ALITHEA
GENOMICS

MERCURIUS™

DRUG-seq v2
Library Preparation kit
for 96 samples
(MGI)

PN 10941

User Guide

v.0.1.4.revA March 2023

Related Products

Kit name	Kit PN	Module name	Module PN
DRUG-seq v2 Library Preparation 96 kit (MGI)	10941	Barcoded Oligo-dT Adapters Module 96 samples (MGI)	10533
		DRUG-seq v2 Library Preparation Module 96 samples (MGI)	10641
		Index Adapters Module (MGI)	10611

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Kit Components

Reagents supplied

Barcoded Oligo-dT Set 96 MGI V1A10 Module (PN 10533)

Component Name	Label	96 samples (PN 10941)	Storage
Plate with 96 barcoded oligo-dT primers, Set A (PN 10433)	96 MGI V1A10 OdT	1 plate	-20°C
Aluminium Seal	-	3 pcs	-20°C/RT

DRUG-seq v2 Library Preparation Module (PN 10641)

Component Name	Label	Cap colour	Volume (µL)	Storage
RT Enzyme	RTE	magenta	18	-20°C
RT Buffer	RTB	magenta	620	-20°C
Exonuclease I Enzyme	EXO	purple	10	-20°C
Exonuclease Buffer	EXB	purple	20	-20°C
Second Strand Enzyme	SSE	orange	20	-20°C
Second Strand Buffer	SSB	orange	45	-20°C
Fragmentation Enzyme	FE	red	44	-20°C
Fragmentation Buffer	FB	red	22	-20°C
End Repair Enzyme	EE	brown	14	-20°C
End Repair Buffer	EB	brown	30	-20°C
Ligation Enzyme	LE	black	44	-20°C
Ligation Buffer	LB	black	135	-20°C
Library Amplification Mix	LAM	green	200	-20°C
Library PCR Primer Mix	LPM	transparent	25	-20°C
Control Human RNA	Ctrl hRNA	blue	10	-20°C
Nuclease-Free Water	NFW	transparent	1800	-20°C
Cell Lysis Buffer	CLB	yellow	450	-20°C
RNase Inhibitor	INH	yellow	140	-20°C

Index Adapters Module (PN 10611)

Component Name	Label	Cap colour	Volume per 20 kits, (µL)	Storage
Index Adapter 1	MGI.AD.01	transparent	100 (x2)	-20°C
Index Adapter 2	MGI.AD.02	transparent	100 (x2)	-20°C
Index Adapter 3	MGI.AD.03	transparent	100 (x2)	-20°C
Index Adapter 4	MGI.AD.04	transparent	100 (x2)	-20°C
Index Adapter 5	MGI.AD.05	transparent	100 (x2)	-20°C
Index Adapter 6	MGI.AD.06	transparent	100 (x2)	-20°C
Index Adapter 7	MGI.AD.07	transparent	100 (x2)	-20°C
Index Adapter 8	MGI.AD.08	transparent	100 (x2)	-20°C

Additional required reagents and equipment (supplied by the user)

Plasticware	Manufacturer	Product number
15 mL conical tubes	Greiner	188271
0.2ml 8-Strip Non-Flex PCR Tubes	Starlab	I1402-3700
25 mL reservoir for spin columns	Zymo	C1039-25
Disposable Pipetting Reservoir 25mL polystyrene	Integra	4382
Solution reservoir 150 mL polystyrene (optional)	Integra	6318

Reagents	Manufacturer	Product number
DNA Clean and Concentrator-5 kit	Zymo	D4014
MGIEasy DNA Clean Beads	MGI	1000005278
Qubit™ dsDNA HS Assay Kit	Invitrogen	Q32851
Qubit™ ssDNA Assay Kit	Invitrogen	Q10212
MGIEasy Circularization Kit	MGI	1000005259
High Sensitivity NGS Fragment Analysis Kit	Agilent	DNF-474
SYBR Green	Thermofisher	S7563
Ethanol, 200 proof	-	-

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
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
Vacuum manifold Qiagen Qiavac 24 Plus or similar	Qiagen	19413
Vacuum pump	-	-
Real-Time PCR Instrument (optional)	-	-
VIAFLO instrument (optional)	Integra	6001
VIAFLO 96 channel pipetting head, 0.5-12.5 µL (optional)	Integra	6101

Protocol Overview and Timing

The MERCURIUS™ DRUG-seq v2 kit allows the preparation of 3'RNA sequencing libraries for 96 RNA samples for MGI instruments in a time and cost-efficient manner. The kit includes a mild cell lysis buffer to prepare crude cell lysates that can be used directly in RT reaction, skipping the tedious RNA purification step.

The 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 BRB-seq technology can be used to generate high-quality sequencing data starting from 10'000 to 50'000 mammalian cells per well. The kit can be used for the preparation of up to 4 libraries from up to 96 samples 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.

The libraries are indexed following the ligation procedure and provided adapters. The kit contains four adapters. Every adapter can be used to prepare an individual BRB-seq library. Libraries with different adapters can be pooled and sequenced in a single flow cell.

Protocol Workflow
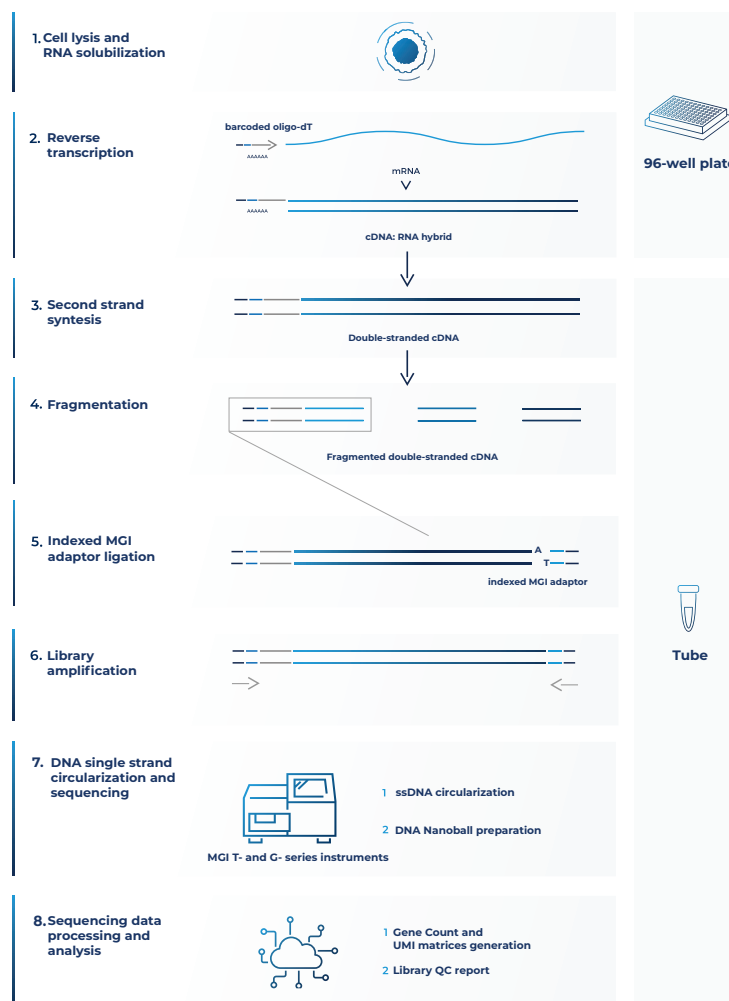
Figure 1 Schematic illustration of the protocol workflow

Part 1. PREPARATION OF CELL LYSATE SAMPLES

1.1. Essential considerations for preparing the cell lysates

- Adherent and suspension cell cultures could be used as the input material for cell lysis protocol.

- The recommended input range of cells is 10'000-50'000/well of a 96-well plate.

- Cells must be seeded a few days in advance for the best results.

- 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 potential effect of the treatment on a 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. For this, we suggest using automatized cell seeding instruments or double-verified cell count.

1.2. Cell culture preparation

Procedure for adherent cells

1.2.1.   Seed the cells in a flat bottom 96-well plate at a density that will enable to harvest of 10'000-50'000 cells per well.

1.2.2.   On the day of the cell lysis preparation, gently aspirate culture media from the plate and wash cells by adding 100 µL DPBS in each well.

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

1.2.4.   Immediately proceed to step 1.3 of this protocol.

Procedure for suspension cells

1.2.5.   Seed the cells in a flat bottom or U-shaped 96-well plate at a density that will enable to harvest of 10'000-50'000 cells per well.

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

1.2.7.   On the day of the cell lysis preparation, gently aspirate culture media from the plate without disturbing the cell pellet and wash cells by adding of 100 µL of DPBS in each well.

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

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

1.2.10.   Immediately proceed to step 1.3 of this protocol.

1.3. Cell lysis procedure

At this step, plated cells are lysed directly in a 96-well plate by adding a 1x Cell Lysis Buffer to pre-washed cells. The lysates can be directly used for the reverse transcription reaction.

Preparation

1.3.1.   Prepare a working solution of 1x Cell Lysis Buffer with RNase Inhibitor:

Reagent	Per well, µL	96 wells +10%, µL
CLB	3.30	350
INH	0.83	90
NFW	5.90	664
TOTAL	10	1104

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

1.3.3.   Prepare and label two new 96-well PCR plates and two seals.

Procedure

1.3.4.   Using a multi dispenser, distribute 10 µL of prepared CLB working solution in every well.

1.3.5.   Gently tap the plate to ensure that the lysis buffer uniformly covers the surface of each well.

1.3.6.   Incubate the plate on ice for 15 min, slightly agitating it from time to time.

1.3.7.   Using a multichannel pipette, transfer the entire volume of cell lysate from every well to a corresponding well of the new labeled 96-well PCR plate. Avoid intensive pipetting to reduce bubbles.

1.3.8.   Seal the plate with a provided Aluseal and centrifuge the plate at 600x g for 5 minutes.

1.3.9.   Remove the seal and carefully transfer the supernatant to a new labeled 96-well plate (avoid transfer of any cell debris).

1.3.10.   Discard the first plate, seal the plate with the cleared lysate, and briefly spin it down.

1.3.11.   The cleared lysates can be used directly for library preparation or safely stored at -80°C.

NOTE: If several plates must be prepared, 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

2.1. Reverse transcription

At this step, each individual cell lysate sample is reverse-transcribed using barcoded oligo-dT primers provided in a 96-well plate. Subsequently, all barcoded samples can be pooled in one tube.

Control RNA can be used as a quality control to assess the library preparation process at each step. The control RNA library should be prepared in parallel with the rest of the samples and should not be pooled with them. See Appendix 1 for the details.

Preparation

- Thaw the cell lysate samples on ice.
- Thaw the **RTB** reagent at room temperature and mix well before use.
- Briefly spin down the 96-well plate(s) containing dried oligo-dT primers. This plate will be referred to as the RT plate(s).
- Prepare Program 1_RT on the thermocycler (set the lid at 90°C):

Step	Temperature, °C	Time
Incubation	50	30 min
Inactivation	85	10 min
Keep	4	pause

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

Procedure

2.1.1.   Keep the RT plate on ice; using a multichannel pipette, transfer 10 µL of cell lysate directly to the corresponding wells and pipette 3-5 times to ensure proper reconstitution of dried oligo-dT.

2.1.2.   Carefully re-seal the RT plate(s) and briefly spin it in the centrifuge.

2.1.3.   Leave the RT plate(s) on ice for 5 min.

2.1.4.   Prepare Master Mix for RT reaction (+10%) as follows:

Reagent	Per well, µL	96 wells +10%, µL
RTB	5.0	528.0
RTE	0.1	10.6
NFW	4.9	517.5
TOTAL	**10.0**	**1056.1**

2.1.5.   Keep the RT plate(s) on ice and, using a multichannel pipette, transfer 10 µL of Master Mix to each well containing the cell lysate sample.

2.1.6.   Carefully re-seal the RT plate(s) and briefly spin it in the centrifuge.

2.1.7.   Transfer the plate to the thermocycler and start Program 1_RT.

Safe stop: After this step, the RT plate(s) can be kept at 4°C overnight.

2.2. Samples pooling and column purification

After pooling, the barcoded RT samples can be purified with either column-based Zymo Clean & Concentration Kit (Zymo, D4014) or MGIEasy DNA Clean Beads (MGI, 1000005278). Both approaches produce comparable outcomes and can be used interchangeably. Depending on the availability of 3rd party reagents and instruments, the corresponding method should be applied.

NOTE: A pool may contain some cell debris, which could block a column membrane during purification leading to a long waiting time. To avoid this, it is recommended to perform a pre-cleaning of the RT pool by passing it through the Zymo column (see below) before mixing it with 7x DNA Binding buffer.

The procedure of cDNA pre-cleaning and purification using the column-based method

After the cDNA from each well is pooled in a reservoir, mix it with a 7x volume of DNA binding buffer (Zymo, D4004-1-L). We strongly recommend using a vacuum manifold for the cDNA purification to avoid column membrane damage due to multiple centrifugation rounds.

Pooling volumes	Per well, μL	For 96 wells (mL)
First-strand cDNA	20	1.92
DNA Binding Buffer	140	13.44
TOTAL	160	15.36

Table 1 Reagent volumes to be used depending on the number of pooled samples

Preparation

- Add Ethanol to Zymo DNA Wash buffer.

Procedure

2.2.1.   Using a multichannel pipette, or pipetting robot, transfer the entire RT volume of each sample into a reservoir (Integra, 4382 or 6318).

2.2.2.   Mix the pool well and transfer it to a falcon tube with a pipette.

2.2.3.   For the RT pool pre-cleaning, place a Zymo column in a new 2 mL tube, add 800 μL of the collected pool, and briefly centrifuge.

2.2.4.   Collect a cleaned flowthrough in a new falcon tube. Repeat step 2.2.3 until all pool passes through the Zymo column. Discard the column.

2.2.5.   Using a pipette, measure the volume of the pool after cleaning, transfer it to a 50 mL falcon tube, and add a 7x DNA Binding buffer accordingly (see Table 1).

2.2.6.   Connect the 25 mL funnel (Zymo, C1039-25) to a Zymo column and place it on a vacuum manifold.

2.2.7.   Gently mix the cDNA in the binding buffer mixture in a reservoir and, using a pipetboy, transfer it to a 25 mL funnel.

2.2.8.   Turn on the vacuum pump and let the liquid pass through the column.

2.2.9.   Transfer any remaining volume to the funnel, do not let the membrane overdry.

2.2.10.   After the entire pool mix passes through the column, add 200 μL of DNA Wash buffer (with Ethanol added) directly to the membrane of the column.

2.2.11.   Repeat step 2.2.10 once the wash buffer passes through the filter.

2.2.12.   Remove the column from a vacuum manifold, put it in a 1.5 mL tube, and centrifuge for 1 min to remove leftovers from the washing buffer.

2.2.13.   Add 37 μL of NFW to the column matrix and incubate for 1 min.

2.2.14.   Transfer the column into a new labeled 1.5 mL tube and centrifuge for 30 sec.

2.2.15.   The expected volume of the eluate is 35 μL.

2.2.16.   Immediately proceed to step 2.3.

The procedure of cDNA purification using the MGIEasy DNA Clean beads-based method

Perform cDNA purification with MGIEasy DNA Clean beads at a 1:1 (DNA:beads slurry) ratio. The purification of large volumes (i.e., 2 mL of the pool from a 96-well plate) requires three 1.5 mL tubes and a corresponding magnetic stand (Permagen, MSR06).

If the volume of the pool is higher than 750 μL, split it equally in the required amount of 1.5 mL tubes and add the identical volume of beads (i.e., a pool of 1 mL divided into 2 tubes with 500 μl per tube and add 500 μL of beads per tube).

Preparation

Pre-warm MGIEasy DNA Clean beads at room temperature for 30 minutes and vortex them vigorously before pipetting.

Procedure

2.2.17.   Pool and pre-clean (optionally) the RT samples as described in steps 2.2.1 - 2.2.4.

2.2.18.   Transfer the collected pool to a 2 mL or 15 mL tube, depending on the pooled volume. Consider that the final volume will be twice higher due to adding the beads.

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

2.2.20.   Incubate for 5 min at room temperature.

2.2.21.   Place the tube on the magnetic stand, wait 5 min or until a solution becomes completely clear, then carefully remove and discard the supernatant.

2.2.22.   To wash the beads, pipette 1 mL of freshly prepared 80% ethanol into the tube.

2.2.23.   Incubate for 30 sec.

2.2.24.   Carefully remove the ethanol without touching the beads pellet.

2.2.25.   Repeat step 2.2.22 for a total of two washes.

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

2.2.27.   Resuspend the beads in 37 μL of NFW.

2.2.28.   Incubate for 1 min.

2.2.29.   Place tubes on the magnetic stand and wait for 5 min.

2.2.30.   Gently aspirate 35 μL of supernatant and transfer it into a new tube. Avoid beads carry-over.

2.2.31.   Immediately proceed to step 2.3.

If the RT pool was split into several tubes for MGIEasy DNA Clean beads purification, at step 2.2.27, use one of the following options:

   • **[Two tubes only]**, resuspend the beads in **both tubes** in 20 μL/tube, and combine both elutions in one tube;

   • **[Two and more tubes]**, resuspend the beads in the **first tube** in 40 μL of NFW. 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;

   • **[Two and more tubes]**, resuspend **every** tube in 37 μL. Combine all elutions in one tube and perform one additional purification of the pool adding beads slurry accordingly to the pool volume (steps 2.2.19 - 2.2.30). Elute in 37 μL of NFW and collect 35 μL in a new tube.

2.3. Free primer digestion

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

Preparation

- Label 0.2 mL PCR tubes corresponding to the number of pools prepared.
- Thaw **EXB** at room temperature.
- Keep **EXO** on ice.
- Prepare the Program 2_FPD on the thermocycler (set the lid at 90°C):

Step	Temperature, °C	Time
Incubation	37	30 min
Incubation	80	20 min
Keep	4	pause

Procedure

2.3.1.   Depending on the cDNA volume obtained from step 2.2, transfer 35 µL of the eluate from each tube into a new labeled 0.2 mL PCR tube.

2.3.2.   Prepare the EXO reaction mix as follows (with 10% excess):

Reagent	Per reaction, µL
EXB	4.0
EXO	1.0
TOTAL	5.0

2.3.3.   According to the table, transfer 5 µL of **EXO** reaction mix into each PCR tube with purified cDNA.

2.3.4.   Mix by pipetting up and down 5 times.

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

2.3.6.   Incubate in thermocycler Program 2_FPD.

2.3.7.   Proceed immediately to step 2.4. or keep the tube at 4°C overnight.

Safe stop: After this step, the tube(s) can be kept at 4°C overnight.

2.4. Second strand synthesis

At this step, the double-stranded full-length cDNA is generated and purified using magnetic beads.

Preparation

- Pre-warm MGIEasy DNA Clean beads at room temperature for ~30 min.
- Prepare 5 mL of 80% ethanol.
- Thaw the **SSB** reagent at room temperature and mix well before use.
- Keep **SSE** reagent constantly on ice.
- Prepare the Program 3_SSS on the thermocycler (set the lid at 70°C):

Step	Temperature, °C	Time
Incubation	37	20 min
Incubation	65	30 min
Keep	4	pause

Second strand synthesis reaction setup

2.4.1. Prepare the SSS reaction mix for the second strand synthesis as follows (with 10% excess):

Reagent	Per reaction, µL
SSB	7.0
SSE	3.0
TOTAL	10.0

2.4.2. Transfer 10 µL of SSS reaction mix to the tube from step 2.3.7 and mix well by pipetting up and down 5 times.

2.4.3. Incubate in thermocycler Program 3_SSS.

2.4.4. Proceed immediately to step 2.4.5.

cDNA clean-up with MGIEasy DNA Clean beads

Perform the double-stranded cDNA purification with MGIEasy DNA Clean beads using a 0.6x ratio (i.e., 30 µL of bead slurry plus 50 µL of cDNA).

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

2.4.5. Complement the final volume to 50 µL with NFW.

2.4.6. Add 30 µL of beads and mix by pipetting up and down 10 times.

2.4.7. Incubate for 5 min at room temperature.

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

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

2.4.10. Incubate for 30 sec.

2.4.11. Carefully remove the ethanol without touching the beads pellet.

2.4.12. Repeat step 2.4.9 for a total of two washes.

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

2.4.14. Resuspend the beads in 21 µL of NFW.

2.4.15. Incubate for 1 min.

2.4.16. 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.

2.4.17. Use 2 µL to measure the concentration with Qubit.

2.4.18. [Optional] Assess the cDNA profile via TapeStation or Fragment Analyzer.

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

2.5.   Fragmentation and Index Adapter Ligation

At this step, the full-length cDNA is fragmented, and MGI library indexing adapters are ligated. The adapter index sequences are indicated in Appendix 2.

Depending on the amount of cDNA used in fragmentation indexing primers should be added to the PCR amplification mix at different stock concentrations. It is recommended to use 20 ng of cDNA for fragmentation to obtain a higher library complexity with less PCR amplification.

To ensure proper balancing of bases in the sequencing run, the indexes of all eight provided Adapters should be used to index the libraries subjected to sequencing on the same flow cell lane. If only one library will be sequenced per flow cell, use a pooled Index Adapter prepared by mixing an equal volume of each of the 8 Adapters provided. See Table 22 for different pooling scenarios depending on the number of sequencing libraries to be sequenced on the same lane.

1 library	2 libraries	3 libraries	4 libraries	5 libraries	6 libraries	7 libraries	8 libraries
MGI.AD.01 MGI.AD.02 MGI.AD.03 MGI.AD.04 MGI.AD.05 MGI.AD.06 MGI.AD.07 MGI.AD.08	MGI.AD.01 MGI.AD.02 MGI.AD.03 MGI.AD.04	MGI.AD.01 MGI.AD.02 MGI.AD.03	MGI.AD.01 MGI.AD.02	MGI.AD.01 MGI.AD.02	MGI.AD.01 MGI.AD.02	MGI.AD.01 MGI.AD.02	MGI.AD.01
							MGI.AD.02
			MGI.AD.03 MGI.AD.04	MGI.AD.03 MGI.AD.04	MGI.AD.03 MGI.AD.04	MGI.AD.03	MGI.AD.03
		MGI.AD.04 MGI.AD.05 MGI.AD.06				MGI.AD.04	MGI.AD.04
	MGI.AD.05 MGI.AD.06 MGI.AD.07 MGI.AD.08		MGI.AD.05 MGI.AD.06	MGI.AD.05 MGI.AD.06	MGI.AD.05	MGI.AD.05	MGI.AD.05
					MGI.AD.06	MGI.AD.06	MGI.AD.06
		MGI.AD.07 MGI.AD.08	MGI.AD.07 MGI.AD.08	MGI.AD.07	MGI.AD.07	MGI.AD.07	MGI.AD.07
				MGI.AD.08	MGI.AD.08	MGI.AD.08	MGI.AD.08

Table 2 Index Adapter mix preparation to ensure the best balancing

Preparation

- Pre-warm MGIEasy DNA Clean beads at room temperature for ~30 min.
- If needed, prepare fresh 5 mL of 80% ethanol.
- Thaw the **FB, EB,** and **LB** reagents at room temperature and mix well before use.
- Keep the **FE, EE,** and **LE** reagents constantly on ice.
- Thaw the required number of **Index Adapters (MGI.AD.01 - MGI.AD.08)** at room temperature and briefly spin them before use.
- Depending on the amount of cDNA used for fragmentation, pre-dilute **Index Adapters** (15 µM stock) to the indicated concentration (0.6 µM or 3 µM).

Procedure

2.5.1.   Prepare the **Fragmentation Mix** on ice in a PCR tube. If several cDNA samples are fragmented, prepare the Master mix as follows (with 10% excess):

Reagent	Per reaction, µL
FB	5.0
FE	10.0
cDNA	X
NFW	35 – X
TOTAL	50.0

2.5.2.   Keep the mix on ice and pipette up and down 10 times. Vortex gently to ensure thorough mixing. Spin down briefly.

2.5.3.   Incubate for 10 min at 37°C in the PCR machine (pre-cool it to 4°C before starting the incubation). Keep the tube on ice or at 4°C.

2.5.4.   Prepare the **End Repair Mix** on ice in a PCR tube. If several cDNA samples are fragmented, prepare the Master mix as follows (with 10% excess):

Reagent	Per reaction, µL
EB	7.0
EE	3.0
TOTAL	10.0

2.5.5.   Add 10 µL of the **End Repair Mix** to the reaction mix from step 2.5.3.

2.5.6.   Pipette and vortex gently to ensure thorough mixing, and spin down briefly.

2.5.7.   Incubate for 30 min at 65°C in the PCR machine. Keep the tube on ice or at 4°C.

2.5.8.   Prepare the **Ligation Mix** as follows. If several libraries are to be prepared and sequenced in one run, use different adapters. Depending on the amount of cDNA used at step 2.5.1, dilute the Adapter to the indicated concentration and use 5 µL of prepared dilution.

Reagent	Per reaction (µL), for cDNA inputs		
	<1 ng	1-10 ng	10-20 ng
LB	30.0	30.0	30.0
LE	10.0	10.0	10.0
Index Adapter or Adapter Mix	5.0 (of 0.6 µM stock)	5.0 (of 3 µM stock)	5.0 (of 15 µM stock)
NFW	5.0	5.0	5.0
TOTAL	50.0	50.0	50.0

2.5.9.   Add 50 µL of the **Ligation Mix** to the reaction mix from step 2.5.7

2.5.10.   Pipette and vortex gently to ensure thorough mixing, and spin down briefly.

2.5.11.   Incubate for 15 min at 20°C in the PCR machine. Keep the tube on ice or at 4°C.

Fragmented cDNA clean-up with MGIEasy DNA Clean beads

Purify the fragmented cDNA with MGIEasy DNA Clean beads using a 0.8x ratio.

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

2.5.12.   Add 88 µL of beads to 110 µL of fragmented cDNA and mix by pipetting up and down 10 times.

2.5.13.   Incubate for 5 min at room temperature.

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

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

2.5.16.   Incubate for 30 sec.

2.5.17.   Carefully remove the ethanol without touching the beads pellet.

2.5.18.   Repeat steps 2.5.15 - 2.5.17 for a total of two washes.

2.5.19.   Remove tubes from the magnetic stand and let the beads dry for 1-2 minutes.

2.5.20.   Resuspend the beads in 21 µL of NFW.

2.5.21.   Incubate for 1 min.

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

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

2.6.   Library amplification

At this step, the 5' terminal fragments are amplified using the **Library PCR Primer mix**.

The number of amplification cycles required for library preparation is usually in the range of 12-17. We recommend performing 15 cycles for the 10-20 ng of input cDNA. The optimal number depends on the RNA samples and the input cDNA amount used for fragmentation. To determine the optimal number of cycles, follow the Library Quantification Protocol in Appendix 3.

Preparation

- Pre-warm the MGIEasy DNA Clean beads at room temperature for ~30 mins.
- Prepare 10 mL of 80% ethanol.
- Thaw the **LAM** reagent on ice and mix well before use.
- Thaw the required number of **LPM** at room temperature and briefly spin before use.
- Prepare the Program 4_AMP (set the lid at 100°C) on the thermocycler (* The exact number of PCR cycles should be determined following Appendix 3):

Step	Temperature, °C	Time	Cycles
Initial denaturation	98	30 sec	
Denaturation	98	10 sec	
Annealing	63	30 sec	15 or TBD*
Extension	72	1 min	
Final extension	72	5 min	
Keep	4	pause	

Library amplification reaction setup

2.6.1.   Prepare the PCR amplification reaction as follows:

Reagent	Per reaction, µL
LAM	25.0
LPM	5.0
Fragmented cDNA	20.0
TOTAL	50.0

2.6.2.   Pipette up and down 5 times.

2.6.3.   Put the tube in the PCR machine and start Program 4_AMP with the cycles shown in Appendix 3 to determine the optimal number of amplification cycles.

Indexed cDNA library clean-up and double-side size selection with MGIEasy DNA Clean beads

For optimal results, the library should be double-side size selected with MGIEasy DNA Clean beads to preserve the fragments in a range of 200-800 bp.

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

2.6.4.   Adjust the library volume to 50 µL with NFW.

2.6.5.   Add 25 µL of beads and mix by pipetting up and down 10 times.

2.6.6.   Incubate for 5 min at room temperature.

2.6.7.   Place the tubes on the magnetic stand, wait 5 min, and carefully transfer 75 µL of the supernatant to the new labeled tube.

2.6.8.   Discard tubes with beads.

2.6.9.   Add 15 µL of beads slurry to the tube containing the supernatant obtained from step 2.6.7. to obtain the final ratio of 0.8x.

2.6.10.   Incubate for 5 min at room temperature.

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

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

2.6.13.   Incubate for 30 sec.

2.6.14.   Carefully remove the ethanol without touching the beads pellet.

2.6.15.   Repeat step 2.6.12 for a total of two washes.

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

2.6.17.   Resuspend the beads in 21 µL of NFW.

2.6.18.   Incubate for 1 min.

2.6.19.   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.

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

2.7.   dsDNA library quality control

Pooled library quality control

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

NOTE: The final library yield should be >1 pmol (in 48 µL) to allow successful downstream circularization. In case of lower yield, a few extra PCR cycles can be performed. Follow steps 2.6.1 – 2.6.19.

A successful library contains fragments in the range of 200 – 800 bp with a peak at 400-700 bp; see Figure 2 for an example of a standard BRB-seq library profile.

Underfragmented libraries have a broader fragment range distribution with a peak at 1200-2000 bp (Figure 3). Only a fraction of such libraries contains fragments that can be efficiently sequenced; therefore, for the best results, it is recommended to re-fragment the cDNA.

Importantly, fragments >1000 bp will impact the downstream library circularization and DNB preparation procedure (Figure 4). Therefore, it is strongly recommended to perform a double-side size selection (see step 2.6.4).
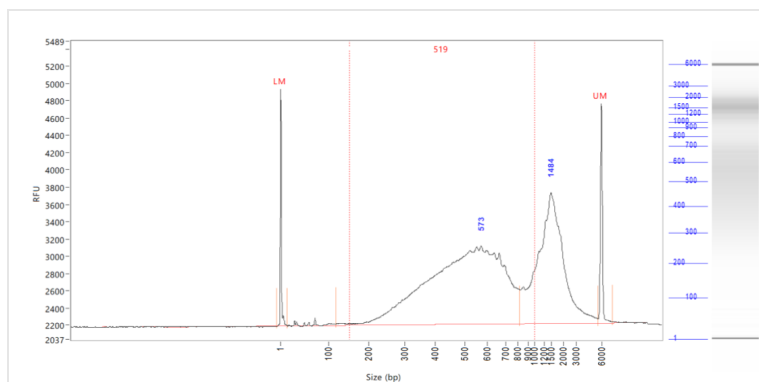
Pre-sequencing library QC:

- 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.

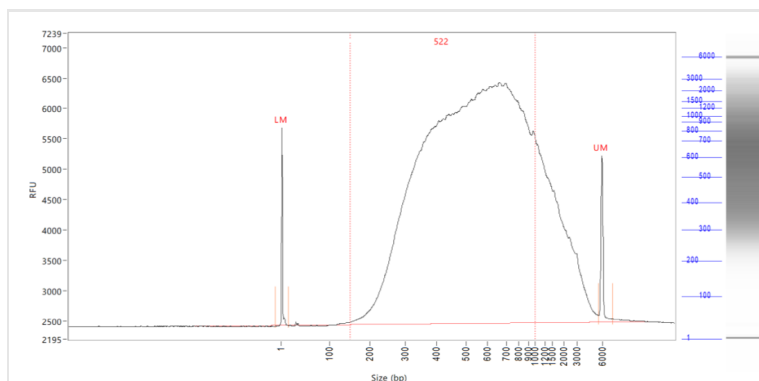**Figure 2** The successful library profile with fragments 200-1000 bp.
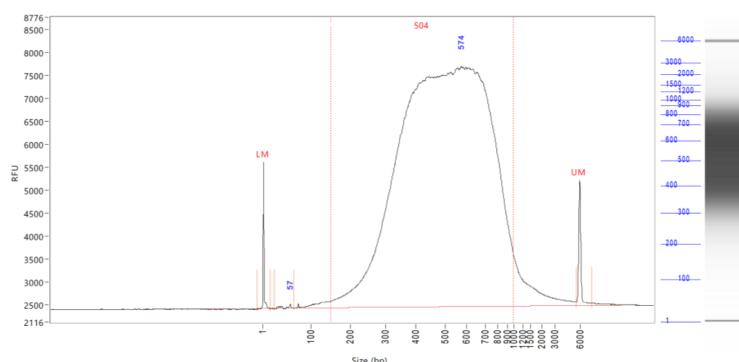
Figure 3 The example of an under-fragmented library profile with an extra peak >1000 bp.

Figure 4 The example of library profile after a single purification (0.8x) with a >1000bp smear.

Assessing uniformity of reads distribution across the samples.

For projects involving highly heterogeneous RNA samples, it is recommended to validate the uniformity of read coverage across the samples by shallow library sequencing (see step 2.2). This approach ensures that every sample will obtain enough reads required for the analysis. BRB-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.

2.8. Library circularisation and DNA Nanoball (DNB) preparation

Follow the manual of the MGIEasy Circularization kit (MGI, 1000005259) to prepare DNB using a double-stranded fragmented cDNA library from step 2.6 as the input.

Part 3.   LIBRARY SEQUENCING

Each DNB library prepared with MERCURIUS™ DRUG-seq v2 kit for MGI is indexed with a single Index Adapter or a mix of Adapters to ensure proper MGI Barcode (Index) balancing (see Appendix 2).

The DNB libraries prepared with different MGI Barcode indexing pools can be sequenced on the same lane of the flow cell. The sequencing structure is provided in Table 3.

Read	Cycles number	Comment
Read1	23	• Sample barcode: cycles 1-14 (12 + 2 "dark mode" cycles) • UMI: cycles 15-23 (9 cycles) **NOTE:** Cycles 13 and 14 should be done in a "dark mode".
MGI Barcode Read	10	MGI Library Barcode (index) read
Read2	80-150	Gene fragment

Table 3 Sequencing structure of BRB-seq libraries

NOTE: Sequencing depth

• The recommended sequencing depth is 1-5 M reads per sample. Deeper sequencing can also be performed to enable the detection of very low expressed genes.

• If only one library is sequenced on a lane of a flow cell, the MGI Barcode Read can be skipped.

• The loading molarity for the library depends on the sequencing instrument and should be discussed with the sequencing facility or an experienced person.

Part 4.　SEQUENCING DATA PROCESSING

Following MGI 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 genes, and UMI read count matrices from raw *fastq* files.

4.1.　OPTION 1: Cloud-based platform for data processing

The users of BRB-seq kits are offered free access to the devoted cloud-based BRB-seq data processing pipeline. This easy-to-use workflow enables the data processing to generate gene and UMI count matrices for the downstream RNA-seq analysis from the raw fastq files. The pipeline also produces a QC report and can be operated by a user without bioinformatics or coding skills.

The pipeline, still in the developer beta testing mode, is accessible via the following link (https://gcw-portal-ali.genomsys.com/). Please request credentials to access the pipeline by email at info@alitheagenomics.com

Currently, only the human, mouse, and fly genomes are supported.

4.2.　OPTION 2: Manual data processing

To obtain the data read for analysis, the user needs to align the sequencing reads to the genome and perform the gene/UMI read count generation that can be done in parallel with the sample demultiplexing.

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

4.2.1.　Required software

- **fastQC** (version v0.11.7 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 2.7.10a)**.** Software for reads 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
- **FeatureCounts** (v.1.6.0 or greater). Software for counting genome-aligned reads for genomic features, such as genes and exons.
- **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 from: https://github.com/DeplanckeLab/BRB-seqTools.

4.2.2.　Merging *fastq* files from individual lanes and/or libraries

After sequencing each library will result in generating 8 individual Read1 and Read2 (R1/R2) fastq files corresponding to library barcodes. For the optimal downstream processing those fastq files should be merged in a single R1.fastq and R2.fastq files.

4.2.2.1　Below is an example of four R1/R2 *fastq* files

```
>    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
```

4.2.2.2   Use the `cat` command in a terminal to merge the files. Following example 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
```

4.2.2.3   Move these 2 *fastq* files in 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 or lanes of the same flow cell.

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.

4.2.3. Sequencing data quality check

4.2.3.1   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
```

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

NOTES:

- Report for 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.

4.2.4. 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 the 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 complete in advance and the index files can be stored on the server for subsequent analyses.

4.2.4.1   Download the right genome assembly fasta file (e.g. Homo_sapiens.GRCh38.dna.primary_assembly.fa) and gene annotation file in gtf format (e.g. Homo_sapiens.GRCh38.102.gtf) from Ensembl or UCSC repository. Below is an example for a human assembly:

```
>    wget ftp:// ftp.ensembl.org/pub/release-
     102/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz
>    gzip -d Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz # unzip
>    wget ftp:// ftp.ensembl.org/pub/release-
     102/gtf/homo_sapiens/Homo_sapiens.GRCh38.102.gtf.gz
>    gzip -d Homo_sapiens.GRCh38.102.gtf.gz # unzip
```

---

[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

> **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.

4.2.4.2 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.GRCh38.102.gtf --runThreadN 8
```

> **NOTES:**
>
> - The --runThreadN parameter can be modified depending on the number of cores available on your machine. The larger is this number, the more parallelized/fast will be the indexing.
>
> - Depending on the genome assembly, STAR can use up to 32-40Gb of RAM. So, you should use a machine that has this RAM capacity.

4.2.5. 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_MGI V1A10_ star.txt":

```
>   TGGATTAGCAAC
>   CGATAAGTTCAC
>   CCATCTTAACGG
>   TTGCCTAAGTGA
>   ACTCAGACGTAT
```

- *--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 barcodes plate set V5 the default value is 12.
- *--soloUMIstart*: Start position of the UMI, the default value is 15.
- *--soloUMIlen*: The length of UMI. This parameter depends on the version of the oligo-dT barcodes in the kit and number of sequencing cycles performed for the Read1. For barcodes plate set V5 the default value is 10.
- --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 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. Following parameters will enable to generate the output of interest.

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

--soloUMIdedup **NoDedup 1MM_All,** 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 12 --
    soloUMIstart 15 --soloUMIlen 10 --soloUMIdedup NoDedup 1MM_All --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).

4.2.6. 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 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.

4.2.7. Demultiplexing bam files (Optional)

Generation of demultiplexed bam files, i.e., individual bam file 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 dir 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_MGI_V1A10.txt:

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

NOTE: this file is different from the list of barcodes file 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)

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

After splitting (demultiplexing) the *bam* file into files for each sample, produce a read counting matrix with per-sample statistics using the featureCounts tool with the following code:

```
>   #!/bin/bash
>
>   gtf=homo_sapience.gtf                  ### GTF genome annotation file
>   output_name=read_counts.txt            ### Name of the final count output file
>   bam_dir=mypath/bam_demult              ### Directory with demultiplexed bam
    files
>
>   featureCounts [options] -t exon -g gene_id -s 1 -a $gtf -o $output_name
    ${bam_dir}/*.bam
```

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

Appendix 1.   Assessing the kit performance with in-line Control Human RNA

Introduction

Control Human RNA (Ctrl hRNA) is provided in the BRB-seq kits as a positive control to assess the kit's performance during the library preparation. The tube provided in the kit contains 10 µL of 0.5 µg/µL (5 µg) of human total RNA from HEK293T cells. It can be used at various amounts per well for RT reaction (e.g., at 50 ng/well for 96 wells or 1 µg/well for 5 wells). The section below describes the best practices and the expected results from using 0.5-5 µg of RNA per well and following the detailed protocol in Part 2 of this User Guide.

Expected results during library preparation

The RT was performed using the reagents from a BRB-seq kit (PN 10811) in 20 µL per well using 0.5-5 µg of total RNA. Each sample was either directly purified using DNA Clean and concentrator kit (Zymo, D4014), or wells were pooled (RT from 5 wells with 1 µg of RNA each) before column purification. After elution, each sample was treated with Exo and subjected to the second strand synthesis. Samples were then purified using AMPure beads (0.6x ratio, Beckman Coulter CNGS-0050) and eluted in 20 µL with water. cDNA was quantified using Qubit and profiled with a Fragment Analyzer (Agilent, model 5200). Figure 54A shows the estimation of cDNA yield, and Figure 54B demonstrates the expected cDNA profile.
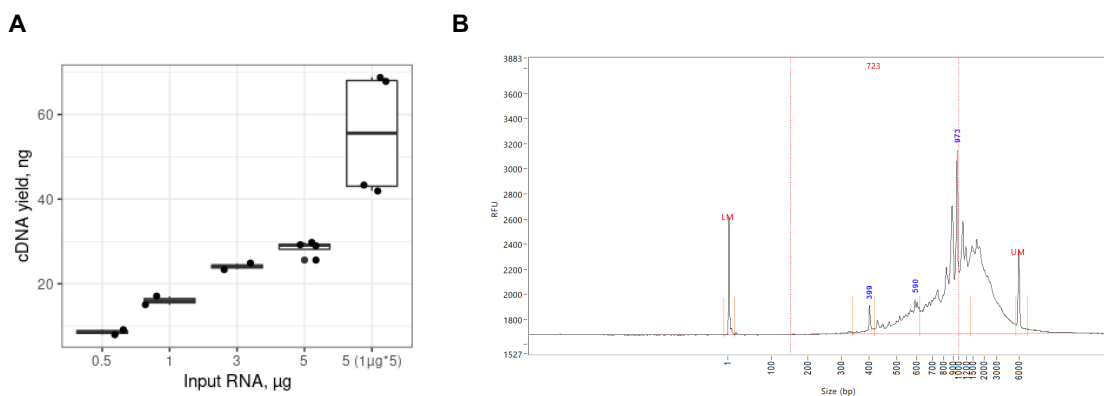
Figure 5 A. Expected cDNA yield from total human RNA used in a single RT reaction (0.5-5 µg/reaction) or 5 RT reactions with 1 µg/well (n= 4). **B**. Expected cDNA profile.

Various amounts (1-20 ng) of cDNA prepared from control RNA were used for the library preparation following the fragmentation, purification with AMPure beads (0.6x), PCR amplification, and a double-side size of purification with AMPure beads.

The amount of cDNA for fragmentation can vary, and we recommend keeping some cDNA for a backup. Typically, 10 ng of cDNA is sufficient to obtain 60-80 ng of DNA library after 12 cycles of amplification. Table 45 shows the approximate amount of amplification cycles and expected library yield, and Figure 6 shows the fragment size distribution.

| Fragmented cDNA, ng	# PCR cycles	Expected library yield, ng/µL	Library purification (Right-side/Left-side)
1	15	3-6	
10	12	4-8	0.5x / 0.8x
20	11	4-8	

Table 4 Expected yield of BRB-seq libraries from different amounts of fragmented cDNA

The final yield of the library and the profile may vary depending on RNA quality and species of origin as well as on the pipetting manipulations during the purification steps.

Figure 6 BRB-seq library profiles prepared by fragmentation of 1 ng (blue), 10 ng (green) or 20 ng (red) of cDNA after a double-side size selection
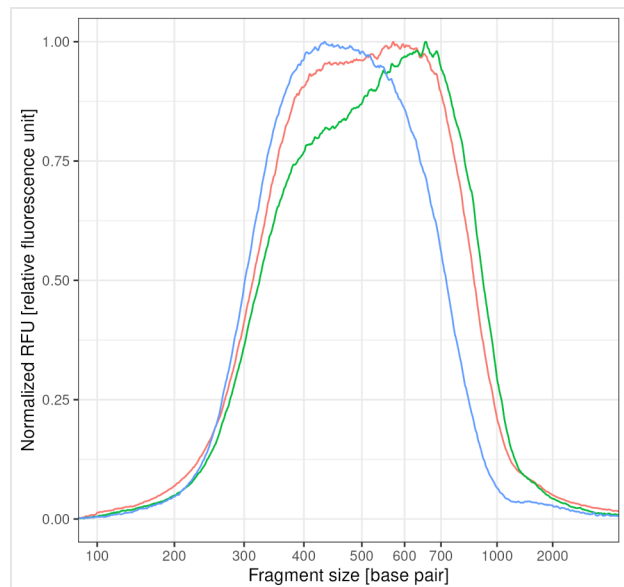
Appendix 2. MGI Adapter sequences

Adapter name	Barcode Name	Barcode 2 sequence in oligo	Barcode 2 sequence in the list
Adapter 1	MGI.AD.01	ATACTCACGC	GCGTGAGTAT
Adapter 2	MGI.AD.02	ATGCTCCGCG	CGCGGAGCAT
Adapter 3	MGI.AD.03	TGTGAACTTG	CAAGTTCACA
Adapter 4	MGI.AD.04	GAGAGGTGCT	AGCACCTCTC
Adapter 5	MGI.AD.05	TGCACTGTAA	TTACAGTGCA
Adapter 6	MGI.AD.06	GCCTAGGCAA	TTGCCTAGGC
Adapter 7	MGI.AD.07	CCATCATAGC	GCTATGATGG
Adapter 8	MGI.AD.08	CATGGTAATT	AATTACCATG

Table 5 MGI adapter sequences

Appendix 3. Library quantification with qPCR

To determine the optimal number of cycles, the following library quantification procedure using real-time PCR is recommended at step 2.6.3.

- After purification of the fragmented library set up the PCR amplification reaction and perform 5 cycles of library preamplification (Program 4_AMP).
- Put the tube on ice.
- Use 5 µL aliquot from the PCR reaction to prepare a qPCR reaction mix in the appropriate PCR tube or plate as follows:

| Reagent	Per reaction, µL	N +10%, µL
5 cycles pre-amplified library	5.0	
SYBR 100X*	0.1	
LAM	2.5	
NFW	2.4	
TOTAL	10.0	

* Prepare 100X dilution with NFW from 10'000X stock

- Put it in the qPCR machine and run with the following program:

Step	Temperature, °C	Time	Cycles
Initial denaturation	98	1 min	
Denaturation	98	10 sec	
Annealing	63	30 sec	25
Extension + acquisition	72	1 min	

- Determine the cycle number depending on the growth curve in the multicomponent plot as in Figure 76. Of note, we recommend adding extra 4 cycles to the number corresponding to 1/3 of the growth curve (e.g., if a graph shows 10 cycles, perform PCR with 14 cycles).
- Perform PCR with a determined additional number of cycles with the tube containing the remaining 45 µL of the pre-amplified library.

Figure 7 Determination of the additional number of amplification cycles with qPCR
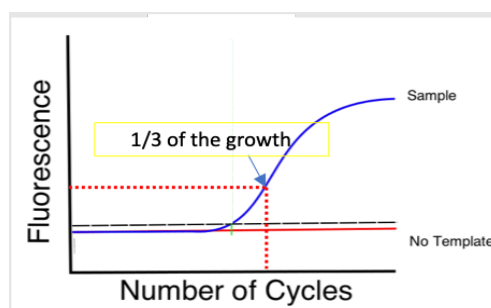
Contact information

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