ALITHEA GENOMICS

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Single-Cell FLASH-seq Service

Sample Preparation and Submission Guidelines

Single-Cell Protocol for
96- and 384-well Plate Format

July 2025

(Early-Access)

Single-Cell FLASH-seq for 96- and 384-well Plate Format

Sample Submission Guidelines at a glance

1. Prepare the samples as described and store them in the provided plates containing Cell Lysis Buffer (CLB) at -80°C prior to shipment.
2. Fill the Sample Submission Form **(SSF)** and **check all the boxes** in the Sample Submission Checklist below; send both files to **orders@alitheagenomics.com**. Please be aware that any inconsistencies may result in delays or additional fees.
3. Request the **shipping address** from your sales specialist.
4. Ship the samples on dry ice, ensuring the plates are placed between layers of dry ice to maintain a consistent freezing temperature throughout transit. Please provide us with the shipment tracking number.

Sample Submission Checklist

- ☐ The Sample Submission Form (SSF) must be completed correctly, including a unique sample ID. Consider adding a suffix for technical replicates (e.g., XX_rep1, XX_rep2, etc.). Ensure that the SSF provides information about all the shipped samples.
- ☐ The **minimum number** of samples to be processed at once is **48** (for a 96-well plate) and **192** (for a 384-well plate).
- ☐ Sample plates are labeled with the same Plate ID as in the Sample Submission Form.
- ☐ Plates are well sealed with the adhesive Aluminum or plastic seal.

Supplied reagents

Reagents	Label	Format		Storage
		96 samples	384 samples	
Tube with 2x Cell Lysis Buffer, 96 (PN 10609)	96 FS CLB 2x	1 tube	-	-20°C
Empty 96-well PCR plate	-	1 plate	-	-20°C/RT
Plate with Cell Lysis Buffer, 384 (PN 10617)	384 FS CLB	-	1 plate	-20°C
Adhesive Aluminium Seal (Aluseal)	-	2 pcs	3 pcs	-20°C/RT

1. Essential considerations for cell input

1.1 A cell suspension must be prepared before sorting, with a viability of greater than 70% and a low debris content. Otherwise, the quality of the obtained data will be low.

1.2 Obtaining such suspension requires some cell-type-specific optimization before the experiment. Contact your FACS expert to get the right cell concentration and buffer. If cells clump, add 0.04% bovine serum albumin (BSA) to the sorting buffer.

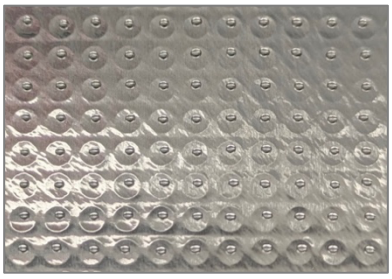
1.3 Please note that the lysis buffer does not inactivate pathogens.

1.4 The protocol is **not optimized for nuclei** sorting.

2. FACS sorting

General guidelines

- First, align the FACS flow so that the cells are dispensed in the middle of the wells. The alignment should be preferentially performed on multiple wells throughout the plate (at least A1-4 and P21-P24 positions for the 384-well plate) to ensure the cells are dispensed in the liquid.
- As a test, place an empty 96- or 384-well plate sealed with transparent plastic or aluminum foil on the FACS stage and dispense droplets (*see the image on the right*). Inspect the plate to ensure the droplets are as centered as possible throughout. This process can take several attempts, but it is crucial to ensure proper FACS alignment.



- We recommend performing a FACS alignment check every three sorted plates, as some instruments can lose calibration during the sorting process.
- We recommend cooling the sorting stage to +4°C whenever possible to prevent further evaporation and RNA degradation.
- When processing rare cells or during prolonged sorting sessions, we recommend sorting 25% or 50% of the plate instead of an entire one. Additional spare plates can be provided on demand.
- The following instruments have been tested and can be recommended for cell sorting: *FACSDiscover S8 Cell Sorter* (BD, ideal for all experiments); *FACSAria II* (BD, suitable for all experiments); and *F.SIGHT* (Cytena, with no fluorescence, suitable for low-debris preparations only).
- **Cell Sorting and Lysis Plate** format:
    - **96-well plate:** provided in a tube, as 2x Cell Lysis Buffer;
    - **384-well plate:** provided as a plate with 1.25 µL of Cell Lysis Buffer per well with an inert oil overlay to prevent evaporation.
- For troubleshooting, follow **Section 3**.

Preparation

- Upon reception, store the **Cell Sorting and Lysis Plate or Tube** at -20°C until the day of the sorting. Avoid repeating freeze-thaw cycles before sorting.
- Prepare the Aluminium seal and the buckets with wet and dry ice.

Procedure

2.1 Based on the plate format, proceed as follows:

- **96WP:** Thaw and vortex the **2x Lysis Buffer**, briefly spin down, dilute to 1x with cold nuclease-free water. Dispense 5 µL per well. Seal the plate, spin down, and keep on **wet** ice. The plate can be further stored at -20°C if needed.
- **384WP:** Remove the **Cell Sorting and Lysis Plate** from -20°C and thaw it on **wet** ice for 1 min.

NOTE: It is essential that the lysis buffer is completely thawed in all wells before starting the sorting. If the cells are deposited on the frozen lysis buffer, the RNA will degrade and become unusable.

2.2 Spin down the plate at 800x g for 30 sec. This step is obligatory.

2.3 Place the plate back on wet ice.

NOTE: The plate should not be kept at + 4°C for more than 30 min. If condensation droplets stick to the lid, use a higher centrifugation speed (1'000–2'000x g). However, in some instances, these microdroplets cannot be recovered. If this is the case, proceed to the next step. This won't affect the outcome.

2.4 Set up the gating strategy. We recommend always including a doublet discrimination step and removing dead cells with a fluorescent dye (e.g., Propidium Iodine).

2.5 Sort one cell per well. We recommend adding a negative control (no cell, e.g., in A01 well) and a positive control (10 cells, e.g., in P24 well) in each plate. Ideally, the sorting step should not take more than 10-20 min per plate.

CRITICAL: Quickly seal the plate with the provided Aluseal, applying sufficient pressure to the entire surface to ensure a secure seal. Centrifuge the plate at 800x g for 30 sec.

2.6 Seal the plate well with an Aluseal and immediately transfer it to a -80°C freezer for storage before shipment.

3. Troubleshooting during FACS sorting

Efficient FACS sorting requires careful optimization to ensure high cell viability and accurate sorting. The table below provides common issues, possible causes, and recommended solutions.

Step	Description	Potential outcome	Recommended solution
Cell dissociation	The cells are not properly dissociated leading to an excess of clumps and/or debris	Large clumps can block the FACS nozzles. Debris can be mistaken for cells. The number of wells passing QC after RT-PCR is lower than expected.	Review the cell dissociation procedure to ensure uniform single cell distribution prior to FACS sorting. Always check the cell suspension under the microscope before FACS. Consider adding extra filtering steps (40 µM, gradient, etc) to remove excess
High number of dropouts	FACS alignment or sorting gating strategy was incorrect. Cells were not dispensed in the lysis buffer but on the walls of the well or only debris was sorted.	Heterogenous distribution of the cDNA yield in the plate. Some wells are <0.5 ng/µL while other display appropriate yields for the assayed cell type. FA profile shows a vague cDNA trace or no trace at all with often primer dimer/leftovers.	Review the FACS settings. Ensure that the FACS is well aligned. Proceed to the alignment on multiple wells (corners & center). Use an empty 384-well plate, sealed with a plastic foil. Dispense drops onto it at different positions and make sure they are well centered.
FACS sorting doublets	FACS or sorting gating strategy was incorrect. More than one cell was sorted per well.	cDNA yield is 1.5 to 2.5x higher than expected. Typically, only in a few wells per plate.	Exclude these wells after sequencing. True doublets are often characterized by a significantly higher number of genes/reads (1.5 to 2.5x) and/or display markers from different cell types. Review the cell dissociation procedure to ensure single-cell suspension.
Spin down plate	The plate with cells has not been spun down after FACS sorting.	The cells have not hit the lysis buffer and many / most will be counted as dropouts.	Make sure that the FACS sorting protocol is followed.

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