ALITHEA GENOMICS



MERCURIUS™

Single-Cell FLASH-seq Library Preparation Kit for 96 and 384 Samples

PN 10921, 10923

User Guide

November 2026

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Related Products

Kit name	Kit PN	Modules	Module PN
Mercurius™ Single-Cell FLASH-seq Library Preparation 96 Kit	10921	Cell Sorting and Lysis Module 96 samples	10609
		Flash UDIs Plate Module 96 samples set 1	10096
		Flash UDIs Plate Module 96 samples set 2	10097
		Flash UDIs Plate Module 96 samples set 3	10098
		Flash UDIs Plate Module 96 samples set 4	10099
		Single-Cell FLASH-seq Library Preparation Module 96 samples	10630
Mercurius™ Single-Cell FLASH-seq Library Preparation 384 Kit	10923	Cell Sorting and Lysis Module 384 samples	10617
		Flash UDIs Plate Module 384 samples	10384
		Single-Cell FLASH-seq Library Preparation Module 384 samples	10637

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Kit Components

Reagents supplied

Cell Sorting and Lysis Module

Component Name	Label	Amount provided per kit		Storage
		96 samples (PN 10921)	384 samples (PN 10923)	
Tube with 2x Cell Lysis Buffer, 96 (PN 10609)	96 FS CLB 2x	1 tube	-	-20°C
Plate with Cell Lysis Buffer, 384 (PN 10617)	384 FS CLB	-	1 plate	-20°C
Aluminium Seal	-	-	3 pcs	-20°C/RT

Indexed Adapters Plate Module

Component Name	Label	Amount provided per kit		Storage
		96 samples (PN 10921)	384 samples (PN 10923)	
Flash UDIs Plate Module 96 samples set 1 (PN 10096)	96 UDI set1	1 plate	-	-20°C
Flash UDIs Plate Module 96 samples set 2 (PN 10097)	96 UDI set2	This listing is for one plate from one of four available sets. Please contact us if you would like a specific set.	-	-20°C
Flash UDIs Plate Module 96 samples set 3 (PN 10098)	96 UDI set3		-	-20°C
Flash UDIs Plate Module 96 samples set 4 (PN 10099)	96 UDI set4		-	-20°C
Flash UDIs Plate Module 384 samples (PN 10384)	384 UDI	-	1 plate	-20°C

IMPORTANT INFORMATION: Four different UDI barcode sets are available for 96-well plates. The barcode set included in a kit is not fixed and may vary between kits. If you require a specific barcode set, please contact your Sales Representative.

Single-Cell FLASH-seq Library Preparation Module

Component Name	Label	Cap colour	Volume (µL)		Storage
			96 samples (PN 10630)	384 samples (PN 10637)	
RT Enzyme	FS RTE	magenta	28	28	-20°C
RT Buffer	FS RTB	magenta	2x 1100	2x 1100	-20°C
TSO	FS TSO	magenta	45	45	-20°C
RNase Inhibitor	FS INH	magenta	45	45	-20°C
Tagmentation Enzyme	FS TAE	red	10	10	-20°C
Tn5 Dilution Buffer	DIB	orange	450	450	-20°C
Tagmentation Buffer	FS TAB	red	425	850	-20°C
Inactivation Mix	INACT Mix	blue	1200	1200	-20°C
Library Amplification Buffer	FS LAB	green	900	1700	-20°C
Library Amplification Enzyme	FS LAE	green	25	45	-20°C

Additional recommended reagents and equipment (supplied by the user)

Plasticware	Manufacturer	Product number
Low-binding 96-well PCR plate	Eppendorf	0030129512
Low-binding 384-well PCR plate	Eppendorf	0030129547
Disposable Pipetting Reservoir 25mL polystyrene	Integra or equivalent	4382

Reagents	Manufacturer	Product number
SPRI Magnetic beads (one of the following)		
• cleanNGS, or	cleanNA	CNGS-0050
• Sera-Mag™ Carboxylate-Modified Magnetic Beads, or	Cytiva	65152105050350
• AMPure XP Reagent, or	Beckman Coulter	A63881
• SPRIselect	Beckman Coulter	B23319
Qubit™ dsDNA HS Assay Kit	Invitrogen	Q32851
High Sensitivity NGS Fragment Analysis Kit	Agilent	DNF-474
Ethanol, 200 proof	-	-
Nuclease-free water	Thermo Fisher	A57775
DPBS, Cell culture grade	Gibco	10010023

Equipment	Manufacturer	Product number
Liquid Handling robots (or equivalent instruments supporting 96- or 384-well heads):		
• VIAFLO 96/384, or	Integra	6031
• Firefly, or	SPTlabtech	3276-00006
• Tecan Fluent	Tecan	30187625
Nanodispencers (optional):		
• Dragonfly	SPTlabtech	ISPT-DRAGONFLY
• I.DOT	Dispendix	I.DOT.LT
Benchtop centrifuge for plates	-	-
Benchtop centrifuge for 1.5 mL tubes	-	-
Single and Multichannel pipettes	-	-
Fragment Analyser / Bioanalyzer / TapeStation	Agilent	M5310AA
Qubit™	Invitrogen	Q33238
Permagen 384 low elution magnet	Permagen	MSP384LE
96-well side pull bar magnet PCR separation plate	Permagen	MSP750

Protocol Overview and Timing

The MERCURIUS™ Single-cell FLASH-seq is a plate-based full-length single-cell RNA sequencing (scRNA-seq) protocol designed for high sensitivity and user-friendly application. Compared to other scRNA-seq protocols, FLASH-seq enables increased gene detection while reducing time and cost constraints.

The Single-cell FLASH-seq kits facilitate the preparation of Illumina-compatible sequencing libraries for up to 384 single-cell samples, providing a scalable solution for large-scale transcriptomic studies. The protocol requires fluorescence-activated cell sorting (FACS) for prior cell selection, which is critical for optimal performance in downstream RNA sequencing workflows. Ensuring high cell viability and proper sorting conditions directly impacts library quality, gene detection sensitivity, and overall sequencing efficiency.

With its optimized workflow and enhanced sensitivity, Single-cell FLASH-seq is an efficient and accessible choice for researchers conducting single-cell transcriptomic analysis.

The kits are provided in the following formats:

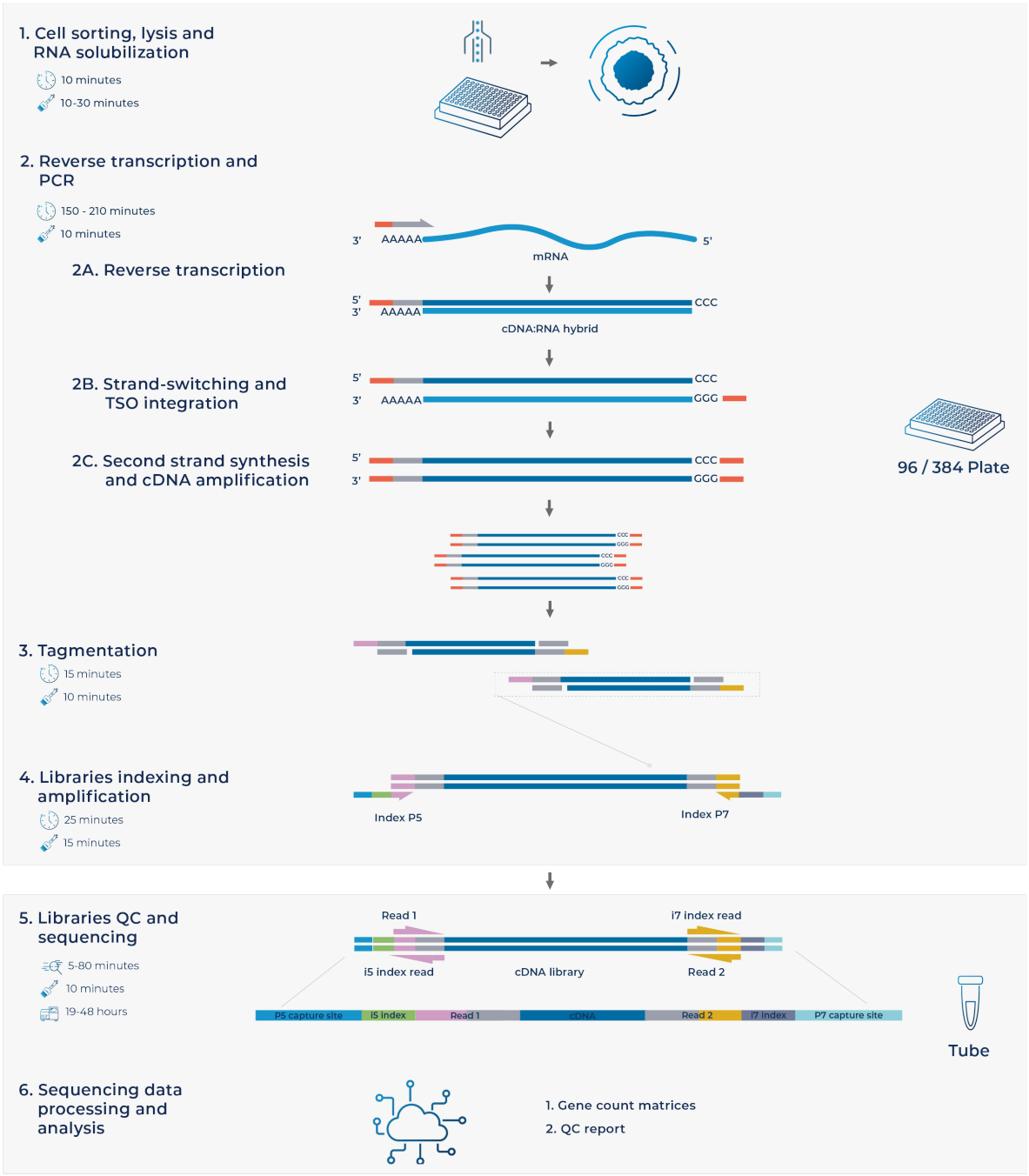
Kit format	PN	PCR plate format	Maximum number of samples processable
96-sample	10921	96WP	96
384-sample	10923	384WP	384

Each kit contains either 96 or 384 dual-indexed adapters, allowing for the efficient pooling of library samples from different experimental groups into a single tube. This simplifies sequencing library preparation while ensuring accurate sample identification and multiplexing.

The Single-cell FLASH-seq technology can generate high-quality sequencing data from a single cell. Notably, the kit can be used to proceed with any number of samples up to the capacity of the provided plate (96 or 384).

Figure 1 provides an estimation of the time required to accomplish each step of the protocol.

Protocol Workflow



Overall time

Incubation time: 3h20-4h20.
Hands-on time: 1h05-1h35.
QC time: 5min-1h20 (depending on the instrument used: Qubit or Fragment Analyzer).
Sequencing time: 19-48 hours (depending on instrument and sequencing run settings).

Figure 1 Schematic illustration of the protocol workflow

Part 1. CELLS PREPARATION AND SORTING

NOTE: Before starting each step, briefly spin down the tubes and plates to ensure that all liquid or particles are collected at the bottom of the tube or plate.

CRITICAL: This low-input protocol is sensitive to temperature and timing, and any deviations can potentially lead to RNA degradation before and during the reverse transcription (RT) step. **Therefore, it is imperative to adhere strictly to the protocol and ensure that incubation time and temperature limits are not exceeded**!

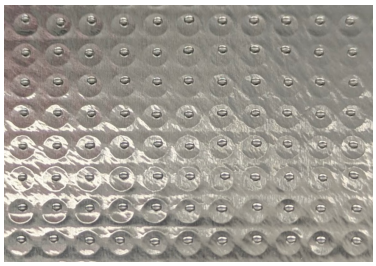
1.1. Essential considerations for cell input

- 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.
- 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.
- Please note that the Single-cell FLASH-seq lysis buffer does not inactivate pathogens.
- The kit protocol is **not optimized for nuclei** sorting.

CRITICAL: To maximise success rate, use hard-shell plates for FACS sorting such as Twin.tec® Low binding (Eppendorf) or Hard-Shell 384-Well PCR Plates (Bio-Rad) or Armadillo plates from Thermofisher.

1.2. FACS sorting

- 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 position 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** format:
  - **96WP:** provided in a tube, as 2x Cell Lysis Buffer;
  - **384WP:** 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 Appendix 1.

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

1.2.1. 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: The lysis buffer must be completely thawed in all the wells before starting the sorting. If the cells are deposited on the frozen lysis buffer, the RNA will degrade and become unusable.

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

1.2.3. 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.

1.2.4. 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).

1.2.5. 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. The sorting step should ideally 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 for 30 sec at 800x g.

1.2.6. Place the plate directly on **dry** ice or at -80°C before proceeding to the next step.

Safe stop: After this step, plates can be stored for 6 months at -80°C. Longer storage can lead to RNA degradation.

Part 2. LIBRARY PREPARATION PROTOCOL

2.1. Reverse transcription and PCR

At this step, each cell lysate sample is reverse-transcribed.

NOTE: All manipulations with cell lysates and RT enzyme should be performed in an RNase-free environment, using RNase-free consumables and filter tips, on ice, and with gloves.

Preparation

- Thaw all tubes on ice, mix well before use (pipette up and down, without vortexing), and then quickly spin them down.
- Prepare Program 1_DNT on the thermocycler (set the lid at 105°C):

Step	Temperature, °C	Time
Incubation	72	3 min
Keep	4	pause

- Prepare Program 2_RT-PCR on the thermocycler (set the lid at 105°C):

Step	Temperature, °C	Time	Cycles
Incubation	50	60 min	1
Initial denaturation	98	3 min	1
Denaturation	98	20 sec	18* or 21**
Annealing	63	20 sec	
Extension	72	6 min	
Final extension	72	3 min	1
Keep	4	pause	1

* 18 cycles are advised for the large and/or active cells (cell lines, i.e., HEK 293T, CHO)

** 21 cycles – for small and/or quiescent cells (i.e., PBMC)

Procedure

2.1.1. Prepare the RT Master Mix for the expected number of reactions (+10%) as follows:

Reagent	96WP, µL		384WP, µL	
	Per well	96 wells +10%	Per well	384 wells +10%
FS RTB	18.4	1943	4.6	1943
FS RTE	0.24	26	0.06	26
FS TSO	0.4	43	0.1	43
FS INH	0.4	43	0.1	43
Water	0.56	60	0.14	60
TOTAL	20.0	2115	5.0	2115

2.1.2. Slowly pipette up and down 10-15 times, then keep it on ice.

2.1.3. Take the plate containing the sorted cells from the -80°C freezer and keep it at room temperature (RT) for 1 min.

NOTE: If the seal has partially detached due to freezing at -80°C, apply firm, even pressure across the entire plate to reseal it before proceeding further. If necessary, a new seal can be used; however, it will adhere properly only if the plate has sufficiently thawed.

When frozen at -80°C the dye present in the lysis buffer can undergo a color shift. As a result, some wells may appear yellow or pink. This change does not affect the reaction.

2.1.4. Spin it down at 800x g for 10 seconds and place it on ice.

2.1.5. Transfer the plate to the thermocycler and start Program 1_DNT.

2.1.6. Immediately put a plate on ice for at least 3 min.

2.1.7. If evaporation or spilling traces are observed, spin down at 800x g for 10 sec.

2.1.8. Using a multichannel pipette, dispense the following volume of the RT Master Mix to each well containing the cell lysate sample:

- **96WP:** 20 µL per well
- **384WP:** 5 µL per well

2.1.9. From this point, the plate will be referred to as an RT-PCR plate.

2.1.10. Carefully re-seal the plate and briefly spin it in the centrifuge.

2.1.11. To homogenize the solution, vortex the plate for a few seconds and spin it down at 800x g, 30 sec.

2.1.12. Transfer the plate to the thermocycler and start Program 2_RT-PCR.

Safe stop: After this step, the RT plate can be kept at +4°C for a few days or at -20°C for at least 1 month.

2.2. Sample purification

The RT samples can be purified using SPRI magnetic beads (see Table 1 for suggested providers and ratios). We suggest using a 96-well side pull bar or a 384-low elution magnet (Permagen, MSP750 and MSP384LE, respectively). We highly recommend using an automation system for the bead clean-up. In case of <48 samples, the purification can be done manually.

Beads	Supplier	Serial number	Recommended ratio
cleanNGS	cleanNA	CNGS-0050	0.7x
Sera-Mag™ Carboxylate-Modified Magnetic Beads*	Cytiva	65152105050350	0.8x
AMPure XP Reagent	Beckman Coulter	A63881	0.75x
SPRIselect	Beckman Coulter	B23319	0.75x

* with homemade 18% PEG buffer, supplied without buffer.

Table 1 Overview of the recommended supplier of the SPRI beads to be used.

Preparation

- Before pipetting, pre-warm beads at RT (for at least 15 min) and vortex vigorously (30 sec).
- The procedure below describes volumes for the 0.7x beads ratio. If the range is different, adjust the volumes correspondingly.

Procedure

2.2.1. Thaw the RT-PCR plate at room temperature and spin it down.

2.2.2. Add the following volume of magnetic beads, pre-warmed at room temperature.

- **96WP:** 17.5 µL per well
- **384WP:** 4.4 µL per well

NOTE: In 384WP, the oil overlay does not influence the purification. Perform the cleanup assuming a 0.7x ratio based on the RT-PCR and lysis reaction volume.

2.2.3. Seal the plate and vortex the RT-PCR plate until the beads are fully homogenized. Avoid spilling liquid on the plastic seal.

2.2.4. Incubate the plate for 5 min at RT.

2.2.5. If necessary, centrifuge the plate at 300x g for 10 sec to collect all liquid.

2.2.6. Place the plate on the magnet and wait for the beads to settle. Due to the oil's additional viscosity, this step may take up to 10 min.

2.2.7. Remove as much supernatant as possible (~20 µL for 384WP and ~43µL for 96WP), without disturbing the beads. Leftovers will not interfere with the subsequent enzymatic reactions, but could impact QC measurements if kept at more than 2 µL.

2.2.8. **CRITICAL**: Do not let the beads dry.

2.2.9. **CRITICAL:** We **do not recommend** washing with ethanol, as it can lead to up to 10% material loss. If you still plan to do so, perform a single ethanol wash using freshly prepared 80% ethanol. After removing the ethanol, leave the beads to dry at RT for a maximum of 2 min.

2.2.10. Pipette water per well, preferentially directly on the beads, as follows:

- **96WP:** 15 µL per well
- **384WP:** 15 µL per well

2.2.11. Seal the plate. Vortex the plate until the beads are well resuspended.

2.2.12. Incubate for 5 min at RT.

2.2.13. Place the plate on the magnet and wait for the beads to settle (~2 min).

2.2.14. Carefully transfer the following volume of supernatant without touching the bead pellets to a new 96- or 384-well PCR plate:

- **96WP:** 14 µL per well
- **384WP:** 14 µL per well

NOTE: Oil leftovers may make the solution appear slightly murky or whitish. This will not interfere with subsequent reactions.

Safe stop: The cDNA can be safely kept at -20°C for a few weeks at this step.

2.3. cDNA quality control (QC)

Individual cDNA quality control

Before further processing, the cDNA should be subjected to quantification and fragment analysis. This information is required to validate the success of the RT reaction and estimate the number of drop-out cells. And most importantly, the samples should be further diluted to 100-200 pg/µL.

Assessing the cDNA yield across the samples

We recommend assessing the cDNA yield using Qubit (Thermofisher, Q33231) or Quant-it Picogreen (Thermofisher, P7589).

Using the current protocol, the cDNA yield is expected to range from **0.5 ng/µL to 5 ng/µL** and may vary depending on the cell type and chosen amplification rate. High-yield outliers can sometimes indicate doublets. The number of drop-out cells depends on sorting efficiency and should ideally be kept below 20%.

Depending on the availability of the plate fluorimeter, we suggest two different strategies to measure the cDNA yield:

- **Option A – All samples** (using the Plate Fluorimeter)

  Measure the cDNA yield from each well individually. This will ensure the cDNA dilution is accurate and uniform, leading to more uniform sequencing depths.

- **Option B – A few samples** (if the Plate Fluorimeter or a liquid handling robot/nanodispenser is not available)

  Measure the cDNA yield in a random fashion ~5% of the wells (but minimum 8).

Assessing the cDNA profile across the samples

When using Single-cell FLASH-seq on new cell types, we recommend measuring the cDNA length distribution of 3-4 samples after cDNA quantification on a 2100 Bioanalyser (Agilent), Fragment Analyser 5200 (M5310AA, Agilent) or TapeStation system 4150 (G2992AA, Agilent).

Typical Single-cell FLASH-seq cDNA ranges from 400 bp to >7 kb, with an average of 1,600 to 2,100 bp, depending on the cell type. See Figure 2 for an example of a standard cDNA profile obtained from 293 cells.

Primer dimers/leftovers (at ~47 bp) can sometimes be observed (see Figure 3). This peak can appear due to an incorrect cDNA-to-bead ratio during the purification step. If the problem persists, we suggest decreasing it (by 0.05x increments). If this is not possible, proceed further with tagmentation.

Figure 2 A successful cDNA profile with most of the fragments between 400-7000 bp

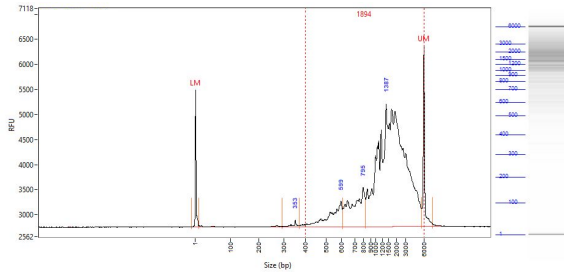
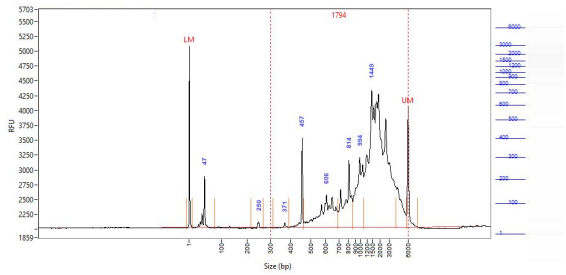


Figure 3 An example of a cDNA profile demonstrating the dimer peak at 47 bp



2.4. cDNA dilution in plates

Using the Tn5 transposase, single-cell RNA-sequencing libraries can be generated from 10 to 400 pg of cDNA (Picelli et al., 2014). Variations in the dilution will impact the uniformity of the sequencing depths. Therefore, using the most accurate quantities for the subsequent reactions is essential.

The Mercurius™ Single-cell FLASH-seq kit is optimized for cDNA input ranging from 150 to 400 pg (384WP) or 300-800 pg (96WP). Do not exceed these values. Low-binding plates are absolutely necessary for this step.

Preparation

- Prepare a new low-binding 96- or 384-well plate.

Procedure

2.4.1. Thaw the plate with the cDNA on ice and briefly spin it down.

2.4.2. Using 2 µL of the cDNA from each well, adjust the dilution based on the sample quantification method in step 2.3:

- **Option A (All samples)**: dilute all samples to 100-150 (**maximum**) pg/µL.
- **Option B** (**A few samples)**: use the average cDNA yield as the reference and dilute each sample to <200 pg/µL.

Option A ensures a more uniform sequencing depth with a standard deviation around +/- 1.5 to 2.5.

Option B is a faster and more straightforward approach; however, it can significantly increase the variation in sequencing depth. While these variations are typically low in cell lines, they can reach up to 10x differences in heterogeneous populations. These outliers can be removed later during analysis if necessary.

2.4.3. **CRITICAL:** Measure a few wells after dilution to ensure that the cDNA yields are in the expected range.

Safe stop: At this step, the cDNA can be safely stored at -20°C for up to a month.

2.5. Tagmentation

At this step, the full-length cDNA is tagmented using a Tn5 transposase pre-loaded with adapters for library amplification.

This step is a key reaction to ensure the correct fragment size distribution. The size of the fragment is determined by the amount of Tn5 enzyme and cDNA in the reaction. FLASH-seq tagmentation reaction should be undertaken with cDNA input of 150-400 pg (384WP) or 300-800 pg (96WP).

Preparation

- Thaw the **DIB**, **FS TAB,** and **INACT Mix** reagent at room temperature and mix well before use.
- Keep the **FS TAE** reagent constantly on ice.
- Set the PCR machine to a 55°C incubation (with the lid heated to >90°C).

Procedure

2.5.1. **CRITICAL:** For first-time use only! Dilute the **FS TAE** with **DIB** as follows:

- Pipette 10 µL of **FS TAE** into a separate 1.5 mL tube and keep it on ice.
- Add 190 µL of **DIB** to the tube with **FS TAE**.
- Set the pipette to 200 µL and carefully pipette the mix up and down (12-15 times)
- Diluted **FS TAE** should be kept at -20°C for up to 3 months after preparation.

2.5.2. Prepare the Tagmentation Master Mix on ice in a PCR tube as follows (with 10% excess):

Reagent	96WP, µL		384WP, µL	
	Per well	96 wells +10%	Per well	384 wells + 10%
FS TAB	3.6	396	1.8	774
FS TAE (diluted)	0.4	44	0.2	86
TOTAL	4.0	440	2.0	860

2.5.3. Keep the mix on ice and pipette up and down 10 times. Pay attention to thoroughly mixing the reaction volume.

2.5.4. Pipette the Tagmentation Master Mix into every well of the new plate, kept on ice:

- **96WP:** 4 µL per well
- **384WP:** 2 µL per well

2.5.5. Seal the plate with Aluminum foil and spin it down.

2.5.6. Transfer the diluted cDNA from step 2.4.3 to the plate with the Tagmentation Master Mix:

- **96WP:** 4 µL per well
- **384WP:** 2 µL per well

2.5.7. Pipette the plate up and down (2-5 times) or gently vortex it (seal it before use).

2.5.8. Seal the plate and spin it down.

2.5.9. Incubate for 8 min at 55°C in the PCR machine.

2.5.10. Proceed immediately to the following step.

Inactivation

The inactivation of the Tn5 transposase is required for the subsequent library amplification step.

NOTE: Use the Inactivation mix (INACT Mix), which has been pre-warmed at room temperature. The solution will typically be yellow. Briefly spin it before use.

2.5.11. Put a plate from step 2.5.10 on ice for 1 min.

2.5.12. Gently remove the seal from the plate (it can be kept for the next step).

2.5.13. Transfer the plate to room temperature and pipette the Inactivation mix into every well as follows:

- **96WP:** 4 µL per well
- **384WP:** 2 µL per well

2.5.14. Seal the plate with a new seal, briefly vortex the plate to homogenize the solution, and spin it down. A change of color from yellow to pink should be observed.

2.5.15. Incubate at room temperature for 3-5 min; afterward, the plate can be placed on ice.

2.5.16. Proceed immediately to step 2.6.

2.6. Library indexing and amplification

The Single-cell FLASH-seq protocol utilizes Unique Dual Indexing (UDI). At this step, the cDNA fragments are amplified using the Indexing Adapter primers, which are provided in a 96-well plate.

The i7 and i5 index sequences are indicated in Appendix 2.

NOTE: Four different UDI barcode sets are available for 96-well plates. The barcode set included in a kit is not fixed and may vary between kits. If you require a specific barcode set, please contact your Sales Representative.

The number of amplification cycles required for library preparation typically ranges from 8 to 12 (see below for details). The precise number may depend on the samples and the amount of input cDNA used for tagmentation.

Preparation

- Thaw the **LAB** reagent on ice and mix well before use.
- Keep the **LAE** reagent constantly on ice.
- Thaw the plate with **UDI Adapters** at room temperature and briefly spin before use.
  - Note the part number (PN) associated with the selected UDI set.

- Prepare the Program 4_AMP (set the lid at 105°C) on the thermocycler (*The exact number of PCR cycles should be determined following the library quantification protocol below)

Step	Temperature, °C	Time	Cycles
Incubation	72	3 min	1
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	8-12*
Annealing	55	30 sec	
Extension	72	30 sec	
Final extension	72	1 min	
Keep	4	pause	

* 8 cycles are advised for 384 samples;
10 cycles - for 96-200 samples;
12 cycles – for less than 100 pg of cDNA input or less than 24 samples

Procedure

2.6.1. Prepare the Amplification Master Mix as follows:

Reagent	96WP, µL		384WP, µL	
	Per well	96 wells +10%	Per well	384 wells + 10%
FS LAB	7.8	858	3.9	1677
FS LAE	0.2	22	0.1	43
TOTAL	8.0	880	4.0	1720

2.6.2. Pipette the mix well, spin it down briefly, and keep the tube on ice.

2.6.3. Remove the seal from the plates with **UDI Adapters** and tagmented cDNA (from step 2.5.14).

2.6.4. Pipette the UDI Adapters into the corresponding wells with tagmented cDNA. Ensure that the layouts of both plates match each other.

- **96WP:** 4 µL per well
- **384WP:** 2 µL per well

2.6.5. Add the prepared Amplification Master Mix to each well:

- **96WP:** 8 µL per well
- **384WP:** 4 µL per well

2.6.6. Seal the plate and briefly vortex it to homogenize the reaction.

2.6.7. Briefly spin it down.

2.6.8. Put the plate in the PCR machine, set the number of amplification cycles, and start Program 4_AMP.

Safe stop: The plate with libraries can be safely stored at +4°C overnight or at -20°C for up to 6 months.

2.7. Indexed libraries pooling and clean-up with SPRI beads

At this step, samples are pooled (see below the recommendations) and further purified using SPRI magnetic beads.

Perform library purification using SPRI magnetic beads (see Table 1) with a 1:0.7 library pool and beads slurry ratio (35 µL of bead slurry for 50 µL of cDNA library).

Preparation

- Pre-warm the SPRI beads at room temperature for ~30 min.
- Prepare 2 mL of 80% ethanol.

Procedure

2.7.1. Using automation or a multichannel pipette, pool an equal volume from every well from the plate (step 2.6.8) to the reservoir as follows:

- **96WP:** 4 µL per well
- **384WP:** 2 µL per well

2.7.2. Gently mix the pool and transfer it to a 1.7 mL tube.

NOTE: Use pre-warmed beads and vortex them vigorously before pipetting (min 30 sec).

2.7.3. Pipette the required volume of beads slurry into the pool to obtain a 0.7x bead-to-library ratio (for instance, for a full 384-well plate, pipette 537 µL of beads slurry into 768 µL of pooled libraries).

2.7.4. Close the tube and vortex vigorously to homogenize the solution with beads.

2.7.5. Incubate for 5 min at room temperature.

2.7.6. Place the tube on the magnetic stand, wait 5 min, then carefully remove and discard the supernatant.

2.7.7. To wash the beads, pipette 750 µL of freshly prepared 80% ethanol into the tube.

2.7.8. Incubate for 30 sec.

2.7.9. Carefully remove the ethanol without touching the bead pellet.

2.7.10. Repeat step 2.7.6 for a total of two washes.

2.7.11. Remove the tube from the magnetic stand and let the beads dry for 1-2 min (do not overdry!).

2.7.12. Resuspend the beads in 52 µL of water or vortex the tube.

2.7.13. Incubate for 5 min at room temperature.

2.7.14. Place the tube on the magnetic stand, wait 5 min, and carefully transfer 50 µL of the supernatant into a new low-binding tube to avoid bead carry-over.

2.7.15. Proceed to the Library quality control (step 2.8). If the library shows the presence of the primer dimers, perform a second purification (steps 2.7.3 - 2.7.15).

Safe stop: At this stage, the libraries can be safely stored at -20°C for several months.

2.8. Pooled libraries quality control

Before sequencing, the libraries should be subjected to fragment analysis (with a Fragment analyzer, Bioanalyzer, or TapeStation) and quantification (with Qubit). This information is required to assess the libraries' molarity and prepare the appropriate library dilution for sequencing.

A successful library contains fragments between 300 and 700 bp, with a peak at 300 to 500 bp; see Figure 4 for an example of a standard Single-cell FLASH-seq library profile. Occasionally, a sharp peak around ~750 bp may appear after library purification (Figure 5). Such a library can be sequenced, and this peak does not compromise data quality. In these cases, we recommend measuring the smear size between 100 and 700 bp.

Overtagmented libraries have shifted to the lower size profile and peak at 100-200 bp (Figure 6). Only a fraction of such libraries contains fragments that can be efficiently sequenced; therefore, it is recommended to re-prepare the library from the cDNA for the best results. Make sure that the diluted cDNA concentration is between 100 and 200 pg/µL. Decrease the cDNA input in the library preparation if needed.

Undertagmented libraries have a broader fragment range distribution with a peak at >700 bp (Figure 7). Only a fraction of such libraries contains fragments that can be efficiently sequenced; therefore, it is recommended to re-tagment the cDNA for the best results.

Importantly, libraries with primer dimer peaks at 150 bp will likely produce lower-quality sequencing data with a reduced proportion of demultiplexed reads (Figure 8). Therefore, it is strongly recommended to remove those peaks by performing an additional round of SPRI bead purification with the 0.7x ratio (see steps 2.7.3 - 2.7.14).

Pre-sequencing library QC:

- Use 2 µL of the library pool to measure the concentration with Qubit (typically, the 384 cells will result in 0.5 - 20 ng/µL library concentration (depending on the type of cells);
- Use 2 µL of the library to assess the profile using the Fragment Analyzer or a similar instrument.
- If necessary, re-purify the libraries by following the steps (2.7.3 - 2.7.14) to remove the peaks <50 bp.

Figure 4 A successful library profile with fragments between 300-1000 bp

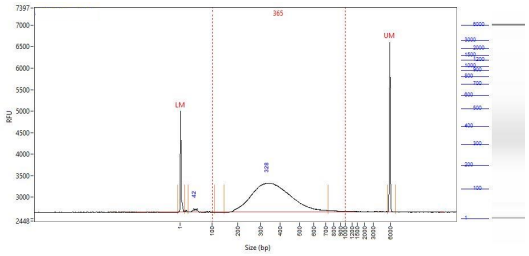


Figure 5 A library profile with a peak ~750 bases bp

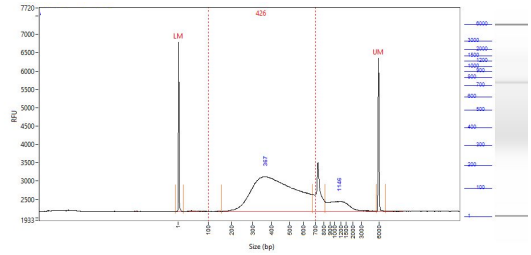


Figure 6 An example of an over-tagmented library profile with a peak at 187 bp and an adapter peak at 42 bp

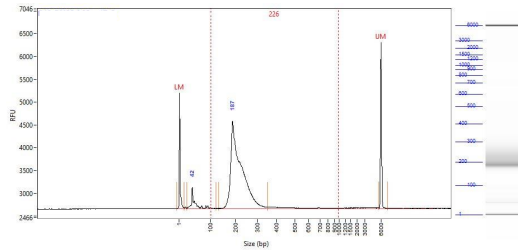


Figure 7 An example of an under-tagmented library profile with a major peak at 1160 bp

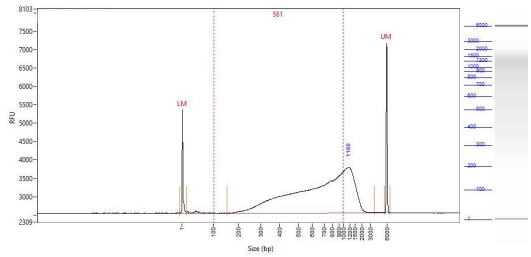
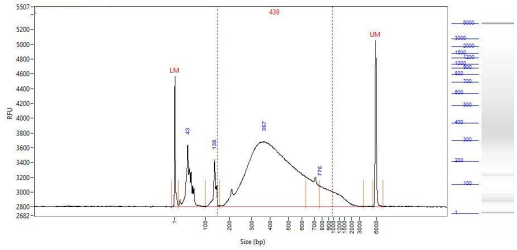


Figure 8 An example of a library with primer dimers and leftovers at <150 bp



Part 3. LIBRARY SEQUENCING

The libraries prepared with the MERCURIUS™ Single-cell FLASH-seq kit carry Illumina- and AVITI-compatible adapter sequences. They can be processed on any Illumina instrument (e.g., HiSeq, NextSeq, MiSeq, iSeq, and NovaSeq) or in the Element AVITI System with Adept Workflow.

The MERCURIUS™ Single-cell FLASH-seq libraries are indexed with unique dual-indexes and can potentially be pooled in a sequencing run with other libraries if the sequencing structure is compatible. Please refer to Table 2 for the optimal sequencing structure and Appendix 2 for the i5 and i7 index sequences list.

We recommend sequencing FLASH-seq libraries with at least 75 bp single-end (SE) reads for gene expression and 75 bp paired-end (PE) reads for detecting isoforms.

Read	Length (cycles)	Comment
Read 1	>50	Gene fragment
Index 1 (i7) read	8 or 10	Library Index
Index 2 (i5) read	8 or 10	Library Index
Read 2 (facultative)	>50	Gene fragment

Table 2 Sequencing structure of FLASH-seq libraries

NOTE: Sequencing depth

1. The recommended sequencing depth is 0.25 Mio reads per sample. Deeper sequencing (1 Mio reads/sample) can also be performed to detect very lowly expressed genes or isoforms and to reach sequencing saturation with most cell types.

2. The library's loading molarity depends on the type of sequencing instrument (see 3.1 and 3.2) and should be discussed with the sequencing facility or an experienced person.

3.1. Sequencing on the Illumina instruments

Table 3 indicates the loading concentration for the Illumina instruments. For the list of compatible Illumina instruments with forward or reverse workflow, please refer to Appendix 2.

Instrument	Final loading concentration	PhiX
MiSeq	20 pM	1 %
iSeq	100 pM	1 %
NextSeq 500/550/550Dx	2.2 pM	1 %
NextSeq 2000, manual denature	85 pM	1 %
NextSeq 2000, onboard denature	850 pM	1 %
NovaSeq Standard Workflow*	160 pM	1 %
NovaSeq XP Workflow	100 pM	1 %
HiSeq4000	270 pM	1 %

* - adjusted molarity for FLASH-seq libraries sequencing. We recommend a prior dilution of the libraries to 0.8 nM before denaturation.

Table 3 Reference loading concentrations for various Illumina instruments

3.2. Sequencing on the Element AVITI instrument

For the most optimal results, the MERCURIUS™ Single-cell FLASH-seq libraries can be sequenced with the Element Biosciences AVITI System using Cloudbreak AVITI 2x75 High Output sequencing kits (#860-00004).

Libraries must be converted with the Adept PCR-Plus module (#830-00018) for linear loading (Table 4).

Type	Loading molarity, pM	Library starting amount for denaturation, nM	PhiX control	PhiX, %
Cloudbreak Adept	14	1*	PhiX Control Library, Adept	2 %

* - requires 4nM of library before conversion

Table 4 Loading concentration for Cloudbreak AVITI 2x75 High Output sequencing kit

NOTE: Please note that the **Cloudbreak AVITI 2x75 High Output sequencing** yields 1'000 Mio reads. Therefore, for the 384-sample library, the sequence depths would be limited to a maximum of 2.5 Mio reads/sample.

Part 4. SEQUENCING DATA PROCESSING

Following Illumina sequencing and standard library index demultiplexing, the user obtains either raw read1 alone (= single-end) and both read1 and read2 *fastq* sequencing files (paired-end, e.g., *mylibrary_R1.fastq.gz* and *mylibrary_R2.fastq.gz*).

This section explains how to generate ready-for-analysis gene, and read count matrices from raw *fastq* files.

To obtain the data ready for analysis, the user needs to align the sequencing reads to the genome and generate counts, which can be done in parallel with sample demultiplexing.

4.1. Recommended software

- **fastQC** (version v0.11.9 or greater). Software for QC of *fastq* or *bam* files. This software is used to assess the quality of the sequencing reads, such as the number of duplicates, adapter contamination, repetitive sequence contamination, and GC content. The software is freely available from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. The website also contains informative examples of good and poor-quality data.
- **STAR** (version 2.7.9a)**.** Software for read alignment on reference genome (Dobin et al., 2013). It can be downloaded from Github (https://github.com/alexdobin/STAR). STAR can only be run on UNIX systems and requires:
- **FeatureCounts** (v.1.6.5 or greater). Software for counting genome-aligned reads for genomic features. **https://subread.sourceforge.net/**
- **Samtools** (v.1.9 or greater). Collections of command-line utilities to manipulate with BAM files. R Software (version 3 or greater).
- **tidyverse** (v 2.2.0 or greater), **R** (v 4.0.0 or greater) library
- **RSeQC** (v 4.0.0 or greater)

4.2. Data processing

4.2.1. Merging fastq files from individual lanes and/or libraries (Optional)

Depending on the type of instrument used for sequencing, one or multiple R1/R2 *fastq* files per library may result from individual lanes of a flow cell. The *fastq* files from individual lanes should be merged into single *R1.fastq* and single *R2.fastq* files to simplify the following steps. This is an example of *fastq* files obtained from HiSeq 4 lane sequencing:

```
>  mylibrary_L001_R1.fastq.gz, mylibrary_L002_R1.fastq.gz,
   mylibrary_L003_R1.fastq.gz, mylibrary_L004_R1.fastq.gz
>  mylibrary_L001_R2.fastq.gz, mylibrary_L002_R2.fastq.gz,
   mylibrary_L003_R2.fastq.gz, mylibrary_L004_R2.fastq.gz
```

To merge the *fastq* files from different lanes use a `cat` command in a terminal. This will generate two files: *mylibrary_R1.fastq.gz* and *mylibrary_R2.fastq.gz*, containing the information of the entire library.

```
>  cat mylibrary_L001_R1.fastq.gz mylibrary_L002_R1.fastq.gz
   mylibrary_L003_R1.fastq.gz mylibrary_L004_R1.fastq.gz >
   mylibrary_R1.fastq.gz
>  cat mylibrary_L001_R2.fastq.gz mylibrary_L002_R2.fastq.gz
   mylibrary_L003_R2.fastq.gz mylibrary_L004_R2.fastq.gz >
   mylibrary_R2.fastq.gz
```

Move these 2 *fastq* files into a new folder, which will be referenced in this manual as $fastqfolder.

NOTE: This step can also be done if you sequenced your library in multiple sequencing runs.

Warning: The order of merging files should be kept the same (for e.g., L001, L002, L003, L004, not L002, L001 ...) to avoid issues when demultiplexing the samples.

4.2.2. Sequencing data quality check

Run fastQC on either R1 and R2 fastq files. Use –outdir option to indicate the path to the output directory. This directory will contain HTML reports produced by the software.

```
> fastqc --outdir $QCdir/ mylibrary_R1.fastq.gz
```

Check fastQC reports to assess the quality of the samples (see Software and materials). Typical quality checks up include stable base quality accross the read sequence, absence of excessive left-over adapter contents or appropriate balance in A/T/C/G proportions along the read length.

4.2.3. Preparing the reference genome

The *fastq* files must be aligned (or "mapped") on a reference genome. The STAR (Dobin et al., 2013[1]) aligner is one of the most efficient tools for RNA-seq reads mapping. The STAR aligner requires a genome assembly together with a genome index file. The index file generation is a time-consuming process that is only performed once on a given genome assembly so that it can be completed in advance and the index files can be stored on the server for subsequent analyses.

Download the correct genome assembly fasta file (e.g., Homo_sapiens.GRCh38.dna.primary_assembly.fa) and gene annotation file in gtf format (e.g., Homo_sapiens.GRCh38.108.gtf) from Ensembl or UCSC repository. Below is an example of a human assembly:

```
> wget https://ftp.ensembl.org/pub/release-
  108/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz
> gzip -d Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz # unzip
> wget https://ftp.ensembl.org/pub/release-
  108/gtf/homo_sapiens/Homo_sapiens.GRCh38.108.gtf.gz
> gzip -d Homo_sapiens.GRCh38.108.gtf.gz # unzip
```

NOTE: It's recommended to download the primary_assembly fasta file when possible (without the 'sm' or 'rm' tags). If not available, download the top_level assembly. For the *gtf*, download the one that does not have the 'chr' or 'abinitio' tags.

Use STAR to create an index for the genome assembly. Indicate the output folder name containing the index files using *--genomeDir* option:

```
> STAR --runMode genomeGenerate --genomeDir /path/to/genomeDir --
  genomeFastaFiles Homo_sapiens.GRCh38.dna.primary_assembly.fa --sjdbGTFfile
  Homo_sapiens.GRCh38.108.gtf --runThreadN 8 --sjdbOverhang 74
```

NOTE:

- The --runThreadN parameter can be modified depending on the number of cores available on your machine. The larger this number is, the more parallelized/fast the indexing will be.
- For optimal generation of the reference genome, modify the *--sjdbOverhang* parameter so that it matches your read length –1.
- STAR can use up to 32-40Gb of RAM depending on the genome assembly. So, you should use a machine that has this RAM capacity.
- Alternative aligners to STAR include HISAT2 (lighter-weight, Pertea *et al*, 2016) or Kallisto (pseudo-alignment, Bray *et al,* 2016). It should be noted that pseudo-aligners do not produce accurate BAM files and are therefore not recommended if visualization or detailed QC of your data is required.

4.2.4. Aligning to the reference genome

After the genome index is created, both R1 and R2 *fastq* files can be aligned to this reference genome.

[1] Alexander Dobin, Carrie A. Davis, Felix Schlesinger, Jorg Drenkow, Chris Zaleski, Sonali Jha, Philippe Batut, Mark Chaisson, Thomas R. Gingeras, STAR: ultrafast universal RNA-seq aligner, *Bioinformatics*, Volume 29, Issue 1, January 2013, Pages 15–21, https://doi.org/10.1093/bioinformatics/bts635

For optimal processing, we recommend processing each FLASH-seq sample separately inside a loop sequence. The following parameters should therefore be set for each sample:

- --**readFilesIn**: full path to the input *fastq* files (*'$R1'* and *'$R2'*). Either R1 (= single-end) or R1 and R2 (= paired-ends) separated by a white space.
    - --**outFileNamePrefix** *'$ID'_*: Where *'$ID'_* corresponds to the prefix of the output.

The following parameters should be adjusted according to the sequencing information and stay the same for every sample:

- --**genomeDir**: a path to the genome indices directory generated before ($genomeDir).
- --**readFilesCommand** zcat: Assumes that the fastq files are provided zipped. If not, change the readmode from *zcat* to *cat*.
- $bamDir: Full path to the output directory where the output from STAR should be placed.

This step will output *bam* files and STAR mapping log files into the folder $bamdir.

```
> STAR --runThreadN 30 --limitBAMsortRAM 20000000000 --genomeLoad LoadAndKeep
  --genomeDir $genomeDir --readFilesIn $R1 $R2 --readFilesCommand zcat --
  limitSjdbInsertNsj 2000000 --outFilterIntronMotifs
  RemoveNoncanonicalUnannotated --outSAMtype BAM SortedByCoordinate --
  outFileNamePrefix $bamdir/'$ID'_
```

The alignment quality and performance metrics can be found in the "*$bamdir/Log.final.out*" file.

NOTE: The most important statistic at this step is the proportion of "Uniquely mapped reads" which is expected to be greater than 70% (for human, mouse or drosophila).

4.2.5. Data Visualization (Recommended)

After mapping the reads, we recommend using the **Integrated Genome Viewer** (IGV) to visualize the results and assess their validity. For an initial quality check, examine a few housekeeping genes (e.g., ACTB, GAPDH) and cell-specific markers to ensure proper mapping to exons, introns, and exon-intron junctions. Pay particular attention to anomalies such as read accumulation in intergenic or centromeric regions.

While no single-cell RNA sequencing protocol is flawless, rare occurrences of non-specific priming or genomic DNA contamination may arise. Additionally, recurrent soft-clipping could indicate residual sequencing adaptors, which might impact the mapping rate and could be further trimmed with Trimmomatic (Bolger, A. M., Lohse, M., & Usadel, B., 2014), BBDuk (Bushnell B.) or similar tools.

Although not mandatory, we recommend filtering out the unmapped/multimapped reads from the BAM file, to improve the visualization experience:

```
> samtools view -b -F 260 $bamDir/'$ID'_Aligned.sortedByCoord.bam >
  $bamDir/'$ID'_Aligned.sortedByCoord.filtered.bam
```

4.2.6. Generating the count matrix

The next step consists in converting the read positions from the BAM file into the number of reads associated to each gene and the matrix of counts. We recommend using **FeatureCounts** (Liao Y *et al*, 2014).

Similarly, to read mapping, this step can be performed in parallel and featureCounts should be implemented inside a loop. The following parameters should be adapted for each sample:

- *'$ID'_*: Where *'$ID'_* corresponds to the prefix of the output. The full path to the BAM file should be provided.

The following parameters should be set for all sample:

- **-a**: *'$GTF', full-path to the GTF file used for mapping*.
    - **-t exon**: the feature that is counted. Can be either set to *exon* or *gene.b* We recommend the former.
    - **-g gene_name**: The column in the GTF file used to represent the feature ID, gene_name or gene_id can typically be used.
    - **--fracOverlap 0.25**: Ensures that 25% of the read is overlapping with the counted feature.
    - **-T 1**: Number of threads to use for the analysis, typically 1 as the analysis is relatively fast on small BAM files.
    - **-o** *$countDir*: Full path to the output folder.

```
featureCounts -T 1 -t exon -g gene_name --fracOverlap 0.25 -a "$GTF" -o
$countDir/"$ID".featureCounts.txt $bamDir/'$ID'_Aligned.sortedByCoord.bam
```

The resulting individual "*$ID*".featureCounts.txt files can be merged into a single matrix of count using the following R script:

```
library(tidyverse)

# Get the paths to the featureCount individual count matrices
sample.path <- list.files("/path/to/countDir/", pattern =
"featureCounts.txt", recursive = TRUE, full.path = TRUE)

# Get their associated sample IDs
sample.ids <- basename(FeatureCounts.path) %>%
str_replace(".featureCounts.txt", "")

# Define the reading function
featureCounts.reads <- function(path, id){
ft <- read_tsv(path, show_col_types = FALSE, comment = "#") %>%
  select(1, last_col())

colnames(ft) <- c("geneID", id)
return(ft)
}

# Read the files
ft.counts <- lapply(seq_along(sample.ids), function(x)
featureCounts.reads(sample.path[x], sample.ids[x]))

# Collapse the results
# THIS FUNCTION ASSUMES THAT ALL FEATURECOUNTS FILES CONTAIN THE SAME NUMBER
OF ROWS / GENES

ft.counts.all <- bind_cols(sapply(ft.counts, function(x) x[,2]))
ft.counts.all$geneID <- ft.counts[[1]]$geneID
ft.counts.all <- select(ft.counts.all, geneID, everything())

# The final matrix of count is stored in ft.counts.all
```

4.2.7. Additional QC (Optional)

In addition to the previous scripts and QC, we recommend assessing the data quality of the samples using the RSeQC tools, such as:

- ***geneBody_coverage.py****:* Assess the uniform full-length coverage of a handful of cell per run.
- ***read_distribution.py***¶: Explore the distribution of the reads between exon, introns, *etc.* FLASH-seq data should be dominated by exonic reads. High intergenic reads may suggest tagmentation of left-over genomic DNA. These values can greatly vary depending on the cell type.
- ***junction_saturation.py***¶: Function to assess the saturation in sequencing depth when looking for isoforms.

4.2.8. Post-processing steps

- Post-processing steps will depend on the specific research question. The online resource *Orchestrating Single-Cell Analysis with Bioconductor* (Amezquita R., Lun A., Hicks S., Gottardo R. O'Callaghan C., available at Bioconductor, https://bioconductor.org/books/release/OSCA/) offers a wealth of information to help you design customized pipelines. Alternatively, Popular tools such as *Seurat* (R) or *scanpy* (Python) are compatible with FLASH-seq data and can be utilized effectively. Due to the methodological similarities, Smart-seq2 guidelines are currently recommended for processing FLASH-seq data.

- When working with FLASH-seq data, it is crucial to normalise for the individual sequencing depth, either using traditional size factors or regressing out the read counts (e.g., Seurat).

- Recommended data curation procedures include filtering out:
    - Cells with high mitochondrial read counts often indicate dead/dying cells.

- Cells with an outlier number of uniquely mapped reads.
- Cells with an outlier number of detected genes, lower and higher.

These parameters must be fine-tuned based on the analyzed cell type. For instance, PBMC typically display a lower mapping rate, higher mitochondrial content, and fewer expressed genes than neurons. We recommended setting up experiment-specific filtering procedures using a mixture of hard thresholds (e.g., mitochondria) and dynamic ones (X-times the median absolute deviation, mapped reads/detected genes).

- Most tools dedicated to doublet removal have not been extensively benchmarked on full-length scRNA-seq data. Doublets can typically be found among cells expressing a higher number of genes (1.5 - 2.5x) and identified by the expression of markers from different subpopulations. The doublet rate is directly influenced by cell dissociation and the FACS gating strategy and should be relatively low.

- Due to the nature of the sorting procedure with FACS, ambient RNA removal procedures are usually not required for FLASH-seq data and have not been extensively benchmarked on full-length scRNA-seq.

Appendix 1. TROUBLESHOOTING DURING FACS SORTING

Efficient fluorescence-activated cell sorting (FACS) requires careful optimization to ensure high cell viability and accurate sorting. Table 5 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 excess of clumps and/or debris	Large clumps can block the FACS nozzles. Increased number of doublets. 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 debris 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 not correct. 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.

Table 5 Troubleshooting for the FACS step

Appendix 2. INDEXING ADAPTER SEQUENCES

The 10 bp Unique Dual Indexing (UDI) strategy provides robust library sequencing and accurate sample demultiplexing while following Illumina best practices for indexed sequencing.

The complete list of index sequences is available on the MERCURIUS™ Single-Cell FLASH-seq kit webpage under the **Resources** tab: https://alitheagenomics.com/kits/mercurius-single-cell-flash-seq-kits-extraction-free-library-preparation-kits/

Notes:

- The **i7 index** sequences are provided in **reverse-complement orientation**, while the **i5 index** sequences are provided in **forward orientation**.

  For sequencing workflows that require reverse-complement i5 index sequences (e.g., NovaSeq 6000 or NextSeq 500/550), use the reverse complement of the provided i5 sequences when configuring your sample manifest.

- UDI sequences are 10 bp in length. For compatibility with workflows or kits that use 8 bp indexes, the last two bases can be trimmed from the manifest file (see Ressource file). Although this generally has minimal impact on sequencing performance, the minimum Hamming distance between i5 or i7 indexes is reduced to 1. Consequently, you may need to adjust the demultiplexing parameters in your manifest file to accommodate the reduced index separation.

  Bcl2fastq (Illumina): `--barcode-mismatches 0`

  Bases2Fastq Manifest (Element Bio):

  - `I1MismatchThreshold 0`
  - `I2MismatchThreshold 0`

- Up to four 96-well plate UDI sets are available for MERCURIUS™ Single-Cell FLASH-seq. Ensure that the manifest file corresponding to the UDI set used for sample indexing is selected.

Appendix 3. ILLUMINA-COMPATIBLE INSTRUMENTS

Illumina instruments can use two workflows for sequencing i5 index (see the details in Indexed Sequencing Overview Guide on Illumina's website).

Forward strand workflow instruments:

- NovaSeq 6000 with v1.0 reagents
- MiSeq with Rapid reagents
- HiSeq 2500, HiSeq 2000

Reverse strand workflow instruments:

- NovaSeq 6000 with v1.5 reagents
- iSeq 100
- MiniSeq with Standard reagents
- NextSeq
- HiSeq X, HiSeq 4000, HiSeq 3000

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