ALITHEA GENOMICS

MERCURIUS™

Low-Input FLASH-seq Library Preparation Kit for 96 Samples

PN 10931

User Guide

Januray 2026
(Early-Access)

Related Products

Kit name	Kit PN	Modules	Module PN
Mercurius™ Low-Input FLASH-seq Library Preparation 96 Kit	10931	Indexed Adapters Plate Module 96 samples	10620
		Low-Input FLASH-seq Library Preparation Module 96 samples	10633

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Kit Components

Reagents supplied

Indexed Adapters Plate Module

Component Name	Label	96 samples (PN 10931)	Storage
Plate with 96 Indexed Adapters (PN 10620)	96 CDI setB2	1 plate	-20°C

Low-Input FLASH-seq Library Preparation Module

Component Name	Label	Cap colour	Volume, µL	Storage
Resuspension Buffer	FS RES	yellow	300	-20°C
RT Enzyme	FS RTE	magenta	28	-20°C
RT Buffer	FS RTB	magenta	2x 1100	-20°C
TSO	FS TSO	magenta	45	-20°C
RNase Inhibitor	FS INH	magenta	45	-20°C
Tagmentation Enzyme	FS TAE	red	10	-20°C
Tn5 Dilution Buffer	DIB	orange	450	-20°C
Tagmentation Buffer	FS TAB	red	425	-20°C
Inactivation Mix	INACT Mix	blue	1200	-20°C
Library Amplification Buffer	FS LAB	green	900	-20°C
Library Amplification Enzyme	FS LAE	green	25	-20°C

Additional required reagents and equipment (supplied by the user)

Plasticware	Manufacturer	Product number
Low-binding 96-well PCR plate	Eppendorf	0030129512
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

Equipment	Manufacturer	Product number
Liquid Handling robots (or equivalent instruments supporting 96-well head):		
• 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
96-Well Side Pull Bar Magnet PCR Separation Plate	Permagen	MSP750
12-channel pipette, 0.5-12.5 µL VIAFLO or similar	Integra	4631
12-channel pipette, 5-125 µL VIAFLO or similar	Integra	4632
8-channel adjustable tip spacing pipette, VOYAGER, 2 – 50 µL	Integra	4726

Protocol Overview and Timing

The MERCURIUS™ Low-input FLASH-seq is a full-length RNA sequencing protocol optimized for low-input samples (1 pg - 1 ng of RNA). Designed for high sensitivity and user-friendly application, FLASH-seq outperforms other low-input RNA-seq methods, enabling increased gene detection while reducing time and cost constraints.

The Low-input FLASH-seq kits enable the preparation of Illumina-compatible sequencing libraries for up to 96 RNA samples, making it ideal for high-throughput transcriptomic studies.

With its streamlined workflow and robust sensitivity, low-input FLASH-seq is a reliable and efficient choice for low-input RNA sequencing projects.

The kits are provided in the following formats:

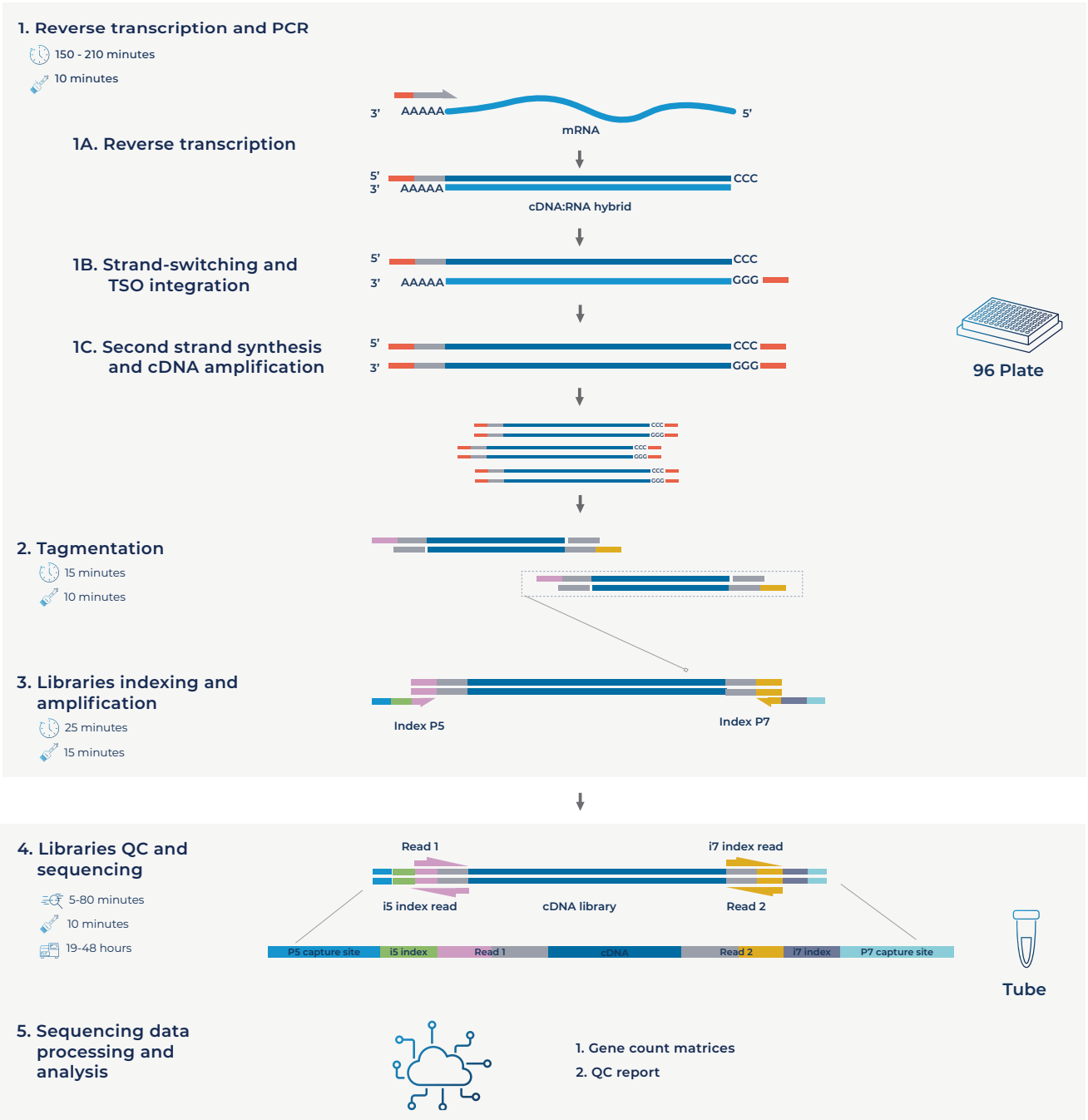
Kit format	PN	PCR plate format	Maximum number of samples processable
96-sample	10931	96WP	96

Each kit contains 96 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 low-input FLASH-seq technology can generate high-quality sequencing data from 1 pg to 1 ng of total purified RNA per sample. The kit can be used to pool any number of samples up to the capacity of the provided plate.

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

Protocol Workflow



Overall time

Incubation time: 3h10-4h10.
Hands-on time: 1h-1h25.
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. PREPARATION OF RNA SAMPLES

Guidelines for RNA samples

The Low-input FLASH-seq protocol is based on sub-nanogram quantities of RNA; therefore, it is crucial to ensure that the quantity, purity, and quality of RNA are uniform across all samples before initiating library preparation.

Quantity

The recommended range of total RNA amount per well is 1 pg – 1 ng. Usually, when more RNA is used in the RT reaction, the higher the library complexity is observed after sequencing.

Purity and Integrity

Whenever possible, RNA purity and integrity should be assessed using a spectrophotometer (e.g., Nanodrop) or by capillary electrophoresis (e.g., Fragment Analyzer or Bioanalyzer, Agilent).

- RNA samples extracted using TRIzol™, phenol, or chloroform compounds are prone to residual contamination with organic solvents that may inhibit the reverse transcription reaction. This typically results in low cDNA library yields, loss of sequencing reads for a portion of the pooled samples, and uneven read distributions. To ensure the high purity of RNA, assess the 260/230 ratio of at least a few samples from the same RNA isolation batch using a spectrophotometer (i.e., Nanodrop). The 260/230 ratio values should be >1.5.
- Leftover traces of genomic DNA can compromise the reaction, leading to an increase in reads mapping to intergenic regions. We recommend working with DNase-treated RNA.
- The integrity of the RNA, and more specifically, the poly-A tail, is key to generating high-quality data. We recommend using the Low-input FLASH-seq protocol on samples with a RIN value of 6 or higher. Samples with lower quality can still be used, but may yield lower-quality results due to RNA degradation.

Uniformity

To ensure an even distribution of reads after sequencing, the RNA amount, integrity (as indicated by the RIN number), and 260/230 values of the starting RNA samples must be as uniform as possible, i.e., 1 ng (±10%) of starting material with an RIN of 6 for every sample. To obtain such uniform amounts, we therefore recommend:

- Measure the RNA concentration of all samples using a dye-based method (e.g., Qubit Quant-iT or RiboGreen) for a large number of samples.
- Dilute samples to obtain the same RNA concentration in all wells (±10%).
- Re-measure the RNA concentration of all samples to confirm uniform concentrations across the samples.
- Ensure the 260/230 ratios are >1.5 and similar across the samples.
- Ensure the RIN values are similar across the samples, preferably ≥ 6.

If the samples cannot be homogenized, we recommend splitting them into separate homogeneous batches to adjust the number of PCR cycles according to the RNA input.

Contact info@alitheagenomics.com for technical support.

Part 2. LIBRARY PREPARATION PROTOCOL

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

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**!

2.1. RNA samples preparation

At this step, RNA samples are diluted and pipetted into a 96-well low-binding PCR plate.

Preparation

- Thaw the **FS RES** tube on ice, mix well before use, and quickly spin it down.
- Thaw the RNA samples on ice.
- Prepare a 96-well low-binding PCR plate, which will be referred to as the “RNA plate”.

Procedure

2.1.1. If necessary, dilute all RNA samples with nuclease-free water to the required concentration and use 2.5 µL of the diluted sample for the next step.

2.1.2. Pipette 2.5 µL of the resuspension buffer into all wells of the 96-well PCR plate (“RNA plate”), according to the number of samples to be used for library preparation. Keep the RNA plate on ice.

2.1.3. Using a multichannel pipette, transfer 2.5 µL of purified RNA directly to the corresponding wells and pipette up and down 3-5 times to ensure proper resuspension.

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

2.1.5. Proceed immediately to step 2.2.

2.2. Reverse transcription and PCR

At this step, each individual RNA sample is reverse-transcribed.

Preparation

- Thaw all tubes on ice, mix well before use (pipette up and down, without vortexing), and 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	see table below
Annealing	63	20 sec	
Extension	72	6 min	
Final extension	72	3 min	1
Keep	4	pause	1

- Adjust the number of PCR cycles based on RNA quality:

RNA input (per well)	Number of PCR cycles
1 ng	12-13
100 pg	16-17
10 pg	18-19
5 pg	21-22
1 pg	23-24
Non-purified / degraded	Titration recommended*

* For non-purified samples, perform a titration. Aim to generate ≥ 200 pg of cDNA for library preparation and avoid overamplification.

Procedure

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

Reagent	Per well, µL	96 wells +10%, µL
FS RTB	18.4	1943
FS RTE	0.24	26
FS TSO	0.4	43
FS INH	0.4	43
Water	0.56	60
TOTAL	20.0	2115

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

2.2.3. Transfer the plate containing the resuspended RNA to the thermocycler and start Program 1_DNT.

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

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

2.2.6. Using a multichannel pipette, pipette 20 µL of the RT Master Mix per well into each well containing the RNA sample in resuspension buffer.

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

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

2.2.9. Vortex the plate for a few seconds at 500 rpm and spin it down at 800x g, 30 sec. The solution should display a homogeneous pinkish color.

2.2.10. 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.

2.3. 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 (Permagen, MSP750). 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

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

2.3.2. Add 17.5 µL of magnetic beads per well, pre-warmed to room temperature.

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

2.3.4. Incubate the plate for 5 min at RT.

2.3.5. Centrifuge the plate at 300x g for 10 sec to collect all liquid.

2.3.6. Place the plate on the magnet and wait for the beads to settle.

2.3.7. Remove as much supernatant as possible (~43 µL), 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.

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

2.3.9. **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.

2.3.10. Pipette 15 µL of water per well, preferably directly onto the beads.

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

2.3.12. Incubate for 5 min at RT.

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

2.3.14. Carefully transfer 14 µL per well of supernatant without touching the bead pellets to a new 96-PCR plate.

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

2.4. 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 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 RNA quality of the samples and the chosen amplification rate.

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

- **Option A – All samples, highly preferable** (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, less recommended** (if not all samples can be measured due to lack of equipment)

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

Assessing the cDNA profile across the samples

When using low-input FLASH-seq, 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 Low-input FLASH-seq cDNA from high-quality RNA will range from 400 bp to >7kb, with an average of 1,600 to 2,100 bp, depending on the RNA origin. See Figure 2 for an example of a standard cDNA profile obtained from RNA, purified from 293 cells.

Primer dimers/leftovers (at ~47 bp) can sometimes be observed (see Figure 3) and could produce lower-quality sequencing data with a reduced proportion of mappable reads. This peak can appear due to the wrong 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 the tagmentation.

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

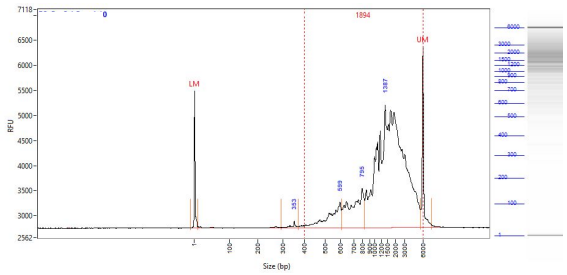
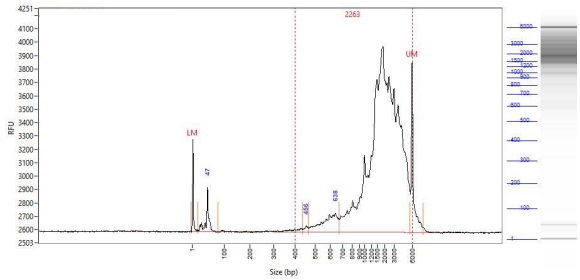


Figure 3 An example of a cDNA profile demonstrating the dimer peak at 47 bp



2.5. cDNA dilution in plates

Using the Tn5 transposase, Low-input FLASH-seq 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™ Low-input FLASH-seq kit is optimized for cDNA input ranging from 300 to 800 pg. Do not exceed these values. Low-binding plates are absolutely necessary for this step.

Preparation

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

Procedure

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

2.5.2. 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, they can reach up to 10x differences when handling samples with varying inputs or quality. **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.

2.6. Tagmentation

At this step, the full-length cDNA is tagmented for subsequent 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 enzyme and cDNA in the reaction. FLASH-seq tagmentation reaction should be undertaken with at most 300-800 pg of cDNA input.

Preparation

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

Procedure

2.6.1. **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.

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

Reagent	Per well, µL	96 wells +10%, µL
FS TAB	**3.6**	**396**
FS TAE (diluted)	**0.4**	**44**
TOTAL	**4.0**	**440**

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

2.6.4. Pipette the 4 µL of Tagmentation Master Mix into every well of the new plate, kept on ice:

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

2.6.6. Transfer 4 µL of the diluted cDNA from step 0 to the plate with the Tagmentation Master Mix.

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

2.6.8. Seal the plate and spin it down.

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

2.6.10. 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.

2.6.11. Put a plate from step 2.6.10 on ice for 1 min.

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

2.6.13. Transfer the plate to room temperature and pipette 4 µL of the Inactivation mix into every well. The inactivation solution should turn pink when it comes into contact with the previous reaction.

2.6.14. Seal the plate with a new Aluminium seal, briefly vortex the plate to homogenize the solution, and spin it down.

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

2.6.16. Proceed immediately to step 2.7.

2.7. Library indexing and amplification

The low-input FLASH-seq protocol utilizes Combinatorial Dual Indexing (CDI). At this step, the cDNA fragments are amplified using the Indexing Adapter primers, which are provided in liquid form in a 96-well plate.

The i7 and i5 index sequences for both plate formats are indicated in Appendix 1.

The number of amplification cycles required for library preparation typically ranges from 8 to 14 (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 **CDI Adapters** at room temperature and briefly spin before use.
- 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	10-16*
Annealing	55	30 sec	
Extension	72	30 sec	
Final extension	72	1 min	1
Keep	4	pause	1

*10 cycles – for 48 - 96 samples
12 cycles - for 24 - 48 samples;
14 cycles – for less than 24 samples
16 cycles – for samples with cDNA that cannot be measured

Procedure

2.7.1. Prepare the Amplification Master Mix as follows:

Reagent	Per well, µL	96 wells +10%, µL
FS LAB	7.8	858
FS LAE	0.2	22
TOTAL	8.0	880

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

2.7.3. Remove the seal from the plates with **CDI Adapters** and tagmented the cDNA (from step 2.6.14)

CRITICAL: To prevent cross-contamination between CDI adapters, handle the PCR plate with care. If only a few samples are being processed, we recommend **partially peeling back the foil seal from the edge** to expose only the required wells, rather than removing the entire seal. After accessing the needed adapters, reseal the foil tightly with tape or another seal to protect the remaining wells.

2.7.4. Pipette 4 µL of the CDI Adapters into the corresponding wells with tagmented cDNA. Ensure that the layouts of both plates match each other.

2.7.5. Add 8 µL of the prepared Amplification Master Mix to each well.

2.7.6. Seal the plate and briefly vortex it (500 rpm or at speed 5-6 for 5 sec) to homogenize the reaction.

2.7.7. Briefly spin it down.

2.7.8. 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.

2.8. 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 5 mL of 80% ethanol.

Procedure

2.8.1. Using automation or a multichannel pipette, pool 4 µL from every well from the plate (step 2.7.8) into the reservoir.

2.8.2. 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).

2.8.3. Pipette the required volume of beads slurry into the pool to obtain a 0.7x bead-to-library ratio.

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

2.8.5. Incubate for 5 min at room temperature.

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

2.8.7. To wash the beads, pipette 200 µL of freshly prepared 80% ethanol into the tube.

2.8.8. Incubate for 30 sec.

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

2.8.10. Repeat step 2.8.6 for a total of two washes.

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

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

2.8.13. Incubate for 5 min at room temperature.

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

2.8.15. Proceed to the Library quality control (step 2.9). If the library shows the presence of the primer dimers, perform a second purification (steps 2.8.3 - 2.8.15).

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

2.9. 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 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 recommended to remove those peaks by performing an additional round of SPRI bead purification with a 0.7x ratio (see steps 2.8.3 - 2.8.14).

Pre-sequencing library QC:

- Use 2 µL of the library pool to measure the concentration with Qubit (typically, starting from 200 pg of cDNA from 24 samples will result in 0.5 - 20 ng/µL library concentration);
- 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 (2.8.3 - 2.8.14) to remove the peaks <50 bp.

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

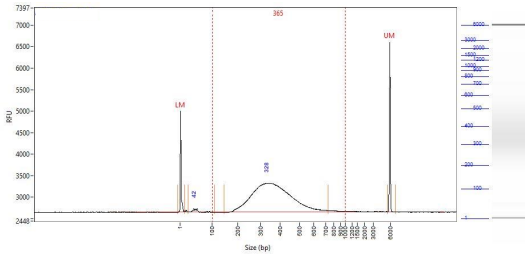


Figure 5 A library profile with a peak ~750 bases bp

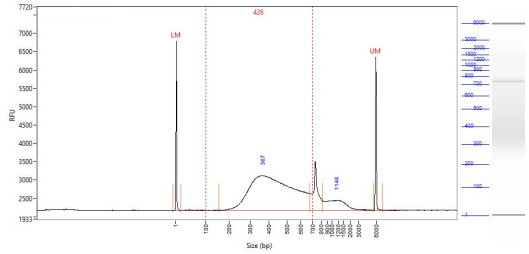


Figure 6 An example of an over-tagmented library profile with a peak at 187 bp and an adapter peak at 42 bp

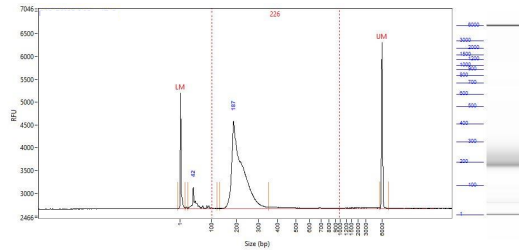
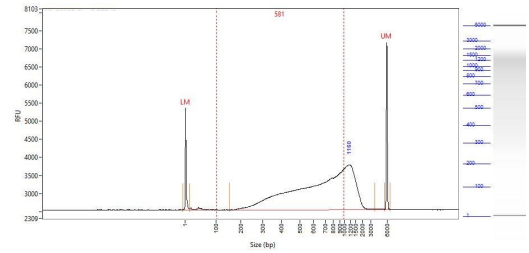


Figure 7 An example of an under-tagmented library profile with a major peak at 1160 bp



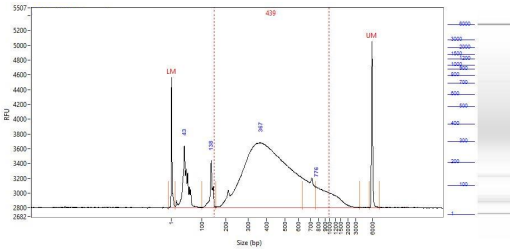


Figure 8 An example of a library with primer dimers and leftovers at <150 bp

Part 3. LIBRARY SEQUENCING

The libraries prepared with the MERCURIUS™ Low-input 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™ Low-input FLASH-seq libraries are Combinatorial Dual-Indexed 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 1 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 the detection of isoforms.

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

Table 2 Sequencing structure of FLASH-seq libraries

NOTE: Sequencing depth

1. The recommended sequencing depth depends on the RNA input concentration and integrity. We recommend sequencing each sample at 1 Mio reads. Typically, samples with <20 pg RNA will reach saturation at this depth. Higher inputs (~1 ng) can require upto 2-4 Mio reads per sample.

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™ 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	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.

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 perform the generate count generation, which can be done in parallel with the 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
```

NOTES:

- 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 RNA sequencing protocol is flawless, rare occurrences of non-specific priming or genomic DNA contamination may arise, especially when working with low quality RNA samples or crude cell extracts. 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**: *'$GTF', full-path to the GTF file used for mapping*.
    - **-t exon**: the feature that is counted. Can be either set to *exon* or *gene.* We recommend the former.
    - **-g gene_name**: The column in the GTF file used to represent the feature ID, gene_name or gene_id can typically be used.
    - **--fracOverlap 0.25**: Ensures that 25% of the read is overlapping with the counted feature.
    - **-T 1**: Number of threads to use for the analysis, typically 1 as the analysis is relatively fast on small BAM files.
    - **-o** *$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", recurvise = 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 RNA samples 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 RNA origin.
- ***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:
    - Samples with an outlier number of uniquely mapped reads.

- Samples with an outlier number of detected genes, lower and higher.

These parameters must be fine-tuned based on the analyzed samples. For instance, RNA samples displaying higher degradation levels may exhibit 3' or 5' bias in gene-body coverage, harbor a lower mapping rate and more reads associated to mitochondrial or ribosomal features. We recommend following the general best practises used to analyse RNA-sequencing data.

Appendix 1. INDEXING ADAPTER SEQUENCES

The Combinatorial Dual Indexing (CDI) strategy ensures high-quality library sequencing and demultiplexing accuracy, complying with best practices for Illumina platform sequencing.

Below is the list of indexes and their well locations in the provided 96-well plates (Table 5).

Row	Column	i7 index sequence	i5 index sequence Forward Workflow	Row	Column	i7 index sequence	i5 index sequence Forward Workflow
A	1	CTCGATAC	GAGCCTTA	A	7	CTTGGATG	GAGCCTTA
B	1	CTCGATAC	CGACCATT	B	7	CTTGGATG	CGACCATT
C	1	CTCGATAC	CTCTCTAT	C	7	CTTGGATG	CTCTCTAT
D	1	CTCGATAC	ACCAGCTT	D	7	CTTGGATG	ACCAGCTT
E	1	CTCGATAC	GTCAGTTG	E	7	CTTGGATG	GTCAGTTG
F	1	CTCGATAC	ACCAATGC	F	7	CTTGGATG	ACCAATGC
G	1	CTCGATAC	TGAGGTGT	G	7	CTTGGATG	TGAGGTGT
H	1	CTCGATAC	CGCTAGTA	H	7	CTTGGATG	CGCTAGTA
A	2	TCCGTGAA	GAGCCTTA	A	8	CTCCTAGA	GAGCCTTA
B	2	TCCGTGAA	CGACCATT	B	8	CTCCTAGA	CGACCATT
C	2	TCCGTGAA	CTCTCTAT	C	8	CTCCTAGA	CTCTCTAT
D	2	TCCGTGAA	ACCAGCTT	D	8	CTCCTAGA	ACCAGCTT
E	2	TCCGTGAA	GTCAGTTG	E	8	CTCCTAGA	GTCAGTTG
F	2	TCCGTGAA	ACCAATGC	F	8	CTCCTAGA	ACCAATGC
G	2	TCCGTGAA	TGAGGTGT	G	8	CTCCTAGA	TGAGGTGT
H	2	TCCGTGAA	CGCTAGTA	H	8	CTCCTAGA	CGCTAGTA
A	3	TAGAGCTC	GAGCCTTA	A	9	CAACGGAT	GAGCCTTA
B	3	TAGAGCTC	CGACCATT	B	9	CAACGGAT	CGACCATT
C	3	TAGAGCTC	CTCTCTAT	C	9	CAACGGAT	CTCTCTAT
D	3	TAGAGCTC	ACCAGCTT	D	9	CAACGGAT	ACCAGCTT
E	3	TAGAGCTC	GTCAGTTG	E	9	CAACGGAT	GTCAGTTG
F	3	TAGAGCTC	ACCAATGC	F	9	CAACGGAT	ACCAATGC
G	3	TAGAGCTC	TGAGGTGT	G	9	CAACGGAT	TGAGGTGT
H	3	TAGAGCTC	CGCTAGTA	H	9	CAACGGAT	CGCTAGTA
A	4	TGACTGAC	GAGCCTTA	A	10	TGGCTATC	GAGCCTTA
B	4	TGACTGAC	CGACCATT	B	10	TGGCTATC	CGACCATT
C	4	TGACTGAC	CTCTCTAT	C	10	TGGCTATC	CTCTCTAT
D	4	TGACTGAC	ACCAGCTT	D	10	TGGCTATC	ACCAGCTT
E	4	TGACTGAC	GTCAGTTG	E	10	TGGCTATC	GTCAGTTG
F	4	TGACTGAC	ACCAATGC	F	10	TGGCTATC	ACCAATGC
G	4	TGACTGAC	TGAGGTGT	G	10	TGGCTATC	TGAGGTGT
H	4	TGACTGAC	CGCTAGTA	H	10	TGGCTATC	CGCTAGTA
A	5	TAGACGTG	GAGCCTTA	A	11	CGGTCATA	GAGCCTTA
B	5	TAGACGTG	CGACCATT	B	11	CGGTCATA	CGACCATT
C	5	TAGACGTG	CTCTCTAT	C	11	CGGTCATA	CTCTCTAT
D	5	TAGACGTG	ACCAGCTT	D	11	CGGTCATA	ACCAGCTT
E	5	TAGACGTG	GTCAGTTG	E	11	CGGTCATA	GTCAGTTG
F	5	TAGACGTG	ACCAATGC	F	11	CGGTCATA	ACCAATGC
G	5	TAGACGTG	TGAGGTGT	G	11	CGGTCATA	TGAGGTGT
H	5	TAGACGTG	CGCTAGTA	H	11	CGGTCATA	CGCTAGTA
A	6	CCGGAATT	GAGCCTTA	A	12	TCCAATCG	GAGCCTTA
B	6	CCGGAATT	CGACCATT	B	12	TCCAATCG	CGACCATT
C	6	CCGGAATT	CTCTCTAT	C	12	TCCAATCG	CTCTCTAT
D	6	CCGGAATT	ACCAGCTT	D	12	TCCAATCG	ACCAGCTT
E	6	CCGGAATT	GTCAGTTG	E	12	TCCAATCG	GTCAGTTG
F	6	CCGGAATT	ACCAATGC	F	12	TCCAATCG	ACCAATGC
G	6	CCGGAATT	TGAGGTGT	G	12	TCCAATCG	TGAGGTGT
H	6	CCGGAATT	CGCTAGTA	H	12	TCCAATCG	CGCTAGTA

Table 5 List of the indexes and their positioning in the 96-well plate

Appendix 2. ILLUMINA-COMPATIBLE INSTRUMENTS

Illumina instruments can use two workflows for sequencing the i5 index (see the details in the 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

ALITHEA GENOMICS

info@alitheagenomics.com

www.alitheagenomics.com

Alithea Genomics SA
Batiment Serine
Rue de la Corniche 8, level -2
1066 Epalinges
Switzerland
Tel: +41 78 830 3139

Alithea Genomics Inc.
321 Ballenger Center Drive
Suite 125 C/O Alithea Genomics Inc.
Frederick, MD 21703
USA
Tel: +1 240 656 3142