a P1000 set to 750 µL. Store on ice.
7. Record the addition of ●RNase Inhibitor by marking the cap of the ●Cell Buffer tube.

8. Record today's date on the Cell Fixation Reagents kit box.

Note: These reagent mixes can be stored at -20°C for up to 1 month and freeze-thawed once. If samples need to be fixed over more than 2 separate sessions, aliquot the reagent mixes.

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 Buffer. Reagents are added to fix and permeabilize the cells, which are then neutralized. Samples are either processed immediately, or DMSO is added prior to freezing at -80°C.

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. If the samples will not be barcoded immediately after fixation, place a Mr. Frosty Freezing Container (or equivalent) at room temperature.
5. Gather the following items and handle them as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
Cell Prefixation Buffer	Cell Fixation Reagents (-20°C)	5 mL	Thaw and store on ice. Mix by inverting 3x.
Cell Buffer	Cell Fixation Reagents (-20°C)	2 mL	
Cell Fixation Solution	Cell Fixation Reagents (-20°C)	1.5 mL	
Cell Permeabilization Solution	Cell Fixation Reagents (-20°C)	1.5 mL	Thaw at room temperature then store on ice. Mix by inverting 3x.
Cell Neutralization Buffer	Cell Fixation Reagents (-20°C)	5 mL	
DMSO	Cell Fixation Reagents (-20°C)	1.5 mL	Thaw and store at room temperature. Mix by inverting 3x.
40 µm Strainer (or alternative size)	Cell Fixation Reagents (-20°C)	2 per samples	Keep at room temperature.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
7.5% Gibco BSA Fraction V (optional)	Not supplied	50 µL per sample	Store on ice.

CRITICAL! Ensure the reagent mixes have been prepared as described in Section 1.2 by verifying marks on the tube caps of the ●Cell Prefixation Buffer, ●Cell Buffer, and ●Cell Fixation Solution. Additionally, confirm that these mixes were prepared within the last month by checking the preparation date of the reagents listed on the kit box. Longer storage or more than one freeze-thaw will compromise data quality.

6. 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.

7. If the sample has less than 1 million cells or is prone to aggregation (such as PBMCs), we recommend preparing Cell Prefixation Buffer + BSA as described below. Mix thoroughly by pipetting 5x and store on ice. This mix should be prepared fresh and used the same day.

CELL PREFIXATION BUFFER + BSA				
Number of Samples	1	2	3	4
● Cell Prefixation Buffer	750 µL	1.5 mL	2.25 mL	3.0 mL
Gibco Bovine Albumin Fraction V (7.5% solution)	50 µL	100 µL	150 µL	200 µL
Total Volume	800 µL	1.6 mL	2.4 mL	3.2 mL

8. Transfer 100,000 to 4 million cells into a 15 mL polypropylene centrifuge tube (or BSA-coated polypropylene centrifuge tube).

9. Centrifuge the 15 mL tube in a swinging bucket rotor for **5-10 minutes** at 200-600 x g at 4°C.

CRITICAL! Ideal centrifugation speed and duration for each sample type should be established through empirical testing to maximize both retention and resuspension efficiencies. Overly aggressive centrifugation can damage cell integrity and decrease data quality. See the Appendix for details on optimizing centrifugation speeds.

10. Remove and discard the supernatant. Fully resuspend the pellet in **750 µL** of cold ●Cell Prefixation Buffer (or Cell Prefixation Buffer + BSA, if prepared in step 7) with a P1000 set to 750 µL.

11. Pipette cells through a cell strainer with an appropriately sized mesh into a new 15 mL tube (or BSA-coated 15 mL tube) with a P1000 and store on ice.

CRITICAL! If the sample has cells larger than 40 µm, the provided strainers should be replaced with the appropriate size mesh (70 µm or 100 µm) throughout the protocol.

Note: To ensure that all 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.

12. Add **250 µL** of ●Cell Fixation Solution to the 15 mL tube and mix immediately by pipetting exactly 3x with a P1000 set to 250 µL.

CRITICAL! Do not perform additional mixing at this step.

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

14. Add **80 µL** of ●Cell Permeabilization Solution to the 15 mL tube and mix thoroughly by pipetting 3x with a P1000 set to 250 µL.

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

16. Mix the ●Cell Neutralization Buffer by inverting the tube 5x. Do not vortex.

17. Add **1 mL** of ●Cell Neutralization Buffer to the 15 mL tubes. Gently pipette 3x with a P1000 set to 1000 µL.

18. Centrifuge the 15 mL tube in a swinging bucket rotor for **5-10 minutes** at 200-600 x g at 4°C.

19. Remove and discard the supernatant. Fully resuspend each pellet in **150 µL** cold ●Cell Buffer with a P1000 set to 150 µL and store on ice.

20. Pipette cells through a cell strainer with an appropriately sized mesh into a new 1.5 mL tube with a P1000 and store on ice.

21. Count the number of cells in the sample with a hemocytometer or alternative counting device and record the count. Keep cells on ice during counting and work quickly to minimize the time that fixed cells are out.

Note: Downstream Evercode Whole Transcriptome processing can be streamlined by diluting and aliquoting samples at this step. See Important Guidelines for recommendations.

22. Proceed to the appropriate user guide if immediately processing samples with an Evercode Whole Transcriptome kit. Otherwise, proceed to the next step.
23. Add **2.5 µL** of ●DMSO. Gently flick the tube 3x to mix.
24. Incubate on ice for **1 minute**.
25. Repeat steps 22 and 23 twice for a total addition of **7.5 µL** of ●DMSO.
26. Gently mix by pipetting up and down 5x with a P200 set to 75 µL. Avoid creating bubbles.
27. Store tubes in a Mr. Frosty Freezing Container (or equivalent) 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.

Appendices

Appendix A: Centrifugation Optimization

Centrifugation conditions should be optimized for each sample type to maximize retention and downstream success with Evercode kits. This appendix provides guidelines for optimization, suggestions for common sample types, and a protocol to optimize centrifugation speeds. After determining the appropriate centrifugation conditions, we recommend using the same speed and duration throughout this and downstream Evercode User Guides.

Speed

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

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

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.

Duration

If cells are damaged by increased centrifugation speed, sometimes the 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, myeloid-derived suppressor cells, and macrophages are sensitive to cold temperatures and should be processed at 25°C until the addition of the Cell Fixation Buffer. 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.

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
Mammalian nuclei	300-400 x g	10 min	4°C

Appendix B: Centrifugation Optimization Protocol

When processing a new sample type, follow this protocol to fix samples and determine the optimal cell retention and fixed sample quality. This protocol should be used in place of Section 2. If possible, we recommend processing 1-2 samples to optimize centrifugation conditions prior to processing samples of interest.

To optimize cell retention and 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. If the samples are not barcoded immediately after fixation, place a Mr. Frosty Freezing Container (or equivalent) at room temperature.
5. Gather the following items and handle them as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
Cell Prefixation Buffer	Cell Fixation Reagents (-20°C)	5 mL	Thaw and store on ice. Mix by inverting 3x.
Cell Buffer	Cell Fixation Reagents (-20°C)	2 mL	
Cell Fixation Solution	Cell Fixation Reagents (-20°C)	1.5 mL	
Cell Permeabilization Solution	Cell Fixation Reagents (-20°C)	1.5 mL	Thaw at room temperature then store on ice. Mix by inverting 3x.
Cell Neutralization Buffer	Cell Fixation Reagents (-20°C)	5 mL	
DMSO	Cell Fixation Reagents (-20°C)	1.5 mL	Thaw and store at room temperature. Mix by inverting 3x.
40 µm Strainer (or alternative size)	Cell Fixation Reagents (-20°C)	2 per sample	Keep at room temperature

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
7.5% Gibco BSA Fraction V (optional)	Not Supplied	50 µL per sample	Store on ice

Note: Ensure the reagent mixes have been prepared as described in Section 1.2 by verifying marks on the tube caps of the ●Cell Prefixation Buffer, ●Cell Buffer, and ●Cell Fixation Solution. Additionally, confirm that these mixes were prepared within the last month by checking the preparation date of the reagents listed on the kit box. Longer storage or more than one freeze-thaw will compromise data quality.

6. 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.

7. If the sample has less than 1 million cells or is prone to aggregation (such as PBMCs), we recommend preparing Cell Prefixation Buffer + BSA as described below. Mix thoroughly by pipetting 5x and store on ice. This mix should be prepared fresh and used the same day.

CELL PREFIXATION BUFFER + BSA				
Number of Samples	1	2	3	4
● Cell Prefixation Buffer	750 µL	1.5 mL	2.25 mL	3.0 mL
Gibco Bovine Albumin Fraction V (7.5% solution)	50 µL	100 µL	150 µL	200 µL
Total	800 µL	1.6 mL	2.4 mL	3.2 mL

8. Transfer 100,000 to 4 million cells into a 15 mL polypropylene tube (or BSA-coated polypropylene tube).

9. Centrifuge the 15 mL tube in a swinging bucket rotor for **5-10 minutes** at 200-600 x g at 4°C.

CRITICAL! Ideal centrifugation speed and duration should be determined empirically for each sample type to optimize retention and resuspension efficiencies. Overly aggressive centrifugation can damage cell integrity and decrease data quality. See the Appendix for details on optimizing centrifugation speeds

10. Remove and discard the supernatant. Fully resuspend the pellet in **750 µL** of cold ●Cell Prefixation Buffer (or Cell Prefixation Buffer + BSA, if prepared in step 7) with a P1000 set to 750 µL.

11. Pipette cells through a cell strainer with an appropriately sized mesh into a new 15 mL tube (or BSA-coated 15 mL tube) with a P1000 and store on ice. If the sample has cells larger than 40 µm, replace the provided strainers with the appropriate size mesh (70 µm or 100 µm) throughout the protocol.

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

12. Add **250 µL** of ●Cell Fixation Solution to the 15 mL tube and mix immediately by pipetting exactly 3x with a P1000 set to 250 µL.

CRITICAL! Do not perform additional mixing at this step.

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

14. Add **80 µL** of ●Cell Permeabilization Solution to the 15 mL tube and mix thoroughly by pipetting 3x with a P1000 set to 250 µL.

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

16. Mix the ●Cell Neutralization Buffer by inverting the tube 5x. Do not vortex.

17. Add **1 mL** of ●Cell Neutralization Buffer to the 15 mL tubes. Gently pipette 3x with a P1000 set to 1000 µL.

18. Centrifuge the 15 mL tube in a swinging bucket rotor for **5-10 minutes** at 200-600 x g at 4°C.

19. Remove the supernatant and store it in a new 15 mL tube on ice.

20. Fully resuspend each pellet in **100 µL** of cold ●Cell Buffer with a P1000 set to 100 µL and store on ice.

21. Count the number of cells in the sample with a hemocytometer or alternative counting device and record the count. Keep cells on ice during counting and work quickly to minimize the time that fixed cells are out.

22. If the number of cells recovered is ≥ 50% of the number transferred during step 8, proceed to step 25. Otherwise, proceed to step 23.

23. If there is < 50% retention, centrifuge the reserved supernatant again, increasing the centrifugation speed by 50-100 x g each time until the total number of recovered cells is ≥ 50% or the cells appear aggregated and/or damaged.

24. To balance cell recovery and minimize aggregation, lysis, and sample loss from difficult to resuspend pellets, combine the high quality samples in cold ●Cell Buffer and store on ice.

Note: For this sample type, the optimal centrifugation speed should be used for all centrifugation steps in Evercode workflows.

25. If required, add additional cold ●Cell Buffer to a final volume of **150 µL**.

26. Pipette cells through a cell strainer with an appropriately sized mesh into a new 1.5 mL tube with a P1000 and store on ice.

27. Count the number of cells in the sample with a hemocytometer or alternative counting device and record the count. Keep cells on ice during counting and work quickly to minimize the time that fixed cells are out.

Note: Downstream Evercode Whole Transcriptome processing can be streamlined by diluting and aliquoting samples at this step. See Storing Fixed Samples in the Introduction for recommendations.

28. If immediately processing samples with an Evercode Whole Transcriptome kit, proceed to the appropriate user guide. Otherwise, proceed to the next step.

29. Add **2.5 µL** of ●DMSO. Gently flick the tube 3x to mix.

30. Incubate on ice for **1 minute**.

31. Repeat steps 22 and 23 twice for a total addition of **7.5 µL** of ●DMSO.

32. Gently mix by pipetting 5x with a P200 set to 75 µL. Avoid creating bubbles.

33. Store tubes in a Mr. Frosty Freezing Container (or equivalent) at -80°C, according to the manufacturer's instructions. Samples are stable for up to 6 months at -80°C.

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

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