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USER MANUAL

Evercode™ Fixation

FOR USE WITH

Evercode Cell Fixation v2
Evercode Nuclei Fixation v2

Evercode Cell Fixation v2 (SKU: ECF2001)
Evercode Nuclei Fixation v2 (SKU: ECF2003)

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Cell Fixation Parts List

Cell Fixation Reagents (-20°C) WF300

Label	Component	Format	Quantity	Part Number
	Cell Prefixation Buffer	5 mL tube	1	WF301
	Cell Buffer	2 mL tube	1	WF302
	Cell Fixation Solution	1.5 mL tube	1	WF303
	Cell Fixation Additive	1.5 mL tube	1	WF304
	Cell Permeabilization Solution	1.5 mL tube	1	WF305
	Cell Neutralization Buffer	5 mL tube	4	WF306
	RNase Inhibitor	1.5 mL tube	1	WF307
	DMSO	1.5 mL tube	1	WF308

Fixation Accessory Box (Room Temp) WF200

Label	Component	Format	Quantity	Part Number
	40 µm strainers	Plastic Bag	8	WF201

Nuclei Fixation Parts List

Nuclei Fixation Reagents (-20°C) WN300

Label	Component	Format	Quantity	Part Number
	Nuclei Buffer	5 mL tube	1	WN301
	Nuclei Fixation Solution	1.5 mL tube	1	WN302
	Nuclei Permeabilization Solution	1.5 mL tube	1	WN303
	Nuclei Neutralization Buffer	5 mL tube	4	WN304
	RNase Inhibitor	1.5 mL tube	1	WN305
	DMSO	1.5 mL tube	1	WN306

Fixation Accessory Box (Room Temp) WN200

Label	Component	Format	Quantity	Part Number
	40 µm strainers	Plastic Bag	8	WN201

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. Any questions regarding these items can be directed to support@parsebiosciences.com.

Required Equipment

Item	Supplier	Part Number	Notes
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Capable of reaching 4°C. Compatible with 15 mL centrifuge tubes and 96-well plates.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. We recommend validating alternatives relative to a hemocytometer.
Single Channel Pipettes: P20, P200, P1000	Various Suppliers	Varies	Use with RNase/DNase-free pipette tips. See “Required Consumables” (next page).

Optional Equipment

Item	Supplier	Part Number	Notes
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific®	5100-0001	If not immediately processing fixed samples with a Whole Transcriptome kit. Or an equivalent device that cools samples at about -1°C/minute. Faster freezing times will lead to excess cell damage.

Required Consumables			
Item	**Supplier**	**Part Number**	**Notes**
Falcon® High Clarity PP Centrifuge Tubes,15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes as it will lead to substantial cell loss.
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	22431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Pipette Tips TR LTS 20 µL, 200 µL, 1,000 µL	Rainin®	17014961 17014963 17014967	Or appropriate sterile, DNA low-binding, and filtered pipette tips. We do not recommend using wide bore tips. Autoclaved pipette tips are not RNase and DNase free.
Trypan Blue	Various Suppliers	Varies	Or alternative dyes that can be used to assess cell viability, such as AOPI.
Gibco™ Bovine Albumin Fraction V (7.5% solution)	Thermo Fisher Scientific	15260037	For fixing nuclei, PBMCs, or cell types prone to clumping. This bovine serum albumin (BSA) was chosen due to its low RNase activity. We do not recommend substitutions.

Optional Consumables			
Item	**Supplier**	**Part Number**	**Notes**
TrypLE™ Express Enzyme (1X), phenol red OR TrypLE Select	Thermo Fisher Scientific	12605010 OR 12563011	If performing fixation on adherent cells. Trypsin is NOT recommended due to variable levels of RNase contamination.
Isopropyl alcohol	Various Suppliers	Varies	If using a Mr. Frosty Freezing Container to store samples.
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
Corning Cell Strainer (70 µm or 100 µm)	Corning	431751 (70 µm) 431752 (100 µm)	For cells larger than 40 µm, the 40 µm strainer should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm).

Notes Before Starting

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 single cell suspension. If you are fixing cells that were previously frozen, ensure the suspension is thawed before beginning. For cell samples, we recommend suspensions with >70% viability, <5% aggregation/debris, and 100,000 or more cells. For nuclei samples, we recommend suspensions with 100,000 or more nuclei. We recommend minimizing the length of time samples are stored on ice prior to fixation, as it can negatively impact results. If you have questions about your starting material, please contact us at support@parsebiosciences.com.

Cell Detachment

If dissociating 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.

Centrifugation

Use a swinging bucket rotor for all high-speed centrifugation steps in this protocol. Use of a fixed-angle rotor will lead to substantial cell/nuclei loss. Although the recommended centrifugation speeds are appropriate for most sample types, they can be adjusted to improve retention. Ideal centrifugation speed and duration should be empirically determined to optimize retention and resuspension efficiencies.

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. RNases are not inactivated by ethanol or isopropanol but can be inactivated by products such as RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific). These can be sprayed on benchtops and pipettes. Filtered pipette tips should be used to reduce RNase contamination from pipettes.

Addition of BSA

BSA can prevent aggregation of nuclei and some cell types prone to clumping, including PBMCs. For nuclei, BSA should be added to the Nuclei Buffer as described in the protocol. If your cell type is prone to clumping, we strongly recommend adding BSA to the Cell Prefixation Buffer as described in the protocol. If you have lower cell numbers or you are unsure if your cell type fits this category, we also recommend adding BSA. We strongly recommend using Gibco Bovine Albumin Fraction V (7.5% solution) (Thermo Fisher Scientific), which was chosen based on its very low RNase activity.

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 that 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 40 µm strainer should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm).

Reagent Stability

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

Cell/Nuclei Counting and Quality Assessment

We recommend a hemocytometer for cell counting, but alternative cell counting devices can also be used. If possible, we recommend validating counts from alternative devices to a hemocytometer when first using Evercode Fixation kits. To assess sample quality, we also recommend use of viability stains like trypan blue or acridine orange and propidium iodide (AOPI). As debris and cell/nuclei clumping can impact counts and can be difficult to assess, the figure below shows some samples of varying quality. When first using Evercode Fixation kits, we suggest saving images from each counting step.

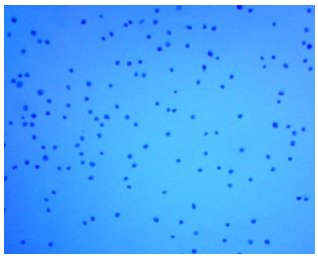
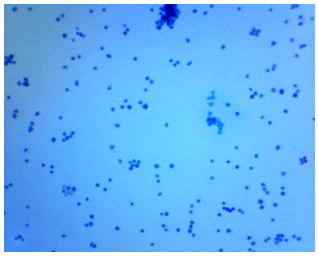
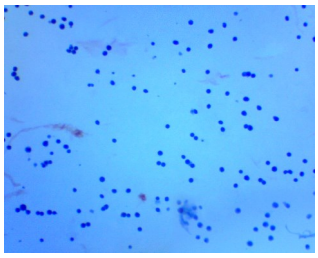
High Quality Sample		Single cells are easily distinguishable and no debris can be seen. Count each cell and proceed with your experiment as instructed.
Cell Clumps Have Formed		High quality cell samples should consist of > 95% single cells. Lower fractions of single cells may lead to elevated doublets.
Presence of Cell Debris		This is not a cause for concern assuming single cells are visible and debris is not causing cells to aggregate. Do not include any debris in your counting and continue your experiment as instructed.

Figure 1: Photos Representative of Varying Qualities of Cell Samples Observed While Counting in the Presence of the Trypan Blue Stain.

Optimizing Cell/Nuclei Recovery

It is critical that cells/nuclei are thoroughly resuspended after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to cell adherence to tubes, we also recommend carefully pipetting up and down along the bottom and sides of tubes to minimize cell loss. Similarly, we do not recommend wide bore pipette tips as they make it difficult to adequately resuspend cell/ nuclei pellets.

Cell/nuclei adherence to plastic can impact cell recovery throughout the protocol and impact sequencing data. Ensure that the 15 mL centrifuge tubes that will be used are **polypropylene** and not polystyrene. Polystyrene tubes will lead to substantial cell loss. BSA can also prevent cell adhesion to plastics. Thus, we recommend blocking 15 mL polypropylene centrifuge tubes with BSA to increase cell retention, especially for samples with fewer cells. See the Appendix for a blocking protocol.

Ensure the correct cell strainer is used based on the diameter of the cells you are processing (see "Cell Strainers" in Notes Before Starting for more details).

For the first few times you use Evercode Fixation kits, we recommend retaining the supernatants removed in steps 1.2.15 and 1.2.13 (for cell fixation) or 2.2.5 and 2.2.13 (for nuclei fixation). In the unlikely event of very high sample loss, these supernatants can be analyzed to identify points for optimization.

SECTION 1

CELL FIXATION

1.1 SETUP

1.2 PROTOCOL

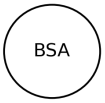
1.1 Cell Fixation Setup

This protocol is designed for fixing single cell suspensions which will be prepared in step 1.2.1.

Note: *If you are fixing nuclei, proceed to the Nuclei Fixation Protocol.*

1. *(Optional)* To maximize cell retention, prepare two BSA coated 15 mL centrifuge tubes per sample being fixed, according to the protocol in the Appendix.
2. *(Optional)* If you do not plan to immediately process samples with an Evercode Whole Transcriptome kit after fixation, place a Mr. Frosty Freezing Container (or equivalent) at room temperature.
3. Cool the centrifuge with swinging bucket rotor to 4°C.
4. Fill a bucket with ice and proceed to the next step.

5. Gather the following items and handle as indicated below. It is important that all solutions (except DMSO) are kept on ice after thawing.

Item		Location	Quantity	Format	After taking out
	40 µm Strainer	Fixation Accessory Box (Room Temp)	2 per number of samples	In plastic bag	Keep at room temperature.
	7.5% Gibco BSA Fraction V **(optional and not supplied)**	User Stored Location (4°C)	50 µL per number of samples	100 mL bottle	Keep at 4°C.
	Cell Prefixation Buffer	Cell Fixation Reagents (-20°C)	1	5 mL tube	Thaw, then place on ice.
	Cell Buffer	Cell Fixation Reagents (-20°C)	1	2 mL tube	Thaw, then place on ice.
	Cell Fixation Solution	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Cell Fixation Additive	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Cell Permeabilization Solution	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Cell Neutralization Buffer	Cell Fixation Reagents (-20°C)	1 per number of samples	5 mL tube	Thaw, then place on ice.
	RNase Inhibitor	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Place directly on ice.
	DMSO	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw and keep at room temperature (Warning: do NOT put on ice!).

CRITICAL! All items should be fully thawed before moving to the next step. Ensure that **DMSO** is not stored on ice.

6. If using this set of reagents for the first time, proceed to step 1.1.7. Otherwise, check the date on the Cell Fixation Reagents kit box. If less than 1 month has elapsed, proceed to step 1.1.11.

   *Note: Evercode Cell Fixation kits previously mixed by the user should have a date on the Cell Fixation Reagents kit box and a mark on the caps of the **Cell Fixation Solution**, **Cell Fixation Buffer**, and **Cell Buffer** tubes. After mixing reagents, Evercode Cell Fixation kits should only be freeze-thawed once and stored for up to 1 month at -20°C. Longer storage or additional freeze-thaws will compromise data quality.*

7. Add **550 µL** of **Cell Fixation Additive** directly into the **Cell Fixation Solution**. Mix thoroughly by pipetting up and down 5x with a P1000 set to 750 µL.

   ☐ To record the addition of **Cell Fixation Additive**, mark the cap of the **Cell Fixation Solution** tube, and store on ice.

8. Add **50 µL** of **RNase Inhibitor** directly into the **Cell Prefixation Buffer** tube. Mix thoroughly by pipetting up and down 5x with a P1000 set to 750 µL.

   ☐ To record the addition of **RNase Inhibitor**, mark the cap of the **Cell Prefixation Buffer** tube, and store on ice.

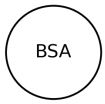
9. Add **17 µL** of **RNase Inhibitor** directly into the **Cell Buffer** tube. Mix thoroughly by pipetting up and down 5x with a P1000 set to 750 µL.

   ☐ To record the addition of **RNase Inhibitor**, mark the cap of the **Cell Buffer** tube, and store on ice.

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

    Note: After mixing reagents, Evercode Cell Fixation kits should only be freeze-thawed once and stored for up to 1 month at -20°C. Longer storage or additional freeze-thaws will compromise data quality.

11. *(Optional)* If your sample is cell-limited or prone to clumping (such as PBMCs), it is recommended to add 7.5% Gibco BSA Fraction V to the Cell Prefixation Buffer. For each sample being fixed, prepare **Cell Prefixation Buffer + BSA** according to the table below. **Cell Prefixation Buffer + BSA** should be prepared fresh and used the same day. Mix thoroughly by pipetting up and down 5x and store on ice.

(Optional addition of BSA)		**Volume to Add by Number of Samples (µL)**			
# Samples		**1**	**2**	**3**	**4**
	Cell Prefixation Buffer	750	1,500	2,250	3,000
	7.5% Gibco BSA Fraction V (not supplied)	50	100	150	200
Total (µL)		**800**	**1,600**	**2,400**	**3,200**

CRITICAL! Ensure the **Cell Prefixation Buffer** contains **RNase Inhibitor**, as marked on the tube cap.

1.2 Cell Fixation Protocol

Section 1.1 should have been completed before proceeding. Ensure tube caps have been marked when reagents were mixed and no more than 1 month has elapsed since the time of mixing, as dated on the Cell Fixation Reagents kit box.

1. Create a single cell suspension for the samples you plan to fix and store them on ice. When possible, avoid prolonged incubation on ice prior to fixation.

2. Count the number of cells in your sample 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.

3. Transfer up to 4 million cells into a 15 mL polypropylene centrifuge tube and store on ice.

CRITICAL! No more than 4 million cells should be used for any single sample. Exceeding this number may result in substantially elevated doublet rates. The minimum recommended number of cells to proceed with is 100,000. It is possible to be successful with fewer cells, but it is not recommended as pelleting cells becomes more difficult.

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

Cell Prefix ☒

5. Remove and discard the supernatant. Fully resuspend the pellet in **750 µL** of cold **Cell Prefixation Buffer** or (if prepared in step 1.1.11) **Cell Prefixation Buffer + BSA** with a P1000 set to 750 µL.

CRITICAL! Failure to fully resuspend cells may result in substantially elevated doublet rates. For this reason, do NOT use a wide bore pipette tip as it makes it difficult to fully resuspend cells.

6. Pipette cells through a 40 µm strainer into a new 15 mL polypropylene centrifuge tube with a P1000 and store on ice.

   Note: For cells larger than 40 µm, the 40 µm strainer should be replaced throughout the protocol with the appropriate size mesh (70 µm or 100 µm).

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

Cell Fix ☒

7. Add **250 µL** of **Cell Fixation Solution** to the 15 mL tube and mix immediately by pipetting up and down exactly 3x with a P1000 set to 250 µL. Return the tube to ice.

CRITICAL! Do NOT perform additional mixing at this step. Also, ensure the **Cell Fixation Solution** contains **Cell Fixation Additive**, as indicated by a mark on the tube cap.

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

Cell Perm

9. Add **80 µL** of **Cell Permeabilization Solution** to the 15 mL tube and mix thoroughly by pipetting up and down 3x with a P1000 set to 250 µL. Return the tube to ice.

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

*Note: Do NOT vortex the **Cell Neutralization Buffer**. Prior to use, invert the tube 5x to mix.*

Cell Neut

11. Add **4 mL** of **Cell Neutralization Buffer** to the 15 mL tubes. Gently invert the 15 mL tube once to mix and return to ice.

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

Cell Buffer ☒

13. Remove and discard the supernatant. Fully resuspend each pellet in **150 µL** of cold **Cell Buffer** with a P1000 set to 150 µL and return to ice.

14. Pipette cells through a 40 µm strainer into a new 1.5 mL tube with a P1000 and store on ice.

15. If immediately processing samples with an Evercode Whole Transcriptome kit, proceed to the appropriate user guide. Otherwise, proceed to step 1.2.16.

DMSO

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

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

DMSO

18. Repeat steps 16 and 17 two more times for a total addition of **7.5 µL** of **DMSO**.

19. Mix gently by pipetting up and down 5x with a P200 set to 75 µL. Avoid creating bubbles.

CRITICAL! Do NOT vortex cells.

20. Count the number of cells in your 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.

21. *(Optional)* If your sample has more than 500,000 cells, we recommend splitting it into two 1.5 mL tubes prior to storage.

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

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

SECTION 2

NUCLEI FIXATION

2.1 SETUP

2.2 PROTOCOL

2.1 Nuclei Fixation Setup

This protocol is designed for fixing single nuclei suspensions which will be prepared in step 2.2.1.

Note: *If you are fixing cells, refer back to the Cell Fixation Protocol.*

1. *(Optional)* To maximize cell retention, prepare two BSA coated 15 mL centrifuge tubes per sample being fixed, according to the protocol in the Appendix.

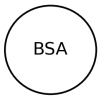
   Note: *Although step 1 is optional, 7.5% Gibco BSA Fraction V is required for other parts of the protocol.*

2. *(Optional)* If you do not plan to immediately process samples with an Evercode Whole Transcriptome kit after fixation, place a Mr. Frosty Freezing Container (or equivalent) at room temperature.

3. Cool the centrifuge with swinging bucket rotor to 4°C.

4. Fill a bucket with ice and proceed to the next step.

5. Gather the following items and handle as indicated below. It is important that all solutions (except DMSO) are kept on ice after thawing.

Item		Location	Quantity	Format	After taking out
	40 um Strainer	Fixation Accessory Box (Room Temp)	2 per number of samples	In plastic bag	Keep at room temperature.
	7.5% Gibco BSA Fraction V **(required and not supplied)**	User Stored Location (4°C)	100 µL per number of samples	100 mL bottle	Keep at 4°C.
	Nuclei Buffer	Nuclei Fixation Reagents (-20°C)	1 per number of samples	1.5 mL tube	Thaw, then place on ice.
	Nuclei Fixation Solution	Nuclei Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Nuclei Permeabilization Solution	Nuclei Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Nuclei Neutralization Buffer	Nuclei Fixation Reagents (-20°C)	1 per number of samples	5 mL tube	Thaw, then place on ice.
	RNase Inhibitor	Nuclei Fixation Reagents (-20°C)	1	1.5 mL tube	Place directly on ice.
	DMSO	Nuclei Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw and keep at room temperature (Warning: do NOT put on ice!).

CRITICAL! All items should be fully thawed before moving to the next step. Ensure that **DMSO** is not stored on ice.

6. If using this set of reagents for the first time, proceed to step 2.1.7. Otherwise, check the date on the Nuclei Fixation Reagents kit box. If less than 1 month has elapsed, proceed to step 2.1.8.

   *Note: Evercode Nuclei Fixation kits previously mixed by the user should have a date on the Nuclei Fixation Reagents kit box and a mark on the of the **Nuclei Buffer** tube. After mixing reagents, Evercode Nuclei Fixation kits should only be freeze-thawed once and stored for up to 1 month at -20°C. Longer storage or additional freeze-thaws will compromise data quality.*

7. Add **63 µL** of **RNase Inhibitor** directly into the **Nuclei Buffer** tube. Mix thoroughly by pipetting up and down 5x with a P1000 set to 750 µL.

   - To record the addition of **RNase Inhibitor**, mark the cap of the **Nuclei Buffer** tube and store on ice.
   - Record today's date on the Nuclei Fixation Reagents kit box**.**

   Note: After mixing reagents, Evercode Nuclei Fixation kits should only be freeze-thawed once and stored for up to 1 month at -20°C. Longer storage or additional freeze-thaws will compromise data quality.

8. For each sample being fixed, prepare **Nuclei Buffer + BSA** in a new tube. You will need **Nuclei Buffer** (without BSA) for later use. **Nuclei Buffer + BSA** should be prepared fresh and used the same day. Mix by pipetting up and down 5x with a P1000 set to 750 µL and store both **Nuclei Buffer + BSA** and **Nuclei Buffer** (without BSA) on ice.

	Volume to Add by Number of Samples (µL)			
# Samples	**1**	**2**	**3**	**4**
Nuclei Buffer (**RNase Inhibitor** added)	750	1,500	2,250	3,000
BSA 7.5% Gibco BSA Fraction V (**not supplied**)	50	100	150	200
Total (µL)	**800**	**1,600**	**2,400**	**3,200**

CRITICAL! Ensure the **Nuclei Buffer** contains **RNase Inhibitor**, as marked on the tube cap.

2.2 Nuclei Fixation Protocol

Section 2.1 should have been completed before proceeding. Ensure tube caps have been marked when reagents were mixed and no more than 1 month has elapsed since the time of mixing, as dated on the Nuclei Fixation Reagents kit box.

1. Create a single nuclei suspension for the samples you plan to fix and store them on ice. When possible, avoid prolonged incubation on ice prior to fixation.
2. Count the number of nuclei in your sample with a hemocytometer or alternative counting device and record the count. Keep nuclei on ice during counting and work quickly to minimize time on ice prior to fixation.
3. Transfer up to 4 million nuclei into a 15 mL polypropylene centrifuge tube and store on ice.

CRITICAL! No more than 4 million nuclei should be used for any single sample. Exceeding this number may result in substantially elevated doublet rates. The minimum recommended number of nuclei to proceed with is 100,000. It is possible to be successful with fewer nuclei, but it is not recommended as pelleting nuclei becomes more difficult.

4. Centrifuge the 15 mL tube in a swinging bucket rotor for **10 minutes** at **200 x *g*** at **4°C**.
5. Remove and discard the supernatant. Fully resuspend the pellet in **750 µL** of cold **Nuclei Buffer + BSA** with a P1000 set to 750 µL.

CRITICAL! Failure to fully resuspend nuclei may result in substantially elevated doublet rates. For this reason, do NOT use a wide bore pipette tip as it makes it difficult to fully resuspend nuclei.

6. Pipette nuclei through a 40 µm strainer into a new 15 mL polypropylene centrifuge tube with a P1000 and store on ice.

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

Nuclei Fix

7. Add **250 µL** of **Nuclei Fixation Solution** to the 15 mL tube and mix immediately by pipetting up and down exactly 3x with a P1000 set to 250 µL. Return the tube to ice.

CRITICAL! Do NOT perform additional mixing at this step.

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

Nuclei Perm

9. Add **80 µL** of **Nuclei Permeabilization Solution** to the 15 mL tube and mix thoroughly by pipetting up and down 3x with a P1000 set to 250 µL. Return the tube to ice.
10. Incubate on ice for **3 minutes**.

*Note: Do NOT vortex the **Nuclei Neutralization Buffer**. Prior to use, invert the tube 5x to mix.*

Nuclei Neut

11. Add **4 mL** of **Nuclei Neutralization Buffer** to the 15 mL tube. Gently invert the 15 mL tube once to mix and return to ice.

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

Nuclei Buffer

13. Remove and discard the supernatant. Fully resuspend the pellet in **150 µL** of cold **Nuclei Buffer** (without BSA but with **RNase Inhibitor** added) with a P1000 set to 150 µL and return to ice.

14. Pipette nuclei through a 40 µm strainer into a new 1.5 mL tube with a P1000 and store on ice.

15. If immediately processing samples with an Evercode Whole Transcriptome kit, proceed to the appropriate user guide. Otherwise, proceed to step 2.2.16.

DMSO

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

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

DMSO

18. Repeat steps 24 and 25 two more times for a total addition of **7.5 µL** of **DMSO**.

19. Mix gently by pipetting up and down 5x with a P200 set to 75 µL. Avoid creating bubbles.

CRITICAL! Do NOT vortex nuclei.

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

21. *(Optional)* If your sample has more than 500,000 nuclei, we recommend splitting it into two 1.5 mL tubes prior to storage.

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

    Note: Storing samples directly in the freezer without controlled cooling may lead to nuclei damage and compromise data quality.

APPENDIX

TUBE BLOCKING WITH BSA

Appendix: Tube Blocking With BSA

65 min

Blocking the 15 mL polypropylene centrifuge tubes used in the Cell or Nuclei Fixation Protocols with BSA can increase cell/nuclei yield. This is especially helpful for cells prone to sticking to plastic or when working with low cell/nuclei counts.

1. Prepare a fresh **1% BSA Master Mix** as follows, depending on the number of tubes you want to block.

	Volume to Add by Number of Tubes (mL)			
# Tubes	**2**	**4**	**6**	**8**
Water — Nuclease-free water (**not supplied**)	26	52	78	104
BSA — 7.5% Gibco BSA Fraction V (**not supplied**)	4	8	12	16
Total (mL)	**30**	**60**	**90**	**120**

Note: Two 15 mL polypropylene centrifuge tubes are needed for each sample.

2. Fill each 15 mL tube with the **1% BSA Master Mix** and cap the tubes.
3. Incubate the tubes for **30 minutes** at room temperature.
4. Decant and discard the **1% BSA Master Mix**. Remove any remaining solution from the bottom of the tube with a P1000.
5. With the caps removed, incubate the tubes for **30 minutes** in a biosafety cabinet at room temperature.
6. Proceed to the appropriate Cell or Nuclei Fixation Protocol, or store BSA coated tubes at 4°C for up to 4 weeks.

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