

Prepare Cell Suspension

Use this protocol to prepare single-cell suspensions from mammalian fresh or cryopreserved cells. Adjustments to centrifuge speed and times might be required for other cell types. If you are using your own cell preparation protocol, the final cell dilution step must be performed with the Cell Suspension Buffer provided in the kit.

If you are preparing single cell suspensions from tissues or fragile cell types, additional dissociation or cell handling steps might be required. These steps are not provided in the following protocol.

Consumables

- Cell Suspension Buffer
- **[Cryopreserved cells]** Thawing media
- **[Fresh cells]** 5 ml conical tube
- **[Cryopreserved cells]** 15 ml conical tube
- 1000 µl wide bore, low retention, pipette tips
- Cell counting materials
- **[Optional]** Cell strainer

Preparation

- Thawing media—Warm to 37°C in a water bath.

Procedure

Thaw Cryopreserved Cells

If you are starting from fresh cells, proceed to [Prepare Cell Suspension on page 19](#).

1. Place the cryopreserved cell vial and an aliquot of Cell Suspension Buffer in a water bath set to 37°C and let stand in the water bath until a small amount of ice remains (~1 minute).
2. Remove the vial from the water bath, decontaminate the outside with 70% isopropyl alcohol, and then move it into the biosafety cabinet.

The remaining ice thaws over the next 30–60 seconds at room temperature.

3. Use a wide-bore 1000 µl pipette to slowly pipette to mix, and then transfer the cell suspension from the cell vial to the conical tube.
4. Add thawing media to the cell suspension as follows.
 - a. Slowly add 2 ml thawing media (~30 seconds).
 - b. Add the remaining 7 ml thawing media.
 - c. Invert five times to mix.

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1. Centrifuge cells at 200 × g for 5 minutes at room temperature to pellet.
Use a swinging bucket rotor for pelleting to minimize cell shearing and loss. Optimize minimum speed and time required for pelleting your specific cell type. For example, you can use up to 350 g × 5 minutes for smaller cell types.
2. Without disturbing the pellet, remove and discard the supernatant.
If it is not possible to leave less than 100 µl supernatant remaining, increase the wash volume using the following table as guidance.

 The wash step removes reagents inhibitory to the assay from the cell suspension (eg, FBS, BSA > 1%, high salt or calcium, carryover organic solvents). Failure to remove inhibitory reagents can cause cDNA QC failure.

3. Add the appropriate volume of Cell Suspension Buffer, and then place the remaining Cell Suspension Buffer on ice.

Remaining Supernatant Volume (µl)	Cell Suspension Buffer Volume (ml)
< 100 µl	1
100–200 µl	2

4. Using a wide-bore 1000 µl pipette tip, slowly pipette 5 times to mix.
5. Centrifuge at 200 × g for 3 minutes at room temperature to pellet.
Optimize minimum speed and time required for pelleting your specific cell type. For example, you can use up to 350 g × 5 minutes for smaller cell types.
6. Without disturbing the pellet, remove and discard the supernatant.
7. Using a standard-bore pipette tip, add 200–400 µl chilled Cell Suspension Buffer, and then gently pipette 10–15 to mix.
If clumps are visible, increase the amount of force used during pipette mixing. Use the minimum force and number of pipette mixes necessary to create a homogeneous cell suspension.
8. **[Optional]** To remove debris and dead cells, pass the cell suspension through a strainer as follows.

 Filtering can cause cell stress and up to 30% cell loss. It is recommended only for cell types prone to forming aggregates.

- a. Use a wide-bore pipette tip to withdraw 200 µl cell suspension.
 - b. Slowly dispense the cell suspension through the cell strainer into a fresh 1.5 ml tube.
Use minimal force to push the cell suspension through the strainer to avoid unwanted debris in the cell suspension.
 - c. Repeat [a](#) and [b](#) until all cell suspension has been passed through the cell strainer.
9. Use a cell counter to determine cell concentration.
Cells should have > 90% viability to proceed. If necessary, perform additional wash steps to remove debris and improve viability. Always perform a final count.
 10. Using a wide-bore low retention pipette tip, prepare 3,400 live cells/µl suspension in Cell Suspension Buffer to achieve an input volume of 5 µl (17,000 live cells).
If it is not possible to concentrate cells to 3,400 cells/µl, a larger volume can be input as long as the total loading volume including 40 units of RNase inhibitor is ≤ 6 µl.
 11. Repeat the cell count described in step [9](#) to confirm the final concentration in the prepared suspension. Adjust concentration and repeat count as necessary.
 12. After the required concentration is achieved, place the cells on ice and proceed immediately to [Capture and Lysis on page 22](#).

Prepare Nuclei Suspension

Illumina recommends that you prepare nuclei suspensions using the Illumina Single Cell Nuclei Isolation Kit to ensure consistent, high-quality nuclei across frozen mammalian tissues. Refer to the [Illumina Single Cell Nuclei Isolation Kit Product Documentation](#) for instructions.

If using an alternative nuclei isolation protocol, use the provided NSB to prepare NSB working solution (refer to [Prepare NSB Working Solution on page 21](#)) for the final nuclei suspension. Substitute NSB working solution for the wash steps in the alternative nuclei isolation protocol (at least 1 ml of NSB working solution in up to two wash steps). This kit only includes enough BSA for the final nuclei suspension, so make sure to add user-supplied BSA (1% final concentration) and RNase inhibitor (0.8 U/µl final concentration) if needed for the wash steps.

Alternative Nuclei Isolation Protocols

- When using alternative nuclei isolation protocols, it is necessary to include 0.8 U/µl RNase inhibitor final concentration and 1X protease inhibitor into the nuclei extraction buffer/nuclei lysis buffer during tissue lysis.
 - After nuclei extraction is done, centrifuge nuclei suspension (500 × g for 5 minutes at 4°C) to pellet and aspirate supernatant. Use a 200 µl pipette to carefully remove as much supernatant as possible without disturbing the pellet.
-  Using a fixed angle centrifuge for pelleting nuclei for wash steps can result in excessive sample loss. Use tubes compatible with swinging bucket rotor centrifuges to ensure a flat pellet.

- Add 1 ml of NSB working solution, mix well and spin at 500 × g for 5 minutes at 4°C.
- Aspirate supernatant without disturbing the pellet and resuspend nuclei using NSB working solution.
- If unable to leave less than 100 µl supernatant remaining, increase the wash volume to 2 ml.

 The wash step removes reagents inhibitory to the assay from the nuclei suspension (eg, FBS, BSA > 1%, high salt or calcium, carryover organic solvents). Failure to remove inhibitory reagents can cause cDNA QC failure.

Refer to [Prepare NSB Working Solution on page 21](#) for instruction on preparing Nuclei Suspension Buffer for various applications. Refer to the Illumina Single Cell Nuclei Isolation Kit product documentation for recommendations on the resuspension volume per mg tissue for various tissue types. It is not recommended to process more than 50 mg of tissue per sample at once to achieve optimal lysis.

After preparing the nuclei suspension, place samples on ice and proceed immediately to [Capture and Lysis on page 22](#). Do not freeze isolated nuclei. If not proceeding directly to capture and lysis, it is recommended to fix nuclei to prevent mRNA degradation. Refer to [Prepare Nuclei Suspension on page 20](#) for details.

Prepare NSB Working Solution

To prepare 1 ml of NSB working solution, combine the volumes from the following table. Prepare sufficient volume for the final resuspension with NSB working solution per sample. Exact volumes required might be sample size and type specific to achieve optimal nuclei concentration.

Refer to [DSP-Methanol Fixation for Nuclei Protocol Instructions](#) for the volume to use for the final suspension before fixation.

Component Name	NSB Working Solution Volumes for Fresh Nuclei (µl)	NSB Working Solution Volumes for Fixed Nuclei (µl)
Nuclei Suspension Buffer	167	167
Nuclease-Free Water	773	833
25% BSA	40	Not applicable
RNase Inhibitor (40 U/µl)	20*	Not applicable
Total	1000	1000

* Alternatively, you can use 40 µl RNase inhibitor if provided at 20 U/µl and 753 µl nuclease-free water. Refer to RNase inhibitor recommendations in [User-Supplied Consumables & Equipment on page 5](#).