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HD POOL
LUTHOR

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

User Guide

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

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CONTACT INFORMATION

Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
www.lexogen.com
E-mail: support@lexogen.com

Support
E-mail: support@lexogen.com
Tel. +43 (0) 1 3451212-41
Fax. +43 (0) 1 3451212-99

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1. Overview

Low-throughput, Ultra-low input T7 High-resolution Original RNA (LUTHOR HD Pool) 3' mRNA-Seq Library Prep Kits use Lexogen's proprietary THOR (T7 High-resolution Original RNA) Amplification technology and library preparation methods. LUTHOR HD Pool 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 Unique Molecular Identifier (UMI, 12 nt), an i1 sample-barcode (12 nt), 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 *in vitro* transcription step, the original mRNA template is copied repeatedly by linear amplification. 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 Pool 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, complete adapter sequences, required for cluster generation on Illumina instruments, are added by non-indexed primers. For higher multiplexing capability Unique Dual Indexing is recommended for LUTHOR HD Pool, 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. 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 plate format.

LUTHOR HD Pool 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 of maximum 24 nt in Read 2 is required for i1 read-out (12 nt required) and UMI read- out (additional 12 nt, optional).

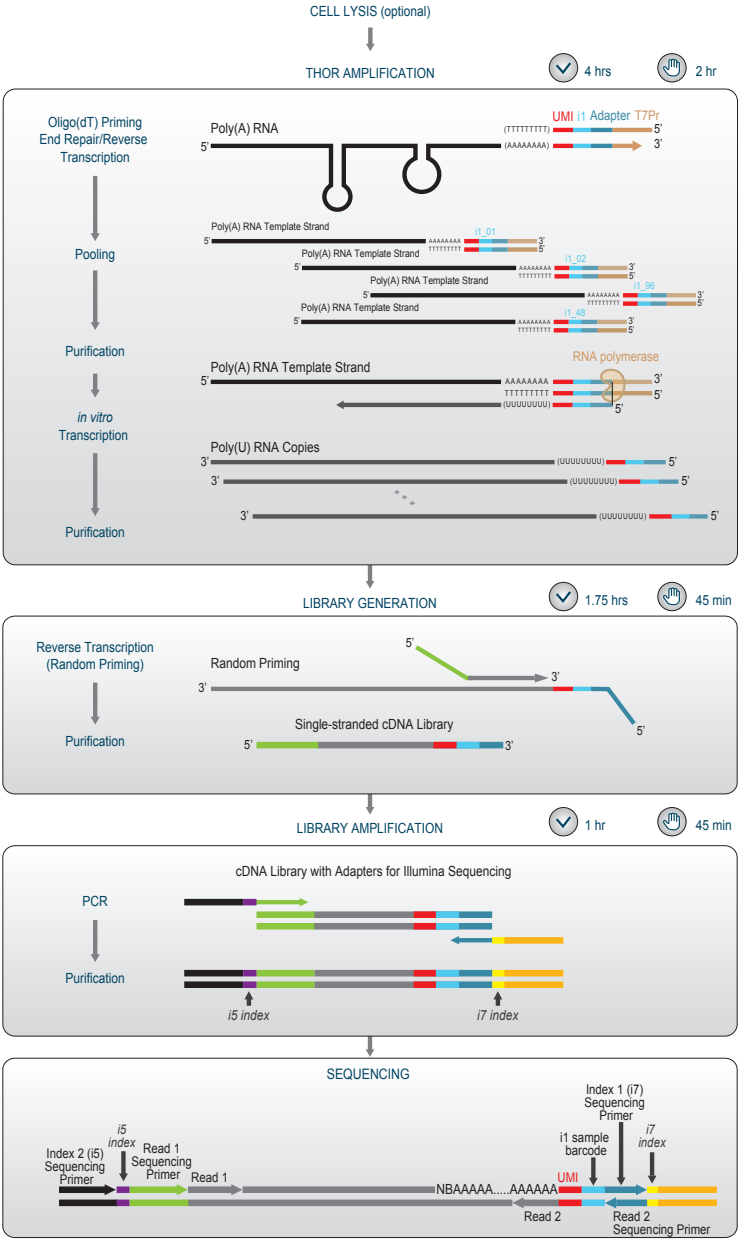


Figure 1. Schematic overview of the LUTHOR HD Pool 3' mRNA-Seq library preparation workflow (Cat. No. 205). Partial P7 sequences are shown in dark blue, partial P5 sequences in green, Unique Molecular Identifier (UMI) in red, i1 sample-barcode in light blue, and T7 promoter sequences in light brown. Sequencing read orientation for LUTHOR HD Pool is depicted, Read 1 reflects the mRNA sequence. A 24 nt long Read 2 is required for i1 and UMI read-out.

2. Kit Components and Storage Conditions

Kit Component	Tube / Plate Label	Volume*	Storage
		96 preps	
i1 Primer Set (96-well plate, dried-in)		1 i1/ rxn	-20 °C /+4 °C
Lysing Agent	**LA** ●	28 µl	-20 °C
Molecular Biology Grade Water	**H_2O** ●	777 µl	-20 °C /+4 °C
Nucleotides (dNTPs)	**NT** ●	114 µl	-20 °C
DTT	**DTT** ●	85 µl	-20 °C
THOR Reagent	**TR** ●	106 µl	-20 °C
Enzyme Mix 1	**E1** ●	60 µl	-20 °C
Enzyme Mix 2	**E2** ●	53 µl	-20 °C
End Repair Enhancer - dilute before use!	**ERE** ●	14 µl	-20 °C
Enzyme Mix 3	**E3** ●	53 µl	-20 °C
In Vitro Transcription Buffer	**IVT** ●	33 µl	-20 °C
Organic Solvent	**OS** ●	33 µl	-20 °C
Enzyme Mix 4	**E4** ●	20 µl	-20 °C
Ribonucleotides (rNTPs)	**RN** ●	40 µl	-20 °C
Primer 2	**P2** ●	14 µl	-20 °C
Reverse Transcription Buffer	**RT** ●	40 µl	-20 °C
Library Amplification Module			
PCR Mix	**PM** ○	93 µl	-20 °C
P5 Primer	**P5** ●	66 µl	-20 °C
P7 Primer	**P7** ●	66 µl	-20 °C
PCR Enzyme Mix	**PE** ○	14 µl	-20 °C
Purification Module			
Purification Beads	**PB**	990 µl	+4 °C
Purification Solution	**PS**	1716 µl	+4 °C
Elution Buffer	**EB**	1452 µl	+4 °C

*including ≥10 % surplus

Upon receiving the LUTHOR HD Pool 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**, **PS**, and **TR**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

It is preferable to avoid thawing and freezing **DTT** ● multiple times. If you plan to use **DTT** ● in different experiments, please aliquot it accordingly, and store frozen aliquots, then use a fresh aliquot for each new experiment.

ATTENTION: Non-index primers P5 and P7 are provided with this kit. For higher multiplexing capability Lexogen's UDI 12 nt Sets A1 – A4, or B1 (Cat. No. 101-104, 156, or 105) containing up to 384 pre-mixed i5/i7 Unique Dual Indices in 96-well format may be used. Lexogen UDIs are 12 nucleotides long and are recommended for LUTHOR HD Pool 3' mRNA-Seq Library Prep Kits due to their superior error correction capacity.

Lexogen UDI 12 nt Sets (optional).			
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**).
- 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 and Reamplification Kit V2 (Cat. No. 208), for qPCR assay.
- SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or Thermo Fisher, Cat. No. S7585), diluted to 2.5x in DMSO, for qPCR assay.

Equipment

- Magnetic plate e.g., 96S Super Magnet Plate,Cat. No. 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 RNase*Zap*, Thermo Fisher Scientific) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNase*Zap* residue with RNase-free water after usage. Residues of RNase*Zap* 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.
- For steps requiring a thermocycler, 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, 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 6 for 96 preps use:
+ 105.6 µl **TR** ● (= 1.0 µl x 96 rxn x 1.1)
+ 52.8 µl **E1** ● (= 0.5 µl x 96 rxn x 1.1)
+ 52.8 µl **E2** ● (= 0.5 µl x 96 rxn x 1.1)
+ 52.8 µl **E3** ● (= 0.5 µl x 96 rxn x 1.1)
+ 52.8 µl 1: 10 diluted **ERE** ● (= 0.5 µl x 96 rxn x 1.1)
+ 211.2 µl **H_2O** ● (= 2 µl x 96 rxn x 1.1)

resulting in a total of 528 µl, which is sufficient for multi-channel pipetting.

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

5. Detailed Protocol

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

ATTENTION: Step 15 contains an optional overnight incubation step.

5.1 Cell Lysis

Preparation

Cell Lysis	For Each Sample cell suspension	For Each Sample FACS sorted cells	Temperature
i1 Primer Plate – thawed at +4 °C	dried in	dried in	Thermocycler: 50 °C, 10 min
LA ● – keep on ice or at -20 °C	0.26 µl	0.26 µl	80 °C, 10 min
NT ● – thawed at +4 °C	1 µl	1 µl	35 °C, 15 sec
DTT ● – thawed at RT	0.74 µl	0.74 µl	+4 °C, ∞
H_2O ● – thawed at +4 °C	1 µl	3 µl	
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**).

A mimimum of 8 samples must be processed in one experiment. **ATTENTION:** Avoid cross-contamination! When processing less than 96 samples at a time, only open those wells needed for the experiment. Dissolve the dried-in i1 Primer provided in the 96-well PCR plate (**i1 Primer plate**) in freshly prepared CLB, and transfer the reaction into a fresh PCR plate. Take care to avoid cross-contamination when transferring the samples!

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.

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 Molecular Biology Grade Water (**H_2O** ●) 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** ●), and 3 µl Molecular Biology Grade Water (**H_2O** ●).

Prepare **CLB** as a mastermix for multiple samples to ensure accurate pipetting of low volumes. Process a minumim of 8 reactions at a time.

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2 **For cell suspensions**: Mix 3 µl of **CLB** with 2 µl of the cell suspension. Transfer CLB / cell suspension in a volume of 5 µl to the desired dried-in sample-barcoded **i1 Primer** provided in the 96-well PCR plate (**i1 Primer plate**). Mix well to dissolve the primer. Seal the plate or close the tube.
For FACS-sorting of cells: Add 5 µl of **CLB** per well to the desired dried-in sample-barcoded **i1 Primer** provided in the 96-well PCR plate (**i1 Primer plate**), mix well to dissolve the primer, spin down briefly and directly sort cells into the buffer. Seal the plate or close the tube.

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3 Mix gently by vortexing or shaking, and centrifuge for 30 seconds at 1250 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.

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4 Lyse cells by incubating the plate / tube for 10 minutes at 50 °C followed by 10 minutes at 80 °C and 15 seconds at 35 °C. Cool to +4 °C, briefly spin down and place plate / tubes on ice.
OPTIONAL: In case frozen cells are used, thaw the suspensions on ice, centrifuge again briefly for 30 seconds at 1250 x g before starting with cell lysis.

5.2 THOR Amplification

Preparation

THOR reaction	In Vitro Transcription	Purification
NT ● – thawed at +4 °C **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 at +4 °C	**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 65 °C, 20 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 a Unique Molecular Identifier (UMI), an i1 sample-barcode, 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.

The minimum number of samples to be processed and pooled at a time is 8 i.e., one column of the dried-in **i1 Primer plate**. **ATTENTION:** Avoid cross-contamination!

The minimum recommended input amount is 10 pg total RNA or one single cell per sample.
The maximum recommended input amount is 1 ng total RNA or 100 cells per sample.
When using purified RNA ensure that the input amount per sample is quantified accurately, as variations in input RNA amounts will influence the distribution of sequencing reads between individual samples. **NOTE:** Higher input amounts are possible, however they require protocol modifications. Please reach support@lexogen.com for additional information.

Input Amount per Sample	Min. No. of Samples Pooled	Max. No. of Samples Pooled
10 pg / 1 cell	8 samples	96 samples
100 pg / 10 cells	8 samples	96 samples
1 ng / 100 cells	8 samples	8 samples

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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.

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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.

Add up to 4 µl **RNA** and 1 µl **NT** ● to the dried in sample-barcoded **i1 Primer** provided in the 96-well PCR plate (**i1 Primer plate**). If a lower RNA volume is used, bring the total volume per reaction to 5 µl with **H_2O** ●. If not the entire plate is used, transfer the RNA / **NT** / **i1 Primer** solution into a separate PCR plate (or 8-well strip) for further processing. Seal the plate (or close the tube). **ATTENTION:** Avoid cross-contamination, open only wells needed for the experiment.

Incubate samples as follows: 5 seconds at 23 °C, 90 seconds at 70 °C, 15 seconds at 35 °C, cool to +4 °C, briefly spin down and place plate / tubes on ice.

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** / **i1 Primer** 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.

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Incubate with the following temperature program: 10 minutes at 37 °C, 5 minutes at 42 °C, 20 minutes at 65 °C, then cool to 25 °C and hold for 1 minute.

OPTIONAL: ☞ Safe stopping point. Individual or pooled THOR reactions may be stored at -80 °C or -20 °C for up to 2 weeks.

Pooling and Purification of Sample-Barcoded RTs

After reverse transcription, each reaction contains a sample specific barcode (i1), enabling pooling and processing of all samples in one reaction. The pooled THOR reactions are 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.

NOTE: Before starting the purification, determine the amounts of Purification Solution (**PS**) by using the table below.

No. of Reactions Pooled	Volume after Pooling	Volume of PB to add	Volume of PS to add
8 reactions	80 µl (8x 10 µl)	30 µl	50 µl
16 reactions	160 µl (16x 10 µl)		130 µl
24 reactions	240 µl (24x 10 µl)		210 µl
48 reactions	480 µl (48x 10 µl)		450 µl

For 96 rxn please purify 2x 48 rxn pools and combine during the purification at step 11.

8 Pool 10 µl of each RT reaction (a minimum of 8 RT reactions) in a 1.5 ml tube and note the final volume of the pool. **ATTENTION:** If evaporation occurs and the total volume of the pool is lower than expected, fill up to the expected volume using Elution Buffer (**EB**).

9 Premix 30 µl of Purification Beads (**PB**) and the required volume of Purification Solution (**PS**), then add to the pooled reaction. Mix well, spin down briefly, and incubate for 5 minutes at room temperature.

10 Place the plate (if only 8 RT reactions were pooled) or 1.5 ml tube onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear. **NOTE:** When using 1.5 ml tubes, look vertically into the tubes to ensure collection of **PB** on the magnet.

11 Remove and discard the clear supernatant without removing the tube from the magnet. **ATTENTION:** Make sure that accumulated beads are not disturbed. Spin down the tube briefly, and place back onto the magnet. Carefully remove the remaining liquid with a 10 µl pipette. Avoid bead loss! **NOTE**: If more than 48 RT reactions (e.g., 96 RT reactions) are to be processed in one *in vitro* transcription reaction, two parallel purifications are required. To combine the samples again, remove the supernatant from both parallel purifications, until ~80 µl are left in each tube (e.g., for 2x 48 reactions, you remove 880 µl). Resuspend the beads in the remaining liquid (~80 µl) and combine both purification reactions in one of the tubes (~160 µl). Place the tube back on the magnet and wait for another 5 - 10 min for the supernatant to become clear again. Remove and discard the supernatant. The purification beads now contain cDNA from both purifications.

12 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.

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

14 Carefully transfer 20 µl of the clear supernatant into a fresh plate / tube.
OPTIONAL: ☞ Safe stopping point. Pooled purified THOR reactions may be stored at -80 °C or -20 °C for up to 2 weeks.

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.

15	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 from step 14. Seal the plate / tube, vortex well and spin down.
16	Use thermocycler to incubate 2 hours at 37 °C, cool down to 25 °C. **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

17	Premix 20 µl Purification Beads (**PB**) and 16 µl Purification Solution (**PS**), then add to each reaction product from step 16. Gently mix and incubate for 5 minutes at room temperature.
18	Place the plate / tube onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear.
19	Remove and discard the clear supernatant without removing the PCR plate / tube from the magnet. Make sure that accumulated beads are not disturbed.
20	Add 120 µl 80 % **EtOH** and incubate for 30 seconds. Leave the plate / tube in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
21	Repeat this washing step once for a total of two washes. To remove the residual ethanol, briefly centrifuge the reaction tube, place it back on the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
22	Keeping the reaction tube on the magnet, air dry the beads for 1 minute at room temperature or until there are no droplets of ethanol left on the walls of the tube. **IMPORTANT:** Do not over-dry by prolonged incubation. Over-drying significantly decreases the elution efficiency.
23	Add 20 µl of Elution Buffer (**EB**) per well / tube, remove the plate from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

24 Place the plate / tube onto a magnet and let the beads collect for 5 - 10 minutes, or until the supernatant is completely clear.

25 Transfer 20 µl of the clear supernatant into a fresh PCR plate / tube. Do not transfer any beads. **OPTIONAL:** ☞ Safe stopping point. Purified aRNA can be stored at -20 °C before proceeding to step 26.

5.3 Library Generation

Preparation

Reverse Transcription		Purification	
NT ●	– thawed at +4 °C	**PB**	– stored at +4 °C
P2 ●	– thawed at RT	**PS**	– stored at +4 °C
RT ●	– thawed at RT	**80 % EtOH**	– provided by user; **prepare fresh!**
E1 ●	– keep on ice or at -20 °C	**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.

26 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.

27 Add 5 µl of the **H_2O / NT / P2** mastermix to 20 µl purified *in vitro* transcription product from step 25. 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 28. **NOTE:** Prepare the mastermix for step 28 while incubating samples at 16 °C for 5 min.

28 Prepare a mastermix of 3 µl RT buffer (**RT** ●), 1.5 µl **H_2O** ●, and 0.5 µl Enzyme Mix 1 (**E1** ●) per sample. Mix thoroughly and spin down briefly. Add 5 µl of the **RT / H_2O / E1** mastermix to the samples from step 27. Mix gently by vortexing or tapping the tube / plate and quickly spin down.

29 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.

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.

30	Add 15 µl Purification Beads (**PB**) and 15 µl Purification Solution (**PS**) to each reaction product. Mix thoroughly and incubate for 5 minutes at room temperature
31	Place the plate / tube onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear.
32	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.
33	Add 20 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads fully in **EB**. Incubate for 2 - 5 minutes at room temperature.
34	Place the plate / tube onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear.
35	Transfer 20 µl of the supernatant into a fresh PCR plate or tube. ☞ Safe stopping point. After elution, libraries can be stored at -20 °C before proceeding to step 36.

5.4 Library Amplification

Preparation

PCR			Purification (Cat. No. 022)
PM ○ **P5*** ● **P7*** ● **PE** ○	- thawed at RT - thawed at RT - thawed at RT - 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 - 15x Endpoint cycle number as determined by qPCR (Cat. No. 208), see Appendix B, p.27.	Equilibrate all reagents to room temperature for 30 minutes prior to use.

* Primers P5 and P7 may be replaced by barcoded primers containing 12 nt UDIs (Cat. No. 101 - 105, 156) for increased multiplexing capabilities.

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. 208) is required, if you have 7 or more pools per kit. For qPCR assay details see Appendix B, p.27.

Generic PCR primers (P5 and P7) without i5 and i7 Indices are included in the kit. Without additional indexing, 96 samples can be combined in one NGS run and demultiplexed according to their respective i1 sample-barcodes (i1 Index sequences). The provided PCR mix is also compatible with barcoded primers that are available for purchase in addition to this kit. For multiplexing of more than 96 samples, Lexogen offers UDI 12 nt Sets A1 – A4, or B1 (Cat. No. 101 – 104.96, 156.384, or 105.96) with superior error-correction. Alternatively, dual indexing primer pairs for partial, i.e., stubby TruSeq adapters from other vendors can also be used.

- 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. Re-seal 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 39 at room temperature to give it at least 30 minutes to equilibrate.

36 Prepare a mastermix containing 7 µl Dual PCR Mix (**PM** ○), 5 µl non-indexed PCR primer **P5** ●, 5 µl non-indexed PCR primer **P7** ●, and 1 µl PCR Enzyme Mix (**PE** ○) per pool.
OPTIONAL: Non-indexed PCR primer **P5** and non-indexed PCR primer **P7** may be substituted using 10 µl of pre-mixed i5 / i7 12 nt UDI primers available as Cat. No. 101 - 105, 156 at Lexogen.

37 Add 18 µl of the **PM / PE** / **P5** / **P7** mastermix to 17 µl of the eluted library.

38 Conduct 6 - 15 cycles of PCR (determine the required cycle number by qPCR) with the following program: Initial denaturation for 60 seconds at 95 °C, then 6 - 15 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C and 60 seconds at 72 °C, and a final extension for 6 minutes at 72 °C, 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.

39	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 38. Mix well, and incubate for 5 minutes at room temperature.
40	Place the plate onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear.
41	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads.
42	Add 30 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.
43	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.
44	Place the plate onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear.
45	Remove and discard the clear supernatant without removing the plate from the magnet. Do not disturb the beads.
46	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.
47	Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.
48	Leave the plate in contact with the magnet, and let the beads dry for up to 5 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.
49	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.
50	Place the plate onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear.
51	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.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! 4 hrs RNA Amplification and IVT

Cell Lysis and i1 Primer Hybridization		i1 Primer Hybridization only
Sorted Cells	**Cell suspension**	**Ultra-low Input Purified RNA (10 pg - 1 ng)**
☐ Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl **LA** ●, 1 µl **NT** ●, 0.74 µl **DTT** ●, and 3 µl **H_2O** ● per reaction.	☐ Prepare Cell Lysis Buffer (**CLB**) by combining 0.26 µl **LA** ●, 1 µl **NT** ●, 0.74 µl **DTT** ● and 1 µl **H_2O** ● per reaction.	☐ Add 4 µl RNA and 1 µl **NT** ● into the desired dried-in sample-barcode RT primer (**i1 Primer**) provided in the 96-well plate and dissolve the primer.
☐ Dispense 5 µl **CLB** into the desired dried-in sample-barcode RT primer (**i1 Primer**) provided in the 96-well plate and dissolve the primer.	☐ Add 3 µl **CLB** into the desired dried-in sample-barcode RT primer (**i1 Primer**) provided in the 96-well plate and dissolve the primer.	
☐ Sort cells directly into the buffer / primer plate.	☐ Add 2 µl cell suspension* to the 3 µl **CLB** / **i1 Primer** mix	
☐ Incubate for 10 min at 50 °C, 10 min at 80 °C, and 15 sec at 35 °C then cool to +4 °C. Briefly spin down, place on ice, then proceed to THOR reaction.		☐ Incubate for 5 sec at 23 °C, 90 sec at 70 °C, 15 sec at 35 °C, then cool to +4 °C. Briefly spin down, place on ice. Proceed to THOR reaction.

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, 20 min at 65 °C, then cool for 1 minute at 25 °C. ☞ Safe stopping point (storage up to 2 weeks).

***ATTENTION:** When using cell suspensions, make sure to remove the cell culture media and wash the cells a least once with 1x PBS before adding 2 µl cell suspension in PBS to the **CLB** / **i1 Primer** mix.

	Pooling and Purification of Sample-Barcoded RTs
☐	Pool 10 µl of each RT reaction into a 1.5 ml tube. **Pool at least 8 samples** and **adjust the maximum number of samples pooled according to the input amount!**
☐	Premix 30 µl of **PB** and required volume of **PS** (see p.16), add to sample, mix well, and incubate 5 min at RT.
☐	Place on magnet for 5 - 10 min, discard supernatant. **ATTENTION:** Do not disturb the beads! Avoid beads loss! **NOTE:** Parallel purifications (e.g., 2x 48 rxn pools) may be combined again at this step (see p.16).
☐	Remove from magnet, add 20 µl **EB**, mix well and incubate 2 min at RT.
☐	Place on magnet for 5 - 8 min, transfer the supernatant into a fresh PCR plate / tube. ☞ Safe stopping point (storage up to 2 weeks).
	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. **OPTIONAL:** ☞ Safe stopping point. After incubation at 37 °C, cool down to +4 °C for overnight incubation, or storage at -20 °C is possible.
	Purification
☐	Premix 20 µl **PB** + 16 µl **PS** per reaction, add to sample, mix well, incubate 5 min at RT.
☐	Place on magnet for 5 - 10 min, discard supernatant.
☐☐	Rinse beads twice with 120 µl 80 % **EtOH**, 30 sec.
☐	Air dry beads for up to 1 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 the supernatant into a fresh PCR plate or tube. ☞ Safe stopping point.

1 hr 40 Reverse Transcription

	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.

Purification

- ☐ Add 15 µl **PB** + 15 µl **PS** per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 5 - 10 min, discard supernatant.
- ☐ Remove from magnet, add 20 µl **EB**, mix well, incubate 2 min at RT.
- ☐ Place on magnet for 5 - 10 min, transfer 20 µl of the supernatant into a fresh PCR plate or tube.
  OPTIONAL: Perform a qPCR assay to determine the exact endpoint cycle number (see p.27)
  ☞ Safe stopping point.

1 - 1.5 hrs (+qPCR) Library Amplification

qPCR [Strongly Recommended! Requires PCR Add-on Kit (Cat. No. 208) for ≥7 pools / kit]

- ☐ 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 of 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.27)
  72 °C, 6 min
  10 °C, ∞..
- ☐ Calculate the optimal cycle number for Endpoint PCR (see Appendix B, p.27).

Endpoint PCR

- ☐ Prepare a mastermix with 7 µl **PM** ○, 5 µl **P5** ●, 5 µl **P7** ●, 1 µl **PE** ○,per reaction.
  OPTIONAL: replace P5 and/or P7 with 10 µl of one Unique Dual Index (UDI) Primer pair.
- ☐ Add 18 µl of the **PM / PE / P5 / P7** mastermix to 17 µl of the eluted library.
- ☐ PCR: 95 °C, 60 sec

  95 °C, 15 sec
  60 °C, 15 sec
  72 °C, 60 sec
  } **6 - 15x** (see p.20)
  72 °C, 6 min, then 10 °C, ∞. ☞ Safe stopping point.

Purification

- ☐ Premix 10 µl **PB** + 25 µl **PS** per reaction, add to sample, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 5 - 10 min, discard supernatant.
- ☐ Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- ☐ Add 24 µl **PS**, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 5 - 10 min, discard supernatant.
- ☐☐ Rinse the beads twice with 120 µl 80 % **EtOH**, 30 sec.
- ☐ Air dry beads for up to 5 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 5 - 10 min, transfer 17 µl of the supernatant into a fresh PCR plate.
  ☞ Safe stopping point.

7. Appendix A: Input and Cell Preparation Guidelines

Input Guidelines

The minimum recommended input amount for LUTHOR HD Pool is 10 pg purified RNA or 1 cell per sample.

The maximum recommended input amount or LUTHOR HD Pool is 1 ng purified RNA or 100 cells per sample. **ATTENTION:** When using cell suspensions, make sure to remove the media and wash the cells a least once with 1x PBS before LUTHOR cell lysis. When using the maximum input amount of 1 ng / 100 cells, a maximum of 8 samples should be pooled. Alternatively, reduce the input material (diluting RNA, less cells), when intending to pool more reactions.

NOTE: Higher input amounts are possible, however they require protocol modifications. Please reach support@lexogen.com for additional information.

Accurate quantification of the RNA input and exact pipetting are required to ensure equal read depth during sequencing. Prepare RNA dilutions with care and use a 10 µl pipette for addition of the RNA to the i1 Primer plate. Variations in input RNA amount will influence the distribution of sequencing reads among individual samples.

Cell Sorting

Prepare single-cell suspensions in 1x Phosphate Buffered Saline (PBS, Ca^{2+}- / Mg^{2+}- free) or Hank's Balanced Salt Solution (HBSS). Do not prepare single-cell suspension in media containing phenol red. Phenol red will increase background fluorescence. It is recommended 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 (diameter)	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.

Cells may also be fixed prior to LUTHOR HD Pool. A protocol for fixation is available in our online Frequently Asked Questions (FAQs).

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 HD Pool 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.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. If you have 7 or more pools per kit, 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 have 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 Pool 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 for 60 seconds at 95 °C, then 35 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C and 60 seconds at 72 °C, and a final extension for 6 minutes at 72 °C, 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. 3).

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. 3).

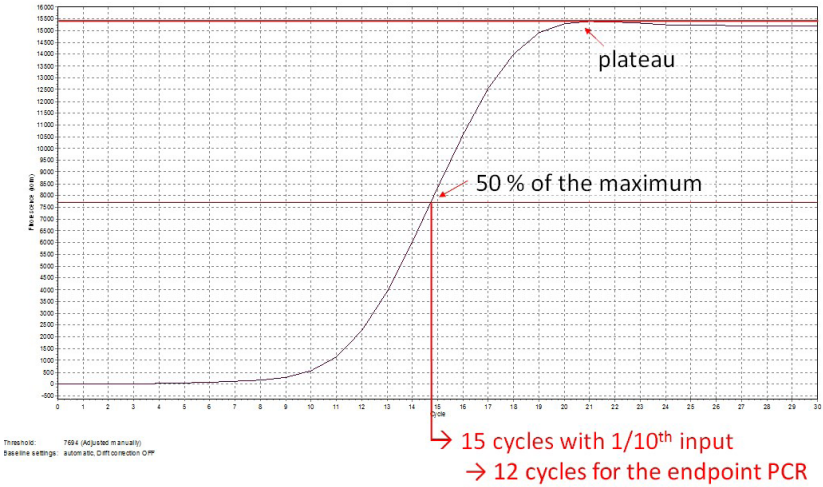


Figure 3. Calculation of the number of cycles for the endpoint PCR.

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 i1 sample-barcoded Libraries (without UDI)

If your library yields are too low, reamplification can be performed using the PCR Mix (**PM** ○), the Enyzme Mix (**PE** ○), the P5 Primer (**P5** ●), and the P7 Primer (**P7** ●) provided in this kit.

Prepare a mastermix of 7 µl PCR Mix (**PM** ○), 5 µl **P5** ●, 5 µl **P7** ●, 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 38). Yield roughly doubles with each additional PCR cycle.

If reagents are not sufficient, you can purchase the PCR Add-on and Reamplification Kit V2 (Cat. No. 208). **NOTE:** For unindexed (no UDI) libraries, you can also use **RE** ○ instead of **P5** ● and **P7** ●.

Reamplification of Dual-Indexed (Lexogen 12 nt UDI) Libraries

If your library yields are extremely low, 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 7 µl 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 38). Yield roughly doubles with each additional PCR cycle.

10. Appendix D: Library Quality Control

Quality control of finished LUTHOR HD Pool 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 Pool 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 Pool 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 a 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 Pool 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.

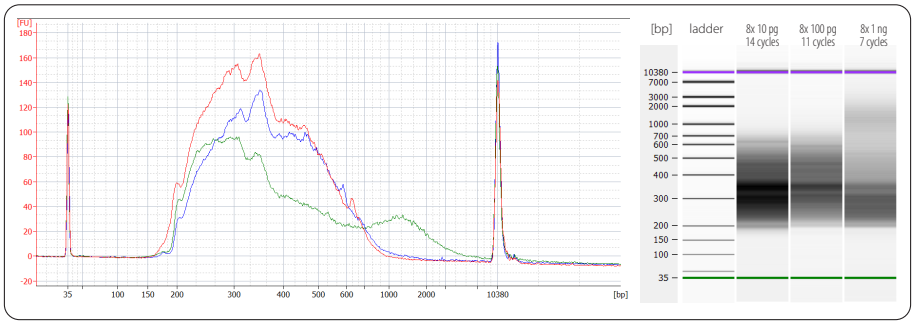


Figure 4. Bioanalyzer traces of LUTHOR HD Pool libraries prepared from different input amounts of purified total RNA (UHRR). Libraries were prepared using 8x 10 pg (red trace, 14 PCR cycles), 8x 100 pg (blue trace, 11 PCR cycles), and 8x 1 ng (green trace, 7 PCR cycles) total RNA input (UHRR). Endpoint PCR was performed using i5 / i7 dual indexing.

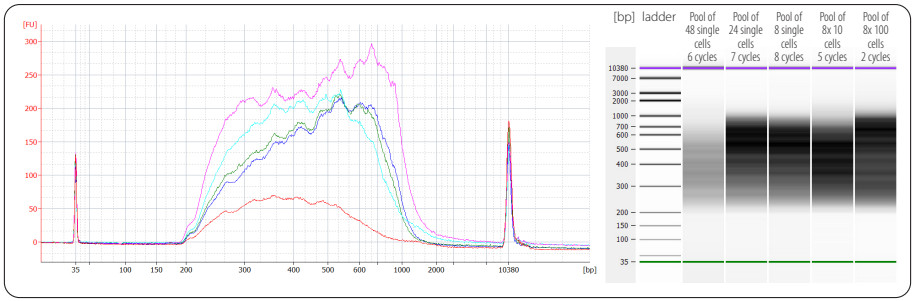


Figure 5. Bioanalyzer traces of LUTHOR HD Pool libraries generated from DU-145 cells. Libraries were prepared using 48x 1 DU-145 cell (red trace, 6 PCR cycles), 24x 1 DU-145 cell (blue trace, 7 PCR cycles), 8x 1 DU-145 cell (green trace, 8 PCR cycles) or 8x 10 DU-145 cells (turquoise trace, 5 PCR cycles) and 8x 100 DU-145 cells (pink trace, 2 PCR cycles) as input.

Overcycling

A second peak in high molecular weight regions (between 2,000 - 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

Generic PCR primers (**P5** ●, **P7** ●) without i5 and i7 Indices are included in the kit. Without additional indexing, up to 96 samples can be combined in one NGS run and demultiplexed according to their respective i1 sample-barcode Indices.

Single Indexing - i1 sample-barcodes (i1 Indices)

i1 sample-barcodes allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit (LP_i1 12 nt Primer Set for LUTHOR HD Pool, 96-well plate). i1 sample-barcodes are 12 nucleotides long. i1 sample-barcodes are succeeded by a 12 nucleotide Unique Molecular Identifier (UMI) and are read out at the end of Read 2, hence to read out i1 and UMIs Read 2 should be 24 cycles. **NOTE:** i1 sample-barcodes contain a nested design allowing for different index read-out lengths: they support read-out of either 8, 10, or 12 nucleotides. You can choose to only read 8 or 10 nt of each i1, in which case you will not be able to read the UMI (located after the i1 Index). For optimal demultiplexing resolution, please consider reading all 12 nucleotides of the i1 sample-barcodes.

The location of the respective i1 sample-barcodes within the 96-well plate is shown below. The minimum pooling requirement is 8, so one column of the i1 Primer plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	LP_i1_0001	LP_i1_0009	LP_i1_0017	LP_i1_0025	LP_i1_0033	LP_i1_0041	LP_i1_0049	LP_i1_0057	LP_i1_0065	LP_i1_0073	LP_i1_0081	LP_i1_0089
B	LP_i1_0002	LP_i1_0010	LP_i1_0018	LP_i1_0026	LP_i1_0034	LP_i1_0042	LP_i1_0050	LP_i1_0058	LP_i1_0066	LP_i1_0074	LP_i1_0082	LP_i1_0090
C	LP_i1_0003	LP_i1_0011	LP_i1_0019	LP_i1_0027	LP_i1_0035	LP_i1_0043	LP_i1_0051	LP_i1_0059	LP_i1_0067	LP_i1_0075	LP_i1_0083	LP_i1_0091
D	LP_i1_0004	LP_i1_0012	LP_i1_0020	LP_i1_0028	LP_i1_0036	LP_i1_0044	LP_i1_0052	LP_i1_0060	LP_i1_0068	LP_i1_0076	LP_i1_0084	LP_i1_0092
E	LP_i1_0005	LP_i1_0013	LP_i1_0021	LP_i1_0029	LP_i1_0037	LP_i1_0045	LP_i1_0053	LP_i1_0061	LP_i1_0069	LP_i1_0077	LP_i1_0085	LP_i1_0093
F	LP_i1_0006	LP_i1_0014	LP_i1_0022	LP_i1_0030	LP_i1_0038	LP_i1_0046	LP_i1_0054	LP_i1_0062	LP_i1_0070	LP_i1_0078	LP_i1_0086	LP_i1_0094
G	LP_i1_0007	LP_i1_0015	LP_i1_0023	LP_i1_0031	LP_i1_0039	LP_i1_0047	LP_i1_0055	LP_i1_0063	LP_i1_0071	LP_i1_0079	LP_i1_0087	LP_i1_0095
H	LP_i1_0008	LP_i1_0016	LP_i1_0024	LP_i1_0032	LP_i1_0040	LP_i1_0048	LP_i1_0056	LP_i1_0064	LP_i1_0072	LP_i1_0080	LP_i1_0088	LP_i1_0096

The Lexogen i1 sequences are available for download at www.lexogen.com/docs/indexing.

The provided PCR mix is also compatible with index primers that are available for purchase in addition to this kit. Lexogen offers various indexing systems that are fully compatible. For multiplexing of more than 96 samples, Unique Dual Indices (UDIs) for the individual pools (containing up to 96 sample-barcoded libraries) is recommended. In this way, multiple pools can be sequenced in one run increasing the multiplexing capacity significantly. The resulting libraries are thus triple indexed with unique i5 and i7 Indices to distinguish the different pools and i1 sample-barcodes to differentiate between individual samples within one pool.

Lexogen's 12 nt Unique Dual Indexing Sets for up to 384 UDIs (Cat. No. 101 - 105, 156) with pre-mixed i5 and i7 Indices in a convenient 96-well format are available and offer superior error correction capacity for massive multiplexing. For more information on indexing, please visit our website and our Frequently Asked Questions (FAQs) or contact support@lexogen.com.

The complete lists of i1, i5, and i7 Index sequences for all Lexogen index Sets are available at www.lexogen.com/docs/indexing.

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).

2. Determine the average library size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to include the whole size distribution and exclude any linker-linker (LL) artifacts. For generic P5 / P7 primers this would be 180 - 2,000 bp (LL peak at ~170 bp) and for 12 nt unique dual indexing use 200 - 2,000 bp (LL peak at ~197 bp). An elevated baseline that extends past the upper marker is an indication of overcycling, and quantification will be biased.

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 ~170 bp (for P5 / P7 amplified non-indexed libraries), or ~197 bp (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), is 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 39 onwards (p.21).

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 Pool 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 12 nt long i1 sample-barcode followed by a 12 nt long Unique Molecular Identifier (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 24 nt in Read 2 are not recommended as a poly(T) stretch corresponding to the oligo(dT) primer is located right after the UMI. 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 Complement 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.

LUTHOR HD Pool libraries:

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

          UMI                 5'-(Index 1 (i7) Sequencing Primer)-3'
…Insert)-N[12]-i1(12 nt) AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
…Insert)-N[12]-i1(12 nt) 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 Pool libraries:
Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Read 2 for LUTHOR HD Pool libraries (required for i1 sample-barcode and UMI read-out):
Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

NOTE: LUTHOR HD Pool libraries are oligo(dT)-primed. Therefore, the poly(T) stretch is located at the beginning of the insert following i1 Index and the UMI sequence in Read 2. Read 2 should therefore be restricted to a maximum of 24 nt and a minimum of 8 to 12 nt (UMI read-out is optional). Read 2 can be shortened to 8 or 10 nt thanks to the nested design of i1 sample-barcodes (see p.32).

Index reads are optional. When multiplexing more than 96 samples per lane, additional i5 / i7 Indices are required and need to be read out in order to distinguish the different pools.

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

Index 2 Read (i5): i5 Index 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 HD Pool 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 Pool data and is kept as general as possible for integration with your standard pipeline.
LUTHOR HD Pool 3' mRNA-Seq (Cat. No. 205) 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 i1

LUTHOR HD Pool libraries need to be demultiplexed according to their i1 sample-barcodes located at the beginning of Read 2 at position 1 to 12. Optionally i1 read-out can be shortened to 8 or 10 nt, if UMI read-out is skipped during sequencing. The Lexogen i1 sequences are available for download at www.lexogen.com/docs/indexing. Demultiplexing of i1 sample-barcoded libraries can be performed using Lexogen's Demultiplexing Tool (available free of charge at https://github.com/Lexogen-Tools). Please contact support@lexogen.com for more information.

Demultiplexing i5 / i7

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 Demultiplexing Tool (available free of charge at https://github.com/Lexogen-Tools) 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, 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 Pool 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 Pool 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 Pool UMI Data Analysis

LUTHOR libraries contain 12 nt long Unique Molecular Identifiers (UMI), located after the i1 sample-barcodes on Read 2. In order to analyze the UMI information, all 12 nt of the UMI sequence need to be extracted and can then be 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
205UG514V0110 Nov. 19, 2026	Changed IVT composition and mastermix generation.	6, 16, 23
	Recommendation to aliquot DTT.	7
	Replaced "stored at" with "thawed at" in small insets.	12, 14, 18
205UG514V0100 Jul. 9, 2024	Initial Release.	

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Associated Products:

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)

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

Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
Telephone: +43 (0) 1 345 1212-41
Fax: +43 (0) 1 345 1212-99
E-mail: support@lexogen.com
© Lexogen GmbH, 2026

Lexogen, Inc.
51 Autumn Pond Park
Greenland, NH 03840, USA
Telephone: +1-603-431-4300
Fax: +1-603-431-4333
www.lexogen.com