

The Lexogen logo features the word "LEXOGEN" in a bold, sans-serif font. The letters "L", "E", and "X" are green, while "O", "G", "E", and "N" are dark blue. The background of the entire page is white with a pattern of semi-transparent, light blue spheres of various sizes and thin, light blue lines connecting them, creating a molecular or network-like structure.

LEXOGEN

Enabling complete transcriptome sequencing

CORALLTM

Cover all bases

Total RNA-Seq Library Prep Kit User Guide

Catalog Numbers:

095 (CORALL Total RNA-Seq Library Prep Kit)

096 (CORALL Total RNA-Seq Library Prep Kit with RiboCop)

095UG190V0130

FOR RESEARCH USE ONLY. NOT INTENDED FOR DIAGNOSTIC OR THERAPEUTIC USE.

INFORMATION IN THIS DOCUMENT IS SUBJECT TO CHANGE WITHOUT NOTICE.

Lexogen does not assume any responsibility for errors that may appear in this document.

PATENTS AND TRADEMARKS

The CORALL RNA-Seq kits are covered by issued and/or pending patents. CORALL™, SIRV™, and RiboCop™ are trademarks of Lexogen. The SIRVs are covered by issued and/or pending patents. Lexogen is a registered trademark (EU, CH, USA).

Illumina® is a registered trademark of Illumina, Inc.; RNasin® is a registered trademark of Promega Corporation. Bioanalyzer®, Fragment Analyzer™, and TapeStation® are trademarks of Agilent Technologies, Inc.; SYBR® Green I is a registered trademark of Molecular Probes, Inc.; LabChip® GX II is a registered trademark of Perkin Elmer. RNaseZap™, RNAlater®, and ERCC are registered trademarks of Ambion, Inc.; ThermoMixer® is a registered trademark of Eppendorf AG. Qubit™ and NanoDrop™ are registered trademarks of Thermo Fisher Scientific.

All other brands and names contained in this user guide are the property of their respective owners.

Lexogen does not assume responsibility for violations or patent infringements that may occur with the use of its products.

LIABILITY AND LIMITED USE LABEL LICENSE: FOR RESEARCH USE ONLY

This document is proprietary to Lexogen. The CORALL RNA-Seq kits are intended for use in research and development only. They need to be handled by qualified and experienced personnel to ensure safety and proper use. Lexogen does not assume liability for any damage caused by the improper use or the failure to read and explicitly follow this user guide. Furthermore, Lexogen does not assume warranty for merchantability or suitability of the product for a particular purpose.

The purchase of the product is subject to Lexogen general terms and conditions (<https://www.lexogen.com/terms-and-conditions/>) and does not convey the rights to resell, distribute, further sub-license, repack-age, or modify the product or any of its components. This document and its content shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way without the prior written consent of Lexogen.

For information on purchasing additional rights or a license for use other than research, please contact Lexogen.

WARRANTY

Lexogen is committed to providing excellent products. Lexogen warrants that the product performs to the standards described in this user guide up to the expiration date. Should this product fail to meet these standards due to any reason other than misuse, improper handling, or storage, Lexogen will replace the product free of charge or issue a credit for the purchase price. Lexogen does not provide any warranty if product components are replaced with substitutes.

Under no circumstances shall the liability of this warranty exceed the purchase price of this product.

LITERATURE CITATION

When describing a procedure for publication using this product, please refer to it as Lexogen's CORALL™ Total RNA-Seq Kit.

We reserve the right to change, alter, or modify any product without notice to enhance its performance.

CONTACT INFORMATION

Lexogen GmbH

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

Support

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

Table of Contents

1. Overview	4
2. Kit Components and Storage Conditions	6
3. User-Supplied Consumables and Equipment	8
4. Guidelines	9
5. Detailed Protocol	12
5.1 Library Generation	12
5.2 Library Amplification - Single Indexing (i7 only)	16
6. Short Procedure	19
7. Appendix A: General RNA Requirements	21
8. Appendix B: RNA Input	23
9. Appendix C: qPCR	25
10. Appendix D: Library Reamplification	27
11. Appendix E: Library Quality Control	28
12. Appendix F: Multiplexing	30
13. Appendix G: Sequencing	32
14. Appendix H: Data Analysis	34
15. Appendix I: Automation	36
16. Appendix J: Revision History	37
17. Notes	38

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.

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. The CORALL Total RNA-Seq Library Prep Kit is also available as a bundle with RiboCop (Cat. No. 096).

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.

During PCR second strand synthesis is performed and the double-stranded cDNA is amplified. In doing so, i7 and (optional) i5 indices as well as complete adapter sequences required for cluster generation on Illumina instruments are added (see also Appendix F, p.30). All purification steps are based on magnetic beads, rendering the protocol highly suitable for automation.

Multiplexing of libraries can be carried out using up to 96 i7 indices (included in the kit). Additional i5 indices can also be introduced using the Lexogen i5 6 nt Dual Indexing Add-on Kit (Cat. No. 047). Used together, Lexogen's 96 i7 and 96 i5 indices enable up to 9,216 different index combinations for sequencing (Appendix F, p.30). Alternatively, CORALL libraries can be barcoded with pre-mixed unique dual indices (UDIs) using the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits (Cat. No. 107-111, 120). For convenience, CORALL Total RNA-Seq Library Prep Kits with Unique Dual Indices are also available (Cat. No. 117-119, 132-134).

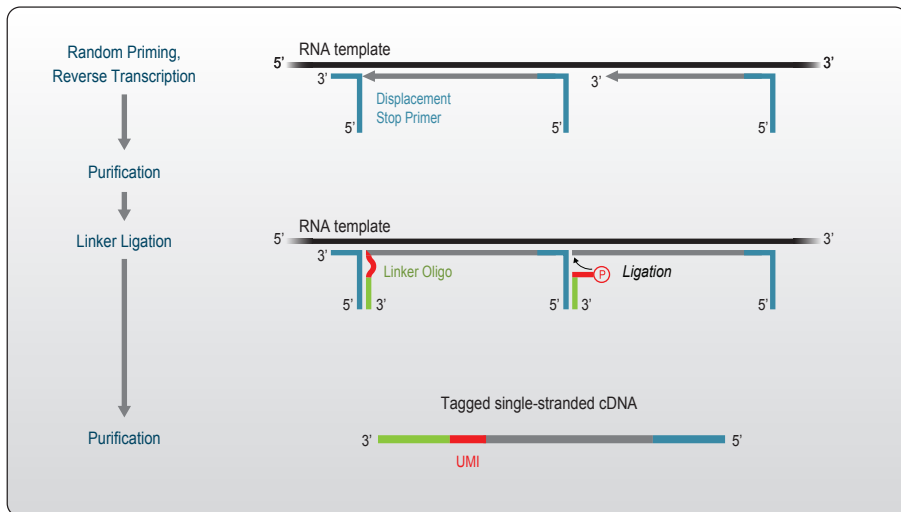
CORALL Total RNA-Seq libraries are compatible with single-read and paired-end sequencing on Illumina instruments (Appendix G, p.32). Read 1 contains the UMI and represents the transcript sequence. Hence the UMI information is directly accessible also in the cost-efficient single-read mode. Data can be analyzed with a number of standard bioinformatic pipelines. Information on read orientation and trimming is provided in Appendix H, p.34.

RiboCop rRNA Depletion Kit (optional)
or
Poly(A) RNA Selection Kit (optional)

LIBRARY GENERATION

3.5 hrs

1.5 hrs



LIBRARY AMPLIFICATION

1 hr

45 min

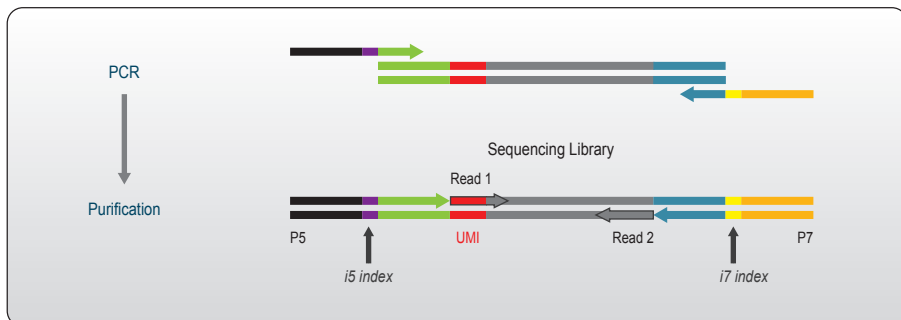


Figure 1. Schematic overview of the CORALL workflow.

2. Kit Components and Storage Conditions

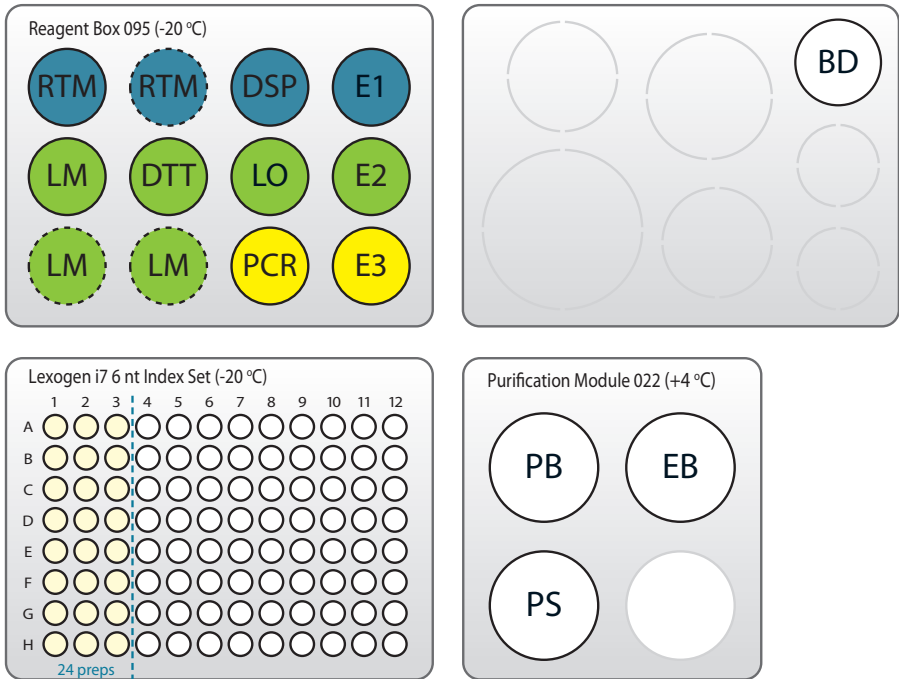


Figure 2. Location of kit components. For the 24 prep kit the dotted RTM and LM tubes are missing and the Lexogen i7 6 nt Index Set is only filled with indices 7001-7024 (up to the blue dotted line). All kits include Purification Modules.

Kit Component	Tube Label	Volume*		Storage
		24 preps	96 preps	
Reverse Transcription Mix	RTM ●	524 µl	2,094 µl	-20 °C
Displacement Stop Primer	DSP ●	30 µl	117 µl	-20 °C
Enzyme Mix 1	E1 ●	27 µl	106 µl	-20 °C
Ligation Mix	LM ●	1,047 µl	4,185 µl	-20 °C
Dithiothreitol	DTT ●	30 µl	117 µl	-20 °C
Linker Oligo	LO ●	30 µl	117 µl	-20 °C
Enzyme Mix 2	E2 ●	59 µl	234 µl	-20 °C
PCR Mix	PCR ●	204 µl	814 µl	-20 °C
Enzyme Mix 3	E3 ●	30 µl	117 µl	-20 °C
Lexogen i7 6 nt Index Set (96-well plate)		5 µl / reaction		-20 °C
Bead Diluent	BD ○	2,086 µl	8,343 µl	-20 / +4 °C
Purification Module (Cat. No. 022) included in the kit				
Purification Beads	PB	1,200 µl	4,800 µl	+4 °C
Purification Solution	PS	1,920 µl	7,680 µl	+4 °C
Elution Buffer	EB	3,168 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 purification 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 **E3** ●) 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 at least 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 (except Enzyme mixes) 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.

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 at least 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.

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:**

- 4 Proceed immediately to purification. **REMARK:** At this point we recommend placing the Purification Reagents (**PB**, **BD O**, 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 O**, 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 O**) 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 μ l Purification Beads (**PB**) and 50 μ l Bead Diluent (**BD**) per sample and add 59 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 - Single Indexing (i7 only)

This section describes single indexing PCR (i7 indices only) for multiplexing and unique indexing of up to 96 libraries using the Lexogen i7 6 nt Index Set included in the kit. Lexogen also offers i5 Dual Indexing Add-on Kits (Cat. No. 047), which enable dual indexing for up to 9,216 different i5 / i7 index combinations, or preparation of 96 libraries with unique dual indexing. For details, please refer to the Lexogen i5 6 nt Dual Indexing Add-on Kits Instruction Manual (047IM109).

NOTE: CORALL is compatible with the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits. For details, please refer to the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits Instruction Manual (107IM223).

Preparation

PCR		Purification	
PCR ●	– thawed at RT	PB	– stored at +4 °C
E3 ●	– keep on ice or at -20 °C	PS	– stored at +4 °C
i7 6 nt Index Set	– thawed at RT; spin down before opening!	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, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use.	
	} 10 - 20x see Appendix C, p.25		

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 i7 6 nt Index Set. Spin down the plate before opening and visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices only. Reseal opened wells of the plate after use to prevent cross contamination.
- Each well of the Lexogen i7 6 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.

OPTIONAL: For dual indexing please follow the protocol for endpoint PCR as indicated in the i5 6 nt Dual Indexing Add-on Kits (Cat. No. 047) Instruction Manual (047IM109) or in the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits (Cat. No. 107 - 111, and 120) Instruction Manual (107IM223). Do not use **PCR**!

28 Prepare a mastermix containing 7 μ l of PCR Mix (**PCR**) and 1 μ l of Enzyme Mix 3 (**E3**) per reaction.

29 Add 8 μ l of the **PCR / E3** mastermix to 17 μ l of the eluted library.

30 Add 5 μ l of the respective i7 index (**7001-7096**, in 96-well plate). Mix well by pipetting. Seal the PCR plate and quickly spin down. **ATTENTION:** Reseal opened wells of the Lexogen i7 6 nt Index Set after use to prevent cross contamination.

31 Conduct 10 - 20 cycles of PCR (as determined by qPCR assay, see Appendix C, p.25) with the following program: Initial denaturation at 98 °C for 30 seconds; 10 - 20 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.

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.

If dual indexing using the Lexogen i5 6 nt Dual Indexing Add-on Kits has been used, please refer to the Instruction Manual (047IM109). For unique dual indexing with the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits, please refer to the Instruction Manual (107IM223).

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

32 Add 27 μ l of fully resuspended Purification Beads (**PB**) to each reaction.
REMARK: For **CORALL libraries generated from FFPE RNA**, add 36 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 O, 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 O, 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 μ l of **EB** to the 17 μ l 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 μ l of cDNA with: 7 μ l **PCR** ●, 5 μ l Primer **7000**, 1 μ l **E** ● (from PCR Add-on Kit), 1.2 μ l of 2.5x SYBR Green I nucleic acid stain, and 14.1 μ l of **EB**, per reaction. Mix well.
- PCR: 98 °C, 30 sec.

98 °C, 10 sec	}	35x (see p.25)
65 °C, 20 sec		
72 °C, 30 sec		
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 μ l **PCR** ● and 1 μ l **E3** ● per reaction. Mix well.
- Add 8 μ l of **PCR** / **E3** mastermix to 17 μ l of each purified library.
- Add 5 μ l of i7 Primer (**7001-7096**) to each reaction, mix well.
ATTENTION: Reseal opened index wells after usage!
- PCR: 98 °C, 30 sec

98 °C, 10 sec	}	10 - 20x (as determined by qPCR assay, see p.25)	ATTENTION: Increased cycle numbers may be required for low input / low quality / FFPE RNA.
65 °C, 20 sec			
72 °C, 30 sec			
72 °C, 1 min			
10 °C, ∞ . Safe stopping point.			

Standard Input

FFPE RNA

Purification

- | | |
|---|---|
| <ul style="list-style-type: none"> <input type="checkbox"/> Add 27 μl PB per reaction, mix well, incubate 5 min at RT. <input type="checkbox"/> Place on magnet for 2 - 5 min, discard supernatant. <input type="checkbox"/> Add 30 μl EB, remove from magnet, mix well, incubate 2 min at RT. <input type="checkbox"/> Add 30 μl PS, mix well, incubate 5 min at RT. <input type="checkbox"/> Place on magnet for 2 - 5 min, discard supernatant. <input type="checkbox"/> Rinse the beads twice with 120 μl 80 % EtOH, 30 sec. <input type="checkbox"/> Air dry beads for 5 - 10 minutes. ATTENTION: Do not let the beads dry too long! <input type="checkbox"/> Add 20 μl EB, remove from magnet, mix well, incubate 2 min at RT. <input type="checkbox"/> Place on magnet for 2 - 5 min, transfer 15 - 17 μl of the supernatant into a fresh PCR plate.
 Safe stopping point. | <ul style="list-style-type: none"> <input type="checkbox"/> For FFPE RNA: Add 36 μl PB per reaction, mix well, incubate 5 min at RT. |
|---|---|

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 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 μ l), the reaction can be directly used as input for rRNA depletion with Lexogen's RiboCop rRNA Depletion Kits for Human/Mouse/Rat or Bacteria (Cat. No. 037, or 125 - 127, respectively).

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.23.

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 Kits for Human/Mouse/Rat (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 we recommend using Lexogen's RiboCop rRNA Depletion Kits for Human/Mouse/Rat or Bacteria (Cat. No. 037, or 125 - 127, respectively). RiboCop for Human/Mouse/Rat removes cytoplasmic as well as the mitochondrial rRNA, which can otherwise account for more than 40 % of the reads. For sample types other than Human/Mouse/Rat we recommend the use of rRNA depletion methods that also remove mt-rRNAs. RiboCop for Bacteria efficiently removes 23S, 16S, and 5S rRNA from a broad range of bacterial species. CORALL is also compatible with other commercially available rRNA depletion kits. Please contact support@lexogen.com for more information. RiboCop is generally suited for a wide input range from 1 ng to 1 µg. Special input requirements may apply for some applications using RiboCop for Bacteria or for degraded samples, including FFPE RNA. For details please refer to the respective User Guides.

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 qPCR assay details).

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

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 36 μ l of **PB** (instead of 27 μ l).
- **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.28).

The PCR Add-on Kit for Illumina (Cat. No. 020) is required for the following qPCR assay protocol. This assay can be used for dual- or single-indexed libraries.

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

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

1 Dilute the primary library from step 27 to 19 μ l by adding 2 μ l 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 μ l of the diluted cDNA library, 7 μ l of PCR Mix (**PCR ●**), 5 μ l of P7 Primer (**7000 ●**), 1 μ l of Enzyme Mix (**E ●**), and 1.2 μ l of 2.5x SYBR Green I nucleic acid dye. Make the total reaction volume up to 30 μ l by adding 14.1 μ l 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 $^{\circ}$ C for 30 seconds; 35 cycles of: 98 $^{\circ}$ C for 10 seconds, 65 $^{\circ}$ C for 20 seconds, and 72 $^{\circ}$ C for 30 seconds; a final extension at 72 $^{\circ}$ C for 1 minute, and hold at 10 $^{\circ}$ 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 μ l 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 μ l of cDNA for a qPCR, the cycle number corresponding to 50 % of the maximum fluorescence was 15 cycles. The remaining 17 μ l of the template should therefore be amplified with 12 cycles (15 - 3 cycles = 12 cycles).

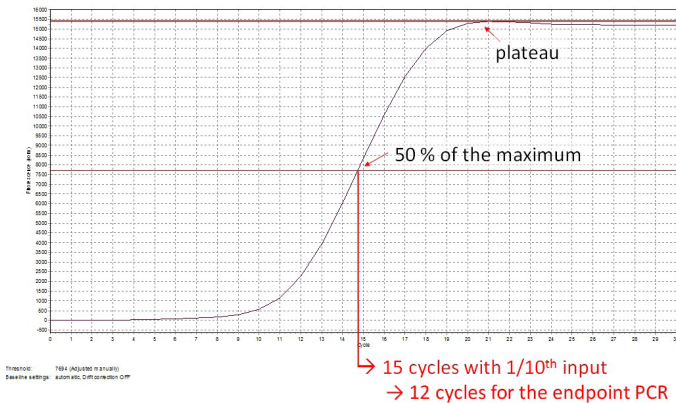



Figure 3. Calculation of the number of cycles for the endpoint PCR.

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 Single-Indexed Libraries (i7 only)

If the cDNA library yield is not sufficient, additional cycles of library amplification may be performed. Where possible, it is desirable to increase the amount of input RNA rather than relying on extra PCR cycles to increase library yield.

Lexogen's PCR Add-on Kit contains an i7 Reamplification Primer (**i7-RE** ) that can be used to reamplify single-indexed (i7) libraries to obtain enough material for sequencing if they were undercycled. For details please refer to the PCR Add-on Kit (Cat. No. 020) Instruction Manual.

Reamplification of Dual-Indexed Libraries (i5 and i7)

For reamplification of dual-indexed libraries the Reamplification Add-on Kit for Illumina (Cat. No. 080) is available on request. Please contact Lexogen at support@lexogen.com.

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 μ l 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 μ l of the finished library may be diluted to the required volume (e.g., 2 μ l sample for TapeStation and 10 μ l for LabChip GX II).

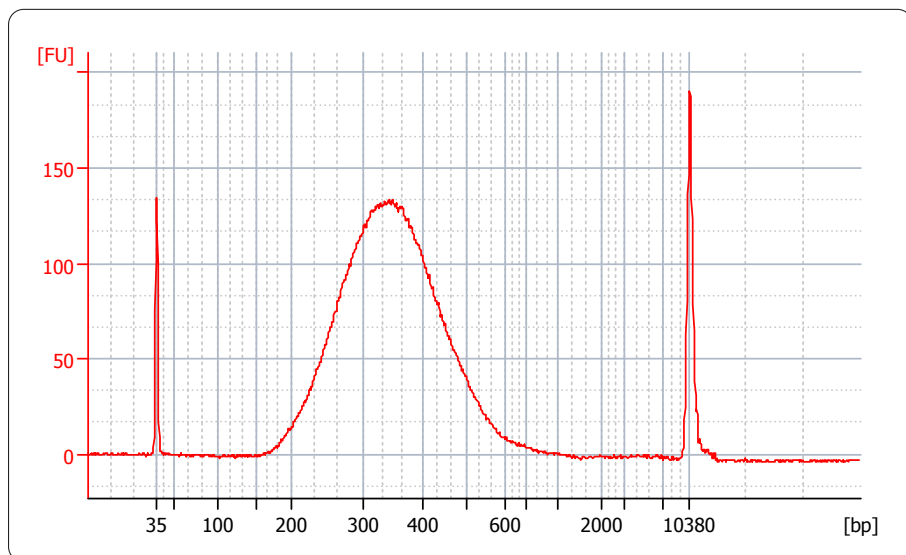


Figure 4. Bioanalyzer trace of a typical CORALL library synthesized from 200 ng Universal Human Reference RNA (UHRR) input for poly(A) enrichment. Approximately ~2 ng purified mRNA were recovered using the Lexogen Poly(A) Selection Kit (Cat. No. 039) and inserted into CORALL library preparation. The 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 C_q 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

REMARK: A shorter side-product representing linker-linker artifacts is sometimes visible at ~155 bp (with single-indexing, i7), 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 (≤ 155 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).

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

 Follow the detailed protocol from step **33** onwards (p.17).

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

CORALL libraries can be multiplexed for sequencing. i7 indices (included in the kit) are introduced during the PCR amplification (step 30). The Lexogen i5 6 nt Dual Indexing Add-on Kits (Cat. No. 047) enable dual indexing, including unique dual indexing of 96 samples or up to 9,216 different indexing combinations. For details on i5 indexing and sample sheet entry, please refer to the Lexogen i5 6 nt Dual Indexing Add-on Kit Instruction Manual (047IM109).

Single-Indexing - i7 Indices

i7 indices allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit (Lexogen i7 6 nt Index Set, 96-well plate). i7 indices are 6 nucleotides long and require an additional index-specific sequencing reaction (Index 1 Read).

	1	2	3	4	5	6	7	8	9	10	11	12
A	7001: CAGCGT	7009: TCAGGA	7017: TCTTAA	7025: TTTATG	7033: AGATAG	7041: CTCTCG	7049: GTGCCA	7057: AGTACT	7065: AAGCTC	7073: GACATC	7081: GCAGCC	7089: CGCGGA
B	7002: GATCAC	7010: CGGTTA	7018: GTCAGG	7026: AACGCC	7034: TTGGTA	7042: TGACAC	7050: TCGAGG	7058: ATAAGA	7066: GACGAT	7074: CGATCT	7082: ACTCTT	7090: CCTGCT
C	7003: ACCACT	7011: TTAACT	7019: ATACTG	7027: CAAGCA	7035: GTTACC	7043: AAGACA	7051: CACTAA	7059: GGTGAG	7067: TCGTTC	7075: CGTCGC	7083: TGCTAT	7091: GCGCTG
D	7004: TGCACG	7012: ATGAAC	7020: TATGTC	7028: GCTCGA	7036: CGCAAC	7044: ACAGAT	7052: GGTATA	7060: TTCCGC	7068: CCAATT	7076: ATGGCG	7084: AAGTGG	7092: GAACCT
E	7005: ACATTA	7013: CCTAAG	7021: GAGTCC	7029: GCGAAT	7037: TGGCGA	7045: TAGGCT	7053: CGCCTG	7061: GAAGTG	7069: AGTTGA	7077: ATTGGT	7085: CTCATA	7093: TTTCAG
F	7006: GTGTAG	7014: AATCCG	7022: GGAGGT	7030: TGGATT	7038: ACCGTG	7046: CTCCAT	7054: AATGAA	7062: CAATGC	7070: AACCGA	7078: GCCACA	7086: CCGACC	7094: AGAATC
G	7007: CTAGTC	7015: GGCTGC	7023: CACACT	7031: ACCTAC	7039: CAACAG	7047: GCATGG	7055: ACAAACG	7063: ACGTCT	7071: CAGATG	7079: CATCTA	7087: GGCCAA	7095: AGGCAT
H	7008: TGTGCA	7016: TACCTT	7024: CCGCAA	7032: CGAAGG	7040: GATTGT	7048: AATAGC	7056: ATATCC	7064: CAGGAC	7072: GTAGAA	7080: AACAAG	7088: AGACCA	7096: ACACGC

i7 index sequences are available for download at www.lexogen.com.

In general, we recommend processing a minimum of eight samples, using a complete set of eight i7 indices for multiplexing (e.g., **7001-7008**). However, if fewer indices are required care should be taken to always use indices that give a well-balanced signal in both lasers (red and green channels) for each nucleotide position. All columns (1 - 12) and rows (A - H) fulfill these criteria when individual libraries are mixed in an equimolar ratio. Use the online Index Balance Checker tool available at <https://www.lexogen.com/support-tools/index-balance-checker/>, to select the ideal combination of indices for optimal color and nucleotide balance.

REMARK: If an 8 nucleotide i7 index (Index 1) needs to be entered into an Illumina sample sheet, add 2 nucleotides from the Illumina adapter sequence to the 3' end of the index. **EXAMPLE:** **7001** would become **CAGCGTAT**, **7002** would become **GATCACAT** and so on. These additional nucleotides are identical for all indices as they are derived from the Illumina adapter.

ATTENTION: Take care to avoid cross contamination! Spin down the index plates before carefully removing the seal. Tightly reseal the plate for later use of the remaining indices.

Dual Indexing - i5 and i7 Indices

Lexogen i5 6 nt Dual Indexing Add-on Kits enable dual indexing of CORALL libraries, for enhanced multiplexing capacity and improved control of index identification accuracy.

The Lexogen i5 6 nt Unique Dual Indexing Add-on Kit (5001–5096, Cat. No. 047.96) provides 96 unique i5 indices in a convenient microplate format. In combination with Lexogen's i7 indices (included in the CORALL Library Prep Kits) up to 96 uniquely dual-indexed libraries can be prepared for sequencing in a single lane or run. Alternatively, up to 9,216 dual-indexed libraries with different i5 / i7 index combinations can be multiplexed in a single sequencing lane or run.

Lexogen also offers the CORALL Total RNA-Seq Library Prep Kits with UDI 12 nt Sets (Cat. No. 117 - 119). These kits follow the same CORALL protocol and include 96 or 384 pre-mixed Unique Dual Index combinations. Minor modifications during library amplification are outlined in the corresponding User Guide (117UG228).

13. Appendix G: Sequencing*

General

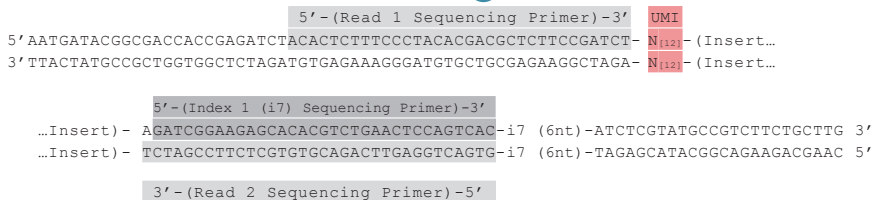
The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. All CORALL libraries can be sequenced using the standard Illumina Multiplexing Read 1 and Read 2 Sequencing Primers. A schematic representation of those libraries is shown below.

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.

Libraries with i7 Indexing

i7 indices (6 nt) are introduced during PCR (step 30).



Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):

5'ACACTCTTTCCTACACGACGCTCTCCGATCT 3'

Index 1 Read (i7): Multiplexing Index 1 Sequencing Primer (not supplied):

5'GATCGGAAGAGCACACGCTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):

5'GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT 3'

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

Multiplexing with Other Library Types

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

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

Due to size differences, libraries prepared with the Lexogen Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with QuantSeq, QuantSeq-Flex, SENSE, or 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's i7 6 nt index sequences are available for download at www.lexogen.com.

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 N₁₂ 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 on 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: Automation

Automating the process of library preparation has the advantage of avoiding sample tracking errors, dramatically increasing throughput, and saving hands-on time. CORALL is ideally suited to automation.

Instrument setups can vary widely, so if you are interested in CORALL automation scripts please contact us at support@lexogen.com.

Dummy reagents that mimic the CORALL reagent properties, designed to assist with the setup of CORALL automation protocols are available upon request. Please email support@lexogen.com for more information.

16. Appendix J: Revision History

The revision history table below shows user guide versions and kit changes made from 2019 onwards. The full revision history is available from www.lexogen.com.

Publication No. / Revision Date	Change	Page
095UG190V0130 March 31, 2020	Legal terms and conditions statements updated.	2
	Recommendations for FFPE samples, BD storage and DNase I treatment updated.	6 - 7, 12, 17, 19 - 21, 24
	Short Procedure steps updated, and reformatted.	19 - 20
	Information on CORALL data analysis and protocol automation added.	35, 36
	References to new products included.	4, 16, 17, 21, 23, 31, 40
095UG190V0120 Sept. 24, 2019	Minor revision of text in the detailed protocol (step 16).	15
	Recommendations for FFPE samples added to Appendix B.	24
095UG190V0110 Feb. 28, 2019	Initial Release.	
	Step 15 incubation time at 37 °C reduced to 30 minutes (from 1 hour).	14, 19
095UG190V0100 Jan. 03, 2019	Early Access Release.	

17. Notes

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, 125 - 127 (RiboCop rRNA Depletion Kits)
039 (Poly(A) RNA Selection Kit)
044 (Lexogen i7 6 nt Index Set (7001-7096))
047 (Lexogen i5 6 nt Dual Indexing Add-on Kit (5001-5096))
080 (Reamplification Add-on Kit for Illumina)
107 - 111, 120 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits)
117 - 119, 132-134 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Sets)

CORALL Total RNA-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, 2020

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