FLEX – Sample Fixation (REV3.2026)

GENERAL NOTES

IMPORTANT: This protocol is compatible with the "GEM-X Flex Sample Preparation v2 Kit" PN-1000781 **ONLY**

This protocol is designed for the fixation of **cell or nuclei suspensions**, whether sorted or unsorted, enabling subsequent processing with the probe-based FLEX kits. This fixation protocol is based on user guides CG000782 and CG000787. This assay is suitable for human or mouse samples only.

The individual aliquoted reagents (🔴 🟢 🟡 ○) are sourced from the GEM-X Flex Sample Preparation v2 Kit (PN-1000781). Nuclease-free filtered 50% Glycerol (🔵) is prepared and provided by the core as well. Nuclease-free water and 'clean' 37% Formaldehyde (e.g. Sigma 252549) should be available in the host lab. If you have no access to 37% formaldehyde, please contact the Single Cell Core.

Use a chemical fume hood to prepare and work with 4% PFA. Work in an RNase-free environment: ensure a clean surface, use RNase-free pipettes, equipment, gloves, and filter tips. Before initiating fixation (or before quenching), verify that all tubes are correctly labeled using a non-erasable black marker and/or a clear printed label: **use a simple and short sample identifier,** avoid full sample descriptions on tubes to avoid mix-ups.

Recommended cell/nuclei number to fix: recommend min. **50K** to **100K** cells per fixation/freezing aliquot. Although FLEX is technically possible with lower cell numbers, inputs below ~25,000 cells lead to increased variability in the final cell recovery. To mitigate this risk, we recommend fixing above 50K cells or nuclei per aliquot and preparing a duplicate fixed aliquot when possible. Please review the addendum (page 3) for direct fixation of low-input samples obtained by FACS, where centrifugation may lead to cell loss. Instead of pelleting, sorted cells are directly fixed in the FACS collection tube by adjusting the volume and adding the correct fixative.

Day I

Prior to fixation, determine cell/nuclei count and viability.

1. Prepare the **4% PFA Fixation Buffer B** in the fume hood:

When making a master mix directly from kit PN1000781:

Buffer	Catalogue number	For 1 sample (500µl)	2 samples (+10%)	4 samples (+10%)	8 samples (+10%)
Conc. Fix & Perm Buffer B (🔴)	10X Genomics PN-2001301	50 µl	110 µl	220 µl	440 µl
Nuclease-free water	(e.g Ambion AM9937)	396 µl	871 µl	1742 µl	3485 µl
Formaldehyde 37%	(e.g. Sigma 252549)	54 µl	119 µl	238 µl	475 µl

2. Spin down your cells at 4°C at 400g - or nuclei at 500g - for 6 min.
3. Remove the supernatant. *Up to 30µl can be left when the cell/nuclei number is low, to ensure the pellet is not touched.*
4. Resuspend the cell/nuclei pellet in 500µl fixation buffer B, carefully pipette-mix 5x.
5. Incubate for 16-24h at 4°C in the dark.
   Ensure that samples within the same experiment are incubated for the same duration.

Day 2

1) Equilibrate the (swinging-bucket) centrifuge at **RT** and two heat blocks (**65°C** and **42°C**). Prepare the following buffers:

   a. Thaw the **Enhancer (** 🟢) for minimum 10 minutes at **65°C** *(100µl/sample needed).*
   Vortex, centrifuge briefly. Keep at 65°C, for a maximum of 1 hour*
   Never put enhancer solution on ice or at RT before use or the solution will precipitate!

   *If the Enhancer solution is intended for use throughout the day (intervals of hours), it is advisable to re-freeze the Enhancer solution at -20°C between uses and then re-heat at 65°C as needed.

   b. Retrieve **50% Glycerol solution** (🔵) from the freezer and put **at RT** *(~275µl/sample).*
   c. Retrieve **Additive C** (○) from the freezer and thaw **at RT** *(~500µl/sample).*

   d. Thaw and dilute the **Concentrated Quenching Buffer B** (🟡) and put on ice:

Buffer	Catalogue number	For 1 sample (1000µl)
Conc. Quenching Buffer B (🟡)	10X Genomics PN-2001300	125 µl
Nuclease-free water	(e.g Ambion AM9937)	875 µl

When making a master mix directly from kit PN1000781:

2 samples (+10%)	4 samples (+10%)	8 samples (+10%)
275 µl	550 µl	1100 µl
1925 µl	3850 µl	7700 µl

2) **Before** centrifugation, add **500µL** (RT) **Additive C** (○) to the cells and carefully pipette-mix 5x.

3) Spin down the fixed cells at **850g** for **10 min** at **RT** with a **swinging-bucket** centrifuge.

4) Carefully remove supernatant.

5) Resuspend first in **200µl (cold) diluted Quenching Buffer B and pipette-mix 5x.** Immediately take an **10µl** aliquot for cell counting (at step 10).

   Important! Post-fixation counts are required for efficient downstream processing.

6) Add the remaining **800µl (cold) diluted** Quenching Buffer B.

7) Transfer Enhancer solution from 65°C to 42°C and incubate Enhancer for minimum **2 min** (at 42°C) - but no longer than 10 minutes - before addition to the fixed cells.
   After 10 min at 42°C, components of the enhancer solution will start to precipitate and the tube needs to be reheated at 65°C

8) Add **100µl** (warm) **Enhancer** (🟢) and pipette-mix 5x.
   Ensure the Enhancer solution is not cooling down to RT.

9) Add **275µl of 50% Glycerol** solution (🔵) *

10) Gently flip eppendorf tube 5 times and store sample at -80°C.
   0.1 volume Enhancer was added to the fixed & quenched cells. 50% glycerol is added to a final concentration of 10%.
   End volume = 1375µl *Optional: samples with high cell numbers (>1.5M) can be split in 2 aliquots (2x690µl) before freezing*

11) Count the cells using the aliquot taken at step 5.

12) Contact the Single Cell Core for planning the scRNAseq experiment and provide us the completed **FLEX sample submission form.**

ADDENDUM – Direct Fixation After Sorting (Low Cell Numbers)

This alternative workflow is intended for cases where low or unpredictable numbers of sorted cells are collected and immediately fixed **without centrifugation**. Cells are sorted directly into a collection tube containing PBS + 0.04% BSA (or 1% BSA for nuclei), brought to the correct volume using 1x PBS, and fixation is initiated directly in the tube.

Always work in a chemical fume hood when handling PFA
Keep all buffers and cells/nuclei on ice at all times

1. Retrieve your FACS collection tubes: use RNase-free, low-binding 1.5 mL Eppendorf tubes, each pre-filled with **100 µL** of **1x PBS + 0.04% ultrapure BSA** for cells; or **1x PBS + 1% BSA** for nuclei (available from the Single Cell Core).

2. Sort the cells directly into the collection tube.

3. After sorting, adjust the sample to a total volume of 396 µL.

   Top up with 1x PBS until the total volume reaches 396 µL. You can do this either by visually comparing your sample to a reference tube containing 396 µL of liquid, or by measuring the current volume using a P1000 pipette.

4. In a chemical fume hood, prepare the fixative mix just before use:
   - 50 µL of Concentrated Fix & Perm Buffer B
   - 54 µL of 37% Formaldehyde

   Mix gently by pipetting 5x (do not vortex).

5. Add **104 µL** of the freshly prepared fixative mix to your 396 µL sample.

   ! The fixation components should always be combined into a premix before addition

6. Pipette-mix gently 5x to ensure homogenous fixation.

7. Incubate for 16–24 h at 4 °C in the dark.
   Ensure consistent incubation time across all samples in the same experiment.

8. Proceed with Day 2 of the standard FLEX v2 fixation protocol.

Summary:

Always work in a chemical fume hood when handling PFA - Keep all buffers and cells/nuclei on ice at all times

Default protocol: Centrifugation and buffer exchange

Pellet cells (400g) or nuclei (500g)
and remove supernatant

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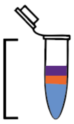
Resuspend cells in **500µl** Fixation buffer B (4% PFA)



Fixation Buffer B		
	50µl	Conc. Fix & Perm Buffer B
	54µl	37% Formaldehyde
	396µl	Nuclease-free water

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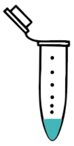
Incubate for 16–24 h at 4 °C in the dark
and proceed with Day 2 of the standard protocol

Direct fixation after sorting (centrifugation-free)



Sort cells into 150µl buffer
in 1.5mL eppendorf tube

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For cells: PBS + 0.04% BSA
For nucei: PBS + 1% BSA

After sort, adjust the total
volume to 396 µl
using 1x PBS

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104 µl → 50µl Conc. Fix & Perm Buffer B
54µl 37% Formaldehyde

396µl (cells in PBS)

- Prepare the mix of **50µl** Conc. Fix & Perm Buffer B with 54µl 37% Formaldehyde
- Pipette-mix 5x
- Add 104µl of the mixture to the cells
- Pipette-mix 5x

↓

Incubate for 16–24 h at 4 °C in the dark
and proceed with Day 2 of the standard protocol