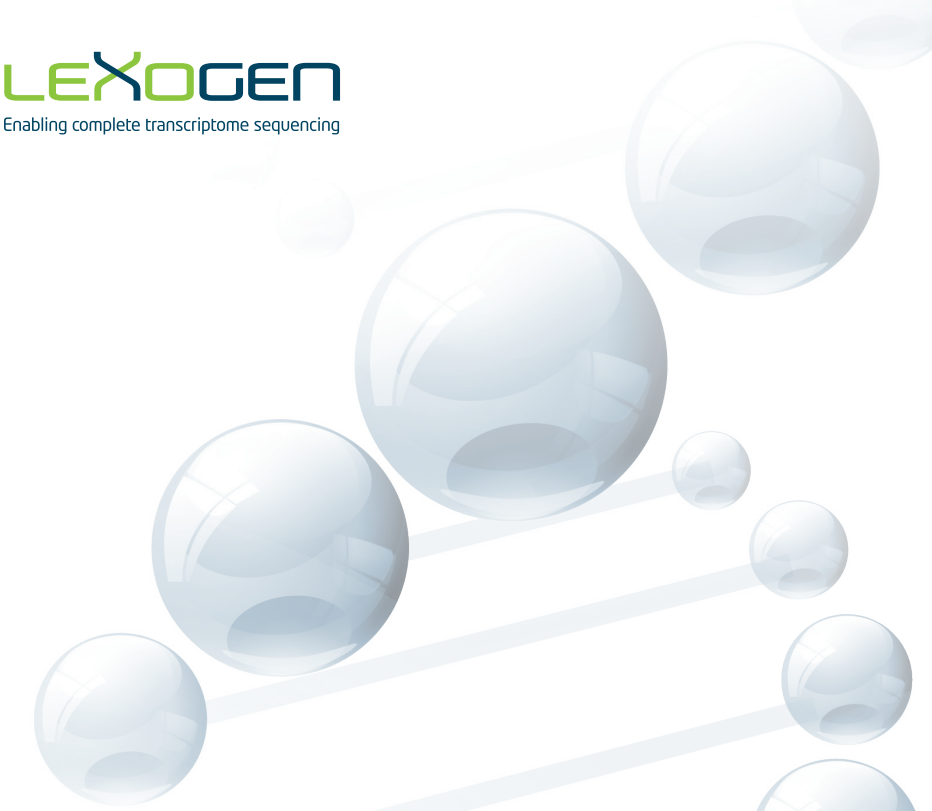
LEXOGEN

Enabling complete transcriptome sequencing



CORALL™

Cover all bases

Total RNA-Seq Library Prep Kit with Unique Dual Indices

User Guide

Catalog Numbers:
117 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Set A1, (UDI12A_0001-0096), 1 rxn/UDI
118 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Set B1, (UDI12B_0001-0096), 1 rxn/UDI)
119 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Sets A1-A4, (UDI12A_0001-0384), 1 rxn/UDI)

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LEXOGEN

Enabling complete transcriptome sequencing

1. Overview

Lexogen's CORALL Total RNA-Seq Kit enables the streamlined generation of Illumina-compatible libraries within 4.5 hours. The fragmentation-free protocol provides for complete transcript coverage including start and end sites. It seamlessly integrates Unique Molecular Identifiers (UMIs) while maintaining protocol-inherent strand specificity (>99 %). CORALL libraries are suitable for cost-efficient single-read as well as paired-end sequencing. The CORALL Total RNA-Seq Library Prep Kits with UDIs include the Lexogen UDI 12 nt Unique Dual Indices (Set A: Cat. No. 117 and 119; or Set B: Cat. No. 118).

Input can be either rRNA-depleted, poly(A)-enriched, or total RNA without prior depletion or enrichment. Information regarding input RNA requirements can be found in Appendices A and B, p.21 - 24. We recommend using Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037) or Poly(A) RNA Selection Kit (Cat. No. 039) upstream of the protocol.

CORALL library generation is initiated by random hybridization of Displacement Stop Primers (DSP) to the RNA template. These primers contain partial Illumina-compatible P7 sequences. Reverse transcription extends each DSP to the next DSP where transcription is effectively stopped. This stop prevents spurious second strand synthesis and thus maintains excellent strand specificity. In addition, no prior RNA fragmentation is necessary, as the insert size is determined by the distance between two DSPs. A highly efficient ligation of Linker Oligos to the 3' ends of first-strand cDNA fragments then introduces partial Illumina-compatible P5 sequences and UMIs. Combined, Lexogen's proprietary Strand Displacement Stop and Ligation technologies empower complete transcript representation including start and end sites.

Lexogen UDI 12 nt Unique Dual Indices for multiplexing (Set A: Cat. No. 117 and 119; or Set B: Cat. No. 118) are included in the kit and are introduced during the PCR amplification step, in which complete adapter sequences required for cluster generation on Illumina instruments are also added (see also Appendix F, p.30). All purification steps are based on magnetic beads, rendering the protocol highly suitable for automation.

CORALL Total RNA-Seq libraries are compatible with single-read and paired-end sequencing on Illumina instruments (Appendix G, p.33). Read 1 contains the UMI and represents the transcript sequence. Hence the UMI information is directly accessible also in the cost-efficient single-read mode. Data can be analyzed with a number of standard bioinformatic pipelines. Information on read orientation and trimming is provided in Appendix H, p.38.

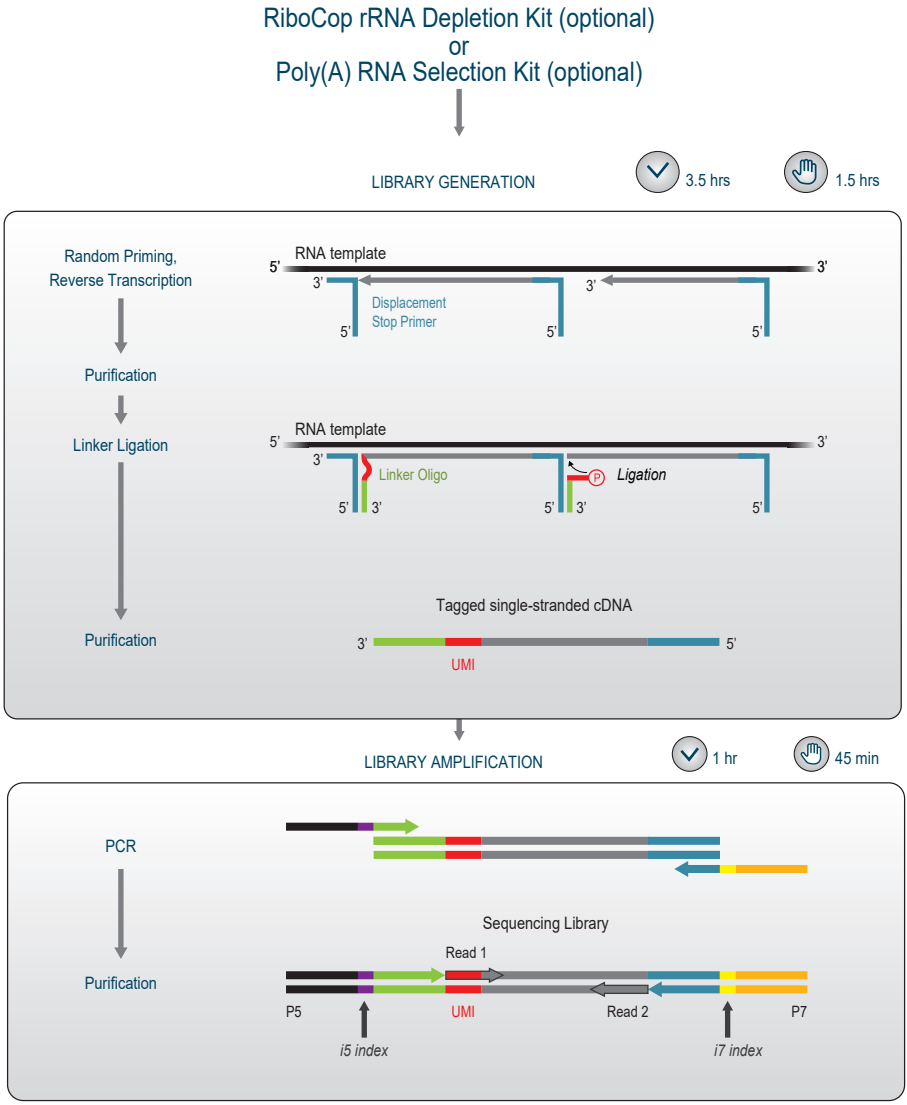


Figure 1. Schematic overview of the CORALL workflow.

2. Kit Components and Storage Conditions

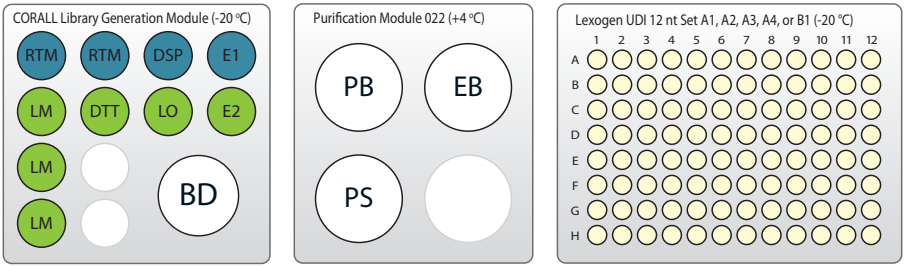


Figure 2. Location of kit components. All kits include Purification Modules. The 96 prep kits are supplied with either the UDI 12 nt Set A1 or B1 96-well plate. The 384 prep kit contains 4 different UDI 12 nt plates (Sets A1-A4), and 4 of each modules.

Kit Component	Tube Label	Volume*		Storage
		96 preps	384 preps	
Reverse Transcription Mix	**RTM** ●	2,094 µl	4x 2,094 µl	-20 °C
Displacement Stop Primer	**DSP** ●	117 µl	4x 117 µl	-20 °C
Enzyme Mix 1	**E1** ●	106 µl	4x 106 µl	-20 °C
Ligation Mix	**LM** ●	4,185 µl	4x 4,185 µl	-20 °C
Dithiothreitol	**DTT** ●	117 µl	4x 117 µl	-20 °C
Linker Oligo	**LO** ●	117 µl	4x 117 µl	-20 °C
Enzyme Mix 2	**E2** ●	234 µl	4x 234 µl	-20 °C
Bead Diluent	**BD** ○	8,343 µl	4x 8,343 µl	-20 °C / +4 °C
Library Amplification Module				
Dual PCR Mix	**Dual PCR** ●	814 µl	4x 814 µl	-20 °C
Enzyme Mix	**E** ●	117 µl	4x 117 µl	-20 °C
Lexogen UDI 12 nt Sets				
Lexogen UDI 12 nt Set A1 or B1	**UDI12A/B_0001-0096**	10 µl / reaction		-20 °C
Lexogen UDI 12 nt Sets A1-A4	**UDI12A_0001-0384**		10 µl / reaction	-20 °C
Purification Module				
Purification Beads	**PB**	4,800 µl	4x 4,800 µl	+4 °C
Purification Solution	**PS**	7,680 µl	7,680 µl	+4 °C
Elution Buffer	**EB**	12,672 µl	12,672 µl	+4 °C

*including ≥10 % surplus

Upon receiving the CORALL kit, store the Library Generation Module at -20°C and the Purification Module (**PB, PS, and EB**) at +4 °C. Prolonged storage of **BD** ○ at -20°C does not affect the stability of the solution. Storage at +4°C together with the Purification Module is suggested for user convenience. Ensure **BD** ○ is fully thawed and equilibrated to room temperature before use.

Before use, check the contents of **BD** ○ and **PS** which may precipitate during storage. If a precipitate is visible or the content appears turbid, incubate at 37 °C until buffer components dissolve completely.

3. User-Supplied Consumables and Equipment

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

Reagents

- 80 % fresh ethanol (EtOH, for washing of Purification Beads, **PB**).
- Recommended: SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585), 10,000x in DMSO for qPCR.

Equipment

- Magnetic rack or plate e.g., for 1.5 ml tubes: BcMag Magnetic separator-24, article# MS-03 from Bioclone; for 96-well plates: 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml tubes or 3,000 x g, rotor compatible with 96-well plates).
- Calibrated single-channel and multi-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath, ice box, ice pellets, or benchtop cooler (-20 °C for enzymes).

Labware

- Suitable 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 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 materials, reagents, and labware necessary for RNA extraction and quality control is not listed. See Appendix A, p.21 for more information on RNA quality and Appendix E, p.28 for information on library quantification and quality control.

4. Guidelines

General

- The Library Generation and purification protocols are primarily designed to be performed in PCR plates or PCR strips, but can also be carried out in 1.5 ml tubes and thermoblocks or thermomixers. This may increase the time requirements for bead separation in purifications steps.
- 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 room temperature, 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. Pre-heat lids to 105 °C, in case this must be adjusted manually.
- Pre-heat thermomixers (dry bath incubators) well in advance.
- Ensure that adequate volumes of all reagents and the necessary equipment are available and set to the proper temperatures 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 thoroughly before use.
- Enzyme mixes 1, 2, and 3 (**E1** ●, **E2** ●, and **E** ●) should not be vortexed! Centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep enzyme mixes at -20 °C until immediately before use or store in a -20 °C benchtop cooler.
- In steps 1, 5, 13, 16, and 28 mastermixes of enzymes and / or 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.
- Reaction mixes must be mixed thoroughly by pipetting or vortexing. Centrifuge briefly with a benchtop centrifuge to collect contents before moving on to the next step.

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 touching equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above open tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Purification Beads, **PB**, 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 fully resuspended, the solution should have a uniform brown color with no visible clumping on the walls or bottom of the tube.
- 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 onto a magnet. The time required for complete separation will vary depending on the strength of your magnets, tube thickness, viscosity of the solution, and the proximity of the 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 tube, depending on the magnet that was used.
- Do not remove the plate from the magnet when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.
- Always store beads in an upright position to ensure that they are covered by liquid.
- In general, beads should not be centrifuged during the protocol. However, should drops of fluid stay on the side of the reaction tube (e.g., after mixing by vortexing), centrifugation at

2,000 x g for 30 seconds should be carried out before placing the tube on the magnetic rack.

- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant, before adding the next reagent. Beads can be resuspended by vortexing but make sure that beads are not deposited on the 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 briefly.

Pipetting and Handling of Viscous Solutions

Enzyme mixes, Reverse Transcription Mix (**RTM** ●), Ligation Mix (**LM** ●), and Purification Solution (**PS**) are viscous solutions which require care to pipette accurately.

- Ensure these solutions are not cooled below room temperature (20 - 25 °C) before use. Cooler temperatures can enhance viscosity and may increase the time and mixing effort required to achieve homogeneity. Follow the specified temperature instructions for thawing and equilibration of these solutions before use!
- 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 an aliquot, 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 well or tube.
- Ensure that pipetting of **RTM** ●, **LM** ●, or **PS** into mixes is performed swiftly, while avoiding bubble formation. Ensure the tip remains under the surface of the liquid. Mix thoroughly until the samples are homogeneous.
- 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.

Automation

CORALL Total RNA-Seq Library preparation is compatible with automation on various platforms. For automation support and inquiries, please contact support@lexogen.com.

5. Detailed Protocol

5.1 Library Generation

Preparation

Reverse Transcription	Ligation	Purification
DSP ● – thawed at RT **RTM** ● – THAWED AT 30 °C, MIX WELL BEFORE USE! **E1** ● – keep on ice or at -20 °C	**LM** ● – THAWED AT 30 °C, MIX WELL BEFORE USE! **DTT** ● – thawed at RT **LO** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **BD** ○ – stored at -20 / +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Thermocycler 94 °C, 3 min, 16 °C, 15 min; 25 °C, 10 min, 37 °C, 40 min, 42 °C, 10 min, 25 °C, 1 min	Thermocycler 37 °C, 30 min, 25 °C, 1min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

Reverse Transcription

The Displacement Stop Primers (**DSP** ●) are hybridized to the RNA and reverse transcription is performed, generating short cDNA fragments with partial adapter sequences at the 5'-ends.

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

EXAMPLE: Step 1 for 24 preps: 475.2 µl RTM (= 18 µl x 24 rxn x 1.1)
+ 26.4 µl DSP (= 1 µl x 24 rxn x 1.1)
resulting in a total of 501.6 µl.

NOTE: Protocol modifications are required while working with FFPE samples. Please refer to Appendix B, p.23 for more information.

1. Prepare a mastermix of 18 µl Reverse Transcription Mix (**RTM** ●) and 1 µl Displacement Stop Primer (**DSP** ●) per sample. Mix thoroughly and spin down briefly.
2. Add 19 µl of the **RTM / DSP** mastermix to 10 µl of RNA sample. If a smaller volume of RNA is used, add RNase-free water to a total volume of 29 µl. Mix thoroughly, quickly spin down. Incubate for 3 minutes at 94 °C, then 15 minutes at 16 °C.
3. Spin down briefly and add 1 µl of Enzyme Mix 1 (**E1** ●). Mix thoroughly and spin down.

4 Incubate with the following temperature program: 10 minutes at 25 °C, 40 minutes at 37 °C, 10 minutes at 42 °C, then cool to 25 °C and hold for 1 minute. **ATTENTION:** Proceed immediately to purification. **REMARK:** At this point we recommend placing the Purification Reagents (**PB, BD ○,** and **EB**) for step 5 at room temperature to equilibrate for at least 30 minutes before use.

Purification

The first strand cDNA is purified using magnetic beads. The purification reagents (**PB, BD ○**, 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.

5 Prepare a mastermix of 9 µl Purification Beads (**PB**) and 29 µl Bead Diluent (**BD ○**) per sample and add 38 µl of **PB / BD** mastermix to each reaction product from step 4. Mix thoroughly and incubate for 5 minutes at room temperature.

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

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

8 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.

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

10 Leave the plate in contact with the magnet and let the beads dry for 5 -10 minutes, or until all the 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.

11 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.

12 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear. Transfer 20 µl of clear supernatant into a fresh PCR plate. **REMARK:** Carry over of residual beads into the ligation will not affect the efficiency of the reaction.
☞ Safe stopping point. After elution, samples can be stored at -20 °C.

Linker Oligo Ligation

Ligation of the Linker Oligo (**LO** ●) adds partial Illumina-compatible adapters at the 3' ends of the first strand cDNA fragments.

ATTENTION: Important notes for Linker Oligo Ligation

- If samples were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before restarting the protocol.
- Before use, thaw the Ligation Mix (**LM** ●) at 30 °C and 1,250 rpm on a thermomixer until dissolved completely.
- **LM** ● and Enzyme Mix 2 (**E2** ●) are viscous solutions! Proper mixing is essential for high yield and excellent reproducibility.
- Thaw DTT at room temperature and mix gently.
- At this point we recommend placing the Purification Module (**PB, PS, BD** ○, and **EB**) for step 16 at room temperature to give it at least 30 minutes to equilibrate.

13	Prepare a mastermix containing 36 µl of Ligation Mix (**LM** ●), 1 µl of Dithiothreitol (**DTT** ●), 1 µl of the Ligation Oligo (**LO** ●), and 2 µl Enzyme Mix 2 (**E2** ●) per sample. Mix thoroughly and spin down briefly. **ATTENTION:** Keep the mastermix at room temperature and proceed immediately to step 14.
14	Add 40 µl of the **LM** / **DTT** / **LO** / **E2** mastermix to the purified reverse transcription product from step 12. Mix thoroughly and spin down briefly.
15	Incubate for 30 minutes at 37 °C, cool to room temperature and proceed immediately to purification.

Purification

The primary library is purified using magnetic beads to remove ligation reaction components. The Purification Reagents (**PB**, **BD** ○, **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.

16 Prepare a mastermix of 9 µl Purification Beads (**PB**) and 50 µl Bead Diluent (**BD** ○) per sample and add 59 µl of **PB / BD** mastermix to the ligation reaction from step 15. Mix thoroughly and incubate for 5 minutes at room temperature.

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

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

19 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.

20 Add 42 µl of Purification Solution (**PS**) to the beads to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

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

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

23 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.

24 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.

25 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.

26 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.

27 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes. Transfer 17 µl of the supernatant into a fresh PCR plate. **ATTENTION:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, add an additional 2 µl of Elution Buffer (**EB**) to the eluted library. For further details please refer to Appendix C, p.25.
☞ Safe stopping point. After elution, libraries can be stored at -20 °C.

5.2 Library Amplification with UDI 12 nt Unique Dual Indices

This section describes unique dual indexing PCR for multiplexing up to 384 libraries using the Lexogen UDI 12 nt Unique Dual Indices included in this kit.

Preparation

PCR			Purification (Cat. No. 022)*
Dual PCR ● (purple) **Lexogen UDI 12 nt Sets** (A1 - A4, or B1) **E** ● (yellow)		- thawed at RT - thawed at RT - keep on ice or at -20 °C } **spin down before opening!**	**from standard library prep kits:** **PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞	} 11- 25x Endpoint cycle number as determined by qPCR (Cat. No. 020), see Appendix C, p.25.	Equilibrate all reagents to room temperature for 30 minutes prior to use.

PCR

The library is amplified to add the complete adapter sequences required for cluster generation and unique indices for multiplexing, and to generate sufficient material for quality control and sequencing.

ATTENTION: Important notes for Library Amplification.

Perform a qPCR assay 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. The PCR Add-on Kit for Illumina (Cat. No. 020) is required. For qPCR assay details see Appendix C, p.25.

- Avoid cross contamination when using the Lexogen UDI 12 nt Indexing 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 using 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 32 at room temperature to equilibrate for at least 30 minutes.

28 Prepare a mastermix containing 7 µl Dual PCR Mix (**Dual PCR** ●) and 1 µl Enzyme Mix (**E** ●) per reaction.

29 Add 8 µl of the **Dual PCR / E** mastermix to 17 µl of the eluted library.

30 Add 10 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 the plates containing the UDIs before opening! Pierce or cut open the sealing foil of the wells containing only the desired UDIs. Reseal opened wells of the UDI plate after use with a fresh sealing foil to prevent cross contamination!

31 Conduct 11 - 25 cycles of PCR (determine the required cycle number by qPCR) with the following program: Initial denaturation at 98 °C for 30 seconds, 11 - 25 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.
☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The final library is purified to remove PCR components that can interfere with quantification. 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.

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

32 For **CORALL** libraries add 31.5 µl of thoroughly resuspended Purification Beads (**PB**) to each reaction.
REMARK: For **CORALL libraries generated from FFPE RNA**, add 42 µl of **PB**. Mix well, and incubate for 5 minutes at room temperature.

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

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

35 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.

36 Add 30 µl of Purification Solution (**PS**) to the **PB** / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

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

38	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads!
39	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.
40	Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.
41	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.
42	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.
43	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
44	Transfer 17 µl of the supernatant into a fresh PCR plate. Do not to transfer any beads.
45	At this point, the libraries are finished and ready for quality control (Appendix E, p.28), pooling (for multiplexing see Appendix F, p.30), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates. Thaw RTM ● and LM ● at 30 °C and 1,250 rpm before use.

3.5 hrs Library Generation

	Reverse Transcription
☐	Prepare a mastermix of 18 µl **RTM** ● and 1 µl **DSP** ● per sample. Mix well.
☐	Add 19 µl of **RTM / DSP** mastermix to 10 µl RNA sample. Mix well.
☐	Incubate for 3 min at 94 °C, then 15 min at 16 °C.
☐	Add 1 µl **E1** ●, mix well.
☐	Incubate: 10 min at 25 °C, 40 min at 37 °C, 10 min at 42 °C, cool to 25 °C. **Proceed immediately to purification!**
	Purification
☐	Add 9 µl **PB** + 29 µl **BD** ○, mix well and incubate 5 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 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
☐	Place on magnet for 2 - 5 min, transfer the supernatant to a fresh PCR plate. ☞ Safe stopping point.
	Linker Oligo Ligation
☐	Prepare a mastermix of 36 µl **LM** ●, 1 µl **DTT** ●, 1 µl **LO** ●, and 2 µl **E2** ● per sample. Mix well.
☐	Add 40 µl of **LM / DTT / LO / E2** mastermix to each sample. Mix well.
☐	Incubate for 30 min at 37 °C, then cool to 25 °C. **Proceed immediately to purification!**
	Purification
☐	Add 9 µl **PB** + 50 µl **BD** ○, mix well and incubate 5 min at RT.
☐	Place on magnet for 2 - 5 min, discard supernatant.
☐	Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
☐	Add 42 µl **PS**, mix well, incubate 5 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 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
☐	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

ATTENTION: Spin down solutions before opening tubes or plates.

1 - 1.5 hrs (+qPCR) Library Amplification

qPCR [Strongly Recommended! Requires PCR Add-on Kit (Cat. No. 020.96)]

- ☐ Add 2 µl of **EB** to the 17 µl of eluted cDNA.

  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).
- ☐ Combine 1.7 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 stain, and 14.1 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.25)

  72 °C, 1 min

  10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (see Appendix E, p.28).

Endpoint PCR

- ☐ Prepare a mastermix with 7 µl Dual PCR Mix (**Dual PCR** ●) and 1 µl Enzyme Mix (**E** ●) per reaction.
- ☐ Add 8 µl of the **Dual PCR / E** mastermix to 17 µl of the eluted library.
- ☐ Add 10 µl of one Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. **ATTENTION:** Reseal opened index wells after use! Use only one UDI / sample.
- ☐ PCR: 98 °C, 30 sec

  98 °C, 10 sec
  65 °C, 20 sec
  72 °C, 30 sec
  } **11 - 25x** (see p.25)

  72 °C, 1 min

  10 °C, ∞. ☞ Safe stopping point.

ATTENTION: Increased cycle numbers may be required for low input / low quality / FFPE RNA (see Appendix A, p.21 and Appendix B, p.23)

Standard Input	FFPE RNA
Purification	
☐ Add 31.5 µl **PB** per reaction, mix well, incubate 5 min at RT.	☐ For FFPE RNA: Add 42 µl **PB** per reaction, mix well, incubate 5 min at RT.
☐ Place on magnet for 2 - 5 min, discard supernatant.	
☐ Add 30 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.	
☐ Add 30 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

Potential 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 agents generates a lower A260/230 ratio. Phenol has an absorption maximum between 250 and 280 nm, which overlaps that of nucleic acid, hence 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 however should be removed by subsequent poly(A) selection or rRNA depletion.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of gDNA, which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many 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. CORALL libraries generated from samples containing gDNA may have an increased number of intergenic reads and lower strandedness.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA (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. DNase I treatment is highly recommended for FFPE RNA. 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. Alternatively, after treatment with DNase I (using DNase I according to manufacturers instructions in a total reaction volume of 20 µl), the reaction can be directly used as input for rRNA depletion (human / mouse / rat) with Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037).

RNA Storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater (Ambion Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Avoid frequent freeze / thaw cycles as RNA might be sheared. Addition of an RNase inhibitor, e.g., RNasin from Promega at a final concentration of 0.4 U/µl, is recommended.

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.). However, RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Best practice is to check the RNA before mRNA selection or rRNA depletion as well as afterwards. After poly(A) selection or rRNA depletion rRNA peaks should not be detectable anymore. rRNA depleted samples will show a high abundance of short RNAs (especially tRNAs) which are not removed by the depletion methods. Using an RNA extraction method that avoids co-purifying those small RNA, such as Lexogen's SPLIT RNA Extraction Kit (Cat. No. 008.48, following the User Guide for extracting large RNA fractions) can be used if small RNAs are not of interest in a particular experiment.

RNA Integrity - FFPE RNA

RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue is often heavily degraded due to the fixation process. As a result of this degradation 28S and 18S rRNA peaks are often completely absent from such RNA samples and hence the calculation of a meaningful RIN value is not possible anymore. A more commonly used reference value for FFPE and degraded RNA is the Distribution Value 200 (DV_{200}), which is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. This typically leads to smaller insert sizes which should be kept in mind when choosing read lengths for the sequencing experiment. For further information on protocol modifications for FFPE samples see Appendix B, p. 24.

8. Appendix B: RNA Input

The recommended input for CORALL Total RNA Library Prep is ribosomal RNA-depleted (ribo-depleted) RNA, or poly(A)-enriched RNA. Total RNA without depletion or enrichment (100 pg to 100 ng) can also be used, if required.
High quality RNA-Seq data relies on high quality input RNA. Ribosomal RNAs (rRNAs) will consume the majority of sequencing reads unless these are removed before CORALL Total RNA-Seq Library Prep. Removal of rRNA can be achieved either by rRNA depletion or by poly(A) selection. The amount of non-ribosomal RNA recovered depends on the method and the starting material. For Universal Human Reference RNA the non-ribosomal RNA fraction usually accounts for 1 % to 5 %. Higher recovery rates may indicate rRNA contamination.

Poly(A) Selection

We recommend using Lexogen's Poly(A) Selection Kit (Cat. No. 039) to prepare poly(A) mRNA as input for CORALL Total RNA-Seq Library Prep. High quality RNA (RIN >8) constitutes an ideal input for poly(A) selection. For samples with RNA integrity or quality scores (RIN, RQN) below 8, we strongly recommend using rRNA depletion (Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037)). For poly(A) selection using Lexogen's Poly(A) Selection Kit and subsequent CORALL library prep, the advised input range is 1 ng - 1 µg of total RNA. If input amounts below 100 ng are used, reduce the elution volume of poly(A)-selected RNA according to the Poly(A) Selection Kit User Guide (039UG069).

Ribo-Depletion

For rRNA depletion of human / mouse / rat RNA samples we recommend using Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037). RiboCop also removes the mitochondrial rRNA (mt-rRNA), which can otherwise account for more than 40 % of the reads. For other sample types we recommend the use of rRNA depletion methods that also remove mt-rRNAs. CORALL is also compatible with other commercially available rRNA depletion kits. Please contact support@lexogen.com for more information. For RiboCop rRNA depletion the recommended input range is 1 ng - 1 µg of high quality total RNA (RIN ≥8), or 100 ng - 1 µg of lower quality RNA (RIN <8).

RNA Input Amount

We recommend performing the protocol initially with 1 - 2 ng of rRNA-depleted RNA or poly(A) RNA (quantified after depletion / selection). This is equivalent to approximately 100 - 200 ng of total RNA. **We strongly recommend performing a qPCR assay to determine the optimal PCR cycle number for your RNA input** (see Appendix C, p.25 for more details).

The CORALL library prep protocol was also tested using RNA from different species, including hamster, mini pig, mouse, *Arabidopsis sp.*, and *E. coli*).

EXAMPLE: 200 ng of Universal Human Reference (total) RNA used for RiboCop rRNA depletion gives ~2 ng of rRNA-depleted RNA, which can be used directly for CORALL Total RNA-Seq library prep, and amplified with 11 - 12 PCR cycles.

FFPE Samples

Protocol modifications are recommended for library preparation from FFPE samples with CORALL library prep kit. The following recommendations have been evaluated with as low as 25 ng total RNA input and sequencing is recommended with SR75:

- Perform DNase I treatment prior to rRNA depletion (without an intermediate cleanup step). Avoid inactivation!
- In step 32 use 42 µl of **PB** (instead of 31.5 µl)
- **Optional:** Add SIRV-Set 3 (0.1 - 0.2 % of target RNA fraction) prior to DNase I treatment.

For further questions, please contact support@lexogen.com.

SIRV Spike-in RNA Variant Control Mixes

The Lexogen SIRV (Spike-In RNA Variant) controls are artificial spike-in transcripts that serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences, hence they can be spiked into any RNA. The SIRV sequences enable bioinformatic algorithms to accurately map, assemble, and quantify isoforms, and provide the means for the validation of these algorithms in conjunction with the preceding RNA-Seq pipelines. SIRVs are available in three sets, SIRV-Set 1 (Cat. No 025) contains the Isoform Mixes E0, E1, and E2. Each mix contains all SIRV transcripts, but at different molar concentrations to each other. SIRV-Set 2 (Cat. No. 050) provides the Isoform Mix E0 only, whereas SIRV-Set 3 (Cat. No. 051) has the SIRV Isoform Mix E0 in a mixture with the ERCC RNA Spike-in controls (Thermo Fisher Scientific Inc., see below). SIRV Mixes can be used as single spike-ins, or by spiking-in different SIRV Isoform mixes to different samples, for the assessment of differential gene expression.

ERCC RNA Spike-in Controls

To enable the hypothesis-neutral calculation of strandedness, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the External RNA Controls Consortium Spike-In controls (ERCCs, Thermo Fisher Scientific Inc.). These sets of RNAs, just as the SIRVs, have a known strand orientation, so the calculation of strandedness based on spike-in sequences is more accurate than calculations based on reads aligning to the genome. ERCC spike-in transcripts are however monoexonic, hence for CORALL we recommend using SIRVs, or SIRV-Set 3, which contains both SIRVs and ERCCs.

9. Appendix C: 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. Variable input types and amounts require optimization of endpoint PCR cycle numbers. **We strongly recommend taking advantage of the qPCR assay to optimize the number of cycles required for the endpoint PCR.** This will prevent both under- and overcycling, the latter of which may bias your sequencing results (see also Appendix G, p.33).

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 additional 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. Enzyme Mix 3 (**E3** ●) supplied in the CORALL Kits, and Enzyme Mix **E** ● from the PCR Add-on Kit can be used interchangeably for qPCR and endpoint PCR. **PCR** ● is also interchangeable between the PCR Add-on and CORALLKits.

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 have to be adjusted manually.

ATTENTION: The use of SYBR Green I-containing qPCR mastermixes from other vendors is not recommended.

1	Dilute the primary library from step 27 to 19 µl by adding 2 µl Elution Buffer (**EB**) or molecular biology-grade water.
2	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 the amplification.
3	For each reaction combine: 1.7 µl of the diluted 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 14.1 µl of Elution Buffer (**EB**) or molecular biology-grade water. **ATTENTION:** Include a no template control!

4 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; a final extension at 72 °C for 1 minute, and hold at 10 °C. **REMARKS:** There is no need to purify or analyze the overcycled PCR product on a Bioanalyzer.

5 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).

REMARK: The qPCR assay can be used for dual- or single-indexed libraries.

Example for Endpoint Calculation

2 ng of rRNA-depleted RNA input (equivalent to ~200 ng total RNA) was used for generating CORALL libraries. Using 1.7 µl of cDNA for a qPCR, the cycle number corresponding to 50 % of the maximum fluorescence was 15 cycles. The remaining 17 µl of the template should therefore be amplified with 12 cycles (15 - 3 cycles = 12 cycles).

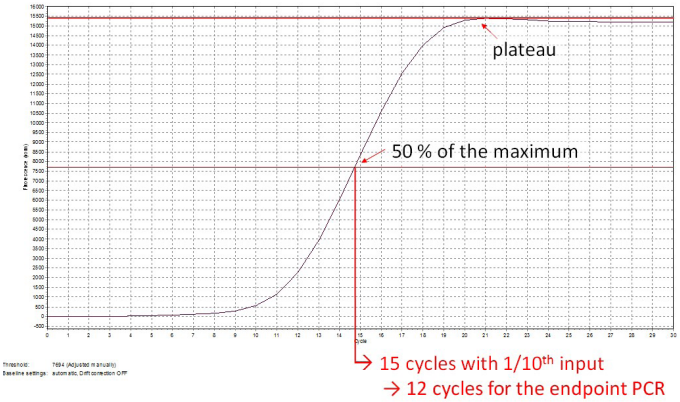


Figure 3. Calculation of the number of cycles for the endpoint PCR.

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

10. Appendix D: Library Reamplification

Reamplification of Uniquely 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.

11. Appendix E: Library Quality Control

Quality control of finished CORALL 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 CORALL 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).

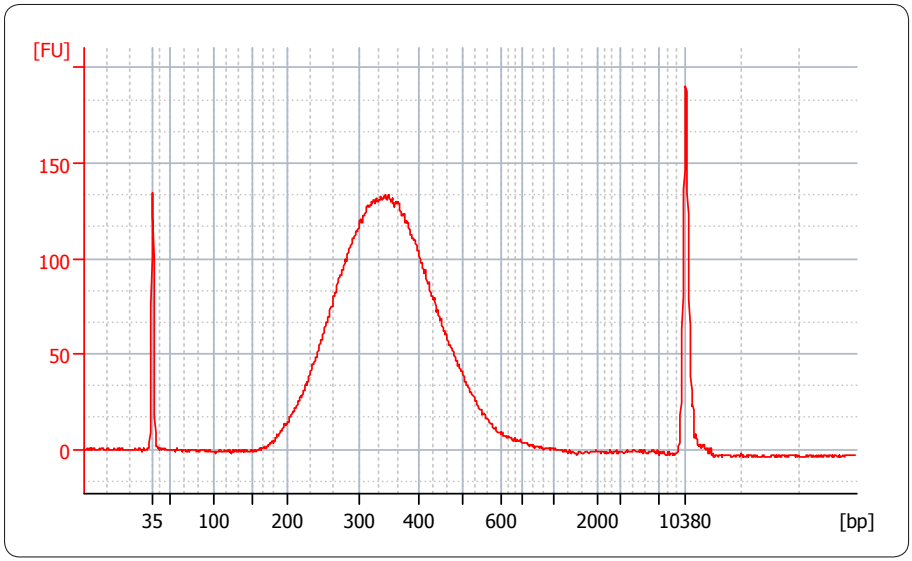


Figure 4. Bioanalyzer trace of a typical CORALL library synthesized from 200 ng Universal Human Reference RNA (UHRR) input for poly(A) enrichment. Approximately ~2 ng purified mRNA were recovered using the Lexogen Poly(A) Selection Kit (Cat. No. 039) and inserted into CORALL library preparation. The primary library was amplified with 11 cycles, purified, and quality controlled using a Bioanalyzer High Sensitivity DNA assay.

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 CORALL 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.

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible at ~175 bp (for dual-indexed libraries), 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 (≤175 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 33 onwards (p.17-18).

Overcycling

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. Performing the qPCR to determine the optimal cycle number for endpoint PCR (as recommended in Appendix C, p.25) prevents overcycling. Overcycled PCRs may still be used for subsequent sequencing. However, gene expression values may be biased. To guarantee accurate quantification of overcycled libraries for lane mixing, we recommend performing a qPCR-based quantification method rather than relying on the Bioanalyzer quantification. For further experiments using the same input RNA, please adjust your cycle number accordingly.

12. Appendix F: Multiplexing

Libraries prepared with the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits 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 nucleotides long UDI sequence while maintaining superior error correction features. The longer the UDI read-out the higher is 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 and flow cell type (paired-end, PE; single-read, SR), i5 indices are sequenced differently.

Illumina Instruments	Flow Cell Type	Work-flow	Lexogen UDI 12 nt Unique Dual Indexing Kits
HiSeq 2000/2500 HiSeq 3000/4000	SR	A	Add-on Kits, **Set A1, A2, A3, and / or A4** (UDI12A_0001-0384), Cat. No. 107 – 110.96 or 120.384 CORALL with Set A1 or A1-A4, Cat. No. 117.96 or 119.384
HiSeq 2000/2500 MiSeq NovaSeq 6000	PE		
MiniSeq NextSeq 500/550 HiSeq 3000/4000	PE	B	Add-on **Set B1** (UDI12B_0001-0096), Cat. No. 111.96 CORALL with Set B1, Cat. No. 118.96

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

The following Illumina instruments read the i5 index according to Workflow A:

- 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)

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 - A4 (UDI12A_0001-0384) for Workflow A (Cat. No. 107 - 110, or 120) are used on Illumina machines with Workflow B 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 optimal error correction all 12 nt should 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 Workflow B

The following Illumina instruments read the i5 index according to Workflow B:

MiniSeq (all (PE) flow cells)

NextSeq 500 / 550 (all (PE) flow cells)

HiSeq 3000 / 4000 (PE flow cells only)

For these instruments the Index 2 Read (i5) is performed after Read 2 Resynthesis, using the Index 2 (i5) Sequencing Primer (see also Appendix B, p.23).

ATTENTION: If Lexogen UDI 12 nt Set B1 (UDI12B_0001-0096) for Workflow B (Cat. No. 111) is used on Illumina machines with Workflow A 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 optimal error correction all 12 nt should 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), in which: 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.

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 175- 1,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (~175 bp), or overcycling bumps (>1,000 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.

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

13. Appendix G: Sequencing*

General

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. All CORALL libraries can be sequenced using the standard Illumina Multiplexing Read 1 and Read 2 Sequencing Primers. A schematic representation of those libraries is shown below.
CORALL libraries can be sequenced using either single-read (SR) or paired-end (PE) formats. The required sequencing depth per sample may vary depending on the intended application and sample type. Unique Molecular Identifiers (UMIs) are contained at the start of Read 1 (see below). Therefore, the complete information is conveniently accessible by cost-efficient single-read sequencing.
For information on loading amounts for the various sequencing instruments please refer to the CORALL online Frequently Asked Questions (FAQs), or contact support@lexogen.com.

Dual-Indexed Library Sequencing Workflows

The workflow for dual-indexed library sequencing differs, depending on the Illumina instrument and flow cell type. Dual indexing can be performed on single-read (SR) and paired-end (PE) flow cells. All HiSeq systems support SR and PE flow cells. NextSeq, MiniSeq, MiSeq, and NovaSeq systems use PE flow cells only, which can also be used in single-read mode. Illumina defines Workflows A and B for dual indexing read-out, which refer to the order of Index 2 read-out in relation to Read 2 Resynthesis. Lexogen defines four different workflows used for Index 2 Read (i5) read-out on Illumina instruments, which take into account also the differences in flow cell types and sequencing primers used for Index 2 (i5) read-out. These are summarized in the table below along with the compatible UDI 12 nt sets recommended for each.

<table>
<tr><th>UDI 12 nt Sets (Illumina Workflow)</th><th>Lexogen Workflow[1]</th><th>Instrument(s)</th><th>Flow Cell Type</th><th>i5 Index Read Primer</th><th>i5 Index Read Orientation</th><th>No. of Chemistry-Only Cycles[2]</th></tr>
<tr><td rowspan="3">A1 - A4
(A)</td><td>1</td><td>HiSeq 2000/2500</td><td rowspan="2">SR</td><td>Multiplexing Index 2 (i5) Sequencing Primer (HP9)</td><td rowspan="3">Forward (i5)</td><td>0</td></tr>
<tr><td>2</td><td>HiSeq 3000/4000</td><td rowspan="2">Grafted P5 Oligo</td><td rowspan="2">7</td></tr>
<tr><td>3</td><td>HiSeq 2000/2500
MiSeq
NovaSeq 6000</td><td rowspan="3">PE</td></tr>
<tr><td rowspan="2">B1
(B)</td><td rowspan="2">4</td><td>MiniSeq
NextSeq 500/550</td><td>Multiplexing Index 2 (i5) Sequencing Primer (HP14)</td><td rowspan="2">Reverse complement (i5rc)</td><td rowspan="2">0</td></tr>
<tr><td>HiSeq 3000/4000</td><td>Multiplexing Index 2 (i5) Sequencing Primer (dual-indexing primer mix)</td></tr>
</table>

[1]Workflows 3 and 4 correspond to Illumina's Dual-Indexed Workflows A and B on paired-end flow cells, respectively.
[2]Additional chemistry-only (no-imaging) cycles are performed before the i5 index is read-out.

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

The order of sequencing for Lexogen Workflows 1 - 3 (Illumina Workflow A) is: Read 1, index read preparation, Index 1 Read (i7), Index 2 Read (i5), Read 2 Resynthesis, and Read 2 for paired-end flow cells / runs.
The order of sequencing for Workflow 4 (Illumina Workflow B) is: Read 1, index read preparation, Index 1 Read (i7), Read 2 Resynthesis, and Index 2 Read (i5), and Read 2 for paired-end runs.

For paired-end runs, Read 2 is read-out after the Index 2 Read (i5). The order of Read 2 Resynthesis with respect to the Index 2 Read (i5) determines whether or not the i5 index is sequenced in forward or reverse complement orientation.

Sequencing Workflows for Dual-Indexed Libraries

The following section depicts the library adapters and sequencing primer binding sites for CORALL libraries. The sequences of the relevant Index 2 Read (i5) Sequencing Primers are also provided.

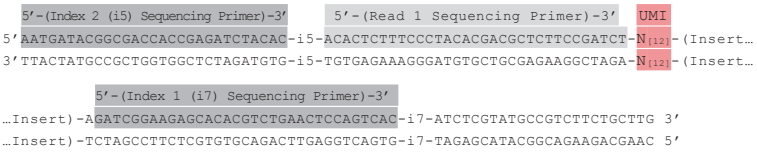
NOTE: All CORALL libraries include 12 nt Unique Molecular Identifiers (UMIs) at the beginning of Read 1.

Workflow 1: Single-Read Flow Cells - HiSeq 2000 / 2500

The Index 2 (i5) Sequencing Primer (included in HP9) is required for Index 2. 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 nt, 10 nt or 8 nt can be read out optionally.

Index 2 Read (i5): Multiplexing Index 2 (i5) Sequencing Primer (not supplied):
5′ AATGATACGGCGACCACCGAGATCTACAC 3′

CORALL libraries:



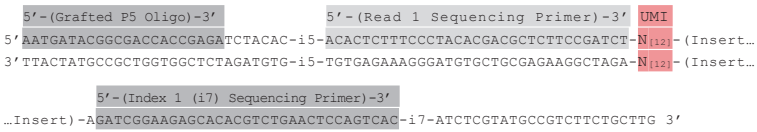
Workflow 2: Single-Read Flow Cells - HiSeq 3000 / 4000

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus 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 nt, 10 nt or 8 nt can be read out optionally.

Index 2 Read (i5): Grafted P5 Oligo on flow cell (not supplied):

5′ AATGATACGGCGACCACCGAGA 3′

CORALL libraries:



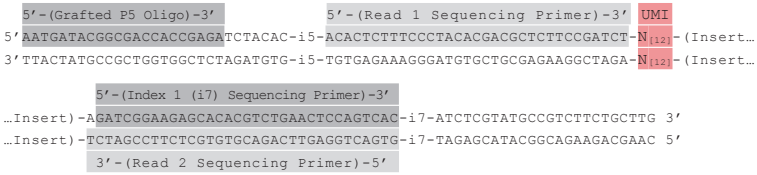
Workflow 3: Paired-End Flow Cells - HiSeq 2000 / 2500, MiSeq, and NovaSeq

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus 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 nt, 10 nt or 8 nt can be read out optionally.

Index 2 Read (i5): Grafted P5 Oligo on Flow Cell (not supplied):

5′ AATGATACGGCGACCACCGAGA 3′

CORALL libraries:



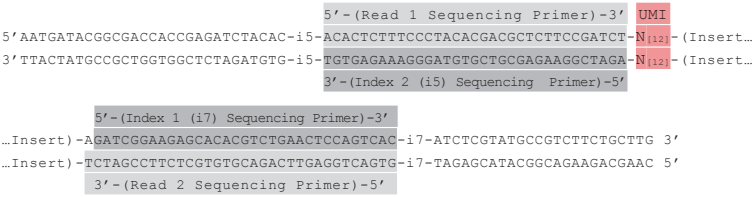
Workflow 4: Paired-End Flow Cells - MiniSeq, NextSeq, and HiSeq 3000 / 4000

All instruments use a Multiplexing Index 2 (i5) Sequencing Primer, which is included in the "Dual-Indexing Primer Mix" for MiniSeq and NextSeq, 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 nt, 10 nt or 8 nt can be read out optionally.

ATTENTION: Index 2 (i5) is read-out after the Read 2 Resynthesis step, hence a reverse complement of the Index 2 (i5) primer sequence is produced (see also Appendix F, p.30).

Index 2 Read (i5): Multiplexing Index 2 (i5) Sequencing Primer (not supplied):
5′ AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3′

CORALL libraries:



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 and flow cell type as outlined previously.

Read 1 for CORALL libraries:
Multiplexing Read 1 Sequencing Primer (not supplied):
5′ ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3′
Index 1 Read (i7): Multiplexing Index 1 (i7) Sequencing Primer (not supplied):
5′ GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3′
Index 2 Read (i5):

Workflow 1: Multiplexing Index 2 (i5) Sequencing Primer (not supplied):
5′ AATGATACGGCGACCACCGAGATCTACAC 3′

Workflows 2 and 3: Grafted P5 Oligo on Flow Cell (not supplied):
5′ AATGATACGGCGACCACCGAGA 3′

Workflow 4: Multiplexing Index 2 (i5) Sequencing Primer (not supplied):
5′ AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3′

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5′ GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 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 CORALL 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).

14. Appendix H: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of CORALL NGS data and is kept as general as possible for integration with your standard pipeline. For more information please contact support@lexogen.com.

In contrast to most other library preparation protocols, **CORALL libraries generate reads in forward orientation**, thus mapping should be performed to the corresponding strand of the genome.

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 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 data set.

Trimming

As CORALL libraries are based on random priming the first 9 nucleotides of Read 2 may have an increased error rate. As random priming may also occur at the junction between the ultimate exon and the poly(A) tail, mapping rates can be increased by trimming of poly(A) sequences at the 3' end of Read 1 and poly(T) sequences the 5' end of Read 2, when analyzing data from paired end runs. Further, CORALL libraries contain N_{12} Unique Molecular Identifiers (UMIs) at the start of Read 1. Hence, the first 12 nucleotides of Read 1 can be trimmed before proceeding to alignment. Alternatively, a less stringent aligner could be used with relaxed settings. Low quality sequences and adapter sequences should be trimmed. In case an adapter sequence is detected at the 3' end of Read 2, an additional 12 nucleotides upstream of the adapter can also be trimmed (i.e., the UMI sequence).

Alignment

After trimming, filtered and trimmed reads can be aligned with a short read aligner to the reference genome or assembled de novo. Please note, that **Read 1 reflects the RNA transcript sequence** not the cDNA sequence. This is important for downstream applications. If data from paired-end runs with read length >100 nucleotides is analyzed, ensure that the aligner used can handle overlaps (e.g., use relaxed settings).

Read Counting and Downstream Analyses

Depending on the intended application different methods for read counting on transcript or gene-level can be applied in order to generate expression data.
The analysis of SIRV spike-in control reads can be performed by aligning the trimmed reads to the SIRVome and evaluating the number and levels of detected isoforms. The SIRVome .fasta and .gtf annotation files are available for download from www.lexogen.com/sirvs/download.

Integrated CORALL Data Analysis Pipeline at BlueBee®

An optimized CORALL Data Analysis Pipeline, which performs read quality control, mapping, Unique Molecular Identifier (UMI) deduplication, and transcript quantification, is available on the BlueBee® Genomics Platform.

The pipeline can be accessed using Activation Codes, that are available from the Lexogen Webstore (Cat. No. 106.24).

To access the Data Analysis Pipeline, visit www.bluebee.com/lexogen, and enter the Activation Code to register an account. For further inquiries, please contact support@lexogen.com.

15. Appendix I: Revision History

Publication No. / Revision Date	Change	Page
117UG228V0100 Dec. 27, 2019	Initial Release of CORALL Total RNA-Seq Library Prep Kits with UDIs	

LEXOGEN

Enabling complete transcriptome sequencing

Associated Products:

020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
025, 050, 051 (SIRVs Spike-in RNA Variant Control Mixes)
037 (RiboCop rRNA Depletion Kit)
039 (Poly(A) RNA Selection Kit)
080 (Reamplification Add-on Kit for Illumina)
095 (CORALL Total RNA-Seq Library Prep Kit)
096 (CORALL Total RNA-Seq Library Prep Kit with RiboCop)
107 - 111 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits, Set A1, A2, A3, A4, or B1)
120 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit, Sets A1-A4)

CORALL Total RNA-Seq Library Prep Kit with UDIs· User Guide

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