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LUTHOR HD
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High-Definition Single-Cell 3' mRNA-Seq Library Prep Kit

User Guide

Catalog Numbers:
204 (LUTHOR High-Definition Single-Cell 3' mRNA-Seq Library Prep Kit)
221 (LUTHOR High-Definition Single-Cell 3' mRNA-Seq Library Prep Kit with UDI Set B1)

204UG513V0100

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1. Overview

Low-throughput, Ultra-low input T7 High-resolution Original RNA (LUTHOR) 3' mRNA-Seq Library Prep Kits use Lexogen's proprietary THOR (T7 High-resolution Original RNA) Amplification technology and library preparation methods. LUTHOR HD reliably generates 3' mRNA-Seq libraries from ultra-low input RNA or intact single cells. RNA is amplified directly from the mRNA template. The technology is template-switch- and ligation-free and maintains a true representation of the original mRNA transcripts. Cell suspensions containing 1 or more cells (up to 100 cells), or FACS-sorted, intact single cells can be used as input. Cells are lysed prior to RNA amplification. For singularization by FACS, cells can be directly sorted into Cell Lysis Buffer. Alternatively, ultra-low amounts of purified total RNA (10 pg - 1 ng) can be used without the need for prior poly(A) enrichment or rRNA depletion.

THOR Amplification is initiated by oligo(dT) primed reverse transcription to stabilize the RNA template (Fig. 1). The primer contains a 12 nt long Unique Molecular Identifier (UMI), a partial Illumina-compatible P7 linker and a T7 promoter sequence. The proprietary THOR reaction removes the single-stranded 3' poly(A) overhang and generates a double stranded T7 promoter sequence for RNA amplification. During the subsequent *in vitro* transcription step, the original mRNA template is copied repeatedly by linear amplification. Antisense-RNA copies are generated, which contain the partial Illumina-compatible P7 linker followed by a poly(U) sequence at the 5' end. As the RNA copies do not contain the promoter sequence required for initiation of *in vitro* transcription, only the original template RNA molecule can be amplified. This prevents systematic errors, i.e., errors that are introduced in an early step of conventional protocols and propagated by amplification of the erroneous copy.

LUTHOR HD library generation is initiated by random priming using Lexogen's proprietary Displacement Stop technology maintaining high strand specificity. No prior RNA fragmentation is necessary. Random Displacement Stop Primers are hybridized to the amplified RNAs. These primers contain partial Illumina-compatible P5 sequences. The reverse transcription enzyme extends the primer. The primer hybridized closest to the 5' end of the antisense RNA copy (which corresponds to the 3' end of the original endogenous RNA molecule) generates a fragment encompassing the partial P5 and P7 linker.

During Library Amplification, only fragments corresponding to the 3' end of the original RNA template are amplified, as these contain both P5 and P7 partial sequences. During PCR, i7 and i5 indices, as well as complete adapter sequences required for cluster generation on Illumina instruments, are added. Lexogen's Unique Dual Indices are provided with the LUTHOR HD kits as a bundle with Set B1 (Cat. No. 221); additional Lexogen UDI 12 nt Sets A1 – A4 are available separately (Cat. No. 101 - 104.96, or 156.384). The Lexogen UDI 12 nt Sets contain up to 384 pre-mixed i5 and i7 indices with superior error correction capacity and are provided in a convenient 96-well format.

LUTHOR HD contains the Read 1 linker sequence in the random primer, hence NGS reads are generated towards the poly(A) tail and directly correspond to the mRNA sequence. A limited paired-end read out (12 nt in Read 2) is required for UMI read out.

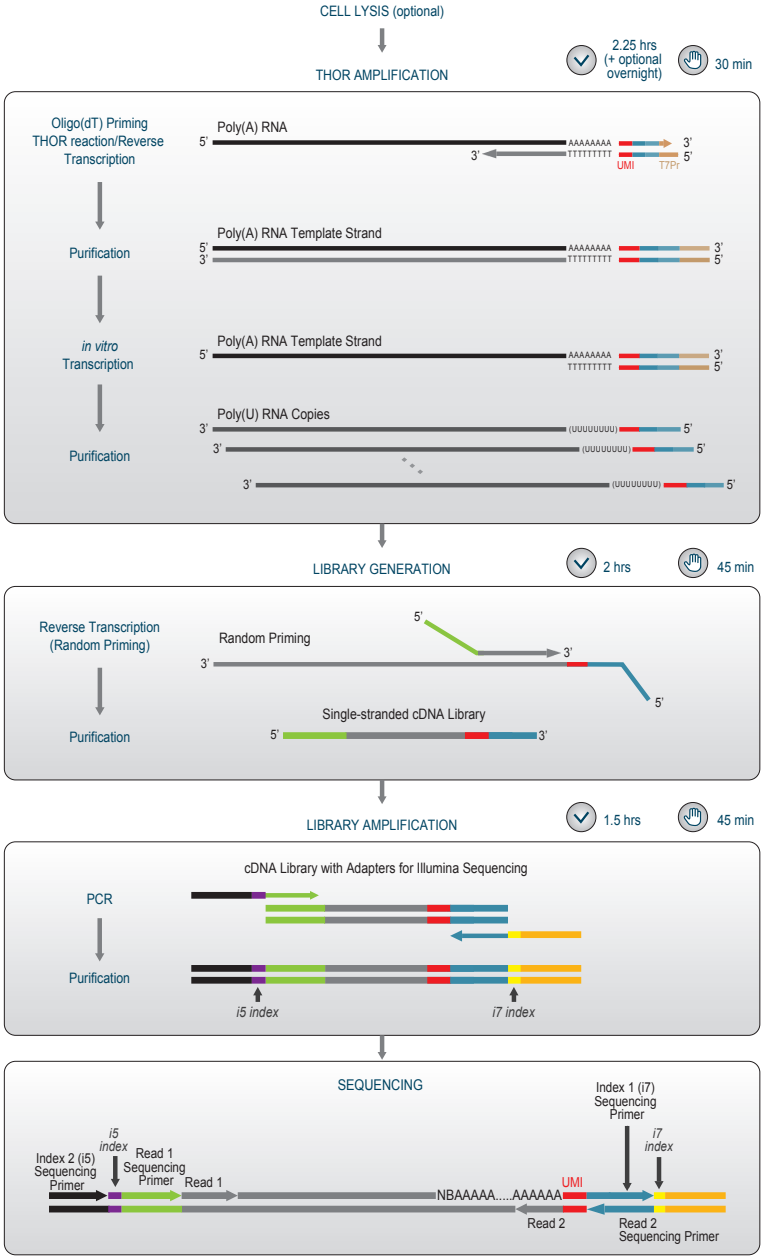

Figure 1. Schematic overview of the LUTHOR HD 3' mRNA-Seq library preparation workflow (Cat. No. 204). Partial P7 sequences are shown in blue, partial P5 sequences in green, Unique Molecular Identifier (UMI) in red, and T7 promoter sequences in light brown. Sequencing read orientation for LUTHOR HD is depicted, where Read 1 reflects the mRNA sequence, while a 12 nt long Read 2 is required for UMI read-out.

2. Kit Components and Storage Conditions

Kit Component	Tube / Plate Label	Volume*		Storage
		24 preps	96 preps	
Lysing Agent	**LA** ●	7 µl	28 µl	-20 °C
Molecular Biology Grade Water	**H_2O** ●	298 µl	1190 µl	-20 °C / /+4 °C
Nucleotides (dNTPs)	**NT** ●	43 µl	169 µl	-20 °C
RNA Amplification Primer	**P1** ●	27 µl	106 µl	-20 °C
THOR Reagent	**TR** ●	27 µl	106 µl	-20 °C
Enzyme Mix 1	**E1** ●	27 µl	106 µl	-20 °C
Enzyme Mix 2	**E2** ●	14 µl	53 µl	-20 °C
End Repair Enhancer - dilute before use!	**ERE** ●	4 µl	16 µl	-20 °C
Enzyme Mix 3	**E3** ●	14 µl	53 µl	-20 °C
In Vitro Transcription Buffer	**IVT** ●	66 µl	264 µl	-20 °C
Organic Solvent	**OS** ●	66 µl	264 µl	-20 °C
Dithiothreitol	**DTT** ●	33 µl	131 µl	-20 °C
Enzyme Mix 4	**E4** ●	40 µl	159 µl	-20 °C
Ribonucleotides (rNTPs)	**RN** ●	80 µl	317 µl	-20 °C
Primer 2	**P2** ●	27 µl	106 µl	-20 °C
Reverse Transcription Buffer	**RT** ●	80 µl	317 µl	-20 °C
Library Amplification Module				
PCR Mix	**PM** ○	185 µl	740 µl	-20 °C
PCR Enzyme Mix	**PE** ○	27 µl	106 µl	-20 °C
Lexogen UDI 12 nt Sets (only Cat. No. 221)				
Lexogen UDI 12 nt Set B1	**UDI12B_0001-0024**	10 µl / rxn		-20 °C
	UDI12B_0001-0096		10 µl / rxn	-20 °C
Purification Module				
Purification Beads	**PB**	1,056 µl	4,224 µl	+4 °C
Purification Solution	**PS**	2,508 µl	10,032 µl	+4 °C
Elution Buffer	**EB**	2,904 µl	11,616 µl	+4 °C

*including ≥10 % surplus

Upon receiving the LUTHOR HD kit, store the Purification Module (Cat. No. 022), containing **PB**, **PS**, and **EB** at +4 °C, and the rest of the kit at -20 °C. **NOTE: H_2O** ● and **EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **IVT** and **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

The PCR Master Mix (**PM** ○) does not include primers hence index primers for both i5 and i7 are required to introduce the complete Illumina-linkers for cluster generation. LUTHOR HD library prep kits are provided either without UDI (Cat. No. 204) or as a bundle with Lexogen UDI 12 nt Set B1 (Cat. No. 221).

Sets A1 – A4 are also available separately (Cat. No. 101-104 or 156) and contain up to 384 pre-mixed i5/i7 unique dual indices in 96-well format. Lexogen UDIs are 12 nucleotides long and are recommended for LUTHOR HD 3' mRNA-Seq Library Prep Kits due to their superior error correction capacity.

Lexogen UDI 12 nt Sets (Cat. No. 101 -105, 156)			
Lexogen UDI 12 nt Sets	**UDI 12 A_0001-0384** **UDI 12 B_0001-0096**	10 µl / rxn	-20 °C

3. User-Supplied Consumables and Equipment

Check to ensure that you have all of the necessary material and equipment before beginning the library preparation. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

Reagents / Solutions

- 80 % freshly prepared ethanol (for washing of Purification Beads, **PB**).
- Lexogen PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208), for qPCR assay.
- SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585), diluted to 2.5x in DMSO, for qPCR assay.
- For Cat. No. 204: suitable i5 / i7 indices, e.g., Lexogen UDI 12 nt Sets A1 - A4, or B1 (Cat. No. 101 -104, 156, or 105).

Equipment

- Magnetic plate e.g., 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates).
- Calibrated single-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Calibrated multi-channel pipettes for handling 1 µl to 200 µl volumes.
- Thermocycler.

- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

Labware

- Suitable certified ribonuclease-free low binding pipette tips (pipette tips with aerosol barriers recommended). We recommend using RPT (Repel Polymer Technology) tips from Starlab (Cat. No. S1183-1810 and Cat. No. S1183-1840) for optimal performance.
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free. e.g., Eppendorf Cat. No. 0030108051 or PCR clean Eppendorf Cat. No. 0030108051.
- 0.5 ml reaction tubes, low binding, certified ribonuclease-free, e.g., Eppendorf Cat. No. 0030108035.
- 200 µl PCR tubes, e.g., Azenta Cat. No. 4ti-0781 or 96-well plates Azenta Cat. No. 4ti-0770/C and caps Cat. No. 4ti-0782 or sealing foil.
- Vortex mixer.

Optional Equipment

- Automated microfluidic electrophoresis station (e.g., Agilent Technologies, Inc., 2100 Bioanalyzer).
- qPCR machine and library standards (for library quantification).
- Benchtop fluorometer and appropriate assays (for RNA quality control and library quantification).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).

The complete set of material, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A, p.24 for more information on RNA quality. Consult Appendix D, p.28 for information on library quantification methods.

4. Guidelines

RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e., RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Well before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage. Residues of RNaseZap may damage the RNA.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Beads are stored at +4 °C and must be resuspended before usage. Beads can be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or bottom of the tube or storage bottle.
- Beads may stick to certain pipette tips, in which case removing the beads from the inside of the tip may be impossible. Avoid resuspending by repeated pipetting and instead resuspend by vortexing if this occurs with your tips.
- Beads are superparamagnetic and are collected by placing the plate / tube in a magnetic plate or stand. The time required for complete separation will vary depending on the strength of your magnets, wall thickness of the wells / tubes, viscosity of the solution, and the proximity of the well / tube to the magnet. Separation time may need to be adjusted accordingly. When fully separated, the supernatant should be completely clear and the beads collected at one point or as a ring along the wall of the well / tube, depending on the magnet that was used.

- To remove the supernatant, the plate / tube containing the beads has to stay in close contact with the magnet. Do not remove the plate / tube from the magnetic plate / stand when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.
- When using a multichannel pipette to remove the supernatant, make sure not to disturb the beads. If beads are disturbed, ensure that no beads are stuck to the pipette tip opening and leave the multichannel pipette in the well for an extra 30 seconds before removing the supernatant. This way all beads can be recollected at the magnet, and the clear supernatant can be removed.
- In general, beads should not be centrifuged during the protocol. However, should drops of fluid stay on the wall of the reaction tube / plate (e.g., after mixing by vortexing), centrifugation at 2,000 x g for 30 seconds should be carried out before placing the tube / plate on the magnetic stand / plate.
- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant and before adding the next reagent. Beads can be resuspended by vortexing, but make sure that beads are not deposited on the well / tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the plate / tube briefly with a suitable benchtop centrifuge.

General

- Unless explicitly mentioned, all centrifugation steps should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined and must be strictly adhered to.
- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep Enzyme Mixes at -20 °C until just before use or store in a -20 °C benchtop cooler.
- When mixing, we recommend vortexing rather than pipetting to avoid RNA adsorption or volume loss due to tip adsorption. If using pipetting to mix, make sure to use RPT (Repel Polymer Technology) tips or similar.

- To maximize reproducibility and avoid cross contamination, spin down the reactions both after mixing and after incubations at elevated temperatures (i.e., before removing the sealing foil from PCR plates or tubes, e.g., step 7).

Pipetting and Handling of (Viscous) Solutions

- Enzyme Mixes, **PB**, and **PS** are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- When drawing up liquid, the tip should be dipped 3 to 5 mm below the surface of the liquid, always at a 90 degree angle. Do not dip the tip in any further as viscous solutions tend to stick to the outside of the pipette tip.
- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles, take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile, whereas the pipette itself is not. If necessary, tilt the bottle to bring the liquid closer to the opening and facilitate pipetting.

Preparation of Mastermixes and Pipetting with Multi-Channel Pipettes

In various steps of the protocol, mastermixes of enzymes and reaction buffers should be prepared. When preparing mastermixes and when using multi-channel pipettes, always include a 10 % surplus per reaction in order to have enough solution available for all reactions.

EXAMPLE: Step 1 for 24 cell suspension preps use:

6.9 µl **LA** ● (= 0.26 µl x 24 rxn x 1.1)
+ 26.4 µl **NT** ● (= 1.0 µl x 24 rxn x 1.1)
+ 19.5 µl **DTT** ● (= 0.74 µl x 24 rxn x 1.1)
+ 26.4 µl **P1** ● (= 1.0 µl x 24 rxn x 1.1)

resulting in a total of 79.2 µl, which is sufficient for multi-channel pipetting.

All reagents of the LUTHOR HD kit include ≥10 % surplus.

5. Detailed Protocol

In case purified RNA (10 pg - 1 ng) is used as input for THOR Amplification, start directly with step 5 of the protocol.

ATTENTION: Always use low binding plastics (tips, tubes, etc.) at any step during the LUTHOR HD protocol.

5.1 Cell Lysis

Preparation

Cell Lysis	For Each Sample cell supension	For Each Sample FACS sorted cells	Temperature
LA ● – stored at - 20 °C **NT** ● – stored at - 20 °C **DTT** ● – thawed at RT **P1** ● – stored at - 20 °C **H_2O** ● – stored at + 4 °C / -20 °C	0.26 µl 1 µl 0.74 µl 1 µl	0.26 µl 1 µl 0.74 µl 1 µl 2 µl	Thermocycler: 50 °C, 10 min 80 °C, 10 min 35 °C, 15 sec 4 °C, ∞
Centrifuge **Ice or Dry Ice**			– at 4 °C – Ice for immediate use, Dry Ice for storage of sorted cells or cell suspensions

Cell Lysis

LUTHOR HD is optimized for cell inputs ranging between 1 and 100 cells.
Cells are lysed prior to THOR Amplification. Prepare cell suspensions in 1x PBS. For singularization by FACS, cells can be directly sorted into Cell Lysis Buffer (**CLB**).

ATTENTION: Pre-cool a centrifuge to 4 °C. All centrifugation steps should be carried out at 4 °C. Keep cells on ice prior first strand synthesis.

For cell suspensions: Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl Lysing Agent (**LA** ●), 1 µl Nucleotides (**NT** ●), 0.74 µl Dithiothreitol (**DTT** ●), and 1 µl RNA Amplification Primer 1 (**P1** ●) per sample.

1 **For FACS-sorting of cells:** Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl Lysing Agent (**LA** ●), 1 µl Nucleotides (**NT** ●), 0.74 µl Dithiothreitol (**DTT** ●), 2 µl Molecular Biology Grade Water (**H_2O** ●), and 1 µl RNA Amplification Primer 1 (**P1** ●) per sample.

Prepare **CLB** as a mastermix for multiple samples to ensure accurate pipetting of low volumes (see p.11). Process a minimum of 8 reactions at a time.

2 **For cell suspensions**: mix 3 µl of **CLB** with 2 µl of the cell suspension. Seal the plate or close the tube.
For FACS-sorting of cells: Add 5 µl of **CLB** per well / tube and directly sort cells into the buffer. Seal the plate or close the tube.

3 Mix gently by vortexing or shaking and centrifuge for 30 seconds at 1,250 x g to collect the cells at the bottom of the plate / tube. Place on ice immediately.
OPTIONAL: To store cells for later use, flash freeze the suspensions on dry ice and store at - 80 °C for 3 - 6 months. ☞ Safe stopping point.

4 Lyse cells by incubating the plate / tube for 10 minutes at 50 °C, 10 minutes at 80 °C and 15 seconds at 35 °C. Then cool to 4 °C, spin down quickly, and place plate / tubes on ice. **OPTIONAL:** In case frozen cells are used, thaw the suspensions on ice, centrifuge briefly for 30 seconds at 1,250 x g and continue with cell lysis.

5.2 THOR Amplification

Preparation

THOR reaction	*In Vitro* Transcription	Purification
NT ● – thawed at RT **H_2O** ● – thawed at RT **TR** ● – thawed at RT **E1** ● – keep on ice or at -20 °C **E2** ● – keep on ice or at -20 °C **ERE** ● – keep on ice or at -20 °C dilute 1:10 before usage, keep on ice **E3** ● – keep on ice or at -20 °C	**IVT** ● – thawed at RT **OS** ● – thawed at RT **DTT** ● – thawed at RT **E4** ● – keep on ice or at -20 °C **RN** ● – thawed on ice	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Purified RNA Thermocycler: 23 °C, 5 sec 70 °C, 90 sec 35 °C, 15 sec 4 °C, 1 min THOR Reaction (Cells and RNA) Thermocycler: 37 °C, 10 min 42 °C, 5 min 25 °C, 1 min	Thermocycler: 37 °C, 2 hrs 25 °C, 1 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

THOR Reaction

An oligo(dT) primer containing an Illumina-compatible linker and a T7 promoter sequence at its 5' end is hybridized to the RNA and reverse transcription is performed to stabilize the RNA template.

5 **ATTENTION:** End Repair Enhancer (**ERE** ●) must be **diluted 1:10** shortly before usage! Mix at least 1 µl **ERE** ● with 9 µl **H_2O** ●, mix well and store on ice until needed. 0.5 µl of 1:10 diluted **ERE** ● will be needed per reaction. Process at least 8 reactions at a time, as **ERE** ● dilutions cannot be stored.
ATTENTION: Before use, check the contents of **TR** ●. If a precipitate is visible, incubate at 37 °C until buffer components dissolve. The buffer may still appear turbid after incubation. Mix well before setting up the reaction. A turbid appearance will not impact the performance of the buffer or the reaction.

6 **For purified RNA:**
Prepare a mastermix containing 2 µl **H_2O** ●, 1 µl THOR Reagent (**TR** ●), 0.5 µl Enzyme Mix 1 (**E1** ●), 0.5 µl Enzyme Mix 2 (**E2** ●), 0.5 µl Enzyme Mix 3 (**E3** ●), and 0.5 µl **1:10 diluted** End Repair Enhancer (1:10 diluted **ERE** ●) per reaction. Keep on ice.

Mix 3 µl **RNA**, 1 µl **NT** ●, 1 µl **P1** ●. If a lower RNA volume is used, bring the total volume per reaction to 5 µl with **H_2O** ●. Prepare a mastermix of 1 µl **NT** ● and 1 µl **P1** ● beforehand when processing more than 3 samples. Incubate samples as follows: 23 °C for 5 seconds, 70 °C for 90 seconds, 35 °C for 15 seconds, 4 °C for 1 minute.

Add 5 µl of the **TR** / **H_2O** / **E1** / **E2 / E3 / 1:10 diluted ERE** mix to the lysed cells from step 4 or the completed RNA / **NT** / **P1** hybridization reaction if purified RNA was used as input and mix well by vortexing. Ensure the plate is tightly sealed, and spin down to collect the liquid at the bottom of the wells.

7 Incubate with the following temperature program: 10 minutes at 37 °C, 5 minutes at 42 °C, then cool to 25 °C and hold for 1 minute. **ATTENTION:** Proceed immediately to **purification.**

Purification

The cDNA / mRNA hetero-duplex is purified using magnetic beads to remove all reaction components.
The Purification Module (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

8 Add 10 µl Purification Beads (**PB**) per sample from step 7. Mix well and incubate for 8 minutes at room temperature.

9 Place the plate / tube onto a magnet and let the beads collect for 5 minutes or until the supernatant is completely clear.

10 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Make sure that accumulated beads are not disturbed. Re-seal the plate, spin down briefly, and place back onto the magnet. Carefully remove the remaining liquid with a 10 µl pipette. **ATTENTION:** Do not disturb the beads! Avoid beads loss!

11 Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB.** Beads will move forward in the EB to the next step. **ATTENTION:** Proceed immediately to ***in vitro* transcription** (step 12).

In Vitro Transcription

During this step linear amplification of the original mRNA template by *in vitro* transcription generates antisense-RNA copies.

ATTENTION: IVT ●, OS ●, and **DTT ●** must be brought to room temperature. **RN ●** must be thawed on ice and kept at 4 °C. **E4 ●** is kept at 4 °C or -20 °C. Before use, check the contents of **IVT ●**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

12 Prepare a mastermix containing 2.5 µl In Vitro Transcription Buffer (**IVT ●**), 2.5 µl organic solvent (**OS ●**), 0.5 µl Dithiothreitol (**DTT ●**), and 1.5 µl Enzyme Mix 4 (**E4 ●**) per reaction, mix gently. Then add 3 µl Ribonucleotides (**RN ●**) per reaction to the mastermix and mix again. **IMPORTANT: RN ●** must be added last to the mastermix.

Add 10 µl of **IVT** / **OS** / **DTT** / **E4** / **RN** mastermix to the purified sample/beads mixture from step 11. Seal the plate / tube, vortex well and spin down.

13 Use thermocycler to incubate at 37 °C for 2 hours, cool down to 25 °C, and **proceed immediately to purification**. **OPTIONAL:** If immediate purification is not feasible cool down the IVT reaction after 2 hours at 37 °C to 4 °C and keep it in the thermocycler overnight. Alternatively, the completed IVT reaction can be stored at -20 °C.

Purification

The amplified RNA (aRNA) is purified using magnetic beads. The Purification Module components, **PS** and **EB,** should equilibrate for 30 minutes at room temperature before use.

ATTENTION: If you have frozen aRNA at the end of step 13, please thaw on ice.

14 Prepare a mastermix containing 10 µl Purification Beads (**PB**) and 26 µl Purification Solution (**PS**) per sample. Add 36 µl of **PB** / **PS** mastermix to each reaction product from step 13. Gently vortex and incubate for 8 minutes at room temperature.

15 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

16 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Make sure that accumulated beads are not disturbed.

17 Add 120 µl of 80 % EtOH and incubate for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

18 Repeat this washing step once for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit subsequent reactions.

19 Seal the plate / close the tube, spin down, place back onto the magnet. After a few seconds, carefully remove remaining liquid by using a 10 µl pipette. Keeping the reaction plate / tube on the magnet, air dry the beads for 1 - 2 minutes at room temperature or until there are no droplets of ethanol left on the walls of the tube. **ATTENTION:** Do not over-dry by prolonged incubation (visible cracks appear). Over-drying significantly decreases the elution efficiency.

20 Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

21 Place the plate / tube onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

22 Carefully transfer 20 µl of the clear supernatant into a fresh plate / tube.
OPTIONAL: ☞ Safe stopping point. Purified aRNA can be stored at -20 °C before proceeding to step 23.

5.3 Library Generation

Preparation

Reverse Transcription	Purification
H_2O ● – thawed at RT **NT** ● – thawed at RT **P2** ● – thawed at RT **RT** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user; **prepare fresh!** **EB** – stored at +4 °C
Thermocycler { 94 °C, 1 min, 16 °C, 5 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.
Thermocycler { 25 °C, 10 min, 37 °C, 40 min, 42 °C, 10 min, 30 °C, 2 min, 25 °C, 1 min	

Reverse Transcription

The random Primer 2 (**P2** ●) is hybridized to the RNA, and reverse transcription is performed, generating 3'- cDNA fragments with partial adapter sequences.

23 **ATTENTION:** If you have frozen aRNA at the end of step 22, please thaw on ice. Prepare a mastermix of 3.4 µl **H_2O** ●, 0.6 µl Nucleotides (**NT** ●) and 1 µl Primer 2 (**P2** ●) per sample. Mix thoroughly and spin down briefly.

24 Add 5 µl of the **H_2O / NT / P2** mastermix to 20 µl purified *in vitro* transcription product from step 22. Mix gently by vortexing or tapping the tube and quickly spin down. Incubate for 1 minute at 94 °C, cool to 16 °C and incubate for 5 minutes. Transfer the samples to room temperature and immediately proceed to step 25. **NOTE:** Prepare the mastermix for step 25 while incubating samples at 16 °C for 5 min.

25 Prepare a mastermix of 1.5 µl **H_2O** ●, 3 µl Reverse Transcription buffer (**RT** ●), and 0.5 µl Enzyme Mix 1 (**E1** ●) per sample. Mix thoroughly and spin down briefly. Add 5 µl of the **H_2O/ RT/ E1** mastermix to the samples from step 24. Mix gently by vortexing or tapping the tube / plate and quickly spin down.

26 Incubate with the following temperature program: 10 minutes at 25 °C, 40 minutes at 37 °C, 10 minutes at 42 °C, 2 minutes at 30 °C then cool to 25 °C and hold for 1 minute. **OPTIONAL:** ☞ Safe stopping point. Libraries can be stored at -20 °C before proceeding to step 27.

Purification

The first strand cDNA is purified using magnetic beads. The purification reagents (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

27	Prepare a mastermix of 10 µl Purification Beads (**PB**) and 20 µl Purification Solution (**PS**) per sample and add 30 µl of **PB** / **PS** mastermix to each reaction product. Mix thoroughly and incubate for 5 minutes at room temperature.
28	Place the plate / tube onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
29	Remove and discard the clear supernatant without removing the PCR plate / tube from the magnet. Make sure that accumulated beads are not disturbed. Re-seal the plate / tube, spin down briefly, and place back onto the magnet. Carefully remove the remaining liquid with a 10 µl pipette.
30	Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 - 5 minutes at room temperature.
31	Place the plate / tube onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
32	Transfer 20 µl of the supernatant into a fresh PCR plate / tube. ☞ Safe stopping point. After elution, libraries can be stored at -20 °C before proceeding to step 33.

5.4 Library Amplification

Preparation

PCR			Purification (Cat. No. 022)
PM ○ - thawed at RT **Indexing Primers, e.g., Lexogen UDI 12 nt Sets (Cat. No. 204: not included!)*** **PE** ○ - keep on ice or at -20 °C		**spin down before opening!**	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Thermocycler	95°C, 60 sec 95 °C, 15 sec 60 °C, 15 sec 72 °C, 60 sec 72 °C, 6 min 10 °C, ∞	6 - 18x Endpoint cycle number as determined by qPCR (Cat. No. 208), see Appendix B, p.26.	Equilibrate all reagents to room temperature for 30 minutes prior to use.

* 12 nt UDIs (Cat. No. 101 - 105, 156). Set B1 (UDI12B_0001-0024) is included in Cat. No. 221.

PCR

The single-stranded cDNA is amplified to add the complete adapter sequences required for cluster generation, to introduce indices for multiplexing, and to generate sufficient material for quality control and sequencing. Indexing primers are required to generate ready-to-sequence libraries. Unique Dual Indices (UDIs) are recommended for indexing of LUTHOR HD libraries, e.g., Lexogen UDI 12 nt Sets A1 – A4 or B1 (Cat. No. 101 – 104.96, 156.384, or 105.96). Alternatively, dual indexing primer pairs for partial, i.e., stubby TruSeq adapters from other vendors can also be used.

ATTENTION: Important notes for Library Amplification.

- **Perform trial reactions to determine the optimal PCR cycle number for endpoint PCR**. The number of PCR cycles for library amplification must be adjusted according to RNA input amount, quality, and sample type. For details see Appendix B, p.26.
- **Alternatively, a qPCR assay can be used to determine the optimal PCR cycle number.** The PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208) is required. For qPCR assay details see Appendix B, p.26.
- **Indexing Primers are required.** Lexogen's UDI 12 nt Index Sets are recommended.
- Avoid cross contamination when using the Lexogen UDI 12 nt Sets. Spin down the Index Set before opening and visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired UDIs only. Reseal opened wells with a fresh sealing foil after use to prevent cross contamination.
- Each well of the Lexogen UDI 12 nt Index Set is intended for single-use only.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, and **EB**) for step 36 at room temperature to give it at least 30 minutes to equilibrate.

33	Prepare a mastermix containing 7 µl Dual PCR Mix (**PM** ○) and 1 µl PCR Enzyme Mix (**PE** ○) per sample.
34	Add 8 µl of the **PM / PE** mastermix and 10 µl UDI primer (Cat. No. 101 - 105, 156) to 17 µl of the eluted library. Only add 1 UDI per PCR reaction.
35	Conduct 6 - 18 cycles of PCR (determine the required cycle number by qPCR) with the following program: initial denaturation at 95 °C for 60 seconds, 6 - 18 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, hold at 10 °C. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Module (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

ATTENTION: If the libraries were stored at -20 °C, ensure that they are thawed, equilibrated to room temperature, and spun down before restarting the protocol.

36	Prepare a mastermix containing 10 µl Purification Beads (**PB**) and 25 µl Purification Solution (**PS**) per sample. Add 35 µl of **PB** / **PS** mastermix to each reaction product from step 35. Mix well and incubate for 5 minutes at room temperature.
37	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
38	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads.
39	Remove the plate from the magnet, add 30 µl of Elution Buffer (**EB**), and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
40	Add 24 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.
41	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
42	Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads.
43	Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
44	Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.
45	Seal the plate / close the tube, spin down, place back onto the magnet. After a few seconds, carefully remove remaining liquid by using a 10 µl pipette. Keeping the reaction plate / tube on the magnet, air dry the beads for 1 minute at room temperature. **ATTENTION:** Do not over-dry by prolonged incubation (visible cracks appear). Over-drying significantly decreases the elution efficiency.
46	Remove the plate from the magnet, add 20 µl of Elution Buffer (**EB**) per well, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
47	Place the plate onto a magnet, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
48	Transfer 17 µl of the supernatant into a fresh PCR plate / tube. Do not transfer any beads. Libraries are now finished and ready for quality control (Appendix D, p.28), pooling (for multiplexing, Appendix E, p.31), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure

ATTENTION: Spin down before opening tubes or plates!

2.25 hrs RNA Amplification

Cell Lysis and P1

Sorted Cells

- ☐ Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl **LA** ●, 1 µl **NT** ●, 0.74 µl **DTT** ●, 2 µl **H_2O** ●, and 1 µl **P1** ● per reaction.
- ☐ Dispense 5 µl **CLB** per well and directly sort cells into the buffer.

Cell suspension

- ☐ Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl **LA** ●, 1 µl **NT** ●, 0.74 µl **DTT** ●, and 1 µl **P1** ● per reaction.
- ☐ Add 2 µl cell suspension to 3 µl **CLB**.

- ☐ Incubate for 10 min at 50 °C, 10 min at 80 °C, and 15 sec at 35 °C then cool to 4 °C. Spin down quickly, and place on ice, proceed to THOR reaction.

P1 Hybridization

Ultra-low Input Purified RNA (10 pg - 1 ng)

- ☐ Prepare a mastermix of 1 µl **NT** ●, and 1 µl **P1** ● per reaction.
- ☐ Add 2 µl of **NT/ P1** mastermix to 3 µl RNA. Mix well.
- ☐ Incubate for 5 sec at 23 °C, 90 sec at 70 °C, 15 sec at 35 °C, then cool to 4 °C for 1 min.

THOR reaction

- ☐ Prepare a fresh 1:10 dilution of **ERE** ●. Use at least 1 µl **ERE** ● for accurate pipetting. Process at least 8 samples at a time to have sufficient undiluted **ERE** ● for all samples. Store the 1:10 **ERE** ● dilution on ice until needed. **ATTENTION:** **ERE** ● dilutions cannot be frozen and/or stored.
- ☐ Prepare a mastermix with 2 µl **H_2O** ●, 1 µl **TR** ●, 0.5 µl **E1** ●, 0.5 µl **E2** ●, 0.5 µl **E3** ●, and 0.5 µl **1:10 diluted ERE** ● per reaction.
- ☐ Add 5 µl **H_2O** / **TR** / **E1 / E2 / E3 / 1:10 diluted ERE** mix to the lysed cells or purified RNA premix. Mix well by vortexing and spin down.
- ☐ Incubate for 10 min at 37 °C, 5 min at 42 °C, then cool to 25 °C for 1 minute. **Proceed immediately to purification**.

Purification

- ☐ Add 10 µl **PB** per reaction, mix well, incubate 8 min at RT.
- ☐ Place on magnet for 5 min, discard supernatant. **ATTENTION:** Do not disturb the beads! Avoid beads loss!
- ☐ Remove from magnet, add 20 µl **EB**, mix well.
- ☐ Keep the beads in and **immediately proceed to *in vitro* transcription.**

	In Vitro Transcription
☐	Prepare a mastermix with 2.5 µl **IVT** ●, 2.5 µl **OS** ●**,** 0.5 µl **DTT** ● and 1.5 µl **E4** ● per reaction. Mix, then add 3 µl **RN** ● per reaction and mix again.
☐	Add 10 µl **IVT / OS / DTT / E4** / **RN** mastermix to the purified sample, vortex well, and spin down.
☐	Incubate 2 hrs at 37 °C, then cool to 25 °C. **Proceed immediately to purification**.
	OPTIONAL: After incubation at 37 °C, cool down to 4 °C for overnight incubation, or storage at -20 °C is possible.
	Purification
☐	Add 10 µl **PB +** 26 µl **PS** per reaction, mix well, incubate 8 min at RT.
☐	Place on magnet for 2 - 5 min, discard supernatant.
☐☐	Rinse beads twice with 120 µl 80 % EtOH, 30 sec.
☐	Air dry beads for 1 - 2 min. **ATTENTION:** Do not let the beads dry too long!
☐	Remove from magnet, add 20 µl **EB**, mix well, incubate 2 min at RT.
☐	Place on magnet for 2 - 5 min, transfer the supernatant into a fresh PCR plate / tube.
	Reverse Transcription
☐	Prepare a mastermix of 3.4 µl **H_2O** ●**,** 0.6 µl **NT** ● and 1 µl **P2** ● per sample. Mix well, spin down.
☐	Add 5 µl of **H_2O / NT / P2** mastermix to 20 µl of purified *in vitro* transcription product. Mix well, spin down.
☐	Incubate for 1 min at 94 °C, then 5 min at 16 °C.
☐	Prepare a mastermix of 1.5 µl **H_2O** ●, 3 µl **RT** ● and 0.5 µl **E1** ● per sample. Mix well, spin down.
☐	Add 5 µl **H_2O / RT / E1,** mix well, spin down briefly.
☐	Incubate: 10 min at 25 °C, 40 min at 37 °C, 10 min at 42 °C, 2 min at 30 °C then cool to 25 °C and hold for 1 min. ☞ Safe stopping point. store at -20 °C.
	Purification
☐	Add 10 µl **PB** + 20 µl **PS** per reaction, mix well and incubate 5 min at RT.
☐	Place on magnet for 2 - 5 min, discard supernatant.
☐	Remove from magnet, add 20 µl **EB**, mix well, incubate 2 - 5 min at RT.
☐	Place on magnet for 2 - 5 min, transfer the supernatant into a fresh PCR plate / tube. **OPTIONAL:** Perform a qPCR assay to determine the exact endpoint cycle number (see p.26). ☞ Safe stopping point.

qPCR [Strongly Recommended! Requires PCR Add-on and Reamplification Kit V2 (Cat. No. 208.96)] - Appendix B, p.26

- ☐ Prepare a 2.5x stock of SYBR Green I nucleic acid stain (i.e., 1:4,000 dilution in DMSO; use Sigma-Aldrich, Cat. No. S9430).
- ☐ For each reaction combine: 1.7 µl cDNA with 7 µl **PM** ○, 5 µl **P5** ●, 5 µl **P7** ●, 1 µl **PE** ○, 1.4 µl of 2.5x SYBR Green nucleic acid stain, and 13.9 µl of **EB**, per reaction. Mix well.
- ☐ PCR: 95 °C, 60 sec.

  95 °C, 15 sec
  60 °C, 15 sec
  72 °C, 60 sec
  } **35x** (see p.26)

  72 °C, 6 min

  10 °C, ∞. .
- ☐ Calculate the optimal cycle number for Endpoint PCR (see Appendix B, p.26).

Endpoint PCR

- ☐ Prepare a mastermix with 7 µl **PM** ○ and 1 µl **PE** ○ per reaction.
- ☐ Add 8 µl of the **PM / PE** mastermix to 17 µl of the eluted library.
- ☐ Add 10 µl of one Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. **ATTENTION:** Reseal opened index wells after use! Use only one UDI / sample.
- ☐ PCR: 95 °C, 60 sec

  95 °C, 15 sec
  60 °C, 15 sec
  72 °C, 60 sec
  } **6 - 18x** (see p.26)

  72 °C, 6 min

  10 °C, ∞. ☞ Safe stopping point.

Purification

- ☐ Add 10 µl **PB** + 25 µl **PS** per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐ Remove from magnet, add 30 µl **EB**, mix well, incubate 2 min at RT.
- ☐ Add 24 µl **PS**, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐☐ Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.
- ☐ Air dry beads for 1 - 2 min. **ATTENTION:** Do not let the beads dry too long!
- ☐ Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

7. Appendix A: General Input Requirements

7.1 RNA Input Requirements

LUTHOR HD is optimized for inputs ranging between 10 pg and 1 ng total RNA per library. If your input is higher than 1 ng, dilute your RNA sample down to ≤1 ng.
Please contact support@lexogen.com for sample inputs lower than 10 pg.

RNA Purity and Chemical Contaminants

RNA samples should be free of salts, metal ions, and organic solvents, which can be carried over from RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260/230 ratio. Phenol has an additional absorption maximum between 250 and 280 nm, which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts. These contaminants may have a negative impact on the efficiency of the protocol.

RNA Integrity

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies, Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN). Quality control of ultra-low input RNA may not be possible, as concentrations below 50 pg/µl total RNA are below the detection limit for most microfluidics assays. For best practice, high quality RNA (RIN >8) is recommended as input. Lower quality samples (RIN <8) may result in lower gene detection or increased presence of rRNA, intronic and intergenic reads.

7.2 Cell Input Requirements

LUTHOR HD is optimized for inputs ranging between 1 cell and 100 cells per library.
Single cell biopsies have also been successfully prepared. Please contact support@lexogen.com for more information.

Cell Viability

As a general rule, cell viability should be monitored prior to isolation (and, if possible, after isolation) and reach >90 %. Lower viability samples may result in lower gene detection or increased presence of rRNA, intronic and intergenic reads.
One method to preserve RNA content in cells is to fix cells before or after isolation. For a more detailed and validated fixation protocol, please contact us at support@lexogen.com or consult our online Frequently Asked Questions (FAQs).

Cell Sorting

Prepare single-cell suspensions in 1x Phosphate Buffered Saline (PBS, Ca/Mg2+ free) or Hank's Balanced Salt solution (HBSS). Please do not prepare single-cell suspension in media containing phenol red. Phenol red will increase background fluorescence if a fluorescence-based analysis is performed (imaging, flow cytometry). It is critical to examine the single-cell suspension under a microscope before sorting. Use cell strainers to filter out cell clumps and check cell viability e.g. using Trypan Blue. Cell viability in 1x PBS buffer needs to be high (>90 %). Cell aggregates additionally should be removed from the sort gate with doublet discriminator.

Cell Concentration

For flow cytometry-assisted cell sorting: ensure correct input sample cell concentration based on the cell size. The proper concentration is a combination of the cell type you are using and the nozzle size of the cytometer. Use the nozzle that is five times the size of the cells being sorted.

Recommended nozzle sizes and cell concentrations:

Nozzle Size	Cell Size (Ø)	Concentration [cells / ml]
70 µM	<10 µm	7 - 12 x 10^6
85 - 100 µM	10 - 15 µm	5 - 7 x 10^6
130 µM	>15 µm	3 - 5 x 10^6

Cell Storage

If immediate RNA extraction is not possible, sorted cells can be flash-frozen using liquid nitrogen or dry ice and stored at -80 °C. Ensure freezing of the cells immediately after sorting to preserve the RNA.

7.3 Mitochondrial Ribosomal RNA

Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library. These mt-rRNAs can make up 0.5 - 5 % of the reads when using a 3' mRNA-Seq protocol, such as LUTHOR HD. Please note that rRNA content can differ depending on the cell type, or when low quality, or degraded RNA samples are used as input for LUTHOR HD.

8. Appendix B: PCR Cycle Assessment and qPCR

Adjusting PCR Cycle Numbers for Sample Type

The mRNA content and quality of total RNA affects the number of PCR cycles needed for the final library amplification step. The mRNA content of RNA samples can vary between species and tissue / cell types. Variable RNA quality may also affect differences in mRNA content between samples. Variable input types and amounts require optimization of PCR cycle numbers. This will prevent both under and overcycling, the latter of which may bias your sequencing results (see also Appendix D, p.28).

Optimal cycle numbers can be established by running trial library preps through the complete LUTHOR HD protocol for each new sample type. Each trial reaction can be amplified using the PCR Master Mix (**PM** ○) and index primers with a different cycle number across the range of suitable cycles to assess the optimal cycle number for endpoint amplification. It is recommended to use increments of 2 – 3 cycles and 4 reactions per sample type to cover the required range. After purification, the individual libraries can be assessed and the optimal cycle number can be determined based on size distribution and concentration (see Appendix D, p.28). Once established, there is no need to repeat trial reactions for the same input type and condition. Alternatively, a qPCR assay can be used to determine the optimal cycle number. The PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208) is required for the following qPCR assay protocol. This assay can be used to determine cycle numbers for subsequent dual or single indexing PCRs.

qPCR to Determine the Optimal Cycle Number for Endpoint PCR

NOTE: SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually.

ATTENTION: The use of SYBR Green I-containing qPCR mastermixes from other vendors is not recommended. Use only PCR Add-on and Reamplification Kit V2 (Cat. No. 208) for LUTHOR HD libraries, in combination with SYBR Green I dye.

1	Prepare a 1:4,000 dilution of SYBR Green I dye in DMSO, for a 2.5x working stock concentration. **ATTENTION:** The final concentration in the reaction should be 0.1x. Higher concentrations of SYBR Green I will inhibit amplification.
2	For each reaction combine: 1.7 µl of cDNA with 7 µl PCR Mix (**PM** ○), 5 µl **P5** ●, 5 µl **P7** ●, and 1 µl Enzyme Mix (**PE** ○), 1.4 µl of 2.5x SYBR Green I nucleic acid stain, and 13.9 µl of **EB**, per reaction. Mix well.
3	Perform 35 cycles of PCR with the following program: initial denaturation at 95 °C for 60 seconds, 35 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, hold at 10 °C. **NOTE:** There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.

4 Using the amplification curves in linear scale, determine the value at which the fluorescence reaches the plateau. Calculate 50 % of this maximum fluorescence value and determine at which cycle this value is reached. As the endpoint PCR will contain 10x more cDNA compared to the qPCR, subtract three from this cycle number. This is then the final cycle number you should use for the endpoint PCR with the remaining 17 µl of the template (see Fig. 2).

Endpoint PCR Cycle Calculation

When using 1.7 µl of cDNA for a qPCR, if the cycle number corresponding to 50 % of the maximum fluorescence is 15 cycles, the remaining 17 µl of the template should therefore be amplified with 12 cycles (15 - 3 cycles =12 cycles, Fig. 2).

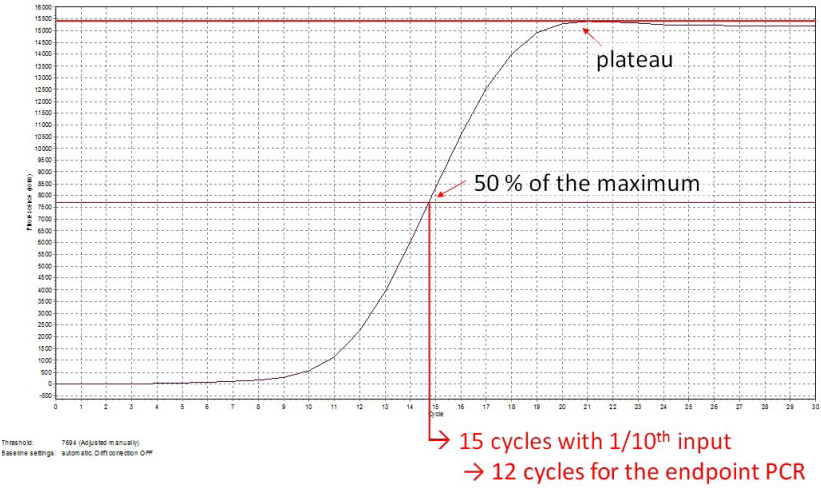

Figure 2. Calculation of the number of cycles for the endpoint PCR.

NOTE: Once the number of cycles for the endpoint PCR is established for one type of sample (same input amount, tissue / cell type, and RNA quality), there is no need for further qPCRs.

9. Appendix C: Library Reamplification

Reamplification of Dual-Indexed Libraries

If your library yields are extremely low and insufficient for pooling, reamplification can be performed using the Reamplification Primer Mix **RE** ○ in the PCR Add-on and Reamplification Kit V2 (Cat. No. 208). Prepare a mastermix of 7µl PCR Mix (**PM** ○), 10 µl Reamplification Primer Mix (**RE** ○), and 1 µl Enzyme Mix (**PE** ○). Mix well and add up to 17 µl purified PCR product. Add 3-6 cycles (depending on the originally determined yields) using the same PCR program used for the endpoint PCR reaction during final library amplification (step 35). Yield roughly doubles with each additional PCR cycle.

10. Appendix D: Library Quality Control

Quality control of finished LUTHOR HD libraries is highly recommended and should be carried out prior to pooling and sequencing. A thorough quality control procedure should include the analysis of library concentration and size distribution (i.e., library shape).

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become standard practice for many NGS laboratories and generates information regarding library concentration and size distribution. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). For high-throughput applications instruments such as the Fragment Analyzer or 2200 TapeStation (Agilent Technologies, Inc.), or LabChip GX II (Perkin Elmer) are recommended. Typically, 1 µl of a LUTHOR HD library produced according to the directions in this manual is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished LUTHOR HD library is calculated by comparing Cq values to a set of known standards. While delivering a more accurate quantification, these assays do not supply the user with information regarding library size distribution. Unwanted side-products such as linker-linker artifacts are not discernible from the actual library in the qPCR assay as both will be amplified. Hence it is highly recommended to combine such an assay for quantification with microcapillary electrophoresis analysis for library size distribution.

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays, e.g., Qubit dsDNA HS assay. Most UV-Vis spectrophotometers (e.g., NanoDrop, Thermo Fisher Scientific Inc.), are not sensitive enough to accurately quantify NGS libraries at these concentrations and should be avoided.

Typical Results

LUTHOR HD libraries do not need to be extensively amplified. Library yield, shape, and average insert size may vary, depending on the type of input sample (e.g., degraded, low quality RNA produces shorter library inserts than high quality RNA). The majority of inserts are greater than 100 bp in size, corresponding to final library fragment sizes ≥300 bp.

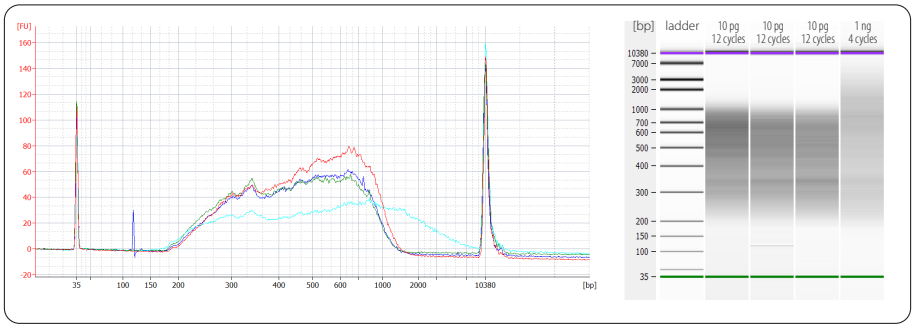

Figure 3. Bioanalyzer traces of LUTHOR libraries generated from purified total RNA (UHRR). Libraries were prepared using the minimum recommended input amount of 10 pg UHRR with either purification and 2nd RT being performed immediately after 2h of IVT (red trace, 12 PCR cycles), or storing the aRNA at -20 °C overnight after 2h of IVT at 37 °C (blue traces, 12 PCR cycles), or cooling the thermocycler to 4 °C after 2h of IVT at 37 °C (green trace, 12 cycles). The maximum recommended input of 1 ng (turquoise traces, 4 PCR cycles) UHRR was processed as the red trace, using the safe stopping point at step 26. Endpoint PCR was performed using unique dual indexing.

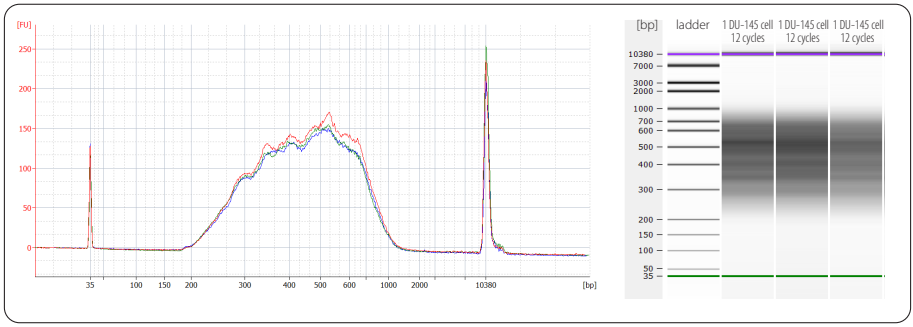

Figure 4. Bioanalyzer traces of LUTHOR HD libraries generated from DU-145 FACS sorted single cells with purification and 2nd RT being performed immediately after 2h of IVT, using the safe stopping point at step 26. Endpoint PCR was performed using 12 cycles and unique dual indexing.

Overcycling

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. This could occur if cycle numbers are increased too much to compensate for lower input material. Prevent overcycling by using either the trial reaction setup or the qPCR assay as described in Appendix B, p.26.

11. Appendix E: Multiplexing

Libraries prepared with the Lexogen UDI 12 nt Unique Dual Indexing Sets are suitable for sequencing on all Illumina instruments listed below. The Lexogen UDI 12 nt Sets enable adjustable read out of 8, 10, or the full 12 nucleotide long UDI sequence while maintaining superior error correction features. The longer the UDI read-out, the higher the error correction capability.

The complete lists of i5 and i7 index sequences for all Lexogen UDI 12 nt Sets are available at www.lexogen.com/docs/indexing.

Depending on the instrument workflow, flow cell type (paired-end, PE; single-read, SR), and chemistry, i5 indices are sequenced differently.

Illumina Instruments	Flow Cell Type	Workflow	Lexogen UDI 12 nt Unique Dual Indexing Sets
HiSeq 2000/2500 HiSeq 3000/4000	SR	Forward Strand (A)	Lexogen UDI 12 nt **Sets A1, A2, A3, and / or A4** (UDI12A_0001-0384), Cat. No. 101 – 104.96, or 156.384
HiSeq 2000/2500 MiSeq NovaSeq 6000 (v1.0 reagent kits)	PE		
iSeq 100 MiniSeq NextSeq 500 – 2000 HiSeq 3000/4000 NovaSeq 6000 (v1.5 reagent kits) NovaSeq X	PE	Reverse Strand (B)	Lexogen UDI 12 nt **Set B1** (UDI12B_0001-0096), 1 rxn/UDI, Cat. No. 105.96

Sets A1 - A4 (UDI12A_0001-0384) for Forward Strand Workflow (A)

For instruments using the Forward Strand workflow, the Index 2 Read (i5) is primed using the Grafted P5 Oligo on the flow cell (or the Index 2 (i5) Sequencing Primer (HP9) for SR HiSeq 2000 / 2500 flow cells).

Set B1 (UDI12B_0001-0096) for Reverse Complement Workflow (B)

For instruments using the Reverse Complement workflow, the Index 2 Read (i5) is performed after Read 2 Resynthesis, using the Index 2 (i5) Sequencing Primer.

ATTENTION: If LUTHOR HD with UDI Set A (UDI12A_0001-0384) are sequenced on Illumina machines using the Reverse Complement Workflow (B), or *vice versa*, the i5 Index will be read out as reverse complement. **In this case, all 12 nt of the i5 Index must be read out if error correction shall be applied**. Additionally, the reverse complement of the i5 index read out needs to be analyzed.

EXAMPLE: i512_0001 is read as GTCTTTGGCCCT instead of AGGGCCAAAGAC. The read out in reverse complement (GTCTTTGGCCCT) shall be used for demultiplexing and error correction.

Lane Mix Preparation

Libraries should ideally be pooled in an equimolar ratio for multiplexed sequencing. It is important to ensure accurate quantification of the individual libraries prior to pooling, as well as for the library pool (lane mix). To quantify your libraries:

1. Measure the concentration of each library, using either qPCR or fluorescence-based assays (e.g., QuBit, Thermo Fisher Scientific Inc.).
2. Determine the average library size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to 160 - 2,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (160 bp), or overcycling bumps (>2,000 bp).

Molarity is then calculated from the average library size and the concentration (ng/µl) using the following equation:

$$\text{Molarity} = (\text{library concentration (ng/µl)} \times 10^6) / (660 \times \text{average library size (bp)})$$

A template for molarity calculation is also available for download from www.lexogen.com.

After pooling the libraries, the prepared lane mix and any dilutions made for denaturing (e.g., 2 nM), should be reanalyzed to ensure the accuracy of the concentration. This can be performed according to steps 1 and 2 as above.

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible between ~150 and 190bp (for dual-indexed libraries with 12 nt UDIs) and should not compose more than 0 - 3 % of the total lane mix for sequencing. If the fraction of linker-linker, or other small fragments (≤150 bp) are too prominent, repurification of the lane mix prior to sequencing is advised.

Libraries or lane mixes can be repurified using the Purification Module with Magnetic Beads (Cat. No. 022) using the following protocol.

1. Measure the volume of the library or lane mix. If the volume is less than 20 µl, adjust the total volume to 20 µl using Elution Buffer (**EB**) or molecular biology-grade water (**H_2O** ●).
2. Add 0.9 volumes (0.9x) of Purification Beads (**PB**). Mix thoroughly and incubate for 5 minutes at room temperature. **EXAMPLE:** For 50 µl of lane mix, add 45 µl **PB.**

Follow the detailed protocol from step 37 onwards (p.20).

12. Appendix F: Sequencing*

General

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. For information on loading amounts for the various sequencing instruments please refer to the LUTHOR HD online Frequently Asked Questions (FAQs), or contact support@lexogen.com. All LUTHOR HD libraries can be sequenced using the standard Illumina Multiplexing Read 1 Sequencing Primer. A length of 75 nt to 100 nt (Read 1) is generally a good starting point. A 12 nt long Unique Molecular Index (UMI) is located at the beginning of Read 2 and can be read out with the standard Multiplexing Read 2 Sequencing Primer. Longer read outs than 12 nt in Read 2 are not recommended as a poly(T) stretch corresponding to the oligo(dT) primer is located right after the UMI. As sequencing through the homopolymer stretch would result in dephasing and drastic reduction of Read 2 quality. A schematic representation of dual indexed libraries is shown below.

Dual Indexed Libraries with Lexogen UDI 12 nt Set B1 for Reverse Strand Workflow Machines / Chemistries

All instruments use a Multiplexing Index 2 (i5) Sequencing Primer, which is included in the "Dual-Indexing Primer Mix" for iSeq, MiniSeq, and NextSeq in the v1.5 reagent kits for NovaSeq, and in HP14 for HiSeq 3000 / 4000. A minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. 12 nucleotides, 10 nucleotides, or 8 nucleotides can be read out optionally.

ATTENTION: In case any other indices than Lexogen UDI 12 nt Set B1 are used, a reverse complement of the Index 2 (i5) sequence is produced as index 2 (i5) is read-out after the Read 2 Resynthesis step. If Lexogen UDI 12 nt Sets A1 - A4 (UDI12A_0001- 0384) for the forward strand workflow are used on Illumina machines with reverse strand workflow, the i5 Index will be read out as reverse complement. In this case all 12 nucleotides of the i5 Index must be read out for error correction. Additionally, the reverse complement of the i5 index read out needs to be analyzed, see Appendix E, p.31 for details.

LUTHOR HD libraries:

```
  5'-(Index 2 (i5) Sequencing Primer)-3'      5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT- (Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert…


          UMI   5'-(Index 1 (i7) Sequencing Primer)-3'
…Insert)-N(12)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
…Insert)-N(12)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
               3'-(Read 2 Sequencing Primer)-5'
```

*Note: Some nucleotide sequences shown in Appendix F may be copyrighted by Illumina, Inc.

Read 1 for LUTHOR HD libraries:
Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Optional: Read 2 for LUTHOR HD libraries (required for UMI read out):
Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

NOTE: LUTHOR HD libraries are oligo(dT)-primed. Therefore, the poly(T) stretch is located at the beginning of the insert following the UMI sequence in Read 2. Read 2 should therefore be restricted to 12 nt only.

Index 1 Read (i7): i7 Index Primer (not supplied):
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Index 2 Read (i5): i5 Index Primer (not supplied):
5' AATGATACGGCGACCACCGAGATCTACAC 3'

Multiplexing with Other Library Types

We do not recommend multiplexing Lexogen libraries with libraries from other vendors in the same sequencing lane.

Though this is possible in principle, specific optimization of index combinations, library pooling conditions, and loading amounts may be required, even for advanced users. Sequencing complex pools that include different library types at different lane shares may have unpredictable effects on sequencing run metrics, read quality, read outputs, and/or demultiplexing performance. Lexogen assumes no responsibility for the altered performance of Lexogen libraries sequenced in combination with external library types in the same lane (or run).

Due to size differences, libraries prepared with the Lexogen Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with QuantSeq, CORALL or LUTHOR HD libraries. Please refer to the sequencing guidelines for each library type (library adapter details, loading amounts to use, and use of custom sequencing primers, etc.), which are provided in our library prep kit User Guides, and online Frequently Asked Questions (FAQs).

13. Appendix G: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of LUTHOR HD data and is kept as general as possible for integration with your standard pipeline.
LUTHOR HD 3' mRNA-Seq (Cat. No. 204) contains the Read 1 linker sequence in the 5' part of the random primer, hence NGS reads are generated towards the poly(A) tail. To pinpoint the exact 3' end, longer read lengths may be required. Read 1 directly reflects the mRNA sequence.

Demultiplexing

Demultiplexing can be carried out by the standard Illumina pipeline. Lexogen i7 and i5 12 nt index sequences are available for download at www.lexogen.com.

Additionally to the standard error-correction included in the Illumina pipeline, Lexogen's iDemux tool is freely available on github: https://github.com/Lexogen-Tools and can be used for higher accuracy in error correction. Please contact support@lexogen.com for more information.

Processing Raw Reads

We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the dataset.

Trimming

The reads should be trimmed to remove adapter sequences (see Appendix F, p.33), poly(A) / poly(T) sequences, and low quality nucleotides. Reads that are too short (i.e., <20 nt) or have generally low quality scores should be removed from the set.
In addition, as LUTHOR HD library generation is based on random priming, there is a higher proportion of mismatches over the first 9 nt of Read 1. We therefore recommend using an aligner that can perform soft-clipping of the read ends (e.g., STAR aligner) during alignment, or increasing the number of allowed mismatches to 11. Alternatively, trimming the first 9 nt of Read 1 can be performed prior to alignment when using a more stringent aligner (e.g., HISAT2). While trimming the read can decrease the number of reads of suitable length for alignment, the absolute number of mapping reads may increase due to the improved read quality.

Alignment

After filtering and trimming, reads can be aligned with a short read aligner to the reference genome. We recommend the use of STAR aligner for mapping LUTHOR HD data. The reads may not be confined exclusively to the last exon and span a junction hence splice-aware aligners should be used. Bowtie2, BBMap, or BWA can also be used for mapping against a reference transcriptome.

Annotations and Read Counting

Mapping only the 3' end of transcripts requires an annotation that covers the 3' untranslated region (UTR) accurately. Conservative annotations might decrease the power of correct gene quantification after mapping. For some gene annotations it might be an advantage to extend the 3' UTR annotation further downstream in order to assign the mapped read correctly.

LUTHOR HD UMI Data Analysis

LUTHOR HD libraries contain 12 nt long Unique Molecular Identifiers (UMI) at the beginning of Read 2. In order to analyze the UMI information, the first 12 nt of Read 2 can be extracted and used to collapse the reads with well-established open source tools for UMI de-duplication, e.g., umi_tools.

14. Appendix H: Revision History

Publication No. / Revision Date	Change	Page
204UG513V0100 Nov. 14, 2023	Initial release.	

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Associated Products:

008 (SPLIT RNA Extraction Kit)
022 (Purification Module with Magnetic Beads)
101 - 104, 156 (Lexogen UDI 12 nt Sets A1-A4 (UDI12A_0001-0384), 1 rxn/UDI)
105 (Lexogen UDI 12 nt Set B1 (UDI12B_0001-0096), 1 rxn/UDI)
208 (PCR Add-on and Reamplification Kit V2 for Illumina)

High-Definition Single-Cell 3' mRNA-Seq Library Prep Kit · User Guide

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