PARSE BIOSCIENCES
A QIAGEN company

User Manual

Version 1.1 – UMFN4300

Evercode™ Nuclei Fixation v4

12 Reactions

For use with

ECFN4300

ECFN4301

Support Suite

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Overview

Workflow

From a single nuclei suspension, the Evercode Nuclei Fixation v4 kit generates fixed and permeabilized nuclei ready for use in the Evercode kits.

This 1.5 mL tube-based workflow is recommended when processing ≤ 12 samples at a time. If processing > 12 samples at a time, we recommend the mid-throughput plate-based workflow which streamlines fixation when processing more samples. If processing ≥ 48 samples, we recommend using the Evercode Nuclei Fixation v4, HT 96 reactions kit and its high-throughput plate-fixation protocol. The HT 96 reactions kit should not be used more than twice.

Fixation maintains nuclei structure, prevents RNA degradation, and locks the RNA inside the nucleus, 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°C, Evercode Nuclei Fixation v4 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.

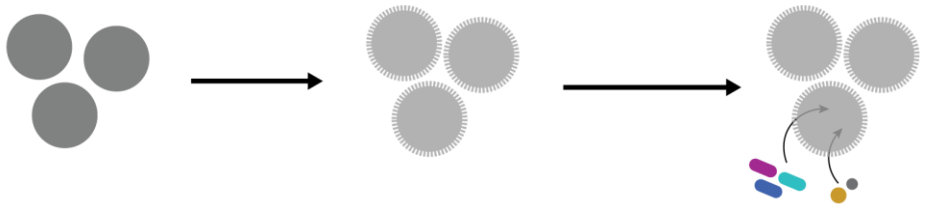


Figure 1: Evercode Nuclei Fixation v4. Nuclei in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

The figure below provides an overview of the fixation workflow. Between 100,000 and 1 million nuclei can be fixed in a single reaction. Note that more than 100,000 nuclei may need to be fixed to fully utilize the capacity of the downstream Evercode kits. See Important Guidelines for additional details. If desired, 1-4 million nuclei can be fixed in a single reaction, see Appendix B for details.

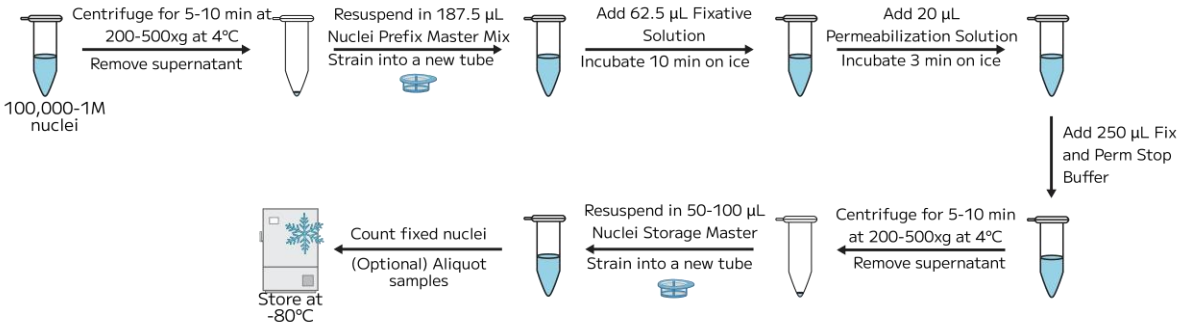


Figure 2: Evercode Nuclei Fixation v4 Workflow

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	STOPPING POINTS
Section 1: Set Up			
1.1 Block tubes/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 Nuclei Fixation	60 min	60 min	-80°C ≤ 6 months

Important Guidelines

These guidelines provide additional information to obtain optimal performance beyond the detailed instructions in the protocol. For additional questions not discussed below, please contact us at support@parsebiosciences.com. We also have a library of additional resources and videos on our support site at https://support.parsebiosciences.com/.

Sample Input

- This protocol begins with a previously prepared single nuclei suspension. We recommend suspensions with <5% aggregation/debris.
- If nuclei were previously frozen, ensure the suspension is completely thawed and in suspension before beginning fixation.
- ·We recommend minimizing the length of time between nuclei isolation and fixation, as it can negatively impact the results. Store the samples on ice the entire time.
- If fixing between 1-4 million nuclei, use the High Input Workflow in Appendix B. Exceeding the maximum number of nuclei 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 in Appendix C 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 nuclei concentrations and volumes, reference the relevant Sample Loading Table.
- Note that retention during fixation varies typically between 40-60%, and some nuclei will be lost when freezing and thawing fixed samples, typically between 5-15%. The final concentration of nuclei post-fixation is also influenced by the resuspension volume used in Section 2. These factors should all be considered when determining how much sample input is needed for fixation. **Refer to Appendices C1 and C2 for detailed cell number and concentration reference tables.**

Avoiding RNase Contamination

- Standard precautions should be taken to avoid introducing RNases into samples or reagents throughout the workflow. Always wear proper laboratory gloves and use aseptic technique.

- Although RNases are not inactivated by ethanol or isopropanol, they are inactivated by products such as RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific). These can be sprayed on benchtops and pipettes.

- Nuclease-free, filtered pipette tips should be used to reduce RNase contamination from pipettes.

Cell Strainers

- To maximize nuclei retention with cell strainers, press the pipette tip 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. An example video can be found on our support site.

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 µm is appropriate for most nuclei, the mesh size should be chosen based on your sample type.

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 Fixation v4 kits.

- We suggest saving images at each counting step, especially when first using Evercode Fixation v4 kits.

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

- 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 nuclei with <5% aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing and may indicate a poor quality nuclei isolation. When quantifying fixed nuclei, it is critical to avoid counting debris to avoid overestimating the number of nuclei.

High Quality Sample | Aggregation | Debris

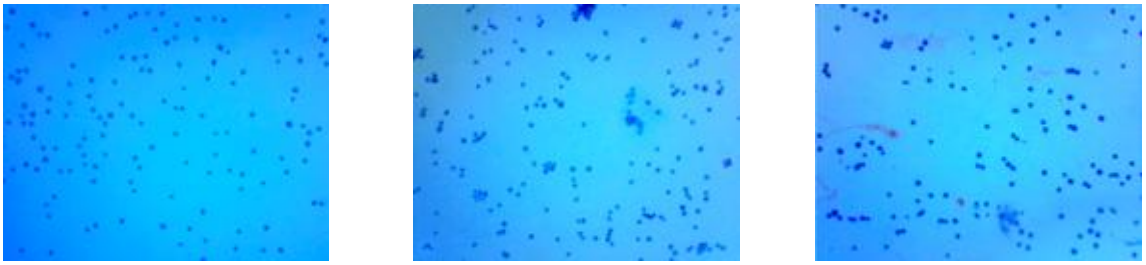


Figure 3: Example of trypan blue stained fixed cells.

Centrifugation

- A range of centrifugation speeds and durations are given in this protocol rather than a single speed. We recommend optimizing centrifugation conditions for each sample type to balance retention and resuspension efficiencies. See Appendix A for 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 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. Ideally this should be verified with microscopy.
- To minimize nuclei loss from nuclei 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 resuspend nuclei pellets adequately.
- Ensure that the 1.5 mL and 15 mL centrifuge tubes are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- When using Evercode Fixation v4 kits for the first few times, we recommend retaining the supernatants removed in Section 2 steps 8 and 18 and if relevant, steps 8 and 18 in Appendix B3. In the unlikely event of very high sample loss, these supernatants can be analyzed to identify points for optimization.

Reagent Stability

- Reagents in the Nuclei Fixation Reagents box should not be frozen and thawed more than 3 times.
- If the kit is going to be used more than 3 times, the reagents should be aliquoted into nuclease-free 1.5 mL tubes and stored at -20°C for up to 1 month. We do not recommend making single use aliquots to minimize the impact of evaporation during storage.
- To avoid pipetting <2 µL of RNase Inhibitor, we do not recommend preparing less than 2 reactions of Nuclei Storage Master Mix. If required, the master mix can be prepared without DMSO, split into aliquots, and stored at -20°C for up to a month. DMSO should be added prior to use in the protocol to a final concentration of 5%.
- With the exception of the partial Nuclei Storage Master Mix described above, 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 21. The aliquots should be ≥20 µL when stored in 1.5 mL tubes.
- We **strongly suggest** making a 20 µL counting aliquot for each sample. This aliquot should be used to update sample concentrations in the Evercode Sample Loading Table before starting the Evercode workflow. The loss after freeze/thaw should match the sample. This will minimize the time between sample thawing and the start of the Evercode workflow.

Part List

The Evercode Nuclei Fixation v4, 12 reactions tube workflow requires Nuclei Fixation Reagents and Nuclei Prefixation Enhancer boxes.

Nuclei Fixation Reagents, 12 reactions. Store at -20°C, PN NF500

LABEL	ITEM	PN	FORMAT	QTY
Prefix	Prefixation Buffer	NF101	8 mL bottle	1
Storage	Storage Buffer	NF102	2 mL tube	1
Fix	Fixative Solution	NF103	1.5 mL tube	1
Perm	Permeabilization Solution	NF113	1.5 mL tube	1
Stop	Fix and Perm Stop Buffer	NF105	8 mL bottle	1
RNase Inhib	RNase Inhibitor	NF106	1.5 mL tube	1
DMSO	DMSO	NF107	1.5 mL tube	1

Nuclei Prefixation Enhancer, 12 reactions. Store at 4°C, PN NF600

LABEL	ITEM	PN	FORMAT	QTY
Prefix Enhan	Prefixation Enhancer	NF201	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	PCS1030	Plastic sleeve	1

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 1.5 mL and 15 mL tubes and capable of reaching 4°C. Compatible with 96 deep well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
1-channel: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other nuclei counting device.
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific®	5100-0001	(Optional) If storing fixed samples before processing with an Evercode Whole Transcriptome. Or an equivalent device that cools samples at about -1°C/minute to minimize nuclei damage.
Water bath	Various Suppliers	Varies	(Optional) If preparing aliquots to count the day before running a downstream Evercode Whole Transcriptome kit. Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.

Consumables

ITEM	SUPPLIER	PN	NOTES
Protein LoBind® Tubes	Eppendorf®	022431081	Or equivalent protein low-binding, nuclease-free 1.5 mL tubes.
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-20 TC70-SWM-40	Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm). We do not recommend FlowMi Cell Strainers (SP Bel-Art).
pluriStrainer® Mini (Cell Strainer)	pluriSelect®	43-10020-40 43-10040-40	
Falcon® Cell Strainer	Corning®	431750	
EASYstrainer™, small	Greiner Bio-One™	542120 542140	
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.
Isopropyl alcohol	Various Suppliers	Varies	(Optional) If using a Mr. Frosty Freezing Container.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning	352097	Or equivalent 15 mL centrifuge tubes. If using the high input protocol in Appendix B, do not substitute polystyrene tubes as it will lead to substantial nuclei loss.
Falcon® High Clarity PP Centrifuge Tubes, 50 mL	Corning	352070	Or equivalent 50 mL centrifuge tubes.
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.

Reagents

ITEM	SUPPLIER	PN	NOTES
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Trypan Blue	Various Suppliers	Varies	Or alternative viability dyes, such as AO/PI.
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.
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 Tubes with BSA

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

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

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)	2.6 mL	31.2 mL
Gibco Bovine Albumin Fraction V (7.5% solution) (not supplied)	400 µL	4.8 mL
Total Volume	3 mL	36 mL

2. For each sample, fill two 1.5 mL tubes with 1.5 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. Remove the 1% BSA with a P1000 and discard.
6. Remove any remaining solution from the bottom of the tube with a P200.
7. With the caps removed, air dry the tubes for **30 minutes** in a biosafety cabinet at room temperature.
8. Proceed to Section 1.2 or store capped BSA-coated tubes at 4°C for up to 4 weeks.

1.2. Prepare Master Mixes

Master mixes should be prepared just prior to fixation.

To prepare master mixes:

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

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	Nuclei 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	Nuclei Fixation Reagents (-20°C)	2 mL tube	
● Fixative Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	
● Permeabilization Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	
○ Fix and Perm Stop Buffer	Nuclei Fixation Reagents (-20°C)	8 mL bottle	
● DMSO	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
● RNase Inhibitor	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Nuclei Prefixation Enhancer (4°C)	1.5 mL tube	
Plate Strainer (30 µm)	Plate Strainer (Room Temperature)	Plastic sleeve	Keep at room temperature

2. Prepare the Nuclei Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting, carefully avoiding to create bubbles, and store on ice.

NUCLEI PREFIXATION MASTER MIX		
Number of Samples	1	12
○ Prefixation Buffer	203.5 µL	2.44 mL
● RNase Inhibitor	2.75 µL	33 µL
● Prefixation Enhancer	13.75 µL	165 µL
Total Volume	220 µL	2.64 mL

CRITICAL! Reagents in the Nuclei Fixation box can be frozen and thawed 3 times. If the kit will be used more than 3 times, aliquots should be made. See Reagent Stability in Important Guidelines for details.

3. Prepare the Nuclei Storage Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

NUCLEI STORAGE MASTER MIX		
Number of Samples	1	12
● Storage Buffer	112.5 µL	1.35 mL
● RNase Inhibitor*	1.5 µL	18 µL
● DMSO	6 µL	72 µL
Total Volume	120 µL	1.44 mL

Note: *To avoid pipetting 2 µL of ● RNase Inhibitor, we do not recommend preparing less than 2 reactions of Nuclei Storage Master Mix. See Reagent Stability in Important Guidelines for additional details.

4. Proceed immediately to Section 2.

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 Prefixation Master Mix. Reagents are added to fix and permeabilize nuclei, and then to stop these reactions. Nuclei are resuspended in Nuclei Storage Master Mix and stored at -80°C or processed immediately with a downstream Evercode kit.

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 nuclei counting device.
4. Place a Mr. Frosty Freezing Container at room temperature.
5. 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.
6. Transfer 100,000 to 1 million nuclei from each sample into a Protein LoBind 1.5 mL tube (or a BSA coated tube if prepared in Section 1.1).
7. Centrifuge the tubes in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

   CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial nuclei loss.

   CRITICAL! Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See Appendix A for details.

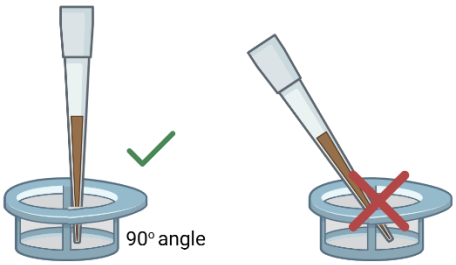
   CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

8. Slowly aspirate then discard the supernatant. Less than 20 µL should be remaining in the tubes.
9. Fully resuspend each pellet in **187.5 µL** of Nuclei Prefixation Master Mix.

10. Pipette **187.5 µL** of each sample through a cell strainer into a new 1.5 mL tube and store on ice.

CRITICAL! Do not directly touch the mesh of cell strainer(s) with gloved hands.

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.



11. Add **62.5 µL** of ● Fixative Solution to each tube and mix immediately by pipetting exactly 3x.

CRITICAL! Do not perform additional mixing at this step.

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

13. Add **20 µL** of ● Permeabilization Solution to each tube. Immediately mix thoroughly by pipetting 3x with a P200 set to 180 µL.

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

15. Mix the ○ Fix and Perm Stop Buffer by inverting the tube 5x. Do not vortex.

16. Add **250 µL** of ○ Fix and Perm Stop Buffer to each tube and gently pipette mix 3x.

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

18. With a P1000 set to 500 µL, slowly aspirate then discard the supernatant. Less than 20 µL should be remaining in the tubes.

19. Fully resuspend each pellet in an additional **50-100 µL** Nuclei Storage Master Mix and store on ice.

Note: Choose a resuspension volume appropriate for the experimental design and downstream Evercode kit. See Appendix C1 for additional details. If using the MAX WT V4 kits, refer to Appendix C2 to choose the most appropriate nuclei concentration for the MAX kits size selected.

20. Pipette the sample through a cell strainer into a new, non BSA-coated 1.5 mL tube and store on ice.

21. While minimizing time on ice, count the number of nuclei in the sample with a hemocytometer or alternative counting device and record the nuclei count.

Note: We recommend preparing 20 µL Counting Aliquot to streamline downstream Evercode processing. Mix the sample 5x before aliquoting. See Important Guidelines for details.

22. Proceed to the appropriate user guide if immediately processing samples with an Evercode kit. Otherwise, proceed to the next step.

Note: If collecting and storing multiple samples over time, we recommend transferring **20 µL** to a new 0.2 mL tube strip. These aliquots can be thawed and counted separately from the remaining sample for downstream processing with Evercode kits.

23. 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.

24. The day before running the downstream Evercode kit, thaw the 20 µL aliquots in a water bath set to 37°C. Count the nuclei in the single nuclei suspension with a hemocytometer or alternative counting device and record the count. Discard any remaining sample from the counting aliquot.

Appendices

Appendix A: Centrifugation Optimization

When using Evercode Nuclei Fixation v4 for the first time or when testing a new sample type, we recommend optimizing centrifugation conditions. This appendix provides guidelines for optimization, suggestions for common sample types, and an example experiment to optimize centrifugation speed. Note that physical properties of nuclei may change after the fixation process, which requires centrifugation conditions to be optimized during fixation.

Important Guidelines

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 nuclei retention and assess any aggregation or morphological changes. After determining the appropriate centrifugation conditions, we recommend using the same speed and duration throughout this and downstream Evercode User Guides.

Typical Sample Retention

Across a range of samples, nuclei retention post-fixation typically varies between 40-60% of the initial input. Retention is impacted by sample type, sample preparation method, centrifugation conditions, and sample handling.

Speed

Increasing centrifugation speeds can improve nuclei retention, but high speeds can complicate the pellet 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 nuclei size. Smaller nuclei need faster speeds, and larger nuclei need slower speeds.

Duration

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

Temperature

For most sample types, the centrifugation should be done at 4°C. However, some sample types may require different temperatures to maximize nuclei quality prior to fixation. After fixation,

the final centrifugation step in this User Guide and all centrifugation steps in the downstream Evercode User Guide should be done at 4°C to maintain nuclei and RNA integrity.

Aggregates After Centrifugation

If the pellet cannot be resuspended back into a single nuclei suspension and there are aggregates where there were previously not, this is an indication that the sample may have been over centrifuged.

Aggregates may also be an indication of insufficient pipette mixing. Gently resuspend the pellet by slowly and repeatedly pipetting until no clumps are visible. This can be visually inspected via microscopy.

Aggregates at this stage may also be a result of the sample preparation method used. If none of the above have been successful in removing the aggregates, a filtering step may help remove aggregates or the sample preparation may require additional optimization.

Debris After Centrifugation

Samples with low quality nuclei may lead to excessive debris in your fixed sample. Ideally, measures should be taken to optimize sample quality prior to proceeding into fixation. The Parse Biosciences applications support team can provide sample preparation optimization techniques.

If a sample with minimal debris has significant debris after centrifugation, this may be an indication that the sample has lysed due to over centrifugation and/or overly aggressive resuspension. The centrifugation speed should be reduced and/or pellets should be less aggressively pipetted.

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	200 x g	10 min	4°C

Centrifugation Optimization Method

When using Evercode Nuclei Fixation v4 for the first time or when testing a new sample type, we recommend using 1-2 samples to optimize centrifugation conditions prior to processing samples of interest. When this is not possible, centrifugation conditions can be determined while fixing samples of interest.

Figure 4 outlines suggested modifications to the fixation protocol to test different centrifugation conditions. This approach starts centrifugation at a low speed, but retains the supernatants after each spin. These supernatants are then centrifuged again to recover additional nuclei. After resuspension, each pellet should be assessed with microscopy to count nuclei, quantify debris, and assess aggregation. Resuspended pellets of high quality (minimal debris, minimal aggregation, and minimal evidence of nuclei damage) are pooled and can be used with downstream Evercode Whole Transcriptome kits. If the retention is below 40-60% after pooling, we recommend contacting our applications support team for additional recommendations.

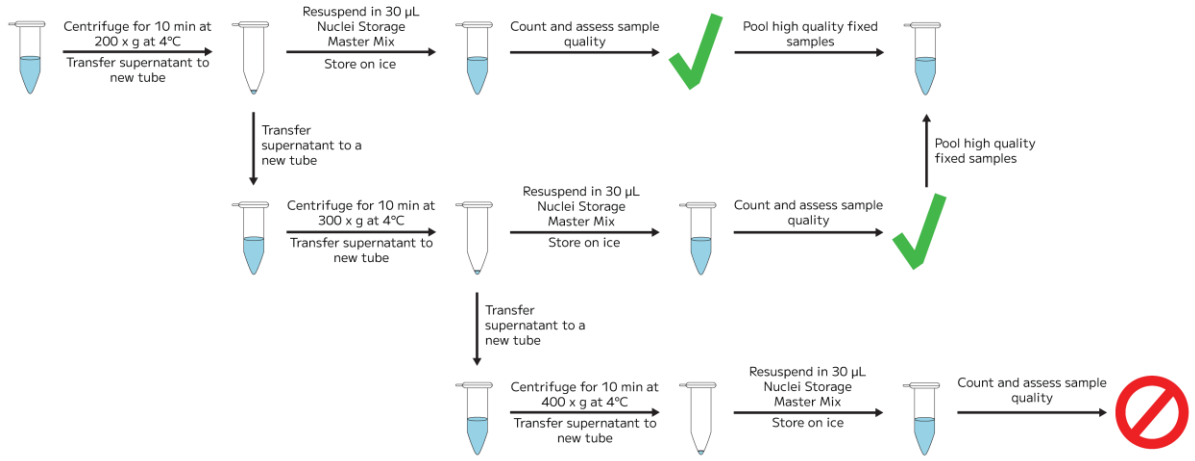


Figure 4: **Example Centrifugation Optimization Experiment.** In this example, the sample is first centrifuged at 200 x g for 10 minutes. The pellet is resuspended in Nuclei Storage Master Mix, and the first supernatant is centrifuged again at 300 x g for 10 minutes. The second pellet is resuspended in Nuclei Storage Master Mix, and the second supernatant is centrifuged again at 400 x g for 10 minutes. This final, third pellet is resuspended in Nuclei Storage Master Mix and the third supernatant is discarded. The three resuspended pellets are then counted with a hemocytometer. In this example, the nuclei centrifuged at 400 x g are aggregated with significant debris, so this resuspended pellet should be discarded. Conversely, the nuclei centrifuged at 200 x g and 300 x g were high quality, so they were pooled together. Once pooled, this sample has ~50% retention. These results suggest that this sample type should be centrifuged at 300 x g in Evercode Nuclei Fixation and Evercode Whole Transcriptome workflows.

To modify the fixation protocol and optimize the centrifugation as suggested, complete all the steps as outlined in Section 1, follow steps 1-16 in Section 2. Then follow steps 17-25 below.

17. Centrifuge in a swinging bucket rotor for **10 minutes** at 200 x g at 4°C.

18. Transfer each supernatant to a new 1.5 mL tube(s).

19. With a P200, fully resuspend each pellet in **30 µL** of Nuclei Storage Master Mix and store on ice.

20. Repeat steps 17-19 between 2-4x increasing the centrifugation speed by 50-100 x g for each centrifugation.

21. While minimizing time on ice, count the nuclei and assess their quality with a hemocytometer or alternative counting device and record the nuclei count.

22. Calculate the retention for each centrifugation condition by comparing the number of nuclei input into fixation and the number of nuclei recovered.

23. Assess the level of debris, aggregation, and nuclei damage in each resuspended nuclei pellet.

24. Pool the high quality resuspended pellets and discard any low quality ones.

Note: If the retention is below 40-60% after pooling, we recommend contacting our applications support team for additional recommendations.

25. Proceed to step 20 in Section 2.

Appendix B: High Input Workflow

If desired, 1-4 million nuclei can be fixed in a single reaction. However, this requires the reagent volume to be scaled up 4x, which reduces the total number of reactions that can be fixed with a 12 reaction kit to 3 reactions. The figure below outlines the protocol for the high input fixation workflow.

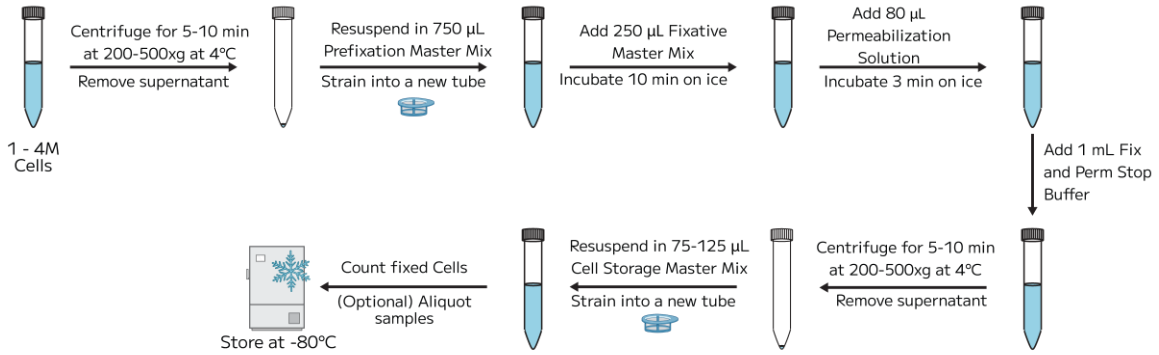


Figure 5: High Input Evercode Nuclei Fixation v4 Workflow

Appendix B1: Block Tubes with BSA

Although not required, blocking tubes with BSA can increase cell retention.

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	2	3
Nuclease-free water (not supplied)	26 mL	52 mL	78 mL
Gibco Bovine Albumin Fraction V (7.5% solution) (not supplied)	4 mL	8 mL	12 mL
Total Volume	30 mL	60 mL	90 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 Appendix B2 or store BSA-coated tubes at 4°C for up to 4 weeks.

Appendix B2: Prepare Master Mixes

Master mixes should be prepared just prior to fixation.

To prepare master mixes:

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

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
○ Prefixation Buffer	Nuclei 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	Nuclei Fixation Reagents (-20°C)	2 mL tube	
● Fixative Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	
● Permeabilization Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	
○ Fix and Perm Stop Buffer	Nuclei Fixation Reagents (-20°C)	8 mL bottle	
● DMSO	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
● RNase Inhibitor	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Nuclei Prefixation Enhancer (4°C)	1.5 mL tube	

2. Prepare the Nuclei Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

NUCLEI PREFIXATION MASTER MIX		
Number of Samples	1	3
○ Prefixation Buffer	740 µL	2.22 mL
● RNase Inhibitor	10 µL	30 µL
● Prefixation Enhancer	50 µL	150 µL
Total Volume	800 µL	2.4 mL

Note: Reagents in the Nuclei Fixation Reagents box can be frozen and thawed 3 times. If the kit will be used more than 4 times, aliquots should be made. See Reagent Stability in Important Guidelines for details.

3. Prepare the Nuclei Storage Master Mix in a new tube as follows. Mix thoroughly by pipetting and store on ice.

NUCLEI STORAGE MASTER MIX		
Number of Samples	1	3
○ Storage Buffer	300 µL	900 µL
● RNase Inhibitor	4 µL	12 µL
● DMSO	16 µL	48 µL
Total Volume	320 µL	960 µL

4. Proceed immediately to Appendix B3.

Appendix B3: Nuclei Fixation

After the initial centrifugation to remove the buffer/medium from the single nuclei suspension, nuclei are transferred to Nuclei Prefixation Master Mix. Reagents are added to fix and permeabilize nuclei, and then stop these reactions. Nuclei are resuspended in Nuclei Storage Master Mix and stored 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 nuclei counting device.
4. Place a Mr. Frosty Freezing Container at room temperature.
5. 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.
6. Transfer 1-4 million nuclei from each sample into a 15 mL polypropylene centrifuge tube (or BSA-coated polypropylene centrifuge tube if prepared in Section 1.1).
7. Centrifuge the tubes in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

   CRITICAL! Use of a fixed-angle rotor in this protocol will lead to substantial nuclei loss.

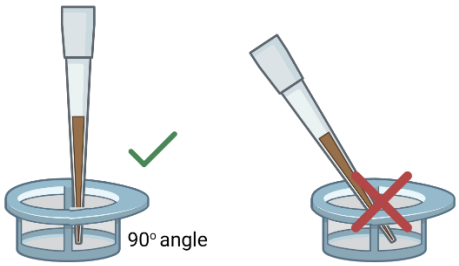
   CRITICAL! Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See Appendix A for details.

   CRITICAL! Move quickly and handle the samples gently to avoid dislodging the pellet, which will impact data quality.

8. Slowly aspirate then discard the supernatant. Less than 20 µL should be remaining in the tubes.
9. Fully resuspend each pellet in **750 µL** of Nuclei Prefixation Master Mix.
10. Pipette **750 µL** of each sample through a cell strainer into a new 15 mL polypropylene centrifuge tube (or BSA-coated polypropylene centrifuge tube) with a P1000 and store on ice.

CRITICAL! Do not directly touch the mesh of cell strainer(s) with gloved hands.

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.



11. Add **250 µL** of ● Fixative Solution to each tube. Immediately mix by pipetting exactly 3x with a P1000 set to 250 µL.

CRITICAL! Do not perform additional mixing at this step.

12. Incubate on ice for **10 minutes**.
13. Add **80 µL** of ● Permeabilization Solution to each tube. Immediately mix thoroughly by pipetting 3x with a P1000 set to 250 µL.
14. Incubate on ice for **3 minutes**.
15. Mix the ○ Fix and Perm Stop Buffer by inverting the tube 5x. Do not vortex.
16. Add **1 mL** of ○ Fix and Perm Stop Buffer to each tube. Gently pipette 3x with a P1000 set to 1000 µL.
17. Centrifuge in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.
18. Remove and discard the supernatant. Less than 20 µL should be remaining in the tubes.
19. Fully resuspend each pellet in an additional **75-125 µL** Nuclei Storage Master Mix and store on ice.
20. Pipette each sample through a cell strainer into a new, non BSA-coated 1.5 mL tube with a P1000 and store on ice.

21. While minimizing time on ice, count the number of nuclei in the sample with a hemocytometer or alternative counting device and record the nuclei count.

Note: We recommend preparing 20 µL Counting Aliquot to streamline downstream Evercode processing. Mix the sample 5x before aliquoting. See Important Guidelines for details.

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

23. 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.

Appendix C: Nuclei Number and Concentration Reference Tables

Appendix C1: Nuclei Concentrations for Evercode WT v4 Kits

The final concentration of nuclei post-fixation is influenced by the resuspension volume used in Step 19 of Section 2. These factors should all be considered when determining how much sample input is needed for fixation.

When using the Evercode WT v4 kits, use the table below to target the post-fixation nuclei concentration.

NUCLEI CONCENTRATIONS FOR EVERCODE WT V4 KITS		
Kit	Target Post-Fixation Concentration	Minimum Post-Thaw Concentration to Fully Load Kit
Evercode WT Mini	>500 nuclei/µL	238 nuclei/µL
Evercode WT	>1,000 nuclei/µL	373 nuclei/µL
Evercode WT Mega	>3,000 nuclei/µL	1,860 nuclei/µL
Evercode Mega 384	>1,000 nuclei/µL	456 nuclei/µL
Evercode WT Penta	4,500-5,000 nuclei/µL	3,720 nuclei/µL
Evercode WT Penta 384	4,200-5,000 nuclei/µL	2,325 nuclei/µL

Note: We do not recommend storing fixed samples at a concentration higher than 5,000 nuclei/µL after fixation. Higher concentrations could lead to clumping in certain sample types, such as HEK and 3T3 cell lines.

Appendix C2: Nuclei Concentrations for Evercode MAX WT v4 Kits

When using the Evercode MAX WT v4 Barcoding kits, the table below shows the range number of nuclei needed for each barcoding kit, and the range of post-fixation nuclei concentration.

Users should confirm both their target nuclei number and their sublibrary format before adding the Nuclei Storage Master Mix in Section 2.1.19, to ensure the fixed nuclei concentration meets both requirements.

We do not recommend preparing less than two sublibraries (2 SL).

LOADING CONCENTRATION GUIDANCE							
KIT	**METRIC**	**2 SL**	**4 SL**	**8 SL**	**12 SL**	**16 SL**	**32 SL**
MAX WT S	**MIN CONC.**	381	762	952	X	X	X
	TARGET CONC.	500	1,000	2,000	X	X	X
	MAX NUCLEI	16,000	32,000	64,000	X	X	X
MAX WT M	**MIN CONC.**	238	476	952	1,429	1,905	X
	TARGET CONC.	500	1,000	2,000	2,000	3,000	X
	MAX NUCLEI	64,000	128,000	256,000	384,000	512,000	X
MAX WT L	**MIN CONC.**	238	476	952	1429	1,905	3910
	TARGET CONC.	500	1,000	2,000	2,000	3,000	5,000
	MAX NUCLEI	128,000	256,000	512,000	768,000	1,024,000	2,048,000
MAX WT L-384	**MIN CONC.**	X	X	238	357	476	952
	TARGET CONC.	X	X	500	500	1,000	1,500
	MAX NUCLEI	X	X	512,000	768,000	1,024,000	2,048,000

Appendix D: Revision History

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

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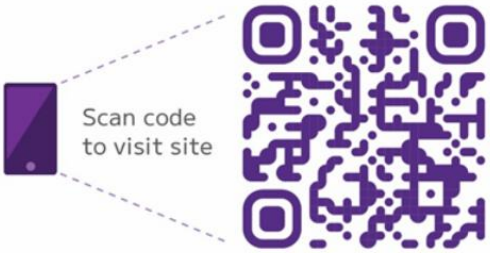

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