User Manual

Version 2.2 – UM0016

Evercode™ Nuclei Fixation v2

For use with

ECF2103

ECF2003

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Patents pending in the U.S. and other countries

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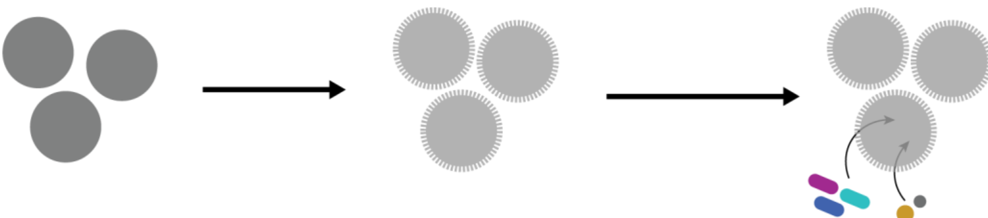
Overview

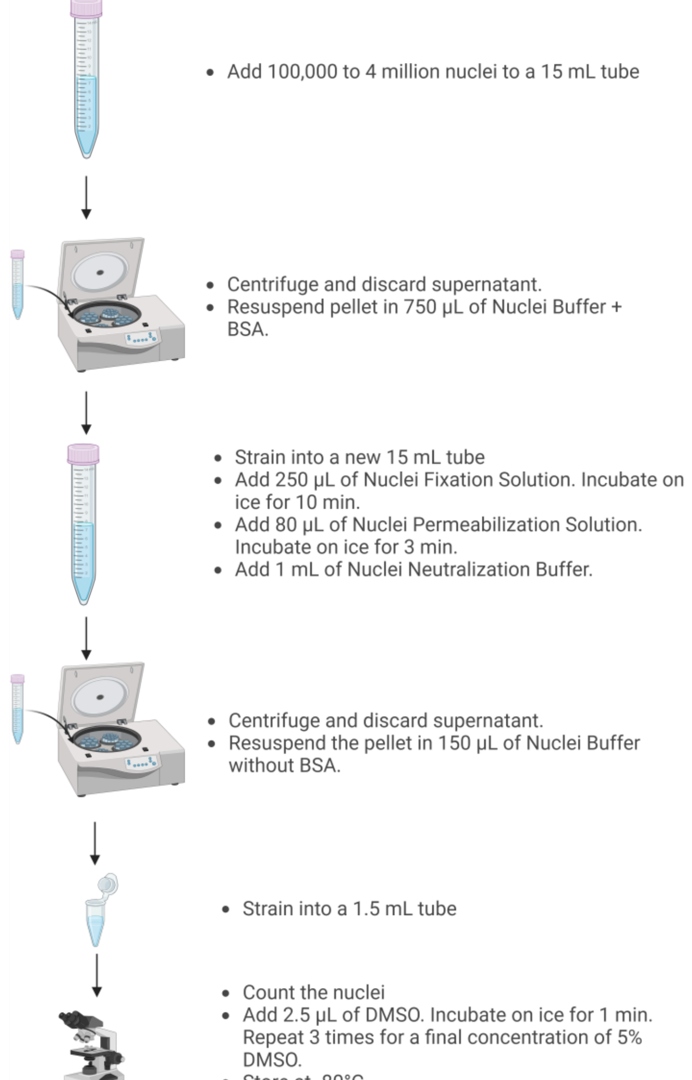
Workflow

From a single nuclei suspension, the Evercode Nuclei Fixation v2 kit generates fixed and permeabilized nuclei ready for use in Evercode Whole Transcriptome kits. Fixation maintains nuclei 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 Nuclei 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 of batch effects.



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

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



Protocol timing

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

SECTION	TOTAL TIME	HANDS-ON TIME	SAFE STOPING POINTS
1.1 Block Tubes with BSA	30 min	5 min	Blocked tubes: 4°C ≤ 1 month
1.2 Prepare Reagent Mixes	15 min	5 min	Reagent mixes: -20°C ≤ 1 month
2.1 Nuclei Fixation	45 min	45 min	-80°C ≤ 6 months

Important Guidelines

Although this user guide provides detailed instructions, these 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 nuclei suspension. We recommend suspensions with <5% aggregation/ debris, and >100,000 nuclei. If more nuclei are available, we recommend using the highest number available up to 4 million total.
- Exceeding 4 million nuclei in a single fixation will result in substantially elevated doublet rates.
- If nuclei 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.

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 an 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.
- Filtered pipette tips should be used to reduce RNase contamination from pipettes.

Cell Strainers

- A 40 µm cell strainer will be used in multiple steps. To maximize nuclei 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.

Nuclei 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 Nuclei Fixation v2 kits.

- When first using Evercode Nuclei Fixation v2 kits, we suggest saving images at each counting step.

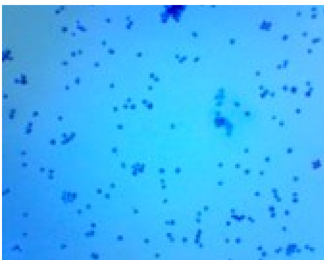
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).

- 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.

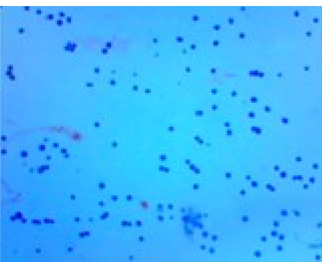
- High quality fixed samples have single distinct nuclei with <5% aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed nuclei, it is critical to avoid counting debris to avoid overestimating the number of nuclei (Figure 1).

High Quality Sample

Aggregation



Debris



Centrifugation

- A range of centrifugation speeds and durations are given in this protocol rather than a single 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 sample types, the centrifuge should be set to 4°C to help maintain nuclei integrity.

- 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 nuclei loss.

Maximizing Nuclei Recovery

- It is critical to thoroughly resuspend the nuclei after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. To minimize nuclei loss from adherence to tubes, carefully pipet 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 that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.

- The 15 mL polypropylene centrifuge tubes can also be blocked with Bovine serum albumin (BSA) to prevent nuclei adhesion to plastics and improve retention. The recommended protocol is described in Section 1.1.

- When using Evercode Nuclei 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 RNase Inhibitor is added to Nuclei Buffer, as indicated in the protocol, the mixed reagent is 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. Fixed samples should not be refrozen after thawing.

- 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 nuclei are lost after thawing, we strongly recommend targeting a higher concentration.

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

Part List

The Evercode Nuclei 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 WN400

LABEL	ITEM	PN	FORMAT	QTY
Nuclei Buffer	Nuclei Buffer	WN301	5 mL tube	1
Nuclei Fix	Nuclei Fixation Solution	WN302	1.5 mL tube	1
Nuclei Perm	Nuclei Permeabilization Solution	WN303	1.5 mL tube	1
Nuclei Neut	Nuclei Neutralization Buffer	WN304	5 mL tube	1
Rnase Inhib	RNase Inhibitor	WN305	1.5 mL tube	1
DMSO	DMSO	WN306	1.5 mL tube	1

Note: An alternative version of Evercode Nuclei Fixation v2 (ECF2003) includes WN300 instead of WN400. This configuration includes 4 Nuclei 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 Material and Equipment

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

LABEL	ITEM	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 sample damage.

Consumables

LABEL	ITEM	PN	NOTES
Falcon® High Clarity PP Centrifuge Tubes, 15 m	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 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 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.
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 nuclei retention. This is especially helpful for low inputs.

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 the Nuclei Buffer reagent mix. Otherwise, proceed to Section 2. This reagent mix is 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
● Nuclei Buffer	Nuclei Fixation Reagents (-20°C)	1.5 mL	Thaw at room temperature then place on ice. Mix by inverting 3x.
● RNase Inhibitor	Nuclei Fixation Reagents (-20°C)	1.5 mL	Store on ice. Briefly centrifuge before use.

2. Add **63 µL** of ●RNase Inhibitor directly into the ●Nuclei Buffer tube. Mix thoroughly by pipetting 5x with a P1000 set to 750 µL. Store on ice.
3. Record the addition of ●RNase Inhibitor by marking the cap of the ●Nuclei Buffer tube.
4. Record today's date on the Nuclei Fixation Reagents kit box.

Note: This reagent mix 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 days, the reagent mixes can be aliquoted.

Section 2: Fixation

2.1 Nuclei Fixation

After the initial centrifugation to remove the buffer/medium from the single nuclei suspension, nuclei are transferred to Nuclei Buffer + BSA. Reagents are added to fix and permeabilize the nuclei, which are then neutralized. Samples are either processed immediately with an Evercode Whole Transcriptome kit, or DMSO is added prior to freezing at -80°C.

To fix nuclei:

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.

<table>
<tr><th>ITEM</th><th>SOURCE</th><th>FORMAT</th><th>HANDLING AND STORAGE</th></tr>
<tr><td>● Nuclei Buffer</td><td>Nuclei Fixation Reagents (-20°C)</td><td>1.5 mL</td><td rowspan="2">Thaw and store on ice. Mix by inverting 3x.</td></tr>
<tr><td>● Nuclei Fixation Solution</td><td>Nuclei Fixation Reagents (-20°C)</td><td>1.5 mL</td></tr>
<tr><td>● Nuclei Permeabilization Solution</td><td>Nuclei Fixation Reagents (-20°C)</td><td>1.5 mL</td><td rowspan="2">Thaw at room temperature then store on ice. Mix by inverting 3x.</td></tr>
<tr><td>● Nuclei Neutralization Buffer</td><td>Nuclei Fixation Reagents (-20°C)</td><td>5 mL</td></tr>
<tr><td>● DMSO</td><td>Nuclei Fixation Reagents (-20°C)</td><td>1.5 mL</td><td>Thaw and store at room temperature. Mix by inverting 3x.</td></tr>
</table>

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
40 µm Strainer (or alternative size)	Nuclei Fixation Reagents (-20°C)	2 per samples	Keep at room temperature.
7.5% Gibco BSA Fraction V (optional)	Not supplied	100 µL per sample	Store on ice.

Note: Ensure the ●Nuclei Buffer and ●RNase Inhibitor mix was prepared as described in Section 1.2 by verifying a mark on the ●Nuclei Buffer tube cap. 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 nuclei in the single nuclei suspension 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.

7. Prepare Nuclei 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.

NUCLEI PREFIXATION BUFFER + BSA				
Number of Samples	1	2	3	4
● Nuclei 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 nuclei 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! An alternative version of Evercode Nuclei Fixation v2 (ECF2003) includes WN300 instead of WN400. This configuration includes 4 Nuclei Neutralization Buffer tubes, but only 1 will be used in this protocol. The other 3 tubes can be discarded.

10. Remove and discard the supernatant. Fully resuspend the pellet in **750 µL** of cold Nuclei Buffer + BSA with a P1000 set to 750 µL.

11. Pipette nuclei 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.

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

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

Note: Do not perform additional mixing at this step.

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

14. Add **80 µL** of Nuclei 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 Nuclei Neutralization Buffer by inverting the tube 5x. Do not vortex.

17. Add **1 mL** of Nuclei 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 Nuclei Buffer without BSA with a P1000 set to 150 µL and store on ice.

20. Pipette nuclei 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 nuclei in the 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.

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

22. If immediately processing samples with an Evercode Whole Transcriptome kit, proceed to the appropriate user guide. 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 23 and 24 twice for a total addition of **7.5 µL** of ●DMSO.

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

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

CRITICAL! Storing samples directly in the freezer without controlled cooling may lead to nuclei 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 retention, but high speeds can make resuspension of pellets challenging 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 nuclei size. Smaller nuclei need faster speeds, and larger 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 nuclei solution. Nuclei should be examined under a microscope before and after centrifugation to calculate retention and assess any aggregation or morphological changes.

Duration

If nuclei are damaged by increased centrifugation speed, sometimes the duration can be adjusted to increase retention without nuclei damage.

Temperature

For most sample types, the centrifugation should be done at 4°C. 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 nuclei 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
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 nuclei 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 nuclei retention and fix nuclei:

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
● Nuclei Buffer	Nuclei Fixation Reagents (-20°C)	1.5 mL	Thaw and store on ice. Mix by inverting 3x.
● Nuclei Fixation Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL	
● Nuclei Permeabilization Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Nuclei Neutralization Buffer	Nuclei Fixation Reagents (-20°C)	5 mL	
● DMSO	Nuclei Fixation Reagents (-20°C)	1.5 mL	Thaw and store at room temperature. Mix by inverting 3x.
40 µm Strainer (or alternative size)	Nuclei Fixation Reagents (-20°C)	2 per sample	Keep at room temperature
7.5% Gibco BSA Fraction V (optional)	Not Supplied	100 µL per sample	Store on ice

CRITICAL! Ensure the ●Nuclei Buffer and ●RNase Inhibitor mix was prepared as described in Section 1.2 by verifying a mark on the ●Nuclei Buffer tube cap. Also, ensure it was prepared less than 1 month ago by verifying the preparation date on the kit box. Longer storage or more than one freeze-thaw will compromise data quality.

6. Count the nuclei in the single nuclei suspension 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.

7. Prepare Nuclei 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.

NUCLEI BUFFER + BSA				
Number of Samples	1	2	3	4
● Nuclei 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 nuclei 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. See the Appendix A for details on optimizing centrifugation speeds.

10. Remove and discard the supernatant. Fully resuspend the pellet in **750 μL** of cold Nuclei Buffer + BSA with a P1000 set to 750 μL.

11. Pipette nuclei 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.

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

12. 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.

CRITICAL! Do not perform additional mixing at this step.

13. Incubate on ice for **10 minutes**.
14. Add **80 µL** of ●Nuclei 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 ●Nuclei Neutralization Buffer by inverting the tube 5x. Do not vortex.
17. Add **1 mL** of ●Nuclei 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 in a new 15 mL tube on ice.
20. Fully resuspend each pellet in **100 µL** of cold ●Nuclei Buffer without BSA with a P1000 set to 100 µL and store on ice.
21. Count the number of nuclei in the 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.
22. If the number of nuclei 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 nuclei is ≥ 50% or the nuclei appear aggregated and/or damaged.

24. To balance recovery and minimize aggregation, lysis, and sample loss from difficult to resuspend pellets, combine the high quality samples in cold Nuclei Buffer without BSA 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 ●Nuclei Buffer without BSA to a final volume of **150 µL**.
26. Pipette nuclei 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 nuclei in the 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.
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.

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

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

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

Safe stopping point: Samples are stable for up to 6 months at -80°C.

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