ALITHEA GENOMICS

MERCURIUS™

Full-Length DRUG-seq Library Preparation kit (mRNA) for 96, 384 and 1’536 samples

PN 10701, 10702, 11651, 11652

User Guide

January 2025
(BETA, B)

Related Products

Kit name	Kit PN	Modules	Module PN
FL DRUG-seq Library Preparation 96 kit (mRNA)	10701	Barcoded Oligo-dT Adapters Module 96 samples	10513
		FL DRUG-seq Library Preparation and UDI Module 96 samples (mRNA)	10601
FL DRUG-seq Library Preparation 384 kit (mRNA)	11702	Barcoded Oligo-dT Adapters Module 384 samples	10515
		FL DRUG-seq Library Preparation and UDI Module 384 samples (mRNA)	10602
FL DRUG-seq Library Preparation 4x 96 kit (mRNA)	11651	Barcoded Oligo-dT Adapters Module 4x 96 samples	10513
		FL DRUG-seq Library Preparation and UDI Module 4x 96 samples (mRNA)	10605
FL DRUG-seq Library Preparation 4x 384 kit (mRNA)	11652	Barcoded Oligo-dT Adapters Module 4x 384 samples	10513
		FL DRUG-seq Library Preparation and UDI Module 4x 384 samples (mRNA)	10606

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Kit Components

Reagents supplied

Barcoded Oligo-dT Adapters Set V5 Module

Component Name	Label	Amount provided per kit				Storage
		96 samples (PN 10701)	384 samples (PN 10702)	4x 96 samples (PN 11651)	4x 384 samples (PN 11652)	
Plate with 96 barcoded oligo-dT primers, set V5C (PN 10400)	96 V5C OdT	1 plate		4 plates	-	-20°C
Plate with 384 barcoded oligo-dT primers, set V5A (PN 10415) or V5C (PN 10401)	384 V5A OdT or 384 V5C OdT	-	1 plate	-	4 plates	-20°C
Aluminium Seal	-	2 pcs	2 pcs	8 pcs	8 pcs	-20°C/RT

FL DRUG-seq Library Preparation and UDI Module (mRNA)

Component Name	Label	Cap colour	Volume (µL)				Storage
			96 samples (PN 10601)	384 samples (PN 10602)	4x 96 samples (PN 10605)	4x 384 samples (PN 10606)	
First Strand Buffer FL	FSB FL	magenta	245	1000	1000	4x 1000	-20°C
Repair/RT Enzyme	RAE	magenta	140	280	2x 280	4x 280	-20°C
Repair/RT Mix	RAM	magenta	930	1900	2x 1900	4x 1900	-20°C
Exonuclease I Enzyme	F-EXO	purple	10	10	10	10	-20°C
Exonuclease Buffer	F-EXB	purple	20	20	20	20	-20°C
Second Strand Enzyme FL	SSE FL	orange	25	25	25	25	-20°C
Second Strand Buffer FL	SSB FL	orange	30	30	30	30	-20°C
Adapter Ligation Buffer	ALB	blue	100	100	100	100	-20°C
BRB-compatible Adapter	BRB.AD	blue	10	10	10	10	-20°C
Library Amplification Mix FL	LAM FL	green	200	200	200	200	-20°C
UDI Adapter Mix 1	MF.UDI.1	transparent	10	10	10	10	-20°C
UDI Adapter Mix 2	MF.UDI.2	transparent	10	10	10	10	-20°C
UDI Adapter Mix 3	MF.UDI.3	transparent	10	10	10	10	-20°C
UDI Adapter Mix 4	MF.UDI.4	transparent	10	10	10	10	-20°C
Cell lysis Buffer	CLB	yellow	710	1500	2x 1500	4x 1500	-20°C
RNase Inhibitor	INH	Yellow	175	375	2x 375	4x 375	-20°C

Additional required reagents and equipment (supplied by the user)

Plasticware	Manufacturer	Product number
0.2 mL 8-Strip Non-Flex PCR Tubes	Starlab	I1402-3700
Disposable Pipetting Reservoir 25 mL polystyrene	Integra	4382
Disposable Pipetting Reservoir 150 mL polystyrene	Integra	6318

Reagents	Manufacturer	Product number
NEBNext Poly(A) mRNA Magnetic Isolation Module, 96 rxns	NEB	E7490L
SPRI AMPure Beads	Beckman Coulter	A63881
or		
CleanNA Beads	CleanNA	CNGS0050D
Qubit™ dsDNA HS Assay Kit	Invitrogen	Q32851
High Sensitivity NGS Fragment Analysis Kit	Agilent	DNF-474
Ethanol, 200 proof	-	-
Nuclease-free water	Thermofisher	A57775
DPBS, Cell culture grade	Gibco	10010023
ERCC RNA Spike-In Mix (recommended)	Thermo Fisher	4456740

Equipment	Manufacturer	Product number
Benchtop centrifuge for plates	-	-
Benchtop centrifuge for 1.5 mL tubes	-	-
Single and Multichannel pipettes	-	-
Fragment Analyser / Bioanalyzer / TapeStation	Agilent	M5310AA
Qubit™	Invitrogen	Q33238
Magnetic stand for 0.2 mL tubes	Permagen	MSR812
Magnetic stand for 1.5 mL tubes	Permagen	MSR06
Magnetic stand for 5 mL tubes	Permagen	
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
Pipetboy	Integra	155 000
VIAFLO instrument (optional)	Integra	6001
VIAFLO 96 channel pipetting head, 0.5-12.5 µL (optional)	Integra	6101

Protocol Overview and Timing

The MERCURIUS™ Full-Length (FL) DRUG-seq kits (mRNA) allow the preparation of Illumina-compatible RNA sequencing libraries for up to 1’536 samples in a time and cost-efficient manner. The kits include a mild cell lysis buffer to prepare crude cell lysates. This protocol is based on the enrichment of the mRNA pool, its further fragmentation with subsequent modifications, and the library preparation using DRUG-seq technology.

The kits are provided in the following formats:

Kit format	PN	PCR plate format	Maximum number of samples in one pool	Maximum number of samples processable	Number of UDI libraries
96-sample	10701	96WP	96	96	4
384-sample	11702	384WP	384	384	4
4x 96-sample	11651	96WP	96	384	4
4x 384-sample	11652	384WP	384	1’536	4

Every kit contains barcoded MERCURIUS™ Oligo-dT primers designed to tag RNA samples with individual barcodes during the first strand synthesis reaction, allowing the pooling of the resulting cDNA samples from each experimental group into a single tube for streamlined sequencing library preparation.

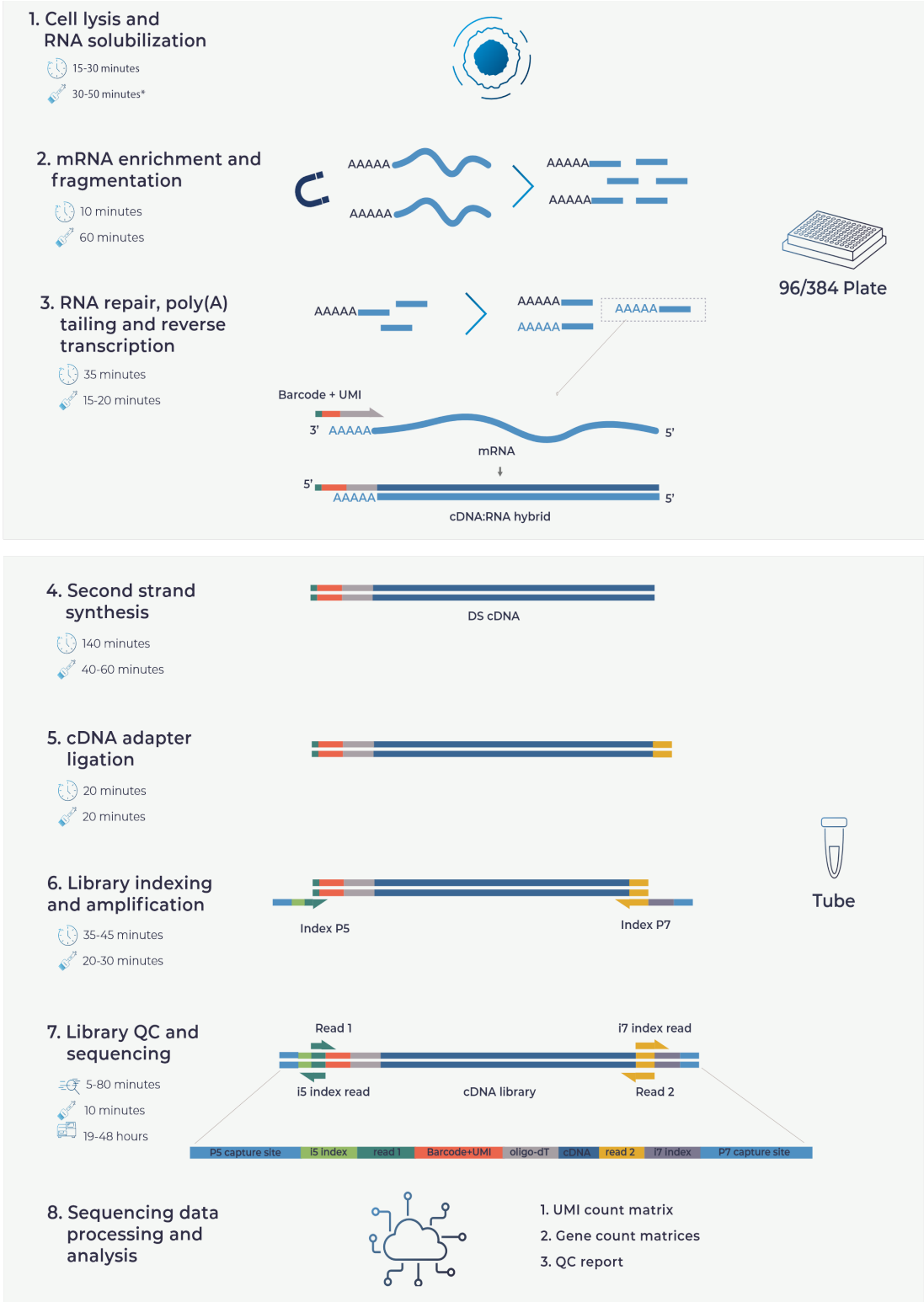
The DRUG-seq technology can be used to generate high-quality sequencing data starting with 2’000 – 25’000 mammalian cells per well. Notably, the kit can be used to pool any number of samples up to 384 with two considerations:

- The total cell number per pool should be at least 80’000.
- Pooling less than eight samples may result in low-complexity reads during sequencing, decreasing the overall sequencing quality. If necessary, the latter can be improved by increasing the proportion of PhiX spike-in control during sequencing (see Part 3).

Each library indexing is performed using a Unique Dual Indexing (UDI) strategy, which minimizes the risk of barcode misassignment after NGS. Every adapter can be used to prepare an individual library. Libraries with different UDI adapters can be pooled and sequenced in a single flow cell.

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

Protocol Workflow



Overall time

Incubation time: 4h20-6h.
Hands-on time: 3h15-4h10.
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 CELL LYSATE SAMPLES

1.1. Essential considerations for input cells

- The recommended input range of cells is 5'000-25'000 cells/well of **96WP** and 2'000-10'000 cells/well of **384WP** (on the day of the sample preparation).
- **NOTE:** Do not use more than 25'000 cells per well, as it will result in a high rRNA content.
- Cells must be seeded a few days in advance for the best results.
- To obtain the best result prior to the experiment ensure that cell viability is >70% (e.g., trypan blue, propidium iodide).
- Depending on the type of cells (human, mouse, cancer, or primary cells) and experimental design (e.g., induction of differentiation or apoptosis, cell cycle arrest, etc.), consider the doubling time of cells after the seeding and the potential effect of the treatment on the cell number during the experiment.
- To ensure an even distribution of reads after sequencing, the amount of starting material must be as uniform as possible. We suggest automatizing cell seeding instruments or double-verified cell counts for this.

1.2. ERCC Spike-in Controls (Optional)

To ensure the evaluation of the sequencing reads uniformity across the samples and to assess the impact of sample and library preparation steps on it, we recommend the addition of External RNA Controls Consortium (ERCC) Spike-Ins to the lysate buffer (Thermo Fisher, 4456740). Please follow Appendix 1 for detailed information before the lysis step.

1.3. Cell pellet preparation

At this step, plated cells are washed with DPBS and frozen at -80°C for at least 5 minutes. If possible, snap-freeze the plate with dry ice or liquid nitrogen beforehand.

NOTE: The freezing step is required to achieve a higher exon mapping.

Procedure for the preparation of adherent cells

1.3.1. Seed the cells in a flat bottom **96WP** or **384WP** at the density that will enable harvesting:
- **96WP:** 5'000-25'000 cells per well
- **384WP:** 2'000-10'000 cells per well

1.3.2. Gently aspirate culture media from the plate and wash cells by adding the following:
- **96WP:** 80-100 µL DPBS in each well
- **384WP:** 30-50 µL DPBS in each well

1.3.3. Gently tap the plate and aspirate as much DPBS as possible without disturbing the cell pellet.

1.3.4. Seal the plate and snap-freeze it on dry ice or liquid nitrogen for at least 5 minutes. Alternatively, the plate can be stored at -80°C for a few weeks.

1.3.5. Proceed to step 1.4.1 for cell lysis.

Procedure for the preparation of suspension cells

1.3.6. Seed the cells in a flat bottom or U-shaped **96WP** or **384WP** at the density that will enable harvesting:
- **96WP:** 5'000-25'000 cells per well
- **384WP:** 2'000-10'000 cells per well

1.3.7. Centrifuge the plate at 300x g for 5 minutes.

1.3.8. Gently aspirate culture media from the plate and wash cells by adding the following:
- **96WP:** 80-100 µL DPBS in each well
- **384WP:** 30-50 µL DPBS in each well

1.3.9. Centrifuge the plate at 300x g for 5 minutes.

1.3.10. Gently tap the plate and aspirate as much DPBS as possible without disturbing the cell pellet

1.3.11. Seal the plate and snap-freeze it on dry ice or liquid nitrogen for at least 5 minutes. Alternatively, the plate can be stored at -80°C for a few weeks.

1.3.12. Proceed to step 1.4.1 for cell lysis.

1.4. Cell lysate preparation

At this step, frozen cells are lysed directly in a 96-well plate by adding 1x Cell Lysis Buffer to the wells. The lysates can be used directly for the mRNA selection.

Preparation

- Thaw the CLB and INH tubes on ice.
- Mix well and briefly spin down before use
- Prepare a working solution of 1x Cell Lysis Buffer with RNase Inhibitor:

Reagent	96WP, µL		384WP, µL	
	Per well	96 wells +10%	Per well	384 wells +10%
CLB	6.6	700	3.3	1400
INH	1.6	170	0.8	340
Water	11.8	1250	5.9	2500
TOTAL	20	2120	10	4240

Pipette the prepared mix gently a few times, briefly spin the tube. Keep the mix on ice until further use.

Procedure for cell lysis

1.4.1. Using a multi-dispenser in every well, distribute the prepared CLB:
- **96WP:** 20 µL per well
- **384WP:** 10 µL per well

1.4.2. Centrifuge the plate at 300x g for 1 minute to ensure that CLB is uniformly distributed on the surface of each well.

1.4.3. Incubate the plate at room temperature for 15 min.

1.4.4. Transfer the whole lysate from every well to the corresponding well of the 96- or 384-well PCR plate.

1.4.5. Seal the plate with an aluminum seal provided and briefly spin it down.

1.4.6. The lysates can be used directly for mRNA enrichment (see step 2.1) or safely stored at -80°C for a few weeks.

NOTE: If several plates must be processed, perform the procedure with each plate individually one by one to avoid keeping plates at room temperature for a prolonged time.

Part 2. LIBRARY PREPARATION PROTOCOL

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

2.1. mRNA enrichment and fragmentation

At this step, mRNA molecules are enriched from every sample due to oligo-dT-based purification from the total RNA pool in the lysate. We highly recommend using the pipetting robot to minimize the variation between sample preparations due to different volumes, purification times, drying, elution, etc.

Preparation

- Thaw the cell lysate samples on ice.
- Thaw the **FSB FL** reagent at room temperature and mix well before use.
- Prepare Program 1_Fragmentation on the thermocycler (set the lid at 100°C) and preheat it to 94°C

Step	Temperature, °C	Time
Incubation	94	3 min
Keep	4	pause

NOTE: All the 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 using gloves.

Procedure

We recommend using the NEBNext Poly(A) mRNA Magnetic Isolation kit (NEB #E7490) for the mRNA enrichment procedure. It is essential to follow the guidelines described in Appendix 2.

NOTE: The 384-well plate format includes a modified protocol that must be strictly adhered to.

Proceed with the corresponding protocol until the elution step. For the latter, follow the procedure below:

2.1.1. Prepare the Elution Master mix (+10%) as follows:

Reagent	96WP, µL		384WP, µL	
	Per well	96 wells +10%	Per well	384 wells +10%
FSB FL	2.2	242	2	846
Water	8.8	968	8	3384
TOTAL	11.0	1210	10	4230

2.1.2. Using a multichannel pipette or robot, transfer the following volume Elution Master mix to each well and pipette a few times to ensure a proper resuspension of the beads:

- **96WP:** 11 µL per well
- **384WP:** 10 µL per well

2.1.3. Carefully seal the plate and briefly spin it in the centrifuge.

2.1.4. Incubate in a thermocycler Program 1_Fragmentation. Do not exceed the incubation time, as this can lead to mRNA degradation. Keep the plates at 4°C for a few minutes.

2.1.5. Briefly spin the samples in the centrifuge and open a seal. Place the plate on the magnetic stand and wait 5 min.

2.1.6. Carefully transfer supernatant with fragmented mRNA into a plate with barcoded oligo-dT primers directly (keep it on ice)

- **96WP:** 10 µL per well
- **384WP:** 5 µL per well

2.1.7. Pipette 3-5 times to ensure proper reconstitution of dried oligo-dT. The appearance of red in all wells indicates a proper and uniform reconstitution of oligos.

2.1.8. Carefully seal the plate and briefly spin it in the centrifuge.

2.1.9. Proceed immediately to step 2.2.

2.2. RNA repair, poly (A) tailing, and reverse transcription

At this step, fragmented mRNA molecules are repaired, poly-adenylated, and reverse-transcribed using the barcoded oligo-dT primers provided in a 96- or 384-well plate. Subsequently, all the barcoded samples can be pooled into one tube.

NOTE: Barcoded oligo-dT primers are provided lyophilized with the addition of dye. The dye has no impact on the enzymatic reactions and is used solely to visualize reaction preparation and pooling better.

Despite variations in appearance caused by the drying process, wells may exhibit traces of dried dye ranging from dispersed to solid dots on the bottom. The following addition of the reagents will enable the visualization of red color, confirming the presence of the oligos in all wells.

Preparation

- Thaw all tubes on ice and mix well before use.
- Prepare Program 2_Repair/RT on the thermocycler (set the lid at 90°C) and pre-heat it to 37°C:

Step	Temperature, °C	Time
Incubation	37	30 min
Inactivation	75	5 min
Keep	4	pause

Procedure

2.2.1. Keep the plate with RNA and oligo-dT on ice.

2.2.2. Depending on the number of samples, prepare the following Repair/RT Master mix (+10%) as follows:

Reagent	96WP, µL		384WP, µL	
	Per well	96 wells +10%	Per well	384 wells +10%
RAM	8.75	927.5	4.4	1855
RAE	1.25	132.5	0.6	265
TOTAL	10.0	1060	5	2120

2.2.3. Using a multichannel pipette or robot, pipette the Repairing/RT Master mix to each well:

- **96WP:** 10 µL per well
- **384WP:** 5 µL per well

2.2.4. Carefully seal the plate, vortex well and briefly spin it in the centrifuge.

2.2.5. Incubate in thermocycler Program 2_Repair/RT.

2.2.6. Proceed immediately to step 2.3.

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

2.3. Sample pooling and bead purification

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

NOTE: Library normalization

The volume used for pooling from each well can be adjusted to re-equilibrate the proportion of samples in the pool, helping to improve the distribution of sequencing reads in the library, especially if some samples risk obtaining too many reads.

Shallow sequencing allows for assessing the coverage per sample unequivocally. For this approach, we recommend pooling only a fraction of the RT volume from each well (i.e., 10 µL out of 20 µL) for the library preparation. After the library QC by sequencing (see section 2.8), the volume used for pooling can be re-adjusted to reduce the variation at the sequencing stage.

Perform cDNA purification with SPRI magnetic beads using a 1:1.8 ratio of cDNA pool and beads slurry. Purifying large volumes (i.e., 2 mL of the pool if 20 µL of 96 samples are pooled) requires three to four 1.5 mL tubes and a corresponding magnetic stand (Permagen, MSR06).

If the pool's volume is higher than 500 µL, split it equally in the required number of 1.5- 2 mL tubes and add the identical volume of beads (i.e., a pool of 1 mL split in 2 tubes with 500 µL per tube and add 900 µL of beads per tube).

Preparation

- Pre-warm the SPRI beads at room temperature for ~30 min.
- Prepare 5 mL of 80% ethanol.

Procedure

2.3.1. Using a multichannel pipette or robot, pool the RT samples in the reservoir (Integra, 4382 or 6318).

2.3.2. Transfer the collected pool into a 2 mL or 5 mL tube, depending on the pooled volume. The final volume will be almost three times higher due to the addition of the beads.

2.3.3. Add pre-warmed beads in a 1:1.8 ratio (i.e., for 960 µL of pooled samples, add 1728 µL of beads slurry), and mix by pipetting up and down ten times.

2.3.4. Incubate for 5 min at room temperature.

2.3.5. If needed, split the volume into a few tubes.

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

2.3.7. To wash the beads, pipette 1000 µL of freshly prepared 80% ethanol into the tube.

2.3.8. Incubate for 30 sec.

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

2.3.10. Repeat step 2.3.7 for a total of two washes.

2.3.11. Remove the tube from the magnetic stand and let the beads dry for 1-2 min.

2.3.12. Resuspend the beads in 21 µL of water and incubate for 1 min. If the number of pooled samples exceeds 96, resuspend in 42 µL of water.

2.3.13. Place tubes on the magnetic stand, wait 5 min, and carefully transfer 20 µL (or 40µL) of supernatant to a new tube to avoid bead carry-over.

2.3.14. Immediately proceed to step 2.4

If the RT pool was split into several tubes at step 2.3.5, resuspend the beads in the **first tube** in 22 µL of water. Keep other tubes closed to avoid over-drying of the beads. Transfer obtained elution to the next tube and resuspend beads. Repeat this step for every tube.

2.4. Free primer digestion

It is recommended to perform non-incorporated primer digestion immediately after pooling.

Preparation

- Pre-warm the SPRI beads at room temperature for ~30 min.
- Prepare 5 mL of 80% ethanol.
- Label 0.2 mL PCR tubes corresponding to the number of pools prepared.
- Thaw the **F-EXB** reagent at room temperature.
- Keep the **F-EXO** reagent constantly on ice.
- Prepare Program 3_FPD on the thermocycler (set the lid at 90°C):

Step	Temperature, °C	Time
Incubation	37	6 min
Incubation	80	1 min
Keep	4	pause

Procedure

2.4.1. Depending on the cDNA volume obtained from step 2.3.13, transfer **17 µL** or **34 µL** of the eluate into a new labeled 0.2 mL PCR tube.

2.4.2. Prepare the F-EXO reaction Master mix as follows (with 10% excess):

Reagent	Per reaction (µL)	
	For 17 µL elution	For 34 µL elution
F-EXB	2.2	4.4
F-EXO	1.1	2.2
TOTAL	3.3	6.6

2.4.3. According to the table, transfer **3 µL** or **6 µL** of the F-EXO reaction mix into each PCR tube with purified cDNA.

2.4.4. Mix by pipetting up and down 5 times.

2.4.5. Briefly spin down in the bench-top centrifuge.

2.4.6. Incubate in thermocycler Program 3_FPD.

2.4.7. Proceed immediately to step 2.5.1 or keep the tube at 4°C overnight.

2.5. Second-strand synthesis and DNA repair

At this step, double-stranded full-length cDNA is generated and repaired.

Preparation

- Pre-warm the SPRI beads at room temperature for ~30 min.
- Prepare 5 mL of 80% ethanol.
- Thaw the **SSB FL** reagent at room temperature and mix well before use.
- Keep the **SSE FL** reagent constantly on ice.
- Prepare Program 4_SSS on the thermocycler (set the lid at 90°C):

Step	Temperature, °C	Time
Incubation	16	60 min
Incubation	70	20 min
Keep	4	pause

Procedure

2.5.1. Add **11 µL** (17 µL elution) or **22 µL** (34µL elution) of water to the tube from step 2.4.7

2.5.2. Prepare the SSS FL Master mix for the second-strand synthesis as follows (with 10% excess):

Reagent	Per reaction (µL)	
	For 17 µL elution	For 34 µL elution
SSB FL	5.5	11.0
SSE FL	4.4	8.8
TOTAL	9.9	19.8

2.5.3. According to the table, transfer **9 µL or 19 µL** of the SSS reaction mix to the tube from step 2.4.7 and mix well by pipetting up and down 5 times.

2.5.4. Briefly spin down in the bench-top centrifuge.

2.5.5. Incubate in thermocycler Program 4_SSS.

2.5.6. Proceed immediately to step 2.5.7

cDNA clean-up with SPRI beads

Perform the cDNA purification with SPRI magnetic beads using a 1.8x beads:cDNA ratio (i.e., 90 µL of bead slurry plus 50 µL of cDNA).

NOTE: Use pre-warmed beads and vortex them vigorously before pipetting.

2.5.7. Complement the final reaction volume to 50 µL with water (if necessary).

2.5.8. Add 90 µL of beads (50µL sample) or 144 µL (80 µl sample) and mix by pipetting 10 times.

2.5.9. Incubate for 5 min at room temperature.

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

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

2.5.12. Incubate for 30 sec.

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

2.5.14. Repeat step 2.5.11 for a total of two washes.

2.5.15. Remove the tube from the magnetic stand and let the beads dry for 1-2 min.

2.5.16. Resuspend the beads in 21 µL of water.

2.5.17. Incubate for 1 min.

2.5.18. Place tubes on the magnetic stand, wait 5 min, and carefully remove 20 µL of supernatant into a new tube to avoid bead carry-over.

2.5.19. Use 2 µL to measure the concentration with Qubit (recommended).

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

2.6. cDNA adaptor ligation

At this step, the BRB-compatible adaptor is ligated to the cDNA fragments to facilitate the following amplification of the library with Unique Dual Indexing (UDI) primers.

Preparation

- Pre-warm the SPRI beads at room temperature for ~30 mins.
- Prepare 5 mL of 80% ethanol.
- Thaw the ALB and BRB.AD reagents on ice and mix well before use.
- Prepare Program 5_ADL on the thermocycler (set the lid at 90°C):

Step	Temperature, °C	Time
Incubation	20	15 min
Keep	4	pause

Procedure

2.6.1. Complement every sample from step 2.5.18 to 18µl with water (if necessary). Then pipette BRB.AD, and then add ALB as indicated in the table below. It is **not recommended** to prepare a master mix for all samples.

Reagent	Per reaction (µL)
BRB.AD	1.25
ALB	20.0
TOTAL	21.25

2.6.2. Mix well by pipetting up and down 10 times.

2.6.3. Briefly spin down the tube in the bench-top centrifuge.

2.6.4. Incubate in thermocycler Program 5_ADL.

2.6.5. Proceed immediately to step 2.6.6

cDNA clean-up with SPRI beads

Perform the cDNA purification with SPRI magnetic beads using 0.9x beads:cDNA ratio (i.e., 45 µL of bead slurry plus 50 µL of cDNA).

NOTE: Use pre-warmed beads and vortex them vigorously before pipetting.

2.6.6. Complement the final reaction volume to 50 µL with water.

2.6.7. Add 45 µL of beads and mix by pipetting 10 times.

2.6.8. Incubate for 5 min at room temperature.

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

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

2.6.11. Incubate for 30 sec.

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

2.6.13. Repeat step 2.6.10 for a total of two washes.

2.6.14. Remove the tube from the magnetic stand and let the beads dry for 1-2 min.

2.6.15. Resuspend the beads in 21 µL of water.

2.6.16. Incubate for 1 min.

2.6.17. Place tubes on the magnetic stand, wait 5 min, and carefully remove 20 µL of supernatant into a new tube to avoid bead carry-over.

2.6.18. Proceed to step 2.7.1.

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

2.7. Library indexing and amplification

At this step, the library undergoes amplification using Unique Dual Indexing (UDI) primers. The kit contains four Illumina-compatible primer pairs to generate up to four UDI libraries. The index sequences are indicated in Table 2.

The number of amplification cycles required for library preparation is usually 10-14, depending on the number and quantity of RNA samples.

It is strongly recommended that the final library bead clean-up be performed twice to remove primer dimer fragments.

Preparation

- Pre-warm the SPRI beads at room temperature for ~30 mins.
- Prepare 10 mL of 80% ethanol.
- Thaw the **LAM FL** reagents on ice and mix well before use.
- Thaw the required number of **MF.UDI Adapters** at room temperature; briefly spin them before use.
- Prepare Program 6_AMP (set the lid at 100°C) on the thermocycler:

Step	Temperature, °C	Time	Cycles
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	5-20*
Annealing and Extension	65	75 sec	
Final extension	65	5 min	1
Keep	4	pause	

*The required number of PCR cycles can be estimated based on the amount of cDNA used for adapter ligation (preferably) or the total RNA input used for the protocol. Follow the guidelines below.

Library amplification reaction setup

2.7.1. Prepare the PCR amplification reaction as follows:

Reagent	Per reaction (µL)
LAM FL	25.0
MF.UDI Adapter	5.0
Ligated cDNA	20.0
TOTAL	50.0

2.7.2. Pipette up and down 5 times.

2.7.3. Put the tube in the PCR machine and start Program 6_AMP.

2.7.4. Set the required number of PCR cycles based on the amount of cDNA used for adaptor ligation (step 2.5.19).

cDNA used for library prep, ng	Number of cycles
80	6-7
40	8
20	9
10	10
5	11
2.5	12
1.25	13
0.6	14

Indexed cDNA library clean-up with SPRI beads

Purify the final cDNA library with SPRI magnetic beads using a 0.9x ratio (45 µL of bead slurry for 50 µL cDNA library).

NOTE: Use pre-warmed beads and vortex them vigorously before pipetting.

2.7.5. Adjust the library volume to 50 µL with water.

2.7.6. Add 45 µL of beads and mix by pipetting up and down 10 times.

2.7.7. Incubate for 5 min at room temperature.

2.7.8. Place the tubes on the magnetic stand, wait 5 min, carefully remove, and discard the supernatant.

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

2.7.10. Incubate for 30 sec.

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

2.7.12. Repeat step 2.7.9 for a total of two washes.

2.7.13. Remove tubes from the magnetic stand and let the beads dry for 1-2 min.

2.7.14. Resuspend the beads in 21 µL of water.

2.7.15. Incubate for 1 min.

2.7.16. Place tubes on the magnetic stand, wait 5 minutes and carefully remove 20 µL of supernatant into a new tube to avoid bead carry-over.

2.7.17. Perform the bead clean-up once again by repeating the procedure from step 2.7.5.

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

2.8. Library quality control

Pooled library 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 in the 300 – 700 bp range with a peak at 400-500 bp; see Figure 2 for an example of a standard FL DRUG-seq (mRNA) library profile.

Importantly, the bead clean-up must be performed twice to remove primer dimer fragments, likely producing lower-quality sequencing data with reduced mappable reads (Figure 3). Therefore, it is strongly recommended that those peaks be removed by performing an additional round of SPRI bead purification with the 0.9x ratio (see step 2.7.5).

Library quantification can also be done unbiasedly by qPCR using standard Illumina library quantification kits (i.e., KAPA HiFi, Roche).

Pre-sequencing library QC:

- Use 2 µL of the library to measure the concentration with Qubit.
- Use 2 µL of the library to assess the profile with the Fragment Analyzer instrument or similar.
- If necessary, re-purify the libraries by following steps 2.7.5 – 2.7.16 to remove the peaks <300 bp.

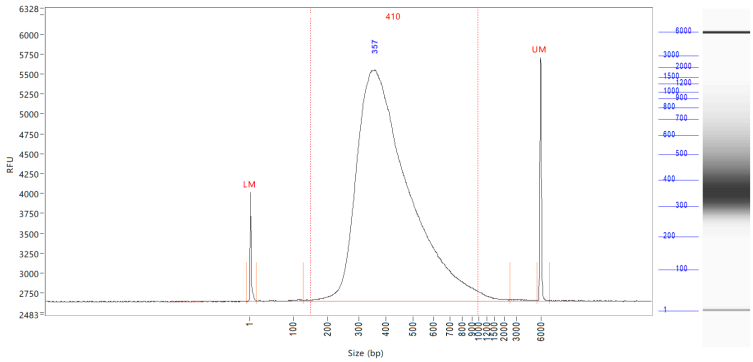


Figure 2 A successful library profile with fragments between 300-700 bp

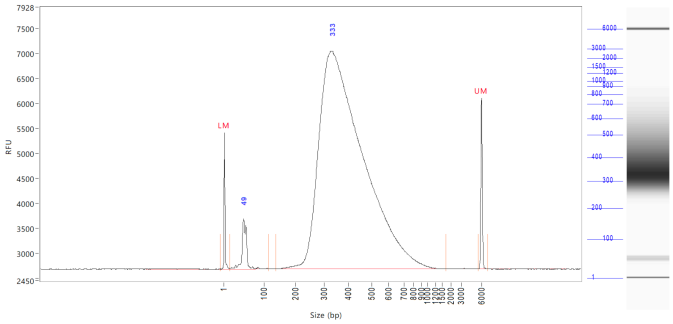


Figure 3 An example of a library profile after a single purification, demonstrating the small peak at 50 bp

Assessing uniformity of read distribution across the samples

For projects involving highly heterogeneous samples, it is recommended to validate the uniformity of read coverage across the samples by shallow library sequencing (see step 2.3). This approach ensures that every sample will obtain enough reads required for the analysis. DRUG-seq libraries can be added as spike-ins to the compatible sequencing run (see Part 3). For this validation, 0.5-1M sequencing reads per library is sufficient to assess the fraction of reads attributed to every sample.

Part 3. LIBRARY SEQUENCING

The libraries prepared with the MERCURIUS™ FL DRUG-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™ FL DRUG-seq libraries are Unique Dual-Indexed and can potentially be pooled in a sequencing run with other libraries if the sequencing structure is compatible. Please refer to Table 1 for the optimal sequencing structure and Table 2 for the list of i5 and i7 index sequences.

Given the DRUG-seq library structure, the optimal number of cycles for Read 1 is 28 (and 29 for AVITI). The following cycles, 29-60, will cover the homopolymer sequence, which may result in a significant drop of Q30 values reflecting sequencing quality. Therefore, standard Illumina or AVITI run setups (e.g., 100 PE or 150 PE) are not recommended.

Read	Length (cycles)		Comment
	for Illumina	for AVITI	
Read 1	28	29	Sample barcode (14 nt) and UMI (14 nt); +1 extra base for AVITI
Index 1 (i7) read	8	8	Library Index
Index 2 (i5) read	8*	8*	Library Index (*optional and valid for UDI libraries)
Read 2	60-90	101	Gene fragment

Table 1 Sequencing structure of DRUG-seq libraries

The Unique Dual Indexing (UDI) strategy ensures the highest library sequencing and demultiplexing accuracy and complies with the best practices for Illumina sequencing platforms. UD-indexed libraries have distinct index adapters for i7 and i5 index reads (Table 2).

Name	Type	i7 index sequence	i5 index sequence Forward Workflow	i5 index sequence Reverse Workflow
MF.UDI.1	UDI (i7/i5)	GCTTGTCA	AGGCGAAG	CTTCGCCT
MF.UDI.2	UDI (i7/i5)	CAAGCTAG	TAATCTTA	TAAGATTA
MF.UDI.3	UDI (i7/i5)	AGTTCAGG	CAGGACGT	ACGTCCTG
MF.UDI.4	UDI (i7/i5)	GACCTGAA	GTACTGAC	GTCAGTAC

Table 2 UDI adapter sequences

NOTES: Sequencing depth

1. The recommended sequencing depth is 5-10 Mio reads per sample (see Figure 4). Overall, the higher the input, the more genes can be detected at the same sequencing depth (compare 10 ng and 100 ng RNA samples in Figure 4). We recommend getting 15-20 Mio reads per sample to detect very lowly expressed genes.

2. If only one library is sequenced in a flow cell, the Index reads can be skipped.

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

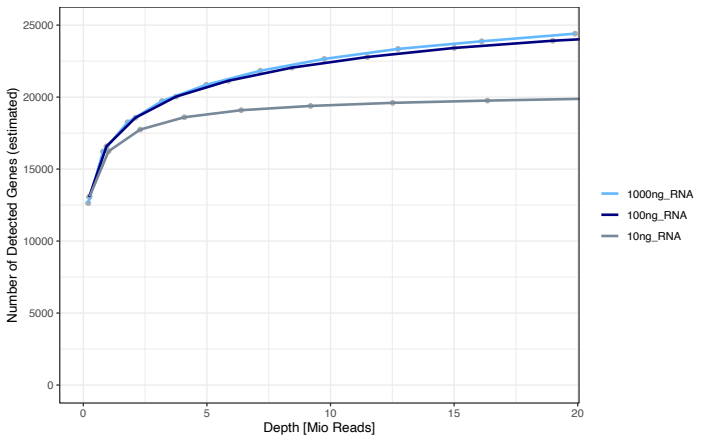


Figure 4 Number of detected genes as a function of the sequencing depth for a different quantity of starting RNA per well (Universal Human Reference RNA, Thermofisher, QS0639)

3.1. Sequencing with Illumina instruments

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

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 DRUG-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 with Element AVITI instrument

For the most optimal results, the MERCURIUS™ FL DRUG-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: Sequencing depth

Please note that the **Cloudbreak AVITI 2x75 High Output sequencing** yields 1 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 raw read1 and read2 *fastq* sequencing files (e.g., *mylibrary_R1.fastq.gz* and *mylibrary_R2.fastq.gz*).

This section explains how to generate ready-for-analysis gene, and UMI 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 gene/UMI read count generation, which can be done in parallel with the sample demultiplexing.

For manual data processing, the user requires a terminal and a server or powerful computer with an installed set of standard bioinformatic tools.

4.1. Required 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.
- **STARsolo from 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:
- x86-64 compatible processors
- 64-bit Linux or Mac OS X.
- ~30-40Gb of RAM
- **FastReadCounter** (v.1.1 or greater). Software for counting genome-aligned reads for genomic features.
- **Picard** (v.2.17.8 or greater) and **Samtools** (v.1.9 or greater). Collections of command-line utilities to manipulate with BAM files. Note: Picard requires **Java version 8 or higher** to be installed.
- R Software (version 3 or greater).
- (Optional) **BRBseqTools** (version 1.6). The software suite for processing BRB-seq libraries is available at https://github.com/DeplanckeLab/BRB-seqTools.

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

4.1.1.1 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
```

4.1.1.2 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
```

4.1.1.3 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 (i.e., L001, L002, L003, L004, not L002, L001 ...) to avoid issues when demultiplexing the samples.

4.2. Sequencing data quality check

4.2.1.1 Run fastQC on both 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
> fastqc --outdir $QCdir/ mylibrary_R2.fastq.gz
```

4.2.1.2 Check fastQC reports to assess the quality of the samples (see Software and materials).

NOTES:

- The report for the R1 *fastq* file may contain some "red flags" because it contains barcodes/UMIs. Still, it can provide useful information on the sequencing quality of the barcodes/UMIs.
- The main point of this step is to check the R2 *fastq* report. Of note, *per base sequence content* and *kmer content* are rarely green. If there is some *adapter contamination* or *overrepresented sequence* detected in the data, it may not be an issue (if the effect is limited to <10~20%). These are lost reads but most of them will be filtered out during the next step.

4.2.2. 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. It contains a "soft-clipping" tool that automatically cuts the beginning or the end of reads to improve the mapping efficiency, thus allowing the user to skip the step of trimming the reads for adapter contamination. Moreover, STAR has a mode called STARsolo, designed to align multiplexed data (such as BRB-seq) and directly generate count matrices.

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.

4.2.2.1 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.

4.2.2.2 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
```

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.
- 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.

[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

4.2.3. Aligning to the reference genome and generation of count matrices

After the genome index is created, both R1 and R2 *fastq* files can be aligned to this reference genome. For this step, use the "solo" mode of STAR, which not only aligns the reads to the reference genome but also creates the gene read count and UMI (unique molecular identifier) count matrices.

The following parameters should be adjusted according to the sequencing information:

- *--soloCBwhitelist*: a text file with the list of barcodes (one barcode sequence per lane) which is used by STAR for demultiplexing. This file is provided according to version of the MERCURIUS kit used. Example of "barcodes_96_V5C_star.txt":

```
> TACGTTATTCCGAA
> AACAGGATAACTCC
> ACTCAGGCACCTCC
> ACGAGCAGATGCAG
```

- *--soloCBstart*: Start position of the barcode in the R1 fastq file, equal to 1.
- *--soloCBlen*: Length of the barcode. This value should match the length of the barcode sequence in the file specified by –soloCBwhitelist. The barcode length depends on the version of the oligo-dT barcodes provided in the kit. For the barcode plate set V5, the default value is 14.
- *--soloUMIstart*: Start position of the UMI, it's *soloCBlen* + 1 since the UMI starts right after the barcode sequence.
- *--soloUMIlen*: The length of UMI. This parameter depends on the version of the oligo-dT barcodes in the kit and the number of sequencing cycles performed for Read1. For the barcode plate set V5 the default value is 14.
- --readFilesIn: name and path to the input *fastq* files.

The order of the fastq files provided in the script is important. The first *fastq* must contain genomic information, while the second the barcode and UMI content. Thus, files should be provided for STARsolo in the following order: --readFilesIn mylibray_R2 mylibrary_R1.

- --genomeDir: a path to the genome indices directory generated before ($genomeDir).

Output count matrix parameters:

By default, STARsolo produces a UMI count matrix, i.e., containing unique non-duplicated reads per sample for each gene. This type of count data is a standard for single-cell RNA-seq analysis. For bulk RNA-seq analysis, a gene read count matrix is usually used. The following parameters will enable the generation of the output of interest.

--soloUMIdedup **NoDedup**, will generate a read count matrix output

--soloUMIdedup **NoDedup 1MM_Directional,** will generate both UMI and read count matrices in mtx format.

This step will output *bam* files and count matrices in the folder $bamdir.

```
> STAR --runMode alignReads --outSAMmapqUnique 60 --runThreadN 8 --
  outSAMunmapped Within --soloStrand Forward --quantMode GeneCounts --
  outBAMsortingThreadN 8 --genomeDir $genomeDir --soloType CB_UMI_Simple --
  soloCBstart 1 --soloCBlen 14 --soloUMIstart 15 --soloUMIlen 14 --
  soloUMIdedup NoDedup 1MM_Directional --soloCellFilter None --soloCBwhitelist
  barcodes.txt --soloBarcodeReadLength 0 --soloFeatures Gene --
  outSAMattributes NH HI nM AS CR UR CB UB GX GN sS sQ sM --
  outFilterMultimapNmax 1 --readFilesCommand zcat --outSAMtype BAM
  SortedByCoordinate --outFileNamePrefix $bamdir --readFilesIn
  mylibrary_R2.fastq.gz mylibrary_R1.fastq.gz
```

The demultiplexing statistics can be found in the "*bamdir/Solo.out/Barcodes.stats*" file.

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).

4.2.4. Generating the count matrix from .mtx file

STARsolo will generate a count matrix (*matrix.mtx* file) located in the *bamdir/Solo.out/Gene/raw* folder. This file is a sparse matrix format that can be transformed into a standard count matrix using an R script provided below:

```
> #Myscript.R
> library(data.table)
> library(Matrix)
> matrix_dir <- "$bamdir/Solo.out/Gene/raw"
> f <- file(paste0(matrix_dir, "matrix.mtx"), "r")
> mat <- as.data.frame(as.matrix(readMM(f)))
> close(f)
> feature.names = fread(paste0(matrix_dir, "features.tsv"), header = FALSE,
  stringsAsFactors = FALSE, data.table = F)
> barcode.names = fread(paste0(matrix_dir, "barcodes.tsv"), header = FALSE,
  stringsAsFactors = FALSE, data.table = F)
> colnames(mat) <- barcode.names$V1
> rownames(mat) <- feature.names$V1
> fwrite(mat, file = umi.counts.txt, sep = "\t", quote = F, row.names = T,
  col.names = T)
```

The resulting UMI/gene count matrix can be used for a standard expression analysis following conventional bioinformatic tools.

4.2.5. Generating the read count matrix with per-sample stats (Optional)

Given a multiplex BAM file obtained with STARsolo and a set of barcodes, the software FastReadCounter produces a read count matrix with per-sample statistics with the following code:

```
> #!/bin/bash
>
> gtf_file=homo_sapience.gtf                ### GTF genome annotation file
> output_folder=counts/              ### Name of the final count output file
> bam_dir=mypath/bam_demult          ### Directory with demultiplexed bam
  files
> barcode_file=v5C_96_frc.txt        ### Barcode reference file
>
> FastReadCounter-1.0.jar" --bam ${bam_dir}/${bam_dir}.bam \
>                          --gtf "${gtf_file}" \
>                          --umi-dedup none \
>                          --barcodeFile \${barcode_file} \
>                          -o ${output_folder}
```

The resulting read count matrices can be used for subsequent gene expression analysis using established pipelines and tools.

NOTE: Please contact us at info@alitheagenomics.com in case you don't have the barcode sequences (in your email, please indicate the name of the barcode set and the PN of the barcode module).

4.2.6. Demultiplexing bam files (Optional)

Generation of demultiplexed bam files, i.e., individual bam files for each sample, might be needed in some cases, for example, for submitting the raw data to an online repository that does not accept multiplexed data (for example, GEO or ArrayExpress), or for running an established bulk RNA-seq data analysis pipeline.

For this purpose, the Picard tool can be used with the following parameters:

- $out_dir, The output directory for demultiplexed bam files
- $path_to_bam, the path to multiplexed single bam file
- $barcode_brb.txt, tab-delimited file containing 2 columns: sample_id and barcode seq. Example of barcode_96_V5C_brb.txt:

```
> Sample1     TACGTTATTCCGAA
> Sample2     AACAGGATAACTCC
> Sample3     ACTCAGGCACCTCC
> Sample4     ACGAGCAGATGCAG
```

NOTE: This file is different from the list of barcode files provided to STAR.

Run the following Picard script:

```
> #!/bin/bash
> demultiplexed_bam_out_dir=$out_dir
```

```
>  input_bam=$path_to_bam
>  barcode_info=$barcode_brb.txt
>
>  while IFS=$'\t' read -r -a line
>  do
>    sample_id="${line[0]}"
>    tag_value="${line[1]}"
>
>  java -jar /path/to/picard.jar FilterSamReads  \
>          I=${input_bam} \
>          O=${demultiplexed_bam_out_dir}/${sample_id}.bam \
>          TAG=CR TAG_VALUE=${tag_value}  \
>          FILTER=includeTagValues
>  done < "$barcode_info"
```

NOTE: Please contact us at info@alitheagenomics.com in case you don't have the barcode sequences (in your email, please indicate the name of the barcode set and the PN of the barcode module).

Appendix 1. ERCC Spike-In Control

The current protocol includes the addition of External RNA Controls Consortium (ERCC) Spike-Ins to the lysate buffer.

Prepare a 1:100 dilution of the ERCC RNA Spike-In mix in nuclease-free water. Mix 990 µL of pre-chilled water with 10 µL of ERCC. Pipette well and aliquot the dilution into 50 µL aliquots, keeping them at -20°C.

The working solution of 1x Cell Lysis Buffer with ERCC Spike-In controls consists of the following:

Reagent	96WP, µL		384WP, µL	
	Per well	96 wells +10%	Per well	384 wells +10%
CLB	6.6	700	3.3	1400
INH	1.6	170	0.8	340
Water	11	1166	5.8	2457.6
ERCC* (1:100)	0.8	84	0.1	42.4
TOTAL	20	2120	10	4240

**The final ERCC is 1:1000 in a 384-type well (equal to 50 ng of RNA/well) or 1:250 in a 96-type well (150-200 ng of RNA/well).*

Cell Lysis Buffer (CLB) preparation with ERCC

1. Thaw the CLB and ERCC tubes on ice and avoid their long-term storage.
2. Keep the nuclease-free water on ice to maintain a cold temperature.
3. Spin down all the tubes before pipetting.
4. First, add the water to a 15 mL falcon tube, then the CLB, INH, and the ERCC (in this particular order).
5. Pipette the prepared mix a few times and briefly spin the tube. Keep it on ice until further use.
6. Follow the main protocol for cell lysis procedure (step 1.4.1)

Appendix 2. mRNA enrichment protocol on beads

For the mRNA enrichment procedure, we recommend using the **NEBNext Poly(A) mRNA Magnetic Isolation kit (NEB #E7490**).

For the **96-well plate format**, we suggest following the **Express protocol** of the mentioned kit and proceeding until the elution step (to be done with FL DRUG-seq protocol, step 2.1.1). Before starting, make sure to add 30 µL of nuclease-free water to each sample to start with 50 µL for mRNA enrichment protocol.

For the **384-well plate format**, proceed with the modified version as follows. The procedure is meant to be done at room temperature. Prepare all buffers in advance and mix well before using them. If some precipitation occurs, pre-warm buffer(s) at 37°C.

Beads preparation:

1. Add a required amount of **NEBNext oligo-d(T) beads** (10 µL per sample) to a 1.5 mL tube. Consider adding 10% more and using a 5 mL tube (if needed).
2. Place the tube on the magnetic stand, wait 5 min, and carefully remove the supernatant without touching the bead pellet.
3. Remove the tube from the magnetic rack.
4. Add **2x NEBNext RNA binding buffer** (25 µL per sample) to the beads and mix by pipetting up and down at least 6 times.
5. Place the tube on the magnetic stand, wait 5 min, and carefully remove the supernatant without touching the bead pellet.
6. Remove the tube from the magnetic rack.
7. Add **2x NEBNext RNA binding buffer** (17 µL per sample) to the beads and mix by pipetting up and down at least 6 times
8. Add **nuclease-free water** (7 µL per sample) to the prepared beads.
9. Pool the beads from all tubes in one (if prepared in a few tubes).

mRNA selection:

10. Prepare the program RNA_Slct on the thermocycler (set the lid at 100°C and preheat it to 80°C). It will be used twice during the procedure.

Step	Temperature, °C	Time
Incubation	80	2 min
Incubation	25	5 min
Keep	25	pause

11. Mix beads well and add 24 µL beads to each lysate sample in a 384-well plate.
12. Seal the plate with aluminum foil and mix by vortexing. Briefly spin down in a centrifuge.
13. Incubate the plate in the thermocycler using the program RNA_Slct.
14. Remove the plate after the program is finished.
15. Place the plate on a plate magnet at RT for 5 min.
16. Remove the supernatant without disturbing the beads.
17. Keep the plate on the magnet. Gently rinse the beads by adding **FL DRUG-seq Wash buffer** (30 µL per sample) without disturbing the beads.
18. Remove the supernatant without disturbing the beads.
19. Repeat step 17 two more times with a total of three washes.
20. Remove the supernatant without disturbing the beads.
21. Remove the plate from the magnet.
22. In a separate 15 mL tube, prepare a mix of:
    - **NEBNext Tris buffer,** 15 µL per sample
    - **2x NEBNext RNA binding buffer**, 15 µL per sample
23. Mix it thoroughly and add 30 µL to each sample in a 384-well plate.
24. Seal the plate with aluminum foil and mix by vortexing. Briefly spin down in a centrifuge.

25. Place the plate in a preheated thermal cycler and run the program RNAslct.
26. Remove the plate after the program is finished.
27. Place the plate on a plate magnet at RT for 5 min.
28. Remove the supernatant without disturbing the beads.
29. Keep the plate on the magnet. Gently rinse the beads by adding **NEBNext wash buffer** (30 µL per sample) without disturbing the beads.
30. Remove the supernatant without disturbing the beads.
31. Remove the plate from the magnet and directly continue with Elution (**step 2.1.1 of the User Guide**).

Appendix 3. Compatible Illumina 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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