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LUTHOR 3' mRNA-Seq Library Prep Kit

User Guide

Catalog Numbers:
143 (LUTHOR 3' mRNA-Seq Library Prep Kit for Illumina)

143UG290V0100

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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 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, 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 partial Illumina-compatible P7 linker and a T7 promoter sequence. The proprietary end repair step removes the single-stranded 3' poly(A) overhang and generates a double stranded T7 promoter sequence for RNA amplification. During the subsequent overnight *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 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. Indices are not included in the LUTHOR kits and must be purchased separately. Unique Dual Indexing is recommended for LUTHOR, e.g., Lexogen UDI 12 nt Sets A1 – A4 (Cat. No. 101 - 104.96, or 156.384), or UDI 12 nt Set B1(Cat. No. Cat. No. 105.96). The 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 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. LUTHOR libraries should be sequenced in Single Read (SR)-mode. A poly(T) stretch corresponding to the oligo(dT) primer is located at the beginning of Read 2, therefore paired-end sequencing is not recommended.

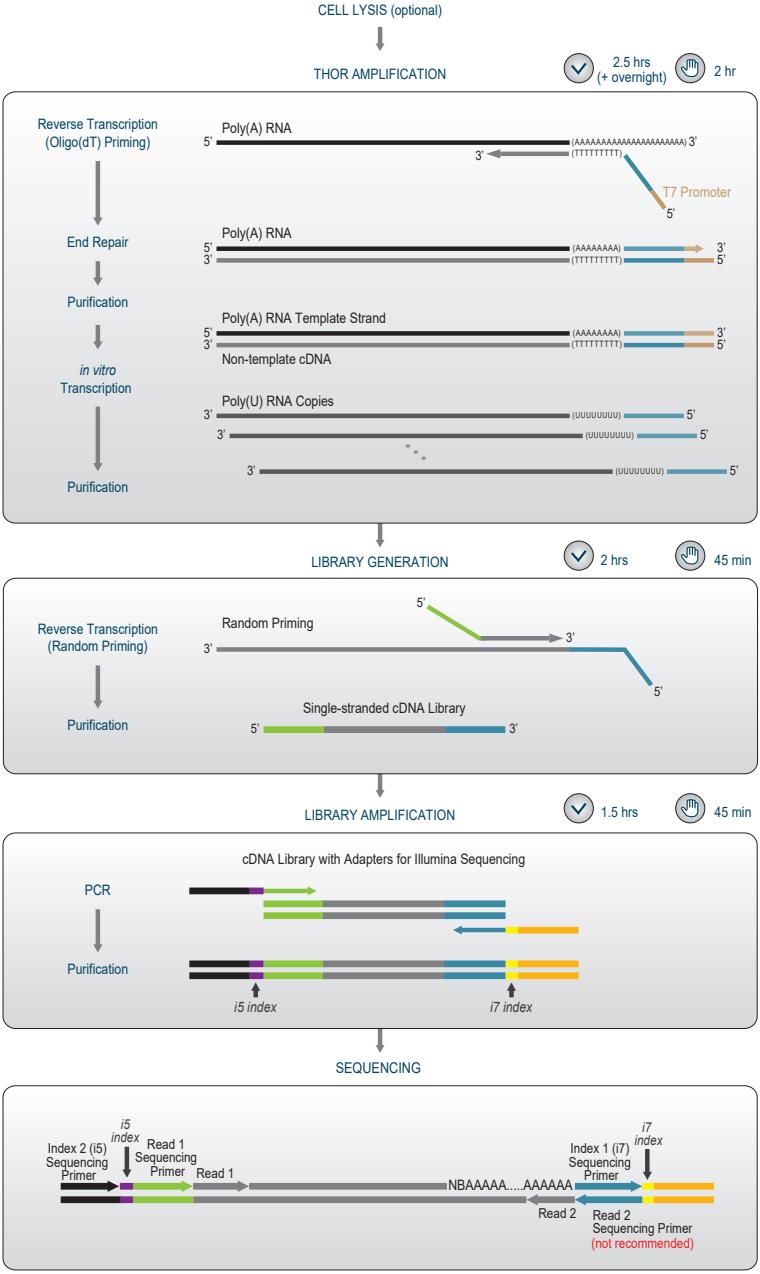


Figure 1. Schematic overview of the LUTHOR 3' mRNA-Seq library preparation workflow (Cat. No. 143). Partial P7 sequences are shown in blue, partial P5 sequences in green, and T7 promoter sequences in light brown. Sequencing read orientation for LUTHOR is depicted, Read 1 reflects the mRNA sequence. Paired-end sequencing is not recommended.

2. Kit Components and Storage Conditions

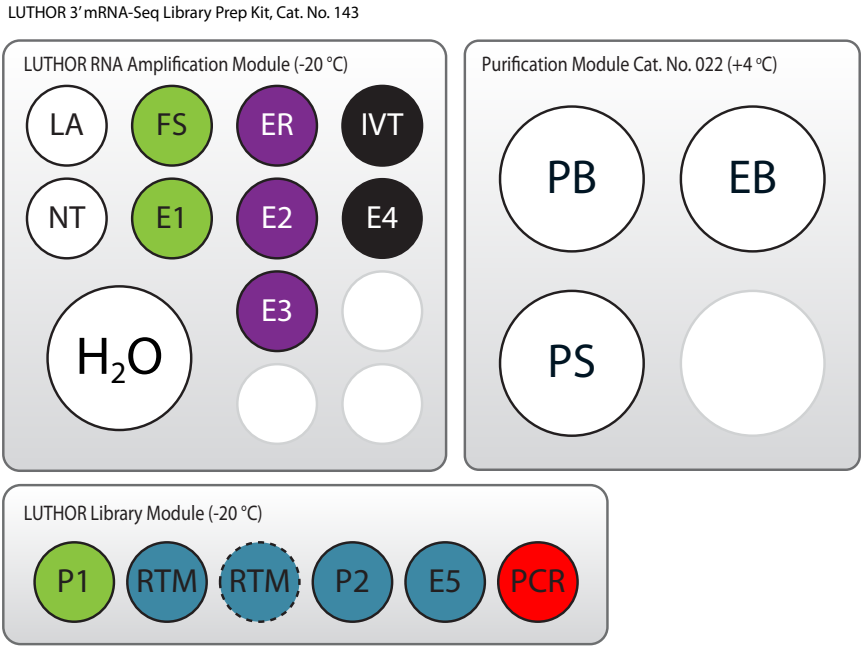


Figure 2. Location of kit components. LUTHOR kits include RNA Amplification Modules, Library Generation Modules, and Purification Modules. LUTHOR is available for 24 and 96 reactions, the larger kit size includes a second vial of RTM (dotted line) and bottles for H_2O and PB which are provided in vials for the 24 prep kit. Please note that indexing primers are required for successful library preparation. Indexing primers are not included in the kit and need to be purchased separately. See p.7 for details.

Kit Component	Tube Label	Volume*		Storage
		24 preps	96 preps	
RNA Amplification Module				
Lysing Agent	**LA** ○	6.9 µl	27.5 µl	-20 °C
Nucleotides (dNTPs)	**NT** ○	26.4 µl	105.6 µl	-20 °C
Molecular Biology Grade Water	**H_2O** ○	996.3 µl	3,985.4 µl	+4 °C / -20 °C
First Strand cDNA Synthesis Mix	**FS** ●	79.2 µl	316.8 µl	-20 °C
Enzyme Mix 1	**E1** ●	26.4 µl	105.6 µl	-20 °C
End Repair Mix	**ER** ●	105.6 µl	422.4 µl	-20 °C
Enzyme Mix 2	**E2** ●	26.4 µl	105.6 µl	-20 °C
Enzyme Mix 3	**E3** ●	26.4 µl	105.6 µl	-20 °C
In Vitro Transcription Mix	**IVT** ●	237.6 µl	950.4 µl	-20 °C
Enzyme Mix 4	**E4** ●	26.4 µl	105.6 µl	-20 °C
Library Generation Module				
RNA Amplification Primer 1	**P1** ●	26.4 µl	105.6 µl	-20 °C
Reverse Transcription Mix	**RTM** ●	475.2 µl	1,900.8 µl	-20 °C
Primer 2	**P2** ●	26.4 µl	105.6 µl	-20 °C
Enzyme Mix 5	**E5** ●	26.4 µl	105.6 µl	-20 °C
PCR Master Mix	**PCR** ●	396.0 µl	1584.0 µl	-20 °C
Purification Module				
Purification Beads	**PB**	792 µl	3,168 µl	+4 °C
Purification Solution	**PS**	3,036 µl	12,144 µl	+4 °C
Elution Buffer	**EB**	1,452 µl	5,808 µl	+4 °C / -20 °C

*including ≥10 % surplus

Upon receiving the LUTHOR 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. **REMARK: H_2O** and **EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **FS**, **ER**, **IVT**, and **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

ATTENTION: Index primers for i5 and i7 are not provided with this kit but required for library amplification. The PCR Master Mix (**PCR** ●) does not include primers and thus index primers for both i5 and i7 are required to introduce the complete Illumina-linkers for cluster generation. Lexogen's UDI 12 nt Sets A1 – A4, or B1 (Cat. No. 101-104, 156, or 105) 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 3' mRNA-Seq Library Prep Kits due to their superior error correction capacity.

Indices are required (not included)! Lexogen UDI 12 nt Sets are recommended (Cat. No. 101 -105, 156).			
Lexogen UDI 12 nt Sets	**UDI 12 A_0001-0384** **UDI 12 B_0001-0096**	6 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**).
- Suitable i5 / i7 indices, e.g., Lexogen UDI 12 nt Sets A1 - A4, or B1 (Cat. No. 101 -104, 156, or 105)
- Lexogen PCR Add-on Kit for Illumina (Cat. No. 020), 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.

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).
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96-well plates and caps 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.25 for more information on RNA quality. Consult Appendix D, p.30 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.
- Steps requiring a thermocycler have been tested with a maximum ramp speed of 5 °C/sec before denaturation and extension, and 2.5 °C/sec during primer annealing. While these ramp speeds are typical for most modern thermocyclers, some models can exceed these rates, and ramp speed may need to be decreased to ensure efficient annealing. Ramp speeds may be reduced even further in some steps of the protocol to ensure better hybridization. Preheat lid to 105 °C, in case this has to be adjusted manually.
- 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 by pipetting, set the pipette to a larger volume. For example after adding 5 / 6 µl in step 5 use a pipette set to 15 µl or 30 µl, respectively, to ensure proper mixing.
- 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 6).

Pipetting and Handling of (Viscous) Solutions

- Enzyme Mixes, **RTM** ●, **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 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 **H_2O** ○ (= 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 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: Step 14 contains an overnight incubation step.

5.1 Cell Lysis

Preparation

Cell Lysis	For Each Sample	Temperature
LA ○ – stored at - 20 °C **NT** ○ – stored at - 20 °C **H_2O** ○ – stored at + 4 °C / -20 °C **P1** ● – stored at - 20 °C	0.26 µl 1 µl 0.74 µl 1 µl	Thermocycler: 50 °C, 10 min 80 °C, 10 min 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

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 to first strand synthesis.

1. **For cell suspensions:** Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl Lysing Agent (**LA** ○), 1 µl Nucleotides (**NT** ○), 0.74 µl Molecular Biology Grade Water (**H_2O** ○), and 1 µl RNA Amplification Primer 1 (**P1** ●) per sample.
   For FACS-sorting of cells: Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl Lysing Agent (**LA** ○), 1 µl Nucleotides (**NT** ○), 2.74 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).
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 10 seconds at 900 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 followed by 10 minutes at 80 °C. Cool to 4 °C and place plate / tubes on ice. **OPTIONAL:** In case frozen cells are used, thaw the suspensions on ice, centrifuge briefly for 30 seconds at 900 x g and continue with cell lysis.

5.2 THOR Amplification

Preparation

First Strand cDNA Synthesis	End Repair	In Vitro Transcription	Purification
NT ○ – thawed at RT **H_2O** ○ – thawed at RT **FS** ● – thawed at RT **P1** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**ER** ● – thawed at RT **E2** ● } keep on ice **E3** ● } or at -20 °C	**IVT** ● – thawed at RT **E4** ● – 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: 42 °C, 20 min 52 °C, 10 min 80 °C, 10 min 25 °C, 1 min	22 °C, 45 min	Thermocycler: 37 °C, 12 - 24 hours 25 °C, 1 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

First Strand cDNA Synthesis - Reverse Transcription

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.

ATTENTION: Before use, check the contents of **FS** ●. 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.

5 **For lysed cells:** prepare a mastermix containing 3 µl First Strand cDNA Synthesis Mix (**FS** ●), 1 µl **H_2O** ○, and 1 µl Enzyme Mix 1 (**E1** ●) per reaction. Add 5 µl of the **FS** / **H_2O** / **E1** mix to the lysed cells from step 4.

For purified RNA: prepare a mastermix containing 3 µl **FS** ●, 1 µl **P1** ●, 1 µl **NT** ○, and 1 µl **E1** ● per reaction. Keep the RNA on ice. Mix 10 pg - 1 ng of total RNA in a volume of 4 µl, with 6 µl **FS** / **P1 / NT / E1** mix in a PCR plate. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate is tightly sealed, and spin down to collect the liquid at the bottom of the wells.

6 Incubate with the following temperature program: 20 minutes at 42 °C, 10 minutes at 52 °C, 10 minutes at 80 °C, then cool to 25 °C and hold for 1 minute. **ATTENTION:** Proceed immediately to end repair.

End Repair

During this step single-stranded 3' poly(A) overhangs are removed from the mRNA, a double stranded promoter sequence for RNA amplification is generated, and the template is prepared for *in vitro* transcription.

ATTENTION: Before use, check the contents of **ER** ●. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

7	Prepare a mastermix containing 4 µl End Repair Mix (**ER** ●), 34 µl **H_2O** ○, 1 µl Enzyme Mix 2 (**E2** ●), and 1 µl Enzyme Mix 3 (**E3** ●) per reaction. Mix gently by pipetting up and down to avoid formation of air bubbles. Add 40 µl of the **ER** / **H_2O / E2 / E3** mix to each sample and mix gently by pipetting, seal the plate, and spin down briefly.
8	Incubate for 45 minutes at 22 °C in a thermocycler or at room temperature. **ATTENTION:** Proceed immediately to purification. **REMARK:** At this point we recommend placing the Purification reagents (**PB**, **PS**, and **EB**) at room temperature to equilibrate for at least 30 minutes before use.

Purification

The cDNA / mRNA hetero-duplex is purified using magnetic beads to remove all reaction components. The beads are retained in the samples for the subsequent reactions steps 13 - 36 and are removed by transfer of the eluted cDNA in step 37.

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.

9	Prepare a mastermix containing 15 µl Purification Beads (**PB**) and 45 µl Purification Solution (**PS**) per sample, and add 60 µl of **PB** / **PS** mastermix to each reaction product from step 8. Mix well, and incubate for 8 minutes at room temperature.
10	Place the plate onto a magnet and let the beads collect for 5 minutes or until the supernatant is completely clear.
11	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.
12	Add 10 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads fully in **EB**. Proceed immediately to step 13.

In Vitro Transcription

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

ATTENTION: Before use, check the contents of **IVT** ●. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely. Step 14 contains an overnight incubation step.

13	Prepare a mastermix containing 9 µl In Vitro Transcription Mix (**IVT** ●) and 1 µl Enzyme Mix 4 (**E4** ●) per reaction, mix gently by pipetting. Add 10 µl of **IVT** / **E4** mastermix to the purified end repair product including beads from step 12 . Mix by pipetting gently, seal the plate, and spin down. **REMARK:** Use a pipette set to 30 µl for efficient mixing.
14	Use thermocycler to incubate overnight (12 - 24 h) at 37 °C, cool down to 25 °C , and proceed immediately to purification.

Purification

The amplified RNA is purified using magnetic beads added previously in step 9. The Purification Module components **PS** and **EB** should equilibrate for 30 minutes at room temperature before use.

15	Add 24 µl of Purification Solution (**PS**) to each reaction product from step 14. Mix well, and incubate for 10 minutes at room temperature.
16	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
17	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Make sure that accumulated beads are not disturbed.
18	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.
19	Repeat this washing step once for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit subsequent PCR reactions.
20	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
21	Add 10 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet and resuspend the beads fully in **EB**. Proceed immediately to step 22. **ATTENTION:** Keep **PS** and **EB** at room temperature, purification reagents will be needed again in step 26.

5.3 Library Generation

Preparation

Reverse Transcription		Purification	
P2 ●	– thawed at RT	**PB**	– stored at +4 °C
RTM ●	– THAWED AT 30 °C, MIX WELL BEFORE USE!	**PS**	– stored at +4 °C
E5 ●	– keep on ice or at -20 °C	**80 % EtOH**	– provided by user; **prepare fresh!**
		EB	– stored at +4 °C
Thermocycler	94 °C, 1 min, 16 °C, 10 min,	Equilibrate all reagents to room temperature for 30 minutes prior to use.	
on ice	4 °C, 5 min;		
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.

ATTENTION: Reverse Transcription Mix (**RTM** ●) is viscous! Thaw on a thermomixer at 30 °C with agitation at 1,250 rpm until completely dissolved and mix thoroughly before use. For the preparation of mastermixes include 10 % surplus per reaction.

22 Prepare a mastermix of 4 µl Reverse Transcription Mix (**RTM** ●) and 1 µl Primer 2 (**P2** ●) per sample. Mix thoroughly and spin down briefly.

23 Add 5 µl of the **RTM / P2** mastermix to 10 µl purified *in vitro* transcription product from step 21. Mix gently by pipetting and quickly spin down. Incubate for 1 minute at 94 °C, cool to 16 °C and incubate for 10 minutes. Transfer the samples to ice immediately and incubate for 5 minutes on ice.

24 Prepare a mastermix of 14 µl Reverse Transcription Mix (**RTM** ●) and 1 µl Enzyme Mix 5 (**E5** ●) per sample. Mix thoroughly and spin down briefly. Add 15 µl of the **RTM / E5** mastermix to the samples from step 23. Mix gently by pipetting and quickly spin down.

25 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. **ATTENTION:** Proceed immediately to purification.

Purification

The first strand cDNA is purified using magnetic beads added previously in step 9. The purification reagents (**PS** and **EB**) should equilibrate for 30 minutes at room temperature before use.

26	Add 10 µl of Purification Solution (**PS**) to each reaction product from step 25. Mix thoroughly and incubate for 5 minutes at room temperature.
27	Place the plate onto a magnet and let the beads collect for 10 minutes, or until the supernatant is completely clear.
28	Remove and discard the clear supernatant without removing the plate from the magnet. **ATTENTION:** Do not disturb the beads!
29	Add 20 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
30	Add 20 µl of Purification Solution (**PS**) to the beads to reprecipitate the cDNA. Mix thoroughly and incubate for 5 minutes at room temperature.
31	Place the plate onto a magnet and let the beads collect for 5 minutes, or until the supernatant is completely clear.
32	Remove and discard the clear supernatant without removing the plate from the magnet. **ATTENTION:** Do not disturb the beads!
33	Add 120 µl of freshly prepared 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.
34	Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely, as traces of ethanol can inhibit the subsequent PCR.
35	Leave the plate in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and hence the resulting library yield.
36	Add 15 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 - 5 minutes at room temperature.
37	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes. Transfer 13 µl of the supernatant into a fresh PCR plate. **ATTENTION:** Proceed immediately to qPCR in case the qPCR assay is used to determine the optimal cycle number for endpoint amplification, see Appendix B, p.27. ☞ Safe stopping point. After elution, libraries can be stored at -20 °C before proceeding to step 38.

5.4 Library Amplification

Preparation

PCR			Purification*	
PCR ●	- thawed at RT	**spin down before opening!**	**PB**	– stored at +4 °C
Indexing Primers, e.g., Lexogen UDI 12 nt Sets (not included!)	- thawed at RT		**PS**	– stored at +4 °C
			80 % EtOH	– provided by user **prepare fresh!**
			EB	– stored at +4 °C
Thermocycler	98 °C, 30 sec 98 °C, 15 sec 60 °C, 30 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞	6 - 18x Endpoint cycle number as determined by qPCR (Cat. No. 020), see Appendix B, p.27.	Equilibrate all reagents to room temperature for 30 minutes prior to use.	

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 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.27.
- **Alternatively, a qPCR assay can be used to determine the optimal PCR cycle number.** The PCR Add-on Kit for Illumina (Cat. No. 020) is required. For qPCR assay details see Appendix B, p.27.
- **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 41 at room temperature to give it at least 30 minutes to equilibrate.

38 Mix 15 µl PCR Master Mix (**PCR ●**) and 9 µl of the eluted library.

39 Add 3 µl i5 and 3 µl i7 indexing primer or 6 µl of the respective Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. Use only one UDI per sample! **ATTENTION:** Spin down UDI plates before opening! Open only the wells containing the desired UDIs. Reseal opened wells after use with a fresh sealing foil to prevent cross contamination!

40 Conduct 6 - 18 cycles of PCR (determine the required cycle number by trial reaction or qPCR) with the following program: Initial denaturation at 98 °C for 30 seconds, 6 - 18 cycles of 98 °C for 15 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, 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.

41 Prepare a mastermix containing 15 µl Purification Beads (**PB**) and 15 µl Purification Solution (**PS**) per sample. Add 30 µl of **PB** / **PS** mastermix to each reaction product from step 40. Mix well, and incubate for 5 minutes at room temperature.

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

43 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads.

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

45 Add 20 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

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

47 Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads.

48 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.

49	Repeat this washing step once for a total of two washes. Remove the supernatant completely.
50	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
51	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
52	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
53	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads. Libraries are now finished and ready for quality control (Appendix D, p.30), pooling (for multiplexing, Appendix E, p.32), 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!

2.5 hrs (+overnight) RNA Amplification

Cell Lysis

Sorted Cells

- ☐ Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl **LA** ○, 1 µl **NT** ○, 2.74 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 **H_2O** ○, 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, then cool to 4 °C. Spin down quickly, and place on ice, proceed to First Strand Synthesis.

First Strand cDNA Synthesis

Cells (Sorted or Cell Suspension)

- ☐ Prepare a mastermix with 3 µl **FS** ●, 1 µl **H_2O** ○, and 1 µl **E1** ● per reaction.
- ☐ Add 5 µl **FS** / **H_2O** / **E1** mix to the lysed cells. Mix well and spin down.

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

- ☐ Prepare a mastermix with 3 µl **FS** ●, 1 µl **NT** ○, 1 µl **P1** ● **,** and 1 µl **E1** ● per reaction.
- ☐ Mix 4 µl RNA and 6 µl **FS** / **P1** / **NT** / **E1.** Mix well and spin down.

- ☐ Incubate for 20 min at 42 °C, 10 min at 52 °C, 10 min at 80 °C, then cool to 25 °C for 1 minute. **Proceed immediately to end repair**.

End Repair

- ☐ Prepare a mastermix with 4 µl **ER** ●, 34 µl **H_2O** ○, 1 µl **E2** ●, and 1 µl **E3** ● per reaction.
- ☐ Add 40 µl of **ER** / **H_2O** / **E2** / **E3** mastermix to each sample. Mix well and spin down briefly.
- ☐ Incubate 45 min at 22 °C. **Proceed immediately to purification**.

Purification

- ☐ Add 15 µl **PB** + 45 µl **PS** per reaction, mix well, incubate 8 min at RT.
- ☐ Place on magnet for 5 min, discard supernatant.
- ☐ Add 10 µl **EB**, remove from magnet, mix well. **Proceed immediately to in vitro transcription.**

In Vitro Transcription

- ☐ Prepare a mastermix with 9 µl **IVT** ● and 1 µl **E4** ● per reaction.
- ☐ Add 10 µl **IVT** / **E4**, to the purified sample / beads mixture, mix well, and spin down.
- ☐ Incubate 12 - 18 hrs at 37 °C, then cool to 25 °C. **Proceed immediately to purification**.

Purification

- ☐ Add 24 µl **PS** per reaction, mix well, incubate 10 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 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!
- ☐ Add 10 µl **EB**, remove from magnet, mix well. **Proceed immediately to reverse transcription**.

Reverse Transcription

- ☐ Prepare a mastermix of 4 µl **RTM** ● and 1 µl **P2** ● per sample. Mix well, spin down.
- ☐ Add 5 µl of **RTM / P2** mastermix to 10 µl in vitro transcription product / beads mixture. Mix well, spin down.
- ☐ Incubate for 1 min at 94 °C, then 10 min at 16 °C, move to ice and incubate for 5 min.
- ☐ Prepare a mastermix of 14 µl **RTM** ● and 1 µl **E5** ● per sample. Mix well, spin down.
- ☐ Add 15 µl **RTM / E5** ●, mix well, spin down briefly.
- ☐ Incubate: 10 min at 25 °C, 40 min at 37 °C, 10 min at 42 °C, 2 minutes at 30 °C, then cool to 25 °C and hold for 1 min. **Proceed immediately to purification.**

Purification

- ☐ Add 10 µl **PS**, mix well and incubate 5 min at RT.
- ☐ Place on magnet for 10 min, discard supernatant.
- ☐ Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Add 20 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 5 - 10 minutes. **ATTENTION:** Do not let the beads dry too long!
- ☐ Add 15 µl **EB**, remove from magnet, mix well, incubate 2 - 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, transfer 13 µl of the supernatant into a fresh PCR plate. **OPTIONAL:** Immediately proceed to qPCR assay.
  ☞ Safe stopping point.

OPTIONAL: qPCR [Requires PCR Add-on Kit (Cat. No. 020.96)]

- ☐ 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 or ThermoFisher, Cat. No. S7585).
- ☐ Combine 1 µl of cDNA with: 7 µl **PCR** ●, 5 µl Primer **7000**, 1 µl **E** ● (from PCR Add-on Kit), 1.2 µl of 2.5x SYBR Green I nucleic acid dye, and 14.8 µl of **EB**, per reaction. Mix well.
- ☐ PCR: 98 °C, 30 sec

  98 °C, 10 sec
  65 °C, 20 sec
  72 °C, 30 sec
  } **35x** (see p. 27)

  72 °C, 1 min

  10 °C, ∞. Calculate the optimal cycle number for endpoint PCR (see Appendix B, p.27).

ATTENTION: Spin down before opening tubes or plates! 1.5 hrs Library Amplification

Endpoint PCR

- ☐ Add 15 µl **PCR** ● to 9 µl of the eluted library.
- ☐ Add 3 µl i5 and 3 µl i7 indexing primer or 6 µl of one Unique Dual Index Primer pair to each sample. **ATTENTION:** Reseal opened index wells after use! Use only one UDI / sample.
- ☐ PCR: 98 °C, 30 sec

  98 °C, 15 sec
  60 °C, 30 sec
  72 °C, 30 sec
  } **6 - 18x** (see p.27)

  72 °C, 1 min

  10 °C, ∞. ☞ Safe stopping point.

Purification

- ☐ Add 15 µl **PB** + 15 µl **PS** per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, discard supernatant.
- ☐ Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Add 20 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 5 - 10 minutes. **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 15 - 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

7. Appendix A: General RNA Requirements

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.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of genomic DNA (gDNA), which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids more intensively than double-stranded, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel gDNA can appear as either a dark mass, which remains in the slot if relatively intact, or as a high molecular weight smear if it has been sheared during extraction. LUTHOR libraries generated from samples containing gDNA may have an increased number of intergenic reads or lower strandedness.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that minimizes gDNA content (e.g., Lexogen's SPLIT RNA Extraction Kit, Cat. No. 008). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. If samples must be DNase treated, heat inactivation should be avoided, and the enzyme should be deactivated by other means such as phenol / chloroform extraction or silica column purification.

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). For ultra-low input RNA, quality control may not be possible as concentrations below 50 pg/µl total RNA are below the detection limit for most microfluidics assays. LUTHOR 3' mRNA-Seq Kits can also be used with low quality RNA samples. Protocol modifications do not apply. For best practice, high quality RNA or fresh, intact cells are recommended as input.

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 1 - 2 % of the reads when using a 3' mRNA-Seq protocol, such as LUTHOR. 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.

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. 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 (85-100 %). Cell aggregates additionally should be removed from the sort gate with doublet discriminator.

Cell Concentration

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 Cell Concentrations

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

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.30).

Optimal cycle numbers can be established by running trial library preps through the complete LUTHOR protocol for each new sample type. Each trial reaction can be amplified using the PCR Master Mix (**PCR** ●) 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.30). 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 Kit for Illumina (Cat. No. 020) 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

The PCR Add-on Kit provides PCR Mix (**PCR** ●), Enzyme Mix (**E** ●), and the P7 Primer (**7000** ●) required for the qPCR assay. In addition, SYBR Green I nucleic acid dye (Sigma Aldrich, S9430 or ThermoFisher, Cat. No. S7585) is also needed and must be supplied by the user.

ATTENTION: Do not use the 12 nt UDIs with the PCR Mix (**PCR** ●) from the PCR Add-on Kit for Illumina (Cat. No. 020)!

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.

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 µl of the cDNA library, 7 µl of PCR Mix (**PCR** ●), 5 µl of P7 Primer (**7000** ●), 1 µl of Enzyme Mix (**E** ●), and 1.2 µl of 2.5x SYBR Green I nucleic acid dye. Make the total reaction volume up to 30 µl by adding 14.8 µl of Elution Buffer (**EB**) or molecular biology-grade water. **ATTENTION:** Include a no template control!

3 Perform 35 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 35 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C **REMARK:** 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. Subtract eight cycles from the cycle number obtained from the previous calculation (see Fig. 3). This is then the final cycle number you should use for the endpoint PCR with 9 µl of the template and the PCR Master Mix (**PCR ●**) from the LUTHOR kit.

Endpoint PCR Cycle Calculation

ATTENTION: The qPCR Add-on Kit uses a different PCR system than the one provided with the LUTHOR kit. Determine the cycle number at 50 % of the maximum fluorescence and subtract 8 cycles to for the endpoint amplification.

10 pg purified total RNA was used for generation of LUTHOR libraries. Using 1 µl of cDNA for a qPCR, the cycle number corresponding to 50 % of the maximum fluorescence was 19 cycles. The remaining 9 µl of the template should therefore be amplified with 11 cycles (19 - 8 cycles = 11 cycles, Fig. 3). Ensure to use the PCR Master Mix (**PCR ●**) provided with the LUTHOR kit for endpoint amplification.

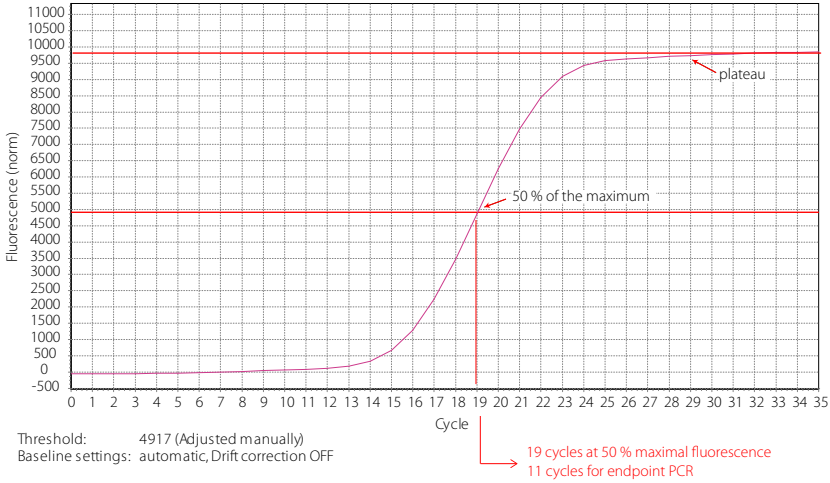


Figure 3. Calculation of the number of cycles for the endpoint PCR.

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 Add-on Kit for Illumina (080.96). This kit is available only upon request. Please contact Lexogen at support@lexogen.com for more information.

Please note that the PCR Add-on Kit (Cat. No. 020) **cannot** be used for reamplification of dual-indexed libraries.

10. Appendix D: Library Quality Control

Quality control of finished LUTHOR 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 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 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 libraries are intended for a high degree of multiplexing, and hence 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.

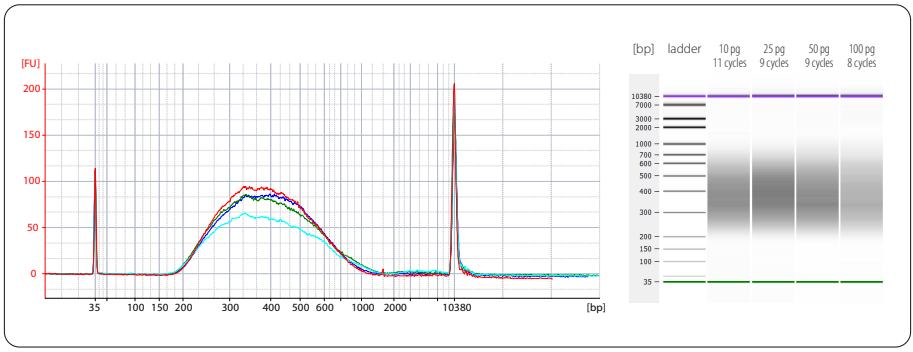


Figure 4. Bioanalyzer traces of LUTHOR libraries prepared from different input amounts of purified total RNA (UHRR). Libraries were prepared using 10 pg (red trace, 11 PCR cycles), 25 pg (blue trace, 9 PCR cycles), 50 pg (green trace, 9 PCR cycles), and 100 pg (cyan trace, 8 PCR cycles) of UHRR input. Endpoint PCR was performed using i5 / i7 dual indexing.

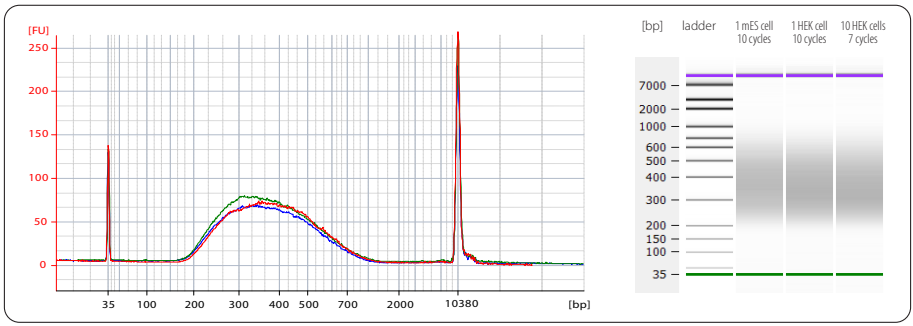


Figure 5. Bioanalyzer traces of LUTHOR libraries generated from mES and HEK293T cells. Libraries were prepared using 1 mES cell (red trace, 10 PCR cycles), 1 HEK293T cell (blue trace, 10 PCR cycles), and 10 HEK293T cells (green trace, 7 PCR cycles) as input.

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.27.

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)	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)

The following Illumina instruments read the i5 index according to the forward strand workflow:

- HiSeq 2000 / 2500 (all SR and PE flow cells)
- HiSeq 3000 / 4000 (SR flow cells only)
- MiSeq (all (PE) flow cells)
- NovaSeq 6000 (all (PE) flow cells) with v1.0 reagent kits

For these instruments 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).

ATTENTION: If Lexogen UDI 12 nt Sets A1, A2, A3 and / or A4 (UDI12A_0001-0384, Cat. No. 101 – 104.96, or 156.384) 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 nt 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.

EXAMPLE: i512A_0001 is read as TTAGTAACTGGG instead of CCCAGTTACTAA. For error correction all 12 nt must be read out and the index read should be analyzed as the reverse complement in this case CCCAGTTACTAA again.

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

The following Illumina instruments read the i5 index according to the reverse strand workflow:

- iSeq 100 (all (PE) flow cells)
- MiniSeq (all (PE) flow cells)
- NextSeq 500 / 550 / 2000 (all (PE) flow cells)
- HiSeq 3000 / 4000 (PE flow cells only)
- NovaSeq 6000 (all (PE) flow cells) with v1.5 reagent kits

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

ATTENTION: If Lexogen UDI 12 nt Set B1 (UDI12B_0001-0096, Cat. No. 105.96) for the reverse strand workflow is used on Illumina machines with forward strand workflow, the i5 Index will be read out as reverse complement. In this case all 12 nt 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.

EXAMPLE: i512B_0001 is read as TTAGTAACTGGG instead of CCCAGTTACTAA. For error correction all 12 nt must be read out and the Index read should be analyzed as the reverse complement in this case CCCAGTTACTAA again.

Index Balance

In general, it is important that each nucleotide (A, C, G, and T) is present at each position of the index reads (Index 1 Read, i7; and Index 2 Read, i5), and that the signal intensity of each nucleotide is perfectly balanced to maintain optimal base calling accuracy and read quality. This is particularly critical for instruments that use two-channel detection (e.g., NextSeq, MiniSeq, and NovaSeq) and one-channel detection (iSeq). For two-channel detection: T is labeled with a green fluorophore, C is labeled with red, half of A is labeled red and the other half is labeled green, and G is unlabeled. One-channel detection uses one dye, two chemistry steps, and two images for detection. In the first image, A and T are labeled with a fluorophore, C and G are unlabled. The Chemistry 2 step leads to an exchange of the fluorophore from A to C, thus in the second image C and T are labeled, and A and G are unlabled. Each nucleotide is determined by the signal pattern across both images.

In general, using the UDIs in numerical order as the number of libraries to multiplex increases, or column-wise for increasing multiples of 8 samples, will result in optimal nucleotide balance. For smaller numbers of samples we can also suggest the following:

- **Four libraries:** Use UDI12A / B_0001 - 0004 as these contain almost perfect nucleotide balance at each position of the index read.
- **Eight libraries:** Use column 1 of the Lexogen UDI 12 nt Set A or B (UDI12A / B_0001 - 0008).

NOTE: Individual libraries within a lane or run should always be pooled at an equimolar ratio to preserve perfect nucleotide balance at each position of the index read.

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 at ~150 bp (for dual-indexed libraries with 12 nt UDIs), or at ~140 bp (for dual-indexed libraries with 6 nt i5 / i7 indices) 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 42 onwards (p.19-20).

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 online Frequently Asked Questions (FAQs), or contact support@lexogen.com. All LUTHOR libraries can be sequenced using the standard Illumina Multiplexing Read 1 Sequencing Primer. A poly(T) stretch corresponding to the oligo(dT) primer is located at the beginning of Read 2. As sequencing through the homopolymer stretch would result in dephasing and drastic reduction of Read 2 quality, paired-end sequencing is not recommended for LUTHOR libraries. 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.32 for details.

LUTHOR libraries:

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

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

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

Sequencing Primers

Standard Illumina sequencing primers are used for all dual-indexed libraries.
The Multiplexing Read 1 Sequencing Primer is always used for Read 1 sequencing, and the Index 1 (i7) Sequencing Primer is always used for Index 1 Read (i7) sequencing. The Index 2 Read (i5) is initiated using different sequencing primers specific to the instrument, flow cell type, and reagent kit as outlined previously.

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

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

Index 2 Read (i5): Multiplexing Index 2 (i5) Sequencing Primer (not supplied):
5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 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 LUTHOR 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 data and is kept as general as possible for integration with your standard pipeline.
LUTHOR 3' mRNA-Seq (Cat. No. 143) 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 Error Correction Tool (available free of charge) can be used for higher accuracy in error correction. Please visit our website or 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.35), 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 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 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.

14. Appendix H: Revision History

Publication No. / Revision Date	Change	Page
143UG290V0100 Oct. 21, 2020	Initial Release of LUTHOR 3' mRNA-Seq Library Prep Kits	

LEXOGEN

Enabling complete transcriptome sequencing

Associated Products:

008, 099 (SPLIT RNA Extraction Kits)
020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
080 (Reamplification Add-on Kit for Illumina)
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)

LUTHOR 3' mRNA-Seq Library Prep Kit · User Guide

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